DEVELOPMENT AND CHARACTERIZATION OF SELECTIVE AND DIFFERENTIAL CULTURE MEDIUM FOR THE POULTRY TRANSMITTED PATHOGEN, *ESCHERICHIA ALBERTII*

A Dissertation

By

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2023

Major Subject: Food Science and Technology

ABSTRACT

Escherichia albertii is an emerging foodborne enteropathogen. It was first isolated in 1991 from a 9-month-old febrile Bangladeshi girl with diarrhea, and retrospectively found to be the causative agent in a human foodborne disease outbreak occurring at the end of May 2011 in Kumamoto, Japan. Previous phenotypic, biochemical, genetic, and microbiological studies have misidentified this under-researched bacterial foodborne pathogen, and this has impeded accurate assessment by food safety scientists and epidemiologists of the burden borne by this organism to U.S. and/or global food safety. Despite enjoying increased attention paid to its pathogenesis, global dissemination, and antimicrobial resistance capacity, this foodborne pathogen remains difficult to identify from human foods. The primary objective of this study was to develop and test a selective and differential plating medium for the isolation of *E. albertii* from enteric pathogens commonly transmitted via fresh poultry meat, namely *E. coli* and *Salmonella enterica*. MacConkey agar was supplemented with α-D-+-melibiose and the lactose analogue X-gal and utilized to differentially enumerate *E. albertii*, *Salmonella*, and *E. coli* from inoculated ground chicken meat. The medium, MXgMac agar, differentiated the inoculated pathogens with a greater degree of efficiency than did the previously developed *E. albertii*-selective medium xylose–rhamnose–melibiose (XRM) MacConkey agar, based on differential usage of the lactose analogue and melibiose. Chicken-derived feces and litter samples were subsequently tested using the medium and found not to contain *E. albertii* by 16S rRNA gene amplification. In conclusion, MXgMac agar facilitates improved differential recovery of *E. albertii* and other enteric pathogens from poultry meat versus other *E. albertii* selective/differential media. It is hoped that this novel cultural medium could be utilized to explore further this pathogen's

virulence mechanisms and potentials, nutrient utilization, stress tolerance capacity, and their regulation.

DEDICATION

This dissertation is dedicated to my wife, Victoria A. Annor, who has been a source of strength, support, patience, and motivation for me throughout this entire experience. I am truly blessed to have you as my partner for life. It's You and Me, Honey.

I Love You with All My Heart!

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Taylor, and my committee members, Dr. Lawhon, Dr. Kerth, and Dr. Pillai, for their guidance and support throughout the course of this research.

I am grateful to Dr. Jason J. Gill, Dr. Sapna C. Dass, and Karla S. Salazar for assistance with technical advice relating genetic screening of presumptive identification of *E. albertii*.

Finally, I acknowledge the assistance I received from Allison Milby, a Ph.D. student at the Department of Poultry Science. She graciously sent me information relating to the chicken fecal/litter samples.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supported by a dissertation committee consisting of Professor Matthew Taylor (advisor), Professor Suresh Pillai of the Department of Food Science and Technology, Professor Christopher Kerth of the Department of Animal Science, and Professor Sara Lawhon of the Department of Veterinary Pathobiology.

The 16S sequencing analysis for Chapter 3 was provided in part by Professor Jason J. Gill of the Department of Animal Science. All other work conducted for the dissertation was completed by the student independently.

Funding Sources

This work was supported by the USDA National Institute of Food and Agriculture (Agriculture and Food Research Initiative Award No. 2017-67017-26227).

NOMENCLATURE

A/E…….. Attaching/Effacing.

BPW…… Buffered peptone water.

CFU/g……. Colony forming units per gram.

CFU/mL……. Colony forming unit per milliliter.

DEC……. Diarrheagenic *Escherichia coli.*

DNA……. Deoxyribonucleic acid.

EHEC…… Enterohemorrhagic *Escherichia coli.*

EPEC ……... Enteropathogenic *Escherichia coli.*

FSIS …… Food Safety and Inspection Service.

kGy…… kiloGray.

LEE…… Locus of enterocyte effacement.

MeV……. Million electron volts.

MLST…….. Multilocus sequence typing.

mRNA…… . Messenger RNA.

PCR…… . Polymerase chain reaction.

PBS…… . Phosphate buffered saline.

RNA…… Ribonucleic acid.

rRNA…… Ribosomal RNA

mTSB…… Modified TSB

TSB……Tryptic soy broth

USDA……United States Department of Agriculture

WGS……. Whole Genome Sequence

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CHAPTER 1: COMPREHENSIVE REVIEW OF RELEVANT LITERATURE

1.1 Introduction

Escherichia albertii is a Gram-negative, rod-shaped, non-spore forming, facultative anaerobe, and a recently recognized member of the family *Enterobacteriaceae*. It is now considered one of the five species belonging to the genus *Escherichia* (van der Putten et al. 2021). This organism was isolated for the first time from a 9-month-old febrile Bangladeshi girl with diarrhea and described as an atypical *eae*-positive *Hafnia alvei* by routine biochemical identification tests, as well as by Edwards and Ewing's criteria for the classification of *Enterobacteriaceae* (Albert et al. 1991; Edwards et al. 1986; Nimri 2013; Oaks et al. 2010). Subsequent phenotypic, genetic, biochemical, and molecular studies (e.g., 16S rRNA sequencing, DNA-DNA hybridization), however, demonstrated that these unique *Hafnia alvei*-like isolates were more physiologically like members of the genus *Escherichia.* In 2003, the name *Escherichia albertii* was proposed to honor John Albert who, with his collaborators, originally isolated and identified the organism in human stool samples in Bangladesh in 1991 (Abbott et al. 2003; Huys et al. 2003). It was retrospectively found to be the likely causative agent in a human foodborne gastroenteritis outbreak occurring at the end of May 2011 in Kumamoto, Japan (Konno et al. 2012; Ooka et al. 2013). Since then, *E. albertii* has been isolated from patients of many foodborne illness outbreaks, including one which occurred in Okinawa, Japan, in 2016, where 217 persons suffered from diarrhea and/or abdominal pain after consumption of a salad (Ikeda et al. 2020; Muchaamba et al. 2022). Presently, infectious disease outbreaks and sporadic cases attributed to this organism have been reported in many countries including Bangladesh, Belgium, Brazil, China, Germany, Guinea-Bissau, Iran, Japan, Mexico, Nigeria, Poland, and the United States, indicating a worldwide distribution (Lima et al. 2019;

Lindsey et al. 2019; Sulaiman et al. 2021; Wang et al. 2021). The consumption of food contaminated with this organism can induce fever, vomiting, abdominal pain, diarrhea, dehydration, and abdominal distention (Albert et al. 1991; Huys et al. 2003; Masuda et al. 2020; Ooka et al. 2012). This effectively classifies *E. albertii* as a foodborne pathogen.

The identification of *E. albertii* is challenging because it shares various biochemical characteristics and some virulence-related genes with DEC. These biochemical features include the inability of both microorganisms to produce hydrogen sulfide (H_2S) in triple sugar iron (TSI) agar, inability to assimilate 2-ketogluconate, the ability to reduce nitrate, and utilize glucose, mannose, and galactose (Abbott et al. 2003; Hinenoya et al. 2019; Stock et al. 2005). *E. albertii* encodes the *eae*-gene, one of the components of the total toxin production, and which encodes for intimin, an integral membrane protein which is also present in EHEC and EPEC. However, in contrast to *E. coli*, almost all reported *E. albertii* isolates carry a *cdt*ABC locus which encodes only the cytolethal distending toxin B gene (c*dt*B). This gene has been shown to encode for cell arrest during the cell cycle, leading to cell distention and, ultimately, cell death (Hinenoya, et al. 2019). In addition, both organisms have similar antimicrobial susceptibility profiles, especially as it relates to drugs like tetracycline, doxycycline, penicillin, oxacillin, amoxicillin, ampicillin, rifampicin, and chloramphenicol (Mac Vane et al. 2017; Stock et al. 2005; Vranic et al. 2016).

E. albertii has also been misidentified either as *E. coli*, *H. alvei* or *Yersinia ruckeri* due to the absence of unique phenotypic markers (Abbott et al. 2003; Albert et al. 1991). Molecular approaches like sequencing analysis of the 16S rRNA gene, which has been previously used to identify this organism, have not been shown to be sufficiently discriminative (Lukjancenko et al. 2010). In terms of whole genome analysis, no large-scale genomic comparisons of multiple *E.*

albertii strains have been carried out and genomic differences between *E. albertii* and other *Escherichia* species have not yet been well elucidated.

With respect to its motility, *E. albertii* has historically been described as non-flagellated. However, a study by Ikeda et al. (2020) showed that swimming motility occurs in *E. albertii* strains when cultured at low osmotic pressure. These researchers further reported that *E. albertii* cells produce flagella and exhibit swimming ability when they are grown at 20 ^{o}C. Another interesting finding of these researchers was that the addition of glutamic acid enhanced the motility of *E. albertii*. They concluded that motility is a prevalent trait among *E. albertii* strains, and that osmotic pressure regulates motility in their cells.

Atmospheric growth needs (e.g., optimal and minimal/maximal growth temperatures) have also been explored in *E. albertii*. Previously, it was believed that growth of *E. albertii* strains was optimum at temperatures ranging from 37 ^{o}C to 42 ^{o}C. However, a recent study by Wakabayashi et al. (2021) showed incubation at 44 ^{o}C offered the best compromise between selection efficiency and robust growth of *E. albertii*. In this study, *E. albertii* strain #24H18 was cultivated in mTSB at 37, 40, 42, 44 or 46 °C. Researchers observed that the most vigorous growth occurred at 40 and 42 °C, followed by 37 and 44 °C. Growth was completely inhibited at 46 °C. The experiment was repeated with other strains of *E. albertii* and different strains of *Enterobacteriaceae*. Based on these data, they concluded that incubation at 44 °C would be desirable for growth as well as selection efficiency of this pathogen.

Does *E.albertii* have the capacity to activate stress responses like acid tolerance, cold shock protein, cold tolerance, etc.? To answer this question, Sharma et al. (2007) conducted studies to evaluate *E. albertii*'s tolerances to heat, acid and pressure and found them to be diverse with respect to processing intervention between experimental strains, and significantly less

tolerant to processing interventions than isolates of *E. coli* O157:H7. This was interpreted as an indication that measures used to kill *E. coli* O157:H7 should be sufficient to inactivate *E. albertii*. Citing Bhagwat et al. (2006), these researchers further argued that the diversity of acid tolerance responses in strains of *E. albertii* suggests that the acid response may be based on the functional heterogeneity of stress response genes.

During food production and storage, many foodborne bacteria encounter adverse environmental conditions and so have developed an array of mechanisms for coping with stress and adapting to such adverse environments. One of these mechanisms is the induction of cold shock proteins (Csps), multifunctional RNA/DNA binding proteins that are characterized by the presence of one or more cold shock domains (CSD). Many bacteria produce small cold shock proteins as a response to rapid temperature downshift. During cold shock, the cell membrane fluidity and enzyme activity decrease, and the efficiency of transcription and translation is reduced due to stabilization of nucleic acid secondary structures. Csps are believed to counteract hostile environmental effects by acting as nucleic acid chaperones that can prevent the formation of secondary structures in mRNA at low temperatures. They have been found to be linked to osmotic, oxidative, starvation, pH and ethanol stress tolerance as well as to host cell invasion (Keto-Timonen et al. 2016). On the other hand, to survive a heat shock, organisms activate a process known as the heat shock response (Lindquist and Craig 1988). Bacterial cells sense elevated temperatures and mount an adaptive heat shock response that involves changes in gene expression. It is believed that this stress response process represses expression of housekeeping proteins and promotes production of stress-protective proteins (Causton et al. 2001; Gasch et al. 2000). The heat shock response (HSR) refers to the activation of the expression of HSPs, and, like the cold shock response, it involves changes in the level of both transcription and translation.

All bacterial cells possess an internal stress response to cope with environmental and pathophysiological challenges. The cold/heat-response concept has been a central issue and studied extensively in *E. coli,* a member of the *Enterobacteriaceae* family. It describes a specific pattern of gene expression in response to abrupt shifts to lower or higher temperatures. This pattern includes the induction of cold-shock proteins, synthesis of proteins involved in transcription and translation, and repression of heat-shock proteins. Given that both Csps and HSPs are central to the cellular proteostasis network and that all organisms ranging from bacteria to plants and mammals have genes encoding for them, it is assumed that *E. albertii* cells encode and express the genes for these proteins even though there is currently no known research on this.

Capsules are important virulence determinants which enable pathogenic bacteria to evade or counteract unspecific host defenses during the early (preceding the immune response) phase of infection. They act by interfering with the action of complement and phagocytes. Extraintestinal *E. coli* are generally encapsulated. Curli, on the other hand, are a class of highly aggregated, extracellular fibers expressed by *Escherichia* and *Salmonella* spp. that are involved in cell aggregation, biofilm formation, mediation of host adhesion, and invasion (Austin et al. 1998; Vidal et al. 1998) and in the mediation of binding to a variety of host proteins (Ben Nasr et al. 1996). They were first discovered in the late 1980s in *E. coli* strains that caused bovine mastitis and have since been implicated in many physiological and pathogenic processes of *E. coli* and *Salmonella* spp. (Barnhart et al. 2006). Currently, not much is known about the ability of *E. albertii* to produce curli.

1.2 Biochemical Properties

Biochemically, *E. albertii* closely resembles *E. coli* in that they are both oxidase and sucrose negative, ferment D-glucose to acid (with gas), utilize D-mannitol, D-arabinose, D-fructose, D-galactose, D- mannose, and L-arabinose. Strains of both *E. albertii* and *E. coli* do not produce lipase, protease, or pectinase (Abbott et al. 2003; Nimri 2013). On the other hand, both differ in some biochemical properties, and this helps to differentiate the two. For example, unlike *E. coli*, *E. albertii* is reportedly indole negative but ribose positive (Abbot et al. 2003; Murakami et al. 2019). Oaks et al. (2010) reported isolates of *E. albertii* do not utilize citrate as a sole carbon source and do not produce arginine decarboxylase, hydrogen sulfide, urease, tryptophan deaminase, acetoin, or gelatinase. Also, some strains of *E. coli* can ferment D-sorbitol within 24 hours (the only one among the *Escherichia* genus) while *E. albertii* strains do not. *E. albertii* strains can be biochemically distinguished/differentiated from other *Escherichia* species by other tests. For instance, strains of *E. albertii* are unable to grow in potassium cyanide (KCN) broth, utilize malonate, and produce gas from D-xylose and D-arabitol (Abbott et al. 2003; Grillova et al. 2018; Nimri 2013). A unique biochemical characteristic of *E. albertii* is that after prolonged incubation of 3 to 7 days at 35-37 ^{o}C, some strains produce acid from glycerol (Abbott et al. 2003). A specific distinguishing characteristic is *E. albertii's* general inability to ferment lactose, D-sorbitol, D-xylose, L-rhamnose, melibiose and dulcitol (Oaka et al. 2015). In addition, *E. albertii* does not produce beta-D-glucuronidase (Donnenberg et al. 1993). The most distinguishing biochemical process between the two *Escherichia* species is *E. albertii*'s inability to ferment D-sorbitol, a trait that is strongly associated with *E. coli* (Grillova et al. 2018). Some other metabolic processes of *E. albertii* are its ability in some strains to ferment sucrose. It was previously known that *E. albertii* tested negative for sucrose

fermentation; however, recently it was found that close to 19% tested positive for sucrose fermentation and beta-galactosidase (Oaka et al. 2015). The strains also fermented D-arabinose, D-fructose, D-galactose, D-mannose, and ribose, but were unable to fertilize D-fucose, palatinose, sedoheptulose anhydride, L-sorbose, D-tagatose, D-turanose, and xylitol (Grillova et al. 2018).

1.3 Pathogenesis and Molecular Characteristics

E. albertii belongs to the so-called A/E group of pathogens, like the EPEC. This group possesses a LEE-encoded type III secretion system and can form A/E lesions on intestinal epithelial cell surfaces by the combined action of intimin (a 94-kDa outer membrane protein which mediates intimate attachment of bacteria to epithelial cells), an *eae* gene-encoded outer membrane protein, and type III secretion system effectors. Researchers believe that the pathogenesis of this organism depends on its ability to adhere to epithelial cells with the formation of A/E lesions; this is achieved through the dual activity of the type III secretion system (T3SS) effectors and intimin (Gomes et al. 2020; Ooka et al. 2013). The A/E lesions formed on human intestinal epithelial cells are generally believed to be the cause of diarrhea (Asoshima et al. 2003; Oaka et al. 2012; Yamamoto et al. 2017). It has been hypothesized that *E. albertii* initiates disease through the formation of A/E lesions in a similar fashion to *E. coli* O157:H7 (Huys et al. 2003). After the formation of A/E lesions, the LEE encodes a T3SS which is a molecular machinery that injects several effector proteins into the host cell cytosol and this is believed to drive infection forward, producing a risk for more severe disease (Gomes et al. 2020). Over 44 potential virulence factors have been discovered in *E. albertii* (Luo et al. 2021; Masuda et al. 2020; Ooka et al. 2015; Wang et al. 2022) and are believed to contribute towards

invasion, cytolysis, stress tolerance, immune response evasion, and virulence factor translocation.

In addition to intimin, *E. albertii* possesses cytolethal distending toxin (CDT), a conserved bacterial genotoxin that works by blocking cell cycle progression, leading to apoptosis (programmed cell death) of a broad range of mammalian cell lineages (Toth et al. 2003; Wang et al. 2016). The *E. albertii* cytolethal distending toxin (Eacdt) genes encode CDT which comprises 3 different subunits: CdtA, CdtB, and CdtC (Grillova et al. 2018). Studies have shown that CdtA and CdtC are necessary for translocating the virulence factor CdtB into the host cell (Yamasaki et al. 2006). However, it is not clearly stated in the literature if all three are needed to form a functional CDT even though genetic studies have indicated that all three genes are necessary to transfer full activity to a noncytotoxic *E. coli* strain (Lara-Tejero et al. 2001). Although the importance and role played by CDT in *E. albertii* pathogenesis is not yet fully understood, studies have shown that it is linked with persistent colonization and invasion, which, in turn, affects disease severity (Lima et al. 2019). Scuron at al. (2016) proposed that Cdt is a unique and potent virulence factor capable of acting as a tri-perditious toxin that impairs host defenses by: (1) disrupting epithelial barriers; (2) suppressing acquired immunity; and (3) promoting pro-inflammatory responses. This well-known virulence factor has also been demonstrated to disrupt tight junctions between gut epithelial cells, which ultimately results in diarrhea onset (Donato et al. 2008; Pickett et al. 2004; Yamamoto et al. 2017). Shiga toxins (*Stx)* are probably the most important virulence factors of *Stx*-producing *E. coli* in human infections. *E. albertii* is a potential Shiga toxin 2 (stx_{2a} and stx_{2f})-producing bacterium (Bhatt et al. 2018; Brandal et al. 2015). Brandal et al. (2015) demonstrated that *E. albertii* has the capability to

carry virulence characteristics (including *stx*$_{2a}$) that are associated with severe illness in infected patients.

Biofilms are defined as complex communities of microorganisms that adhere to biotic or abiotic surfaces and are confined in an extracellular matrix (Costerton et al. 1999; Fleming et al. 2010). Biofilm formation is an important adaptation and survival strategy commonly employed by bacteria. Bacteria form them in response to environmental stresses such as UV radiation, desiccation, limited nutrients, extreme pH, extreme temperature, high salt concentrations, high pressure, and antimicrobial agents. Microbes living in biofilms can resist the actions of antibacterial agents such as antibiotics, antibodies, and phagocytic cells and the mechanical movements exerted by intestinal peristalsis (Beloin et al. 2008). The production of biofilms can assist in niche colonization and persistence in food processing plants. Studies have revealed that some, but not all, strains of *E. albertii* produce biofilms (Hernandes et al. 2013; Lima et al. 2019; Oaks et al. 2010).

1.4 Antimicrobial Resistance Characteristics

Antimicrobial resistance is a phenomenon encountered often among the family *Enterobacteriaceae* (Laxminarayan et al. 2013). Multiple strains of *E. albertii* have been demonstrated to be resistant to significant number of important antibiotics including tetracycline, macrolides (except for azithromycin), ampicillin, penicillin G, oxacillin, gentamicin, ciprofloxacin, trimethoprim, sulfamethoxazole, fusidic acid, rifampicin, meropenem, and norfloxacin (Jafri et al. 2014; Li et al. 2018; Luo et al. 2021; Vranic et al. 2016). Li et al. (2018) reported that *E. albertii* demonstrated antibiotic resistance, with the greatest percentage of strains being tetracycline resistant. On the other hand, Perez et al. (2013) previously reported that *E.*

albertii isolates were resistant to tetracycline and that strains cultured on raw ground beef were sensitive to cephalosporins and chloramphenicol. MacVane (2017) also reported an increasing prevalence of resistance to β-lactam antibiotics among members of the family *Enterobacteriaceae*. β-lactam antibiotics are a class of antibiotics consisting of agents that contain a beta-lactam ring in their molecular structures. This includes penicillin derivatives, cephalosporins, monobactams, carbapenems and carbacephems. Li et al. (2018) investigated the antibiotic resistance and resistance genes *in E. albertii* and were able to identify the co-occurrence of β-lactamase and mobilized colistin resistance (MCR-1) encoding genes in this organism. They reported that some isolates of *E. albertii* from humans, animals, and raw retail meats exhibited resistance to four antimicrobials: piperacillin, ampicillin, cefotaxime, and cefepime. MCR gene confers plasmid-mediated resistance to colistin, one of several last-resort antibiotics for treating Gram-negative infections. MCR-1, the original variant, is capable of horizontal transfer between different strains of bacterial species. Like *E. albertii*, *E. coli* is known to be highly resistant to antibiotics like ampicillin and amoxicillin (Jafri et al. 1014; Vranic et al. 2016). The similarities in antibiotic resistance and susceptibility patterns between species of *Eschericia* make it unlikely that antimicrobial drugs would be useful in the formulation of differential and selective culture media for *E. albertii.*

1.5 Identification and Characterization Methodologies

The accurate isolation and identification of enteropathogenic *E. albertii* has been challenging primarily because it has similar biochemical and molecular characteristics with DEC. Historically, it has been isolated, identified, and characterized by utilizing biochemical tests and performing genetic identification based on 16S rRNA gene sequencing and PCR

analysis targeting the *stx, eae, uidA, lysP, mdh, and cdt* genes (Konno et al. 2012; Lindsey et al. 2017; Oh et al. 2012). Other diagnostic PCR methods developed to differentiate *E. albertii* strains from EPEC were not to be specific enough and so were not optimal for the detection of the pathogen in food samples (Maeda et al. 2014). For example, Hyma et al. (2005) used multiplex PCR to distinguish members of the *E. albertii* lineage from *E. coli*. Based on nucleoside polymorphisms of tested housekeeping genes (*lysP* and *mdh* in the *E. albertii* lineage), multiplex PCR was once a widely used molecular biology technique for amplification of multiple targets in a single PCR experiment. This method is independent of biochemical and antigenic characteristics and therefore can provide a more reliable method of screening *E. albertii* strains (Hyma et al. 2005). However, Lindsey et al. (2017) reported that this technique was inherently complex in its design, implementation, and optimization. In addition, successful design of multiplex PCR assays required investing time and resources toward optimizing and validating the assay.

To identify and detect *E. albertii* rapidly from other *Enterobacteriaceae* and directly screen for *E. albertii* in various samples such as food, water, and human and animal feces, Ooka et al. (2015) developed a nested PCR, a technique that involves the use of two primer sets and two successive PCR reactions. The first set of primers are designed to anneal to sequences upstream from the second set of primers and so are used in an initial PCR reaction. It is thus a modification of PCR intended to reduce non-specific binding in products due to the amplification of unexpected primer binding sites. In this study, the researchers also sequenced the genome of 29 *E. albertii* strains (3 complete and 26 draft sequences) isolated from multiple sources and performed intraspecies and intragenus genomic comparisons. Even though nested PCR is a modification of PCR that was designed to improve sensitivity and specificity, this technique has

a major limitation; the increased potential for contamination, particularly if the first round of PCR products is handled manually for dilution and transfer.

To facilitate rapid identification and differentiate *E. coli*, *E. albertii*, and *E. fergusonii* in a single tube, Lindsey et al. (2017) developed a rapid multiplex PCR. In this study, species-specific primers were incorporated into a conventional multiplex PCR assay and its performance was assessed with a collection of 97 *Enterobacteriaceae* strains. Primers were generated by analyzing the WGS assemblies of 150 *Enterobacteriaceae* genomes and found to be 100% specific when tested in the laboratory against 97 known isolates of *Enterobacteriaceae* species. Based upon these results, they concluded that this technique was much faster, more sensitive, and less labor intensive than the existing methods (i.e., the use of multiple phenotypic and genetic tests). However, it was still considered not sufficiently discriminative for the differentiation of *E. albertii* from other *Enterobacteriaceae*. Other techniques to differentiate *E. albertii* from *E. coli* have been suggested as well. Murakami et al. (2014) reported that genotyping was helpful in the identification of *E. albertii* and that species-specific PCR based on MLST was particularly useful for the confirmation of *E. albertii* strains. Additionally, Hinenoya et al. (2017) reported that multilocus sequence analysis can differentiate *E. albertii* from *E. coli*. According to Muchaamba et al. (2022), WGS is the gold standard for identification and further characterization of *E. alberrtii* and must always be utilized especially in outbreak situations.

1.6 Detection of *E. albertii* from Food Products and Food Processing Environments

E. albertii has been isolated from human hosts living on virtually every continent (Huys et al. 2003). It has been isolated from domesticated cats, pigs, and environmental samples (including a drinking water distribution system of a hospital in Budapest, Hungary) and has been

found as a contaminant of various raw materials (carcasses) of animal origin, mainly poultry-based products (Felfoldi et al. 2010; Konno et al. 2012; Nimri 2013). In China, Wang et al. (2016) isolated this enteropathogen from a variety of retail meat samples and from chicken and duck intestines collected in Zigong City, Sichuan Province. Maeda et al. (2015) isolated *E. albertii* from retail poultry food and concluded "this study supports the hypothesis that chicken products might be a potential vehicle for *E. albertii* transmission". Multiple other studies have demonstrated that this pathogen can be transmitted through other food vehicles including lettuce, turkey, raw chicken, ground beef, and dairy products (Lindsey et al. 2014; Saad et al. 2012). Despite all these findings, the contribution of *E. albert*i to foodborne illness in the United States is yet not fully understood, primarily because of the inherent difficulties in discriminating it from other *Enterobacteriaceae*, especially *E. coli* (Bhatt et al. 2018; Ooka et al. 2012).

Lindsey et al. (2015) reported that in the United States this organism has been recovered in a small percentage (1.6%) of chicken carcass rinse samples at slaughter, an indication that poultry may be a vehicle for human exposure. They described a study which was designed to determine if *E. albertii* was present in chicken carcass rinse samples from federally inspected poultry harvesting facilities and to characterize recovered isolates. 1.0 mL of chicken carcass rinse was diluted with 9.0 mL of BPW and a sterile cotton swab was used to streak it directly onto Chromagar EEC (Hardy Diagnostics, Santa Maria, CA) plates and incubated at 42 ^{o}C for 18 to 24 hours. Colonies isolated were tested by PCR for the presence or absence of *clpX*, *lysP*, *mdh*, and *eae* genes.

Maeda et al. (2015) conducted a study to detect *E. albertii* from chicken meat and giblets by using PCR followed by MLST (to confirm positive isolates of *E. albertii*). Chicken meat samples, collected from 10 supermarkets, were incubated in 9.0 mL BPW; aliquots (of each

BPW culture) were then centrifuged at 13,800 × g for 5 min. The supernatant was used as a template for a PCR assay to detect *E. albertii* using primer pair *lys*P107F/*lys*P358R. To isolate the pathogen, the remainder of the BPW cultures of the PCR-positive samples were streaked onto deoxycholate-hydrogen sulfide-lactose agar and incubated at 37 °C overnight. Presumptive *E. albertii* colonies (white, due to the inability of the organism to ferment the sugars lactose and sucrose) were isolated on nutrient agar plates and then re-tested using PCR. The PCR-positive isolates were subsequently examined for fermentation of glucose, motility, and H_2S production on TSI agar and sulfide indole motility medium agar. Isolates with positive glucose fermentation, negative motility, and negative H_2S production test results were then confirmed as *E. albertii* using MLST.

Currently, the most widely used protocol for identifying *E. albertii* is to initially screen isolates by PCR for some specific genes (e.g., *lysP* and *mdh*) and find those that are positive. *E. albertii* is usually positive for the *eae* and *cdtB* genes but negative for *stx1*, *stx2*, and *sta* (Hyma et al. 2005; Lindsey et al. 2014; Toth et al. 2003). Other employed techniques rely on the application of a variety of phenotypic and genetic tests. For example, to facilitate accurate identification of *E. albertii* from *E. coli*, Lindsey et al. (2017) developed a multiplex PCR targeting conserved, species-specific genes. These researchers believed that, not only was the assay 100% sensitive and specific for detecting the expected species, but it also offered a quick and accurate strategy for differentiating the two organisms. In previous studies, the *uidA* gene had been used for the identification of *E. coli* strains since this gene is a specific housekeeping gene in *E. coli* (Konno et al. 2012). The phenotypic features among some strains of *E. albertii* appear to be significantly variable and, for this reason, classic phenotypic identification is unlikely to be reliably completed.

1.7 Microbiological Tools for Culture-Based Recovery and Detection of *E. albertii*

Even though various biochemical properties of *E. albertii* have been characterized, there are currently only a handful of known microbiological media designed for selective and differential identification for this foodborne pathogen. One is the chromogenic mEA (*E. albertii* medium) agar, a selective and differential medium reportedly developed by Maheux et al. (2018) for the recovery of both lactose-positive and -negative *E. albertii* strains from clinical samples. This medium, which contained all the basic constituents of a microbiological medium (e.g., peptone as a source of carbon, nitrogen, vitamins, bile salts, etc.), also contained cellobiose (a carbohydrate that can be fermented at elevated temperatures). An advantage of this medium was that it was designed to isolate both lactose-positive and -negative and indole-positive and -negative *E. albertii* strains. Also, the inclusion of cellobiose and peptones as carbon sources circumvents the lactose and sucrose fermentation challenges. A big drawback, however, was that an indole spot test and a specific PCR were required to select and identify lactose-positive and-negative-*E. albertii* strains in clinical specimens. Most importantly, *E. albertii* and EHEC O157:H7 could not be differentiated by this medium.

Hinenoya et al. (2020) recently developed XRM-MacConkey agar, a selective medium for the isolation of *E. albertii*. XRM-MacConkey is a modified MacConkey agar supplemented with xylose (X), rhamnose (R), and melibiose (M) instead of lactose. It was developed based on the utilization of different sugars between *E. albertii* and *E. coli*. This new selective medium offered some advantages; it showed much better isolation efficiency than MacConkey and mEA agars and it could differentiate *E. albertii* from *E. coli* including EHEC O157:H7. Notable limitations of XRM-MacConkey agar, however, were that it could not clearly distinguish *E. albertii* from *Shigella spp*. and that it was not 100% specific. As a result of these limitations, the

researchers recommended that both XRM-MacConkey and *E. albertii*-specific PCR be used for the isolation and accurate identification of *E. albertii*.

1.8 Gaps in the Literature Relating to *E. albertii*

E. albertii is an emerging foodborne enteropathogen but very little is known about its pathogenic potential and clinical relevance. Many knowledge gaps, which are needed to adequately identify and mitigate the risks posed by this organism, still exist. Critical information like mortality and morbidity rates, infectious dose, predisposing factors, and epidemiology (prevalence, new case incidence rates, etc.), full characterization of *E. albertii* strains growth capacity on different food products, and hurdle technology resistance, are largely unavailable. Protocols that employ PCR-based techniques have been published using a small number of strains from limited resources and/or geographical regions. Therefore, primers used for these PCR techniques might be only selective for these strains, and thereby missing strains from other alleles of the target genes (Muchaamba et al. 2022). To solve these problems, a more diverse strain collection needs to be included in future studies and publications. Much remains to be explored regarding this pathogen's virulence mechanisms and potentials, nutrient utilization, stress tolerance capacity, and their regulation.

1.9 Rationale and Significance of Research

The Centers for Disease Control and Prevention (CDC) estimates that 48 million cases of foodborne illnesses (equivalent to 1 in 6 Americans), 128,000 hospitalizations, and 3,000 deaths occur annually in the United States (CDC 2020). The World Health Organization (2015) estimated the global burden of foodborne disease at more than 600 million cases and 420,000

deaths yearly. The implication is that every year, nearly 10% of people around the world fall ill after eating contaminated food. Additionally, the World Bank (2019) reported on the economic burden of the foodborne diseases, indicating the total productivity loss associated with foodborne disease was estimated at US$95.2 billion annually. Clearly, *E. albertii* represents a largely under-studied emerging pathogen with respect to U.S food safety. While various studies relating to the biochemical characteristics useful for identification of *E. albertii* have been undertaken, much more needs to be done about this organism. Firstly, no selective and/or differential microbiological culture medium with high specificity and sensitivity has been reported for the isolation and presumptive identification of this foodborne pathogen. As a result, this emerging pathogen has been routinely misidentified by standard identification techniques as EPEC, atypical *Hafnia alvei*, or *Shigella* spp. (Abbott et al. 2003; Lindsey et al. 2014). Commercial identification kits relying on its biochemical attributes have often misidentified *E. albertii* as *E. coli* or even *Salmonella enterica* (Huys et al. 2003). Secondly, there are currently only limited data on the prevalence and/or geographical distribution of this organism in the U.S food system which makes it hard to establish the full extent of its public health significance. A comprehensive *E. albertii* surveillance is needed to provide valuable knowledge for the development of intervention and control strategies. Therefore, the hypothesis for this study is that misidentification of *E. albertii* does not allow for the accurate assessment by food safety scientists and epidemiologists of the burden borne by this organism to U.S food safety.

In recognition of the potential role this recently recognized pathogen could play in human food production and processing systems and the limits of currently used techniques for its isolation and identification, it is imperative that reliable techniques be developed to aid in its identification from foods. Therefore, there is the need to develop tools for the rapid and accurate

method(s) for the identification and epidemiological studies of *E. albertii*. Better detection methods could increase the probability of finding additional subpopulations of this pathogen from various sources: human, environmental, and food animals. This study aims at formulating a novel medium coupled to 16S gene-based identification of *E. albertii* to accurately isolate and identify the organism from differing food animal production environment sample types. It is hoped that this medium could also support subsequent molecular detection and confirmation.

CHAPTER 2: DEVELOPMENT OF MELIBIOSE-X-GAL-MACCONKEY (MXgMac) AGAR[*]

2.1 Introduction

The bacterium *Escherichia albertii*, a Gram-negative facultative anaerobic bacillus, has been previously identified and/or implicated in the occurrence of multiple foodborne disease outbreaks in various locations in the globe (Asoshima et al. 2014; Konno et. al. 2012; Ooka et al. 2012). Muchaamba et al. (2022) recently reviewed critical aspects of its global distribution, microbial physiology, the various identified and possible routes of its transmission into the human food supply. These authors also summarized the unique biochemical properties of this pathogen that have facilitated its early misidentification after its first report on human disease, as well as more recent attempts to develop culture-dependent and -independent tools to differentiate *E. albertii* from members of the genus *Escherichia* and family *Enterobacteriaceae* (Abbott et al. 2003; Huys et al. 2003). The pathogen has been previously recovered from human fecal specimens collected from those suffering diarrheal disease (Hinenoya et al. 2020; Huys et al. 2003), human blood from a bacteremic patient (Inglis et al. 2015), ground and surface water (Maheaux et al. 2014), wild birds (Oaks et al. 2010), poultry GI tracts (Hinenoya et al. 2021), and poultry carcass rinse fluid from commercially harvested chickens (Lindsey et al. 2015). Isolates of *E. albertii* recovered and characterized by these and studies have been demonstrated to possess multiple pathogenesis effectors, including intimin and other components of the locus of enterocyte effacement (LEE) (Ooka et al. 2012; Lacher et al. 2006), cytolethal distending toxin B subunits (CDT), and Shiga toxin 2 variants 2a and 2f (Brandal et al. 2015; Hyma et al. 2005; Lacher et al. 2006; Murakami et al. 2014; Ooka et al. 2012).

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Various plating media have been reported in the literature in recent years providing differing degrees of utility for distinguishing *E. albertii* from E. coli and other enteric Gram-negative bacteria, as well as yielding colonies for subsequent molecular identifications (e.g., multiplex PCR, multilocus sequence typing (MLST)) (Lindsey et al. 2015; Murakami et al. 2014; Wang et al. 2016). Maheux et al. (2018) reported mEA agar for recovery of lactose-positive and -negative *E. albertii* isolates from human feces and differentiation from other *Escherichia* spp. The authors reported 19/19 *E. albertii* isolates were able to be recovered on the medium, and demonstrated indole-positive results, followed by *E. albertii* confirmation with PCR. Nonetheless, many other isolates belonging to various genera within the family *Enterobacteriaceae*, as well as other Gram-negatives, also demonstrated growth on the medium with no differences in appearance. Additionally, 13 isolates with *E. albertii*-typical appearance and indole test results from human-recovered diarrheal stool samples could not be confirmed as *E. albertii* (Maheaux et al. 2018). Hinenoya et al. (2020) reported the development of xylose–rhamnose–melibiose MacConkey (XRM-MacConkey) agar for the selective differentiation of *E. albertii* from clinical specimens, though the authors reported some isolates of *Shigella* could not be visually differentiated from *E. albertii* due to similar fermentation capabilities. *E. albertii* were consistently reported as unable to utilize the supplemented carbohydrates, whereas *E. coli* and *Salmonella enterica* routinely used at least one of the carbohydrates, producing red-tinted colonies. The authors further compared XRM-MacConkey to mEA and MacConkey agars, reporting 100% specificity of XRM-MacConkey for *E. albertii* presumptive identification versus other tested plating media. However, these research reports did not provide any characterization of the developed medium's utility for *E. albertii* differential detection from human food samples.

The U.S. Department of Agriculture Food Safety Inspection Service (USDA-FSIS) previously identified need for assessment of the distribution of *E. albertii* in the US meat and poultry supply (USDA-FSIS, 2023). To that end, the primary purpose of this study was to develop and evaluate a microbiological medium for the selective differentiation of *E. albertii* from *E. coli* and *Salmonella* from fresh non-intact poultry meat inoculated with a blend of isolates belonging to the three pathogens. The medium was then screened for the isolation and presumptive recovery of *E. albertii*-typical colonies from poultry animal feces samples for subsequent 16S rRNA-based identification, to gain preliminary assessment of its utility for pathogen isolation during poultry animal production.

2.2 Materials and Methods

2.2.1 Bacterial Culture Preparation

Bacterial organisms used in the current study are reported in **Table 1** and were obtained from various sources. *E. albertii* isolates were either revived or obtained via material transfer agreement (MTA) from the US Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) and stored upon receipt at –80 °C in the Texas A&M University Food Microbiology Laboratory (FML). Other isolates were either obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) or from the Texas A&M University FML culture collection. Isolates from the ATCC or CDC were revived according to instructions provided by the organism source. Other organisms were revived from –80 °C cryo-preservation in tryptic soy broth supplemented with 0.6% (w/v) yeast extract (TSB-YE; Becton, Dickinson and Co., Sparks, MD, USA) with 24 h of incubation at –37 °C. All cultures were then passed a second time in TSB-YE with a second 24 h incubation at 37 °C prior to further experimentation.

Table 2.1 Bacterial strains used, sources, and typical appearance on MXgMac agar.

Organism	Strain No./Source	Appearance
E. albertii	3033/CDC[1]	Colorless
	4180/CDC	Colorless
	4750/CDC	Colorless
	3449/CDC	Colorless
	3866/CDC	Light Blue
	3542/CDC	Colorless
	4143/CDC	Colorless
	4312/CDC	Light Blue
	5188/CDC	Colorless
	4085/CDC	Colorless
E. coli	1823-B/CDC	Blue-Green
	O157:H7 700278/ATCC	Blue-Green
	P41/TAMU FML	Blue-Green
	O145:NM 83-75/TAMU FML	Blue-Green
	O103 P50/TAMU FML	Blue-Green
	O104 P53/TAMU FML	Blue-Green
	O145/TAMU FML	Blue-Green
S. enterica Anatum	BAA-1427/ATCC	Pink/Red-Centered
S. enterica Agona	BAA-1592/ATCC	Pink/Red-Centered
S. enterica Enteritidis	100/TAMU FML	Pink/Red-Centered
Listeria	707/TAMU FML	NG[2]
monocytogenes	LIS 0089/TAMU FML	NG
Staphylococcus aureus	S101/TAMU FML	NG
Enterococcus faecium	NRRL-B2354/USDA-ARS	NG

[1]CDC: Centers for Disease Control and Prevention (Atlanta, GA, USA): ATCC: American Type Culture Collection (Manassas, VA, USA); TAMU FML: Texas A&M University Food Microbiology Lab (College Station, TX, USA). [2]NG: No growth observed.

2.2.2 Melibiose–X-Gal–MacConkey (MXgMac) Agar Formulation

Preliminary experiments designed to identify carbohydrate(s) and/or their analogues giving useful differentiation of *E. albertii* from *E. coli* and *Salmonella* indicated melibiose and the lactose analogue 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) provided good differentiation of the three organisms from one another. *E. albertii* has been reported as unable to metabolize melibiose due to no production of an α-galactosidase to cleave the α (1→6) glycosidic bond between the galactose and glucose moieties (Hinenoya et al. 2020; Arai et al. 2021). Lactose non-utilization by *E. albertii*, like *Salmonella*, has been reported to occur by

multiple research groups (Abott et al. 2003; Huys et al. 2003). Unlike *E. albertii*, *Salmonella* and *E. coli* utilize melibiose and lactose, respectively. Consequently, X-gal (Teknova, Inc., Hollister, CA, USA) and α-D-+-melibiose (Thermo Fisher Scientific, Inc., Waltham, MA, USA), each at 0.5% (w/v), were filter sterilized (0.22 μm) and added to already sterilized, tempered (48–50 °C) MacConkey agar base. The medium was stirred for 1 min to homogenize and then Petri dishes (100 × 15 mm) were filled prior to experimental use. Individual isolates of cultures were grown up in tryptic soy broth (TSB; Becton, Dickinson and Co.) for 24 h at 37 °C and then streaked for isolation onto surfaces of MXgMac agar. Inoculated plates were incubated thereafter for 24–36 h at 37 °C and inspected at 24 and 36 h for colony development, appearance, and any changes occurring in colony formation or appearance between 24 and 36 h of incubation.

2.2.3 Preparation of Microorganisms for Inoculated Chicken Meat Experiments

To determine the capacity of the experimental medium to facilitate differentiation of *E. albertii* from *E. coli* and *Salmonella* organisms when all were present in a food sample, individual isolates of each pathogen were revived and grown as described in **Section 2.2**, and then mixtures of isolates for each organism were subsequently prepared. *E. albertii* isolates were mixed in equal volumes, as were *E. coli* and *Salmonella* enterica isolates, in sterile 50 mL conical tubes. Tubes were then centrifuged at 2191× *g* for 15 min at 25 °C, after which the supernatant was carefully poured off and one volume of phosphate buffered saline (PBS) was added. The bacterial pellet was then vortexed vigorously in applied PBS for 1–2 min, after which the cells were centrifuged again in identical fashion to wash cells of any remaining biomatter and provide for inoculum preparation. Following the second centrifugation, the

supernatant was again poured off gently and discarded. The resulting pellet was hydrated with one volume of PBS and mixed thoroughly by vortexing. Cell preparations were then placed in ice to suspend growth prior to subsequent dilution and chicken meat sample inoculation.

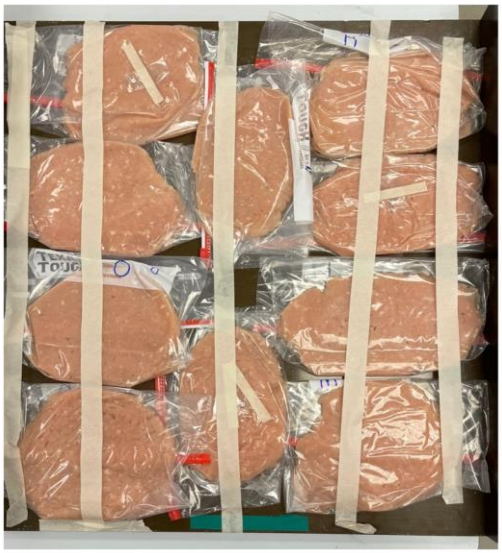
To determine the capacity of MXgMac to differentiate isolated *E. albertii* from *E. coli* and *Salmonella* from an inoculated "spiked" chicken meat sample when *E. coli* and *Salmonella* were present at higher counts than *E. albertii*, three differing cocktails of the three pathogens were prepared. Cocktail 1 contained all three organisms, each at a target of 10^2 CFU/g of chicken meat following inoculation. Cocktail 2 was devised to deliver a final count of 10^3 each of *E. coli* and *Salmonella*, while Cocktail 3 was devised to produce counts of *E. coli* and *Salmonella* of ~10^4 each in inoculated chicken. *E. albertii* target counts were kept at 10^2 CFU/g chicken meat for Cocktails 2 and 3, yielding 10- and 100-fold higher numbers of other pathogens versus *E. albertii*, respectively. Each pathogen mixture was serially diluted in PBS and counts enumerated on TSA following 24 h incubation at 37 °C to quantify the ingoing load of each organism (*E. albertii*, *E. coli*, or *Salmonella*) for each cocktail that was applied to a chicken sample and allow for subsequent comparison of a pathogen's recovery from spiked chicken versus inoculated numbers.

2.2.4 Preparation of Ground Chicken Meat Samples and Inoculation with Pathogens

Refrigerated ground chicken meat (97% lean) was purchased from a College Station, TX, USA retail grocer and immediately returned to the FML. Upon return, 250 g aliquots of chicken were aseptically weighed and placed in polyethylene refrigerator/freezer bags and flattened. Bags were transported to the National Center for Electron Beam Research (Texas A&M

AgriLife, College Station, TX, USA) and subjected to electron beam pasteurization to reduce numbers of background microorganisms prior to inoculation of pathogen mixtures/cocktails. Two hundred and fifty-gram samples packed in Ziploc pouches were arrayed as depicted in Figure 2.1. Chicken packages were irradiated to a target of at least 10.0 kGy via the Tower accelerator (10.0 MeV), positioning the electron beam horn above the chicken samples; samples were passed through the accelerator once. A dose absorption study was completed using three alanine pellets positioned at differing locations within the packaged chicken array. Resulting minimum and maximum dose absorptions were 10.09 and 11.39 kGy, respectively. The mean absorbed dose was 10.71 ± 0.51 kGy, and the dose uniformity ratio (DUR) was 1.13 (11.39 kGy/10.09 kGy).

Figure 2.1: Orientation and array of ground chicken pouches for electron beam device processing/irradiation to pasteurize chicken meat prior to inoculation with microorganisms.



Following irradiation, sample bags were returned to the FML and placed under frozen (-20 ^{o}C) storage or prepared for immediate use. Chicken sample portions (25 g each) were aseptically weighed from irradiated aliquots of chicken meat and inoculated with 1.0 mL of Cocktails 1, 2, or 3 of *E. albertii, E. coli,* and *Salmonella*. Samples were hand-massaged for I min and then allowed to rest for 30 min to facilitate microbial attachment to meat. Thereafter, inoculated samples were serially diluted in PBS and pathogens selectively/differentially enumerated on MXgMac and XRM-Mac (Hinenoya et al. 2021) agars in order to compare the two media for their ability to presumptively discriminate *E. albertii* from *E. coli* and *Salmonella*. Inoculated plates were incubated at 36 ± 1 ^{o}C for 24-30 h prior to inspection and counting.

2.2.5 Experimental Design and Statistical Analysis of Data

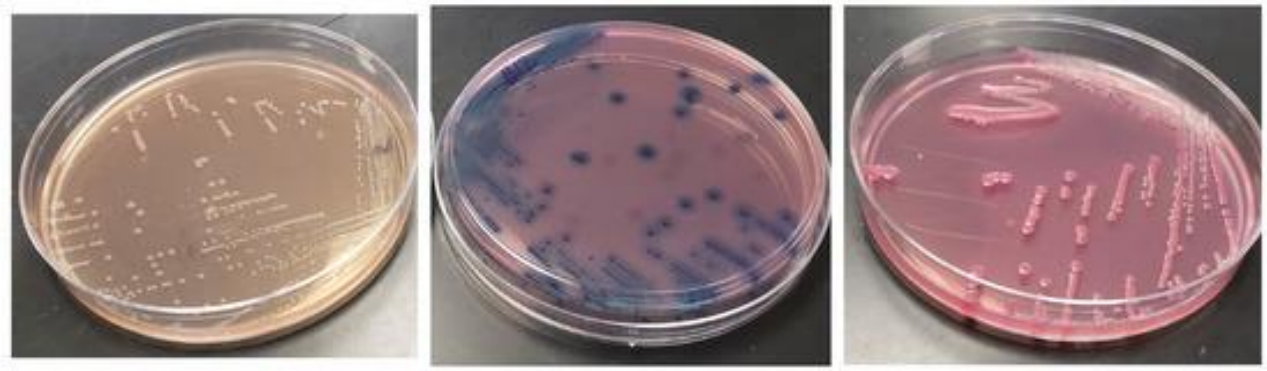
Experiments testing the selective and differential recovery of inoculated *E. albertii, E. coli,* and *Salmonella* from irradiated ground chicken with the three Cocktails (i.e., Cocktail 1, 2, and 3) were completed as a complete block and replicated three times on differing dates. Each replicate possessed three independently completed samples derived from differing 250 g sample packs of irradiated chicken (N=9). Data were analyzed by the general linear method by two-way analysis of variance (ANOVA) for the main effects of Cocktail, Medium (MXgMac or XRM-Mac agar), and their interaction for recovery of *E. albertii*. A similar analysis was completed wherein the counts of *E. coli* and *Salmonella* from MXgMac were first summed together for each sample and then compared to the count of *E. coli* and *Salmonella*-typical colonies from XRM-Mac, again testing the main effects of medium and Cocktail, and their interaction. Means were separated post-ANOVA using Bonferroni's method with significance set at P<0.05. Statistical analyses were completed with Prism v9.4.1 (GraphPad Software, LLC, San Diego, CA, USA).

2.3 Results

2.3.1 Differential Identification of *E. albertii* from *E. coli* and *Salmonella enterica* on MXgMac Agar Surfaces

As indicated in Table 1, isolates of *E. albertii* did not effectively hydrolyze the lactose analogue X-gal or melibiose, resulting in colonies displaying colorless growth. Figure 2 depicts the typical appearance of the three inoculated pathogenic organisms when streaked individually onto MXgMac agar surfaces and incubated as described in Section 2.2.

Figure 2.2 Typical appearance of (a) *E. albertii*, (b) *E. coli*, and (c) *Salmonella* on surfaces of MXgMac agar following 24 h incubation at 36±1°C.



Isolates of *E. albertii* on MXgMac, unable typically to utilize melibiose or lactose, appeared colorless with at times small zones of precipitated bile salts surrounding the colonies. *E. coli* isolates routinely appeared bluish green from the degradation of the X-gal, whereas *Salmonella*, negative for lactose use but positive for melibiose usage, took on a reddish/pink tinge in the colony center following plate incubation with colorless edges (Figure 2.1). Zones of bile precipitation were intermittently observed for all three organisms but were most pronounced for *E. coli*.

2.3.2 Comparisons of Recoveries of Inoculated Pathogens on MXgMac and XRM-Mac from Ground Chicken

Mean numbers of *E. albertii*, *E. coli*, and *Salmonella enterica* isolates following mixing together, prior to final Cocktails preparation for chicken inoculation, were 7.76±0.18, 7.82±0.12, and 7.82±0.07 $\log_{10}$ CFU/mL; counts of organisms did not statistically differ from one another (*P*=0.498). Figure 2.3 depicts the recoveries of *E. albertii* on MXgMac and XRM-Mac agars as a function of the three Cocktail setups (1, 2, and 3), wherein the targeted number of the organism was kept at 10^2 CFU/g chicken meat while numbers of *E. coli* and *Salmonella* were systematically increased for the three Cocktails. Least squares means of *E. albertii* on MXgMac ranged from 1.8 to 2.5 $\log_{10}$ CFU/g chicken meat across the three Cocktail setups, and from 2.2-2.5 $\log_{10}$ CFU/g chicken meat on XRM-Mac, but did not statistically differ as a function of the medium (MXgMac vs. XRM-Mac), Cocktail setup, or their interaction (*P*=0.600).

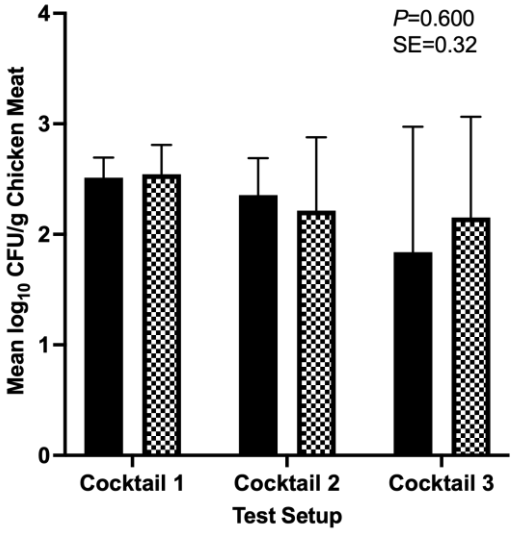


Figure 2.3 Least squares means of *Escherichia albertii* on MXgMac (solid black bars) and XRM-Mac (checkered bars) recovered from inoculated ground chicken meat, following incubation of inoculated plates for 24-36 h at 36+1°C. Bars indicate the mean of triplicate identically completed replicates, with each replicate possessing three independently completed samples (N=9). Error bars depict one sample standard deviation. SE: pooled standard error.

Counts of *E. coli* and *Salmonella* summed from MXgMac as well as collectively on XRM-Mac are presented below (Table 2.2). Means of these pathogens did not statistically differ by medium, when *E. coli* and *Salmonella* counts were summed together, allowing comparison of *E. coli* and *Salmonella* counts on MXgMac with the total count of these organisms on XRM-Mac (P=0.060 for the main effect). The summed counts of these pathogens must be compared to recovered counts from XRM-Mac, given the latter medium's lack of differentiation of *E. coli* from *Salmonella.* Likewise, the interaction of Cocktail setup x medium did not result in one main effect significantly influencing the other main effect with respect to resulting mean counts (P=0.511).

Table 2.2 Least squares means (log10 CFU/g chicken meat) of E. coli, Salmonella from MXgMac agar, summed counts of Escherichia coli+Salmonella enterica Cocktails on MXgMac and XRM-Mac agar

Cocktail Setup	***E. coli*-MXgMac**	***Salmonella*-MXgMac**	**MXgMac Sum Count**	**XRM-Mac**
1	2.65±0.14	2.74±0.20	3.12±0.11[1]	2.87±0.32
2	3.73±0.25	3.81±0.28	4.16±0.21	4.12±0.55
3	4.61±0.58	4.76±0.44	5.19±0.19	4.83±0.75
P=0.511; SE=0.20				

[1] Values present means±one sample standard deviation from triplicate identically completed replicates, each with three independently prepared samples (N=9). Following plate inoculation, plates from each medium were incubated at 37°C for 24-36 h prior to colony enumeration. E. coli and Salmonella colonies on MXgMac were individually counted and resulting plate counts summed prior to log-transformation for MXgMac Sum Count.

2.4 Discussion

The detection of human enteric pathogens from foods, despite the advent of modern genomic analyses, still frequently employs the use of selective enrichment and/or plating media for purposes of isolating the pathogen from background microorganisms. These procedures facilitate researchers and regulatory technicians' efforts to confirm the identity of the

presumptively detected pathogen, allowing for execution of regulatory food safety requirements and/or pathogen surveillance from foods (USDA-FSIS 2023; USDA-FSIS 2015). The emerging pathogen *E. albertii* has become of increased interest due to its being identified as the causative agent of multiple human foodborne disease outbreak and its global dissemination (Muchaamba et al. 2022).

While mEA and XRM-Mac agars have been described as useful for the differentiation of *E. albertii* from other enteric bacteria from clinical samples, to date no medium other than the MXgMac described herein is known to the authors allowing for differential identification of *E. albertii* from other pathogens from a food sample (Hinenoya et al. 2020; Maheaux et al. 2018). This study focused on the differentiation of *E. albertii* from *Salmonella enterica* and *E. coli*, including both non-human-pathogenic and human-pathogenic O157 and non-O157 Shiga-toxin producing *E. coli* (STEC), given *E. albertii*'s previous recovery from US poultry production and commercial poultry meat samples (Hinenoya et al. 2021; Lindsey et al. 2015). In the first set of experiments, the use of melibiose and X-gal demonstrated useful differentiation of the typically lactose-negative and melibiose-negative *E. albertii* from the lactose-positive/melibiose-negative *E. coli* and lactose-negative/melibiose-positive *Salmonella* (Section 2.2). Nevertheless, subsequent testing of the medium should incorporate a far broader range of isolates than those we were able to access during the project, to further screen its differential capabilities.

In experiments testing selective/differential enumeration of *E. albertii* from *E. coli* and *Salmonella* in irradiated ground chicken meat, MXgMac and XRM-Mac agars did not differ in their ability to support the presumptive identification of *E. albertii* across the three cocktail setups. Even as numbers of inoculated *E. coli* and *Salmonella* were systematically increased over the three cocktail setups, *E. albertii* numbers did not differ between the two

selective/differential media, demonstrating both were sufficiently useful for *E. albertii* recovery and enumeration from poultry meat. Nonetheless, the MXgMac demonstrated additional utility versus XRM-Mac due to its ability to allow presumptive discrimination of other possible human pathogens in addition to *E. albertii*, facilitating those organisms' subsequent identification (Table 2.2). The USDA-FSIS implements mandatory performance standards for young chicken carcasses and fabricated chicken parts testing the prevalence of *Salmonella* and has proposed new standards that will include a quantitative maximum allowable *Salmonella* count on certain not ready-to-eat poultry products (USDA-FSIS 2022; USDA-FSIS 2022; USDA FSIS 2016). MXgMac is expected to be of enhanced utility versus other *E. albertii*-differentiating media when seeking to simultaneously identify other poultry-borne human pathogens in addition to *E. albertii*, or when used for enumerating the pathogen from other poultry-borne pathogens.

CHAPTER 3: PRELIMINARY EVALUATION OF MXGMAC AGAR FOR *E. ALBERTII* RECOVERY FROM CHICKEN FECAL/LITTER SAMPLES*

3.1 Introduction

E. albertii has a broad range of hosts. Humans may be exposed to it through several routes including foods derived from livestock and poultry and drinking water. Multiple studies have identified heterologous *E. albertii* in both domestic and wild birds, underscoring the fact that food animals such as poultry and associated meat products could be a source of human infection. Studies have also shown that chicken can be an important reservoir or immediate host of *E. albertii* if exposed to the organism through channels such as contaminated food and water, or feces of other animals. Owing to its distribution in birds, it is important to determine *E. albertii*'s presence in poultry birds.

The gastrointestinal tract of birds is known to be the home to a diverse group of bacteria in which each is adapted to its own ecological niche and synergistically lives with other species in the same community. The taxonomic composition of these families of bacteria is believed to vary depending on the age of the birds, the dietary components, and the location in the GI tract (Apajalahti et al. 4004; Oakley et al. 2014; Pan et al. 2014; Rehman et al. 2007). This diverse and complex microbiota has been shown to play a vital role in digestion and absorption of nutrients, immune system development, and pathogen exclusion (Shank et al. 2018). Bacteria in the gut of birds produce vitamins (e.g., vitamins K and B groups), short chain fatty acids (acetic acid, butyric acid, and propionic acid), organic acids (e.g., lactic acid), and antimicrobial compounds (e.g., bacteriocins), and induce non-pathogenic immune responses, which is believed

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to provide both nutrition and protection for the bird (Jeurissen et al. 2002; Yegani et al. 2008; Dibner et al. 2005). The GI microbiome of birds can also be a source of pathogenic bacteria such as *Salmonella* and *Campylobacter* which can act as a pool for antibiotic resistance and transmission, thereby posing a public health threat (Kumar et al. 2018; Mancabelli et al. 2016; Zhou et al. 2012).

In this study, fecal grabs of birds were evaluated for the presence or absence of *E. albertii* using MXgMac agar as the isolation medium. The overall objective was to evaluate the prevalence of *E. albertii* in some selected U.S. food processing sectors, with special focus on poultry production and poultry-derived food products. Another objective was to characterize the taxonomic composition of avian bacterial communities, focusing on *E. albertii*. Data compiled may be useful to epidemiologists and food safety specialists to further quantify the risk and burden to foodborne disease attributable to this pathogen. This will aid in the analysis of food processing intervention efficacy for the control of *E. albertii* and reduction of foodborne disease risks.

3.2 Materials and Methods

3.2.1 Chicken Fecal/Litter Samples Preparation

Dropped fecal grab samples were collected (50–100 g each) from four cages (n = 16 hens) containing white leghorn egg-laying hens at the Texas A&M University Department of Poultry Science Research, Teaching, and Extension Center (College Station, TX, USA), placed in sterile whirl-pack plastic bags, and then returned to the Food Microbiology Laboratory. Chickens were fed a standard diet and managed according to the Texas A&M University Institutional Animal Care and Use Committee (IACUC), Animal Use Permit 2019-0171 (Principal Investigator: M. Farnell, Department of Poultry Science, Texas A&M AgriLife

Research, College Station, TX, USA). Upon return to the laboratory, 10 g sample material was diluted in 90 mL sterile PBS and 10 µL streaked onto surfaces of MXgMac agar-containing Petri dishes. Following a 24–36 h incubation at 37 °C, plates were removed and visually inspected for the presence of colorless colonies without haloes, typical of *E. albertii.* Colonies with this appearance were picked onto MXgMac plates for re-isolation. From these plates, following incubation, colonies were picked and transferred to TSA slants for subsequent identification via 16S rRNA gene amplification.

3.2.2 Identification of *E. albertii*-Typical Colonies Picked by 16S rRNA Sequence Typing

A pure culture of each isolate from a TSA slant (Section 3.2.1) was grown overnight in TSB at 36 ± 1 °C. Each tube was then centrifuged at 5000× g for 10 min at 4 °C and the resulting pellet resuspended in 2 mL PBS, and then transferred to two microcentrifuge tubes (1.0 mL each) and centrifugated at 10,000× g for 15 min at 4 °C. One of the microtubes was stored at – 80 °C and the other was used for DNA extraction and purification (Quick-DNA™ Fungal/Bacterial Miniprep Kit, Zymo Research Co., Orange, CA, USA) per manufacturer instructions. For each bacterial isolate, the obtained pellet was resuspended in 200 µL of PBS and transferred to a labeled ZR Bashing Bead™ Lysis Tube (0.1 × 0.5 mm) to which 750 µL Bashing Bead™ Buffer was added. Samples were vortexed for 2 min, sonicated for 1 min at 25 ◦C, vortexed for 2 min, sonicated again for 2 min at 25 ◦C, and vortexed individually for 30 s. The samples were then centrifuged at 10,000× g for 1 min at 4 ◦C, and 400 µL of the supernatant was transferred to a Zymo-Spin™ III-F Filter in a collection tube and then centrifuged at 8000× g for 1 min. A volume of 1.2 mL of Genomic Lysis Buffer was then added to the filtrate in the

collection tube and mixed thoroughly. Eight hundred microliters of the mixture were then transferred to a Zymo-Spin™ IICR Column in a collection tube and centrifuged at 10,000× g for 1 min at 4 ◦C. After this, the flow through in the collection tube was discarded and the remaining 800 µL of the mixture was transferred to the Zymo-Spin™ IICR and centrifuged at 10,000× g for 1 min. The Zymo-Spin™ IICR Column was then transferred to a new collection tube, where 200 µL of the DNA Pre-Wash Buffer was added and the microtubes were centrifuged at 10,000× g for 1 min, followed by the addition of 500 µL of DNA Wash Buffer and centrifugation at 10,000× g for 1 min, both times at 4 °C. For elution of DNA, each Zymo-Spin™ IICR Column was transferred to a clean 1.5 mL microcentrifuge tube, 100 µL of DNA Elution Buffer was added directly to the column matrix and centrifuged at 10,000× gfor 30 s at 4 °C. The DNA concentration of the samples was then quantified using a Qubit® dsDNA HS Assay kit (Thermo Fisher Scientific).

All DNA samples for PCR were stored at − 20 °C and thawed on ice before use. Amplification of 16S rRNA genes was conducted in a total reaction volume of 25 µL, using universal primers 27F (5′ AGA GTT TGA TCC TGG CTC AG 3 ′) and 1492R (5′ ACG GCT ACC TTG TTA CGA CTT 3′) (Integrated DNA Technologies, Coralville, IA, USA). The 25 µL PCR mixture was prepared by mixing 12.5 µL of 2× KAPA2G Fast Hot Start ready mix (Sigma-Aldrich Co., St. Louis, MO, USA), 1.25 µL of the forward and reverse primers, 8 µL PCR-grade water, and 2 µL sample DNA. Amplification was undertaken in a programmable thermocycler (Biometra Tone 96 G, 230 V, Analytik Jena, Konrad-Zuse-Str. Germany), under the following conditions: 1 initial cycle of denaturation at 95 °C for 3 min; 35 cycles of denaturation at 95 ◦C for 15 sec, annealing at 60 ◦C for 15 sec, and extension at 72 ◦C for 1 min; and a final extension at 72 ◦C for 1 min.

To corroborate the molecular weight of the PCR products, agarose gel electrophoresis was performed using 1% agarose (CulGenex Agarose LE, Molecular Biology Grade, Hardy Diagnostics, Santa Monica, CA, USA). For this, 2 μL of each PCR product was mixed with dye (Gel Loading Dye, Purple (6×), no SDS, New England Biolabs, Ipswich, MA, USA) and electrophoresed in 1× TAE buffer (Omega Bio-Tek, Inc., Norcross, GA, USA) through a 1% agarose gel containing 5 μL GelGreen® Nucleic Acid Gel Stain (Biotium, Inc., Fremont, CA, USA). Bands of the appropriate size were identified by comparison with a 100 bp DNA ladder (Quick-Load Purple 100 bp DNA Ladder, New England Biolabs). A sample was considered appropriate if a signal band corresponding to 1500 bp was visualized under UV light.

Vials containing the different PCR products were sent for Sanger sequencing to Eton Bioscientific (Eton Bioscience, Inc., San Diego, CA, USA). The sequences obtained in ab1 format were converted to FASTQ, quality controlled with FastQC, and the first 20 bases removed from all sequences by Fastq Trimmer, followed by filtering to remove sequences less than 200 bp using filtlong; these analyses were conducted at usegalaxy.eu [21]. The 16S sequences were exported from Galaxy and analyzed in the Ribosomal Database Project (RDP 11) Classifier to obtain taxonomic assignments to the genus level [22,23]. The sequences identified as belonging to family *Enterobacteriaceae* were then checked and manually edited using the BioEdit 7.2 Sequence Alignment editor. These sequences were then searched by BLASTn against the nt database at the National Center for Biotechnology Information (NCBI), for identification to species level.

3.3 Results

Following completion of colony isolation, DNA extraction, and 16S rRNA gene

sequencing by described procedures, no isolated organisms displaying non-fermentation on MXgMac from chicken feces/litter samples were identified as *E. albertii.* Of 22 NCBI-submitted isolate sequences, five isolates were identified by the RDP Classifier as belonging to the *Escherichia*/*Shigella* genus grouping, with one being identified by BLAST alignment as most closely related to *S. sonnei*, with the remaining four most closely related to *E. coli* (Table 3.1).

Table 3.1 Taxonomic identification and National Center for Biotechnology Information (NCBI) accession numbers for 16s rRNA sequences of poultry litter/feces-recovered bacterial isolates on MXgMac agar surfaces.

Taxonomic ID (RDP 11 > 70% Confidence	**Sequence ID**	**NCBI Accession**
Cronobacter sp.	27	OQ283624
Escherichia sp.	30	OQ283625
Cronobacter sp.	28	OQ283626
Salmonella sp.	26	OQ283627
Citrobacter sp.	24	OQ283628
Escherichia sp.	23	OQ283629
Enterobacter sp.	22	OQ283630
Escherichia sp.	21	OQ283631
Salmonella sp.	20	OQ283632
Escherichia sp.	19	OQ283633
Escherichia sp.	18	OQ283634
Enterobacter sp.	16	OQ283635
Empedobacter sp.	15	OQ283636
Myroides sp.	14	OQ283637
Acinetobacter sp.	13	OQ283638
Myroides sp.	7	OQ283639
Enterococcus sp.	5	OQ283640
Acinetobacter sp.	4	OQ283641
Gammaproteobacteria bacterium	3r	OQ283642
Gammaproteobacteria bacterium	3f	OQ283643
Acinetobacter sp.	2r	OQ283644
Acinetobacter sp.	2f	OQ283645

Figure 3.1 below depicts phylogenetic relatedness of isolates from chicken litter/feces samples. This analysis illustrates that it was difficult to unambiguously assign strains to the species level based on available partial 16S rDNA sequences, with the possible exceptions of strain 22 (*E. cloacae*), strain 16 (*E. hormaechei*), and strain 28 (*C. sakazakii*). The necessity for improved

techniques for *E. albertii* isolation is clearly apparent, such as the pre-application of rigorous selective enrichment to prohibit growth of undesirable microbes at the expense of *E. albertii* isolation.

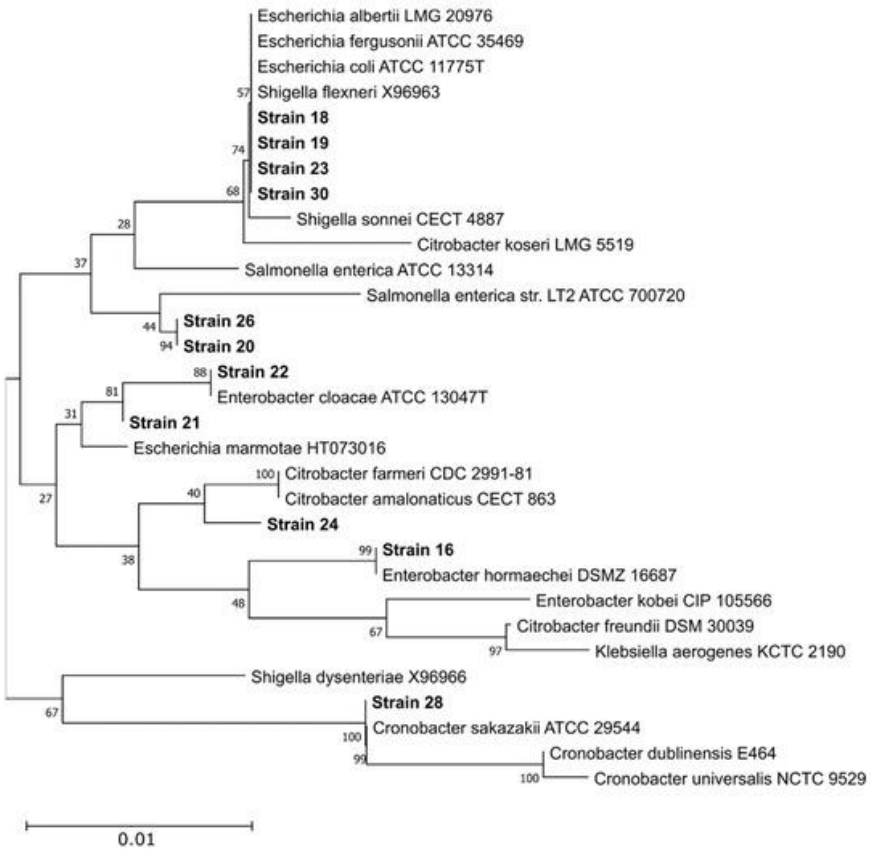

Figure 3.1 Phylogenic relatedness of enterobacterial chicken litter/feces-recovered isolates from 16S sequence analysis.

A reference set of 16S rDNA sequences from species most related to the enterobacterial isolate sequences (based on BLASTn results vs. the NT database) were retrieved from the RDP and aligned with 11 partial 16S rDNA sequences using the ClustalW algorithm at default parameters. Sample 27 was not included in this alignment due to excessive gaps. A neighbor-joining tree was generated from this alignment using the Jukes-Cantor method and omitting all positions containing gaps or ambiguous bases, leaving 521 positions in the final dataset. Numbers at each branch indicate the percentage score from a 500-replicate bootstrap analysis. All analyses were conducted in MEGA11 v11.0.13 (Tamura et al. 2021).

3.4 Discussion

Testing of feces/litter samples from chickens located at the Texas A&M University Department of Poultry Science's Teaching, Research, and Extension Center did not yield any confirmed *E. albertii*. This was likely a result of having only a small number of chickens for which testing could be completed, as compared to commercial establishments that may house several thousand chickens together, such as that reported by other researchers (Hinenoya et al. 2021). The presence of multiple non-lactose- or non-melibiose-using organisms from feces leading to needs for genetic identification of the organism is not surprising, and multiple culture media integrated into routine testing procedures are known to support the growth of multiple organisms other than the targeted microorganism(s). The use of antibiotics to suppress other Gram-negatives in addition to the presence of bile salts in MacConkey agar could improve the selectivity of *E. albertii*; the base of knowledge regarding the organism's resistance to various antibiotics is growing (Huys et al. 2003; Ooka et al. 2012; Ooka et al. 2015). Likewise, the use of *E. albertii*-specific selective enrichment would improve opportunity for pathogen recovery on culture media or by molecular testing, with *E. albertii*-specific selective enrichment formulae only very recently being reported in the literature (Hinenoya et al. 2020; Hirose et al. 2022).

Historically, selective/differential culture-based methodologies have been utilized to identify and characterize the microbial diversity of the avian gut. In recent times, the use of bacterial 16S rRNA gene sequencing has greatly improved our understanding of the composition and diversity of the chicken GI microbiota but not actually the feces/litter from avian sources. Modern high-throughput sequencing approaches are capable of rapidly obtaining a complete knowledge of the bacterial population and are, thus, a useful technique that can point to a significant new insight into the microbiology and ecological roles of feces/litter samples from

avian sources. In combination with continued optimization of *E. albertii*-specific multiplex PCR and/or MLST analyses, such efforts should improve efforts for *E. albertii* surveillance and improved food safety protection.

CONCLUSION

In recent years, significant progress has been made in understanding the burden borne by *E. albertii*, an emerging foodborne pathogen, in United States and global food systems. However, much remains to be explored regarding this pathogen's virulence mechanisms and potentials, nutrients utilization, stress tolerance capacity, and their regulation. Food scientists and epidemiologists are still limited in their comprehensive understanding of the pathobiology, mechanisms of colonization, and survival of this organism.

A handful of selective and differential media agars (e.g., mEA and XRM) have been shown to be effective for the differentiation of *E. albertii* from other enteric bacteria like *E. coli* from clinical samples. However, to date, no known medium other than MXgMac has been proven to allow for differential identification of *E. albertii* from other pathogens from a poultry food sample. Clearly, the study on fresh non-intact poultry meat inoculated with a blend of isolates (Chapter 2) has shown that MXgMac is useful for the isolation and presumptive recovery of *E. albertii*, *E. coli*, and *Salmonella* from poultry food products. This is significant when it comes to subsequent 16S rRNA-based identification. In the study involving chicken feces/litter, MXgMac was again utilized to evaluate the prevalence of *E. albertii* and characterize the taxonomic composition of avian bacterial communities. Even though no isolated organisms displaying non-fermentation of lactose were identified as *E. albertii* following 16S rRNA gene sequencing, it became apparent that this technique offered improved techniques for isolation of *E. albertii* in food samples. Molecular techniques like WGS are useful techniques that can point to a significant new insight into the microbiology of poultry food products. When combined with continued optimization of *E. albertii*-specific molecular analyses, improved efforts for *E.*

albertii surveillance and food safety protection will be easy to achieve. This is not to state that this novel agar has no limitations. First, this medium cannot differentiate lactose-positive from lactose-negative *E. albertii* strains. Also, this medium is by no means 100 % efficient. For these reasons, it is recommended that it be coupled to molecular techniques like multiplex PCR or WGS. There are opportunities for MXgMac agar to be adapted to routine use when utilized in this manner.

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APPENDIX A DIFFERENT EXPERIMENTS CONDUCTED

A1 INITIAL EVALUATION OF CARBOHYDRATES AND ANALOGUES

Different experiments were conducted prior to the final development of MXgMac. Initially, basal MAC was individually supplemented with glycerol, mannose, sucrose, maltose, raffinose, maltose, and ribose (each at a concentration of 1 g/L) (Tables A1 & A2). Following this experimentation involving carbohydrates, 3-Hydrobutryric acid (3-HBA), in different concentrations (20%, 10%, 5%, 4%, 2%, and 1% w/v), was used to replace lactose as a carbohydrate source. The formulation with 3-HBA did not yield any differentiation between any of the three pathogens. The next attempt involved blending basal MAC with isopropyl β-d-1-thiogalactopyranoside (IPTG), 3-HBA, and X-gal. IPTG is an analog of galactose that is non-metabolizable and inactivates the *lac* repressor to induce synthesis of β-galactosidase in *E. coli*. It is also a substrate for thiogalactoside transacetylase and has been reported to induce penicillinase in bacteria. Using IPTG alone or in combination with X-gal as carbohydrate source did not make the novel medium either selective or differential. Therefore, it could not be used as a supplement in this formulation either.

A2 EVALUATION OF ANTIMICROBIALS FOR SELECTIVE RECOVERY

The determination of pathogens' properties with respect to their reactions with specific sugars and antibiotics has always been helpful in the development of differential and selective plating media. Antibiotics like penicillin, streptomycin or malachite green are typically included in culture media to serve as selective agents (i.e., allow the growth of a specific type or types of microbes, while inhibiting others). Trimethoprim (TMP) is an antibiotic used mainly in the

treatment of bladder infections. This antibacterial agent has dihydrofolate reductase inhibitor activity with selectivity for the prokaryote enzyme (Schweitzer et al. 1990). Sulfamethoxazole, on the other hand, is a sulfonamide that induces its therapeutic effects by interfering with the synthesis of folate inside microbial organisms. It does this by competing with *p*-aminobenzoic acid (PABA) in the biosynthesis of dihydrofolate (Wormser et al. 1982). Trimethoprim and sulfamethoxazole have been shown in studies to have a greater effect when administered together than when given separately (i.e., they act synergistically). Based upon these findings and those of Daniels (2021), TMP/SMX combinations in varying concentrations were supplemented in the formulation to act as selective agents. These antibiotics were added just after MAC agar has been sterilized (autoclaved) and tempered down to 50 °C in a water bath. They were first filter-sterilized with 0.2 μm sterile syringe filter (VWR) before being added to the autoclaved agar. The isolates were then streaked on the supplemented Mac agar and incubated for 24 h at 36 ±1 ^{o}C. No phenotypic differences between the isolates were noted after the incubation time.

Table A1: Phenotypes of isolates plated on basal MAC supplemented with different carbohydrates.

Organism	Strain No	Glycerol	Mannose	Sucrose	Raffinose	Maltose	Ribose
E. albertii	3033	Pink	Colorless	Colorless	Colorless	Pink	Colorless
	4180	Pink	Colorless	Colorless	Colorless	Pink	Pink
	3449	Pink	Colorless	Colorless	Colorless	Colorless	Pink
	07-3866	Colorless	Colorless	Colorless	Colorless	Pink	Pink
	4143	Pink	Pink	Colorless	Colorless	Pink	Pink
	4312	Colorless	Colorless	Colorless	Colorless	Pink	Pink
	4085	Colorless	Colorless	Colorless	Colorless	Pink	Pink
	1823-B	Pink	Pink	Colorless	Colorless	Pink	Pink
	4750	Pink	Colorless	Colorless	Colorless	Pink	Pink
E. coli	O157:H7						
	700728	Colorless	Colorless	Colorless	Colorless	Pink	Pink
	P41	Pink	Colorless	Colorless	Colorless	Pink	Pink
	O145:NM						
	83-75	Pink	Colorless	Pink	Colorless	Pink	Pink
	O103 P50	Pink	Pink	Colorless	Pink	Pink	Pink
	O104 P53	Pink	Pink	Colorless	Colorless	Pink	Pink
	O145	Pink	Pink	Colorless	Colorless	Pink	Pink
S. enterica Anatum	BAA 1427	Colorless	Colorless	Pink	Colorless	Pink	Pink
S. enterica Agona	BAA 1592	Pink	Pink	Colorless	Colorless	Pink	Pink
S. enterica Enteritidis	100	Pink	Pink	Colorless	Colorless	Pink	Pink
Listeria monocytogenes	707	NG	NG	NG	NG	NG	NG
Staphylococcus aureus	LIS 0089	NG	NG	NG	NG	NG	NG
Enterococcus faecium	S101	NG	NG	NG	NG	NG	NG

Table A2: Phenotypes of isolates plated on basal MAC supplemented with a combination of carbohydrates.

Organism	Strain No	Melibiose Only	Rhamnose Only	Melibiose + Rhamnose	Melibiose + Rhamnose + X-gal
E. albertii	3033	Colorless	Colorless	Colorless	Colorless
	4180	Colorless	Colorless	Colorless	Colorless
	3449	Colorless	Colorless	Colorless	Colorless
	3866	Colorless	Colorless	Colorless	Colorless
	4143	Colorless	Colorless	Colorless	Colorless
	4312	Colorless	Colorless	Colorless	Colorless
	4085	Colorless	Colorless	Colorless	Colorless
	1823-B	Colorless	Pink	Colorless	Green
	4750	Colorless	Colorless	Colorless	Colorless
E. coli	O157:H7 700728	Colorless	Colorless	Pink	Green
	P41	Colorless	Colorless	Pink	Blue
	O145:NM 83-75	Colorless	Colorless	Pink	Green
	O103 P50	Colorless	Colorless	Pink	Green
	O104 P53	Colorless	Pink	Pink	Blue
	O145	Pink	Pink	Pink	Blue
S. enterica Anatum	BAA 1427	Colorless	Pink	Pink	Pink
S. enterica Agona	BAA 1592	Colorless	Pink	Pink	Pink
S. enterica Enteritidis	100	Colorless	Pink	Pink	Pink
Listeria monocytogenes	707	NG	NG	NG	NG
Staphylococcus aureus	LIS 0089	NG	NG	NG	NG
Enterococcus faecium	S101	NG	NG	NG	NG