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Prevalence, characteristics and antibiogram profile of *Escherichia coli* O157:H7 isolated from raw and fermented (nono) milk in Benin City, Nigeria

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Abstract:

Background: Most *Escherichia coli* strains are harmless commensals, but some serotypes can cause serious food poisoning in their hosts, and are infrequently responsible for product recalls due to food contamination. The present study was carried out to determine the occurrence of *E. coli* O157:H7 and other *E. coli* strains from raw and fermented (nono) milk in Benin City, Nigeria.
Methodology: A total of 66 (33 raw and 33 nono) milk samples were obtained from retailers from 3 different stations in Aduwawa market, Benin City, Nigeria between January and June, 2017. Samples were analysed by cultural methods for faecal coliforms using M-Fc agar, *E. coli* using Chromocult coliform agar, and *E. coli* O157:H7 using sorbitol MacConkey agar supplemented with cefixime and potassium tellurite. Presumptive *E. coli* and *E. coli* O157:H7 isolates were confirmed by polymerase chain reaction (PCR) assay using specific primers. Antimicrobial susceptibility profile of confirmed isolates was performed using the Kirby-Bauer disk diffusion method, with zones of inhibition interpreted according to the guidelines of Clinical and Laboratory Standards Institute (CLSI). Data were analysed using the SPSS version 21.0.
Results: From the 66 nono and raw milk samples assessed in this study, all (100%) were phenotypically positive for *E. coli* O157:H7. A total of 19 *E. coli* O157:H7 and 41 other strains of *E. coli* were confirmed by PCR. The resistance profile of the 19 *E. coli* O157:H7 isolates showed 100% (19/19) resistance to penicillin G and ampicillin; 94.7% (18/19) to chloramphenicol; 89.5% (17/19) to erythromycin; and 78.9% (15/19) to sulfamethoxazole and oxytetracycline, while the sensitivity profile showed that 100% (19/19) *E. coli* O157:H7 isolates were sensitive to gentamicin and ofloxacin. The resistance profile of other 41 *E. coli* isolates showed 100% (41/41) resistance to penicillin G and ampicillin; 97.6% (40/41) to chloramphenicol; and 92.7% (38/41) to erythromycin, while 97.6% (40/41) were sensitive to gentamicin and kanamycin. Ten *E. coli* O157:H7 isolates (52.6%) showed extensive drug resistance pattern to 11 antibiotics in 7 antimicrobial classes with multiple antibiotic resistance (MAR) index of 0.46.
Conclusion: Findings from the present study clearly indicated that the safety and quality of fresh and fermented milk were not satisfactory and could be of public health concern.

Key words: Nono, *Escherichia coli*; Pathotypes, Resistance index, Public health, Milk

Prévalence, caractéristiques et profil antibiogramme d'*Escherichia coli* O157:H7 isolé à partir de lait cru et fermenté (nono) à Benin City, Nigéria

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Abstrait:

Contexte: La plupart des souches d'*Escherichia coli* sont des commensaux inoffensifs, mais certains sérotypes peuvent causer de graves intoxications alimentaires chez leurs hôtes et sont rarement responsables de rappels de produits en raison de la contamination des aliments. La présente étude a été réalisée pour déterminer la présence d'*E. coli* O157:H7 et d'autres souches d'*E. coli* provenant de lait cru et fermenté (nono) à Benin City, au Nigeria.

Méthodologie: Un total de 66 échantillons de lait (33 cru et 33 nono) ont été obtenus auprès de détaillants de 3 stations différentes sur le marché d'Aduwawa, Benin City, Nigeria entre janvier et juin 2017. Les échantillons ont été analysés par des méthodes de culture pour les coliformes fécaux en utilisant de la gélose M-Fc, E. coli en utilisant de la gélose Chromocult coliforme et *E. coli* O157:H7 en utilisant de la gélose MacConkey au sorbitol supplémentée en céfixime et tellurite de potassium. Des isolats présomptifs d'*E. coli* et d'*E. coli* O157:H7 ont été confirmés par un test de réaction en chaîne par polymérase (PCR) en utilisant des amorces spécifiques. Le profil de sensibilité aux antimicrobiens des isolats confirmés a été réalisé en utilisant la méthode de diffusion sur disque de Kirby-Bauer, avec des zones d'inhibition interprétées selon les directives du Institut des normes cliniques et de laboratoire (CLSI). Les données ont été analysées à l'aide de la version 21.0 de SPSS.

Résultats: Parmi les 66 échantillons de lait nono et de lait cru évalués dans cette étude, tous (100%) étaient phénotypiquement positifs pour *E. coli* O157: H7. Un total de 19 *E. coli* O157: H7 et 41 autres souches d'*E. coli* ont été confirmés par PCR. Le profil de résistance des 19 isolats d'*E. coli* O157: H7 a montré une résistance de 100% (19/19) à la pénicilline G et à l'ampicilline; 94,7% (18/19) au chloramphénicol; 89,5% (17/19) à l'érythromycine; et 78,9% (15/19) au sulfaméthoxazole et à l'oxytétracycline, tandis que le profil de sensibilité a montré que 100% (19/19) des isolats d'*E. coli* O157: H7 étaient sensibles à la gentamicine et à l'ofloxacine. Le profil de résistance des 41 autres isolats d'*E. coli* a montré une résistance de 100% (41/41) à la pénicilline G et à l'ampicilline; 97,6% (40/41) au chloramphénicol; et 92,7% (38/41) à l'érythromycine, tandis que 97,6% (40/41) étaient sensibles à la gentamicine et à la kanamycine. Dix isolats d'*E. coli* O157: H7 (52,6%) ont montré un profil de résistance aux médicaments étendu à 11 antibiotiques dans 7 classes d'antimicrobiens avec un indice de résistance aux antibiotiques multiples (MAR) de 0,46.

Conclusion: Les résultats de la présente étude indiquent clairement que la sécurité et la qualité du lait frais et fermenté ne sont pas satisfaisantes et pourraient être préoccupantes pour la santé publique.

Mots clés: Nono, *Escherichia coli*; Pathotypes, Indice de résistance, Santé publique, Lait

Introduction:

Escherichia coli is a Gram-negative, rod-shaped, facultative anaerobic coliform bacterium of the genus *Escherichia*, usually found in the lower intestine of warm-blooded organisms (1). Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts (2), and are infrequently responsible for product recalls due to food contamination. Some types of *E. coli* bacteria cause disease when they make a toxin called Shiga-toxin. The most common type of Shiga toxin-producing *E. coli* (STEC) is *E. coli* O157 while other STECs are called non-O157. STEC has been known to produce two major classes of toxins, shiga-toxin 1 and shiga-toxin 2. These strains produce verotoxin 1 and 2 and their variants. They can catalytically inactivate the 60s ribosomal subunits of most eukaryotic cells, blocking mRNA translation, which leads to cell death (3).

They also possess intimin which is a protein that enables the intimate attachment of enterohaemorrhagic *E. coli* (EHEC) to the epithelial cells of the intestine, causing attachment and effacement (AE) lesions of the intestinal cells (4). The death of these cells leads to intestinal function disruption and intestinal bleeding. The toxic damage to the intestines can lead to kidney damage, anaemia, platelet segregation and death (3). The prominence of *E. coli* O157:H7 serotype dates back to 1982 when it was first discovered in an outbreak traced to contaminate hamburgers (5). Bovine food products and produce contaminated with bovine waste are a reservoir of *E. coli* O157:H7. Consumption of contaminated raw milk and unpasteurized dairy products made from raw milk is a known risk factor for *E. coli* infections (6).

Escherichia coli and other pathogens which are shed in the faeces of livestock such as cows and goats can contaminate milk during the milking process. Milk is an important source of nutrients for humans and animals (7), but milk for human consumption should be free from any pathogenic organisms (8). Contamination of milk can occur through various means such as during the milking process of an infected lactating animal with the use of unsanitary equipment, improper milk handling, transportation, and storage (6). Contamination can also occur through improper handling and storage of milk (7). About 90% of all dairy-related human diseases are as a result of ingestion of contaminated milk, even though milk and milk products are minor constituents in most diets (9).

Escherichia coli O157:H7 has been known to survive well in the environment and can adapt to a variety of conditions. This is possible because it possesses certain virulence factors such as shiga toxins, which are products of the pathogenicity island, *locus of enterocyte effacement* (4), and products of the F-like plasmid pO157 (10). The extensive use of antibiotics in both human medicine and agricultural settings, especially in disease

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prevention and promotion of growth in animal production, is the major cause of selection of antibiotic resistant *E. coli* (11).

Multi-drug resistant *E. coli* isolates characteristically exhibit non-susceptibility to at least one agent in three or more antibiotic categories (12). Surveillance data on antibiotic-resistant *E. coli* shows clearly a higher resistance for older generation human and veterinary antibiotics including ampicillin, streptomycin, and tetracycline and an increasing resistance to new generation antibiotics such as fluoroquinolones and cephalosporins (13). Monitoring the emergence and spread of resistant pathogens in animal reservoirs is very important, especially for those with zoonotic importance (14). The aim of this study is to characterize *E. coli* O157:H7 isolates of raw and "nono" milk samples retailed by street vendors in Benin City, Edo State, Nigeria, using conventional microbiological and molecular methods.

Materials and method:

Study setting and sample collection

Raw (n=33) and fermented (nono) (n=33) milk samples were purchased from retail sellers in an open market (Aduwawa market) in Benin City, Edo State, Nigeria between January and June, 2017. Aduwawa market is the major market where raw and fermented milk are sold to retailers who then sell to other markets in Benin City. Three randomly selected locations within the market were sampled and described as stations (station 1, station 2, and station 3). A total of 22 samples (11 raw and 11 fermented) were obtained per station.

Milk samples were collected in 50 mL sterile bottles, and transported to the Applied and Environmental Microbiology Research Group Laboratory of the University of Benin, in cooler boxes within two hours of collection, for microbial analyses.

Enumeration and isolation of heterotrophic bacteria, faecal coliform, *Escherichia coli* and *Escherichia coli* O157:H7

One millilitre of the raw and 'nono' milk samples was serially diluted, and 0.1 mL immediately spread-plated on nutrient agar plates (Lab M, UK), Chromocult coliform agar (Lab M, UK), and Sorbitol MacConkey agar (Lab M, UK) supplemented with cefixime (50 µg/L) and potassium tellurite (25mg/L). All plates were incubated aerobically at 37°C for 18-24 hours. Likewise; an aliquot of 0.1mL was also spread-plated on M-Fc agar plates (Merck, Darmstadt, Germany) and incubated at 45°C for 24 hours.

Discrete dark blue to violet colonies from Chromocult coliform agar (probable *E. coli*), colourless or beige colonies from Sorbi-

tol MacConkey agar plates supplemented with cefixime and potassium tellurite (probable *E. coli* O157:H7), and blue colonies from M-Fc agar (faecal coliforms), were enumerated and expressed in colony forming units per millilitre (CFU/mL). Discrete colonies of probable *E. coli* and *E. coli* O157:H7 were purified on nutrient agar and stored on nutrient agar slant at 4°C for further laboratory analysis.

Identification of bacterial isolates

Identification of isolates was carried out using methods outlined in Cheesbrough (15), on the basis of cultural, morphological, Gram reaction, and biochemical tests such as indole, oxidase, catalase, and 3% potassium hydroxide.

Antibiotic susceptibility test (AST) of isolates

Antimicrobial susceptibility testing of isolates was carried out by the Kirby-Bauer disk diffusion method in accordance with the Clinical and Laboratory Standards Institute guidelines (16). A 0.5 MacFarland suspension of each isolates was aseptically streaked on Mueller-Hinton (MH) agar plates, and single antibiotic disks were aseptically placed on the inoculated agar. The disks utilized included; penicillin G (10units), ampicillin (10µg), amoxicillin (25µg), ampicillin/sulbactam (30µg), amoxicillin/clavulanate (30µg), gentamycin (10µg), kanamycin (30µg), streptomycin (25 µg), tobramycin (10µg), doxycycline (30µg), tetracycline (30µg), oxytetracycline (30µg), imipenem (10µg), meropenem (10µg), cephalothin (30µg), cefotaxime (30µg), erythromycin (15µg), trimethoprim (25µg), sulfamethoxazole (30µg), polymyxin (300 units), colistin (20µg), chloramphenicol (30µg), ofloxacin (5µg), and ciprofloxacin (10µg). Five antibiotics were used equidistant apart on each plate.

The agar plates were allowed to dry for about 10 minutes and then incubated aerobically at 37°C for 24 hours. The diameter of the inhibitory zone was measured using a transparent meter rule and interpreted as resistant (R), intermediate resistant (I) or sensitive (S), in accordance with the recommended standards established by CLSI (16).

Genetic confirmation of *E. coli* O157:H7 and non-*E. coli* O157: H7

Extraction of genomic DNA

DNA extraction from each bacterial isolate was done using the boiling method previously described by Igbinosa et al., (17) with modifications. Briefly, purified isolates were inoculated into sterile Tryptone Soy Broth and incubated at 37°C overnight. At the end of the incubation, about 2ml from the previously grown culture was transferred into

sterile 2ml Eppendorf tubes and centrifuged at 11000 rpm for 10min. The obtained pellet was washed twice using sterile distilled water before re-suspending into 200µl of sterile distilled water. The mixture was then boiled for 10 minutes at 100°C. The cell lysate was immediately cooled at -20°C for 10 minutes, followed by centrifugation at 12000g for 5 minutes. The supernatant was then carefully transferred into new sterile microfuge tubes and used as template genomic DNA for PCR assay. *E. coli* O157:H7 ATCC 35150 was used as positive control strain.

Species–specific identification by PCR assay

PCR assay was performed in a 25µl reaction cocktail in a 200µl tube with 12.5µl of Master Mix, 0.25 µl each of forward and reverse primers, 2 µl of nuclease free water and 10µl of template DNA. The amplification was performed in a Peltier-Based Thermal Cycler. Primer pairs used in the amplification are shown in Table 1.

The thermal cycling conditions for *uidA* gene of *E. coli* were initial denaturation at 95°C for 2 min followed by 25 cycles of denaturation at 94°C for 1 min, 58°C annealing for 1 min and 72°C extension for 1 min, final extension at 72°C for 2 min and amplicons held at 4°C after the cycles. The thermal cycling conditions for *rfbE* of *E. coli* O157 as well as *fliC* of H7 were initial denaturation at 95°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 90 sec, extension at 72°C for 90 seconds, and initial extension at 72°C for 5 minutes and the amplicons were held at 4°C until ready for electrophoresis.

Electrophoresis of amplicons was performed with 1% agarose gel (CLS-AG100, Warwickshire, United Kingdom) containing ethidium bromide (Merck, SA) with 0.5 mg/L

for 1 hour at 100 V in 0.5×TAE buffer (40mM Tris-HCl, 20mM Na acetate, 1mM EDTA, pH 8.5) and visualized under UV transilluminator (EBOX VX5, Vilber Lourmat, France), and photographed.

Statistical analysis

All data were analysed using IBM SPSS version 21.0. Population densities of the isolates were analysed using descriptive statistics and expressed as mean ± standard deviation of the mean. One-way analysis of variance (ANOVA) was used to analyse the data across sampling months and sampling points while Duncan Multiple Range test was used to show significant difference between mean. The probability-values less than 0.05 were considered statistically significant.

Results:

Population counts from milk samples

The mean heterotrophic bacterial counts of nono and raw milk samples is presented in Table 2. The mean bacterial counts for nono milk in respective stations range as follows; station 1 ($6.40 \times 10^4 \pm 0.14$ - $2.88 \times 10^{10} \pm 0.02$ CFU/ml), station 2 ($3.10 \times 10^4 \pm 0.28$ - $2.40 \times 10^{10} \pm 0.14$ CFU/ml), station 3 ($5.00 \times 10^4 \pm 0.28$ - $2.58 \times 10^{10} \pm 1.40$ CFU/ml), while that of the raw milk ranges as follows; station 1 ($2.64 \times 10^5 \pm 0.14$ - $2.84 \times 10^{10} \pm 0.14$ CFU/ml), station 2 ($2.16 \times 10^5 \pm 0.01$ - $2.29 \times 10^{10} \pm 0.04$ CFU/ml), station 3 ($2.94 \times 10^5 \pm 0.00$ - $2.56 \times 10^{10} \pm 0.01$ CFU/ml). There was no significant difference ($p > 0.05$) across the months (January - June) for stations 2 and 3 (nono milk). There was also no significant difference ($p > 0.05$) across stations for raw and nono milk (station 1 - 3) for the months of February, April, May and June (Table 2).

Table 1: Primer sequences and expected size of PCR amplified genes targeted in the isolates

Primer	Primer sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
Escherichia coli	F- AAAACGGCAAGAAAAAGCAG R- ACGCGTGGTTAACAGTCTTGCG	*uidA*	147	Bej et al., [37]
FliCH7	F-TACCATCGCAAAAGCAACTCC R-GTCGGCAACGTTAGTGATACC	*fliC_{H7}*	247	Wang et al., [38]
RfbE	F-CTACAGGTGAAGGTGGAATGG R-ATTCCTCTCTTTCCTCTGCGG	*rfbE_{O157}*	327	Nazemi et al., [39]

Table 2: Distribution of total heterotrophic bacterial counts on the milk samples over 6 months period of study

Months	Nono Milk			Raw Milk			p-value
	Station 1	Station 2	Station 3	Station 1	Station 2	Station 3	
January	$6.40\times10^4\pm0.14^{A}_{b}$	$3.10\times10^4\pm0.28^{A}_{a}$	$5.00\times10^4\pm0.28^{A}_{ab}$	$2.64\times10^5\pm0.14^{A}_{d}$	$2.16\times10^5\pm0.01^{A}_{c}$	$2.94\times10^5\pm0.00^{A}_{e}$	0.000
February	$1.50\times10^5\pm0.04^{A}_{a}$	$2.00\times10^5\pm0.14^{A}_{a}$	$2.50\times10^5\pm0.14^{A}_{a}$	$2.70\times10^5\pm0.40^{A}_{a}$	$2.30\times10^5\pm0.14^{A}_{a}$	$3.00\times10^5\pm0.14^{A}_{a}$	0.571
March	$2.88\times10^{10}\pm0.02^{C}_{c}$	$2.40\times10^{10}\pm0.14^{B}_{a}$	$2.58\times10^{10}\pm1.40^{B}_{b}$	$2.84\times10^{10}\pm0.14^{D}_{a}$	$2.88\times10^{10}\pm0.01^{C}_{c}$	$2.56\times10^{10}\pm0.01^{C}_{b}$	0.001
April	$4.07\times10^9\pm0.10^{A}_{a}$	$2.21\times10^{10}\pm0.16^{B}_{b}$	$1.33\times10^{10}\pm0.12^{AB}_{ab}$	$1.43\times10^{10}\pm0.93^{BC}_{ab}$	$1.07\times10^{10}\pm0.11^{AB}_{ab}$	$1.04\times10^{10}\pm0.27^{B}_{ab}$	0.223
May	$9.57\times10^9\pm0.01^{AB}_{a}$	$1.81\times10^{10}\pm1.55^{AB}_{a}$	$1.83\times10^{10}\pm0.11^{AB}_{a}$	$2.50\times10^{10}\pm0.49^{CD}_{a}$	$2.29\times10^{10}\pm0.04^{BC}_{a}$	$1.43\times10^{10}\pm0.48^{B}_{a}$	0.511
June	$2.16\times10^{10}\pm0.04^{BC}_{a}$	$1.18\times10^{10}\pm0.88^{AB}_{a}$	$1.13\times10^{10}\pm0.12^{AB}_{a}$	$8.80\times10^9\pm0.11^{AB}_{a}$	$1.16\times10^{10}\pm0.17^{AB}_{a}$	$1.17\times10^{10}\pm0.47^{B}_{a}$	0.559
p-value	0.015	0.056	0.139	0.002	0.005	0.000	

Values are in mean ± standard deviation of the mean. Values across column with different superscript uppercase alphabets show significant difference across column (months); while values across rows with different subscript lowercase alphabets show significant difference across rows (stations). P-values less than 0.05 were considered statistically significant

Table 3: Distribution of faecal coliform counts on the milk samples over the 6 months period of study

Months	Nono Milk			Raw Milk			p-value
	Station 1	Station 2	Station 3	Station 1	Station 2	Station 3	
January	$2.84\times10^5\pm0.14^{A}_{d}$	$2.80\times10^5\pm0.14^{B}_{d}$	$9.60\times10^4\pm0.14^{A}_{a}$	$1.68\times10^5\pm0.01^{A}_{b}$	$2.10\times10^5\pm0.14^{A}_{c}$	$2.16\times10^5\pm0.01^{A}_{c}$	0.000
February	0^{A}_{a}	0^{A}_{a}	0^{A}_{a}	0^{A}_{a}	$1.20\times10^5\pm0.14^{A}_{c}$	$8.00\times10^4\pm1.41^{A}_{b}$	0.000
March	$2.00\times10^5\pm0.14^{A}_{ab}$	0^{A}_{a}	0^{A}_{a}	$5.00\times10^5\pm1.41^{B}_{c}$	$3.00\times10^5\pm1.41^{A}_{bc}$	0^{A}_{a}	0.011
April	$2.00\times10^5\pm0.10^{A}_{a}$	$2.00\times10^5\pm1.73^{AB}_{a}$	$1.33\times10^5\pm1.52^{A}_{a}$	$1.33\ 10^5\pm1.15^{A}_{a}$	$6.00\times10^5\pm8.71^{A}_{a}$	$2.33\times10^5\pm2.08^{A}_{a}$	0.678
May	$1.87\times10^7\pm1.62^{A}_{b}$	$4.00\times10^3\pm4.0^{A}_{a}$	$9.33\times10^3\pm9.71^{A}_{a}$	$2.06\times10^4\pm3.40^{A}_{a}$	$3.16\times10^4\pm4.89^{A}_{a}$	$2.83\times10^5\pm4.14^{A}_{a}$	0.023
June	$5.50\times10^3\pm6.30^{A}_{a}$	$1.50\times10^4\pm0.14^{A}_{a}$	$5.95\times10^3\pm7.41^{A}_{a}$	$5.00\times10^3\pm1.41^{A}_{a}$	0^{A}_{a}	$1.05\times10^4\pm1.20^{A}_{a}$	0.366
p-value	0.123	0.028	0.294	0.001	0.643	0.136	

Values are in mean ± standard deviation of the mean. Values across column with different superscript uppercase alphabets show significant difference across column (months); while values across rows with different subscript lowercase alphabets show significant difference across rows (stations). P-values less than 0.05 were considered statistically significant

The mean faecal coliform count of the nono and raw milk is presented in Table 3. The mean coliform count for nono milk in respective stations ranges as follows; station 1 (0 - $1.87\times10^7\pm1.62$ CFU/ml), station 2 (0 - $2.80\times10^5\pm0.14$ CFU/ml), station 3 (0 - $1.33\times10^5\pm1.52$ CFU/ml), while that of the raw milk ranges as follows; station 1 (0 - $5.00\times10^5\pm1.41$ CFU/ml), station 2 (0 - $6.00\times10^5\pm8.71$ CFU/ml), and station 3 (0 - $2.83\times10^5\pm4.14$ CFU/ml). There was no significant difference ($p>0.05$) across months (January - June) for stations 1 and 3 (nono milk) as well as stations 2 and 3 (raw milk). In addition, there was no significant difference ($p>0.05$) across stations for raw and nono milk (station 1 - 3) for April, May and June.

The mean *E. coli* O157:H7 count of the nono and raw milk is presented in Table 4. The mean *E. coli* O157:H7 count for nono milk in respective stations ranges as follows: station 1 ($1.00\times10^5\pm0.14$ - $2.82\times10^7\pm0.14$ CFU/ml), station 2 ($4.00\times10^5\pm0.14$ - $3.00\times10^7\pm0.14$ CFU/ml), station 3 ($2.00\times10^5\pm0.14$ - $2.30\times10^7\pm0.14$ CFU/ml), while that of the raw milk ranges as follows; station 1 ($3.55\times10^5\pm4.03$ - $2.35\times10^7\pm0.14$ CFU/ml), station 2 ($5.10\times10^5\pm2.68$ - $2.52\times10^7\pm0.14$ CFU/ml), and station 3 ($2.66\times10^5\pm4.44$ - $2.40\times10^7\pm0.14$ CFU/ml). There was no significant difference ($p>0.05$) across the stations for both raw and nono milk (station 1 - 3) for the months of March, April, May and June.

Table 4: Distribution of *Escherichia coli* counts on the milk samples over the 6 months period of study

Months	Nono Milk			Raw Milk			*p*-value
	Station 1	Station 2	Station 3	Station 1	Station 2	Station 3	
January	$1.00 \times 10^{5} \pm 0.14^{A}_{a}$	$4.00 \times 10^{5} \pm 0.14^{A}_{a}$	$2.00 \times 10^{5} \pm 0.14^{A}_{a}$	$1.24 \times 10^{7} \pm 0.14^{B}_{c}$	$9.60 \times 10^{6} \pm 0.14^{C}_{b}$	$1.44 \times 10^{7} \pm 0.14^{B}_{c}$	0.000
February	$7.00 \times 10^{5} \pm 0.14^{A}_{a}$	$3.00 \times 10^{7} \pm 0.14^{C}_{c}$	$1.50 \times 10^{6} \pm 0.14^{A}_{ab}$	$2.40 \times 10^{6} \pm 0.14^{A}_{b}$	$2.40 \times 10^{6} \pm 0.14^{B}_{b}$	$1.20 \times 10^{6} \pm 0.14^{A}_{ab}$	0.000
March	$2.82 \times 10^{7} \pm 0.14^{B}_{b}$	$2.60 \times 10^{7} \pm 0.14^{B}_{ab}$	$2.30 \times 10^{7} \pm 0.14^{B}_{a}$	$2.35 \times 10^{7} \pm 0.14^{C}_{a}$	$2.52 \times 10^{7} \pm 0.14^{D}_{ab}$	$2.40 \times 10^{7} \pm 0.14^{C}_{ab}$	0.071
April	$8.30 \times 10^{5} \pm 5.96^{A}_{a}$	$1.13 \times 10^{6} \pm 0.71^{A}_{a}$	$6.36 \times 10^{6} \pm 5.43^{A}_{a}$	$1.15 \times 10^{6} \pm 0.84^{A}_{a}$	$1.32 \times 10^{6} \pm 7.52^{AB}_{a}$	$1.08 \times 10^{6} \pm 0.85^{A}_{a}$	0.873
May	$1.44 \times 10^{6} \pm 1.29^{A}_{a}$	$1.26 \times 10^{6} \pm 0.98^{A}_{a}$	$1.70 \times 10^{6} \pm 1.47^{A}_{a}$	$1.68 \times 10^{6} \pm 0.73^{A}_{a}$	$8.56 \times 10^{5} \pm 6.64^{AB}_{a}$	$2.66 \times 10^{5} \pm 4.44^{A}_{a}$	0.497
June	$2.30 \times 10^{5} \pm 0.28^{A}_{a}$	$4.30 \times 10^{5} \pm 3.81^{A}_{a}$	$5.30 \times 10^{5} \pm 0.98^{A}_{a}$	$3.55 \times 10^{5} \pm 4.03^{A}_{a}$	$5.10 \times 10^{5} \pm 2.68^{A}_{a}$	$5.85 \times 10^{5} \pm 4.31^{A}_{a}$	0.862
p-value	0.000	0.000	0.000	0.000	0.000	0.000	

Values are in mean ± standard deviation of the mean. Values across column with different superscript uppercase alphabets show significant difference across column (months); while values across rows with different subscript lowercase alphabets show significant difference across rows (stations). *P*-values less than 0.05 were considered statistically significant

Table 5: Distribution of the counts of other *Escherichia coli* strains on the milk samples over the 6 months period of study

Months	Nono Milk			Raw Milk			*p*-value
	Station 1	Station 2	Station 3	Station 1	Station 2	Station 3	
January	$1.00 \times 10^{5} \pm 0.14^{A}_{a}$	$2.00 \times 10^{5} \pm 0.28^{B}_{a}$	0^{A}_{a}	$1.40 \times 10^{7} \pm 0.14^{B}_{a}$	$1.68 \times 10^{7} \pm 0.14^{B}_{c}$	$2.08 \times 10^{7} \pm 0.01^{D}_{d}$	0.000
February	0^{A}_{a}	$1.60 \times 10^{6} \pm 0.14^{C}_{a}$	$4.00 \times 10^{5} \pm 0.14^{A}_{a}$	$2.80 \times 10^{7} \pm 0.14^{C}_{c}$	$1.80 \times 10^{7} \pm 0.14^{B}_{b}$	$1.82 \times 10^{7} \pm 0.01^{C}_{b}$	0.000
March	$6.00 \times 10^{5} \pm 0.14^{B}_{d}$	0^{A}_{a}	0^{A}_{a}	$1.00 \times 10^{5} \pm 0.14^{A}_{a}$	$2.00 \times 10^{5} \pm 0.14^{A}_{a}$	0^{A}_{a}	0.000
April	$7.00 \times 10^{4} \pm 2.00^{A}_{a}$	$4.66 \times 10^{4} \pm 8.08^{A}_{a}$	$3.40 \times 10^{5} \pm 5.37^{A}_{a}$	$5.33 \times 10^{5} \pm 4.88^{A}_{a}$	$4.76 \times 10^{5} \pm 7.65^{A}_{a}$	$4.33 \times 10^{4} \pm 6.65^{AB}_{a}$	0.559
May	$1.50 \times 10^{5} \pm 2.17^{A}_{a}$	$1.00 \times 10^{4} \pm 1.73^{A}_{a}$	$6.33 \times 10^{4} \pm 10.9^{A}_{a}$	$6.00 \times 10^{4} \pm 10.3^{A}_{a}$	$5.33 \times 10^{4} \pm 9.23^{A}_{a}$	$4.66 \times 10^{4} \pm 7.23^{AB}_{a}$	0.800
June	$1.70 \times 10^{5} \pm 2.12^{A}_{a}$	$6.00 \times 10^{4} \pm 1.41^{AB}_{a}$	$2.15 \times 10^{5} \pm 1.20^{A}_{a}$	$6.00 \times 10^{4} \pm 2.82^{A}_{a}$	$2.40 \times 10^{5} \pm 0.42^{A}_{a}$	$5.00 \times 10^{5} \pm 5.37^{B}_{a}$	0.536
p-value	0.019	0.000	0.525	0.000	0.000	0.000	

Values are in mean ± standard deviation of the mean. Values across column with different superscript uppercase alphabets show significant difference across column (months); while values across rows with different subscript lowercase alphabets show significant difference across rows (stations). *P*-values less than 0.05 were considered statistically significant

The mean counts of the other *E. coli* pathotypes for the nono and raw milk is presented in Table 5. The mean density of other *E. coli* pathotypes for nono milk in respective stations ranges as follows; station 1 (0 - $6.00 \times 10^{5} \pm 0.14$ CFU/ml), station 2 (0 - $1.60 \times 10^{6} \pm 0.14$ CFU/ml), station 3 (0 - $4.00 \times 10^{5} \pm 0.14$ CFU/ml), while that of the raw milk ranges as follows; station 1 ($6.00 \times 10^{4} \pm 2.82$ -$2.80 \times 10^{7} \pm 0.14$ CFU/ml), station 2 ($5.33 \times 10^{4} \pm 9.23$ - $1.80 \times 10^{7} \pm 0.14$ CFU/ml), and station 3 (0 - $2.08 \times 10^{7} \pm 0.01$ CFU/ml). There was no significant difference (*p*>0.05) across months (January - June) for stations 3 (nono milk). In addition, there was no significant difference (*p*>0.05) across stations for raw and nono milk (stations 1-3) for April, May and June.

Prevalence of faecal coliforms, *E. coli* O157:H7 and other *E. coli* pathotypes in raw and fermented milk samples

Of the nono milk samples assessed, all 33 (100%) were positive for *E. coli* O157, 22 (66.7%) were positive for other *E. coli* pathotypes, and 20 (60.6%) were positive for faecal coliforms. For the raw milk samples, all 33 (100%) were positive for *E. coli* O157, and 24 (72.7%) for other *E. coli* pathotypes and faecal coliforms (Table 6). Nineteen *E. coli* O157:H7 and 41 other *E. coli* pathotypes were confirmed by PCR assay (Figs 1, 2 & 3).

Table 6: Prevalence of *Escherichia coli* O157:H7, other *Escherichia coli* pathotypes and faecal coliforms from the milk samples

Milk samples	Stations	Bacteria	Number of samples examined	Number of positive samples	Percentage of positive samples
Nono Milk	Station 1	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	9	81.8
		Faecal coliforms	11	8	72.7
	Station 2	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	6	54.6
		Faecal coliforms	11	5	45.5
	Station 3	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	7	63.6
		Faecal coliforms	11	7	63.6
Raw Milk	Station 1	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	8	72.7
		Faecal coliforms	11	8	73.7
	Station 2	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	8	72.7
		Faecal coliforms	11	7	63.6
	Station 3	*E. coli* O157:H7	11	11	100
		Other *E. coli* pathotypes	11	8	72.7
		Faecal coliforms	11	9	81.8
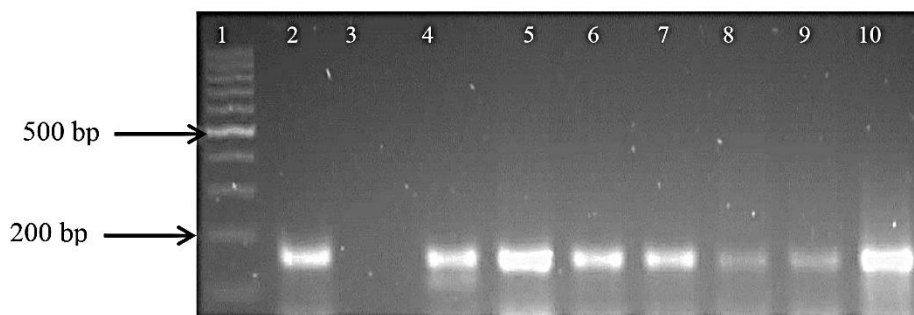
Lane 1: 100 bp Molecular marker; Lane 2: Positive control; Lane 3: Negative control; Lanes 4 to 10: Positive *E. coli* representative isolates obtained from raw and fermented milk samples.

Fig 1: Amplified *uid*A genes (147 bp) of *E. coli* isolates
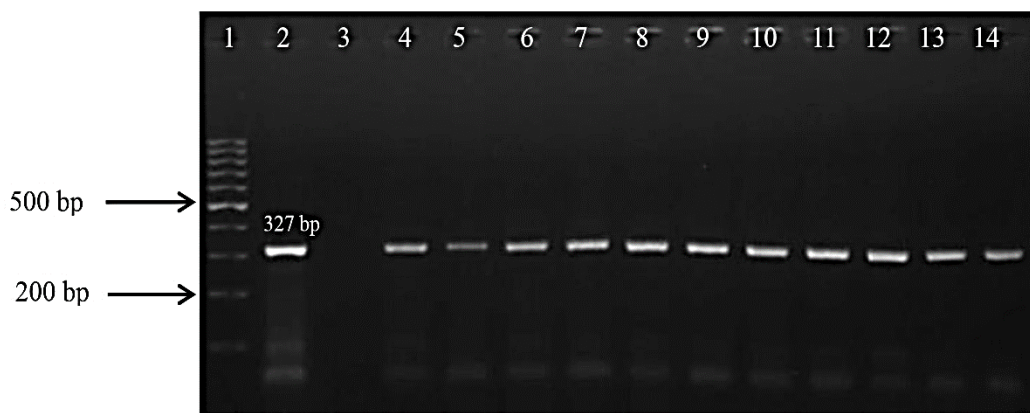
Lane 1: 100 bp Molecular Marker; Lane 2: Positive control; Lane 3: Negative control; Lanes 4 to 14: Positive *E. coli* O157 representative isolates obtained from raw and fermented milk samples

Fig 2: Amplified *rfbE*$_{O157}$ genes (327 bp) of *E. coli* O157 isolates.

Lane 1: 100 bp Molecular marker; Lane 2: Positive control; Lane 3: Negative control; Lanes 4 to 12: Positive *E. coli* H7 representative isolates obtained from raw and fermented milk samples

Fig 3: Amplified *fliC*$_{H7}$ genes (247) of *E. coli* H7 isolates

Table 7: Antimicrobial susceptibility profile of *Escherichia coli* O157:H7 and other *Escherichia coli* isolates

Antimicrobial class	Antibiotics	*E. coli* O157:H7 strain (*n*=19)			Other *E. coli* strain (*n*=41)		
		R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
Penicillin	Penicillin G	19 (100)	-	0	41 (100)	-	0
	Ampicillin	19 (100)	0	0	41 (100)	0	0
	Amoxicillin	12 (63.1)	3 (15.8)	4 (21.1)	34 (82.9)	5 (12.2)	2 (4.9)
β-lactam\Beta lactamase inhibitors	Ampicillin\Sulbactam	10 (52.6)	3 (15.8)	6 (31.6)	25 (61)	5 (12.2)	11 (26.8)
	Amoxicillin\Clavulanate	11 (57.9)	5 (26.3)	3 (15.8)	32 (78)	7 (17.1)	2 (4.9)
Aminoglycosides	Gentamycin	0	0	19 (100)	1 (2.4)	0	40 (97.6)
	Kanamycin	0	1 (5.3)	18 (94.7)	0	1(2.4)	40 (97.6)
	Streptomycin	2 (10.5)	2 (10.5)	15 (80)	3 (7.3)	4(9.8)	34 (82.9)
	Tobramycin	6 (31.6)	1 (5.3)	12 (63.1)	5 (12.2)	7 (17.1)	29 (70.7)
Tetracyclines	Doxycycline	6 (31.6)	1 (5.3)	12 (63.1)	11 (26.8)	3 (7.3)	27 (65.9)
	Tetracycline	7 (36.8)	8 (42.1)	4 (21.1)	21 (51.2)	12 (29.3)	8 (19.5)
	Oxytetracycline	15 (78.9)	4 (21.1)	0	26 (63.4)	10 (24.4)	5 (12.2)
Carbapenems	Imipenem	12 (63.1)	3 (15.8)	4 (21.1)	27 (65.9)	5 (12.2)	9 (22)
	Meropenem	0	3 (15.8)	16 (84.2)	1 (2.4)	1 (2.4)	39 (95.1)
Cephalosporins	Cephalothin	2 (10.5)	2 (10.5)	15 (78.9)	12 (29.3)	2 (4.9)	27 (65.8)
	Cefotaxime	12 (63.2)	7 (36.8)	0	30 (73.2)	8 (19.5)	3 (7.3)
Macrolides	Erythromycin	17 (89.5)	1 (5.3)	1 (5.3)	38 (92.7)	1 (2.4)	2 (4.9)
Folate pathway inhibitors	Trimethoprim	12 (63.2)	2 (10.5)	5 (26.3)	27 (65.9)	5 (12.2)	9 (21.9)
	Sulfamethoxazole	15 (78.9)	4 (21.1)	0	34 (82.9)	4 (9.8)	3 (7.3)
Polymyxins	Polymyxin B	7 (36.8)	10 (52.60	2 (10.5)	10 (24.4)	26 (63.4)	5 (12.2)
	Colistin	3 (15.8)	1 (5.3)	15 (78.9)	6 (14.6)	1 (2.4)	34 (83)
Phenicols	Chloramphenicol	18 (94.7)	0	1 (5.3)	40 (97.6)	0	1 (2.4)
Quinolone	Ofloxacin	0	0	19 (100)	2 (4.9)	1 (2.4)	38 (92.7)
	Ciprofloxacin	0	2 (10.5)	17 (89.5)	0	6 (14.6)	35 (85.4)

GEN: Gentamycin (10µg); KAN: Kanamycin (30µg); STR: Streptomycin (25µg); TOB: Tobramycin (10µg); AMP: Ampicillin (10µg); AMX: Amoxicillin (25µg); SAM: Ampicillin/Sulbactam (30µg); IPM: Imipenem (10µg); MEM: Meropenem (10µg); CTX: Cefotaxime (30µg); CEF: Cephalothin (30µg); SUL: Sulfamethoxazole (30µg); TMP: Trimethoprim (25µg); ERY: Erythromycin (15µg); PEN: Penicillin G (10 units); AMC: Amoxicillin/clavulanate (30µg); CHL: Chloramphenicol (30µg); CST: Colistin (20µg); PMB: Polymyxin B (300 units); DOX: Doxycycline (30µg); OXY: Oxytetracycline (30µg); TET: Tetracycline (30µg); CIP: Ciprofloxacin (10µg); OFX: Ofloxacin (5µg); R: Resistant; I: Intermediate; S: Sensitive; Values in parenthesis represent percentage; MAR: Multiple antibiotic resistance

Antibiotic resistance profiles of *E. coli* isolates

The antibiotic resistance profile of the 19 genetically confirmed *E. coli* O157:H7 isolates is shown in Table 7; all 19 (100%) isolates were resistant to penicillin G and ampicillin, 18 (94.7%) to chloramphenicol, 17 (89.5%) to erythromycin, 15 (78.9%) to sulfamethoxazole and oxytetracycline, 12 (63.1%) to amoxicillin, imipenem, cefotaxime and trimethoprim, 11 (57.9%) to amoxicillin/clavulanate, and 10 (52.6%) to ampicillin/sulbactam. All the 19 (100%) isolates were however sensitive to gentamicin and ofloxacin.

The resistance profile of the 41 other genotypically confirmed *E. coli* isolates is presented in Table 7; all 41 (100%) isolates are resistant to penicillin G and ampicillin, 40

(97.6%) to chloramphenicol, 38 (92.7%) to erythromycin, 34 (82.9%) to amoxicillin and sulfamethoxazole, 32 (78%) to amoxicillin/ clavulanate, 30 (73.2%) to cefotaxime, and 27 (65.9%) to trimethoprim and imipenem. A total of 40 (97.6%) isolates were however sensitive to gentamicin and kanamycin.

Multidrug resistance (MDR) profiles of the 19 *E. coli* O157:H7 isolates shows that all 19 (100%) were resistant to 3 antibiotics (AMP[R], PEN[R], CHL[R]) in 2 antimicrobial classes with multiple antibiotic resistance (MAR) index of 0.13. The extensive drug resistance profiles include resistance of 10 (52.6%) to 11 antibiotics (AMP[R], PEN[R], CHL[R], ERY[R], SUL[R], OXY[R], AMX[R], TMP[R], IMI[R], AMC[R], SAM[R]) in 7 antimicrobial classes with MAR index of 0.46 (Table 8).

Multidrug resistance (MDR) profiles of 41 other *E. coli* isolates shows that 40 (97.6%) were resistant to 3 antibiotics (AMP[R], PEN[R], CHL[R]) in 2 antimicrobial classes with MAR index of 0.13. The extensive drug resistance profiles include resistance of 25 (60.9%) to 11 antibiotics (AMP[R], PEN[R], CHL[R], ERY[R], SUL[R], AMX[R], AMC[R], CTX[R], SAM[R], IMI[R], TMP[R]) in 7 antimicrobial classes with MAR index of 0.46 (Table 8).

Discussion:

Milk is highly nutritious; however, it is a well-thought-out high-risk food as it can serve as a good medium for bacterial growth (18). Several factors can contribute to milk contamination such as unhygienic milking conditions, tainted equipment, and poor hygiene of individual milk handlers (19). The high bacterial counts reported in this study certainly reveal the overall poor conditions of hygiene/cleanliness and temperature control under which the milk was produced and handled. Probable reasons for the high counts could be due to infection of cow udder, use of germ-contaminated equipment, lack of cooling after milking and absence of heat treatment. These can contribute to the low hygiene quality of the raw milk. Consequent on this, training in general milking, hygiene/germ-free practices and keeping of milk at low temperature should be given to the farmers and retailers to circumvent microbial growth and extend the shelf life of milk (20).

The high prevalence of faecal conta-mination reported in our study is higher than the rate reported in Plateau State, Nigeria, where 0.7% (5/350) of the nono samples and 3.0% (21/350) of the raw milk samples had *E. coli* O157 isolated (21). According to a study by Ahmed and Samer (22), where 50 raw and 50 pasteurized milk samples were investigated, *E. coli* O157 was isolated from 33 (66%) raw and 15 (30%) pasteurized milk samples. Another study by Garbaj et al., (23) on milk and dairy products in Libya revealed that *E. coli* O157 (6/28, 21.4%) was iden-tified from fermented cow milk, which is far lower than the rates obtained in our study where 100% of the nono milk samples were positive for *E. coli* O157. Arafa and Soliman (24) in a study conducted in Egypt on raw milk and fresh cream reported that 2.6% and 1% were contaminated with *E. coli* O157:H7 respectively. Allerberger et al., (25) also rep-orted 3% of milk samples tested in Austria to be positive for *E. coli* O157:H7, which is in tandem with the report of Arafa and Soliman (24). On the other hand, Chye et al., (26) detected *E. coli* O157:H7 in 33.5% of raw milk samples in Malaysia. The disparities observed in these studies might be due to

dissimilarity in animal management, milking system, and handling practices in different regions. The ability of *E. coli* O157:H7 to survive in fermented milk samples could be the result of the organism's tolerance to high acidity (23).

The prevalence of *E. coli* from milk samples (66.7%) in our study is higher than the rate of 33.9% reported by Disassa et al., (20) in a study of 380 raw cow milk samples collected from marketed raw cow milk in and around Asosa Town, Western Ethiopia. However, the prevalence rate in our study is similar to those reported by Lingathurai et al., (27) in South India, Ali (28) in Britain, and Shunda et al., (29) in Mekelle town, with reported incidence of *E. coli* from raw milk of 70%, 63% and 44.4% respectively. The presence of *E. coli* is a strong indicator of poor sanitary practices during the milking process, transportation, production methods, and sale of milk and milk products. This portends a potential danger for individuals consuming such products (24). In Nigeria, it is a common practice to manufacture dairy products from raw milk. The isolation of *E. coli* O157:H7, other *E. coli* pathotypes, and faecal coliforms from large proportions of raw and fermented (nono) milk in our study could be a potential source for human diseases following their consumption. However, it is difficult to link these findings with any case of food poisoning that might have occurred in Benin City due to poor documentations of such cases of bacterial food poisoning.

Antimicrobial resistance in enteric bacteria has become a global burden these past years, particularly in developing coun-tries like Nigeria, which has played a crucial role in restricting treatment options with evidential spread of resistant pathogenic strains to humans through food (30). The antibiotic resistance rates in this study is slightly different from an earlier report by Msolo et al., (31) which indicated a resistant rate of 85% for penicillin G, 45% for chlor-amphenicol, 70% for erythromycin, and 74% for sulfamethoxazole. The 100% resistance to penicillin observed in our study agrees with the study of Alam et al., (6) who reported high rate (100%) of resistance to penicillin among *E. coli* O157 isolates cultured from raw milk marketed in Chittagong, Bangla-desh.

The high susceptibility rate (100%) to gentamicin and ofloxacin for genotypically confirmed *E. coli* 0157:H7 isolates obtained in this study is different from a report by Alam et al., (6) where 50% rate to gentamicin and ofloxacin was reported. High resistance rates to penicillin and tetracyclines in our study agrees with the antibiotic susceptibility test study by Reuben and Owuna (32) on *E. coli* O157 isolates recovered from milk samples.

In a similar study by Tadesse et al., (33), *E. coli* showed high resistance rates to ampicillin (70%), sulfamethoxazole-trimethoprim (60%), clindamycin (80%), erythromycin (60%), chloramphenicol (50%), and kanamycin (50%), which is slightly different from the findings of our study. Antibiotic resistance could be due to abuse of antibiotics in both human medicine and for agricultural purposes, predominantly in disease suppression and advancement of growth in animal production. The high susceptibility of *E. coli* to kanamycin in our study is different from the study of Tadesse et al., (33) which reported 50% resistance rate, although the study reported high susceptibility rates to some antibiotics such as gentamicin (100%), ofloxacin (100%), and ciprofloxacin (90%), which is similar to the findings in our study.

Multiple antibiotic resistance (MAR) index is a good tool for health risk assessment which identifies if isolates are from a region of high or low antibiotic use. MAR index values greater than 0.2 indicate high risk source of infection where antibiotics are often used. MAR indexing has been revealed to be a cost effective and usable method of bacteria source tracking. The high MAR index of the isolates from this study could be a potential detriment to human health in our environment. In accordance with the multidrug resistance (MDR) pattern reported by Igwe et al., (5), resistance pattern of their isolates showed that 54.5% were MDR and 18.2% were extensive drug resistance (EDR). This is however different from the high EDR (52.6%) obtained in our study, which might be attributed to misuse of antibiotics within Benin City. Sudda et al., (34) reported 87.5% MDR among *E. coli* isolates, with about 50% resistant to sulfamethoxazole/trimethoprim, penicillin, tetracycline, and amoxicillin/clavulanic acid. The MDR pattern of *E. coli* reported by Haque (35) in Bangladesh and by Memon et al., (36) in Eastern China also showed similar results. This MDR pattern could be the result of amassing of resistance genes on plasmids, each coding resistance to specific antibiotics and/or multi-drug efflux pumps (34). The accumulation of MDR bacteria is a significant threat to public health as this can lead to ineffective treatment of infections and poor recovery of patients (12).

Conclusion:

The findings of our present study clearly indicated that safety and quality of fresh and fermented milk in Benin City were unsatisfactory. The presence of fecal coliform bacteria denotes poor hygiene practices. The pathogenic bacteria such as *E. coli* O157:H7 and other *E. coli* strains may be transferred through the milk to consumers. Fresh and fermented milk is of a special concern since these organisms can proliferate at variable conditions using the milk as reservoir. In addition, the high MAR index observed in *E. coli* O157:H7 and other *E. coli* strains in our study is a threat to consumers' health.

Conflict of interest:

Authors declare no conflict of interest

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