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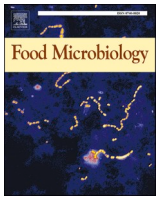

Growth assessment of *Salmonella enterica* multi-serovar populations in poultry rinsates with commonly used enrichment and plating media

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ABSTRACT

Isolation of *Salmonella* from enrichment cultures of food or environmental samples is a complicated process. Numerous factors including fitness in various selective enrichment media, relative starting concentrations in pre-enrichment, and competition among multi-serovar populations and associated natural microflora, come together to determine which serovars are identified from a given sample. A recently developed approach for assessing the relative abundance (RA) of multi-serovar *Salmonella* populations (CRISPR-SeroSeq or Deep Serotyping, DST) is providing new insight into how these factors impact the serovars observed, especially when different selective enrichment methods are used to identify *Salmonella* from a primary enrichment sample. To illustrate this, we examined *Salmonella*-positive poultry pre-enrichment samples through the selective enrichment process in Tetrathionate (TT) and Rappaport Vassiliadis (RVS) broths and assessed recovery of serovars with each medium. We observed the RA of serovars detected post selective enrichment varied depending on the medium used, initial concentration, and competitive fitness factors, all which could result in minority serovars in pre-enrichment becoming dominant serovars post selective enrichment. The data presented provide a greater understanding of culture biases and lays the groundwork for investigations into robust enrichment and plating media combinations for detecting *Salmonella* serovars of greater concern for human health.

1. Introduction

Non-typhoidal *Salmonella enterica* is the most documented cause of bacterial foodborne illness in the United States, and consumption of contaminated poultry is one of the most common sources of *Salmonella* infection in humans (Dewey-Mattia et al., 2018; Snyder et al., 2019; U. S. Centers for Disease Control, 2023; Walter et al., 2021). Routine surveillance for *Salmonella* in poultry is conducted using culture-based methods that include multiple steps and can take up to five days from initial rinse sample to confirmation of an isolated colony (U. S. Department of Agriculture Food Safety Inpection Service, 2023). The general method begins with a Buffered Peptone Water (BPW) rinse sample of poultry carcass or parts that is incubated statically at 35 °C for 18–24 h as a non-selective primary enrichment (or a pre-enrichment). The resulting growth is sub-cultured in parallel into two different selective broths, Tetrathionate Broth (TT) and Rappaport-Vassiliadis Soya Peptone Broth (RVS), and incubated at 42 °C for secondary, selective enrichment. Growth from these secondary cultures is then streaked onto a variety of different differential, selective agars, including Brilliant Green Sulfa (BGS) agar, Double Modified Lysine Iron agar (DMLIA), Xylose Lysine Deoxycholate (XLD) agar, and/or Xylose Lysine Tergitol-4 (XLT-4) agar, which are incubated for 18–24 h at 35 °C. After incubation, one presumptive *Salmonella* colony is selected for confirmation by molecular, serological, and/or biochemical tests (U. S. Department of Agriculture Food Safety Inpection Service, 2023). Whole genome sequencing (WGS) is then performed on the *Salmonella* isolate(s) and the serovar is bioinformatically determined (Zhang et al., 2019).

During handling and/or processing, organisms targeted for surveillance may suffer stress (including temperature stress and exposure to antimicrobials), which makes them less fit for subsequent survival in a selective enrichment. In poultry processing establishments, peracetic acid is the most common antimicrobial used in the carcass chilling step.

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The presence of various antibiotics, bile salts, detergents, and dyes into selective media makes selective enrichment inherently stressful. Therefore, a non-selective, primary enrichment in BPW is used to allow resuscitation of damaged *Salmonella* cells prior to selective enrichment (Busse, 1995; D'Aoust, 1981; Thomason et al., 1977). The selective pressure in RVS broth is due to pH (5.2) and high osmolarity (143 mM $MgCl_2$) that inhibits *E. coli* and *Proteus* spp., and malachite green for general coliform inhibition (Peterz et al., 1989; Rappaport et al., 1956; Vassiliadis, 1983). Part of the selectivity in TT broth is due to an iodine-iodide (I_2-KI) solution that causes tetrathionate production, which reduces levels of coliforms and other bacteria (D'Aoust, 1981; Knox, 1945). Tetrathionate is an electron acceptor for anaerobic respiration in *Salmonella* due to the presence of tetrathionate reductase (Hensel et al., 1999; Winter et al., 2010). TT broth contains bile salts, which cause DNA damage, and some formulations contain brilliant green, which inhibits *E. coli*, *Proteus* spp. and Gram-positive bacteria (Bernstein et al., 1999; Miller and Banwart, 1965). Both RV and TT are used because they were found to be effective in the recovery of *Salmonella* from raw flesh (June et al., 1996). Furthermore, studies have shown that the use of more than one type of enrichment broth increased the chances of recovery and increased the diversity of subtypes isolated (Cox et al., 2019; McEgan et al., 2014). Regarding selective and differential plating media, XLD, XLT-4, and DMLIA agars contain ferric iron compounds that combine with H_2S produced by many *Salmonella* strains to form Fe_2S_3, a black precipitate that darkens the colonies and differentiates them from sulfide non-producers among the Enterobacteriaceae. XLD and XLT-4 contain deoxycholate and tergitol, respectively, which inhibit Gram-positive bacteria, *Pseudomonas* spp., and *Proteus* spp. (Mallinson et al., 2000; Miller et al., 1991). DMLIA agar contains bile salt and novobiocin, which enhance recovery of *Salmonella* (Tate and Miller, 1990). Additionally, DMLIA contains bromocresol purple, and *Salmonella* colonies appear purple with black centers due to decarboxylation of lysine and production of H_2S. The selectivity of BGS agar is due to sodium sulfapyridine and brilliant green, which inhibit Gram-positive bacteria and members of Enterobacteriaceae (Mallinson et al., 2000; Osborne and Stokes, 1955). *Salmonella* strains form pinkish-red colonies with no yellow halo (due to non-fermentation of lactose) on BGS.

The species *S. enterica* contains over 2650 serovars, with approximately 100 causing the most illness in humans (European Food Safety Authority, 2022; Grimont and Weill, 2007; U.S. Centers for Disease Control and Prevention, 2018). Serovars Enteritidis, Typhimurium, and Infantis are among the Top Ten causes of human salmonellosis in the U.S., while *Salmonella* Kentucky rarely causes illness in the U.S (U. S. Centers for Disease Control, 2023). Recent studies indicate that several different serovars can be present in *Salmonella*-positive broiler chickens and parts during various steps of processing (Boubendir et al., 2021; Bourassa et al., 2015; Cox et al., 2020; Rasamsetti and Shariat, 2023). Additionally, several studies indicate that *Salmonella* serovars demonstrate culture bias in enrichments with some serovars outgrowing others in different media (Cox et al., 2019; Gorski, 2012; Harvey and Price, 1967; Larsen et al., 2021; Obe et al., 2021; Singer et al., 2009). For example, serovars Saintpaul and Typhimurium were less dominant in RV broth when grown with strains of serovars Kentucky, Infantis, Give, or Newport (Gorski, 2012). Strains of serovar Kentucky were more frequently isolated from XLT-4 than from BGS, and strains of serovar Enteritidis were more likely to be enriched from TT broth than from RV (Cox et al., 2019). Further, there are reports of some serovars displaying different growth rates in various enrichment media (Chen et al., 1999; Singer et al., 2009), and differential use of nutrients (Gorski and Aviles Noriega, 2023). Culture bias resulting in unequal recovery of serovars is problematic when doing routine surveillance and trace-back investigations.

There are several methods for *Salmonella* serovar determination including agglutination, PCR, and WGS, but these methods are suited for individual colonies post-enrichment (Echeita et al., 2002; Grimont and Weill, 2007; Herrera-Leon et al., 2004; Leader et al., 2009; Zhang et al., 2019). Molecular serotyping of enrichment cultures containing multiple *Salmonella* serovars can be done by the typing of unique spacer sequences between repetitive sequences in *Salmonella* CRISPR regions in a method called CRISPR-SeroSeq (Thompson et al., 2018). The CRISPR spacer sequences are derived from mobile genetic elements, so they are diverse and sufficient for distinguishing subtypes of bacterial strains, including *Salmonella* (Barrangou, 2015; Liu et al., 2011; Shariat and Dudley, 2014). The sequences of *Salmonella* CRISPR spacers correspond well with a specific serovar, as identity is conserved across strains but differs between serovars (Bugarel et al., 2018; Fabre et al., 2012; Liu et al., 2011; Thompson et al., 2018). As a deep serotyping (DST) approach, CRISPR-SeroSeq can detect serovars of *Salmonella* present at low relative abundance (RA) compared to the dominant serovars in a mixed culture (Thompson et al., 2018); thus, it is more sensitive for determining serovar distribution than standard agar plating techniques that rely on strain abilities to grow on given selective media and on selection of small numbers of colonies.

There has been a recent change in the approach to controlling *Salmonella* contamination in foods, because certain serovars are more likely to cause human illness than others. As a result, regulatory bodies such as the Food Safety and Inspection Service (FSIS) are considering classifying these more pathogenic *Salmonella* serovars as adulterants, when present at higher concentrations in food products they regulate (U.S. Department of Agriculture Food Safety Inspection Service, 2022). In this evolving regulatory landscape, it is important to understand the most efficient enrichment and plating media for detecting these serovars of greater concern for human health (also known as Key Performance Indicators, KPI). In the present study, we utilized *Salmonella*-positive BPW rinses from FSIS routine poultry verification programs and compared the serovars retrieved from TT and RVS secondary enrichments using DST to determine if the broths enriched the same levels of serovars. Additionally, the serovar information for *Salmonella* colonies retrieved from broad colony selection on BGS, XLD, and DMLIA agars after TT, and RVS, or IMS-RVS secondary enrichments was assessed to determine if the combination of broths and plating media affected the serovars detected. Finally, the retrieval of serovars in mixed cultures of various serovars in TT and RVS was assessed to determine if culture bias was mediated by *Salmonella* competition or caused by competing microbiota.

2. Materials and methods

2.1. Sample collection and Salmonella isolation

From the processing line, rehang and post-chill carcasses were selected, placed into a sterile bag, and 400 ml of neutralizing Buffered Peptone Water (nBPW) was poured into the carcass cavity. After thoroughly mixing for 1 min, 100–120 ml of the rinsate was poured into sample containers that were sealed, placed on ice, and shipped overnight to USDA-FSIS laboratories where they were cultured for *Salmonella* as described (U. S. Department of Agriculture Food Safety Inpection Service, 2023). Briefly, 30 ml of each rinsate was added to 30 ml BPW (Neogen, Lansing, MI, USA) and incubated overnight at 35 °C. These BPW cultures were screened rapidly for *Salmonella* with the 3M Molecular Detection System (3M, Saint Paul, MN, USA) according to manufacturer's instructions. If positive for *Salmonella*, 0.5 ml and 0.1 ml were transferred into 10 ml TT and 10 ml RVS selective enrichment broths (Neogen), respectively. Samples were cultured overnight at 42 °C. Overnight TT and RVS cultures were vortexed briefly and 1 ml of each was transferred into microcentrifuge tubes. The samples were centrifuged at 17,000×*g* for 3 min and the supernatant was discarded. The remaining pellets were stored at −20 °C. Through a material transfer agreement between USDA-FSIS and the University of Georgia (UGA), these pellets were transferred to UGA for deep serotyping.

2.2. DNA isolation and deep serotyping analysis (DST) by CRISPR-SeroSeq

Total genomic DNA was isolated from the TT and RVS culture pellets using the Genome Wizard kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Genomic DNA was resuspended in 200 μl of molecular grade water and stored at −20 °C. A total of 4 μl of the DNA was used as a template for the single step PCR for CRISPR-SeroSeq. This PCR amplifies *Salmonella* CRISPR spacers by using primers that target the conserved direct repeat sequences within *Salmonella* CRISPR arrays as described (Thompson et al., 2018). The primers also contain dual index barcodes that are based on the Illumina Nextera assay as well as Illumina adaptors to facilitate multiplex sequencing. Amplification of the CRISPR spacers was confirmed by gel electrophoresis of 5 μl of the PCR product on a 2% agarose gel stained with GelRed (Biotium, Fremont, CA, USA). After confirmation, PCR products were purified with AMPure beads (Beckman-Coulter, Indianapolis, IN, USA) following manufacturer's instructions. The purified products were then pooled together in approximate equimolar ratios and sequenced on an Illumina Next-Seq platform (150 cycles, single read, Illumina, San Diego, CA, USA) (Thompson et al., 2018). Sequences were analyzed using the CRISPR-SeroSeq pipeline by means of an R (version 4.04) script that uses BLAST to match experimental sequences to a curated database containing the complete CRISPR profiles for over 150 *Salmonella* types. The pipeline records BLAST matches on an Excel sheet and the relative frequency of each serovar was calculated based on the number of spacer reads corresponding to each serovar (Siceloff et al., 2022). Serovars with a relative frequency greater than 0.5% in a single culture were included in the analysis. The relative frequencies from TT and RVS broths were normalized to show an overall serovar profile for each culture (Shariat et al., 2022).

2.3. Salmonella isolation and molecular serotyping

A set of five *Salmonella*-positive BPW primary enrichments from poultry samples (as determined by 3M Molecular Detection System, and independent from the samples described in Section 2.1) prepared from routine surveillance in FSIS laboratories were stored as frozen glycerol (16% v/v) stocks at −80 °C for shipment to the USDA, ARS laboratory in Clay Center, NE. These frozen glycerol stocks were then subjected to secondary enrichment procedures. The secondary enrichment protocols evaluated included 1) direct subculture of 150 μl BPW primary enrichment into 10 ml of RVS broth (Oxoid, Basingstoke, Hampshire, UK), followed by incubation at 42 °C for 18–24h; 2) direct subculture of 750 μl of BPW cultures into 10 ml of TT broth (Neogen) followed by incubation at 42 °C for 18–24h; and 3) immuno-magnetic separation (IMS) of 1 ml BPW enrichment with 20ul of anti-*Salmonella* magnetic Dynabeads™ (Applied Biosystems, Foster City, CA, USA) as previously described (Bosilevac et al., 2009) with recovered IMS beads transferred to 3 ml of RVS medium, and incubated at 42 °C for 18–22 h (Fig. 1).

Post incubation each secondary enrichment culture was streaked for isolation onto three different selective and differential agar media, including XLD (Oxoid Thermo Fisher, Hampshire, UK), BGS (Difco, Becton Dickinson, Franklin Lakes, NJ, USA) and DMLIA (Remel, Lenexa, KS, USA). Plates were incubated at 37 °C for 20h and then inspected for typical *Salmonella* colony morphologies. Up to eight putative *Salmonella* colonies were picked from each plate for serovar characterization, resulting in a total of 72 colonies picked per primary enrichment sample. Putative *Salmonella* isolates were cultured in Trypticase Soy Broth (TSB, Difco) at 37 °C for 18–20h. These cultures were used to make two different DNA lysates, a BAX (Hygiena, Camarillo, CA, USA) lysate and a proteinase-K lysate. BAX lysates were made as per manufacturers instruction, which briefly entailed transferring 5 μl of TSB culture to 200 μl of BAX lysis buffer, followed by two incubation steps: 1) 37 °C for 20 min, and 2) 95 °C for 10 min. The proteinase-K lysates were made by combining 5 μl of TSB culture with 150 μl of PK-lysis buffer containing

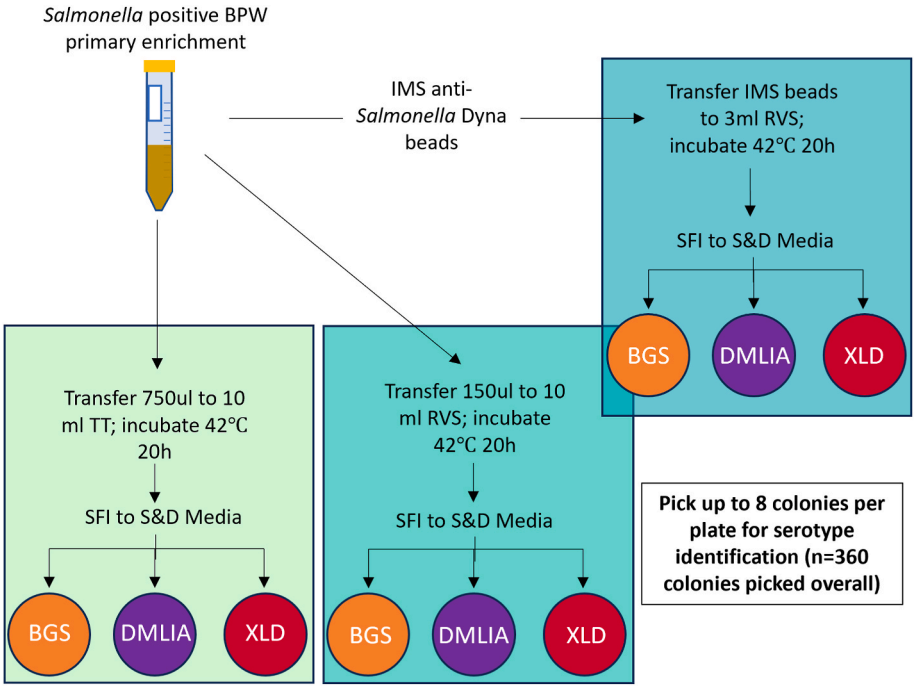

Fig. 1. Schematic overview of the enrichment media, methods, and plating media evaluated in this study. BPW – Buffered Peptone Water; IMS – Immuno-magnetic separation; RVS – Rappaport Vassiliadis Soya peptone medium; TT – Tetrathionate broth; BGS – Brilliant Green Sulfadiazine medium; DMLIA – Double Modified Lysine Iron Agar; XLD – Xylose, Lysine, Deoxycholate medium. The BPW primary enrichments were stored as glycerol stocks [16% (v/v)] and accordingly, increased volumes were used in subculturing to 10 ml RVS (150 μl instead of 100 μl) and 10 ml TT (750ul instead of 500) selective enrichment broths, to account for the addition of glycerol to the samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

1X Tris-EDTA, pH 8.0 (145 μl) and 5 μl proteinase K (20 mg/ml) per reaction, followed by incubation at 95 °C for 15 min. The resulting lysates were stored at 4 °C until used as DNA template in three different multiplex PCR reactions to determine *Salmonella* serovar, as previously described (Echeita et al., 2002; Herrera-Leon et al., 2004, 2007). Finally, a 3 ml aliquot of each secondary, selective enrichment culture was centrifuged at 1900×*g* for 5 min at 4 °C, the supernatant discarded, and the pellets stored at −20 °C. Frozen bacterial pellets were thawed, and total DNA extracted using the Qiagen DNeasy UltraClean 96 Microbial Kit (Qiagen, Germantown, MD, USA), as per manufacturer's instructions. The resulting DNA samples were quantified using a Promega Quantus fluorometer and the QuantiFluor ONE ds DNA system (Promega, Madison, WI, USA). DNA samples were sent to the University of Georgia lab for DST with CRISPR-SeroSeq as described above.

2.4. Direct competition of Salmonella serovars in TT and RVS broths

Six strains, including two each of *Salmonella* serovars Enteritidis, Infantis, and Kentucky (Table 1) isolated from colonies on the agar plates described in Section 2.3 were selected for experiments measuring the competition between serovars in secondary enrichment broth. For competition studies, strains were grown overnight in BPW at 37 °C and adjusted to an A_{600} of 0.1 with fresh BPW. Serial dilutions of these suspensions of 10^{-1} and 10^{-2} were made in BPW. These suspensions were used to inoculate mixtures of strains into TT (Hajna) and RVS broths with dual-strain pairings that consisted of a Kentucky strain with either an Enteritidis or an Infantis strain in mixtures of different ratios of the Kentucky:Enteritidis or Kentucky:Infantis strains of 1:1, 1:10, and 10:1. For TT cultures, 0.5 ml of the appropriate strain mixtures were inoculated into 10 ml of TT, and for RVS cultures, 0.1 ml of the mixtures were inoculated into 10 ml of RVS. TT and RVS cultures were incubated for 22–24h at 42 °C.

Following incubation, the resulting cultures were serially diluted in Phosphate Buffered Saline (PBS, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2), plated onto TSA plates, and incubated overnight at 37 °C. Thirty colonies from the resulting growth were selected for serovar assessment by agglutination reactions using anti-*Salmonella* O Antigen antisera (Difco, Becton Dickinson, Franklin Lakes, NJ, USA). Five microliters of antiserum were pipetted into wells of round bottom 96-well polystyrene microtiter plates (Falcon, Corning, Corning, NY, USA). Serovar Enteritidis was assessed using anti-group D1 (O factors 1, 9, 12) antiserum, serovar Infantis with anti-group C1 (O factor 7) antiserum, and Kentucky with anti-group C2 (O factor 8) antiserum. Two portions of isolated colonies were transferred using toothpicks into antisera corresponding with the strains used in the mixture and observed for agglutination reactions. Occasionally, mixed colonies yielded positive reactions for both antisera, and in these cases both positives were logged. The numbers of colonies of each serovar were logged and the percentage recovery calculated. Experiments were done in triplicate, and averages and standard deviations calculated. Paired t tests were done on strain pairings in the same tube. Unpaired t tests were used to compare different ratio mixtures of a strain in the same medium but different pairings, and one way ANOVA was done to compare the three ratio mixtures of a strain in the same media. P values of <0.05 were considered significant. Statistics were calculated using Prism v. 10.0.2 (GraphPad, San Diego, CA, USA).

Table 1
Identification and source history of strains used in direct serovar competition studies.

Strain ID	Serovar	Source	Medium selected from
0825-IB-1	Enteritidis	Chicken	BGS
0418–2	Enteritidis	Chicken	XLD
0830-IB-1	Infantis	Chicken	BGS
0022–1	Infantis	Turkey	XLD
0863-ID-2	Kentucky	Chicken	DMLIA
0148–2	Kentucky	Chicken	XLD

3. Results

3.1. CRISPR-SeroSeq DST reveals differential recovery of serovars from broiler enrichments

Deep serotyping (DST) was performed on total DNA extracted from overnight TT and RVS culture pellets from 184 broiler carcass samples that were *Salmonella*-positive. Multiple serovars were detected in 76 (41.3%) of these samples, and these samples allowed differences in serovar enrichment under the different conditions to be investigated. There were 21 broiler carcasses where *Salmonella* serovar Enteritidis was detected. Enteritidis was the sole serovar detected in three of these enrichments, and eighteen contained other serovars in addition to Enteritidis. Fig. 2 shows the relative frequency of serovar Enteritidis (light green bars), compared to other serovars, from paired RVS and TT broths in these 18 broiler carcass samples. In 17 (94%) of the mixed serovar samples that contained serovar Enteritidis, its relative abundance was higher in TT than in RVS and it was only detected in six (33%) of the RVS cultures. In three samples (Sample IDs 41-1, 373, and 19-2), >97% of the CRISPR sequences detected from the TT cultures corresponded to serovar Enteritidis; however, the paired RVS cultures resulted in <7% of the sequences corresponding to serovar Enteritidis. In Sample #41-1, serovar Enteritidis was not detected from RVS, but was the sole serovar detected from TT enrichment cultures. However, this result was not because serovar Enteritidis is unable to grow in RVS, since serovar Enteritidis was recovered at high levels from RVS in Samples #4-2 and #48-1.

Fig. 3 shows the DST results for *Salmonella* serovar Infantis (dark blue bars). Of the 184 carcasses, 63 tested positive for serovar Infantis, and 39 of these had multiple serovars. In the RVS and TT media pairings for mixed serovar samples, 29 (74.4%) showed more serovar Infantis sequences from the RVS cultures than the TT cultures. The relative abundance differences for serovar Infantis between RVS and TT were not as severe as those seen for serovar Enteritidis (Fig. 2). There were 14 examples of paired enrichments where Infantis sequences in the two culture types were within 10% of each other.

3.2. Selective broths and agar media show differences in serovar recovery of isolated colonies

CRISPR-SeroSeq analysis revealed the complexity of the serovars present in *Salmonella*-positive enrichment cultures, and differences in TT and RVS broths. However, routine surveillance and outbreak traceback investigations require the isolation of colonies. With the variety of different media available for *Salmonella* enrichment and isolation, we tested if there were differences in serovar recovery when three different plating media (BGS, XLD, and DMLIA) were used after selective enrichments in RVS, TT, and with a magnetic bead recovery (IMS) step using anti-*Salmonella* beads prior to RVS enrichment (IMS-RVS). Examining the serovar distribution for the enrichment samples revealed that all five samples contained multiple serovars, ranging from 2 (n = 1) to 4 (n = 2). DST of the five samples enriched via IMS-RVS, RVS, and TT selective media (Fig. 4), confirmed the colony serotyping data and was a good indicator of the serovars detected on the different plating media. Overall, five serovars were observed, including Enteritidis, which was present in all five samples, Infantis and Kentucky, each found in four, Typhimurium in two, and Schwarzengrund in one sample. In all cases, each enrichment method yielded different serovar profiles (Fig. 4).

The IMS-RVS enrichment method yielded the greatest diversity of serovars, especially when plated on both BGS and DMLIA agars. IMS-RVS selective enrichment yielded 2–4 different serovars on BGS and DMLIA, while fewer serovars were detected on XLD with the same method. False positive, non-*Salmonella* colonies were selected most often from XLD plates, especially after TT selective enrichment.

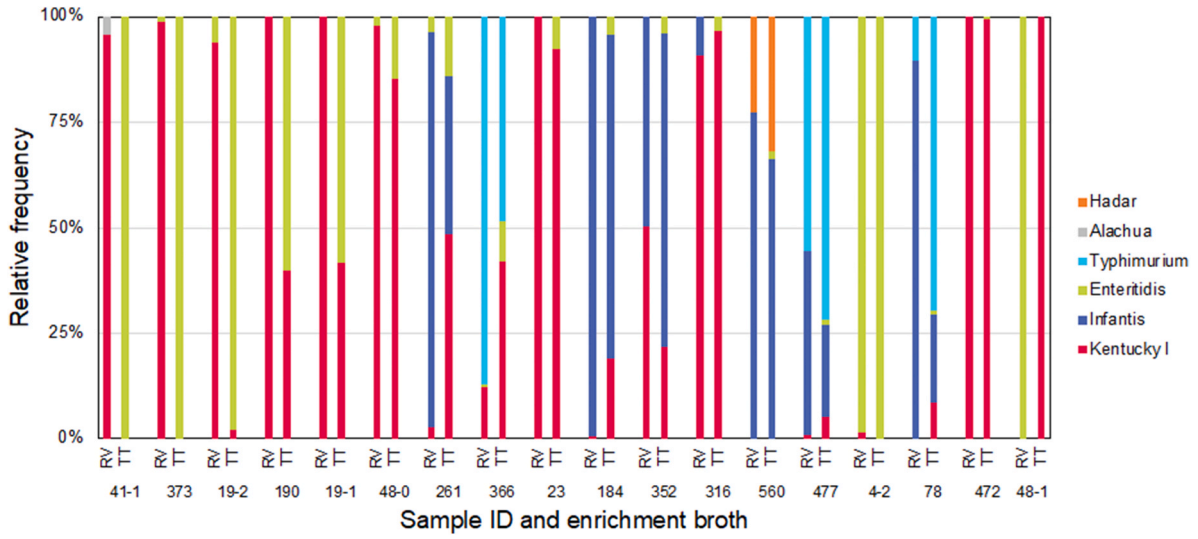

Fig. 2. Serovar Enteritidis is preferentially selected in Tetrathionate broth. Broiler carcass samples were enriched in parallel in RV and TT broths as indicated and the stacked bar graphs show the relative proportion of each serovar as determined by CRISPR-SeroSeq. Serovar Enteritidis is shown in light green and the other serovars are shown as indicated in the color key. An additional three carcasses are not shown as they only had serovar Enteritidis present, so there were no differences between RV and TT. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

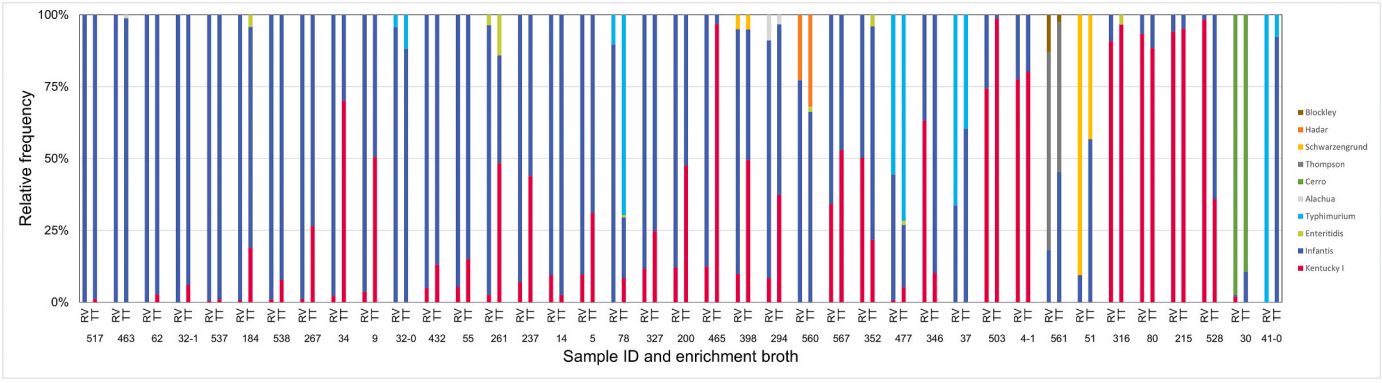

Fig. 3. Serovar Infantis is preferentially selected in Rappaport Vassiliadis broth. Broiler carcass samples were enriched in parallel in RV and TT broths as indicated and the stacked bar graphs show the relative proportion of each serovar as determined by CRISPR-SeroSeq. Serovar Infantis is shown in dark blue and the other serovars are shown as indicated in the color key. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Numbers of serovar Enteritidis colonies were enhanced on XLD from IMS-RVS enrichment. In the case of Sample #737, where serovar Enteritidis was present at a low relative abundance according to DST, more serovar Enteritidis colonies were retrieved through TT and IMS enrichment rather than with RVS enrichment alone. In contrast to serovar Enteritidis, serovar Infantis colonies were detected more often from RVS selective enrichment and plating to DMLIA or BGS agar. DST of the TT broth enrichment of Sample #863 indicated very low levels of serovar Infantis; whereas DST revealed serovar Infantis to make up about 30% of the relative abundance in the IMS-RVS enrichment. Serovar Infantis colonies were readily detectable from IMS-RVS and RVS enrichment, especially on DMLIA agar.

Colonies of serovars Typhimurium (from Samples #737 and #807) and Schwarzengrund (from Sample #825) were isolated more often from DMLIA agar using TT selective enrichment. Serovar Typhimurium made up approximately half of the signals from DST in enrichments from IMS-RVS, RVS, and TT of Sample #807, and was detected on the three media from each enrichment. However, serovar Typhimurium made up the minority of DST signals from Sample #737 enrichments, and serovar Typhimurium colonies were detected only through IMS-RVS and TT enrichment with variation in plating media. Serovar Schwarzengrund made up the majority of the DST signals in the one sample where it was detected (Sample #825), and the majority of serovar Schwarzengrund colonies came from TT enrichment and plating onto BGS and DMLIA.

Retrieval of serovar Kentucky was influenced by the levels of that serovar in the selective enrichments. DST showed that levels of serovar Kentucky were low in Samples #807 and #737 relative to the other serovars in those mixtures. Plating of those samples from the three broths onto the three agars showed low numbers of serovar Kentucky colonies. After TT enrichment of #737, serovar Kentucky was detected in only one of 24 colonies, as part of a mixed colony with serovar Infantis on BGS agar. For Sample #800, DST revealed serovar Kentucky at >25% of the population after IMS-RVS enrichment, but serovar Kentucky colonies were detected only on BGS and DMLIA from IMS-RVS. Serovar Kentucky colonies were recovered on all three plating media after just enrichment in RVS where DST showed serovar Kentucky at approximately half of the population. In contrast, neither serovar Kentucky signals nor colonies were detected from Sample #800 following TT enrichment. However, serovar Kentucky grew well in TT broth from Sample #863 where DST revealed serovar Kentucky sequences dominated in relative abundance by DST analysis.

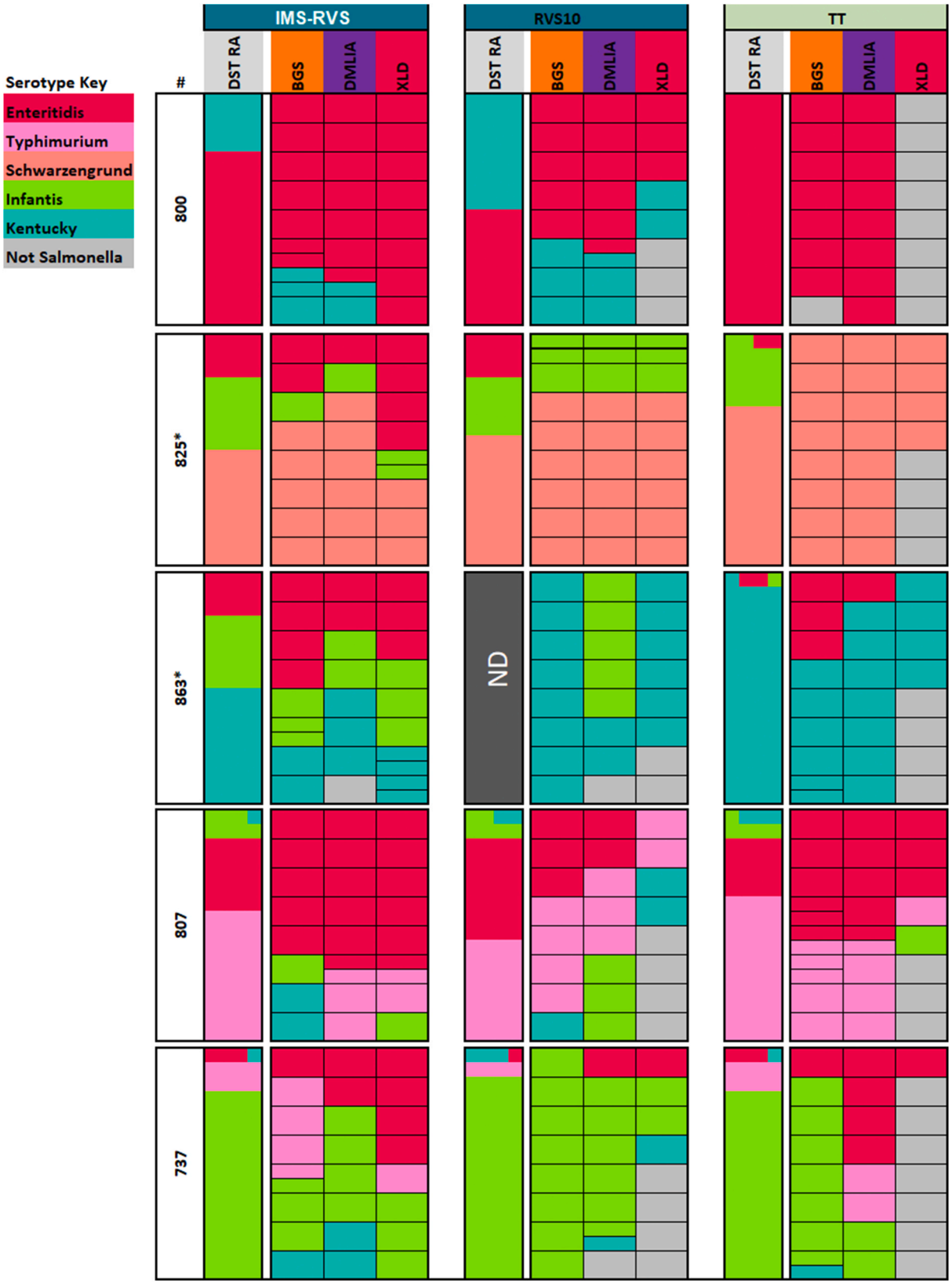

Fig. 4. Overview of the *Salmonella* serovars isolated from each enrichment method (IMS-RVS, TT, and RVS) and plating medium (BGS, DMLIA, XLD), for each sample. Deep Serotyping relative abundance (DST RA) for each sample and secondary enrichment combination is also indicated, with the serovars identified by color code as defined in the figure legend. In some cases, what appeared to be a single colony was selected for analysis, however molecular serotyping results indicated the presence of two different *Salmonella* serovars in the DNA lysate. In those cases, more than 8 serovars were identified from the 8 colonies selected, and data cells were split accordingly to convey this result. * Indicates samples that yielded isolates that were subsequently used in the defined mixed culture competition experiments. ND: No Data, as the DNA extraction for that sample yielded insufficient template for DST. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Serovars show unequal recovery in co-culture in TT and RVS

To study the contribution of relative abundance of serovars in selective enrichments, we tested direct strain pairings of different serovars at different inoculation levels, using strains isolated above in Section 3.2. The most common serovars detected in the enrichments were Enteritidis, Infantis, and Kentucky, so strains of these serovars were selected for these studies. Comparisons in TT and RVS with Kentucky: Enteritidis (Table 2) and Kentucky:Infantis (Table 3) at ratios of 1:1, 1:10, and 10:1 showed some statistically significant differences in recovery of certain strains and serovars in the different media, and some strains demonstrated within serovar differences in recovery. Enteritidis strain 0418-2 was recovered at significantly higher levels in TT (89%–100% of colonies) when co-cultured with either Kentucky strain at any inoculation level ($P < 0.002$). In RVS, recovery of this Enteritidis strain when co-cultured with the Kentucky strains was not significantly different from the 1:1 inoculation (43.5%–77.8%, $P > 0.06$), and was statistically improved when Kentucky was inoculated at 1 log lower levels (92.2%–94.4%, $P < 0.01$). When the Kentucky strains were inoculated at 1 log higher levels in RVS, Enteritidis 0148-2 was recovered at statistically lower levels than Kentucky 0148-2 (10.1%, $P = 0.01$) and was not significantly different against Kentucky 0863-ID-2 (62.2%, $P = 0.26$). The recoveries of the other Enteritidis strain, 0825-IB-1, were slightly different. In TT and RVS broths, Enteritidis 0825-IB-1 recovery was not statistically different from either Kentucky strain when inoculated at a 1:1 level in TT ($P = 0.87$) or RVS ($P = 0.16$). When the Kentucky inoculation level was 1 log lower, Enteritidis 0863-ID-2 recovery levels were higher and significantly different from the Kentucky 0863-ID-2 strain in RVS (68.2%, $P = 0.04$) and TT (67.3%, $P = 0.02$), but not significantly different from the Kentucky 0148-2 strain in RVS (59.0%, $P = 0.36$) and TT (75.3%, $P = 0.29$). Finally, when the Kentucky strains were inoculated at 1 log higher levels than Enteritidis 0825-IB-1 in both media, the Enteritidis recovery was less than 10% of the Kentucky strains ($P < 0.01$).

In the co-culture experiments with serovars Kentucky and Infantis strains, Infantis strain 0830-IB-1 was recovered at higher levels in all pairings with both Kentucky strains in both RVS and TT, but levels were generally higher in TT (Table 3). Infantis strain 0830-IB-1 was recovered at significantly higher levels when co-cultured with either Kentucky strain when inoculated in TT at 1:1 (98%–99%, $P = 0.0005$) and when Infantis was 1 log higher in TT (96.7%–100%, $P < 0.002$). In RVS, this Infantis strain was recovered at significantly higher levels when inoculated in excess with Kentucky strain 0148-2 (98.8%, $P = 0.0005$). Against Kentucky strain 0863-ID-2 in RVS, Infantis 0830-IB-1 was recovered also at significantly higher levels when inoculated at 1:1 (88.9%, $P = 0.01$) and at 1 log higher levels (96.7%, $P = 0.002$). When either Kentucky strain was inoculated at 1 log level higher than Infantis 0830-IB-1, there was no significant difference in recovery between this Infantis strain and either Kentucky strain in TT ($P > 0.09$) and RVS ($P > 0.07$). The other Infantis strain, 0022-1, was recovered at higher levels than either Kentucky strain in both media when inoculated at 1:1 or with Infantis in excess. The difference was significant in TT with both Kentucky strains (83.3%–94.4%, $P < 0.003$), and with Kentucky strain 0148-2 in RVS (72.2%, $P = 0.02$). When the Kentucky strains were inoculated at 1 log higher levels, Infantis strain 0022-1 was recovered at lower levels than the Kentucky strains in TT and RVS; however, this difference was significant with only Kentucky 0863-ID-2 in TT (14.4%, $P = 0.03$) and with both Kentucky strains in RVS (22.2%, $P < 0.04$).

Analysis of the recovery of the serovars in the various media pairings indicated that recovery of any of the strains at any inoculation levels in both media was dependent upon the strain pairings. In general, both Enteritidis strains had more colonies recovered from co-cultures with Kentucky strains in TT than in RVS. However, Enteritidis strain 0418-2 was recovered better than Enteritidis strain 0825-IB-1 across all pairings in both media. The Infantis strains were recovered better in TT than in RVS when inoculated at 1:1 levels, somewhat similar levels in both media when inoculated in excess. Infantis strain 0830-IB-1 was recovered better than Infantis strain 0022-1 when they were inoculated at 1 log lower levels than both Kentucky strains.

Table 2
Percentages of *Salmonella* Enteritidis colonies recovered from serovar Kentucky: Enteritidis strain mixed cultures.

		In competition with Kentucky 0148-2			In competition with Kentucky 0863-ID-2		
		1:1[a]	1:10	10:1	1:1	1:10	10:1
RVS[b]	Enteritidis 0825-IB-1	28.9 ± 16.4[c]	59.0 ± 13.2	10.1 ± 7.0*[d]	48.9 ± 6.3	68.2 ± 6.2*	7.2 ± 4.3*
	Enteritidis 0418-2	43.5 ± 4.2	92.2 ± 7.7*	31.5 ± 4.2*	77.8 ± 21.2	94.4 ± 1.9*	62.2 ± 13.5
TT[b]	Enteritidis 0825-IB-1	53.8 ± 34.5	75.3 ± 30.7	6.5 ± 3.2*	36.7 ± 20.5	67.3 ± 4.3*	4.4 ± 5.1*
	Enteritidis 0418-2	100 ± 0*	100 ± 0*	89.0 ± 3.7*	100 ± 0*	100 ± 0*	97.8 ± 3.9*

[a] The Kentucky:Enteritidis ratios inoculated into TT and RVS.
[b] RVS: Rappaport Vassiliadis Soya Peptone Broth; TT: Tetrathionate Hajna Broth.
[c] Numbers indicate the percentage of Enteritidis colonies resulting from growth co-culture with the indicated *Salmonella* Kentucky strain ±standard deviation.
d * Indicates the there is a statistically significant difference ($P < 0.05$) between the Kentucky strain and the indicated strain in the row.

Table 3
Percentages of *Salmonella* Infantis colonies recovered from Kentucky:Infantis mixed cultures.

		In competition with Kentucky 0148-2			In competition with Kentucky 0863-ID-2		
		1:1[a]	1:10	10:1	1:1	1:10	10:1
RVS[b]	Infantis 0830-IB-1	64.0 ± 6.2	98.8 ± 1.9*	54.4 ± 8.4	88.9 ± 6.9*	96.7 ± 3.3*	67.8 ± 8.4
	Infantis 0022-1	72.2 ± 5.1*	82.2 ± 6.9*	22.2 ± 8.4*	58.2 ± 5.0	75.6 ± 10.7	22.2 ± 10.7*
TT[b]	Infantis 0830-IB-1	99.0 ± 1.9*	100 ± 0*	70.4 ± 11.9	98.9 ± 1.9*	96.7 ± 3.3*	58.9 ± 26.9
	Infantis 0022-1	94.4 ± 5.1*	98.9 ± 1.9*	34.4 ± 30.1	83.3 ± 3.3*	82.2 ± 3.8*	14.4 ± 10.7*

[a] The Kentucky:Infantis ratios inoculated into TT and RVS.
[b] RVS: Rappaport Vassiliadis Soya Peptone Broth; TT: Tetrathionate Hajna Broth.
[c] Numbers indicate the percentage of Infantis colonies resulting from growth co-culture with the indicated *Salmonella* Kentucky strain ±standard deviation.
d * Indicates the there is a statistically significant difference ($P < 0.05$) between the Kentucky strain and the indicated strain in the row.

4. Discussion

Routine surveillance and verification of food and water is conducted for quality control by public health laboratories in order to reduce the incidence of *Salmonella* foodborne illness. The isolation protocols require various culture media, which introduce differential stresses and biases into the isolation process. Here we report on observations of unequal recovery of *Salmonella* serovars when different selective enrichment methods were used to isolate *Salmonella* from a given primary enrichment culture. Using selective media commonly used during enrichment and isolation of *Salmonella* from poultry samples, we observed that certain subtypes could be preferentially isolated after different media combinations. It is routine to find *Salmonella*-positive carcass rinses containing multiple serovars, and this has been described previously in studies characterizing serovars isolated from poultry

carcasses at processing (Boubendir et al., 2021; Bourassa et al., 2015; Cox et al., 2019, 2020; Rasamsetti et al., 2022; Rasamsetti and Shariat, 2023). We found that the type of secondary enrichment method and plating media used strongly impacted the *Salmonella* serovars isolated from the evaluated samples. We also observed the necessity for selecting multiple colonies from selective/differential agar plates, if the goal is to capture the diversity of *Salmonella* present, or serovars of concern that may be present as a minority, in enrichments that contain multiple serovars.

Co-cultures of *Salmonella* serovars in TT and RVS revealed that strain competition differs in the two media, and that the relative concentrations of different strains/serovars in the original sample may influence which subtypes are readily detectable at the end of the process. Further bias in serovar recovery occurred due to influences from selective and differential plating media. Uneven recovery could be caused by a multitude of factors, including differences in fitness of the various strains of *Salmonella* present in a sample, stressed and/or viable but non culturable (VBNC) cells, differences in the concentrations of various *Salmonella* subtypes (if present), and/or competition between serovars or with the resident microbiota (Brandl et al., 2023; Liu et al., 2018; Ottesen et al., 2013; Purevdorj-Gage et al., 2018).

DST and plating results from TT and RVS enrichments of poultry samples revealed that serovar Enteritidis was more likely to be isolated from TT-based selective enrichment. Direct testing of serovar Enteritidis in co-culture with serovar Kentucky reinforced the finding that serovar Enteritidis was more likely to be found after TT enrichment. For one of the strains tested, this observation was dependent upon the relative inoculation concentrations. DST and plating revealed that serovar Infantis strains were found more often after RVS enrichment. However, direct testing of serovar Infantis in co-culture with serovar Kentucky strains indicated serovar Infantis colony retrieval was higher after TT enrichment only when serovar Infantis inoculation levels were equal to or higher than serovar Kentucky inoculation levels. We further observed that serovar Kentucky was more likely to be detected after enrichment in RVS. However, the detection of serovar Kentucky colonies was dependent upon the concentration of that serovar in TT and RVS. When serovar Kentucky was present at equivalent or lower levels than a dominant serovar, as determined by DST, then serovar Kentucky was outcompeted on selective and differential agars and in inoculated TT and RVS (as demonstrated with Sample number 863 in Fig. 4). Since serovar Kentucky predominated when present originally at higher concentrations, the findings presented here suggest that the high levels of serovar Kentucky observed in poultry surveillance in the U.S. may be a result of high levels of serovar Kentucky present originally on the poultry samples.

The co-culture experiments described here contained only two strains, and therefore only measured the direct competition between *Salmonella* strains. This finding suggests that the competition from competing microbiota likely also influences which serovars may be recovered, since the serovar Infantis strains used here were isolated through poultry enrichment cultures and plating onto selective and differential agar. The influence of natural food microbiota was shown to differentially affect the recovery of *Listeria monocytogenes* from various selective enrichment broths (Al-Zeyara et al., 2011; Ottesen et al., 2016). Similarly, microflora associated with food sampling also affects *Salmonella* detection. *Paenibacillus* sp., a common soil bacterium, can inhibit and kill *Salmonella* when present in enrichment cultures from tomatoes (Ottesen et al., 2013). Various bacteriocins and lactic acid bacteria, which are part of the normal microbiota of some foods, have been shown to kill or inhibit *Salmonella* (Higgins et al., 2007; Seo and Kang, 2020). Furthermore, the natural, competing microflora is not consistent between foods, and can differ in the same product between lots and manufacturers (Fleet, 1999; Lusk et al., 2012; Ottesen et al., 2013).

The present study also indicated that there were differences in the serovars isolated on three different selective and differential plating media after TT and RVS enrichment. We observed that the secondary enrichment/agar media combination resulting in the largest diversity of different *Salmonella* serovars was IMS-RVS enrichment followed by plating onto BGS and DMLIA agars. IMS provides an opportunity to concentrate the *Salmonella* present in a sample and can be helpful in detecting *Salmonella* serovars or strains that are weaker competitors in primary or secondary enrichment cultures. IMS has been shown to allow more efficient detection of *Salmonella* from a sample when used in combination with selective and differential plating media, and/or molecular methods (Hanai et al., 1997; Harhay et al., 2021; Hyeon et al., 2019). Hanai et al. (1997) demonstrated that lower concentrations of *Salmonella* serovars were detected as colonies on selective agars from spiked chicken meat enrichments when IMS was used. In a study comparing different enrichment methods and plating media from chicken samples, Cui et al. (2006) showed that the combination of RVS-DMLIA and RVS-BGS yielded more *Salmonella* colonies than TT-DMLIA or TT-BGS; however, they assessed only *Salmonella* spp. and not *Salmonella* serovars. Obe et al. (2021) reported that BGS and XLT-4 agars were more efficient than Hektoen Enteric agar for detection of serovar Enteritidis from broiler carcasses. Another finding in the present study was that more false positive colonies were detected from XLD agar, especially after TT enrichment, which has been reported in other studies (Gorski et al., 2011; Park et al., 2012; van Dijk et al., 2009).

With an increased emphasis on detecting the presence of key performance indicator (KPI) serovars or *Salmonella* of greater concern for human health, it will be important for poultry integrators and third-party diagnostic laboratories to understand how the secondary enrichment and plating media used will enhance or diminish the ability to detect the presence of particular serovars. Follow-up investigations are needed that include more serovars and more strains of each serovar with different sample matrices and microflora to better characterize the impact of inherent biological variability on *Salmonella* detection methodologies. Nevertheless, the results of this study reveal four key findings: 1) IMS-RVS enrichment followed by plating onto BGS and DMLIA agars resulted in the isolation of the largest diversity of *Salmonella* serovars; 2) If the goal is to capture the diversity of *Salmonella* present, or serovars of concern that may be present as a minority in multi-serovar enrichments, it is necessary to select multiple colonies from different selective/differential agar plates; 3) Co-cultures of *Salmonella* serovars in TT and RVS revealed that strain competition differs in the two media, and that the relative concentrations of different strains/serovars in the original sample may influence which subtypes are readily detectable at the end of the process, with Enteritidis favored by TT secondary enrichment, and Infantis favored by RVS enrichment; 4) Serovar Kentucky, when present in greater concentrations in primary enrichment samples, will likely outcompete minority serovars, including Infantis and Enteritidis. This is possibly why sampling of poultry environments predominantly results in the isolation of serovar Kentucky, and not other serovars that may be present but are not as readily detected with the methods used. Implementation of these findings in improved sampling methods will enhance the detection of *Salmonella* serovars of greater concern for human health.

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.

Data availability

Data will be made available on request.

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