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Different *Salmonella* spp. loads to evaluate the performance of chicken sampling methodologies and quantification methodologies.

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Abstract

Global meat production is expected to increase by 2030, driven by factors such as population growth, income levels, and urbanization with poultry leading the growth due to its short production cycle. *Salmonella*, a major cause of gastrointestinal illness, is closely linked to poultry contamination, highlighting the need for its control in the industry. This study evaluated the performance of three different chicken sampling methodologies, comparing the traditional Rinsate method with MicroTally® Swab and MicroTally® Mitt, and the evaluation of three quantification methods (GeneUp® Quant *Salmonella*, Spread Plate, and Spiral Plate) across different concentrations of *Salmonella*. Chicken breasts were inoculated with four different *Salmonella* cocktails, and samples were collected using the three sampling methods. The quantification methods included non-incubation and 4-hour incubation protocols for GeneUp® Quant, and XLT4 agar plating using Spread Plate and Spiral Plate techniques. Statistical analyses were performed using SAS software to determine significant differences between methods at each concentration level, providing insights into reliable sampling techniques for detecting and quantifying *Salmonella* in chicken samples. The study concluded that MicroTally® Mitt was the method more comparable with Rinsate (reference method), which consistently yielded comparable or higher quantifications. While MicroTally® Swab performed similarly behavior, but its results showed more variability. Spread Plating had higher quantifications of bacteria at low concentrations, whereas Spiral Plating at higher concentrations. GeneUp 0 Hours of Incubation had higher quantifications at low concentrations, while GeneUp 4 Hours, at higher concentrations, highlighting the importance of incubation time in molecular quantification.

Keywords: GeneUp, Incubation, MicroTally®, Mitt, Rinsate, Swab.

Resumen

Se espera que la producción global de carne aumente para 2030, como resultado del crecimiento poblacional, niveles de ingreso y la urbanización, con el pollo liderando el crecimiento debido a su ciclo de producción corto. *Salmonella*, una causa principal de enfermedades gastrointestinales, está estrechamente relacionada con la contaminación de aves de corral. Este estudio evaluó el rendimiento de tres metodologías de muestreo de pollo, comparando el método tradicional Rinsate con MicroTally® Swab y MicroTally® Mitt, y tres métodos de cuantificación (GeneUp® Quant Salmonella, Plateo de Extensión en Placa y Plateo en Espiral) en diferentes concentraciones de *Salmonella*. Pechugas de pollo fueron inoculadas con cuatro cócteles diferentes de *Salmonella*, y se recogieron muestras utilizando tres métodos de muestreo. Los métodos de cuantificación incluyeron protocolos de incubación, sin incubación (0 horas) y de 4 horas para GeneUp® Quant, y el recuento en placa XLT4 usando técnicas de Plateo. Se realizaron análisis estadísticos con el software SAS para determinar diferencias significativas entre los métodos en cada nivel de concentración. El estudio concluyó que MicroTally® Mitt fue el método más comparable con Rinsate, mostrando cuantificaciones similares o mayores. Mientras que MicroTally® Swab tuvo un comportamiento similar, pero con mayor variabilidad en los resultados. El Plateo de Extensión en Placa mostró una cuantificación mayor de bacterias a bajas concentraciones, mientras que el Plateo en Espiral las tuvo a concentraciones más altas. GeneUp0 Horas de Incubación mostró una cuantificación mayor de bacterias a bajas concentraciones, mientras que GeneUp4 Horas las mostró a concentraciones más altas, destacando la importancia del tiempo de incubación en la cuantificación molecular.

Palabras clave: Incubación, GeneUp, MicroTally®, Mitt, Rinsate, Swab.

Introduction

According to the Organization for Economic Co-Operation and Development (OECD)/Food and Agriculture Organization of the United Nations (FAO), the meat production forecast for 2030 indicates that there will be a global increase. Poultry meat will continue to have the greatest growth, due to its short production cycle (Font-I-Furnols, 2023). Multiple factors influence increased poultry production and demand including the world population growth, the changing demographics of the world's population, where the middle-income consumer class has significantly grown, urbanization, income levels, price, cultural norms, environmental aspects, animal welfare and health (Logue et al., 2024).

Salmonellosis is the second most reported gastrointestinal disorder in the EU resulting from the consumption of *Salmonella*-contaminated foods. Symptoms include gastroenteritis, abdominal cramps, bloody diarrhea, fever, myalgia, headache, nausea, and vomiting (Ehuwa et al., 2021).According to the Centers for Disease Control and Prevention (CDC), contaminated chicken contributes significantly to foodborne illnesses reported annually in the United States; and *Salmonella* often being the culprit (Tack et al., 2020). *Salmonella* spp. is a gram-negative, oxidase-negative, non-spore-forming bacillus member of the Enterobacteriaceae family (Shaji et al., 2023).

Annually, *Salmonella* causes 200 million to over 1 billion infections worldwide, with 93 million cases of gastroenteritis and 155,000 deaths, and 85% of illnesses that are food-linked (He, 2023). Between 1998 and 2008, poultry accounted for 17.9% of foodborne illnesses in the United States, with *Salmonella* ser. Enteritidis and Typhimurium are responsible for 17.4% and 34% of poultry-related foodborne illnesses, respectively. Given the significant public health impact of *Salmonella*-related illnesses, it is crucial to monitor and reduce contamination in poultry products (Whyte et al., 2002).

Research demonstrates one of the ways to monitor *Salmonella* in poultry is through sampling methodologies. Many sampling techniques can be used to evaluate the quality and safety of food, the

sampling techniques are grouped into two categories: destructive and nondestructive methods (Alnajrani, 2017). This last mentioned includes the one used as the standard during this investigation, Rinsate sampling, which is a common method, USDA approved, used to determine the levels of pathogen contamination on poultry (He et al., 2023). Other methods include MicroTally® Mitt and MicroTally® Swab, that have several advantages over the Rinsate method, including the reduction of labor, costs, time, and more convenience (Fremonta, 2023). However, these last mentioned have been recently used and there is not enough research to completely evaluate if they perform the same as the Rinsate method.

On the other hand, there is also an importance in the enumeration methods for these sampling methodologies. Conventional *Salmonella* detection is usually performed using a culture-based method, which is time-consuming, labor-intensive, and unsuitable for on-site testing and high-throughput analysis. However, technology wants to take over traditional methods. For example, the Spiral plating method, enables automatic and standardized plating of samples, allowing up to 4 dilution series on a single Petri dish (Interscience, 2018), these methods provide faster results and more convenience, although still using traditional agars. This type of automatic plating could potentially be replacing more frequent methods such as pour plating or spread plating.

Currently, there are many detection methods with a unique detection system available for *Salmonella* detection utilizing immunological-based techniques, molecular-based techniques, and others (Awang et al., 2021). During this study, a molecular-based technique called GeneUp Quant *Salmonella,* was used, which employs real-time PCR with dual Fluorescence Resonance Energy Transfer (FRET) hybridization probes to detect *Salmonella* DNA in samples (Adria food expertise, 2022). These types of methods could potentially replace traditional ones because of the convenience they claim to have, such as faster and more accurate results. However, there are certain limitations with this method, when using low concentrations, the machine may not quantify it and give false results manifesting there is no

Salmonella present while there could be. Increasing incubation time, results in increased bacteria counts . In fresh foods like meat, *Salmonella* has a high rate of regeneration, potentially doubling its number every 15-20 minutes (Elika, 2024). For this reason, GeneUp Quant *Salmonella* came up with a GeneUp 4-hour incubation, giving it time to regenerate to have accurate results in its quantification. While conventional methods can take several days, real-time PCR can detect *Salmonella* in just a few hours, allowing for quicker decisions in food safety.

The identification and quantification of specific pathogens are critical to food safety and quality. However, the reliability and accuracy of these measurements can vary significantly depending on the sampling and quantification methods used. As mentioned, there is currently limited information comparing different sampling methods (MicroTally® Mitt, MicroTally® Swab to Rinsates) and different quantification techniques (Gene Up® Quant *Salmonella*, Spread Plating, and Spiral Plating) on poultry. This lack of knowledge hinders the ability to choose the most convenient methods for faster, cheaper but still accurate pathogen detection, which is critical for monitoring and improving food safety practices this is why the objectives of this investigation inlcuded:

To evaluate two new sampling methods (MicroTally® Mitt and MicroTally® Swab) for *Salmonella* on chicken tenders, by comparing them to the Rinsate method, using four cocktails at different concentrations of *Salmonella*.

To compare the performance of two traditional quantification methods, Spiral and Spread Plating, using four cocktails of different concentrations of Salmonella, employing three sampling methods (MicroTally® Mitt, MicroTally® Swab, and Rinsate).

To compare the performance of the GeneUp Quant Salmonella method at two different incubation times, 0 hours and four hours, using four cocktails of different concentrations of Salmonella, employing three sampling methods (MicroTally® Mitt, MicroTally® Swab, and Rinsate).

Methodology

Study Location

Located in Lubbock, Texas, the research study was done at Texas Tech University's Department of Animal and Food Science. Microbiological studies were conducted at the Experimental Sciences Building in the ICFIE Laboratories.

Bacterial Strains and Preparation of Inoculum

Individual frozen isolates *(Salmonella Typhimurium, Salmonella Enteritidis, Salmonella Infantis*) were transferred with an inoculation loop (1 µL) to a testing tube with 5 mL of Brain Heart Infusion Agar (BHI) and incubated at 37 °C for 18-24 hours. The isolates transferred from the BHI culture then were streaked with an inoculation loop (1 µL) onto 2 plates per strain with Tryptic Soy Agar (TSA) and incubated for 18-24 hours at 37 °C. An individual colony from each plate for each strain was transferred with a sterilized cotton swab to 6 testing tubes (two for each strain) with 5 mL of sterilized water. The pathogen concentration was assessed by evaluating the solution turbidity in which a nephelometer was used and was calibrated to 0.5 McFarland, with a McFarland standard. Turbidity of 0.5 McFarland is equivalent to 1-2x10^8 CFU/mL. After that, the concentration with the nephelometer was confirmed, and the 6 tubes with 5 mL of water were combined into a falcon tube, with a 30 mL cocktail of three *Salmonella* strains (*Salmonella* Typhimurium*, Salmonella* Enteritidis*, Salmonella* Infantis) with a final concentration of 1-2x10^8 CFU/mL. Twenty milliliters of the cocktail were transferred to a bottle with 180 mL of PBS (Phosphate buffered saline) to dilute the cocktail by 1 logarithm, creating a solution of 200 tender with a final concentration of 1-2x10^7 CFU/mL. Then, 20 tender of the 180 mL cocktail was transferred to a new 180 tender PBS bottle, to dilute for a final concentration 1-2x10^6 CFU/mL. The last process was repeated until 4 bottles with 4 cocktails at different concentrations (1-2x10^6 CFU/mL, 1-2x10^5 CFU/mL, 1-2x10^4

CFU/mL, 1-2x10^3 CFU/mL) were left. Cocktails with 6, 5, 4, and 3 logarithms were applied to the samples. The final expected concentration on the 3-pound sample was 4 logarithm CFU/mL of rinse for the highest inoculation. Each bottle containing the *Salmonella* cocktail was then poured into spray bottles to use during the inoculation phase. Table 1 includes the concentrations of Salmonella per Cocktail.

Table 1

Concentrations of Salmonella per Cocktail.

Concentration of *Salmonella*	Cocktail
1-2x10^3 CFU/mL	Cocktail 1
1-2x10^4 CFU/mL	Cocktail 2
1-2x10^5 CFU/mL	Cocktail 3
1-2x10^6 CFU/mL	Cocktail 4

Note. Cocktails used with its corresponding concentration. CFU: Colony-forming unit.

Processing Methodology

Four boxes, each containing four ten-pound bags of chicken tenders (16 bags in total), were received from a commercial poultry processing facility, and stored at refrigeration temperature (3-5 ˚C). One pound from each of the ten-pound bags of chicken tenders was collected for the screening step. The collected pounds were sampled using the Rinsate methodology. These samples were then incubated for 24 hours at 37 °C and analyzed following the GeneUp Quant *Salmonella* detection protocol to confirm the presence of *Salmonella*. Eight bags from the sixteen bags were used, these were confirmed to be negative in *Salmonella* prevalence results, so they were selected for inoculation. The process involved combining two bags of the eight previously selected with negative *Salmonella* prevalence results in a single 18-pound bag, repeated per cocktail, and because we had four concentrations of *Salmonella,* it resulted in four bags. Fifteen pounds of each of the four bags of chicken tenders were processed as follows:

During the inoculation process, 9 pounds of chicken tenders were sprayed with 90 mL of prepared bacterial cocktails (ranging from 6 to 3 logarithmic concentrations) in a laminar flow biological safety

cabinet. Each spray bottle dispensed 1.4 mL per spray, requiring 107 sprays per replication for the 15 pounds of chicken tenders. The inoculated tenders were placed in a biological safety cabinet at room temperature for 15 minutes to facilitate bacterial attachment. This process was repeated for each cocktail. *Salmonella* enumeration was determined using the GeneUp® Quant *Salmonella*, GeneUp® Quant Enrichment *Salmonella*, and GeneUp® *Salmonella* detection protocols. Samples were plated on XLT4 agar using both Spread and Spiral Plating methods to facilitate detection and quantification.

Sampling Method

Sampling Method 1 (Rinsate)

One pound of inoculated chicken tenders was transferred (approximately 5 units) to a sterile bag. Then it was mixed with 100 mL of Buffered Peptone Water (BPW) and tampered with both hands for 1 minute. Then it was transferred with the liquid solution to a Whirl Pak sterile bag. This process was repeated per cocktail.

Sampling Method 3 (MicroTally® Mitt and Swab)

For MicroTally Mitt, soil towels were rubbed on both sides vigorously over meat, one minute per side (towels were pre-moistened with 25 mL of Buffered Peptone Water). After rubbing the towels, these were folded, and returned into bags, ensuring the towel was completely submerged in the media. Buffered Peptone Water was added to get to a final volume of 200 mL and mixed. The bags were incubated from 8-24 hours at 42 °C. For MicroTally Swab the same protocol as MicroTally Mitt was followed.

Enumeration Methods

GeneUp Quant Salmonella Non-Incubation and 4 hours Incubation.

The GeneUp Quant *Salmonella* system follows the molecular-based technique that utilizes real-time PCR with dual Fluorescence Resonance Energy Transfer (FRET) probes to rapidly detect and quantify *Salmonella* DNA in various samples. By amplifying specific *Salmonella* DNA sequences and measuring the resulting fluorescence in real-time. For this study two variants for this method were used:

4-hour incubation (GeneUp4H): Incubation at 37 °C for 4 hours before protocol.

0-hour incubation (GeneUp0H): No incubation before protocol.

For these two incubation methods of GeneUp, the GeneUp Quant *Salmonella* Protocol of the manufacturer was followed, as well as its quantification which was done utilizing the automatic calculator the company offered. Further information such as the gene used for amplification remains confidential by the organization.

Spread Plating Method (XLT4 Agar)

XLT4 (Xylose-Lysine-Tergitol 4) agar plates were used as a selective culture medium designed to isolate and identify *Salmonella* from food and environmental samples. The medium contains an enzymatic digest of animal tissue, xylose, L-lysine, lactose, sucrose, sodium chloride, yeast extract, and phenol red. For cocktail 1 and 2, a 10^0 dilution was used, for cocktail 3, 10^1 and for cocktail 4, 10^2. For dilutions 9 mL tubes of BPW were used. For plating, 0.1 mL of sample was used with the help of a pipette, a reusable glass or metal Spreader was flame sterilized by dipping it in alcohol, and even pressure was applied as the plate was spun, either on a turntable or by hand After spreading, the plates were left undisturbed for 10 to 20 minutes and then incubated at 37 °C for 18-24 hours. Results were read using an automatic counter.

Spiral Plating Method (XLT4 Agar)

For the Spiral Plating method, the plate preparation followed the same steps as for Spread Plating. The sample was deposited continuously or in segments, starting from the center and moving outward.

This pattern gradually diluted the sample as it spiraled outwards, resulting in a range of bacterial concentrations across the plate. The dilutions used were the same ones as the Spread Plating Method. Instructions for the Automated Spiral Plater manufacturer were followed.

The plates were then incubated at 37 °C for 18-24 hours, and results were read using an automatic counter.

Experimental Design

A Completely Randomized Design was used, 4 cocktails, each evaluating 3 sampling methods and 4 quantification methods, having a total of 48 treatments, with 4 repetitions, with a total 192 experimental units. Data analysis was conducted using the Statistical Analysis System (SAS®), including ANOVA with Duncan Grouping for mean separation of main factors and LSMeans for interactions between sampling methods and quantification methods.

Results and Discussion

Sampling Methods

As observed in Table 2, a mean separation comparing sampling methods per quantification was made for Cocktail 1. GeneUp0H, revealed statistical differences between Swab and Mitt, however, there were no significant differences between both Swab vs. Rinsate, and Mitt vs. Rinsate. Mitt reported higher CFU counts with a mean of 3.94± 0.32 LogCFU/tender. For GeneUp4H, there were no significant differences between the three sampling methods, in this case, Mitt reported once again the highest CFU counts with a mean of 3.33 ±0.26 LogCFU/tender. For Spiral Plating, there were significant differences between Swab and Mitt, however, there were no significant differences between both Swab and Rinsate, and Mitt and Rinsate. Swab reported higher CFU counts with a mean of 2.90±0.24 LogCFU/tender. Spread Plating reported no significant differences between Rinsate and Swab, however, there were significant differences between Swab and Mitt and between Rinsate and Mitt. Rinsate reported higher CFU counts with a mean of 2.70±0.10 LogCFU/tender, followed by Swab with a mean of 2.72±0.28 LogCFU/tender.

Table 2

Mean Separation for Cocktail 1, comparing Sampling Methods per Quantification Method

	Rinsate Mean± SDLog CFU/tender	Swab Mean ± SD LogCFU/tender	Mitt Mean±SD LogCFU/tender	C.V (%)
GeneUp0H	3.60± 0.24 ^{xy}	3.35±0.29 ^{y}	3.94± 0.32^{x}	8.84
GeneUp4H	3.23±0.30 ^{x}	2.88±0.20 ^{x}	3.33±0.26 ^{x}	9.35
Spiral Plating	2.66±0.28 ^{xy}	2.90±0.24 ^{x}	2.22±0.28 ^{y}	12.17
Spread Plating	2.79± 0.10 ^{x}	2.72±0.28 ^{x}	2.32±0.09 ^{y}	5.82

Note. Means with different letters ^{xy} I on rows indicates significant differences ($p \leq 0.05$) between sampling methods, C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter

As observed in Table 3, a mean separation comparing sampling methods per quantification was made for Cocktail 2. For GeneUp0H, there were no statistical differences reported, with a higher quantification of 3.96±0.46 LogCFU/tender for Rinsate. For GeneUp4H, there were no statistical differences reported, with a higher quantification in Mitt with 4.17±0.46 LogCFU/tender. For Spiral Plating, there were also no statistical differences reported, with a higher quantification of 3.75±0.16 LogCFU/tender for Rinsate. Spread Plating reported significant differences between Rinsate and Swab and between Rinsate and Mitt, however, there were no significant differences between Swab and Mitt, with a higher quantification for Rinsate with a mean of 3.80±0.10 LogCFU/tender.

Table 3

Mean Separation for Cocktail 2, comparing Sampling Methods per Quantification Method

	Rinsate Mean±SDLogCFU/tender	Swab Mean ± SD LogCFU/tender	Mitt Mean ± SD LogCFU/tender	C.V (%)
GeneUp0H	3.96±0.46 ^{x}	3.93±0.31 ^{x}	3.93±0.13 ^{x}	7.36
GeneUp4H	4.01±0.27 ^{x}	3.91±0.23 ^{x}	4.17±0.46 ^{x}	8.3
Spiral Plating	3.75±0.16 ^{x}	3.61±0.25 ^{x}	3.56±0.15 ^{x}	2.99
Spread Plating	3.80±0.10 ^{x}	3.37±0.06 ^{y}	3.50±0.09 ^{y}	2.75

Note. Means with different letters ^{xy} I on rows indicates significant differences ($p \leq 0.05$) between sampling methods, C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter

As shown in Table 4, a mean separation comparing sampling methods per quantification was made for Cocktail 3. For GeneUp0H, there were no statistical differences reported with a higher quantification of 4.87±0.54 LogCFU/tender for Mitt. For GeneUp4H, there were no statistical differences reported between Rinsate and Swab, however, there were significant differences between Rinsate and Mitt, and between Swab and Mitt, with a higher quantification in Rinsate with 5.55 ±0.50 LogCFU/tender. For Spiral Plating, there were no significant differences reported between Swab and Mitt, however, there were significant differences between Rinsate and Swab, and between Rinsate and Mitt, with a higher

quantification of 4.99±0.26 LogCFU/tender for Mitt. Spread Plating reported significant differences between Rinsate and Mitt, and between Swab and Mitt, however, there was no significant difference between Rinsate and Swab. There was a higher quantification for Mitt with 4.81±0.54 LogCFU/tender.

Table 4

Mean Separation for Cocktail 3, comparing Sampling Methods per Quantification Method

	Rinsate Mean±SDLogCFU/tender	Swab Mean ± SD LogCFU/tender	Mitt Mean ± SD LogCFU/tender	C.V (%)
GeneUp0H	4.61±0.09 ^{x}	4.67±0.31 ^{x}	4.87±0.54 ^{x}	6.23
GeneUp4H	5.55±0.50 ^{x}	5.42±0.52^{x}	4.56±0.62^{y}	5.18
Spiral Plating	4.56±0.10 ^{y}	4.92±0.03 ^{x}	4.99±0.26 ^{x}	3.51
Spread Plating	4.45±0.17 ^{y}	4.63±0.24 ^{y}	4.81±0.54 ^{x}	6.70

Note. Means with different letters ^{xy} I on rows indicates significant differences ($p \leq 0.05$) between sampling methods, C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter

As indicated in Table 5, a mean separation comparing sampling methods per quantification was made for Cocktail 4. For GeneUp4H, there were no statistical differences between sampling methods, however, there was a higher quantification of CFU in Swab with 6.86±0.51 LogCFU/tender**.** For Spiral Plating, there were no statistical differences between sampling methods, however, there was a higher quantification for Mitt with a mean of 6.01±0.15 LogCFU/tender. For Spread Plating, there were no significant differences between Rinsate and Swab, and between Swab and Mitt, however, Rinsate and Mitt, had significant differences. There was a higher quantification of CFU in Mitt with a mean of 5.80±0.33 LogCFU/tender. Due to technical difficulties with the GeneUp machine, GeneUp 0-hours could not be calculated for this cocktail.

Table 5

Mean Separation for Cocktail 4, comparing Sampling Methods per Quantification Method

	Rinsate Mean±SDLogCFU/mL	Swab Mean ± SD LogCFU/mL	Mitt Mean ± SD LogCFU/mL	C.V (%)
GeneUp4H	6.80±0.18 ^{x}	6.86±0.51 ^{x}	6.84±0.06 ^{x}	4.60
Spiral Plating	5.37±0.30 ^{x}	5.79±0.56 ^{x}	6.01±0.15 ^{x}	6.44
Spread Plating	5.47±0.18 ^{y}	5.62±0.18 ^{xy}	5.80±0.33 ^{x}	2.40

Note. Means with different letters ^{xy} I on rows indicates significant differences ($p \leq 0.05$) between sampling methods, C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter

A general trend towards greater variability in the results is observed as the concentration of *Salmonella* decreases. This increased variability reflects a reduction in the consistency of the results across the sampling methods (Rinsate, Swab, Mitt). Taking this into account, it becomes evident that as the concentration decreases for each method, the results become less consistent.

Tradition Plating Quantification Methods

A mean separation for traditional Plating Quantification Methods, Spread, and Spiral Plating was conducted per sampling method for Cocktail 1. As observed in Table 6, for both Spiral Plating and Spread plating, there were no significant differences between Rinsate and Swab, and significant differences between Mitt and Swab. However, in Spiral Plating there were no significant differences between Rinsate and Mitt, unlike Spread Plating. There was a higher quantification of CFU for Spiral Plating in Swab with 2.90±0.24 LogCFU/tender, as for Spread Plating there was a higher quantification in Rinsate with 2.79±0.10 LogCFU/tender.

Table 6

Mean separation for Cocktail 1, comparing Traditional Plating Quantification Methods per Sampling Method.

Quantification Method	Sampling Method	Mean ± SD LogCFU/tender
Spiral Plating	Rinsate	2.66 ±0.28 [ab]
Spiral Plating	Swab	2.90±0.24 [a]
Spiral Plating	Mitt	2.22± 0.28 [b]
Spread Plating	Rinsate	2.79±0.10 [a]
Spread Plating	Swab	2.72±0.28 [a]
Spread Plating	Mitt	2.32 ±0.09 [b]
C.V (%)		8.56

Note. Different letters placed vertically [abcd] indicate significant differences ($p \leq 0.05$) between quantification methods, while the same letter placed vertically [abcd] indicates no significant differences ($p \geq 0.05$), C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter.

A mean separation for traditional Plating Quantification Methods, Spread, and Spiral Plating was conducted per sampling method for Cocktail 2. As shown in Table 7, both Spiral and Spread Plating, show different behavior. For Spiral Plating there were no significant differences between Swab and Mitt, and between Swab and Rinsate. In the other side, Spread Plating presented significant differences between Rinsate and Mitt, but these results were statistically closer to the result of Spiral Plating, however, there was a significant difference in Swab – Spread Plating results with all the results of Spiral and Spread Plating techniques. Despite, there were no significant differences between Spread and Spiral Plating Techniques the highest quantification of CFU was Rinsate – Spread Plating with 3.80±0.10 LogCFU/tender.

Table 7

Mean separation for Cocktail 2, comparing Traditional Plating Quantification Methods per Sampling Method.

Quantification Method	Sampling Method	Mean ± SD LogCFU/tender
Spiral Plating	Rinsate	3.75±0.16 [ab]
Spiral Plating	Swab	3.61±0.25 [abc]
Spiral Plating	Mitt	3.56±0.15 [bcd]
Spread Plating	Rinsate	3.80±0.10 [a]
Spread Plating	Swab	3.37±0.06 [d]

Quantification Method	Sampling Method	Mean ± SD LogCFU/tender
Spread Plating	Mitt	3.50±0.09 ^{cd}
C.V (%)		3.96

Note. Different letters placed vertically ^{abcd} indicate significant differences ($p \leq 0.05$) between quantification methods, while the same letter placed vertically ^{abcd} indicates no significant differences ($p \geq 0.05$), C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter.

A mean separation for traditional Plating Quantification Methods, Spread, and Spiral Plating was conducted per sampling method for Cocktail 3. As shown in Table 8, for both, Spiral and Spread Plating, there were no significant differences between Rinsate and Swab, and between Swab and Mitt. However, there were significant differences between Rinsate and Mitt for Spiral Plating, while on Spread Plating these showed no significant difference. Additionally, for Spiral Plating there was a higher CFU quantification on the three sampling methods Rinsate, Swab and Mitt.

Table 8

Mean separation for Cocktail 3, comparing Traditional Plating Quantification Methods per Sampling Method.

Quantification Method	Sampling Method	Mean ± SD LogCFU/tender
Spiral Plating	Rinsate	4.56±0.10 ^{bc}
Spiral Plating	Swab	4.92±0.03 ^{ab}
Spiral Plating	Mitt	4.99±0.26 ^{a}
Spread Plating	Rinsate	4.45±0.17 ^{c}
Spread Plating	Swab	4.63±0.24 ^{abc}
Spread Plating	Mitt	4.81±0.54 ^{abc}
C.V (%)		5.51

Note. Different letters placed vertically ^{abcd} indicate significant differences ($p \leq 0.05$) between quantification methods, while the same letter placed vertically ^{abcd} indicates no significant differences ($p \geq 0.05$), C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter.

A mean separation for traditional Plating Quantification Methods, Spread, and Spiral Plating was conducted per sampling method for Cocktail 4. As indicated in Table 9, for both Spiral and Spread Plating, there were no significant differences between Swab and Mitt and between Rinsate and Swab, however, for Spiral Plating there were significant differences between Mitt and Rinsate, but there were no

significant differences between these two in Spread Plating. Additionally, for Spiral Plating, there was a higher CFU quantification for Mitt, with a mean of 6.01±0.15 LogCFU/tender.

Table 9

Mean separation for Cocktail 4, comparing Traditional Plating Quantification Methods per Sampling Method.

Quantification Method	Sampling Method	Mean ± SD LogCFU/tender
Spiral Plating	Rinsate	5.37±0.30 [b]
Spiral Plating	Swab	5.79±0.56 [ab]
Spiral Plating	Mitt	6.01±0.15 [a]
Spread Plating	Rinsate	5.47±0.18 [b]
Spread Plating	Swab	5.62±0.18 [ab]
Spread Plating	Mitt	5.80±0.32 [ab]
C.V (%)		4.70

Note. Different letters placed vertically [abcd] indicate significant differences ($p \leq 0.05$) between quantification methods, while the same letter placed vertically [abcd] indicates no significant differences ($p \geq 0.05$), C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter.

As the concentration of *Salmonella* decreases, a clear trend towards greater variability in the results is observed for both plating methods (Spiral Plating and Spread Plating) across all sampling techniques (Rinsate, Mitt, Swab). The differences between the values obtained for lower concentrations are more pronounced, indicating that these quantification methods are less accurate in detecting low bacterial concentrations.

GeneUp Quantification Methods

A mean separation for the GeneUp Quantification methods, GeneUp 0 Hours of incubation and GeneUp 4 Hours of incubation, was conducted per sampling method for Cocktail 1. Table 10 indicates for both methods that there were no significant differences between Mitt and Rinsate and between Swab and Rinsate, and significant differences between Swab and Mitt. However, there was a higher quantification of CFU for GeneUp 0 hours of incubations for all three sampling methods (Rinsate, Swab and Mitt).

Table 10

Mean separation for Cocktail 1, comparing GeneUp Quantification Methods per Sampling Method.

Quantification Method	Sampling Method	Mean ± SD LogCFU/tender
GeneUp0H	Rinsate	3.60±0.24 [ab]
GeneUp0H	Swab	3.35±0.29 [b]
GeneUp0H	Mitt	3.94±0.0.32 [a]
GeneUp4H	Rinsate	3.22±0.30 [bc]
GeneUp4H	Swab	2.88±0.20 [c]
GeneUp4H	Mitt	3.33±0.26 [b]
C.V (%)		11.36

Note. Different letters placed vertically [abcd] indicate significant differences ($p \leq 0.05$) between quantification methods, while the same letter placed vertically [abcd] indicates no significant differences ($p \geq 0.05$), C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter.

A mean separation for the GeneUp Quantification methods, GeneUp 0 Hours of incubation and GeneUp 4 Hours of incubation, was conducted per sampling method for Cocktail 2. As observed in Table 11, on both methods, there were no significant differences in any of the sampling methods tested. However, GeneUp 4 hours of incubation reported higher quantification for two out of the three sampling methods (Rinsate and Mitt).

Table 11

Mean separation for Cocktail 2, comparing GeneUp Quantification Methods per Sampling Method.

Quantification Method	Sampling Method	Mean ± SD LogCFU/tender
GeneUp0H	Rinsate	3.96±0.46 [a]
GeneUp0H	Swab	3.93±0.31 [a]
GeneUp0H	Mitt	3.93±0.13 [a]
GeneUp4H	Rinsate	4.01±0.27 [a]
GeneUp4H	Swab	3.91±0.23 [a]
GeneUp4H	Mitt	4.17±0.46 [a]
C.V (%)		7.64

Note: Different letters placed vertically [abcd] indicate significant differences ($p \leq 0.05$) between quantification methods, while the same letter placed vertically [abcd] indicates no significant differences ($p \geq 0.05$), C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter.

A mean separation for the GeneUp Quantification methods, GeneUp 0 Hours of incubation and GeneUp 4 Hours of incubation, was conducted per sampling method for Cocktail 3. As observed in Table 12, for both methods, there were no significant differences between Rinsate and Swab, also inside GeneUp 0 Hours of incubation the results do not show significant differences between the three methods. However, there were significant differences in GeneUp 4 hours of incubation, since Rinsate was statistically different from all the evaluated methods with GeneUp 0 hours of incubation and inside GeneUp 4 hours was different to Mitt methodology. Furthermore, for GeneUp-4 hours of incubation, a higher quantification of the CFU microorganism was achieved.

Table 12

Mean separation for Cocktail 3, comparing GeneUp Quantification Methods per Sampling Method.

Quantification Method	Sampling Method	Mean ± SD LogCFU/tender
GeneUp0H	Rinsate	4.61±0.09 ^{c}
GeneUp0H	Swab	4.67±0.31 ^{c}
GeneUp0H	Mitt	4.87±0.54 ^{bc}
GeneUp4H	Rinsate	5.55 ±0.50 ^{a}
GeneUp4H	Swab	5.42±0.53 ^{ab}
GeneUp4H	Mitt	4.56±0.62 ^{c}
C.V (%)		11.43

Note: Different letters placed vertically ^{abcd} indicate significant differences ($p \leq 0.05$) between quantification methods, while the same letter placed vertically ^{abcd} indicates no significant differences ($p \geq 0.05$), C.V (%) Coefficient of Variation, SD Standard Deviation, LogCFU/tender logarithm colony form unit per milliliter.

A mean separation for the GeneUp Quantification methods, GeneUp 0 Hours of incubation and GeneUp 4 Hours of incubation, was conducted per sampling method for Cocktail 4. However, there was no data for GeneUp 0 Hours of incubation reported due to technical difficulties of the machine, there could not be a comparison between both incubations of GeneUp for this cocktail.

As the concentration of *Salmonella* decreases for the GeneUp 0 Hours and GeneUp 4 Hours methods, an increase in the variability of the results is observed across the three sampling techniques (Rinsate, Mitt, and Swab). This indicates that at lower concentrations, the precision and consistency

quantification methods decrease. At higher concentrations, the results are more repeatable, while at lower levels, greater fluctuations are seen, affecting the accurate detection and quantification of the bacteria. This underscores the importance of considering the variability at low concentrations when evaluating the effectiveness of these methods.

Sampling Methods

During this study, MicroTally Mitt demonstrated strong potential because it presented a similar performance to the Rinsate method for *Salmonella* sampled on chicken tenders, showing comparable or higher quantifications in multiple scenarios. Its consistent performance suggests that it may be a viable alternative in both GeneUp and plating methods. The MicroTally Mitt has been already proven to be an alternative sampling method in the Beef industry, a study conducted to validate a new method of sampling for beef using a Mitt approach, showed there were no significant differences from previous methods used for pathogen detection in this industry (Arthur & Wheeler, 2021). Also, previous research made by (Fremonta, 2024), demonstrated that MicroTally Mitt samplers perform comparably to standard Rinsate sampling for chicken wing and chicken thighs, proving it can be an effective method for sampling poultry.

MicroTally Swab showed less consistent results, with some scenarios where it matched or outperformed Rinsate. Previous studies have found that the MicroTally Swab is effective for pathogen sampling in beef processing. A study based on this showed that the Microtally Swab provides reliable results comparable to traditional methods and is effective even when multiple samples are taken from the same batch (Arthur et al., 2024). It is mentioned that it offers flexible sampling protocols, such as covering less than the entire surface or sampling for shorter times, while still meeting food safety criteria.

Traditional Plating Quantification Methods

Overall, Spiral Plating showed higher bacterial quantifications at higher concentrations (Cocktails 3 and 4), across the sampling methods. A study conducted comparing Spiral to Pour plating, suggested that it is as effective and even better than the conventional pour plate technique, and indicated that the Spiral plating method generally provides higher bacterial counts compared to the pour plate method. The study concluded this method required less time and materials compared to the conventional pour plate procedure (Gilchrist et al., 1973). Another research shows that Spiral Plating Method may be helpful not only directly for food and water but to quantify pathogens in animal feces, such as *Escherichia coli* (Wessels et al., 2021).

In contrast, Spread Plating had higher bacteria quantification at lower concentrations (Cocktails 1 and 2), particularly for Rinsate in this case. Spread Plating has demonstrated its performance in different studies. For example, a study conducted to determine a more accurate method for assessing bacterial counts, comparing the pour plate with the spread plate method for enumerating bacteria in water samples, concluded that the spread method is recommended for more accurate and precise bacterial enumeration in water samples (Taylor et al., 1983).

GeneUp PCR Quantification Methods

GeneUp 0 Hours of Incubation generally showed higher bacterial quantifications at lower concentrations, across all sampling methods for Cocktail 1. However, GeneUp 4 Hours of Incubation showed higher bacterial quantifications at higher concentrations (Cocktail 2 and 3), overall. Data for Cocktail 4 was incomplete due to technical difficulties, preventing a full comparison between incubation times for high concentrations.

A study that compared agars with the GeneUp Method for *Salmonella* quantification in raw ground beef using three concentration levels, showed no significant differences between the methods

(Johnson et al., 2021), however, during this study, there was a separated analysis between traditional and molecular methods. Research has demonstrated the importance of excluding dead cells for an accurate molecular analysis, they mention the failure to limit analysis to viable bacterial cells represent a problem leading to false-positive results in clinical samples (Rogers et al., 2010). This explains the higher CFU counts in the GeneUp quantification methods, which differ from the traditional plating methods which only consider viable cells.

In a study made using PCR for the detection of specific *Salmonella* contamination levels in poultry Rinsate, *Salmonella* strains were used to inoculate at four different concentrations, concluded that PCR accurately detected all concentrations inoculated (Velez et al., 2024). However, during this study, GeneUp 0 Hours of Incubation yielded higher bacterial quantifications at the lowest concentration tested, while GeneUp 4 Hours of Incubation had higher quantifications as the concentrations increased, demonstrating its advantage in detecting Salmonella at elevated levels.

Conclusions

The study concluded that MicroTally® Mitt was the method more comparable with Rinsate (reference method), which consistently yielded higher quantifications. While MicroTally® Swab performed similar behavior, and its results showed more variability. This allows the industry to be open to these new options, allowing for greater convenience when taking samples (less time consumption, quicker results, less expenses).

Spread Plating had higher bacteria quantifications at the lowest concentration tested. In contrast, Spiral Plating showed higher bacterial quantifications as the concentrations increased across the sampling methods.

GeneUp 0 Hours of Incubation yielded higher bacterial quantifications at the lowest concentration tested, while GeneUp 4 Hours of Incubation yielded higher quantifications as the concentrations increased.

As the concentration of *Salmonella* decreases, greater variability and reduced consistency were observed across all sampling methods (Rinsate, Swab, Mitt) and quantification techniques (Spiral Plating, Spread Plating, GeneUp 0-Hour Incubation, GeneUp 4-Hour Incubation). This trend highlights the challenges in detecting and quantifying low bacterial levels. Higher concentrations yielded more accurate and consistent results, unlike lower concentrations. Evaluating methods at different concentrations is crucial to understanding their limitations.

Recommendations

Extend the study to include different chicken cuts (e.g., thighs, wings) or other poultry products to assess if the performance of sampling and quantification methods varies by product type.

Repeat the study with different cocktails, using different concentrations of *Salmonella* (lower), to evaluate the consistency of the results presented during this study.

Repeat Cocktail 4 for the GeneUp 0- Hour incubation, to make the comparison at that concentration with the GeneUp 4-Hour Incubation.

References

Adria food expertise. (2022). *Validation of alternative analytical methods application in food microbiology production environmental samples and for primary production samples (excluding drinking water) qualitative method.* http://www.adria.tm.fr

Alnajrani, M. (2017). *Comparison of swabbing, rinsing, and grinding as sampling methods for the recovery of indicator microorganisms on beef Trimming*. https://ttu-ir.tdl.org/server/api/core/bitstreams/7b129138-f0ea-4576-927e-0271da2476e8/content

Arthur, T. M., Reno, F. J., & Wheeler, T. L. (2024). Validation of a New Method of Sampling Beef Manufacturing Trimmings for Pathogen Testing Using a Manual Sampling Mitt Approach. *Journal of Food Protection*, *87*(3), 100233. https://doi.org/10.1016/J.JFP.2024.100233

Arthur, T. M., & Wheeler, T. L. (2021). Validation of Additional Approaches and Applications for Using the Continuous and Manual Sampling Devices for Raw Beef Trim. *Journal of Food Protection*, *84*(4), 536–544. https://doi.org/10.4315/JFP-20-345

Awang, M. S., Bustami, Y., Hamzah, H. H., Zambry, N. S., Najib, M. A., Khalid, M. F., Aziah, I., & Abd Manaf, A. (2021). Advancement in Salmonella Detection Methods: From Conventional to Electrochemical-Based Sensing Detection. *Biosensors*, *11*(9). https://doi.org/10.3390/BIOS11090346

Ehuwa, O., Jaiswal, A. K., & Jaiswal, S. (2021). Salmonella, Food Safety and Food Handling Practices. *Foods (Basel, Switzerland)*, *10*(5). https://doi.org/10.3390/FOODS10050907

Elika. (2024). *Salmonella*. https://seguridadalimentaria.elika.eus/fichas-de-peligros/salmonella/

Font-I-Furnols, M. (2023). Meat Consumption, Sustainability and Alternatives: An Overview of Motives and Barriers. *Foods (Basel, Switzerland)*, *12*(11). https://doi.org/10.3390/FOODS12112144

Fremonta. (2023). *Sampling methods microtally*. https://MicroTally.com/sampling-methods/

Fremonta. (2024). *Sampling poultry using microtally mitt samplers*. https://MicroTally®.com/wp-content/uploads/2024/08/TechBulletin_Mitt_Poultry_VS-Rinse_FINAL.pdf

Gilchrist, J. E., Campbell, J. E., Donnelly, C. B., Peeler, J. T., & Delaney, J. M. (1973). Spiral plate method for bacterial determination. *Applied Microbiology*, *25*(2), 244–252. https://doi.org/10.1128/am.25.2.244-252.1973

He, Y., Wang, J., Zhang, R., Chen, L., Zhang, H., Qi, X., & Chen, J. (2023). Epidemiology of foodborne diseases caused by Salmonella in Zhejiang Province, China, between 2010 and 2021. *Frontiers in Public Health*, *11*, 1127925. https://doi.org/10.3389/FPUBH.2023.1127925

Interscience. (2018). *Why choose the spiral plating method?* https://www.rapidmicrobiology.com/news/why-choose-the-spiral-plating-method

Johnson, R. L., Mills, J. C., Taylor, N. J., & Bird, P. M. (2021). Evaluation of the GENE-UP®Salmonella Method for the Detection of Salmonella Species in a Broad Range of Foods and Select

Environmental Surfaces: Collaborative Study, First Action 2020.02. *Journal of AOAC International*, *104*(4), 1084–1097. https://doi.org/10.1093/jaoacint/qsab005

Logue, C. M., Cesare, A. de, Tast-Lahti, E., Chemaly, M., Payen, C., LeJeune, J., & Zhou, K. (2024). Salmonella spp. In poultry production-A review of the role of interventions along the production continuum. *Advances in Food and Nutrition Research*, *108*, 289–341. https://doi.org/10.1016/BS.AFNR.2023.11.001

Rogers, G. B., Marsh, P., Stressmann, A. F., Allen, C. E., Daniels, T. V. W., Carroll, M. P., & Bruce, K. D. (2010). The exclusion of dead bacterial cells is essential for accurate molecular analysis of clinical samples. *Clinical Microbiology and Infection : The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases*, *16*(11), 1656–1658. https://doi.org/10.1111/j.1469-0691.2010.03189.x

Shaji, S., Selvaraj, R. K., & Shanmugasundaram, R. (2023). Salmonella Infection in Poultry: A Review on the Pathogen and Control Strategies. *Microorganisms*, *11*(11). https://doi.org/10.3390/MICROORGANISMS11112814

Tack, D. M., Ray, L., Griffin, P. M., Cieslak, P. R., Dunn, J., Rissman, T., Jervis, R., Lathrop, S., Muse, A., Duwell, M., Smith, K., Tobin-D'Angelo, M., Vugia, D. J., Zablotsky Kufel, J., Wolpert, B. J., Tauxe, R., & Payne, D. C. (2020). Preliminary Incidence and Trends of Infections with Pathogens Transmitted Commonly Through Food - Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 2016-2019. *MMWR. Morbidity and Mortality Weekly Report*, *69*(17), 509–514. https://doi.org/10.15585/MMWR.MM6917A1

Taylor, R. H., Allen, M. J., & Geldreich, E. E. (1983). Standard plate count: A comparison of pour plate and spread plate methods. *Journal AWWA*, *75*(1), 35–37. https://doi.org/10.1002/j.1551-8833.1983.tb05055.x

Velez, F. J., Kandula, N., Blech-Hermoni, Y., Jackson, C. R., Bosilevac, J. M., & Singh, P. (2024). Digital PCR assay for the specific detection and estimation of Salmonella contamination levels in poultry rinse. *Current Research in Food Science*, *9*, 100807. https://doi.org/10.1016/j.crfs.2024.100807

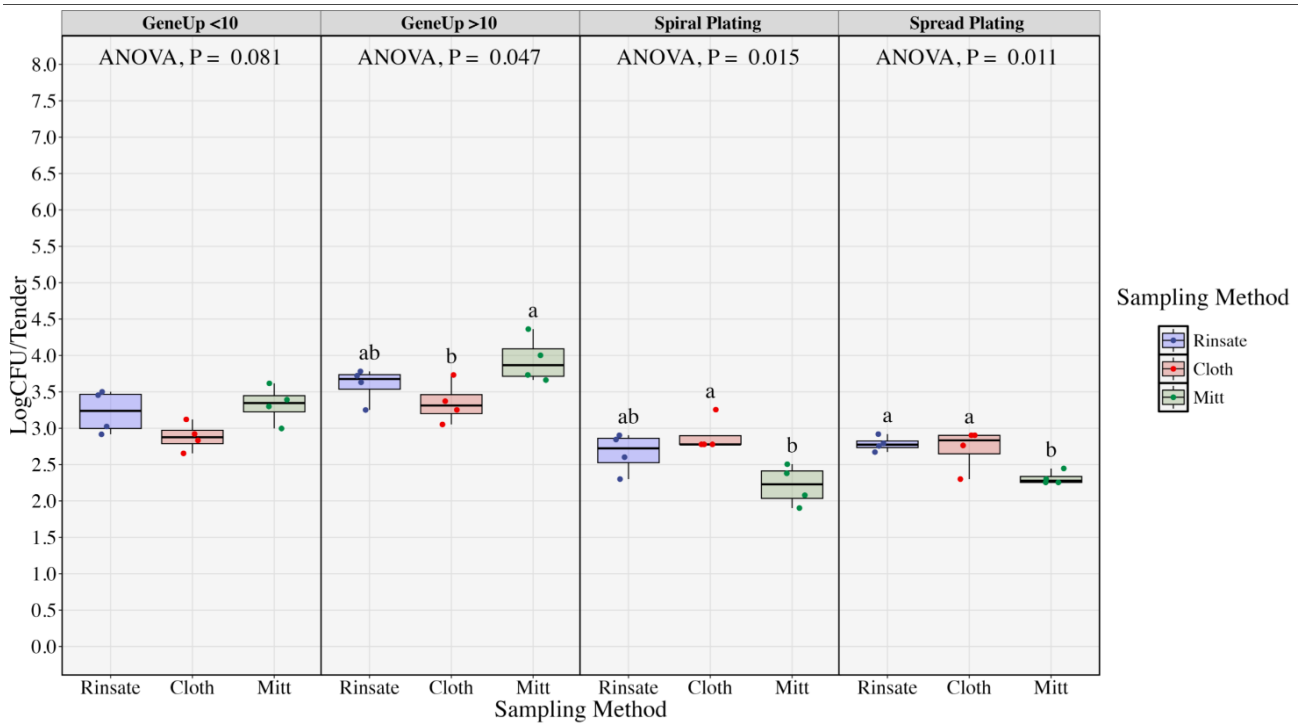
Wessels, K., Rip, D., & Gouws, P. (2021). Salmonella in Chicken Meat: Consumption, Outbreaks, Characteristics, Current Control Methods and the Potential of Bacteriophage Use. *Foods (Basel, Switzerland)*, *10*(8). https://doi.org/10.3390/FOODS10081742

Whyte, P., Mc Gill, K., Collins, J. D., & Gormley, E. (2002). The prevalence and PCR detection of Salmonella contamination in raw poultry. *Veterinary Microbiology*, *89*(1), 53–60. https://doi.org/10.1016/S0378-1135(02)00160-8

Appendices

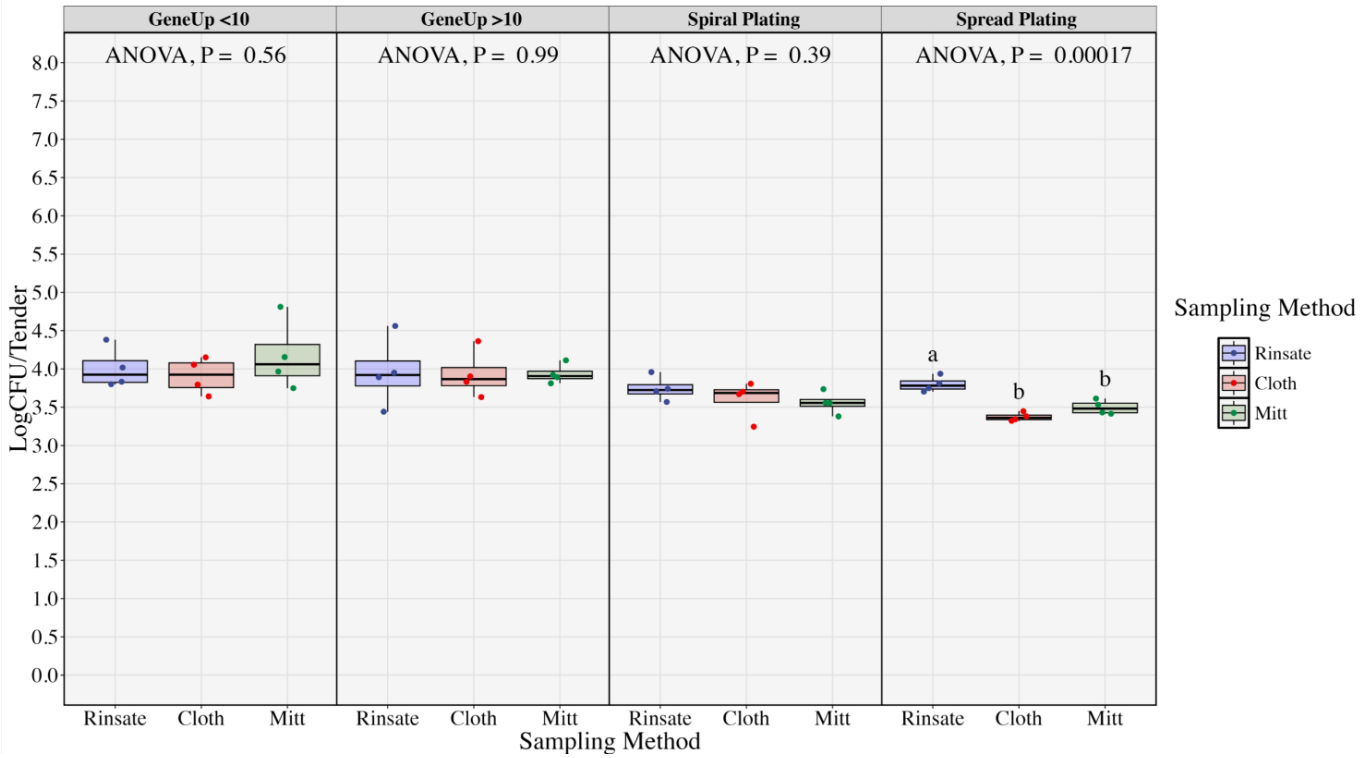
Appendix A

Box Plot of Sampling Methodologies per Quantification Methods for Cocktail 1



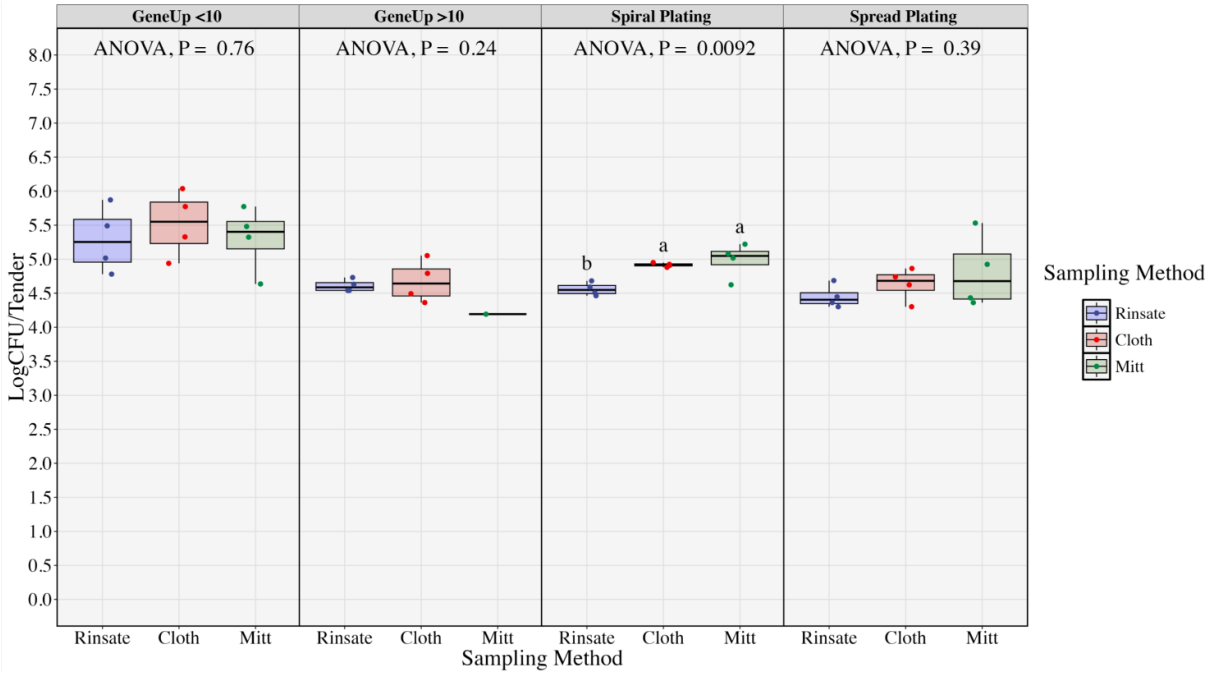
Appendix B

Box Plot of Sampling Methodologies per Quantification Methods for Cocktail 2.



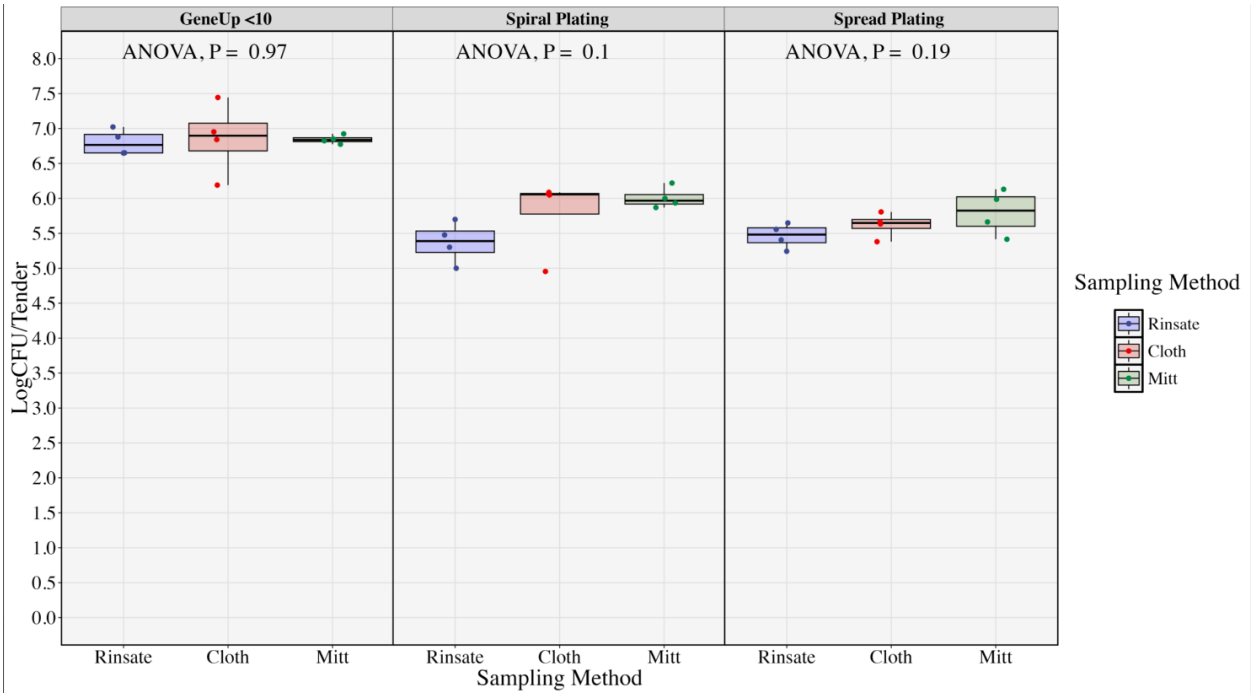
Appendix C

Box Plot of Sampling Methodologies per Quantification Methods for Cocktail 3.



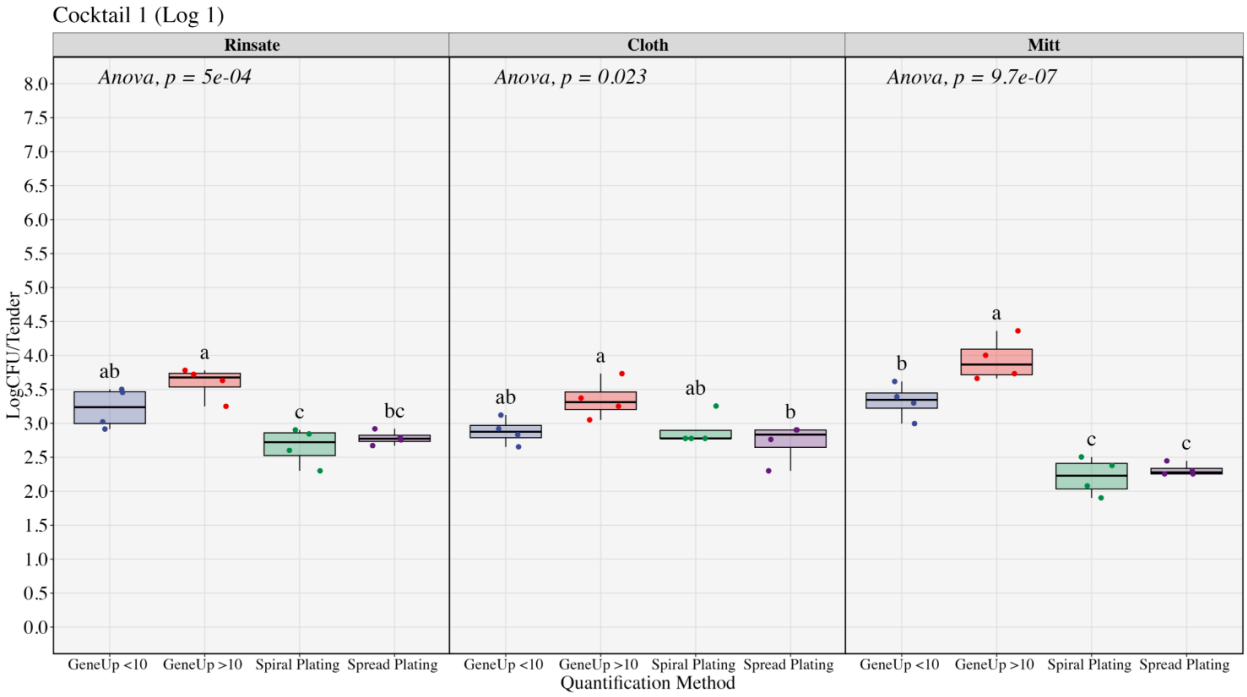
Appendix D

Box Plot of Sampling Methodologies per Quantification Methods for Cocktail 4.



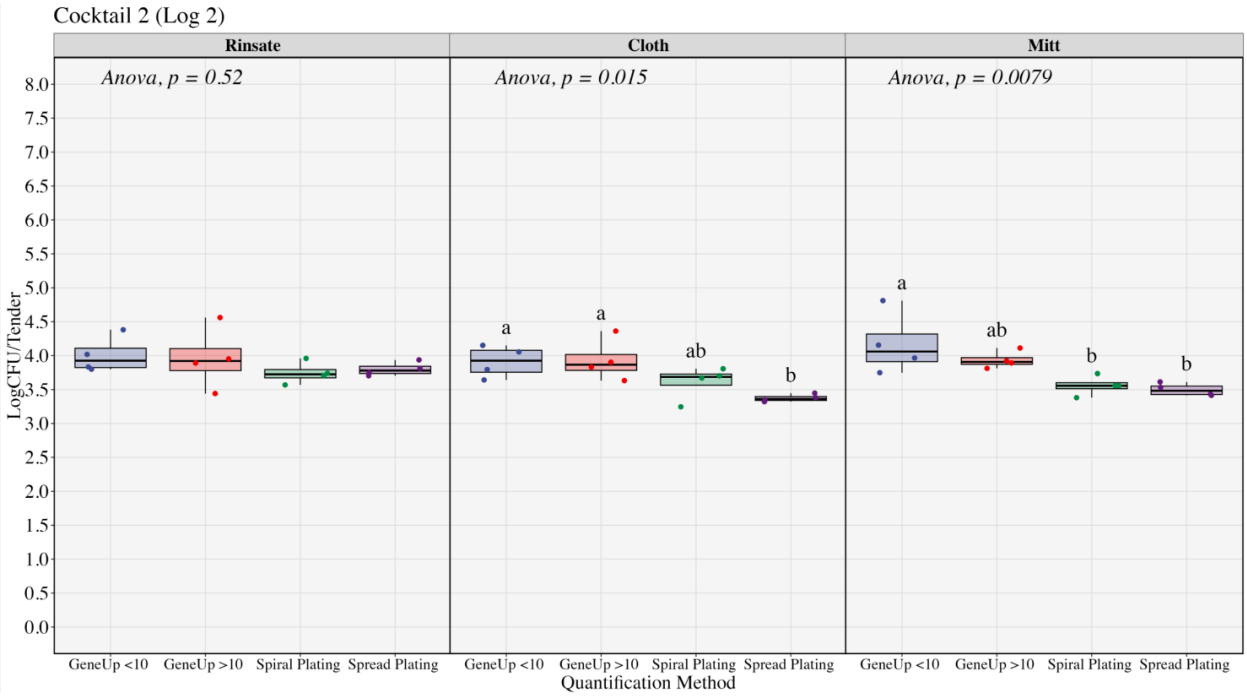
Appendix E

Box Plot of Quantification Methodologies per Sampling Methods for Cocktail 1.



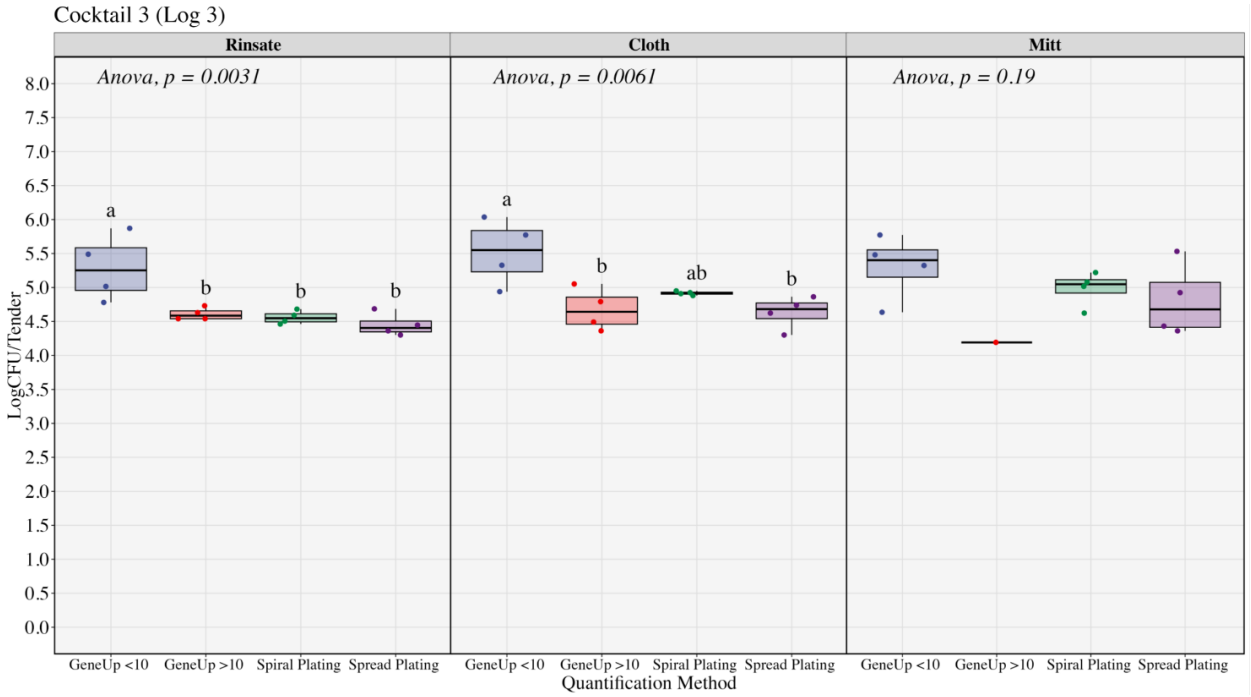
Appendix F

Box Plot of Quantification Methodologies per Sampling Methods for Cocktail 2.



Appendix G

Box Plot of Quantification Methodologies per Sampling Methods for Cocktail 3.



Appendix H

Box Plot of Quantification Methodologies per Sampling Methods for Cocktail 4.