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Original article

Multiple Drug Resistance in *Escherichia coli* Strains with Potential to Produce Shiga Toxins Isolated from Various Chicken Meats Obtained from Selected Vendors in Sri Serdang, Malaysia

Salahaldin Fathalla[1], Alghannay Khalid[2], Shu'aibu Isa[3,4*], Shamsi Shamsi[5,6]

[1] *Department of Food Science, Faculty of Agriculture, Sebha University, Libya*
[2] *Department of Biology, Faculty of Education, Wadi Alshatti University, Libya*
[3] *Department of Microbiology, Gombe State University, P.M.B. 127, Gombe State, Nigeria*
[4] *Center of Excellence for Food Safety Research, Faculty of Food Science and Technology, Universiti Putra Malaysia*
[5] *Faculty of Medical Technology, Sebha University, Sebha, Libya*
[6] *Department of Research and Consultancy, Faculty of Medical Technology, Sebha University, Sebha, Libya*
Corresponding email. shuaibuisa2002@gsu.edu.ng

Abstract
This study aimed to determine the presence, Shiga toxin production, contamination level, and multidrug resistance properties of *Escherichia coli* isolated from chicken meat samples. Overall, 120 chicken parts — 20 samples from each of chicken breast, drumstick, wings, thighs, backs, and legs — were purchased from supermarkets and night market vendors and analyzed immediately after transfer to the laboratory. A bacteriological culture-based technique on MacConkey agar and Eosin Methylene Blue (EMB) agar, Gram staining, and further growth on Sorbitol MacConkey (SMAC) agar, and polymerase chain reaction (PCR) using the Stx2 primer were used to characterize the Shiga toxin-producing *E. coli* (STEC). Antimicrobial susceptibility testing using agar-disc diffusion was performed with commonly used antibiotics on Mueller-Hinton agar. Out of 120 samples, the prevalence of *E. coli* was 25% (n=30). Incidence was highest in chicken legs at 50% (n=10/20) and lowest in drumsticks at 10% (n=2/20). The highest contamination level was in chicken legs at 3.00×10^3 CFU/g, while the lowest was observed in drumsticks at 1.32×10^3 CFU/g. Of the 30 *E. coli* isolates, only 16.6% (n=5) harboured Stx2 genes, indicating the presence of STEC. High resistance was observed to Amoxycillin (100%), Tetracycline (100%), Doxycycline (93.3%), Mecillinam (90%), and Levofloxacin (70%). Low resistance was found to Ciprofloxacin (10%), Streptomycin (10%), Ceftazidime (13.3%), and Ceftriaxone (16.7%). Educating vendors on good hygiene practices is critical to eliminating contamination and preventing outbreaks associated with *E. coli*.
Keywords. Multiple Drug Resistance, Escherichia coli, Strains, Toxins.

Introduction

In both the developed and developing nations of the world, chicken meat is among the most widely consumed meat products globally. Reports revealed that there are almost 19 billion chickens in the world, making it the most common bird species [1]. Data revealed by Uzundumlu and Dilli [2] showed that approximately 118 million tons of poultry were produced worldwide. Due to the lack of strict hygiene practices in the primary and secondary production lines as well as in the final products of the food chain, bacteria—particularly those of public health importance—contaminate chicken and its products. These include *Salmonella spp.* and coliforms, especially *Escherichia coli*, which is among the normal microbial flora of the gastrointestinal tracts of various animals, including chickens and humans. These bacteria are the leading causes of foodborne ailments, including gastroenteritis, and are a major global cause of chronic and acute foodborne diseases in humans and poultry [1].

E. coli is a bacterium that inhabits the intestines of both man and animals, and its presence indicates faecal contamination that usually arises from human faeces via water or food sources. *E. coli* in chicken meat is an indicator of poor hygiene practices at sites where chickens are slaughtered, processed, or vended. *E. coli* has been found to be prevalent in various parts of the world: India, 98% prevalence [3]; Pakistan, 43.5% [4]; Morocco, 48.4% [5]; Nigeria, 99.4% [6]; and Malaysia, 82.3% [7]. The above findings for the contamination of *E. coli* in chickens from different countries are reflections of varying hygiene practices and quality controls. Those recording lower contamination levels are believed to have better hygiene and manufacturing practices in their slaughter processes. High contamination indicates that chicken meat represents an important public health issue with the potential for causing foodborne infections and intoxications in humans that may lead to outbreaks [1].

Due to various food consumption patterns, people worldwide suffer from foodborne ailments throughout the year. Thus, reducing contamination levels at various stages of processing can significantly impact the reduction of incidences associated with consumption of contaminated poultry meats and their products [8,9]. Antibiotics in large quantities have been employed to treat or prevent disease and are also used to promote growth in poultry production [10]. This leads to indiscriminate antibiotic use in the course of poultry production, and almost all antibiotics employed have been utilized in human medicine, most often not for therapy but for prevention. Consequently, there is an increasing emergence of antibiotic-resistant bacteria.

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Methods

Sampling and Sample Processing

A total of 120 samples, 20 from each of chicken breast, drumstick, wings, thighs, backs, and legs, were purchased from vendors including Pasar Malam (night market), Pasar Borong, Pasaraya Borong, and Hero Mart in Sri Serdang, Selangor, Malaysia. Samples were obtained in six visits to each vendor over nine months, from July 2016 to March 2017. The samples were aseptically placed immediately after purchase in sterile plastic bags and transported to the laboratory at the Center of Excellence for Food Safety Research, Faculty of Food Science and Technology, Universiti Putra Malaysia, within two hours for analysis.

Isolation, Identification, and Enumeration of E. coli

The microbial analysis was carried out to determine the prevalence of *E. coli* in the chicken meat. A 25 g portion of each sample was aseptically cut into smaller pieces using a sterile knife, then macerated and homogenized with 225 mL of sterile buffered peptone water in a stomacher bag using a stomacher. Following homogenization, samples were serially diluted to 10^{-1}, 10^{-2}, and 10^{-3} dilutions. To determine bacterial growth, 0.1 mL from each dilution series was spread onto Eosin Methylene Blue (EMB) agar and incubated for 24 hours at 37°C. Colony-forming units per gram (CFU/g) were counted after incubation.

Phenotypic Identification of E. coli

Isolates presumably identified as *E. coli* on EMB agar were further subjected to Gram staining and biochemical tests, including indole production, citrate utilization, urease, ornithine decarboxylase, motility, hydrogen sulphide, gas and acid production, Voges-Proskauer, and methyl red.

Presumptive Screening for Shiga Toxin-Producing E. coli (STEC)

Suspected *E. coli* strains from EMB agar showing colonies with greenish metallic sheen were further subcultured on Sorbitol MacConkey (SMAC) agar. SMAC is selectively used to differentiate STEC from other strains of *E. coli*; STEC cannot ferment sorbitol, so colonies appear colourless, whereas other strains ferment sorbitol, making colonies appear pink or red.

Molecular Detection of Shiga Toxin (Stx2) in E. coli

Isolates negative for sorbitol fermentation were subjected to PCR for the detection of the gene encoding Shiga toxin. These isolates were first subjected to DNA isolation, then PCR, gel electrophoresis, and finally visualization of the electrophoretic PCR products.

DNA Genomic Extraction

The extraction of genomic DNA was conducted using the conventional boiling method. Isolates were subcultured on Luria Bertani (LB) broth in test tubes overnight. Approximately 1 mL of an overnight culture of *E. coli* in LB broth was aseptically transferred to an Eppendorf tube and centrifuged at 13,400 × g for 1 minute at room temperature. The supernatant was discarded and the pellets re-suspended in 200 µL of Tris-EDTA buffer (TE buffer). The mixture was boiled at 100°C for 10 minutes and then cooled at −20°C for 10 minutes. It was further centrifuged at 13,400 × g for 2 minutes. The supernatant containing the extracted DNA was stored at −20°C for further use.

Amplification of the Target Gene using PCR

The primers used in the amplification of the Stx2 target gene were: forward 5'-GCGGTTTTATTTGCATTAGC-3' and reverse 5'-TCCCGTCAACCTTCACTGTA-3', with locations within the gene at positions 1228–1247 and 1342–1323, respectively. The amplicon size was 115 bp [18]. PCR products were prepared in a 25 µL reaction volume consisting of 1 µL of 50 ng genomic DNA, 1 µL each of 10 µM forward and reverse primers, 12.5 µL of 2× PCRBIO Taq Mix Red (PCR Biosystems Ltd, London, UK) containing PCRBIO Taq DNA polymerase, 6 mM $MgCl_2$, and 2 mM dNTPs, and 9.5 µL sterile distilled water. Amplification was performed in a thermal cycler (Model SC300, Kyratec, Australia) with the following conditions: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute (denaturation), 55°C for 1 minute (annealing), and 72°C for 1 minute (extension), and a final extension at 72°C for 5 minutes.

Agarose Gel Electrophoresis and Visualization of PCR Products

Following PCR, the products were subjected to agarose gel electrophoresis. 1.5% agarose (Fisher Scientific, Hampton, New Hampshire, USA) was prepared by dissolving 0.45 g of agarose in 1× TBE (Tris-Borate EDTA) buffer (54 g Tris-base, 27.5 g Boric acid, 20 mL of 0.5 M EDTA, pH 8.3). The mixture was heated on a hot plate (Hanabishi, Japan) until the agarose dissolved completely. The solution was cooled to 50°C, and 1.5 µL of ethidium bromide (EtBr) was added. The solution was dispensed into a casting tray with a comb to form wells. PCR products were loaded into the wells, and the gel was run at 80 V for 40 minutes. Products were visualized under a UV-illuminator using a gel documentation system (SynGene, UK). Bands for the Stx2 gene were observed at 115 bp.

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Antibiotic Susceptibility Testing

Antibiotic susceptibility testing on the isolated *E. coli* was conducted against 10 common antibiotics: ciprofloxacin, levofloxacin, gentamicin, streptomycin, ceftriaxone, ceftazidime, mecillinam, amoxycillin, tetracycline, and doxycycline. The agar-disc diffusion technique on Mueller-Hinton agar (MHA) was employed, following standardization of the inoculum to the 0.5 McFarland turbidity standard equivalent to 1.5 × 10^8 CFU/mL. Standard antibiotic discs were dispensed, and the medium was incubated at 37°C for 24 hours in an inverted position. Zones of inhibition were recorded and interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI) 2024 guidelines as Resistant, Intermediate, or Susceptible.

Determination of Multiple Drug Resistance

Multiple drug resistance (MDR) was defined as resistance to at least one antibiotic from three or more different classes. Strains resistant to more than three antibiotics but from only one or two classes are not considered multidrug resistant. The Multiple Antibiotic Resistance Index (MARI) was calculated as the ratio of the number of antibiotics to which a strain was resistant to the total number of antibiotics tested (MARI = TAR/TAU, where TAR = total antibiotics resistant, TAU = total antibiotics used).

Results

A total of 120 samples were collected from chicken breast, drumstick, wings, thighs, backs, and legs. Out of 120 samples, the highest contamination level of *E. coli* was recorded in chicken legs, while the drumstick accounted for the lowest. Chicken breast and chicken backs had the same level of contamination. Additionally, chicken wings and chicken thighs showed similar contamination levels of 4.17%. Overall, 30 samples were found to be contaminated with *E. coli* from a total of 120, representing 25% of the total samples. This result was based on presumptive isolation and identification on the basis of culture and Gram staining techniques.

Table 1. Prevalence of E. coli from poultry meat samples based on culture and staining properties

S/N	Meat Type	No. of Samples	No. of Samples Positive for *E. coli*	Percentage (%) Positive
1	Chicken breast	20	04	20
2	Drumstick	20	02	10
3	Chicken wings	20	05	25
4	Chicken thighs	20	05	25
5	Chicken backs	20	04	20
6	Chicken legs	20	10	50
7	Total	120	30	25

The prevalence of Shiga toxin (Stx2) and the determination of *E. coli* count from the various poultry meat types, including chicken breast, drumstick, wings, thighs, backs, and legs, are depicted in Table 2. The values of the *E. coli* count that determine the contamination rate are expressed in multiples of × 10^3 CFU/g of sample. The highest *E. coli* count was observed in LEC 9 of the chicken leg samples, followed by LEC 4, LEC 8, WEC 1, and BEC 4 from legs, wings, and backs, respectively. The lowest count was observed in DEC 2 and DEC 1, both of drumstick origin.

Table 2. Determination of total E. coli count and the prevalence of Shiga toxin (Stx2) from poultry meat types

S/N	Sample Identity	Total *E. coli* Count (× 10^3 CFU/g)	Poultry Meat Type	Shiga Toxin (Stx2)
1	CBEC 1	2.52	Chicken breast	−
2	CBEC 2	2.12	Chicken breast	−
3	CBEC 3	2.11	Chicken breast	+
4	CBEC 4	2.61	Chicken breast	−
5	DEC 1	1.50	Drumstick	+
6	DEC 2	1.32	Drumstick	−
7	WEC 1	2.80	Wings	−
8	WEC 2	2.65	Wings	−
9	WEC 3	2.11	Wings	+
10	WEC 4	2.12	Wings	−
11	WEC 5	2.62	Wings	−
12	TEC 1	2.00	Thighs	−
13	TEC 2	2.00	Thighs	−
14	TEC 3	2.61	Thighs	−
15	TEC 4	2.23	Thighs	−
16	TEC 5	2.25	Thighs	+
17	BEC 1	2.22	Back	−

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18	BEC 2	2.32	Back	−
19	BEC 3	2.63	Back	+
20	BEC 4	2.80	Back	−
21	LEC 1	2.52	Legs	−
22	LEC 2	2.24	Legs	−
23	LEC 3	2.52	Legs	−
24	LEC 4	2.70	Legs	−
25	LEC 5	2.21	Legs	−
26	LEC 6	2.20	Legs	−
27	LEC 7	2.50	Legs	−
28	LEC 8	2.80	Legs	−
29	LEC 9	3.00	Legs	−
30	LEC 10	2.20	Legs	−

Key: − = negative; + = positive

Shiga toxin was present among *E. coli* detected across five samples: one from chicken breast (CBEC 3), one from drumstick (DEC 1), one from wings (WEC 3), one from thighs (TEC 5), and one from chicken back (BEC 3). No Shiga toxin-producing *E. coli* (STEC) was detected from samples obtained from chicken legs, despite all those samples being positive for *E. coli*. (Figure 1) displays the amplified PCR products of the five Shiga toxin-producing *E. coli* obtained from chicken breast, drumstick, wings, thighs, and backs. The amplicon size was 115 bp.

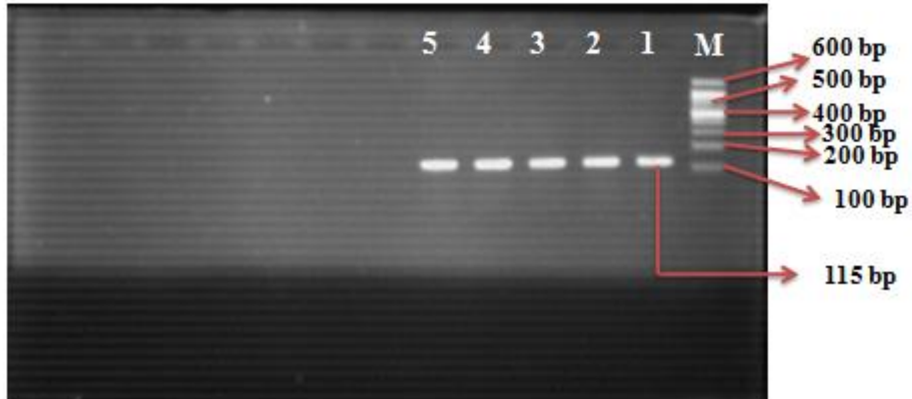

Figure 1. Amplified PCR products of the Stx2 genes of E. coli. Lane M, 100 bp molecular weight marker; Lanes 1–5, Stx2 genes of E. coli isolates of CBEC 3, DEC 1, WEC 3, TEC 5, and BEC 3

The antimicrobial resistance of the 30 *E. coli* strains isolated from the various chicken meat samples obtained in Sri Serdang, Malaysia, is depicted in (Table 3). Out of 30 isolates, 10% (n=3) were resistant to ciprofloxacin, 70% (n=21) to levofloxacin, 30% (n=9) to gentamicin, 10% (n=3) to streptomycin, 16.7% (n=5) to ceftriaxone, 13.3% (n=4) to ceftazidime, 90% (n=27) to mecillinam, and 100% (n=30) to amoxycillin. Additionally, 100% (n=30) were resistant to tetracycline and 93.3% (n=28) to doxycycline. No susceptible isolate was found to mecillinam, amoxycillin, tetracycline, or doxycycline.

Table 3. Antimicrobial sensitivity of E. coli (n=30) isolated from chicken meat samples against commonly used antibiotics

Antibiotics (Conc.)	Resistant		Intermediate		Susceptible	
	Positive Strain n (%)	Zone Diameter (mm)	Positive Strain n (%)	Zone Diameter (mm)	Positive Strain n (%)	Zone Diameter (mm)
Ciprofloxacin (10 µg)	03 (10)	≤21	00 (00)	22–25	27 (90)	≥26
Levofloxacin (10 µg)	21 (70)	≤16	06 (20)	17–20	03 (10)	≥21
Gentamicin (10 µg)	09 (30)	≤14	00 (00)	15–17	21 (70)	≥18
Streptomycin (10 µg)	03 (10)	≤11	03 (10)	12–14	24 (80)	≥15
Ceftriaxone (30 µg)	05 (16.7)	≤22	07 (23.3)	23–25	18 (60)	≥26
Ceftazidime (30 µg)	04 (13.3)	≤17	06 (20)	18–20	20 (66.7)	≥21
Mecillinam (10 µg)	27 (90)	≤11	03 (10)	12–14	00 (00)	≥15
Amoxycillin (10 µg)	30 (100)	≤13	00 (00)	14–16	00 (00)	≥17
Tetracycline (30 µg)	30 (100)	≤11	00 (00)	12–14	00 (00)	≥15
Doxycycline (30 µg)	28 (93.3)	≤10	02 (6.7)	11–13	00 (00)	≥14

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Table 4 displays the multiple antibiotic resistance (MAR) patterns of the 30 isolated *E. coli* strains, showing, for each strain, the number of antibiotics to which it was resistant and the total number of antibiotic classes represented (in parentheses). A strain is considered multidrug resistant (MDR) if it is resistant to at least one antibiotic from three or more different antibiotic classes. It can be observed that CBEC 1 and CBEC 2 were each resistant to seven antibiotics from five and four different classes, respectively. Strains such as CBEC 3, WEC 1, WEC 4, BEC 3, LEC 4, LEC 5, and LEC 8 were resistant to six antibiotics belonging to four different classes. Strain DEC 1 was resistant to six antibiotics, but from three different classes. Strains CBEC 4, DEC 2, WEC 2, WEC 5, TEC 1, TEC 2, TEC 4, BEC 1, BEC 2, BEC 4, LEC 2, LEC 6, LEC 7, and LEC 9 were each resistant to five antibiotics from three different classes (except LEC 9, where the antibiotics belong to four classes). Strains TEC 3, LEC 1, LEC 3, and WEC 3 were resistant to four antibiotics from three different classes, except WEC 3 (two classes), which is therefore non-MDR. Isolate LEC 10 was resistant to only three antibiotics from two classes and is also non-MDR. Strain TEC 5 showed the highest resistance profile, being resistant to nine antibiotics from five different classes. All *E. coli* strains have MARIs ranging from 0.3 to 0.9.

Table 4. Antibiotic resistance profile and multiple antibiotic resistance index (MARI) of individual E. coli isolated from chicken meat samples

Isolate	Antibiotic Resistance Profile	No. of Antibiotics (Classes)	MAR Index
CBEC 1	LEV GEN CRO MEC AMX TCN DCN	7 (5)	0.7
CBEC 2	CIP LEV CAZ MEC AMX TCN DCN	7 (4)	0.7
CBEC 3	LEV CRO MEC AMX TCN DCN	6 (4)	0.6
CBEC 4	LEV MEC AMX TCN DCN	5 (3)	0.5
DEC 1	CIP LEV MEC AMX TCN DCN	6 (3)	0.6
DEC 2	GEN MEC AMX TCN DCN	5 (3)	0.5
WEC 1	LEV GEN MEC AMX TCN DCN	6 (4)	0.6
WEC 2	LEV MEC AMX TCN DCN	5 (3)	0.5
WEC 3	MEC AMX TCN DCN	4 (2)	0.4
WEC 4	LEV GEN S AMX TCN DCN	6 (4)	0.6
WEC 5	LEV MEC AMX TCN DCN	5 (3)	0.5
TEC 1	LEV MEC AMX TCN DCN	5 (3)	0.5
TEC 2	LEV MEC AMX TCN DCN	5 (3)	0.5
TEC 3	GEN AMX TCN DCN	4 (3)	0.4
TEC 4	LEV MEC AMX TCN DCN	5 (3)	0.5
TEC 5	CIP LEV GEN S CRO MEC AMX TCN DCN	9 (5)	0.9
BEC 1	CAZ MEC AMX TCN DCN	5 (3)	0.5
BEC 2	LEV MEC AMX TCN DCN	5 (3)	0.5
BEC 3	LEV CAZ MEC AMX TCN DCN	6 (4)	0.6
BEC 4	LEV MEC AMX TCN DCN	5 (3)	0.5
LEC 1	MEC AMX TCN DCN	4 (3)	0.4
LEC 2	LEV MEC AMX TCN DCN	5 (3)	0.5
LEC 3	LEV MEC AMX TCN	4 (3)	0.4
LEC 4	LEV GEN MEC AMX TCN DCN	6 (4)	0.6
LEC 5	LEV CTR MEC AMX TCN DCN	6 (4)	0.6
LEC 6	LEV MEC AMX TCN DCN	5 (3)	0.5
LEC 7	CAZ MEC AMX TCN DCN	5 (3)	0.5
LEC 8	GEN S CTR AMX TCN DCN	6 (4)	0.6
LEC 9	LEV GEN AMX TCN DCN	5 (4)	0.5
LEC 10	MEC AMX TCN	3 (2)	0.3

CIP=Ciprofloxacin, LEV=Levofloxacin, AMX=Amoxycillin, GEN=Gentamicin, MEC=Mecillinam, CRO/CTR=Ceftriaxone, TCN=Tetracycline, S=Streptomycin, CAZ=Ceftazidime, DCN=Doxycycline.

Discussion

Meats from poultry are vital components of human diets owing to their protein, mineral, and vitamin content. However, these meats are prone to contamination by bacterial pathogens that cause food poisoning [4]. Shiga toxin-producing *Escherichia coli* (STEC) is a highly recognized food pathogen that causes various outbreaks. STEC causes bloody diarrhoea, haemorrhagic colitis, and haemolytic uraemic syndrome (HUS) [19]. STEC can cause the life-threatening HUS due to the production of Shiga toxin by the stx1 gene, stx2 gene, or both [20]. The results obtained from this study showed the highest occurrence of *E. coli* in chicken legs. Legs are the first contact of poultry with the ground where faeces are found, and the scaly nature of the legs makes cleaning difficult. The lowest prevalence was observed in drumsticks, which can be cleaned more easily than other parts. This contradicts the findings of Enver et al. [21], in which drumstick had the highest prevalence of *E. coli* (4.75×10^3 CFU/g) compared to chest, wing, and offal. The high numbers of *E. coli* in the poultry meats represent

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a potential source for human colonization, infection, and spread within the community [22]. The presence of *E. coli* indicates poor hygiene practices and improper handling of meat [23].

Prior to consumption, poultry meat should be absolutely free from *E. coli* due to the severity of infections or intoxications induced by this bacterium in humans [24]. *E. coli* is frequently employed as an indicator of microbiological safety for foodborne pathogens [25]. In this research, the rate of isolation was 25.0%, closely related to that obtained by Rafiq et al. [11] and lower than the 63.5% rate of detection by Rahman et al. [26]. In this study, the *E. coli* counts indicate that isolated strains were not within the acceptable limit (≤100 CFU/g) as per the International Commission on Microbiological Specifications for Foods (ICMSF) specifications for total coliform count. This represents a threat of infections [20] and potential outbreaks [1]. The figures obtained in this study, with regard to *E. coli* and other coliform bacteria, are well above the acceptable limit (>2000 CFU/g). Similar results were obtained by Hassanien et al. [27], Kim et al. [28], and Enver et al. [21].

Coliform counts increase during the course of processing from raw materials to final products [28]. This study shows the low prevalence (16.7%; n=5/30) of Shiga toxin genes in *E. coli* obtained from chicken breast, drumstick, wings, thighs, and backs. No Stx was detected from poultry leg samples, although the highest prevalence of *E. coli* was 100% (n=10/10) from them, showing no association between contamination level and Stx gene detection. The low prevalence of STEC is comparable to studies by Al-Zogibi et al. [29] and Bosilevac and Koohmaraie [30], although those studies used different sample types. STEC strains have various capabilities of causing serious diseases in animals and humans, linked to the amounts and types of stx produced. Only limited information exists regarding the frequency of the stx2 subtype and the combination of virulence factors expressed by STEC from the intestinal tract of healthy animals [16]. In summary, the prevalence of *E. coli* from poultry meat types does not influence the presence of Stx2 in the samples. Antibiotic resistance of *E. coli* to the penicillin family has not been uncommon; Tan et al. [31] recorded 85.71%, while herein 100% and 90% were resistant to amoxycillin and mecillinam, respectively. The reason may be that these antibiotics exhibit antimicrobial activity more effectively against Gram-positive than Gram-negative bacteria by inhibiting peptidoglycan, which accounts for 90% of the Gram-positive bacterial cell wall. High resistance to tetracycline and doxycycline has also been reported by Tan et al. [31] and Akond et al. [32].

Resistance to antibiotics observed in this study is consistent with that reported by Melo et al. [33]. Susceptibility to ciprofloxacin was high as reported in this study, but was low against norfloxacin, although both belong to the fluoroquinolone class. The MARI displayed in (Table 4) represents an important analysis for assessing risk factors associated with antibiotic resistance. Bacterial strains with MARI ≥ 0.2 indicate their origin from high-risk contamination sources—environments where various antibiotics have been extensively used [34,35]. Such strains may harbour plasmids carrying one or more genes encoding phenotypic resistance to antibiotic classes. MARI analysis does not require special training or costly equipment [36,35].

Conclusion

Poultry meat types were screened for the presence of *E. coli*. All samples obtained from chicken legs were positive for *E. coli*. Only five of the *E. coli* strains isolated were positive for Stx2. High *E. coli* counts were observed in samples from chicken legs, and the lowest was from the drumstick. Stx2 genes were present in

Ethical Approval

All study samples were purchased commercially from vendors in Sri Serdang, Selangor, Malaysia, and did not involve human subjects or clinical samples. All laboratory procedures were conducted in accordance with the biosafety guidelines of the Faculty of Food Science and Technology, Universiti Putra Malaysia.

Conflicts of Interest

The authors declare no conflicts of interest.

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