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Prevalence of Multiple Antibiotic Resistant *Escherichia coli* Serotypes in Cow Raw Milk Samples and Traditional Dairy Products in Osun State, Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author TOA was involved in the experimental work and managed the analyses of the study. Author OAO supervised and wrote the paper. Author KDO designed and supervised the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Isolation and characterization of multiple antibiotic resistant *Escherichia coli* serotypes in cow raw milk and traditional dairy products in Osun state, Nigeria was reported.
Study Design: Experimental based study of raw milk of lactating cows, local cheese and yoghurt.
Place and Duration of Study: Samples were collected at different markets in Ile-Ife, Modakeke, Edun-abon, and Akinlalu in Osun State, Nigeria, between June and August, 2011.
Methodology: Samples of cow raw milk, cheese and yoghurt were enumerated bacteriologically on MacConkey agar at 37℃ for the total coliform. Isolation of *E. coli* was done on eosin methylene blue agar at 37℃ using pour plate technique. The isolate identity was further confirmed by

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biochemical tests and serotyping. Antibiotic susceptibility of the isolates was determined by the disk diffusion technique. Molecular typing of *Escherichia coli* was performed using polymerase chain reaction (PCR) technique.

Result: The coliform count ranged from 1.0 to 6.00×105cfu/g in raw milk, 1.30 to 9.60×105cfu/g in cheese and 1.3 to 5.0×105cfu/g in yoghurt. The presence of *E. coli* was significantly low in cow raw milk compared to yoghurt and cheese ($p< 0.05$). Resistance to antibiotics varied among the *E. coli i*solates with the highest resistance to tetracycline (88.6%), cotrimoxazole (77.2%) and nitrofurantoin (6.81%). Most *E. coli* isolates were multiple antibiotic resistant types displaying 17 different multiple antibiotic resistance patterns. All the *E. coli* strains belonged to 9 serogroups ('O') and 17 serotypes ('H'). The 'O' serogroups identified include O26 (2), O55 (4), O86 (3), O111 (11), O114 (1), O119 (1), O127 (5), O128 (1) and O142 (4) with 0111 (35.48%) being the most prevalent serotype.

The flagellin (fliC) gene restriction analysis of *E. coli* serotypes showed that 72.7% were motile with 17 H-type while 27.3% were non-motile (O55 (1), O86 (1), O111 (5), O119 (2), O127 (2), O142 (1)). The polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) patterns for the amplified fliC gene in the *E. coli* strains, produced bands of sizes ranging from 0.9 to 1.8 kb.

Conclusion: The identification of *E. coli* O55:H27, O111:H8, O127:H42 and O142:H6 serotypes in the dairy products is of great public health concern.

Keywords: Cow milk; local cheese; yoghurt; Escherichia coli serotype; antibiotic resistance.

1. INTRODUCTION

Pathogenic bacteria in milk have been a major factor for public health concern since the early days of the dairy industry [1]. Many diseases are transmissible via dairy products; traditionally raw or unpasteurized milk has been a major vehicle for transmission of pathogens [2,3].

Contamination of milk and dairy products by pathogenic micro-organisms can be of endogenous origin, following excretion from the udder of an infected animal and or exogenous origin, through direct contact with infected herds or through the environment for example, water, personnel [4].

Dairy products like yoghurt, ice cream, butter and cheese are widely consumed and market for them has existed in many parts of the world for many generations. Martin et al. [5] reported two cases of haemolytic uremic syndrome which provide evidence that raw milk may be a vehicle of transmission of *E. coli* (*E. coli* 0157:H7) since both affected persons consumed raw milk. In 1971, United States of America faced an outbreak of food poisoning in which 387 persons suffered with enteropathogenic *E. coli*due to the consumption of imported French cheese [6]. The recovery of *E. coli* is indicative of possible presence of enteropathogenic and or toxigenic microorganism which could constitute a public health hazard. Enteropathogenic *E. coli* can cause severe diarrhea and vomiting in infants and young children [7].

The diarrhoea-causing strains of *E. coli* fall into four distinct groups: the Enteropathogenic *Escherichia coli* (EPEC), the Enterotoxigenic *Escherichia coli* (ETEC), the Enteroinvasive *Escherichia coli* (EIEC), and Enterohaemorrhagic *Escherichia coli* (EHEC). Enteropathogenic *Escherichia coli* (EPEC) strains are associated with outbreaks of infantile diarrhoea in bottle-fed babies. Owing to increased awareness of hygienic procedures, the incidence has diminished. Serotypes 0111:B4 and 055:B5 were the first to be identified as causing diarrhoea. The most common serotypes are 026, 055, 0111, 086, 0119, 0128 and 0142

Serotyping of *E. coli* is based on three types of antigens: O, K and H antigens. *Escherichia coli* is known as the "Colon bacillus" because it is the predominant facultative species in the large bowel. More than 181 different O antigens of *E. coli* have been identified by specific agglutination reactions [8].

Escherichia are widely spread throughout nature, and it is possible that strains with O, K or H antigens exist that are similar to the known enteropathogenic varieties. High prevalence of *Escherichia coli* was reported by many researchers. In Egypt, Aly and Galal, [9] showed the presence of *E. coli* in raw milk and the number reduced in the heat treated one. In South

Africa, Lues et al. *[*10] detected a higher percentage of *E. coli* in raw milk. In Malaysia, Chye et al. [11] indicated that 90% of the examined raw milk was contaminated by coliform bacteria of which 65 % were *E. coli* positive. The presence of these potentially pathogenic organisms in milk could be as a result of poor hygiene or contamination from poor handling of the milk samples by workers, particularly carriers of these organisms.

Milk is a highly nutritious food that is ideally suited for the growth of pathogenic and spoilage organisms. Prior to widespread adoption of pasteurization, bacterial infections such as diphtheria, scarlet fever and tuberculosis were often linked to the consumption of raw milk products. Even though dairy products are consumed on a daily basis in the United States, milk, ice cream and cheese have been identified as a vehicle for less than 1.5 % of all food borne illness cases investigated by the center for disease control [12]. Approximately 50 % of the milk produced is consumed as fresh or boiled, one-sixth as yoghurt or curd and the remaining is utilized for manufacturing indigenous varieties of milk products such as ice-cream, butter, Khoa, paner, rabri, kheer, burfi and Gulabjman [13]. The manufacture of these products is based on traditional method without any regard to quality of raw material used and or the hygienic quality of the products. Under such conditions many microorganisms can gain access to the milk products. Among all microorganism *Escherichia coli* is the most frequently contaminating organism and is a reliable indicator of fecal pollution generally in insanitary conditions of water, food, milk and other dairy products [14]. Several studies showed that resistance to antibiotics in *E. coli* is on the increase with cost implication in infection therapy. The present study identified and reported the prevalence of multiple antibiotic resistant *E. coli* serotypes in cow raw milk samples and dairy products in Nigeria.

2. MATERIALS AND METHODS

2.1 Sample Collection

Raw milk samples were collected by direct milking into sterile glassware from selected lactating cows marketed at different markets in Ile-Ife, Modakeke, Edun-abon, and Akinlalu in Osun State, Nigeria. The yoghurt and local cheese (waara) samples collected in clean transparent polythene bags were purchased from the vendors within the localities and transported to the laboratory under refrigeration (4–6ºC) in thermal boxes containing ice packs and were analyzed bacteriologically within one hour of collection. Raw milk was transported to the laboratory immediately after collection at ca. 4ºC Sample collection spanned over a period of three months between June and August 2011.

2.2 Bacteriological Analysis

Enumeration of coliforms in raw milk, yoghurt and cheese samples was carried out on MacConkey agar (Oxoid Ltd., Hampshire, England) plate. For cheese sample, 10g was aseptically transferred into a sterile mortar and pestle, and homogenized in 90 ml sterile distilled water using stomacher. One milliliter of the 10 fold dilutions of the homogenized cheese was cultured. All plates were incubated at 37ºC for 48h. Bacterial counts were expressed as colony forming units per ml. Isolation of *E. coli* was carried out on sterile eosin methylene blue agar ((Oxoid Ltd., Hampshire, England) plate.

2.3 Identification and Characterization of Bacterial Isolates

Preliminary identification of *E. coli* was based on colonial morphology by a characteristic green metallic sheen on EMB agar plates and confirmed by various biochemical tests with reference to Bergey's Manual of Determinative Bacteriology [15].

2.3.1 Antibiotic susceptibility

Antibiotic susceptibility of isolates was performed by the Kirby-Bauer antibiotic disc diffusion technique on Mueller Hinton agar (Hi Media, Vadhani, India) (Clinical and Laboratory Standards Institute [16]. The antibiotics (oxoid, UK), augmentin (30 µg), nitrofurantoin (200 µg), ofloxacin (5 µg), tetracycline (30 µg), gentamicin (10 µg), amoxicillin (20 µg), cotrimoxazole (25 µg), nalidixic acid (30 µg) were firmly placed on the agar plates previously seeded with the test organisms and incubated at 37ºC for 24h. Sensitivity of the isolates to different antibiotics was indicated by the clear zones of inhibition which were measured in milliliter using a calibrated ruler. The diameters of zone of inhibition were compared to CLSI standard.

2.4 Proteolytic Activity

Assaying for proteolytic enzyme was done on sterile lactose egg yolk milk medium containing

0.5ml of the test organisms in a bored well (6 mm) in Petri dishes incubated at 37℃ for 24h. Casein hydrolysis was determined on nutrient agar containing 10% skimmed milk in Petri dishes incubated at 37℃ for 24h. Clearzones around the lines of growth indicated casein hydrolysis and were measured.

2.5 Serology

Determination of the *E. coli* serogroups was performed by agglutination tests using polyvalent and monovalent *E. coli* antisera kits (Statens Serum Institute, Denmark) against O and flagella H antigens (H1 to H 56) according to the manufacturer's instructions.

2.6 DNA Isolation

Isolation of DNA was done by culturing *E. coli* strains overnight in trypticase soy agar (TSA-Merck, German) at 37ºC. One colony was suspended in 100 µL of sterile distilled water. After boiling the suspension for 13 min, this was followed by freezing and subsequently centrifuged at 14,000 rpm for 15 min to pellet the cell debris. The supernatant was used as a template for amplification reaction.

2.6.1 Amplification of flagellin (*Flic)* gene by polymerase chain reaction

Polymerase chain Reaction (PCR) was performed for detection of *E. coli* serotypes by application of specific primers. The coding sequence of the *fliC* gene was amplified by PCR with the primers, fFSA (5′- CAAgTC ATT AATACAAACAgC C-3′) and rFSA (5′- gAC ATA TTGgAC ACT TCg gT-3′) designed for detection of flagella antigen encoded gene in *Escherichia coli* strains [17].

A 50 µl reaction mixture contained 1 µl of template DNA (approximately 50 ng), 25 Pmol of each primer, 200 µ Mdeoxyribonucleotide triphosphate mixture, 8 µl of 10X PCR buffer, 1.5 mM $MgCl_2$, 2.5 U of Taq polymerase. The standard cycling condition after the initial denaturation step of 5 min at 94℃, were 1 min at 94℃, 1 min at 57℃, and 2 min at 72℃ for 35 cycles and final extension step of 5 min at 72℃. For some strains, the annealing temperature was reduced to 54 to 55℃ to increase the amount of product that was amplified. PCR products (10 µl) were separated by electrophoresis in 0.8 % agarose gel in Trisborate buffer (0.089 M Tris-base, 0.089 M boric acid, 2.5 mM EDTA-Na_2, pH 8), with the 1 kbp DNA ladder as a molecular size marker.

The *fli*C PCR product was digested with *Hha*I restriction endonuclease according to the manufacturer's instructions and incubated overnight at 37℃. The restriction fragments were separated on a 2% agarose gel for 5 h at 5 V/cm.

2.7 Statistical Analysis

Data were statistically analyzed using Chi-square tests of the statistical analysis system software (SPSS 16.0 version) to establish the significant relationship in the prevalence of organisms between the samples. P value of < 0.05 was considered significant for all the comparisons.

3. RESULTS

The total coliform count ranged from 1.0 to 6.0×10^5cfu/g in raw milk, 1.3 to 9.6×10^5cfu/g in cheese and 1.3 to 5.1×10^5cfu/g in yoghurt (Table 1). There was no significant difference in the coliform count of raw milk and cheese, and yoghurt's (P=0.05), but comparable between cheese and yoghurt (P=0.05). The presence of *E. coli* was significantly low in raw milk compare to yoghurt and cheese (P= 0.05) (Table 2).

Table 1. Coli form count in cow raw milk, cheese and yoghurt

Coliform count (x10^5 CFU/g)		
Cow raw milk (n=10)	**Cheese (n=10)**	**Yoghurt (n=10)**
4.80	1.50	2.50
1.00	1.40	1.30
2.70	9.60	4.10
0	1.60	5.10
0	3.40	0
1.00	2.70	5.00
1.10	8.0	5.10
6.00	3.60	0
0	3.00	3.60
4.50	1.30	4.20

Escherichia coli developed resistance mostly to tetracycline (88.6%), cotrimoxazole (77.2%) and nitrofurantoin (6.81%) (Table 3).

Table 2. Prevalence of *E. coli* in cow raw milk samples and dairy products

Samples	No of sample positive for *E. coli* (%)
Raw milk	4 (9.2)
Yoghurt	13 (29.5)
Cheese	27 (61.3)

Table 3. Antibiotic resistance profile of *Escherichia coli* isolates in cow raw milk, yoghurt and cheese

Antibiotics(µg)	*E. coli* (44)	
NAL(30 µg)	Resistant	Isolates (%)
Cotrimoxazole(25µg)	14	(31.8)
Amoxicillin (20µg)	34	(77.2)
Nitrofurantoin (300µg)	23	(52.2)
Gentamicin (10µg)	3	(6.81)
Ofloxacin (5µg)	7	(15.9)
Augumentin (30µg)	0	(0)
Tetracycline (30µg)	25	(56.8)
Nalidixic acid(30 µg)	39	(88.6)

Resistance to multiple antibiotics was common among the *E. coli* isolates in cow milk and dairy products (Table 3). The multi-resistance patterns of the isolates to antibiotics are presented in Table 4. A total of 17 different multiple antibiotic resistance patterns were observed. Greater percentage (51.4%) displayed multiple resistance to three classes of antibiotics, 34.2% to four and 17.1% to five classes.

All the isolates showed high proteolytic activity due to their ability to produce clear zones of inhibition around the line of growth on the skimmed milk medium used.

Table 5 shows the *fli C* gene restriction analysis of motile *E. coli* and O-H serotypes. All the forty-four *E. coli* strains belonged to 9 serogroups ('O') and 17 serotypes ('H'). The 'O' serogroups identified include O26 (2), O55 (3), O86 (3), O111 (11), O114 (1), O119 (1), O127 (5), O128 (1) and O142 (4). A common pattern was observed for the flagellin gene in H8, H21, H9, and H2 which has 200 bp restriction fragments in common (Table 6). Molecular weights of the *fliC* gene restriction analysis of *E. coli* serotypes ranged from 0.07 to 1.2 kb.

Molecular screening of *E. coli* serotypes digested with HhaI restriction endonuclease is depicted by Plate 1, and the restriction fragment (bp) of the genes for each serotype at different base pairs is presented in Table 6.

The *fliC* gene restriction analysis of the non-motile *E. coli* serogroup is presented in Table 7. Twelve (27 %) strains were non-motile and belonged to O serogroup viz; O55 (1), O86 (1), O111 (5), O119 (2), O127 (2), O142 (1). Serogroup O111 was the most prevalent serotype (35.48 %), followed by O127 (16.12 %) and O142 (12.90 %).

Table 4. Multiple antibiotic resistance patterns among the *Escherichia coli* isolates in cow raw milk and milk products

No of Antibiotics	Multiple antibiotic resistance pattern	Isolates (n)	Total (%)
3	COT, AUG, TET	8	18 (51.4)
	NAL, AMX, TET	3	
	COT, AMX, TET	2	
	COT, GEN, TET	2	
	AMX, AUG, TET	2	
	NAL, COT, TET	1	
4	AMX, NIT, GEN, TET	1	12 (34.2)
	COT, AMX, AUG, TET	4	
	COT, GEN, AUG, TET	1	
	AMX, GEN, AUG, TET	1	
	NAL, COT, AMX, TET	2	
	COT, NIT, AUG, TET	1	
	COT, AMX, GEN, TET	1	
	COT, AMX, AUG, TET	1	
5	NAL, COT, AMX, NIT, TET	1	6 (17.1)
	NAL, COT, AMX, AUG, TET	4	
	NAL, COT, GEN, AUG, TET	1	

NAL-Nalidixic acid COT- CotrimoxazoleAMX- Amoxicillin NIT- Nitrofurantoin GEN- Gentamicin AUG- Augmentin TET- Tetracycline

Table 5. *FliC* gene restriction analysis of *E. coli* serotypes (motile)

Serogroups No	Percentage (%) occurrence of H antigens	H-type	F-type
O26(2)	6.45	H29(1), H10(1)	F29(1), F10(1)
O55(4)	9.99	H27(2), H12(1), H21(1)	F27(2), F12(1), F21(1)
O86(3)	9.67	H8(1), H32(1), H4(1)	F8(1), F32(1), F4(1)
O111(11)	35.48	H9(1), H8(3), H48(1), H23(1), H32(1), H20(1), H2(1), H19(1), H21(1)	F9(1), F8(3), F48(1), F23(1), F32(1), F20(1), F2(1), F19(1), F21(1)
O114(1)	3.22	H2(1)	F2(1)
O119(1)	3.22	H27(1)	F27(1)
O127(5)	16.12	H19(1), H25(1), H42(2), H6(1)	F19(1), F25(1), F42(2), F6(1)
O128(1)	3.22	H9(1)	F9(1)
O142(4)	12.90	H6(2), H27(1), H48(1)	F6(2), F27(1), F7(1)

H – flagellaantigen; O – Somatic antigen

Table 6. *Flic* gene restriction analysis of motile *Escherichia coli* strains using HhaI

H antigen	Serogroup (no. of strains)	Restriction fragments (bp)
H8	O111(3)	300, 200, 100
H21	O111 (1)	600, 400, 200, 100
	O55 (1)	650, 170, 150, 120, 100
H9	O111 (1)	200, 100, 80, 70
	O128 (1)	
H2	O111(1)	1,200, 200
	O114 (1)	
H32	O111 (1)	400, 250, 230, 100
H48	O111 (1)	500, 190, 100, 50
	O142 (1)	
H19	O111 (1)	300, 140, 100, 50
	O127 (1)	380, 200, 180, 150, 100
H25	O127 (1)	600, 200, 100, 50
H27	O55 (2)	300, 80, 70,
	O119 (1)	
	O142 (1)	
H12	O55 (1)	150, 100, 80, 70

4. DISCUSSION

The presence of coliforms generally in the samples may be due to improper storage on the part of the retailers, environmental conditions and cross transfer of the organisms from workers who are carriers. Meanwhile, the recovery of enteropathogenic strains of *E. coli* serotype is of great public health concern as it signifies gross faecal contamination which may result in bloody diarrhea in man following its consumption.

Escherichia coli were the most common species of facultative anaerobes found in the human gastrointestinal tract and the most commonly encountered pathogen in the *Enterobacteriaceae* family. The present findings confirmed that the dairy products manufactured by traditional methods are more contaminated by *E. coli* than cow raw milk. Hence, agrees with earlier reports [18,3].

According to United States Environmental Protection Agency [19], the presence of *E. coli* in the intestine and faeces of warm-blooded animals is an indicator of faecal pollution. The grazing of cattle and land application of animal wastes may lead to the occurrence of enteric pathogens near the surface and ground waters. This potential contamination due to animal husbandry operations can be a serious threat to public health. The present work reports high level

of *E. coli* in yoghurt and cheese samples, this may be due to careless handling at various stages of processing.

Escherichia coli isolates were resistant to more than three classes of antibiotics used. Resistance of isolates to antimicrobials may however, have been acquired from over exposure to antibiotics particularly in the veterinary sector or to a lesser extent from natural sources especially when some of the antibiotics tested in this study are commonly used in both the health and veterinary sectors. This study reveals that the strains tested were resistant to tetracycline and each of the strains showed multiple resistance to three or more antibiotics. The multiple resistance observed were mostly to those antibiotics frequently employed in veterinary practices. Evidence exists to suggest that not only are such antibiotic resistant strains more difficult to control in terms of human infection, they may also be more resistant to heat processes [20]. It has been reported that *E. coli* and *Enterococcus faecalis* constitute a potential reservoir of resistance genes for pathogenic bacteria and are used internationally as indicator bacteria for antibiotic resistance because of their high prevalence in animal faeces [21,22].

High level of antibiotic resistance in the study may reflect its extensive use and misuse. This resistance has also been recorded in other studies on milk and dairy products [23].

Serotypes O26, O111 and O128 were recovered from the study. This corroborates the work of Allerberger et al. [24] who isolated *E. coli*O26 from animals and humans. Evidences of detection of this pathogen in beef and other foods of animal origin by Allerberger et al. [24] and Murphy et al. [25], support the probability that animals are the main sources of spreading this serogroup.

The serotypes (O26, O111, O128) comprise an important emerging group of zoonotic enteric pathogens of animals and humans, and indeed may be more prominent than O157 [26]. In humans, some *E. coli* infections result in bloody or non-bloody diarrhoea, which may be complicated by haemorrhagic colitis and severe haemolytic uraemic syndrome in humans. *E. coli* with the O111 serotype causes both enteropathogenic and enterohemorrhagic diseases in humans [27].

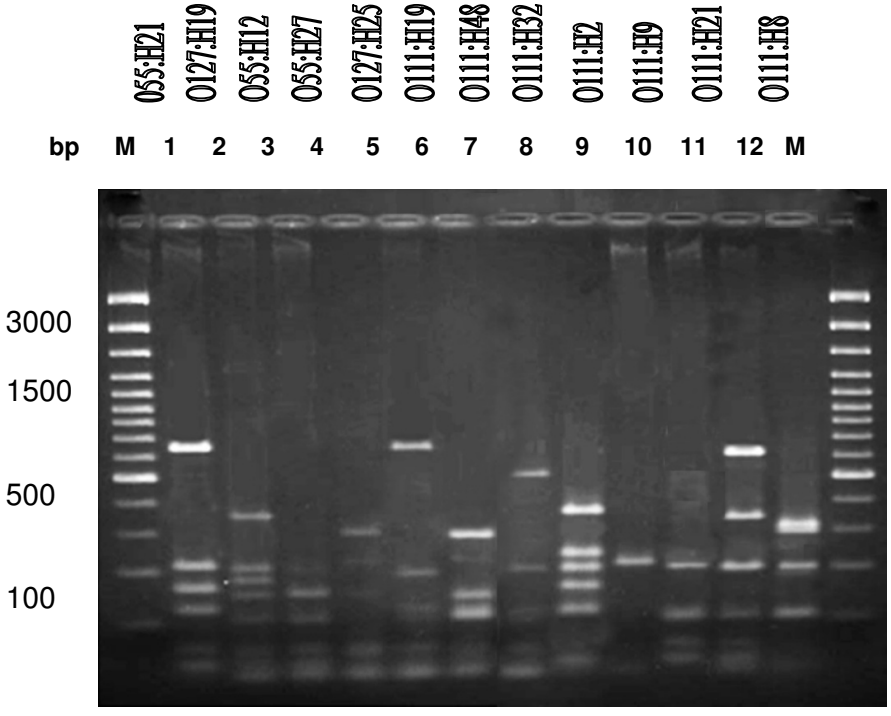

Plate 1. Molecular screening of *E. coli* serotypes digested with Hhal restriction endonuclease
M - 100-bp DNA ladder

Table 7. *FliC* gene restriction analysis of non-motile *E. coli* serotypes in cow raw milk, cheese and yoghurt

Serogroups No	H-type	F-type
O55(1)	H^-(1)	F8(1)
O86(1)	H^-(1)	F12(1)
O111(5)	H^-(5)	F48(1), F9(1), F27(2), F2(1)
O119(2)	H^-(2)	F32(1), F29(1)
O127(2)	H^-(2)	F7(1), F21(1)
O142(1)	H^-(1)	F8(1)

H – flagella antigen; O – Somatic antigen

5. CONCLUSION

This study concluded that variety of *E. coli* serotypes (O26, O111 and O128) are present in the dairy products analyzed, hence calls for great concern, considering the health implications of these serotypes in human. To this extent, regular sterilization of dairy equipment, proper washing of utensils, hands and cow udders, pasteurization and/or boiling of milk before distribution for consumption are highly essential. In addition, Quality control measures should be put in place to checkmate the various contaminations at different stages of dairy product production.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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