COMPARISON OF RECOVERY AND ENUMERATION OF *ESCHERICHIA COLI*, *CRONOBACTER* SPECIES, COLIFORMS, AND *SALMONELLA* TYPHIMURIUM IN GROUND BEEF AND GROUND TURKEY USING CONVENTIONAL METHODS AND A NEW CHROMOGENIC MEDIUM, ECA CHECK® EASYGEL® PLUS

by

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Abstract

ECA Check® Easygel® Plus (ECA) is a pectin-based gelling system that reacts with calcium ions bound to a pre-treated Petri dish, eliminating autoclaving prior to use. It can chromogenically and/or fluorogenically distinguish three organisms: *Escherichia coli, Salmonella* spp., and coliforms. This study compared the recovery of these organisms to conventional media using stock culture, inoculated, and non-inoculated ground beef and ground turkey. ECA was compared to Violet Red Bile Agar (VRB), Violet Red Bile Agar with 4-methylumbelliferyl-β-D-glucuronide (VRB-MUG), Xylose Lysine Desoxycholate Agar (XLD), *Escherichia coli*/Coliform (ECC) Count Plate Petrifilm™, and Tryptic Soy Agar (TSA). The stock culture recovery of *Salmonella* Typhimurium for ECA, TSA, and XLD were 8.62, 8.69, and 6.82 log CFU/ml, respectively. There was very little difference between the media in the recovery of *Escherichia coli* and *Cronobacter* spp., formerly referred to as *Enterobacter sakazakii.* Mean counts of presumptive *E. coli* in ground beef were 7.24 and 7.41 logs for ECA and VRB-MUG. Total coliform mean counts were 7.43, 7.63, and 7.37 logs for ECA, Petrifilm™, and VRB. Presumptive *Salmonella* means were 6.68 and 6.21 logs on ECA and XLD, while total aerobic counts were 7.84 and 6.51 logs on ECA and TSA. At 6.72 logs, ECA recovered considerably more *Salmonella* than XLD (5.71 logs) from the inoculated ground turkey; ECA recovered 7.62 logs total aerobic count which was significantly more than TSA at 6.89 logs. Total counts for both non-inoculated ground meats resulted in significant differences between TSA recovery and all other media. ECA also recovered significantly more than Petrifilm™ from both non-inoculated foods. The randomly selected organisms recovered from ECA were identified using BBL™ Crystal™ Enteric/Nonfermenter ID or Gram-Positive kits,

and correlated precisely to the chromogenic reaction of the colonies. ECA Check® Easygel® was efficient, less labor-intensive, comparable to, and, in some instances, better than conventional media at recovering target organisms.

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Dedication

I would like to dedicate this work to my husband, Jason Jay Boren. You have been my rock, my shoulder, my soul, my saving grace, my devil's advocate, and my one true friend. We couldn't have made it this far alone. I look forward to forever with you. Thank you.

CHAPTER 1 - LITERATURE REVIEW

1.1 Introduction

The need for a simpler and more rapid method to determine the presence of harmful microorganisms and indicators of contamination, such as *Escherichia coli, Salmonella* spp., and coliforms, has led to the increased development and use of chromogenic media as a comparable alternative to conventional methods. Poor specificity in conventional media and using plating techniques that may be harmful to targeted cells may result in false positives or decrease actual counts during recovery (Manafi, 2000, Fung and Chain, 1991). Micrology Laboratories, LLC, developed a medium that is capable of detecting *E. coli*, coliforms, and certain *Salmonella* spp. within a single plate called ECA Check® Easygel® Plus. The technology for the Easygel® uses low methoxyl pectins in lieu of marine algae agar, which react with calcium ions bonded to pre-treated Petri dishes to form a gel. The ability to detect and differentiate several microorganisms using a single medium, requiring no autoclaving and tempering, would significantly increase laboratory efficiency.

In the 1970's Dr. Jonathan Roth of Micrology Labs, began working on a temperature-independent agar substitute, eliminating the need for preparing and tempering agar. Originally known as Redigel®, this medium provided a more "field-friendly" alternative due to the ability to place a desired test sample into the pectin-based nutrient liquid bottle before pouring it into the calcium ion-treated Petri dishes. By adding chromogenic and fluorogenic enzyme substrates to the nutrient liquid, a microorganism can be differentiated by the specific enzymes it produces. Nearly all general coliform members of the family Enterobacteriaceae produce both α-

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galactosidase and β-galactosidase but are negative for β-glucuronidase production. However, more than 95% of *E. coli* strains produce both forms of galactosidase and β-glucuronidase. The β-glucuronidase reaction with certain fluorogenic substrates cause *E. coli* colonies to fluoresce under long wave ultraviolet light (366 nm; De Beaumont *et al*., 2006). Some *Salmonella* spp. can produce α-galactosidase, which results in teal green colonies on the Coliscan® medium, a predecessor of ECA Check® Easygel® Plus. However, when the chromogenic enzyme substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt (X-GLUC), was incorporated into solid media they resulted only in blue colored colonies. A contrasting dye, 6-chloro-3-indolyl-β-D-galactopyranoside (Red-Gal®), was developed and was combined with X-GLUC in a nutrient medium. It was confirmed that coliforms grew as pink/red colonies and *E. coli* grew as blue/purple colonies that were easily distinguishable from each other. Using multiple enzyme substrate dyes ECA Check® Easygel® Plus was developed with the capability of differentiating *E. coli*, certain *Salmonella* spp., and coliforms with one medium.

The objective of this research was to compare the ECA Check® Easygel® Plus to conventional methods for detection, recovery, and identification of *E. coli*, coliforms, *Cronobacter* spp. (formerly *Enterobacter sakazakii*), and *Salmonella enterica* subsp. *enterica* ser. Typhimurium, using pure cultures and inoculated raw ground beef and ground turkey. This study also compares the same media for recovery of natural micro flora in non-inoculated ground beef and ground turkey.

1.2 Pour Plate Technique

Since the contribution of agar-agar by Fanny Hesse and Robert Koch to solid, nutritive culture media in the late 1800's, the pour plate technique has been a staple for bacteriologists for more than a century (Hitchens and Leikind, 1939; Mortimer, 2001). The flexible characteristics of agar include solubility in water at boiling temperatures, gelatinization at temperatures below 43°C, and a melting temperature of ≥85°C (Armisen and Galatas, 1987). The AOAC™ International (AOAC) and the Bacteriological Analytical Manual (BAM) have approved pour plating as the standard recovery procedure for many years based on several studies that have compared pour plating to other plating methods. The basic procedure involves serial dilutions, plating 1.0 ml onto a sterile Petri dish, and pouring tempered (<48°C) selective or non-selective media into the Petri dish, swirling for proper distribution, and allowing the medium to solidify before incubation. An evaluation performed in 2005 used BioBall™ to standardize the amount of inoculum used to compare membrane filtration, most probable number (MPN), standard plate count (SPC) pour and spread plating, Colilert®, Colisure®, and 3M™ Petrifilm™ (Wohlsen *et al.,* 2006). It was determined that the SPC pour plate methodology yielded slightly higher recoveries than spread plating. However, much earlier comparisons of spread and pour plating did result in 70 to 80% higher counts on spread plates using samples of chicken incubated at ≤25°C (Clark, 1967).

Although pour plating has been the most frequently used and approved method for enumerating microbes, it requires a significant amount of time and labor (Ferrati *et al.*, 2005; Maturin and Peeler, 2001). Scientists have continually searched for ways to reduce time and effort without sacrificing quality. The evolution of plating technologies gave rise to an onslaught of evaluations and comparisons to the "tried-and-true" procedure. One comparison by Ferrati *et*

al. (2005) compared more rapid, "ready-to-use systems" (Petrifilm™ and SimPlate® plates) to the conventional pour plate technique for the enumeration of microbial loads in acidic fruit juices. The alternative methods did not require sterilization or media preparation. The pour plate method showed an "excellent" correlation coefficient ($r = 0.9638$) compared to the "good" r values of the SimPlate® total plate count plates and Petrifilm™ Aerobic Count plates ($r = 0.8970$ and $r = 0.8822$, respectively). In most studies pour plating did not often result in the lowest counts achieved and usually resulted in similar or higher sensitivities (Hoben and Somasegaren, 1982; Schmelder *et al.*, 2000). Other methods have been developed to accelerate plating processes including spot plating, spiral plating, gas chromatography, automatic dispensers and diluters, and enzymatic reactions (Gilchrist *et al.,* 1973). Gilchrist *et al.* (1973) compared spiral and pour plating methods, which concluded that the spiral plate method obtained, on average, 14-17% higher counts than pour plating.

Evaluations have also been performed using a combination of spread and pour plating, or overlays. A particular study conducted on coliforms in frozen foods used the spread plate method on Trypticase Soy Agar (TSA) and Violet Red Bile agar (VRB) followed with an overlay of VRB versus pour plating with VRB (Speck *et al.,* 1975). The results showed a drastic increase in counts, from 2.6 log CFU/ml using the pour plate method, to 8.8 and 10.3 log CFU/ml on VRB and TSA, respectively, overlaid with VRB. This method of detection was adopted for coliforms by the BAM, using just the pour plate method or, if resuscitation of injured cells is required, with an initial layer of TSA with a VRB overlay (Feng *et al.*, 2002).

When properly practiced, pour plating has often produced comparable results to newer plating methods. Unfortunately, a lack of training for laboratory technicians and the increasing call for quicker turnarounds, especially with regard to food safety, may result in inaccurate

bacterial counts. If molten agar is poured too hot (>48°C) it can significantly lower actual microbial loads that may be heat sensitive, and may produce life threatening mistakes (Fung and Chain, 1991). Newer advances in pour plating technology have led to the use of prepared and individually bottled nutrient liquids that contain low methoxyl pectin that are poured into sterile Petri dishes pre-treated with calcium ions, such as Easygel. This notably reduces the time and labor required for conventional pour plating by eliminating boiling and autoclave time necessary for preparation, and also provides the ability to take the bottles into the field, utilizing them as transport containers for samples.

1.3 Selective and Differential Media

1.3.1 Xylose Lysine Desoxycholate Agar

Xylose Lysine Desoxycholate agar (XLD) is a more selective version of Xylose Lysine agar, with the addition of sodium thiosulfate and ferric ammonium citrate which allow visualization of hydrogen sulfide production, and sodium desoxycholate to inhibit Gram-positive bacteria (Difco™ and BBL™, 2009). It was initially made to detect *Shigella* and *Providencia* spp., but has been used extensively for the detection of *Salmonella* spp., specifically *Salmonella enterica* subsp. *enterica* ser. Typhimurium, due to the distinctive black colonies it produces from hydrogen sulfide production. *Escherichia coli* can also be detected on the medium, envisaged as yellow colonies. Though its growth is "partially inhibited" on XLD, *E. coli* will usually outgrow *S.* Typhimurium when recommended *Salmonella* enrichment procedures are not followed.

Some problems identified with using XLD agar stem from the differentiating characteristic of H_2S production. Many factors have been identified to interfere with this

unreliable detection attribute: pH of the medium, the amount of iron in the media, bacterial oxygen concentration, and the extent that H_2S is produced by colonies (Rambach, 1990). H_2S-producing *Citrobacter freundii*, lactose-negative, sulfide-positive *Proteus*, *Enterobacter taylorae, Enterobacter agglomerans, Morganella,* and *Pseudomonas fluorescens* have been shown to produce false positives on XLD and cannot be easily discerned from certain strains of *Salmonella* (Schonenbrucher *et al.,* 2008; Rambach, 1990; Bennett *et al.,* 1999). One solution to differentiate *Citrobacter* from *Salmonella* used a pyrrolidonyl peptidase (PYRase) test. All *Citrobacter* produces this enzyme, while most strains (99.6%) of *Salmonella* do not (Bennett *et al.,* 1999). Many studies have compared newer media, including chromogenic media and new formulations such as xylose lysine Tergitol 4 and Miller-Mallinson agar, to more conventional means (e.g. XLD), and found them superior (Mallinson *et al.,* 2000; Maddocks *et al.,* 2002). In 1995, Sherrod *et al.* conducted a comparison of several new selective media for the detection of *Salmonella* to the BAM-recommended agars (bismuth sulfite, Hektoen enteric and XLD). Based on Sherrod's data, the Bacteriological Analytical Manual decided that there was "no advantage in replacing any of the BAM-recommended agars with one or more of the newer agars" (Andrews and Hammack, 2007).

1.3.2 Violet Red Bile Agar

In addition to plating techniques, media differentiation and selectivity has also been a focus for more rapid detection of specific microorganisms. Several inhibitory ingredients and differential dyes were tested in many combinations in the early 20th century. Early comparisons of neutral red bile agar, violet red bile agar, modified Eijkman medium and "medium four" were compared for detection of coliforms in dairy products (Bartram and Black, 1936). Violet Red Bile agar (VRB) was found as one of the more "satisfactory" solid media compared. Today,

VRB contains Bile Salts No. 3 (replacing bile salts) and Crystal Violet to inhibit Gram-positive bacteria, lactose as a source of carbohydrate, and neutral red as the pH indicator (Difco and BBL, 2009). This media is approved for the enumeration of coliforms, including *Escherichia coli*, which turn pink or red from lactose consumption and the neutral red indicator (Feng *et al.*, 2002). A more recent analysis in 1995 in New South Wales comparing the Australian Standard method to a more rapid method stated that "only red colonies with halos should be recorded as presumptive coliforms" (Bloch *et al.,* 1996). This study concluded that all red colonies growing on VRB produced gas and were presumed to be coliforms, and that there was no significant difference between MPN and VRB for coliform enumeration. VRB is the recommended solid medium method for detection of *E. coli* and coliforms in the Bacteriological Analytical Manual.

Dehydrated VRB has been incorporated into a more rapid detection method called *E. coli*/ Coliform Count Petrifilm™ VRB is versatile in its ability to accommodate additives or substitutes for more specificity. Glucose can be used in lieu of lactose as a source of carbohydrate to select for non-lactose-fermenting species of *E. coli, Salmonella,* and *Shigella* (Difco, 2009)*.* The substrate 4-methyl-umbelliferyl-β-D-glucuronide (MUG) may be added for fluorescent differentiation of *E. coli.*

1.3.3 3M™ Petrifilm™

3M™ Petrifilm™ has been a staple in testing for microorganisms in several different food matrices since 1983. Specific types of Petrifilm™ have been manufactured for more targeted applications, including Aerobic Count, Enterobacteriaceae Count, Staph Express Count, Coliform, High Sensitivity Coliform and Rapid Coliform Count, *E. coli*/coliform Count, and Yeast and Mold Count Plates. Petrifilm™ has been included in the AOAC™ *Official Methods of Analysis* as a validated alternative to conventional enumeration and recovery (AOAC, 1995).

A prior comparison of Petrifilm™ performed by Chain and Fung (1991) showed extremely high correlations to other methods including aerobic plate count (APC), Easygel® (formerly known as Redigel®), spiral plating, and Isogrid system in ground meat, chicken and raw milk samples. The correlation dropped, however, when testing spices, nuts, and flour. A subsequent study in Córdoba, Spain in 1994 evidenced much lower correlations when matching Petrifilm™ to conventional plating with no significant differences (Jordano *et al.,* 1995). When evaluated against pour plating in 1996, *E. coli*/coliform (ECC) Petrifilm™ showed superior recovery from fresh and frozen meat samples, similar results in coliform recuperation, and a slight disadvantage in aerobic bacteria recovery (Linton *et al.*, 1997). It has also been found that, in samples containing exceptionally high coliform and natural micro flora populations, the performance of Petrifilm™ decreases due to colony crowding (Townsend *et al.,* 1998).

Along with being an extremely comparable alternative to conventional plating, Petrifilm™ has many advantages. By dehydrating VRB agar and using β-glucuronidase and metabolic indicators, ECC Petrifilm™ is a condensed version of the Petri dish (Schraft and Watterworth, 2005). A film covering allows for gas production from target coliform colonies, eliminating further confirmation steps using Durham tubes. Due to its compact design, ECC Petrifilm™ takes up significantly less space during incubation than traditional Petri dishes. ECC Petrifilm™ has also been found to have elevated specificity when compared to confirmed counts of *E. coli* and fecal coliforms (Schraft and Watterworth, 2005).

Studies have also revealed some disadvantages to the Petrifilm™ method. Sample spreading has been an area of concern when off-centered sample application and excessive pressure causes the sample to disperse outside the allotted area, resulting in inaccurate counts from technician errors (Chain and Fung, 1991). Care must be taken when transporting

Petrifilm™ so that the sample area is not disturbed, possibly producing false positives for gas production or decreasing the countable field by displacement of the medium. Higher incubation times may be necessary for comparable counts to the MPN process (Beuchat *et al.,* 1998). Confirmed *E. coli* colonies are blue associated with gas bubbles; however, one study confirmed *E. coli* using a rapid method kit on blue colonies that did not produce gas (Bloch *et al.,* 1996). The necessity to distinguish fecal coliforms from other strains of *E. coli* is pertinent to determining the quality and possible contaminants in drinking water. Schraft and Watterworth (2005) found that almost 30% of atypical colonies on ECC Petrifilm™ were actually confirmed fecal coliforms. With some types of *E. coli* that are β-glucuronidase negative (approximately 22% in assorted foodstuffs and human isolates) and several anaerogenic varieties, false negatives are possible (Schraft and Watterworth, 2005).

Although there are some disadvantages to Petrifilm™, it has been proven to be comparable to conventional plating methods, accurately differentiating between microorganisms and providing precise enumeration. Its ease of use and compressed design contribute to laboratory efficiency.

1.3.4 Easygel®

Easygel® (Micrology Laboratories, LLC, Goshen, IN), formerly Redigel®, is a low methoxyl pectin-based medium that consists of a nutrient liquid in pre-measured and sterile bottles and Petri dishes that are pre-treated with calcium ions. The calcium ions diffuse through the liquid nutrients creating a bridge and causing gelling of the medium. It can be used as a pour plate or streak plate and eliminates the need for autoclaving and preparing agar. Like many of the previously discussed media, the Easygel® nutrient liquid can support chromogenic, fluorogenic, and selectivity additives to create different media for specific microbe growth and enumeration.

Easygel® is offered in varieties of Coliscan® Easygel® - *E. coli*/Coliform growth medium, Total Count Easygel®, Total Count T-salt Easygel® - General bacterial growth medium, and ECA Check Easygel® - *E. coli*, coliform and *Aeromonas* growth medium.

The pour plating procedure for the Easygel® medium differs from the standard agar pour plate technique due to the fact that Easygel® technology uses specially pre-treated Petri dishes which contain a coating with calcium ions on the bottom of the dish. The liquid Easygel® medium contains a gelling agent (primarily pectin) and calcium ions in the coating of the Petri dish. The proper pour plate technique is to add the inoculum to the liquid Easygel® in the bottle and pour the mix into the pre-treated dish. If the inoculum is dispensed into the dish and the Easygel® medium is poured over it (which is the conventional agar pour plate procedure), there will be an instantaneous "clump" formed and no mixing of the inoculum and the medium will occur. Therefore, this difference in procedure between agar based media and Easygel® media must be carefully noted and followed.

According study by Fung and Chain (1991), total count Redigel® showed a favorable comparison with the standard aerobic plate count method. The correlation coefficient for overall food means was r = 0.964. Beuchat *et al.* (1998) compared Redigel®, Petrifilm™, and SimPlate® Total Plate Count method with conventional pour-plating and found that the SimPlate® compared to the Redigel® and conventional method with correlations of r = 0.97 and r = 0.96, respectively. A study performed by Chain and Fung (1991) compared the Redigel® method to spiral plating, Isogrid, Petrifilm™, and aerobic plate count methods and found very high correlations (r ≥ 0.978) between the methods on chicken breast, ground beef, ground pork, raw milk, pecans, and whole wheat flour. These studies conclude that the new Easygel® system should compare very closely to standard methods.

Images of previously described pour plate media can be seen in Figure 1. The VRB plate is separated into thirds. The top left section is un-inoculated, the top right is inoculated with *Enterobacter aerogenes*, and the bottom section shows typical *E. coli.* The VRB-MUG illustrates the fluorescent properties of *E. coli* as opposed to non-fluorescing enteric bacteria. The image of XLD shows an isolation streak of *S.* Typhimurium, and the TSA plate is un-inoculated. The bottom center ECA picture shows the chromogenic characteristics of the medium: the dark blue colonies are indicative of *E. coli,* pink colonies are coliforms, and the small green colonies are *Salmonella* spp. The bottom right image shows the same plate of ECA seen in the previous picture under long wave ultra violet light (366 nm). It shows that the large dark blue colonies, which are presumably *E. coli,* also fluoresce, resulting in further confirmation.

Figure 1 Solid Pour Plate Media (left to right): Violet Red Bile (VRB), Violet Red Bile-MUG (VRB-MUG), Xylose Lysine Desoxycholate (XLD), Tryptic Soy Agar (TSA), ECA Check® Easygel® Plus (ECA), and ECA Check® Easygel® Plus Under Long Wave Ultra Violet Light (366 nm)
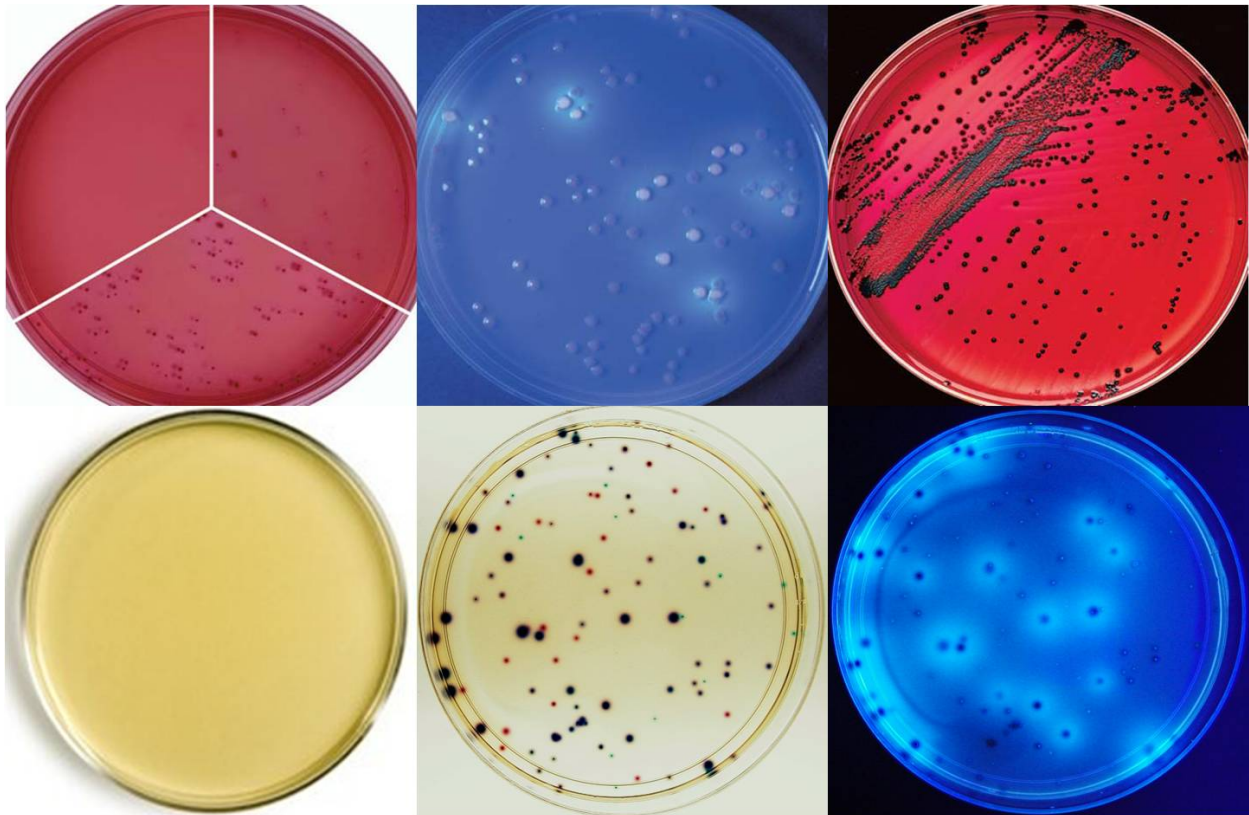
Source: Difco™ and BBL™ Online Manual. 2009

1.4 Chromogenic and Fluorogenic Substrates

1.4.1 Chromogenic Substrates

Chromogenic substrates such as 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt (X-GLUC), phenolphthalein-mono-β-D-glucuronide (PHEGLR), and *p*-nitrophenol-β-D-glucuronide (PNPGLR) for GUD detection; and 6-Chloro-3-indolyl-β-D-galactopyranoside (Red-Gal®), *o*-nitrophenyl-β-D-galactopyranoside (ONPG) for β-galactosidase (β-gal) detection; and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) for α-galactosidase detection can be used in different combinations in solid and liquid media to allow for facilitated discernment of Gram-negative organisms. X-GLUC is cleaved by β-glucuronidase to produce glucuronic acid, which is colorless, and chloro-bromoindigo, a blue/green precipitate (Manafi *et al.,* 1991). Red-Gal® and X-GAL are similarly hydrolyzed, producing a dark pink or light blue color in the presence of coliforms, depending on the amount of enzymes produced. X-GLUC has been shown as efficient as the fluorescent substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) in the detection of *E. coli* in solid agar media, and does not diffuse through the medium as MUG will over extended incubation time (Frampton *et al.,* 1987; Manafi *et al.,* 1991). However, it may be expensive and is not usable in liquid media. A study using GUD activity for detection of *E. coli* concluded that 99.5% of cow, human, and horse isolates tested positive using the Colilert® system (Rice *et al.,* 1990).

1.4.2 Utilization of 4-methyl-umbelliferyl-β-D-glucuronide

In conjunction with the selective nature of certain media like VRB, an addition of the substrate 4-methyl-umbelliferyl-β-D-glucuronide (MUG) can be done after preparation and proper tempering in order to more quickly distinguish most strains of *E. coli* from other

coliforms. MUG is cleaved by GUD, an enzyme produced by more than 95% of *E. coli*, resulting

in 4-methylumbelliferone which displays a blue fluorescence under long wave ultra violet light

(~366 nm; Feng *et al.*, 2002). Due to its versatility and stability, MUG can be used in other solid

and liquid media including MacConkey agar, m-FC agar, lauryl tryptose broth, EC broth, and

Brila broth. It can be incorporated into media and sterilized without losing its functionality

(Manafi, 2000). The addition of MUG to liquid media can be used to distinguish pure or mixed

E. coli cultures in as little as 4 hours, and can detect one cell of *E. coli* in 20 hours (Feng and

Hartman, 1982).

Most members of *Enterobacteriaceae*, excluding approximately 50% of *Shigella* and

about 25% of *Salmonella* (along with select strains of *Yersinia*), do not generate GUD making

this method extremely reliable for the detection and differentiation of *E. coli* (Hartman, 1989;

Manafi, 1991). However, serogroups such as enterohemorrhagic *E. coli* O157:H7, which are

regarded as typically GUD-negative, cannot be detected confidently using this method (Doyle

and Schoeni, 1984; Hayes *et al.,* 1995; Hartman, 1989). MUG is very susceptible and easily

disseminates through solid media (Manafi, 2000). These problems can be avoided by properly

testing the pH of the media before addition of MUG and by decreasing the incubation time for *E.

coli* detection to 18 hours instead of 24 hours. Even though the visual size of colonies may be

smaller than at 24h incubation, the enzymatic reaction will be evident and less diffused under

long wave ultra violet light.

Another problem related to MUG was found in an experiment performed by Robison

(1984) of Ross Laboratories in Columbus, Ohio. Comparing a conventional procedure using

brilliant green bile broth (BGB) to lauryl sulfate broth with MUG (LST-MUG), Robison (1984)

found that, although there were no false negatives, 4.8% of samples falsely tested positive. The

false positives were Gram-positive and thought to be a streptococcal strain. Moberg (1985) performed a more extensive trial using the LST-MUG assay, determining specificity, optimal MUG concentration, and possible inhibition of *E. coli* growth by MUG using two methods: the first used increasing concentrations of MUG in comparison to the growth rates of *E. coli,* and the second incorporated the corresponding standard MPN procedure, one set of LST tubes with MUG and the other without. The findings showed that specificity was very high in that competing coliforms, at a beginning concentration of 2:1, did not inhibit the fluorescence of *E. coli*. The LST-MUG could detect one organism of *E. coli* within 12 hours, and the results yielded no inhibition of *E. coli* by MUG up to 200 μg/ml. Moberg (1985) detected no false negatives in either the conventional MPN procedure or the LST-MUG MPN series. However, the conventional MPN method, identifying presumptive *E. coli* using gas production in EC broth, produced almost twice the number of false positives than the LST-MUG (2.7% and 1.4% respectively). The false positives were identified as *Staphylococcus* spp. in the LST-MUG, and *Klebsiella* spp. and *Enterobacter* spp. in the LST without the addition of MUG. Similar false positives were found in a subsequent study using VRB-MUG where weakly fluorescing strains were found to be *Klebsiella* spp. (Venkateswaran *et al.,* 1996). In these instances, only the colony fluoresced, and did not diffuse into the surrounding agar. False positives may be avoided by streaking onto selective and differential media such as Eosin Methylene Blue (EMB) and looking for metallic green colonies typically indicative of *E. coli.* Although additional selective streaking adds a day to any MUG method, it still decreases the time for *E. coli* detection (compared to conventional confirmation) by 3-5 days; it is more cost effective than rapid method kits and standard biochemical testing (Robison, 1984).

The importance of cost effective rapid testing for fecal contamination in water has been demonstrated by the emergence of many rapid kits that also utilize MUG as an indicator for *E. coli*. The Colilert®® system (Idexx, Warbrook, ME) uses *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and MUG to detect and differentiate β-galactosidase positive coliforms and *E. coli*, and can be used as a presence/absence or enumerated within 24 hours. This system is more convenient and rapid than using traditional fecal coliform methods and EC-MUG (Rice *et al.*, 1990). Although GUD is produced by more than 95% of *E. coli* species, several studies have established that using a probe sequenced for the GUD gene *uidA* is more sensitive in detecting a wider variety of *E. coli* species, including serotype O157:H7 and MUG-negative strains, than using assays with MUG as the primary indicator (McDaniels *et al.*, 1996; Feng *et al.*, 1991).

1.4.3 ECA Check® Easygel® Plus Mode of Action

The ECA Check® Easygel® Plus medium is described in the original patent (US#6,350,588) and several subsequent carry-overs. It consists of a basic nutrient formula, ingredients to reduce undesirable non-target background organisms, and a combination of chromogenic and fluorogenic enzyme substrates. Three types of target organisms are *E. coli*, *Salmonella* spp., and general coliform species. The ability of the medium to differentiate among the three different types of target organisms is due to the different chromogenic and fluorogenic enzyme substrates incorporated in the nutrient liquid. *E. coli* produces three enzymes: β-glucuronidase (β-gluc), β–galactosidase (β–gal), and α-galactosidase (α-gal), while some *Salmonella* spp. produce only α-galactosidase. Most general coliform bacteria produce only two of the enzymes: β-galactosidase and α-galactosidase. Therefore, any colony forming units (CFU) of target bacteria growing on the ECA medium will be colored by the chromogenic or fluorogenic compounds produced. The dark blue/purple color is therefore a combination of the β-

glucuronidase (blue), α-galactosidase (teal green), and the β-gal (pink/magenta) substrate products. Some *Salmonella* spp. appear green in ambient light because they only produce the α-galactosidase enzyme. General coliforms appear as colors that are combinations of the teal green of α-galactosidase and the pink/red of β-galactosidase. If a strain of coliform produces significantly more of one of the two enzymes their color will vary from being blue to a more pink or magenta color. However, they are very distinctive from both *E. coli* and *Salmonella* colonies.

A dual means of verifying the presence of *E. coli* in the ECA medium is provided by the inclusion of a fluorogenic enzyme substrate for β-glucuronidase. When the plated medium containing target *E. coli* colonies is illuminated under long wave ultra violet light (366 nm), the *E. coli* colonies will fluoresce a bright bluish color. This reading is best done at 18-24h incubation time as the fluorescent product is quite water soluble and will diffuse throughout the plate as time passes.

1.5 Pathogens of Concern

1.5.1 Salmonella

Salmonellae are Gram-negative, generally motile using peritrichous flagella, facultative anaerobic bacilli in the same family as *E. coli*. *Salmonella* grows at temperatures between 8-45°C and at a pH of 4-8 (WHO, 2002). It can also grow easily on most media, requiring highly selective media when in matrices that contain multiple microbial populations, such as feces (Yoshikawa *et al.,* 1980). *Salmonella* strains are ubiquitous and found in both cold and warm-blooded animals, including domestic and wild birds, reptiles, and mammals (FDA/CFSAN, 2004). The World Health Organization (WHO, 2002) states that there are approximately 40,000 reported cases of confirmed infections of *Salmonella* every year. Mead *et al.* (2000) showed that the estimated total cases are more than 1.4 million (reported and non-reported, non-typhoidal) and that approximately 95% of the cases reported to the Centers for Disease Control and Prevention (CDC) were foodborne.

There are thousands of *Salmonella* serotypes resulting in a massive discussion to determine proper nomenclature over the years. *Salmonella* consists of two currently recognized species: *enterica,* which includes subspecies *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtene* (IV), and *indica* (VI); and *bongori* (formerly subspecies V), which has been found to be an individual species through DNA-DNA hybridization experiments (Brenner *et al.,* 2000). Like *E. coli, Salmonella* serotypes are identified by somatic, surface, and flagellar antigens. According to the WHO in 2002, there are more than 2,500 serovars in the *Salmonella* genus, causing some controversy regarding proper nomenclature citation. For many years, scientists and public health officials used the serovars as species and cited them as such. During this time, the serovar Typhimurium was typically written *Salmonella typhimurium*, causing

confusion regarding relationships of species and serotypes within the genus. Currently, the nomenclature formula used by the CDC is: genus (italicized), species (italicized), subspecies (italicized), and serovar (first letter capitalized, not italicized). For example, the Typhimurium serovar would be written *Salmonella enterica* subsp. *enterica* ser. Typhimurium. However, this extensive rule may be shortened to genus and serovar: *Salmonella* Typhimurium. For unnamed serovars, the antigenic formula takes the place of serovar name (Brenner *et al.,* 2000). This has reduced confusing serovars with species, while maintaining the importance of serotype differentiation, by removing the italics and capitalizing serovar names.

The adaptability and extensive evolution of *Salmonella* spp. has made it one of the most widely studied bacteria for both clinical and foodborne pathogen investigations. Most serovars are zoonotic and are, in general, non-host adaptive, resulting in widespread infections across species barriers (WHO, 2002). Host specificity has been linked to the ability of the bacterium to cause infection within a developed host system. Most serovars that are not adapted to a particular host tend to cause illness in the young and immuno-compromised, rather than mature, healthy individuals or animals, alluding to the possibility that some types of the bacteria is incapable of surviving within established systems. The serotypes that are host adapted, like *S. enterica* subsp. *enterica* ser. Typhi in humans, have been shown to have increased virulence, thus higher occurrences of death (Baumler *et al.,* 1998).

Symptoms induced by infections of *Salmonella*, also called salmonellosis, include diarrhea, fever, bacteremia, and septicemia. Bacteremia typically occurs after major surgeries, usually dealing with the digestive system or urinary tract, which allows bacteria from constrained sites to enter the blood stream. If there are bacterial toxins released into the blood, it is referred to as septicemia. Symptoms include fever, lowered body temperature, decreased blood pressure,

and could result in death if not treated promptly with antibiotics. In the past 50 years, antibiotic-resistant bacteria have increased the number of cases of bacteremia and septicemia (Britannica Online Encyclopedia, 2009). Salmonellosis is mainly comprised of two disorders: enteric fever, also known as typhoid fever, and severe gastroenteritis (Todar, 2008). Typhoid fever is host adaptive and a result of *S.* Typhi or *S.* Paratyphi infection in the bloodstream. It is typically transmitted from human to human by a fecal-oral route. Typhoid is a major problem for international travelers, and in overcrowded areas, especially those with low hygienic standards and capabilities (CDC, 2005). Gastroenteritis is most often contracted via foods derived from animal origins (poultry, eggs, beef, etc.), through infection or intoxication, and causes the onset of diarrhea, abdominal cramping, fever, and, in some instances, vomiting. The most notable food-borne serotypes are *Salmonella* Enteritidis, commonly found in eggs, and *Salmonella* Typhimurium, which can be easily transmitted between animals and humans. Although not frequently life threatening, Salmonellosis can cause mortality in the elderly, very young, and immuno-compromised. Fluoroquinolones are used to treat Salmonellosis in adults and injected cephalosporins for pediatric illnesses. Alternative antibiotics include ampicillin, amoxicillin, and chloramphenicol. Unfortunately, the increased emergence of drug-resistant *Salmonella* has given rise to infections that may not have normally occurred and failed cures (WHO, 2005). Frequently, humans and animals that have been inflicted with salmonellosis remain carriers and can still secrete the bacterium (Todar, 2008).

Salmonella Typhimurium, originally found in mice with similar symptoms to those of human Typhoid fever, has one of the highest host adaptability ranges of all *Salmonella* serotypes. It is most the most frequent serotype isolated in humans (CDC 2004). Most recently *S.* Typhimurium has been implicated in contamination of peanut products resulting in a multistate

outbreak of 691 reported cases and nine deaths in 46 states. A voluntary recall of all products produced in Blakely, Georgia at the Peanut Corporation of America since January 2007 has been put into effect (CDC, 2009). *S.* Typhimurium has also been the cause of outbreaks in tomatoes in 2006, raw ground beef in 2004, chicken, sausage, and meat paste (CDC, 2006; CDC 2007; Foster 1997). Though it grows normally in the intestinal gut of animals, it causes gastroenteritis when spread to humans. It is capable of producing flagella at multiple antigen sites, and several new strains of *S.* Typhimurium have been discovered that have drug resistance (Todar, 2008). *Salmonella* Typhimurium DT 104, a strain that is resistant to ampicillin, tetracycline, chloramphenicol, and other antimicrobials, caused an outbreak in a veterinarian clinic in 1999 (CDC, 2001). A Danish evaluation performed by Helms *et al.,* (2002) regarding deaths linked with infection of drug-resistant *S.* Typhimurium showed that, up to two years after the infection, 59 deaths occurred out of 2,047 people that were treated. People that had been infected with drug-resistant strains of *S.* Typhimurium were 2.3-10.3 times more likely (depending on the resistance of the strain) to die within two years than the general population in Denmark. The emergence of increasingly dangerous mutations of *S.* Typhimurium requires that on-going testing for public safety in foods and environments be performed.

1.5.2 Escherichia coli

Escherichia coli are Gram-negative, facultative anaerobic, non-spore-forming rods that can utilize glucose or lactose as a carbon source with the production of acid and/or gas. *E. coli* belongs to the *Enterobacteriaceae* family which also includes *Salmonella, Enterobacter, Klebsiella, Shigella,* and *Yersinia,* most often associated with human gastrointestinal diseases. *E. coli* was first discovered in 1885 by Theodor Escherich and is commonly found in the intestinal tracts of mammals. It is extremely adaptable to environmental changes including pH,

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temperature, and the presence or absence of chemicals and oxygen. This microorganism is capable of growing fimbriae to attach to cells and adjusting membrane pore size to accommodate available nutrient particles (Todar, 2008).

Escherichia coli is also included in the coliform group, which is a general, non-taxonomic nomenclature referring to "Gram-negative, facultative anaerobic rod-shaped bacteria that ferments lactose to produce acid and gas within 48 h at 35°C (Feng *et al.*, 2002). Due to this close relationship to other organisms, it was necessary to distinguish fecal from non-fecal coliforms for use as indicator organisms for possible contamination in food and water testing. By increasing the incubation temperature to 44.5°C, fecal coliforms were more easily discerned and comprised of high levels of *E. coli.* However, according to Feng *et al.* (2002), *Klebsiella* and *Enterobacter* are also able to utilize lactose at the higher temperatures, and their presence may not be indicative of contamination. New means of detection and differentiation of *E. coli* made it the most advantageous indicator organism of possible pathogenic contamination.

Pathogenic *E. coli* that cause intestinal diseases are categorized into five classes: enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), and enteroaggregative *E. coli* (EAEC). The class of *E. coli* is determined by site and mode of adherence, whether toxins are produced, invasiveness of the strain, and symptoms of infection (Feng and Weagant, 2002). Certain strains of *E. coli* can colonize the fetal intestinal tract leading to rare cases of neonatal meningitis (Todar, 2008).

Along with the intestinal pathogenic *E. coli,* there are strains that exist outside of the intestines called extraintestinal pathogenic *E. coli* (ExPEC) that cause urinary tract infections. Approximately one half of women will have a bacteria-related urinary tract infection within their lifetime (Ramchandani, 2005). *E. coli* categorized as ExPEC include avian pathogenic (APEC)

and uropathogenic strains (UPEC). Genomic studies have shown that APEC share extensive similarities to UPEC, resulting in a possibility of APEC serovars to cause human urinary tract infections (Johnson *et al.,* 2007). The resemblance of some human and avian strains may provide substantiation that there is a possibility of foodborne transmission of extraintestional *E. coli.* This study utilized *E. coli* O1:K1:H7 which has been isolated in clinical cases involving pyelonephritis, or bacterial infection of the kidney (Vaisanen-Rhen, 1984; MedicineNet, 1998). Another strain of O1:K1:H7, APEC O1, has been found in chickens exhibiting colibacillosis (Johnson *et al.,* 2007). Although food recalls are based on the presence of *E. coli* O157:H7, the evident zoonotic capabilities of ExPEC elevate its importance in food testing as a possible distribution source.

1.5.3 Cronobacter spp., formerly Enterobacter sakazakii

Cronobacter spp., formerly classified as *Enterobacter sakazakii,* is a Gram-negative, rod-shaped, motile bacterium, generally referred to as a coliform that has been a major concern in dehydrated infant formula, causing rare cases of neonatal meningitis, septicemia, and necrotizing enterocolitis (FDA, 2002). Reported cases of infants were found to have low birth weights and a significant portion had onset of symptoms within the hospital (Bowen and Braden, 2006). The high mortality of infants infected with *Cronobacter sakazakii* (ranging from 10-80%) has led to extensive experimentation to find better methods of detection. It grows at temperatures from 6-45°C, with an optimum temperature of 37°C, and can produce gas from lactose, similar to most coliforms. In addition to infant formula, this microbe has been found in dairy, meat, produce, grain products, human bone marrow and blood (Iverson *et al.,* 2004a; Iverson *et al.,* 2007).

After its initial discovery, *Cronobacter* spp. was referred to as "yellow-pigmented *Enterobacter cloacae*" due to its typical yellow appearance on total count agar like TSA and its

relationship, genetically, to *E. cloacae* (FDA/CFSAN 2002; Iverson *et al.* 2007). Subsequently, it was designated *Enterobacter sakazakii* by Farmer *et al.* (1980). Similar to *Salmonella* species, the prevalence of the bacterium has given rise to recent discussions regarding proper taxonomy. Iverson *et al.* (2007) have performed extensive genetic testing including ribotyping, amplified fragment length polymorphisms (AFLP), and gene sequencing to determine the interrelatedness of 16 biogroups within the genus. Based on their findings, new classifications were proposed using *Cronobacter* as the new genus (from the Greek god Cronos, who was said to have swallowed his children; Iverson *et al.*, 2007). The genus includes four species, one genomospecies, and four subspecies. The reclassification of *E. sakazakii* would clearly separate the types known to cause neonatal morbidity and mortality (*Cronobacter sakazakii* subsp. *sakazakii*, *C. sakazakii* subsp. *malonaticus*, and *C. turicensis*) from the likely pathogenic clinical species (*C. muytjensii* and *C. dublinensis*) isolated from generally sterile sites in the human body.

Iverson *et al.* (2004b) determined the D-value of *E. sakazakii* to be 2.4 minutes at 58°C and calculated that "high temperature short time" pasteurization would be more than enough to eradicate the organism from susceptible products. The study also concluded that contamination is most likely the result of non-hygienic post-pasteurization practices. In general, *E. sakazakii* reduces nitrate and utilizes citrate. It is methyl red negative and Voges-Proskauer (VP) positive. It can produce acid from many sugars; it demonstrates chromogenic and fluorogenic properties in the presence of substrates such as 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside and 4-methylumbelliferyl-α-D-glucopyranoside; and, like most coliforms, it is negative for GUD. Biochemical tests that determine differences in species or subspecies of *E. sakazakii* are Dulcitol (Dul), Malonate (Mal), Indole (Ind), and acid from methyl-α-D-glucoside (AMG; Iverson, 2007).

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The variances between strains of *E. sakazakii* can be seen in Table 1, listed as the proposed *Cronobacter* genus and species. The particular strain of *E. sakazakii* used for this study would be reclassified as *C. muytjensii*. Although not determined to be a foodborne species, the importance of its detection is not lessened since a specific reservoir for *E. sakazakii* has not been found.

Table 1 Statistically Relevant Biochemical Tests for the Differentiation of Proposed

***Cronobacter* Species and Subspecies**

Enterobacter sakazakii Proposed Reclassification	Dul	Ind	Malo	AMG
Cronobacter sakazakii subsp. *sakazakii*	-	-	-	+
Cronobacter sakazakii subsp. *malonaticus*	-	-	+	+
Cronobacter muytjensii	+	+	+	-
Cronobacter dublinensis	-	+	v	+
Cronobacter turicensis	+	-	+	+
Cronobacter genomospecies 1	+	-	+	+

Dul, production of acid from dulcitol; Ind, production of indole; Malo, malonate utilization; AMG, production of acid from methyl-α-D-glucoside; +, 85–100% positive; v, 15–85% positive; -, less than 15% positive

Source: Iverson *et al.* (2007)

CHAPTER 2 - Materials and Methods

2.1 Stock Culture Selection and Preparation

All cultures used in this study were purchased from Microbiologics (St. Cloud, MN) in KWIK-STIK™ form, which are traceable to the American Type Culture Collection (ATCC) or National Type Culture Collection (NCTC). Previous studies have shown that the majority of *Escherichia coli* strains will fluoresce in the presence of 4-methylumbelliferyl-β-D-glucuronide (MUG; Feng *et al.,* 2002). Exceptions include *Escherichia coli* O157:H7, which reacts similarly to non-*E. coli* coliforms. To verify reactions on ECA Check® Easygel® Plus (ECA), laboratory testing was performed at Kansas State University under Biohazard Level 2 conditions to determine color variations and fluorescence of different strains of *E. coli*, non-*E. coli* coliforms and *Salmonella enterica* subsp. *enterica.* Two strains of *E. coli* O157:H7 were streaked onto ECA: ATCC #43894 and 35150. The colonies produced were light blue and did not fluoresce, correlating to non-*E. coli* coliform reactions. Subsequently, three generic *E. coli* strains were similarly tested on ECA: ATCC #25922, 35421, and 11775. Each strain produced dark blue colonies that fluoresced under long wave ultra violet light (366 nm). Research was also performed to determine differentiating features for non-*E. coli* coliforms. The organisms tested were: *Klebsiella pneumoniae* (ATCC #13883), *Citrobacter freundii* (ATCC #8090), *Enterobacter aerogenes* (ATCC #3048), *Cronobacter muytjensii* (*Enterobacter sakazakii* ATCC #51329), and *Enterobacter cloacae* (ATCC #23355). They were found to have a light blue to pink color and did not fluoresce on ECA. Three different serovars of *Salmonella* spp. were tested: *Salmonella enterica* subsp. *enterica* ser. Abaetetuba (NCTC #8244), *Salmonella enterica*

subsp. *enterica* ser. Senftenberg (ATCC #43845), and two strains of *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC #13311 and 14028). All *Salmonella* colonies were found to have a consistent teal green color on ECA. Examples of the reactions can be seen in Figure 2. Based on the research performed, three stock cultures were chosen as inoculum for this experiment: *Escherichia coli* (ATCC #11775) which was confirmed as dark blue colonies that fluoresced on ECA; *Cronobacter muytjensii (Enterobacter sakazakii* ATCC #51329) which resulted in dark pink colonies and did not fluoresce on the test medium; and *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC #13311) which showed green colored colonies on ECA. The stock cultures were grown in Bacto™ Tryptic Soy Broth (TSB; Becton, Dickinson and Company, Franklin Lakes, NJ) at 35°C for 24h.

Figure 2 Examples of Chromogenic and Fluorogenic Reactions of Five Bacteria When

Plated on ECA Check® Easygel® Plus
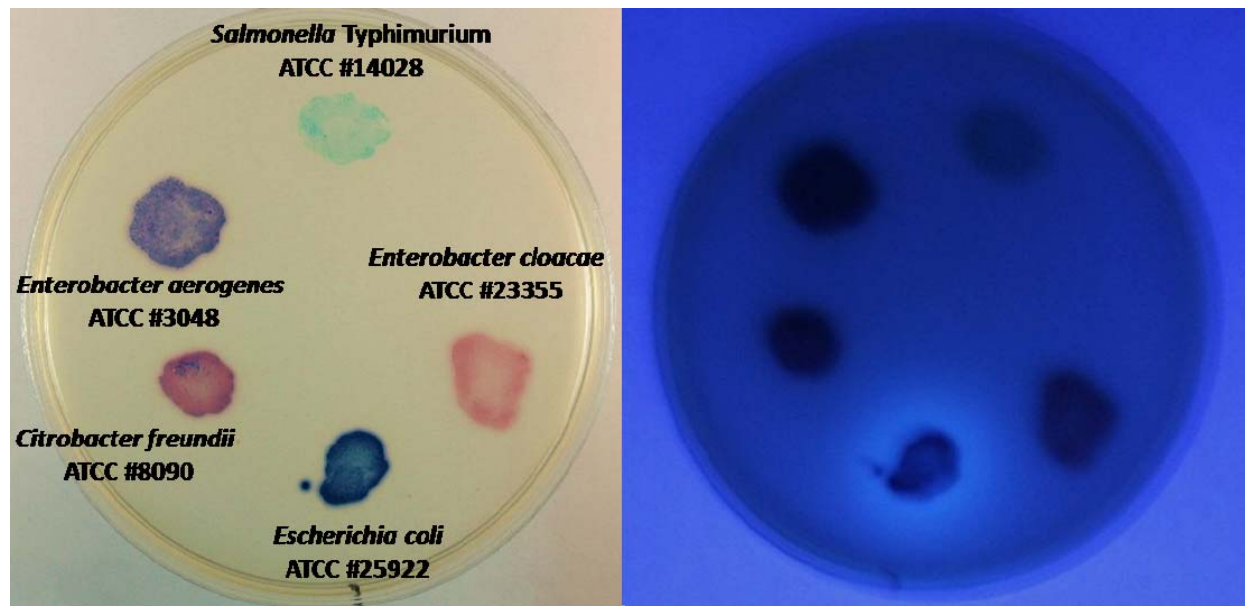
2.2 Media Preparation

Tryptic Soy Agar (TSA; Difco, Becton, Dickinson and Company, Franklin Lakes, NJ), Violet Red Bile (VRB; Difco), and Xylose Lysine Desoxycholate Agar (XLD; Difco) were prepared according to manufacturer instructions and allowed to temper to approximately 45°C before plating. Violet Red Bile Agar with 4-methylumbelliferyl-β-D-glucuronide (VRB-MUG) was made as VRB, cooled to 45°C, and supplemented with MUG (Sigma-Aldrich, Inc, St. Louis, MO) at a level of 0.1 g/L of VRB. The *Escherichia coli*/Coliform Count Plate Petrifilm™ (ECC; 3M, St. Paul, MN) and ECA Check® Easygel® Plus (ECA; Micrology Labs, Ltd, Goshen, IN) were brought to room temperature (23°C) from storage at refrigeration temperature, and required no further preparation.

2.3 Stock Culture Study

The object of this data was to determine actual chromogenic reactions of the stock cultures used on all media, and also in identification kits. Serial 1:10 dilutions of each stock culture were made using 0.1% buffered peptone water (PW). *E. coli* was plated onto ECA, VRB-MUG, ECC, and TSA; *E. sakazakii* was plated onto ECA, VRB, ECC, and TSA; and *S.* Typhimurium was plated onto ECA, XLD, and TSA. The cultures were plated in duplicate (1.0 ml), incubated, and counts were performed. All plates were incubated at 35°C for 24h.

2.4 Inoculated Ground Meat Study

Five samples each of ground beef and ground turkey were purchased at local retailers. The fat content of the ground beef samples varied from 4-20% and the fat content of the ground turkey varied from 1-15%. Between replications, samples were stored in a laboratory freezer at

-15°C. Samples of 25 g of each meat were added to 223.5 ml of 0.1% buffered peptone water and 0.5 ml of each stock culture (grown to 7-9 log CFU/ml), and then placed in a Seward Stomacher® for 2 minutes. Serial 1:10 dilutions were made and plated in duplicate onto ECA, VRB, VRB-MUG, XLD, and TSA, and results were counted after incubation at 35°C for 24h. This experiment was repeated three times for each sample.

2.5 Non-inoculated Ground Meat Study

The non-inoculated part of the research was to determine the ability of the media to recover natural microbial populations in ground beef and ground turkey using the methodology in the previous section. This part of the experiment was performed on the same five ground beef and ground turkey samples used in the inoculated study above. 25 g of each sample was added to 225 ml PW and homogenized in a stomacher for 2 minutes. Serial 1:10 dilutions were made and plated in duplicate onto the media described above. After incubation for 24h at 35°C, counts were performed and recorded. This experiment was also repeated three times.

2.6 Rapid Method Identification

After each study was complete, random typical and atypical colonies were isolated from each medium; Gram stained, and identified using BBL™ Crystal™ Enteric/Nonfermenter ID or Gram-Positive kits (BD Diagnostics, Franklin Lakes, NJ). The kits were incubated at 35°C for 24h and read per manufacturer instructions.

2.7 Statistical Analysis

Plate counts were converted to log CFU/ml and analyzed using SAS (version 9.1.2, 2004). Statistical analysis for this research was a randomized complete block, with the meat

samples as the random block effect and the media as a fixed effect. The Least Squares method

was used to compare mean counts and the significance level was set at p $\leq$0.05.

CHAPTER 3 - Results and Discussion

3.1 Enumeration

Figure 3 is a picture of an inoculated ECA Check® Easygel® Plus Petri dish showing the chromogenic differentiation between typical *Escherichia coli,* coliforms, and *Salmonella.* For enumeration of the inoculated ground beef and ground turkey, presumptive *Escherichia coli* colonies on ECA and Violet Red Bile with 4-methylumbelliferyl-β-D-glucuronide (VRB-MUG) were counted as fluorescent colonies under long wave ultra violet light (366 nm). On ECA, non-fluorescing blue to dark pink colonies were counted as coliforms and small teal green colonies were enumerated as *Salmonella* spp. Data were separated into total aerobic count, comparing ECA with TSA; presumptive *E. coli* counts, comparing ECA to VRB-MUG; total coliform counts, comparing ECA with VRB, VRB-MUG and ECC Petrifilm™; and presumptive *Salmonella* counts*,* comparing ECA and XLD. Total plate counts were recorded for the non-inoculated study since few organisms grew naturally on the meat that would survive in selective and differential media.

Figure 3 Inoculated ECA Check® Easygel® Plus Petri Dish in Normal and Under Long Wave Ultra Violet Light (366 nm)
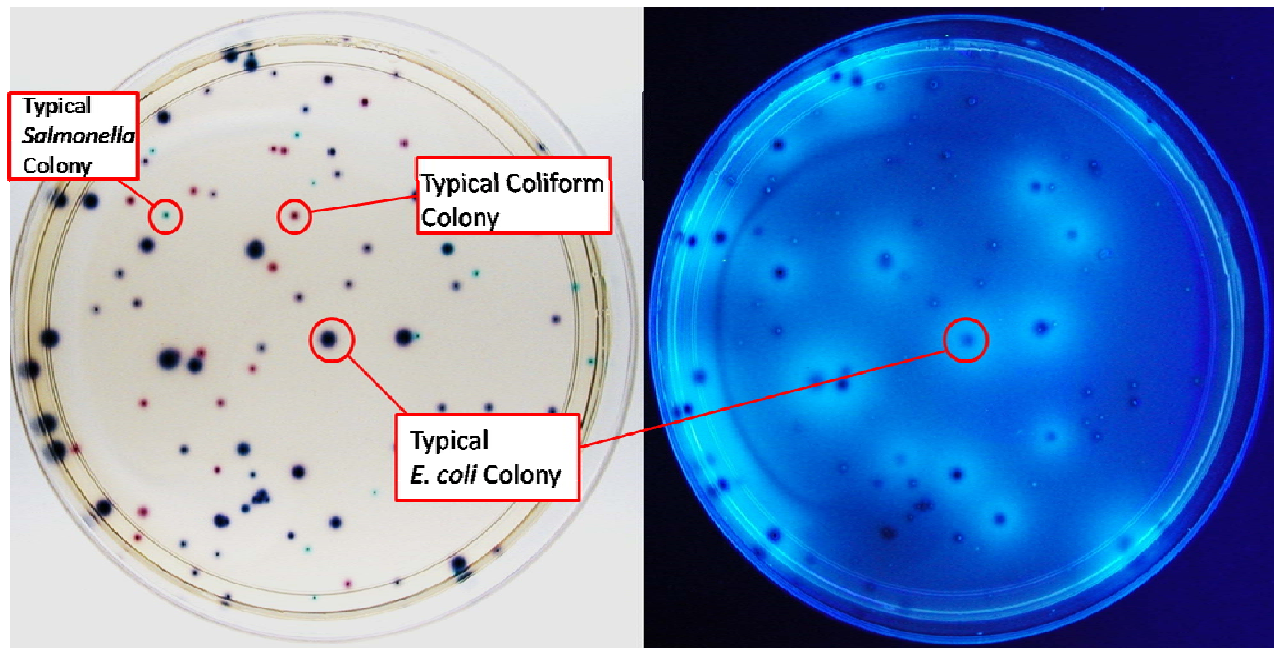
Statistical analysis using SAS could not be performed on this part of the experiment because there was no sample population; each recovery was repeated three times and the averages for each microorganism on each media were compared. However, Table 2 shows the standard deviations for bacterial enumeration on the media plated for each stock culture.

Figures 4, 5, and 6 illustrate the results of the stock culture recovery part of the experiment. The counts did not vary widely during the recovery of *E. coli* and *Cronobacter* spp. but there was a noticeable difference in the results for *Salmonella* Typhimurium. Figure 4 shows the average counts for the recovery of *Salmonella* Typhimurium. *Salmonella* Typhimurium was plated onto Tryptic Soy Agar (TSA), Xylose Lysine Desoxycholate (XLD), and ECA Check® Easygel® Plus (ECA). TSA recovered 8.69 log CFU/ml, which was slightly more than ECA at 8.62 log CFU/ml, and XLD recovered much less at 6.82 log CFU/ml. This may be attributed to the selective ingredients in XLD which may have prevented a portion of the *Salmonella* from growing.

Table 2 Standard Deviations of Media for Stock Culture Recovery

Microorganism	Media	Mean Counts (log CFU/ml)	Standard Deviation
Salmonella Typhimurium	TSA	8.69	0.09
	XLD	6.82	0.09
	ECA	8.62	0.16
Escherichia coli	TSA	9.11	0.75
	VRB-MUG	9.09	0.32
	ECCP	9.04	0.23
	ECA	9.09	0.28
Cronobacter muytjensii	TSA	8.62	0.38
	VRB	8.39	0.81
	ECCP	8.86	0.11
	ECA	8.66	0.12

Figure 4 Comparison of Average Counts for the Stock Culture Recovery of *Salmonella* Typhimurium on Tryptic Soy Agar (TSA), Xylose Lysine Desoxycholate (XLD), and ECA Check® Easygel® Plus (ECA)
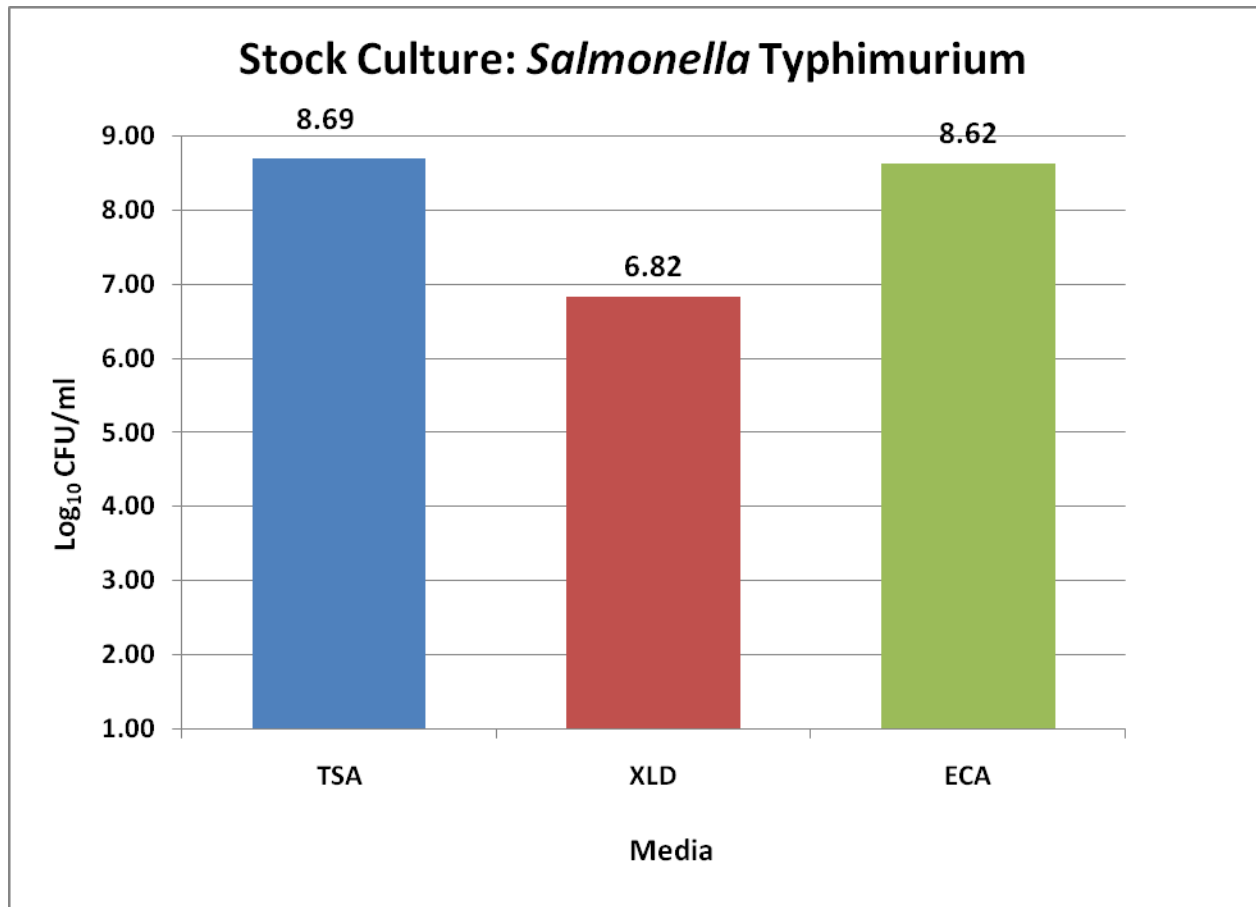
Figure 5 compares the recovery of *E. coli* plated on TSA, Violet Red Bile agar with MUG (VRB-MUG), ECC Petrifilm™ (ECCP), and ECA. The average counts varied only slightly, recovering 9.11, 9.09, 9.04, and 9.09 log CFU/ml, respectively. Similarly, the recovery of *E. sakazakii* on TSA, Violet Red Bile agar (VRB), ECCP, and ECA, shown in Figure 6, varied by less than 0.5 log CFU/ml. The average counts were 8.62 log CFU/ml on TSA, 8.39 log CFU/ml on VRB, 8.86 log CFU/ml on ECCP, and 8.66 log CFU/ml on ECA.

Figure 5 Comparison of Average Counts for the Stock Culture Recovery of *Escherichia coli* on Tryptic Soy Agar (TSA), Violet Red Bile agar with MUG (VRB-MUG), ECC Petrifilm™ (ECCP), and ECA Check® Easygel® Plus (ECA)
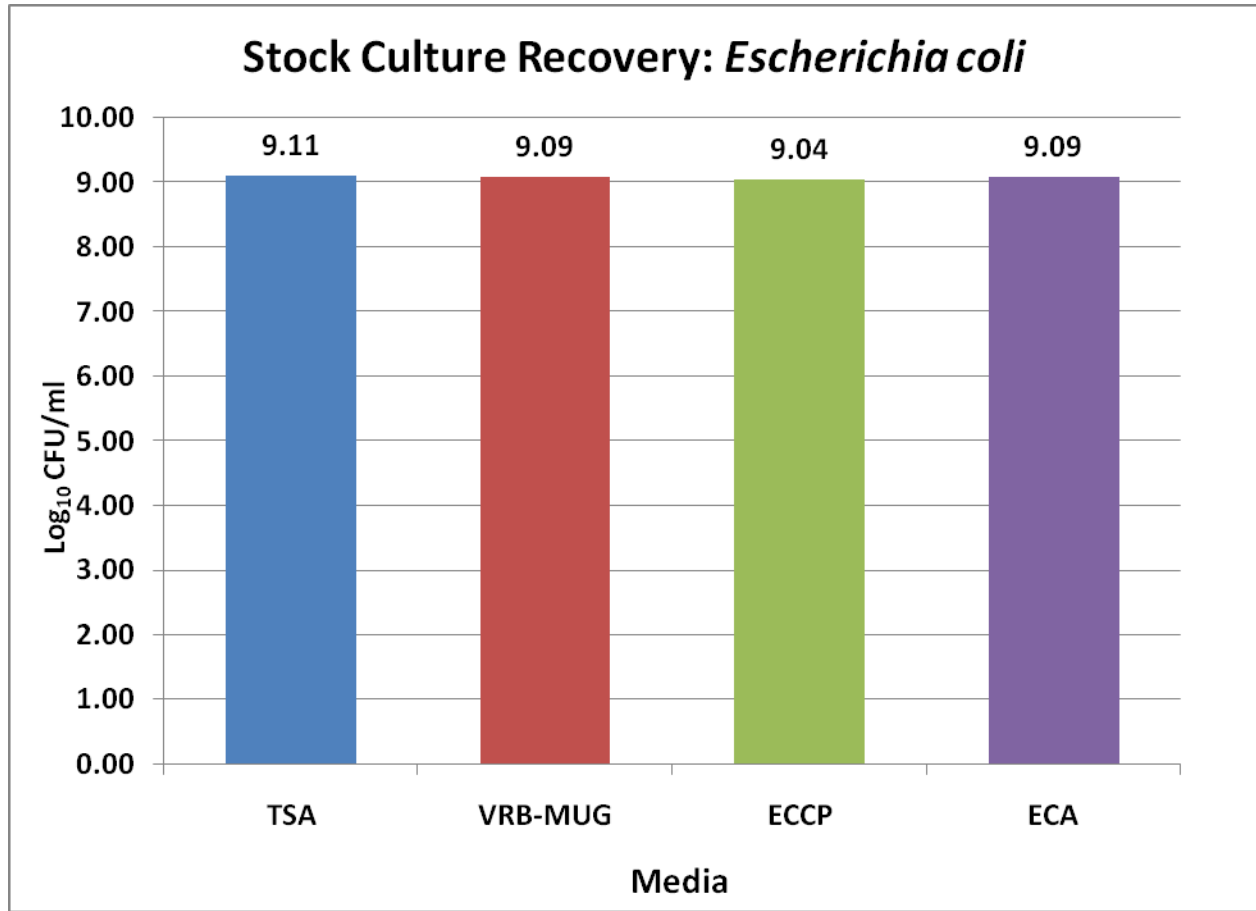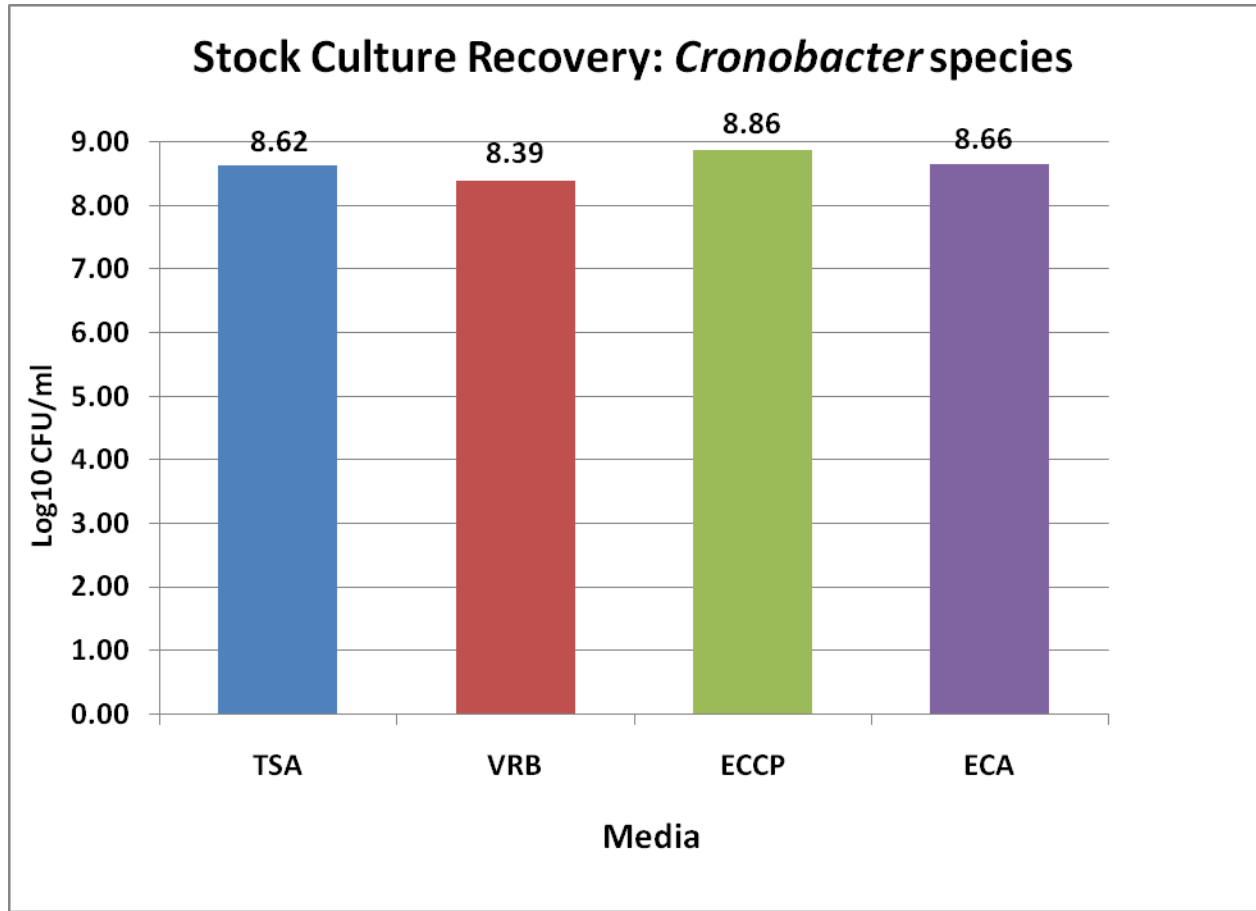
Figure 6 Comparison of Average Counts for the Stock Culture Recovery of *Cronobacter* spp. on Tryptic Soy Agar (TSA), Violet Red Bile (VRB), ECC Petrifilm™ (ECCP), and ECA Check® Easygel® Plus (ECA)

Overall, ECA showed very comparable recovery compared to the conventional media during stock culture recovery. *Escherichia coli* and *Cronobacter* spp. counts were very similar, although ECA showed clear superiority in the recovery of *S.* Typhimurium when compared to XLD.

The inoculated study performed on ground beef samples revealed significant differences between some of the Least Squares (LS) mean counts. Figure 7 shows the comparison of total counts between TSA and ECA. ECA recovery was significantly higher at 7.84 log CFU/ml than TSA which showed a mean of 6.51 log CFU/ml. This may be attributed to the selectivity of the ECA allowing the inoculated enteric bacteria to grow without the competition of the accompanying micro-flora that would be present on TSA.

Figure 7 Comparison of the Total Count Recovery on ECA Check® Easygel® Plus (ECA) and Tryptic Soy Agar (TSA) in Inoculated Ground Beef
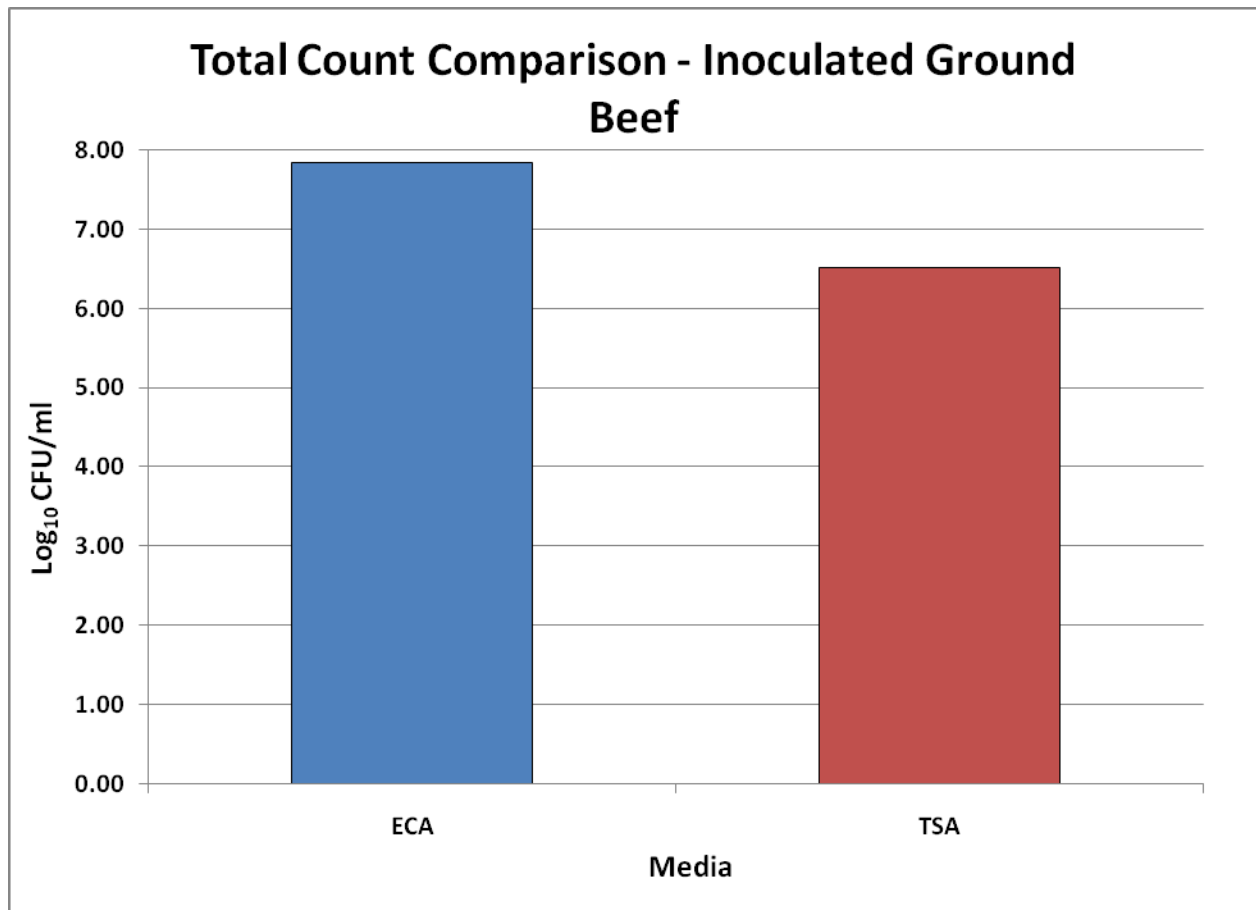
Figures 8, 9, and 10 show *E. coli*, coliform (non-*E. coli*), and total coliform (*E. coli* and other coliforms) count comparisons. Figure 8 compares the recovery of *E. coli* on ECA (blue, fluorescent colonies) and VRB-MUG, with mean counts of 7.24 and 7.41 log CFU/ml, respectively. The diffusion of MUG throughout the VRB medium made it difficult to discern fluorescent and non-fluorescent colonies when growing close together, which may have led to slightly higher counts in VRB-MUG, but was not significantly different. Similarly, the coliform (non-*E. coli*) count on VRB and ECA (pink or blue, non-fluorescent colonies) resulted in a slightly higher mean on VRB (7.37 log CFU/ml) than ECA (7.10 log CFU/ml), since *E. coli* and non-*E. coli* coliform colony appearance on VRB was very similar and difficult to differentiate (Figure 9). According to the statistical data, there was a slight significant difference on these means. In comparing the ECCP to VRB, VRB-MUG and ECA, the ECCP performed slightly better with regard to total coliform recovery than any of the other media. The mean count for ECCP was 7.63 log CFU/ml, followed by 7.43 log CFU/ml on ECA, 7.41 log CFU/ml on VRB-MUG, and 7.37 log CFU/ml on VRB (Figure 10). There were no significant differences between ECCP, ECA, and VRB-MUG; and ECA and VRB-MUG was not significantly different than VRB. However, ECCP was statistically better at total coliform recovery than VRB.

Figure 8 Comparison of the Recovery of Presumptive *Escherichia coli* on ECA Check®

Easygel® Plus (ECA) and Violet Red Bile with MUG (VRB-MUG) in Inoculated Ground
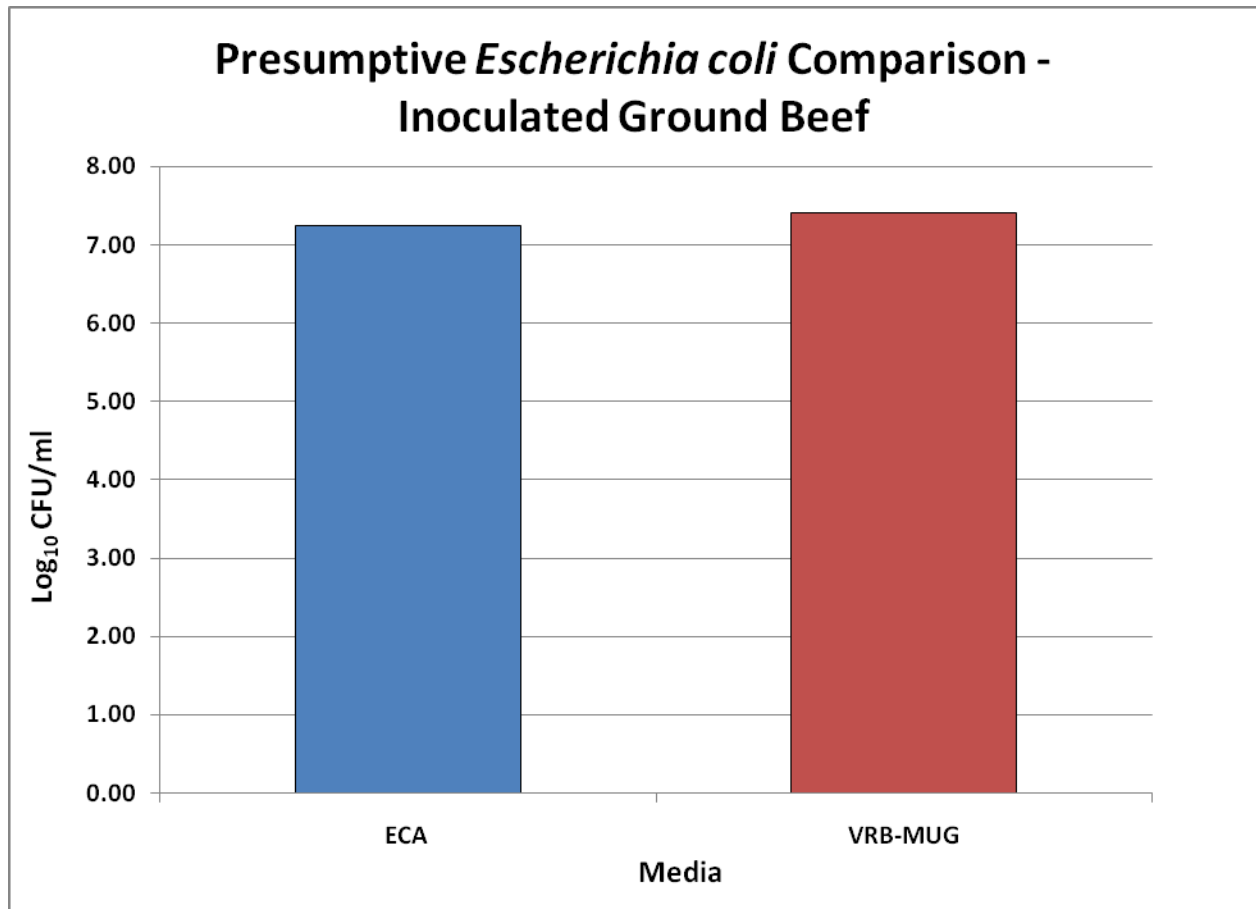
Beef

Figure 9 Comparison of the Recovery of Presumptive Non-*Escherichia coli* Coliforms on

ECA Check® Easygel® Plus (ECA) Violet Red Bile (VRB) in Inoculated Ground Beef
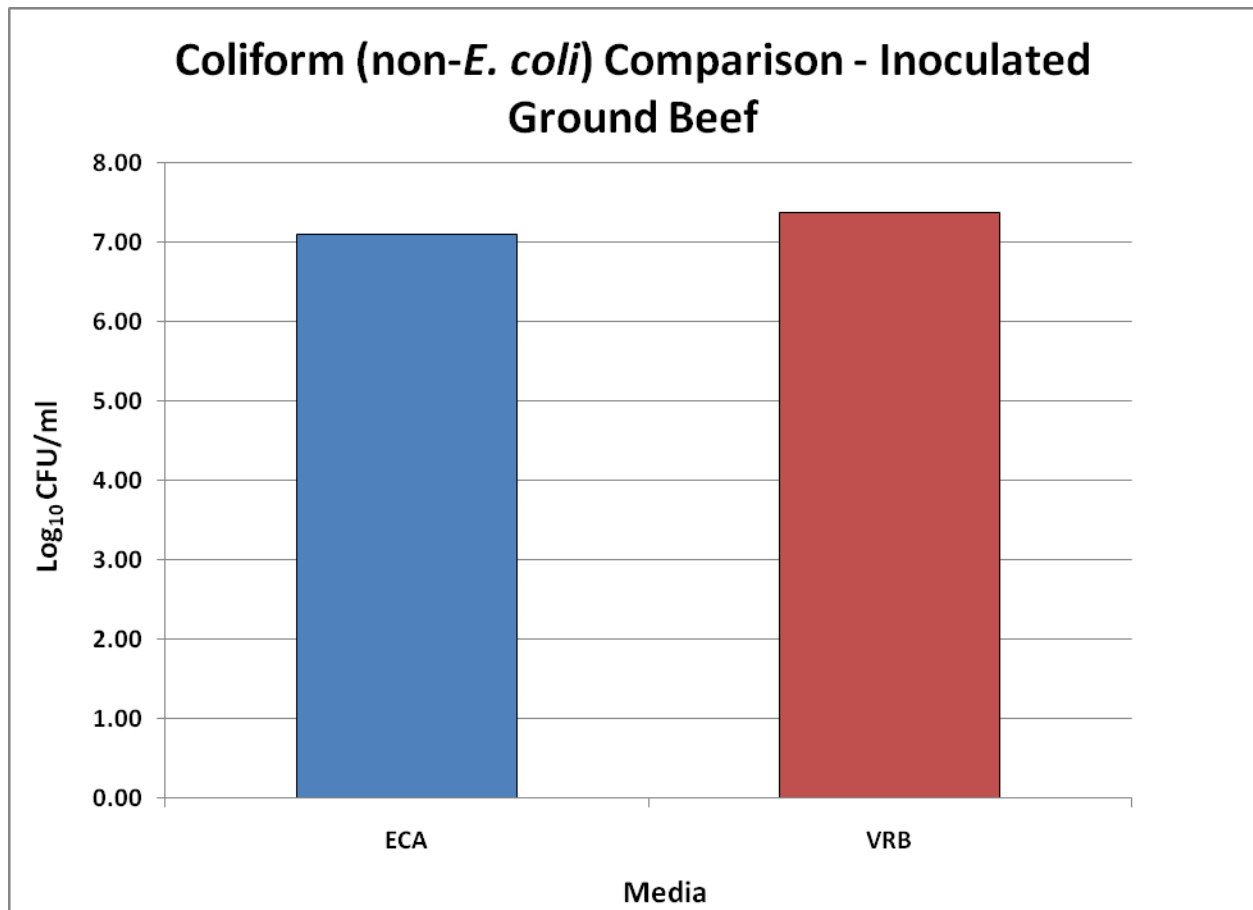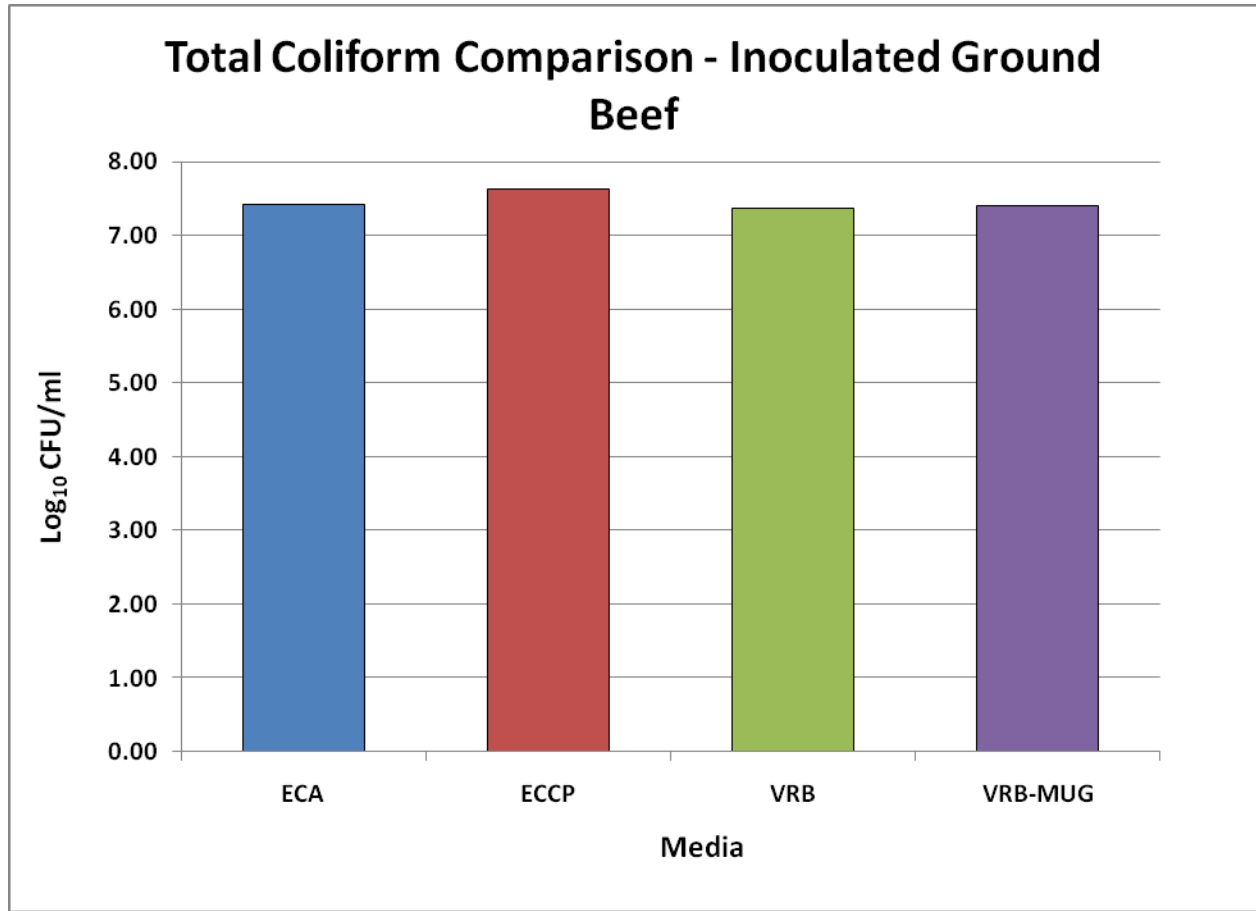
Figure 10 Comparison of the Recovery of Total Coliforms on ECA Check® Easygel® Plus (ECA), ECC Petrifilm™ (ECCP), Violet Red Bile (VRB), and Violet Red Bile with MUG (VRB-MUG) in Inoculated Ground Beef

In contrast, Figure 11 shows the ECA medium was statistically better for the recovery of *Salmonella* Typhimurium compared to XLD with mean counts of 6.68 and 6.21 log CFU/ml. The green colonies were easily differentiated from the coliforms because they were smaller and more separated from other colonies than on XLD, which often grew coliforms (yellow colonies) and inhibiting the typically black colonies indicative of most *Salmonella* spp.

Figure 11 Comparison of the Recovery of Presumptive *Salmonella* Typhimurium on ECA Check® Easygel® Plus (ECA) and Xylose Lysine Desoxycholate (XLD) in Inoculated Ground Beef
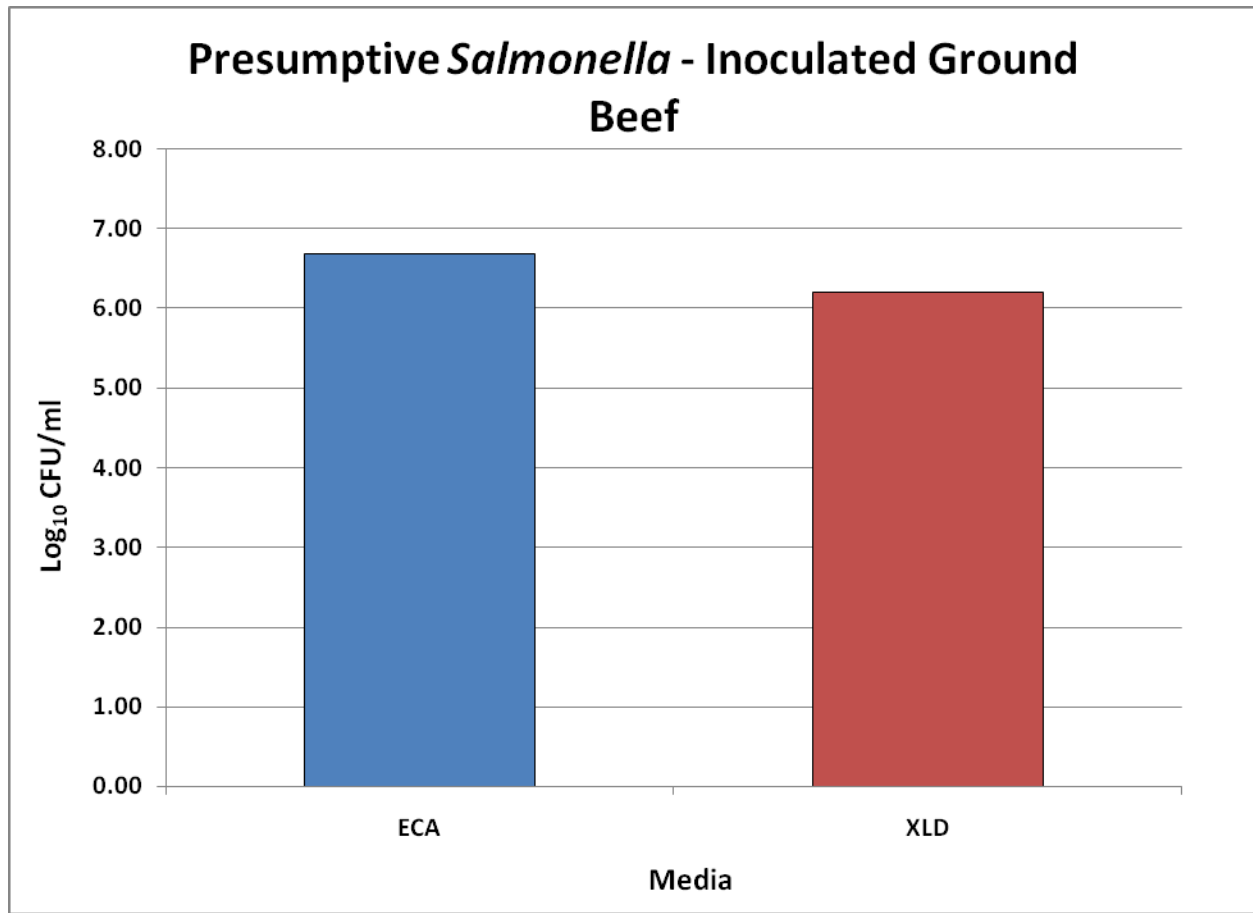
Since there was little microbial growth in the non-inoculated ground beef, total plate counts were performed for all media. In Figure 12, TSA had a significantly higher mean at 3.59 log CFU/ml than any of the other media; and significant differences occurred between ECA and ECCP (1.79 and 1.25 log CFU/ml, respectively) and ECCP (1.25 log CFU/ml) and VRB-MUG (1.90 log CFU/ml). The means for all other comparisons did not result in any statistical disparity. The increased count on TSA was an expected result because of the non-selective nature of the medium. There were no significant differences between the other media, resulting in slightly higher recovery in VRB-MUG and ECA at 1.90 and 1.79 log CFU/ml, than VRB, XLD, and ECCP, recovering 1.63, 1.39, and 1.25 log CFU/ml, correspondingly.

Figure 12 Comparison of the Total Microbial Recovery on Tryptic Soy Agar (TSA), Xylose Lysine Desoxycholate (XLD) and ECA Check® Easygel® Plus (ECA) in Non-Inoculated Ground Beef
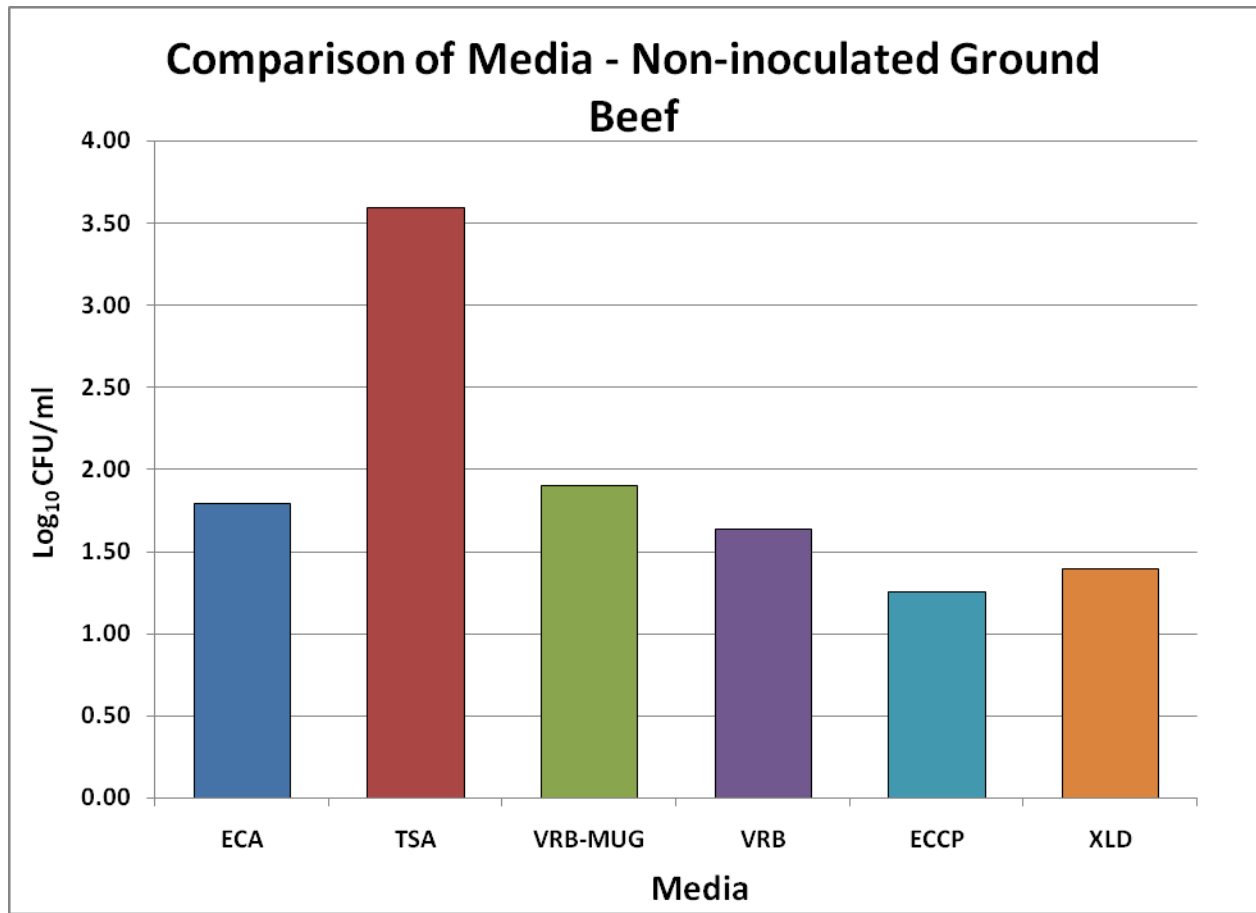
In the ground beef matrix, ECA performed comparably to VRB, VRB-MUG and ECCP in total coliform recovery. ECA also has the ability to visually differentiate the colonies to a greater extent than VRB and VRB-MUG, which may lead to a more accurate count of targeted organisms. ECA also appeared to have more spread between the colonies on the plate, likely from being able to inoculate the room temperature ECA nutrient liquid in the bottle, allowing for more dispersion of the sample, instead of inoculating the Petri dishes and "swirling" the sample using heated agar. Previous studies have indicated that ECC Petrifilm™ may be more capable of recovering *E. coli* from ground meat that was not recovered using the pour plate method, which is supported by the results in this experiment (Linton *et al.*, 1997). *Salmonella* counts are consistently higher using ECA than XLD, and the target colonies did not seem as inhibited by competing bacteria on ECA.

The total recovery in the inoculated ground turkey, shown in Figure 13, again showed significantly higher means on the ECA medium (7.62 log CFU/ml) than on TSA (6.89 log CFU/ml). The same reasons for the ground beef results may also be applied to the ground turkey.

Figure 13 Comparison of the Total Count Recovery on ECA Check® Easygel® Plus (ECA) and Tryptic Soy Agar (TSA) in Inoculated Ground Turkey
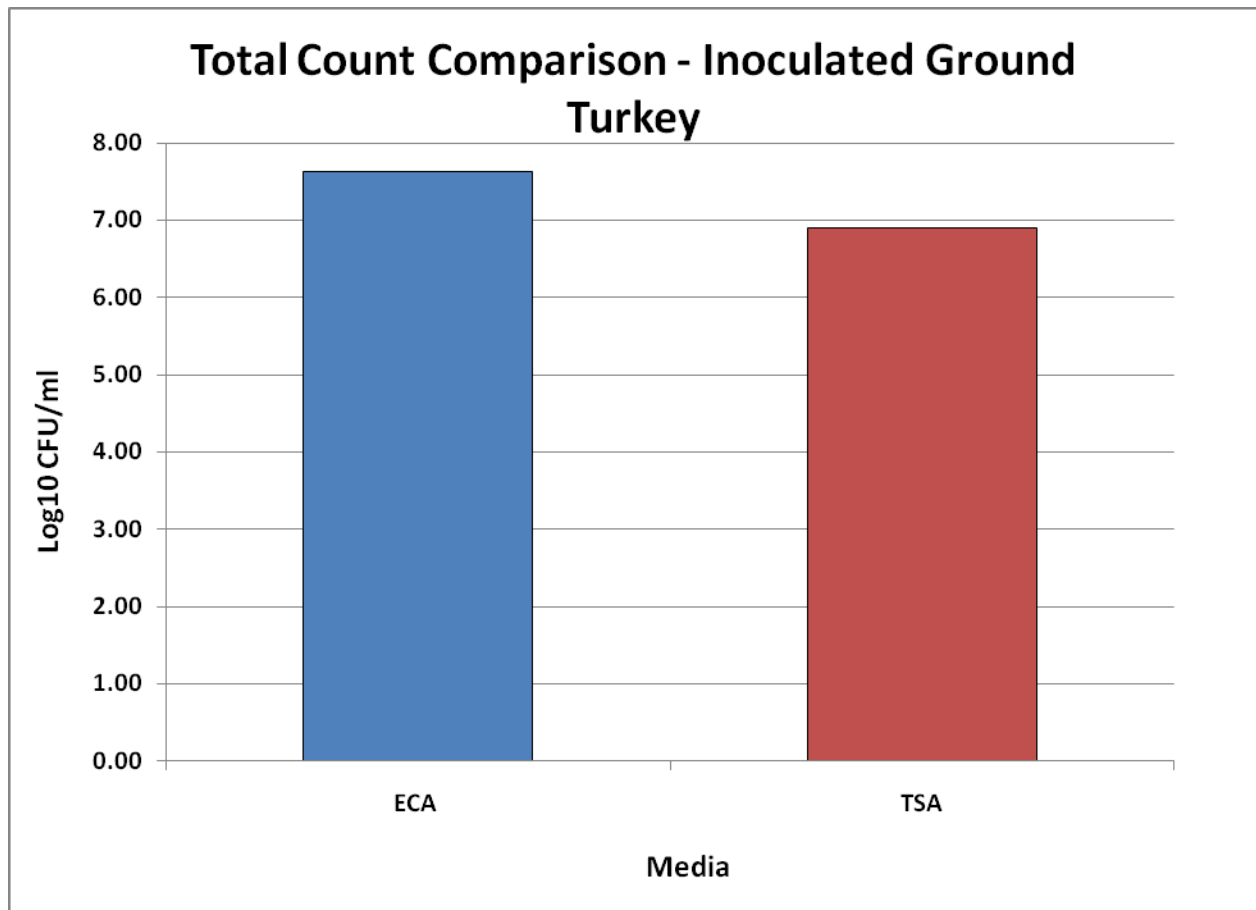
Figure 14, 15, and 16 show correlating results in total coliform recovery on the ground turkey compared to the ground beef samples. Comparison of *E. coli* recovery on ECA revealed slightly higher means by almost 0.5 log CFU/ml than on VRB-MUG, although there was no significant difference statistically (Figure 14). The mean for ECA was 7.15 log CFU/ml and for VRB-MUG was 6.73 log CFU/ml. Non-*E. coli* coliform recovery was a little higher in VRB (6.77 log CFU/ml) than ECA (6.64 log CFU/ml), also with no significant difference (Figure 15). Total coliform recovery on ECA was 7.27 log CFU/ml compared with 7.23, 6.77, and 6.73 log CFU/ml on ECC Petrifilm™, VRB, and VRB-MUG, respectively (Figure 16). There was no statistical difference between ECA and ECCP in total coliform recovery, and no difference between VRB and VRB-MUG; there was a difference between ECA and VRB, and ECA and VRB-MUG.

**Figure 14 Comparison of the Recovery of Presumptive *Escherichia coli* on ECA Check®
Easygel® Plus (ECA) and Violet Red Bile with MUG (VRB-MUG) in Inoculated Ground
Turkey**
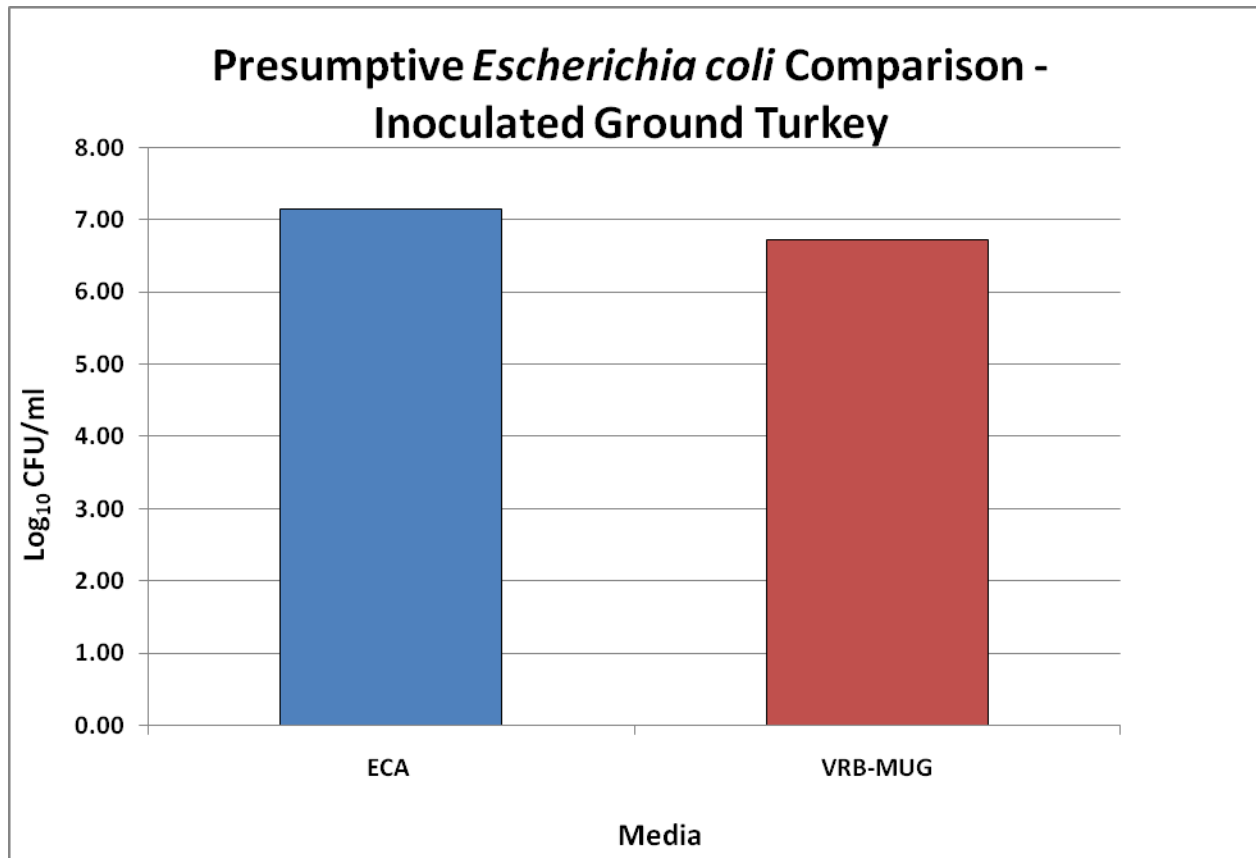
Figure 15 Comparison of the Recovery of Presumptive Non - *Escherichia coli* Coliforms on

ECA Check® Easygel® Plus (ECA) and Violet Red Bile (VRB) in Inoculated Ground
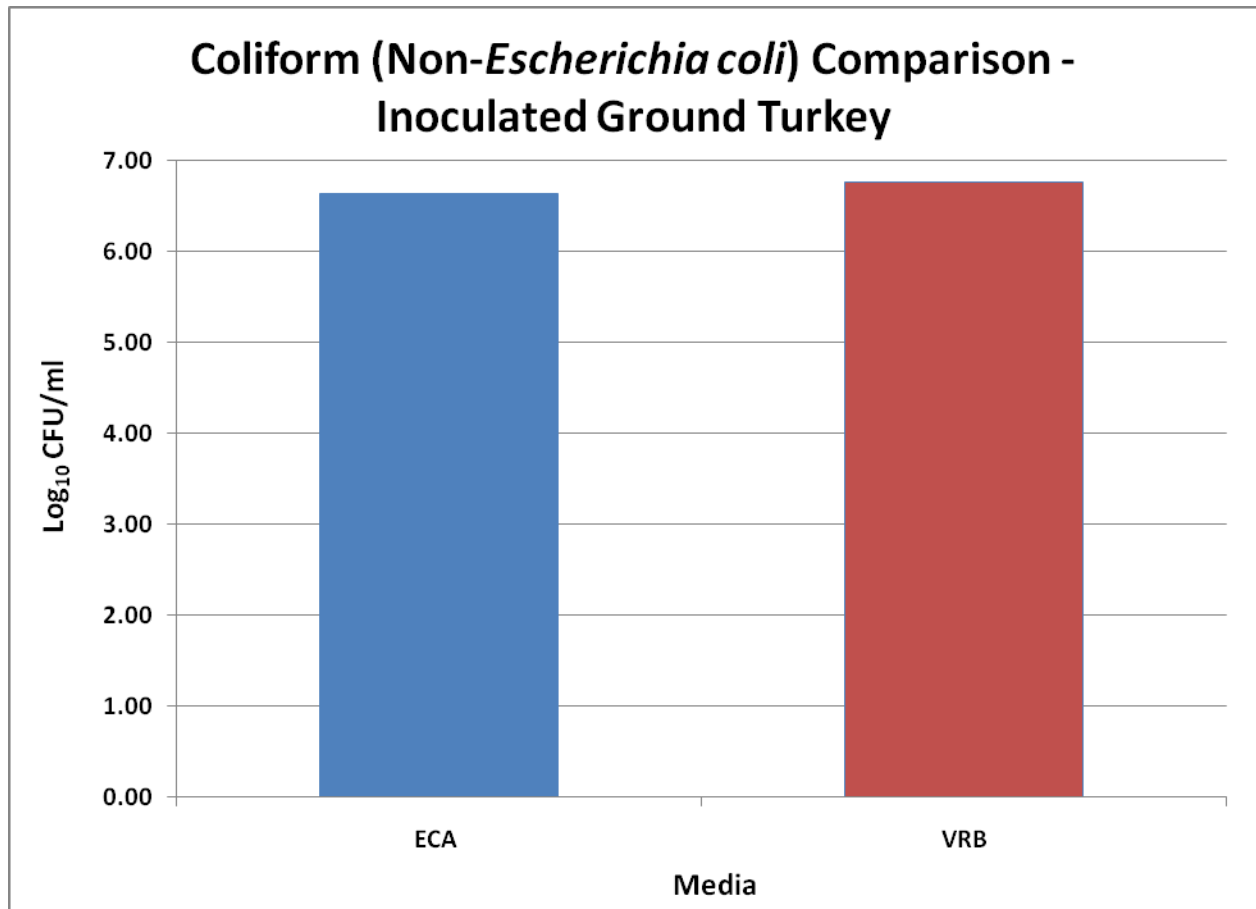
Turkey

Figure 16 Comparison of the Recovery of Total Coliforms on ECA Check® Easygel® Plus (ECA), ECC Petrifilm™ (ECCP), Violet Red Bile (VRB), and Violet Red Bile with MUG (VRB-MUG) in Inoculated Ground Turkey
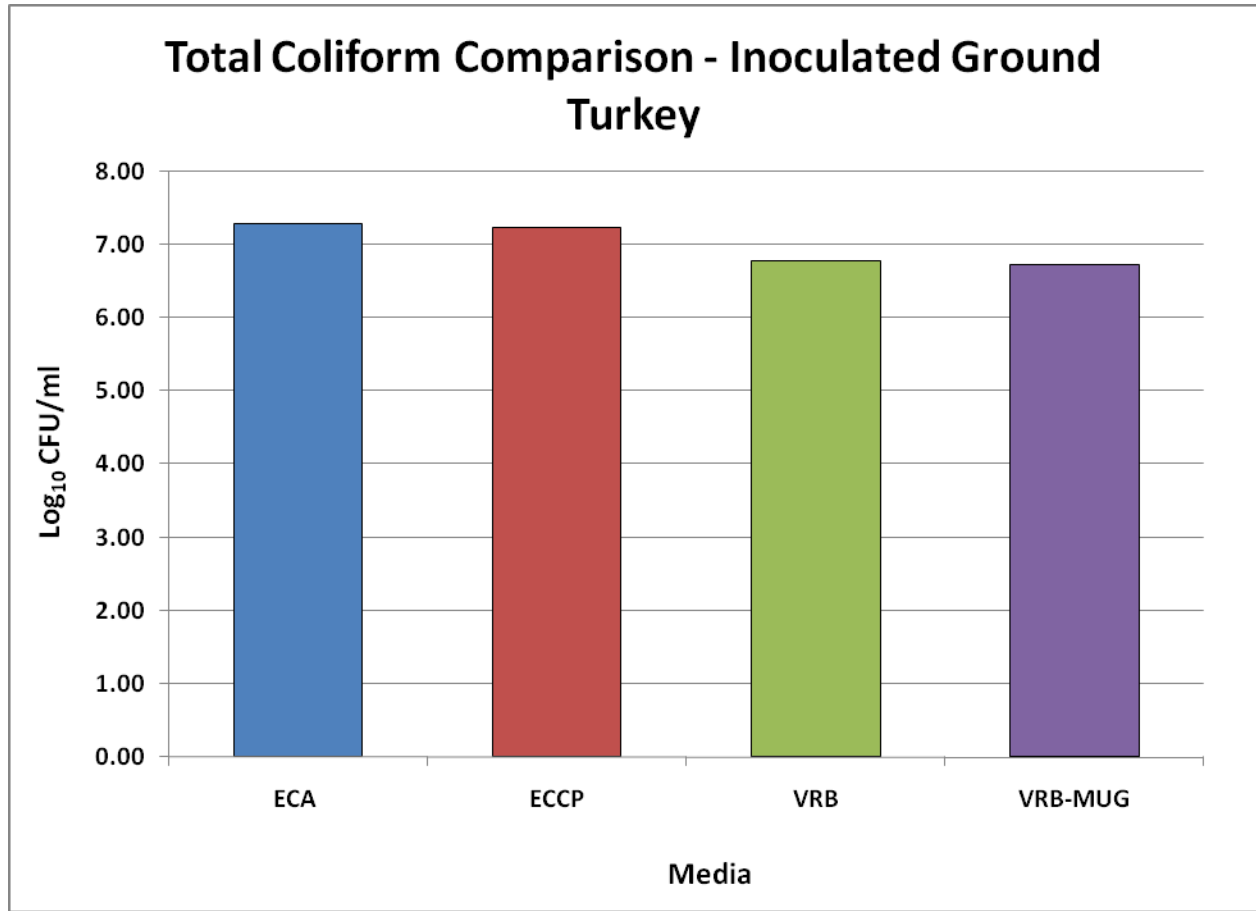
In ground turkey, XLD recovered approximately 1.0 log CFU/ml lower than ECA, which was 6.72 log CFU/ml (Figure 17). This may be contributed to the fact that the competing enteric population would out-compete and overgrow the inoculated *Salmonella* on XLD.

Figure 17 Comparison of the Recovery of Presumptive *Salmonella* Typhimurium on ECA Check® Easygel® Plus (ECA) and Xylose Lysine Desoxycholate (XLD) in Inoculated Ground Turkey
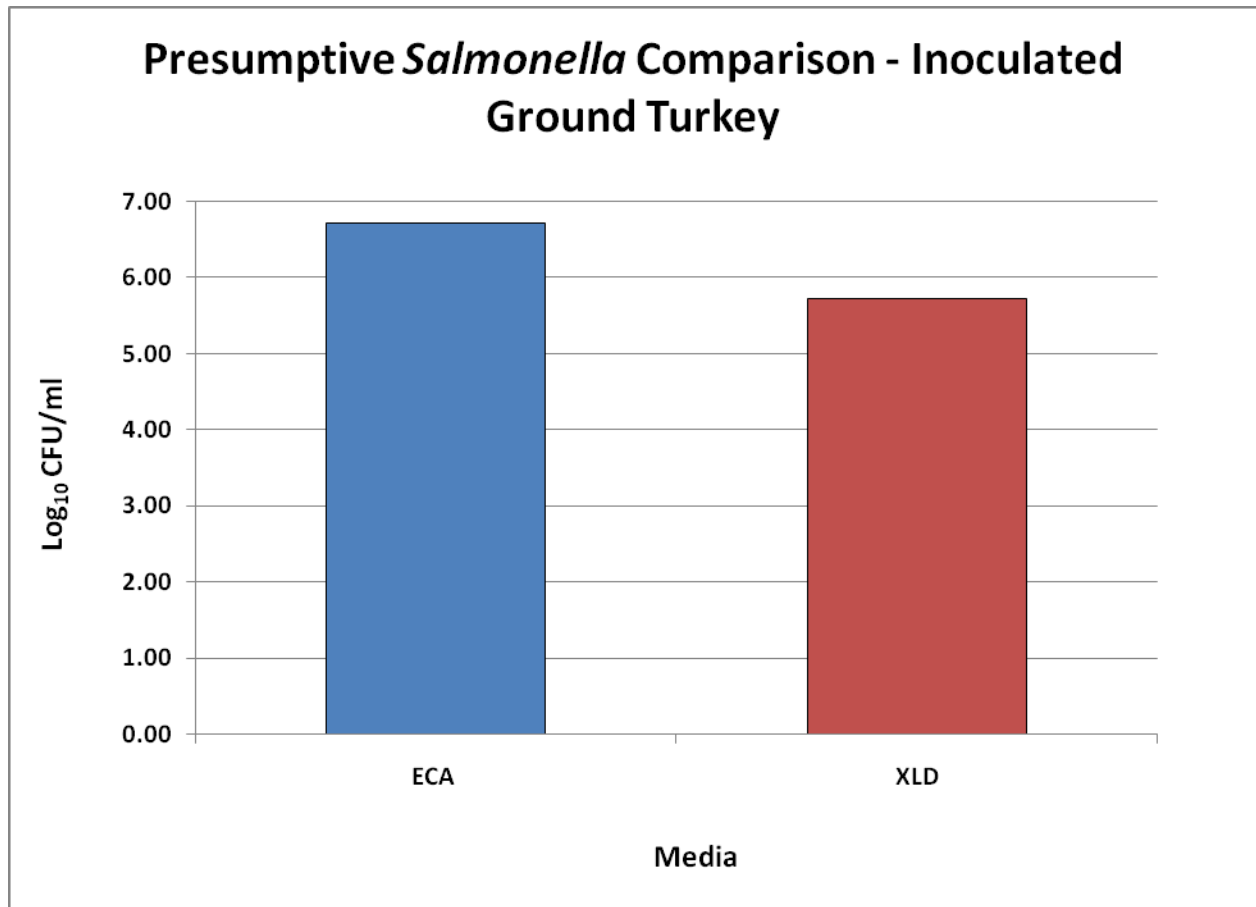
Total plate counts for the ground turkey samples were enumerated for the non-inoculated portion of the study. Figure 18 shows that TSA recovered more bacteria with a mean of 5.78 log CFU/ml, followed by ECA and VRB at 3.19 and 3.13 log CFU/ml, respectively. The means for VRB-MUG, XLD and ECCP were 2.93, 2.48, and 2.13 log CFU/ml. TSA was again significantly higher than all other media tested, and ECA was significantly higher than ECCP. The ECA results for the non-inoculated part of the experiment in both ground beef and ground turkey samples demonstrate that it is capable of growing other bacteria but with greater differentiation.

Figure 18 Comparison of Total Microbial Recovery on Tryptic Soy Agar (TSA), Xylose Lysine Desoxycholate (XLD) and ECA Check® Easygel® Plus (ECA) in Non-Inoculated Ground Turkey
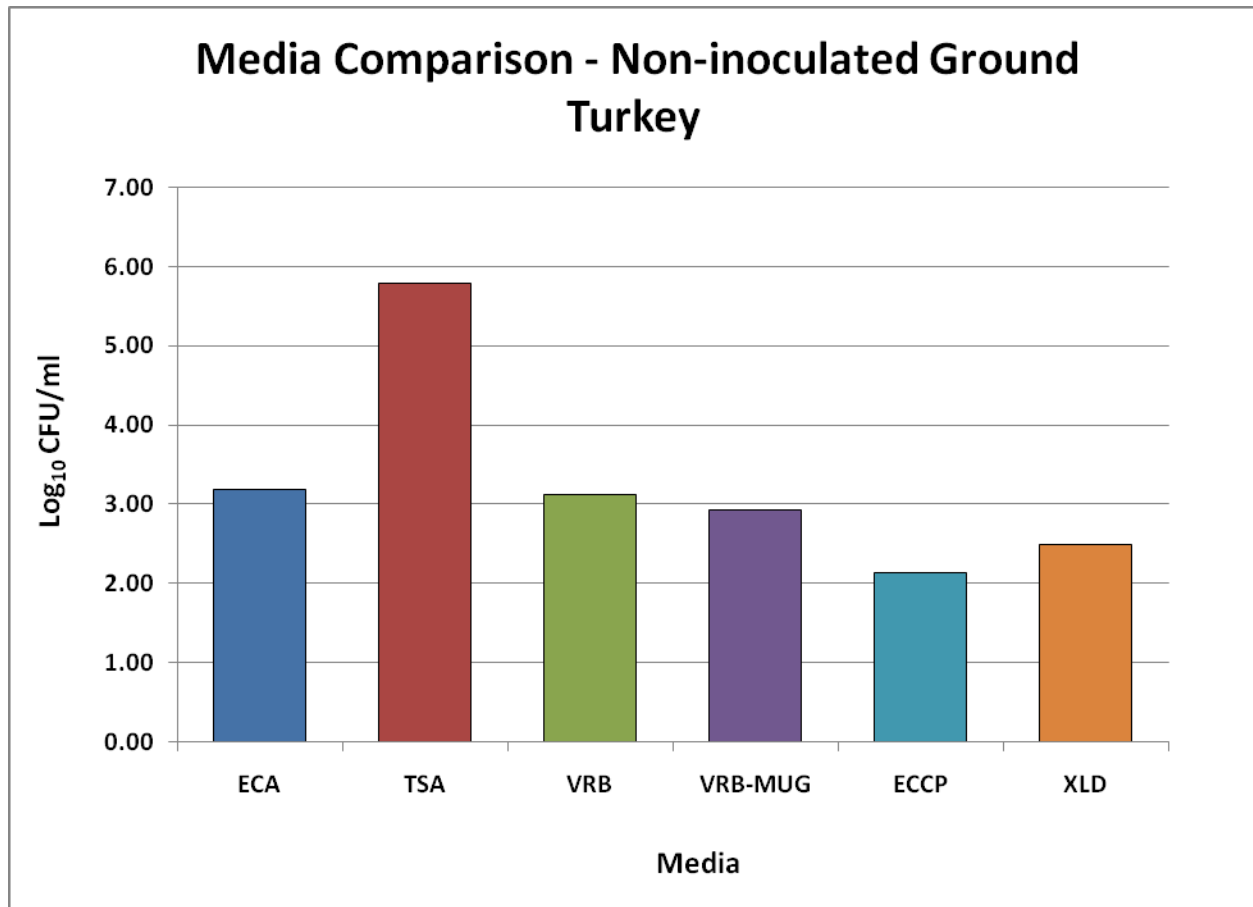
Overall, enumeration on ECA from both food matrices was very comparable to the conventional media. ECA recovered, statistically, as much or more target organisms as the conventional media, and it continuously and significantly recovered more *Salmonella* than XLD throughout the entire study. This may illustrate that *Salmonella* can be easily injured by heat or inhibited by competing microorganisms on XLD.

3.2 Identifications

Identifications performed on select colonies on the ECA medium can be found in Table 3. Out of the typical colonies selected for presumptive *E. coli* confirmation, 100% were confirmed on the inoculated, and 66.7% were confirmed for typical colonies found on the non-inoculated plates. The one exception coded for *Escherichia vulneris*, which may be attributed to a faulty identification kit or environmental mutation. However, if the identification is accurate, *E. vulneris* is an environmental organism found in humans and animals, mainly colonizing around lesions (Senanayake *et al.,* 2006). For both inoculated and non-inoculated parts, 100% of typical coliforms and typical *Salmonella* spp. (inoculated only) were confirmed. One cream-colored colony was selected for confirmation and coded for *Pseudomonas* spp. illustrating that ECA is clearly differential.

Table 3 Identification Results of Colonies Isolated From ECA Check® Easygel® Plus (ECA)

Colony Appearance	Confirmed
Blue color and Blue Fluorescence (inoculated)	(5/5) *Escherichia coli*
Blue color and/or Blue Fluorescence (non-inoculated)	(2/3) *Escherichia coli* (1/3) *Escherichia vulneris*
Purple/Pink (inoculated)	(3/3) *Enterobacter sakazakii* (*Cronobacter* spp.)
Purple/Pink (non-inoculated)	(6/8) *Klebsiella* spp. (1/8) *Enterobacter taylorae* (1/8) *Hafnia alvei*
Green (inoculated)	(3/3) *Salmonella* spp.
Green (non-inoculated)	none found
Other (cream; non-inoculated)	(1/1) *Pseudomonas* spp.

Table 4 contains the results of identification performed on typical and atypical colonies isolated from VRB-MUG. 100% of inoculated and non-inoculated blue fluorescing colonies were confirmed as *E. coli.* Yellow fluorescence was also observed and confirmed as *Klebsiella* spp. and *Serratia marcescens,* which belong to the coliform group and have given false-positives on different fluorescent agars in previous studies (Heizmann *et al.,* 1988)*.* A non-fluorescing colony from a plate with inoculated sample was determined to be either *Enterobacter sakazakii* (*Cronobacter* spp.) or *Enterobacter taylorae.*

Table 4 Identification Results of Colonies Isolated From Violet Red Bile with MUG (VRB-MUG)

Colony Appearance	Confirmed
Blue Fluorescence (inoculated)	(5/5) *Escherichia coli*
Blue Fluorescence (non-inoculated)	(2/2) *Escherichia coli*
Yellow Fluorescence (non-inoculated)	(1/2) *Serratia marcescens* (1/2) *Klebsiella* spp.
Non-fluorescent (inoculated)	(1/1) *Enterobacter taylorae* OR *Enterobacter sakazakii* (*Cronobacter* spp.)

Colonies isolated from the inoculated samples plated on VRB were all confirmed as *E. coli* (Table 5). The non-inoculated colony confirmation yielded *E. coli, Klebsiella* spp., *S. marcescens,* and *Pseudomonas* spp. The *Pseudomonas* spp. is a Gram-negative species that does not belong to the Enterobacteriaceae family, but is a common spoilage organism in refrigerated meat. However, it would be difficult to differentiate the species on VRB without further confirmation with the oxidase test.

Table 5 Identification Results of Colonies Isolated From Violet Red Bile (VRB)

Colony Appearance	Confirmed
Randomly selected Purple and Pink colonies (inoculated)	(8/8) *Escherichia coli*
Randomly selected Purple and Pink colonies (non-inoculated)	(1/6) *Escherichia coli* (3/6) *Klebsiella* spp. (1/6) *Serratia marcescens* (1/6) *Pseudomonas* spp.

All black colonies isolated from XLD were further confirmed as *Salmonella* spp. The yellow colonies that dominated the XLD pour plates with inoculated sample were confirmed as *E. coli* and *E. sakazakii.* Random colonies from the non-inoculated samples were generally confirmed as *Klebsiella* spp., along with *Escherichia* spp. and *S. marcescens* (Table 6).

Table 6 Identification Results of Colonies Isolated From Xylose Lysine Desoxycholate (XLD)

Colony Appearance	Confirmed
Black (inoculated)	(3/3) *Salmonella* spp.
Black (non-inoculated)	none found
Yellow (inoculated)	(6/8) *Escherichia coli* (2/8) *Enterobacter sakazakii*
Randomly selected (non-inoculated)	(1/5) *Escherichia* spp. (1/5) *Serratia marcescens* (3/5) *Klebsiella pneumoniae*

A few isolations were performed on colonies from ECC Petrifilm™ and were confirmed as *E. coli* and *S. marcescens* (Table 7). Random colonies from TSA inoculated samples, which generally appeared cream or white in color, were all confirmed as *E. coli* and a non-inoculated colony was verified as *Klebsiella* spp. (Table 8). Colony confirmation was consistent between the media, and ECA proved extremely accurate in its differentiation.

Table 7 Identification Results of Colonies Isolated From ECC Petrifilm™

Colony Appearance	Confirmed
Blue with gas (inoculated)	(1/1) *Escherichia coli*
Red with gas (non-inoculated)	(1/2) *Escherichia coli* (1/2) *Serratia marcescens*

Table 8 Identification Results of Colonies Isolated From Tryptic Soy Agar (TSA)

Colony Appearance	Confirmed
Randomly selected Cream or White Colonies (inoculated)	(7/7) *Escherichia coli*
Randomly selected Cream or White Colonies (non-inoculated)	(1/1) *Klebsiella* spp.

CHAPTER 4 - Conclusions

The objective of this experiment was to determine if ECA Check® Easygel® Plus is comparable to conventional pour plate media. The stock culture study proved ECA to be as good or better at recovering the targeted microorganisms outside of a food matrix. When recovering organisms in the inoculated experiments using a food matrix, ECA performed equivalent to or better than the typically recommended pour plate media. Rapid method identifications verified the accuracy of the chromogenic and fluorogenic nature of ECA; it was more efficient, easier to prepare and use, and able to provide unmistakable differentiation between colonies. This media eliminates any risk of injuring target cells by excessive heat and reduces the amount of media required to perform testing of multiple enteric organisms.

ECA is designed for use in environmental and food sampling, but has tremendous potential for clinical use as well. Further studies need to be conducted on ECA Check® Easygel® Plus to determine other venues for its application, specificity, and more accurately decide its consistency in recovery. Evaluations using several different food matrices, beverages, various methods for surface sampling, and water quality criteria are recommended. ECA is also able to distinguish *Aeromonas* spp., a major concern in the fish industry and freshwater environments (FDA, 2009). The scope of future experiments should include validation studies to obtain approvals from standardized analytical testing organizations.

Cost for dehydrated selective media used in this study ranged from approximately $0.15-0.50 per plate. However, the time and labor and the amount of media required for multiple organism recovery has made rapid method plating more efficient in the laboratory. ECC

Petrifilm™ costs approximately $1.00 per plate, and ECA Check® Easygel® Plus can be obtained for $1.73 per plate. Although it is more expensive, ECA can differentiate more organisms than ECC Petrifilm™ and only uses one medium as opposed to two or more conventional selective media for recovery of *E. coli, Salmonella* spp., and coliforms.

Rapid identification of pathogenic organisms and organisms that are indicators for potential pathogens is essential for the evolution of food safety and public health. No real limitations of ECA Check® Easygel® Plus came to light during this experiment, and its benefits include ease of preparation, use, enumeration, and differentiation. This study effectively demonstrated its capability and efficiency for use in microbiological testing facilities.

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Appendix A - Alphabetical List of Acronyms and Substrate Definitions

ECA – ECA Check® Easygel® Plus

ECCP – *E. coli*/Coliform Count Petrifilm™

MUG - 4-methylumbelliferyl-β-D-glucuronide (substrate for fluorescence)

Red-Gal® - chromogenic substrate for β-galactosidase activity

TSA – Tryptic Soy Agar

VRB – Violet Red Bile Agar

VRB-MUG - Violet Red Bile Agar with 4-methylumbellifery-β-D-glucuronide

X-GLUC – chromogenic substrate for β-glucuronidase activity

XLD – Xylose Lysine Desoxycholate Agar

X-α-gal – chromogenic substrate for α-galactosidase activity