



Communication

# Melibiose–X-Gal–MacConkey Agar for Presumptive Differentiation of *Escherichia albertii* from *E. coli* and *Salmonella* from Poultry Meat

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**Abstract:** The bacterial foodborne enteropathogen *Escherichia albertii*, despite enjoying increased attention paid to its pathogenesis, global dissemination, and antimicrobial resistance capacity, 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 supplemented with  $\alpha$ -D-+-melibiose and the lactose analogue X-gal was prepared and used 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. MXgMac agar facilitates improved differential recovery of *E. albertii* and other enteric pathogens from poultry meat versus other *E. albertii* selective/differential media.

**Keywords:** *Escherichia albertii*; poultry safety; food safety; culture media; *E. coli*; *Salmonella*; MacConkey Agar



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## 1. Introduction

The bacterium *Escherichia albertii*, a Gram-negative facultatively anaerobic bacillus, has been previously identified and/or implicated in the occurrence of multiple foodborne disease outbreaks in various locations around the globe [1–3]. Muchamba et al. [4] 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 of 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* [5,6]. The pathogen has been previously recovered from human fecal specimens collected from those suffering diarrheal disease [6,7], human blood from a bacteremic patient [8], ground and surface waters [9], wild birds [10], poultry GI tracts [11], and poultry carcass rinse fluids from commercially harvested chickens [12]. Isolates of *E. albertii* recovered and characterized by these and other studies have been demonstrated to possess multiple pathogenesis effectors, including intimin and other components of the locus of enterocyte effacement (LEE) [2,13], cytolethal distending toxin B subunit (CDT), and Shiga toxin 2 variants 2a and 2f [2,13–16].

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 identification

(e.g., multiplex PCR, multilocus sequence typing (MLST)) [12,16,17]. Maheux et al. [18] 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* [18]. Hinenoya et al. [7] 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 [19]. 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. Materials and Methods

### 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  $-80\text{ }^{\circ}\text{C}$  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  $-80\text{ }^{\circ}\text{C}$  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  $-37\text{ }^{\circ}\text{C}$ . All cultures were then passed a second time in TSB-YE with a second 24 h incubation at  $37\text{ }^{\circ}\text{C}$  prior to further experimentation.

### 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- $\beta$ -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  $\alpha$ -galactosidase to cleave the  $\alpha(1\rightarrow6)$  glycosidic bond between the galactose and glucose moieties [7,20]. Lactose non-utilization by *E. albertii*, like *Salmonella*, has been reported to occur by multiple research groups [5,6]. Unlike *E. albertii*, *Salmonella* and *E. coli* utilize melibiose and lactose, respectively. Consequently, X-gal (Teknova, Inc., Hollister, CA, USA) and  $\alpha$ -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.

**Table 1.** Bacterial strains used, sources, and typical appearance on MXgMac agar.

Organism	Strain No./Source	Appearance
<i>E. albertii</i>	3033/CDC <sup>1</sup>	Colorless
	4180/CDC	Colorless
	4750/CDC	Colorless
	3449/CDC	Colorless
	3866/CDC	Colorless
	3542/CDC	Light Blue
	4143/CDC	Colorless
	4312/CDC	Colorless
	5188/CDC	Light Blue
	4085/CDC	Colorless
	1823-B/CDC	Colorless
	<i>E. coli</i>	O157:H7 700278/ATCC
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
BAA-1427/ATCC		Blue-Green
<i>S. enterica</i> Anatum	BAA-1592/ATCC	Pink/Red-Centered
<i>S. enterica</i> Agona	100/TAMU FML	Pink/Red-Centered
<i>S. enterica</i> Enteritidis	707/TAMU FML	Pink/Red-Centered
<i>Listeria monocytogenes</i>	LIS 0089/TAMU FML	NG <sup>2</sup>
<i>Staphylococcus aureus</i>	SA101/TAMU FML	NG
<i>Enterococcus faecium</i>	NRRL-B2354/USDA-ARS	NG

<sup>1</sup> 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).

<sup>2</sup> NG: No growth observed.

### 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  $\sim 10^4$  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.

#### 2.4. 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 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  $10.71 \pm 0.51$  kGy, and the dose uniformity ratio (DUR) was 1.13 (11.39 kGy/10.09 kGy).



**Figure 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 ( $-20\text{ }^{\circ}\text{C}$ ) 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 1 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 [11] 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  $36 \pm 1\text{ }^{\circ}\text{C}$  for 24–30 h prior to inspection and counting.

#### 2.5. Evaluation of MXgMac Agar to Assist Identification of *E. albertii* from Chicken Fecal/Litter Sample

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  $\mu\text{L}$  streaked onto surfaces of MXgMac agar-containing Petri dishes. Following a 24–36 h incubation at  $37\text{ }^{\circ}\text{C}$ , 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 reisolation. From these plates, following incubation, colonies were picked and transferred to TSA slants for subsequent identification via 16S rRNA gene amplification.

#### 2.6. Identification of *E. albertii*-typical colonies picked by 16S rRNA sequence typing

##### 2.6.1. DNA Extraction

A pure culture of each isolate from a TSA slant (Section 2.5) was grown overnight in TSB at  $36 \pm 1\text{ }^{\circ}\text{C}$ . Each tube was then centrifuged at  $5000 \times g$  for 10 min at  $4\text{ }^{\circ}\text{C}$  and the resulting pellet resuspended in 2 mL PBS, and then transferred to two microcentrifuge tubes (1.0 mL each) and centrifuged at  $10,000 \times g$  for 15 min at  $4\text{ }^{\circ}\text{C}$ . One of the microtubes was stored at  $-80\text{ }^{\circ}\text{C}$  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  $\mu\text{L}$  of PBS and transferred to a labeled ZR Bashing Bead™ Lysis Tube (0.1  $\times$  0.5 mm) to which 750  $\mu\text{L}$  Bashing Bead™ Buffer was added. Samples were vortexed for 2 min, sonicated for 1 min at  $25\text{ }^{\circ}\text{C}$ , vortexed for 2 min, sonicated again for 2 min at  $25\text{ }^{\circ}\text{C}$ , and vortexed individually for 30 s. The samples were then centrifuged at  $10,000 \times g$  for 1 min at  $4\text{ }^{\circ}\text{C}$ , and 400  $\mu\text{L}$  of the supernatant was transferred to a Zymo-Spin™ III-F Filter in a collection tube and then centrifuged at  $8000 \times g$  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  $10,000 \times g$  for 1 min at  $4\text{ }^{\circ}\text{C}$ . After this, the flow through in the collection tube was discarded and the remaining 800  $\mu\text{L}$  of the mixture was transferred to the Zymo-Spin™ IICR and centrifuged at  $10,000 \times g$  for 1 min. The Zymo-Spin™ IICR Column was then transferred to a new collection tube, where 200  $\mu\text{L}$  of the DNA Pre-Wash Buffer was added and the microtubes were centrifuged at  $10,000 \times g$  for 1 min, followed by the addition of 500  $\mu\text{L}$  of DNA Wash Buffer and centrifugation at  $10,000 \times g$  for 1 min, both times at  $4\text{ }^{\circ}\text{C}$ .

For elution of DNA, each Zymo-Spin™ IICR Column was transferred to a clean 1.5 mL microcentrifuge tube, 100  $\mu\text{L}$  of DNA Elution Buffer was added directly to the column

matrix and centrifuged at  $10,000\times g$  for 30 s at 4 °C. The DNA concentration of the samples was then quantified using a Qubit<sup>®</sup> dsDNA HS Assay kit (Thermo Fisher Scientific).

#### 2.6.2. PCR, Sequencing, and Organism Identification

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<sup>®</sup> 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.

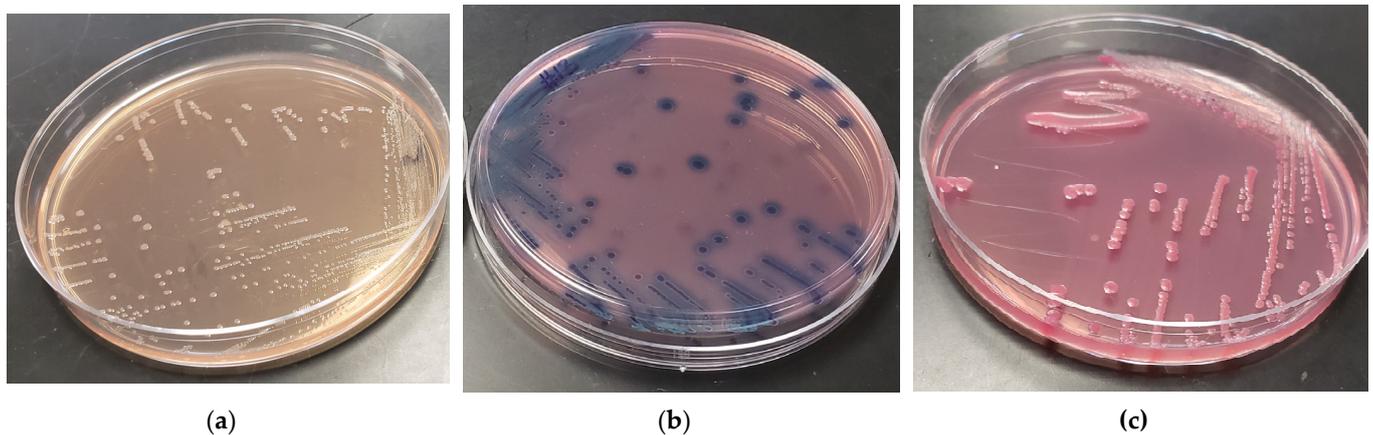
#### 2.7. Experimental Design and Statistical Analysis of Data

Experiments testing the selective/differential recovery of inoculated *E. albertii*, *E. coli*, and *Salmonella* from irradiated ground chicken with the three cocktails (i.e., Cocktails 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 for a two-way analysis of variance 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).

### 3. Results

#### 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.



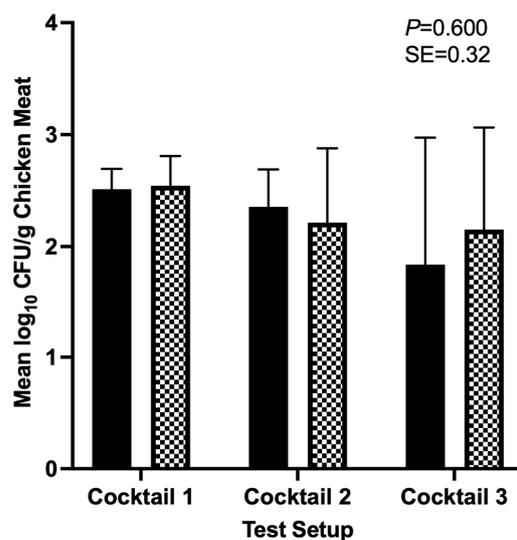
**Figure 2.** Typical appearance of (a) *E. albertii*, (b) *E. coli*, and (c) *Salmonella* on surfaces of melibiose–X-gal–MacConkey (MXgMac) agar surfaces following 36 h incubation at  $36 \pm 1$  °C.

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). Zones of bile precipitation were intermittently observed for all three organisms but were most pronounced for *E. coli*.

#### 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 cocktail preparation for chicken inoculation, were  $7.76 \pm 0.18$ ,  $7.82 \pm 0.12$ , and  $7.82 \pm 0.07$   $\log_{10}$  CFU/mL. Counts of organisms did not statistically differ from one another ( $p = 0.498$ ). Figure 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 inoculated at  $10^2$  CFU/g chicken meat while numbers of *E. coli* and *Salmonella* were systematically increased for the three cocktails from  $10^2$  to  $10^4$  CFU/g chicken meat. 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 ( $p = 0.600$ ).

Counts of *E. coli* and *Salmonella* individually and summed together on MXgMac, as well as collectively on XRM-Mac, are presented in Table 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 ( $p = 0.0595$ ). The summed counts of these pathogens must be compared to recovered counts from XRM-Mac, given the latter's lack of differentiation of *E. coli* from *Salmonella* [7]. Similar to results for *E. albertii*, 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 ( $p = 0.511$ ).



**Figure 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  $36 \pm 1$  °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.

**Table 2.** Least squares means  $\pm$  one standard deviation ( $\log_{10}$  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.

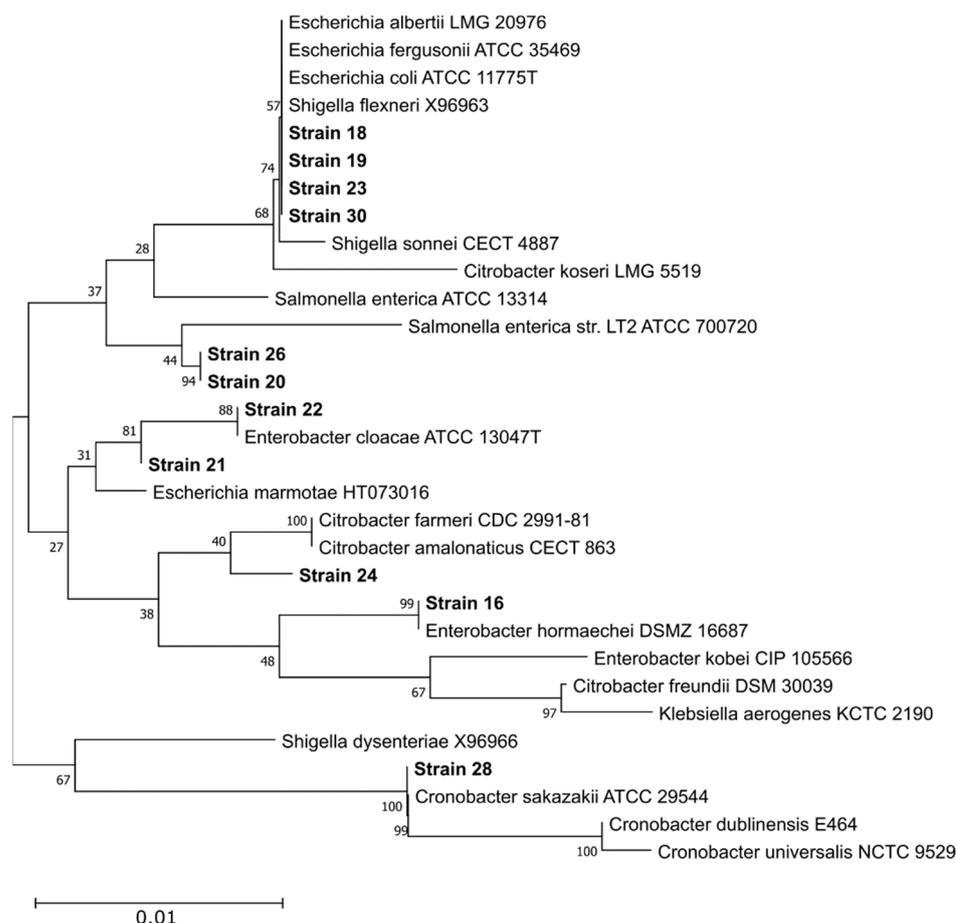
Cocktail Setup	<i>E. coli</i> MXgMac	<i>Salmonella</i> MXgMac	MXgMac Sum Count	XRM-Mac
1	$2.65 \pm 0.14$ <sup>1</sup>	$2.74 \pm 0.20$	$3.12 \pm 0.11$	$2.87 \pm 0.32$
2	$3.73 \pm 0.25$	$3.81 \pm 0.28$	$4.16 \pm 0.21$	$4.12 \pm 0.55$
3	$4.61 \pm 0.58$	$4.76 \pm 0.44$	$5.19 \pm 0.19$	$4.83 \pm 0.75$

$p = 0.511$ ; Pooled Standard Error = 0.20

<sup>1</sup> Values present means  $\pm$  one sample standard deviation from three identically completed replicates, each with three independently prepared samples ( $n = 9$ ). Following inoculation, plates 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.

### 3.3. 16S rRNA Identification of *E. albertii*-Typical Colonies from Chicken Feces/Litter Samples

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 A1). Figure 4 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 4.** Phylogenetic relatedness of enterobacterial chicken litter/feces-recovered isolates from 16S rRNA sequence analysis. A reference set of 20 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 [24].

#### 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 [25,26]. The emerging pathogen *E. albertii* has become of increased interest due to its being identified as the causative agent of at least one human foodborne disease outbreak and its global dissemination [4].

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 [7,18]. Our group 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 [11,12]. 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). 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 [27,28]. 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.

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 [11]. 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 [2,6,29]. 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 [7,30]. 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.

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## Appendix A

**Table A1.** 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
<i>Cronobacter</i> sp.	27	OQ283624
<i>Escherichia</i> sp.	30	OQ283625
<i>Cronobacter</i> sp.	28	OQ283626
<i>Salmonella</i> sp.	26	OQ283627
<i>Citrobacter</i> sp.	24	OQ283628
<i>Escherichia</i> sp.	23	OQ283629
<i>Enterobacter</i> sp.	22	OQ283630
<i>Escherichia</i> sp.	21	OQ283631
<i>Salmonella</i> sp.	20	OQ283632
<i>Escherichia</i> sp.	19	OQ283633
<i>Escherichia</i> sp.	18	OQ283634
<i>Enterobacter</i> sp.	16	OQ283635
<i>Empedobacter</i> sp.	15	OQ283636
<i>Myroides</i> sp.	14	OQ283637
<i>Acinetobacter</i> sp.	13	OQ283638
<i>Myroides</i> sp.	7	OQ283639
<i>Enterococcus</i> sp.	5	OQ283640
<i>Acinetobacter</i> sp.	4	OQ283641
Gammaproteobacteria bacterium	3r	OQ283642
Gammaproteobacteria bacterium	3f	OQ283643
<i>Acinetobacter</i> sp.	2r	OQ283644
<i>Acinetobacter</i> sp.	2f	OQ283645

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