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Microbiological Quality Assessment of Dairy Product: Detection of Extended Spectrum Beta Lactamase Genes in Bacterial Isolates from Yoghurt Sold in Sagamu Metropolis, Ogun State, Nigeria

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Abstract

The presence of pathogenic microorganisms in dairy products including yoghurt is undesirable because of the risk of infectious diseases. This study assessed the microbiological quality of some yoghurt samples in Sagamu metropolis, Ogun State, Nigeria with respect to pH, microbial types and counts and detection of extended spectrum beta lactamase (ESBL) genes. The pH readings ranged between 4.05 and 5.81 while minimum total viable bacterial and minimum total coliform counts ranged from 1.1 x 10^1 to 2.0 x 10^6 CFU/mL and 3.0 X 10^0 to 2.3 x 10^4 CFU/mL, respectively. The samples yielded a total of 40 bacterial isolates including 15 Gram-positive and 25 Gram-negative isolates belonging to eight Genera among which Staphylococcus aureus and Pseudomonas aeruginosa were the most prevalent 10 (25%). Escherichia coli showed the highest resistance to levofloxacin (100%). Gram-negative isolates had higher number of multidrug resistant isolates (92%). ESBL was detected phenotypically in 4 (16%) of Gram-negative bacteria. Only SHV gene was detected in two P. aeruginosa while CTX-M and TEM genes were not detected in any of isolates. The yoghurt samples contained pathogenic microorganisms that are capable of causing health complications. Therefore, rigorous inspection and quality standards are required to ensure Good Manufacturing Practice.

Keywords: Bacterial contaminant, fungal isolate, yoghurt, extended spectrum beta lactamase,

INTRODUCTION

Yoghurt is a dairy product fermented by lactic acid bacteria like *Streptococcus thermophilus* and *Lactobacillus delbruckii* subspecies *bulgaris*, as starter culture (Obi *et al.,* 2016). These bacteria are crucial in the production of yoghurt (Serra *et al*., 2009; Aryana and Olson, 2017). Yoghurt can be made with whole or skim milk and is available in a number of flavours (Oyeleke, 2009).

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The fermentation of milk by lactic acid bacteria also serves to prolong the shelf life of the milk's nutrients (Hui, 1992; Oyeleke, 2009) Yoghurt is a well-balanced food drink that is high in protein and contains almost all of the nutrients found in milk, but in an easier-to-digest form (Cueva and Aryana, 2008). Food infection and intoxication have been attributed to the effect of poor hygiene in the manufacturing, handling, and storage of such foods (Stewart and Humphrey, 2002). Yoghurt, like all milk products, can be contaminated by bacteria and fungi, which are the most prevalent pollutants detected in Nigerian industrially sold yoghurt (Oyeleke, 2009). Yoghurt manufacturing in Nigeria is difficult because of inadequate housing facilities, poor solid waste disposal, a lack of bacteriologically free water and inadequate sanitation, particularly in densely populated regions with low-income residents (Taiwo *et al*., 2018). Environmental sanitation law enforcement agencies are rarely available to issue warnings or prosecute violators. Therefore, these industries are unconcerned about disposing of their wastes properly (Taiwo *et al*., 2018).

Each year, nearly one in every ten people on the planet, or 600 million people, are expected to become ill as a result of contaminated food, with 420 000 people dying as a result (WHO, 2020). Foodborne diseases affect 40% of children under the age of five, resulting in 125 000 deaths each year (WHO, 2020). The relative significance of pathogenic organisms in yoghurt borne infection is quite well recognized in countries in which food-borne illnesses are investigated and documented (Obende, 1999). Some bacteria such as *Staphylococcus aureus, Escherichia coli, Klebsiella* species, other Gram-negative bacteria and fungi, have been linked to yoghurt-borne infections (Okonkwo, 2011; Belli *et al*., 2013; Afolabi *et al*., 2017; Taiwo *et al*., 2018). Enterotoxins, which have been connected to food poisoning, are produced by these microorganisms (Obende 1999; Uzeh 2006; WHO, 2012). Humans are the most common source of *S. aureus* contamination and more than half of all *S. aureus* strains can produce enterotoxin, which is a toxin linked to food poisoning (Payre and Wood, 1974). Diarrhoeal diseases are the most common illnesses caused by contaminated food, with 550 million people falling ill and 230 000 people dying each year (WHO, 2020). The water utilized or the employees or utensils used in the processing are the sources of coliform bacterial contamination (Murphy and Boor, 2000). Fungi can grow and reproduce in an acidic environment with sufficient oxygen (De *et al.,* 2014). *Aspergillus* species have been linked to the formation of toxic and harmful bioactive molecules known as aflatoxins (Issazadeh *et al.,* 2012). The sensory quality and physicochemical characteristics such as colour and texture of yoghurt can be changed or modified when it is contaminated by fungi or moulds (Garnier *et al*., 2017). As a result, humans may be at risk if they eat or drink it.

The transportation of finished items by manufacturers from manufacturing sites to their various consumer sales channels and handling by vendors is a problem and can result in post-production contamination (Samuel and Ifeanyi, 2016; Taiwo *et al*., 2018). Usually, the majority of yoghurts are produced at homestead level in small-scale industries under different brands and sold in kiosks or more commonly hawked along roadways, in parking lots, and in market (Afolabi *et al.,* 2017; Taiwo *et al*., 2018). These consumers do not really take into account the temperature or how the package was handled, so it becomes contaminated if it's dumped in an unsanitary truck or vehicle (Ekanem, 1998; Taiwo *et al*., 2018).

The rise of Gram-negative bacteria expressing extended-spectrum -lactamases (ESBLs) has become a public health problem (Tohoyessou *et al*., 2021). These strains are resistant to a variety of antimicrobial agents and can be difficult to treat due to a lack of therapeutic options (Bradford, 2001). Penicillinases, ESBLs, carbapenemases, and AmpC-type cephalosporinases are the four functional groups of β-lactamases (Bush, 2010). ESBLs are the most common and

widespread of these enzymes, capable of hydrolyzing virtually all penicillins and third generation cephalosporins like cefotaxime and ceftazidime (Livermore, 2012). Many ESBL producers are resistant to multiple antibiotics including non-beta-lactam antibiotics, such as fluoroquinolones, trimethoprim, tetracyclines, sulfonamides, aminoglycosides, and chloramphenicol (Rawat and Nair, 2010). This is because resistance genes to these antibiotics are usually carried on plasmids that carry ESBL leading to few treatment options (Pitout, 2010). Numerous ESBL-genes, carbapenem-resistant genes, and other antibiotic resistance determinant factors occurring simultaneously on mobile elements is a major source of concern, as it could lead to the upsurge of pan-drug resistant bacteria (Bush, 2010). Various researchers have reported the presence of ESBL and antibiotic-resistance in Gram negative bacteria.

The existence of ESBL and antibiotic-resistant Gram-negative bacteria in milk and milk products has been reported by a number of researchers (Stefani *et al.,* 2014; Kurekci *et al.,* 2016; Tekiner and Özpınar, 2016; Tepeli and Zorba, 2018). However, there is little information available regarding the frequency of ESBL-producing Gram negative bacteria in Nigerian yoghurt. Therefore, the purpose of this study was to assess the frequency of bacterial and fungal contaminants in yoghurt sold in Sagamu and ascertain the presence of ESBLs genes (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}9}$) in the Gram negative bacteria isolated.

MATERIALS AND METHODS

Collection of Yoghurt Samples

Thirty yoghurt samples consisting of twenty-one (70%) bottled and nine (30%) sachet samples were purchased from various sellers within Sagamu metropolis, Ogun state. The samples were brought to the Pharmaceutical Microbiology Laboratory, Olabisi Onabanjo University, Sagamu and properly labelled.

Determination of pH of Yoghurt Samples

The pH values of the yoghurt samples were measured with a pH meter (Mettler Toledo, Switzerland). that was standardized with buffer solution pH 7. Samples of yoghurt were aseptically taken in sterile containers. The pH meter was inserted into each sample and the pH values were read and documented.

Determination of Microbial Load in the Yoghurt Samples

One milliliter of each yoghurt sample was added to 9.0 mL of sterile distilled water and diluted up to 10^{-4} using serial dilution method. One milliliter of 10^{-4} dilution was seeded on nutrient agar, MacConkey agar and Potato Dextrose agar (Himedia, India) plates using the pour plate method. Nutrient agar and MacConkey agar plates were incubated at 37 ^{o}C for 24 hours while Potato Dextrose agar plates were incubated at 25 ^{o}C for 5 days. The experiment was performed in triplicates. The mean total colonies count was obtained and expressed as colony forming units per milliliter [CFU/ml] of the yoghurt samples.

Identification of Bacterial Isolates

Using a sterile wire loop, a discrete colony of the bacteria to be identified was collected from cultured plates and subcultured on sterilized Mannitol salt agar, MacConkey agar, Eosin Methylene Blue agar, Salmonella-Shigella agar and blood agar and incubated at 37 °C for 24 hours. Isolated colonies were subcultured on sterilized nutrient agar slants and stored at 4 °C until further use. Gram staining, sugar fermentation on triple sugar iron (TSI) agar, hydrogen

sulphide production, citrate, methyl red, voges-proskauer, oxidase, catalase indole, urease and coagulase tests were all used to identify different bacterial species (Cheesbrough, 2006).

Identification of Fungal Isolates

Colony features such as size, shape, colour and hyphae were observed macroscopically to study the fungal morphology. A small portion of the mycelium was mounted on a slide and stained with Lactophenol-in-cotton blue for microscopic examination of fungi. The conidia, conidiophores and spore arrangement were examined under a compound microscope (Nagamani *et al*., 2006; Gaddeyya *et al*., 2012).

Antimicrobial Susceptibility Test using Agar Diffusion

The antibacterial susceptibility of the isolates to some selected antibiotics was determined using the Kirby-Bauer disc diffusion method. Standard conventional antibiotic discs such as levofloxacin (5 µg), imipenem (10 µg), azithromycin (15 µg), gentamicin (10 µg), cefuroxime (30 µg), carbenicillin (100 µg), cefotaxime (30 µg), amoxicillin/clavulanic acid (30 µg), cefepime (30 µg), cephalexin (30 µg) and ceftazidime (30 µg) were used. All the isolates were subcultured on sterilized nutrient agar plates and incubated at 37 ^{o}C for 18 hours. Then 4 colonies were picked from nutrient agar plates and subsequently suspended in sterile normal saline to attain 1.5 x 10^{8} CFU/mL turbidity (0.5 McFarland standard). Then, 20mL Mueller-Hinton agar was poured to a depth of 4 mm into a 90 mm diameter sterile Petri-dish after cooling to 45 ^{o}C and allowed to solidify. The plates were placed upright in a dry heat oven and allowed to dry for 30 minutes at 36 ^{o}C before being used. The suspension was inoculated on Mueller-Hinton agar surface using a sterile swab stick. With the aid of a sterile pair of forceps, the antibiotic discs were put on the inoculated agar plates' surfaces. The plates were left on the bench for 1 hour before being incubated for 24 hours at 37 °C. Using the Clinical and Laboratory Standards Institute (CLSI) 2021 performance standards for antimicrobial susceptibility testing, the zones of growth inhibition were measured in millimeters, recorded, and interpreted as sensitive, intermediate, or resistant.

Detection of Extended Spectrum Beta- Lactamase

Phenotypic Detection of Extended Spectrum Beta- Lactamase

Extended spectrum beta- lactamase detection (ESBL) was done by using modified double disc synergy test (MDDST). The isolates were sub-cultured on sterilized nutrient agar plates and incubated overnight at 37 ^{o}C. Then, a loopful of each isolate was picked and suspended in sterile normal saline to give turbidity equivalent to McFarland standard (1 x 10^{8} CFU/mL). Then, 20ml of Mueller-Hinton agar after cooling to 45 ^{o}C was poured into 90mm diameter sterile Petri dish to depth of 4 mm. The plates were dried for immediate use, for 30 minutes at 36 ^{o}C by placing them in an upright position in a dry heat oven. The Mueller-Hinton agar plates were streaked with the isolate suspension using a sterile swab stick. Then, amoxicillin-clavulanic acid (30 µg) was placed at the center of the plate, and antibiotic discs containing cefotaxime (30 µg), cefepime (30 µg) and ceftazidime (30 µg) were placed 2cm (center to center) from amoxicillin-clavulanic acid disc with the aid of a sterile pair of forceps. The plates were then incubated at 37 ^{o}C for 24 hours. A clear extension of the edge of the zones of growth inhibition of cephalosporins towards amoxicillin-clavulanic acid disc was interpreted positive for ESBL production. As positive and negative controls, an ESBL-positive strain of *Klebsiella pneumoniae* ATCC 700603 and an ESBL-negative strain of *Escherichia coli* ATCC 25922 were used, respectively.

Extraction of DNA

Chromosomal DNA from bacteria was extracted using the boiling process. Cultures from nutrient agar were used to inoculate 2 mL of Luria Bertani broth (LB), which was then incubated for 24 hours. The membranes of the bacteria were weakened by being suspended in 500 µL of phosphate buffer (100 mM, pH 7), after which the genetic material was released by boiling the bacteria for 15 minutes in a water bath at 100 °C. The DNA was then precipitated in 250 liters of pure alcohol and thrice washed in 1000 mL of 70% alcohol. The DNA was then re-suspended in 100 mL of sterile water (Duru *et al*., 2020).

Detection of ESBL genes in Gram-negative bacterial isolates

The ESBL genes- blaTEM, blaSHV and blaCTX-M- were detected by PCR using the thermal cycler (Applied Biosystems, USA). The PCR final volume of 25 µL, consists of 1 µL of DNA, 12.5 µl of WizPure™ PCR 2X Master (Wizbiosolutions, South Korea), 1 µL of each primer (0.2 pmol/µl) and 9.5 µL molecular grade water.

Two PCR processes were performed; one duplex for blaTEM (F: 5-GAGTATTCAACATTTTCGT-3 and R: 5-ACCAATGCTTAATCAGTGA-3) and blaSHV (F: 5-TCGCCTGTGTATTATCTCCC-3 and R: 5-CGCAGATAAATCACCACAATG-3) annealing at 50 °C, and one simplex for blaCTX-M (F: 5-TTTGCGATGTGCAGTACCAGT AA-3 and R: 5-CGATACGTTGGTGGTGCCATA-3) annealing at 56 °C (Maynard *et al*., 2003; Edelstein *et al*., 2003). The PCR products was loaded on 2% agarose gel stained with ethidium bromide and run at 100 v for hour. As a reference, a marker of 100 bp was used. Following migration, the various bands were visible under Ultraviolet light.

RESULTS

Samples' pH and microbial growth

The pH ranged from 4.05 to 5.81. Table 1 shows the mean colony forming unit per milliliter of each sample on De Man Rogosa and Sharpe agar, nutrient agar, MacConkey agar and potato dextrose agar. On nutrient agar, 26(86.7%) had growth. Twenty-one (70%) and 19 (63.3%) samples had growth on MacConkey agar and potato dextrose agar, respectively. Lactic acid bacteria growth was observed in 16 (53.3%) of the samples. The mean total viable bacterial count ranged from 1.1×10^1 to 2.0×10^6 CFU/mL. Out of the thirty samples, 24 (80.0%) samples had total viable bacterial count of less than or equal to 10^5 CFU/mL. Two (6.7%) samples had total viable bacterial count above 10^5 CFU/mL while 4 (13.3%) samples had no growth. The mean total coliform count ranged from 3.0×10^0 to 2.3×10^4 CFU/mL. Out of the 30 samples, 5 (6.7%) had a total coliform count between 1 and 10 CFU/mL, 16 (or 53.3%) had a total coliform count over 10 CFU/mL, and 9 (30%) had no coliform at all. The mean total yeast and mold counts ranged from 2.0×10^1 to 7.6×10^6 CFU/mL. Three (10) samples had total yeast and mold counts exceeding 10^5 CFU/mL while 11 (36.7%) samples had no growth. Between 1.0×10^3 and 6.0×10^7 CFU/mL were the values for the lactic acid bacterial count. Out of the thirty samples, 14 (46.7%) showed no growth, and 16 (53.3%) contained colonies with fewer than 10^6 CFU/mL

Table` 1 1: Microbial counts and pH of the yoghurt samples

S/N	Samples	MTVBC [CFU/mL]	MTCC [CFU/mL]	MTFC [CFU/mL]	MTLC [CFU/mL]	pH of Samples
1.	A	3.2×10^1	5.0×10^0	9.0×10^1	_	4.97
2.	B	2.1×10^1	_	7.6×10^6	6.0×10^3	5.17
3.	C	2.0×10^1	3.0×10^0	2.5×10^1	2.5×10^5	4.84
4.	D	1.8×10^1	_	7.0×10^1	9.0×10^3	4.98

5.	E	1.0 X 10^2	3.5 X 10^1	5.3 X 10^1	_	4.08
6.	F	_	2.1 X 10^1	3.0 X 10^1	5.0 X 10^3	4.77
7.	G	1.4 X 10^2	9.0 X 10^1	7.0 X 10^1	_	5.81
8.	H	2.2 X 10^2	2.2 X 10^1	1.1 X10^6	5.2 X 10^5	4.07
9.	I	8.0 X 10^4	3.0 X 10^0	1.4 X 10^1	1.0 X 10^3	4.07
10.	J	3.5 X 10^2	2.7 X 10^1	9.9 X 10^1	7.5 X 10^4	4.97
11.	K	3.0 X 10^2	_	1.5 X 10^1	2.0 X 10^3	4.12
12.	L	6.0 X 10^2	3.0 X 10^2	5.0 X 10^6	5.0 X 10^4	4.32
13.	M	2.0 X 10^6	1.5 X 10^1	3.5 X 10^0	4.0 X 10^3	4.95
14.	N	1.6 X 10^2	_	_	1.2 X 10^7	4.17
15.	O	5.0 X 10^0	_	4.0 X10^1	_	4.05
16.	P	3.2 X 10^1	1.0 X 10^1	_	6.5 X 10^4	4.95
17.	Q	6.0 X 10^1	3.2 X 10^1	_	6.0 X 10^7	4.89
18.	R	1.1 x 10^1	1.2 x 10^1	_	-	4.97
19.	S	3.1 x 10^1	1.6 x 10^1	4.0 x 10^1	-	4.82
20.	T	2.0 x 10^2	8.0 x 10^1	_	2.0 x 10^4	4.10
21.	U	-	6.0 x 10^0	2.0 x 10^1	-	4.77
22.	V	1.6 x 10^1	-	-	-	4.99
23.	W	2.3 x 10^1	2.3 x 10^4	6.0 x 10^1	-	5.21
24.	X	6.0 x 10^1	-	-	-	4.11
25.	Y	-	-	-	-	4.90
26.	Z	2.1 x 10^1	4.0 x 10^0	-	-	4.89
27.	Aa	-	-	-	-	4.65
28.	Ba	1.7 x 10^1	1.1 x 10^1	-	6.0 x 10^4	5.05
29.	Ca	1.8 x 10^1	1.6 x 10^1	9.0 x 10^1	-	4.97
30.	Da	4.5 x 10^1	5.0 x 10^1	4.0 x 10^1	3.0 x 10^4	4.89

MTVBC = Mean total viable bacterial count; MTCC = Mean total coliform count; MTFC = Mean total fungal count; MTLC = Mean total lactic Acid bacteria

Occurrence of Bacterial and Fungal Isolates in Yoghurt Samples

A total 40 isolates were obtained from the samples including 15 Gram positive and 25 Gram negative bacterial isolates. Figure 1 shows the percentage of the isolated bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were the most prevalent 10 (25%), followed by *Klebsiella pneumoniae* 5 (13%). The least occurring isolate was *Acinetobacter* species. Twenty-four fungal species belonging to five genera were identified (Figure 2). *Aspergillus* species was the most occurring fungus 13 (54.2%) followed by *Penicillium* species 4 (16.7%).

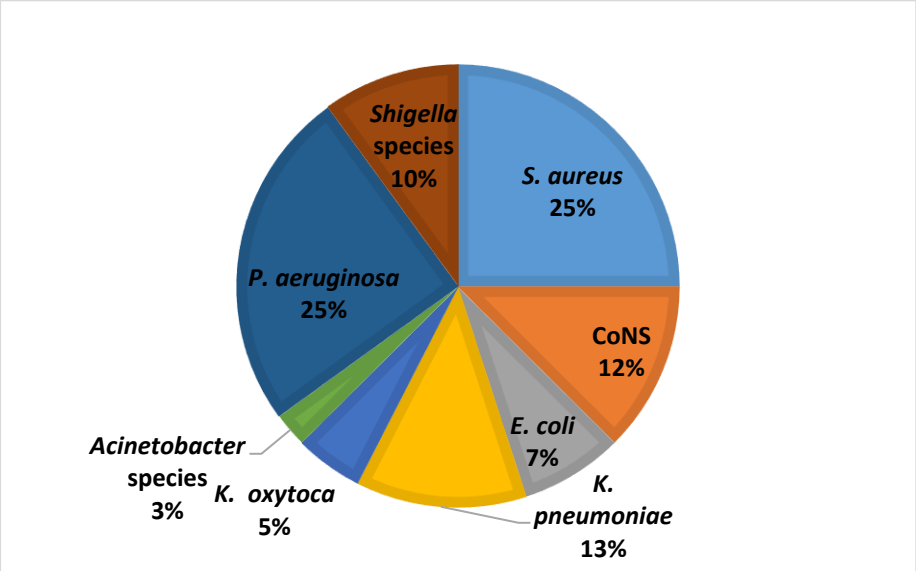

Figure 1: Percentage of bacterial isolates from yoghurt samples

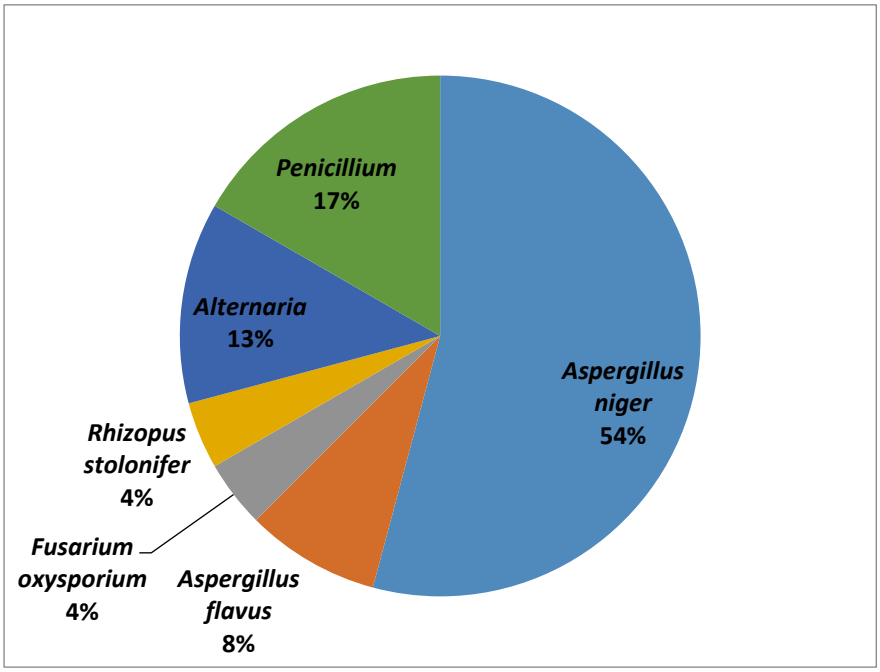

Figure 2. Percentage of fungal isolates in herbal mixtures

Antibiotic Susceptibility Profile of the Isolates

E. coli showed the highest resistance to levofloxacin (100%) followed by *K. oxytoca*. *E. coli* was the most resistant to gentamicin (100%), followed by *K. pneumoniae* (60.0%). *E. coli* and *K. pneumoniae* showed highest resistance rate of 66.7% and 60.0%, respectively to imipenem (Table 2). Table 3 shows the prevalence of multidrug resistance in both Gram-positive and Gram-negative isolates. Gram-negative isolates had higher percentage of multidrug resistant isolates (92%) than Gram-positive isolates (86.7%). In Gram-positive isolates; 90.9% of *S. aureus* was multidrug resistant. All *E. coli, K. oxytoca, Shigella* and *Acinetobacter* species were multidrug resistant

Table 2. Antibiotic resistance profile of bacterial isolates

Isolates (no)	LEV	IMP	AZM	GM	CRX	CAR	CTX	AUG	CFP	CLX	CAZ
S. aureus (10)	2(20)	2(20)	1(10)	3(30)	8(80)	9(90)	1(10)	6(60)	7(70)	9(90)	8(80)
CoNS (5)	0	0	0	2(40)	4(80)	4(80)	3(60)	2(40)	4(80)	4(80)	3(60)
E. coli (3)	3(100)	2(66.7)	1(33.3)	3(100)	3(100)	3(100)	2(66.7)	3(100)	1(33.3)	3(100)	2(66.7)
K. pneumonia (5)	2(40)	3(60)	3(60)	3(60)	4(80)	4(80)	4(80)	2(40)	3(60)	5(100)	4(80)
K. oxytoca (2)	1(50)	0	0	1(50)	2(100)	2(100)	2(100)	1(50)	0	2(100)	2(100)
A. baumannii (1)	0	0	0	0	1(100)	0	1(100)	0	1(100)	1(100)	1(100)
P. aeruginosa (10)	1(10)	0	2(20)	2(20)	8(80)	4(40)	5(50)	7(70)	2(20)	9(90)	4(40)
Shigella species (4)	0	0	1(25)	2(50)	4(100)	4(100)	2(50)	3(75)	3(75)	4(100)	3(75)

Note: CoNS = Coagulase negative staphylococci, LEV=Levofloxacin, IMP=Imipenem, AZM=Azithromycin, GM=Gentamicin, CRX= Cefuroxime, CAR = Carbenicillin, CTX= Cefotaxime, AUG=Augmentin, CFP= Cefepime, CLX= Cephalexin, CAZ= Ceftazidime

Table 3. Antibiotic resistance profile of multidrug resistant bacterial isolates

	Isolate code	Isolate	Resistance profile
1.	A	*S. aureus*	IMP, GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
2.	C1	*S. aureus*	IMP, AZM, GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
3.	F2	*S. aureus*	CRX, CAR, CTX, AUG, CFP, CLX, CAZ
4.	L1	*S. aureus*	GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
5.	P1	*S. aureus*	CRX, CAR, CTX, CFP, CLX, CAZ
6.	Q	*S. aureus*	LEV, CAR, CTX, AUG, CFP, CLX, CAZ
7.	R	*S. aureus*	CRX, CAR, CTX, CFP, CLX, CAZ
8.	T1	*S. aureus*	CRX, CAR, AUG, CLX, CAZ
9.	Ba1	*S. aureus*	LEV, CAR, CTX, CLX
10.	H1	CoNS	GM, CRX, CAR, CTX, CFP, CLX, CAZ
11.	N2	CoNS	CTX, CFP, CLX, CAZ
12.	P2	CoNS	CRX, CAR, AUG, CFP, CLX, CAZ
13.	E3	CoNS	GM, CRX, CAR, CTX, AUG, CFP, CLX
14.	D	*Shigella* sp.	GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
15.	H2	*Shigella* sp.	GM, CRX, CAR, AUG, CFP, CLX, CAZ
16.	L2	*Shigella* sp.	CRX, CAR, AUG, CLX
17.	N1	*Shigella* sp.	AZM, CRX, CTX, CFP, CLX, CAZ
18.	J1	*E. coli*	LEV, GM, CRX, CAR, AUG, CLX
19.	K1	*E. coli*	LEV, IMP, AZM, GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
20.	S	*E. coli*	LEV, IMP, GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
21.	E4	*A. baumannii*	CRX, CTX, CFP, CLX, CAZ
22.	P3	*K. oxytoca*	LEV, GM, CRX, CAR, CTX, AUG, CLX, CAZ
23.	F1	*K. oxytoca*	CRX, CAR, CTX, CLX, CAZ
24.	B1	*K. pneumoniae*	IMP, AZM, GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
25.	I1	*K. pneumoniae*	CTX, AUG, CFP, CLX, CAZ
26.	W	*K. pneumoniae*	LEV, IMP, AZM, GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
27.	Da1	*K. pneumoniae*	LEV, IMP, GM, CRX, CAR, CTX, AUG, CFP, CLX, CAZ
28.	B2	*P. aeruginosa*	AZM, CRX, CAR, CLX
29.	C2	*P. aeruginosa*	AZM, CRX, CTX, CFP, CLX, CAZ
30.	E2	*P. aeruginosa*	GM, CRX, CAR, AUG, CLX, CAZ
31.	G	*P. aeruginosa*	CRX, AUG, CLX
32.	J3	*P. aeruginosa*	CRX, AUG, CLX
33.	L3	*P. aeruginosa*	CRX, AUG, CLX
34.	O	*P. aeruginosa*	CTX, AUG, CFP, CLX, CAZ
35.	U	*P. aeruginosa*	LEV, CRX, CAR, CTX, AUG, CLX
36.	Ca1	*P. aeruginosa*	CRX, CTX, AUG, CLX

Note: CoNS = Coagulase negative *Staphylococcus*, LEV = Levofloxacin, IMP = Imipenem, AZM = Azithromycin, GM = Gentamicin, CRX = Cefuroxime, CAR = Carbenicillin, CTX= Cefotaxime, AUG = Augmentin, CFP = Cefepime, CLX = Cephalexin, CAZ = Ceftazidime

Prevalence of ESBL in Gram Negative Bacterial Isolate from Assayed Yoghurt Samples

ESBL was detected phenotypically in 4 (16%) of Gram-negative bacteria which included two *P. aeruginosa* and one *Shigella* and *Acinetobacter* species. Figure 3 shows the gel picture of amplified SHV genes. Among the isolates, only two *P. aeruginosa* had SHV genes while CTX-M and TEM genes were not detected in any of these isolates.

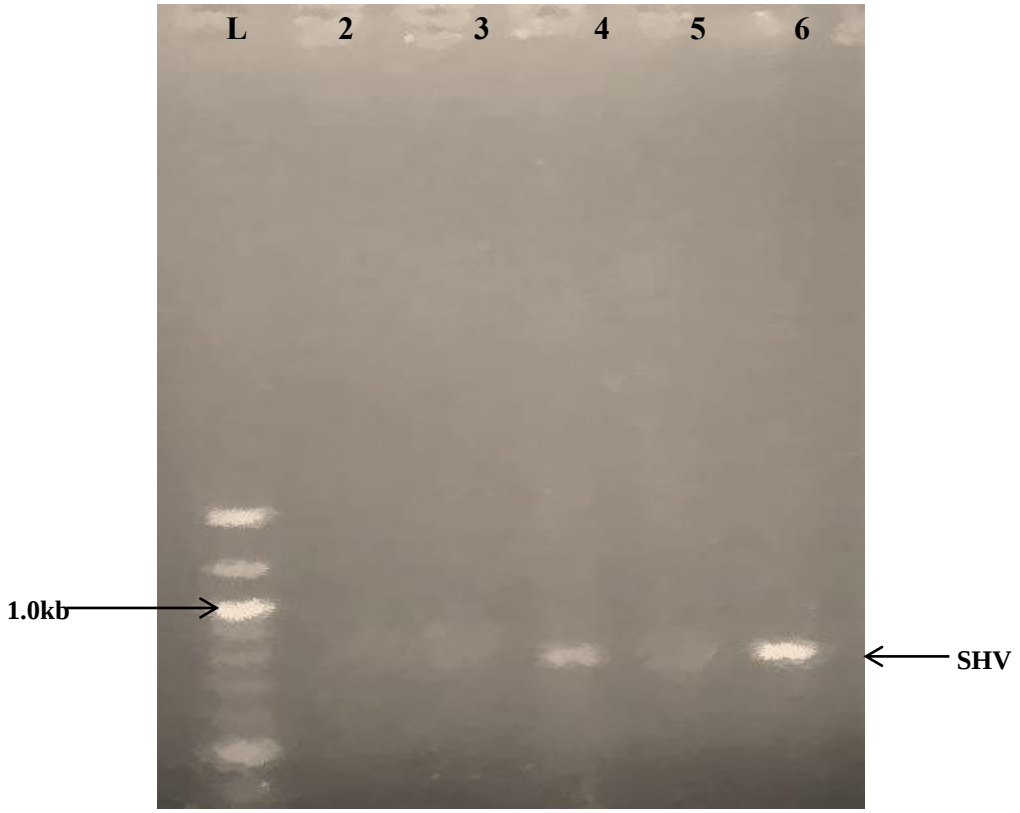

Figure 3. Gel image of amplified bla_{SHV} (768 bp) L = Molecular weight marker; 2-6 = F1, H2, E2, E4 and L3

DISCUSSION

One of the most commonly consumed fermented dairy products which has high nutritional and health benefits as well as wide acceptance worldwide is yoghurt. However, the presence of pathogenic bacterial and fungal species has been reported in yoghurt (Karagul-Yuceer *et al.*, 2002; Keta *et al.*, 2019). This could be due to inadequate production practices or lack of proper storage and handling. The spoilage of yoghurt products is influenced by the unhygienic nature of preparation and manufacturing processes, storage conditions, post-production procedures and environmental conditions surrounding the production (Oyeleke, 2009; Alli *et al.*, 2010). In this study, we detected that 2 (6.7%) of the samples had lactic acid bacteria higher than 10^7 CFU/mL standards as recommended by Rodrigues *et al.* (2010).

Bacterial contaminants were found in 26 (86.7%) of the yoghurt samples and we detected the highest viable bacterial count of 2.0 x 10^6 CFU/mL which was lower than the highest count reported by Samuel and Ifeanyi (2016) in their study. The total coliform count ranged from 3.0 – 2.3 x 10^4 CFU/mL. The acceptable limit for the presence of coliforms in yoghurt was less than 10 CFU/mL reported by (Karagul-Yuceer *et al.*, 2002). In this study, 14 (46.7%) of the samples had coliforms ranged between 0 and 10, which conformed to the recommended standard (Food and Drug Administration, 2015). Highest mean coliform count of 2.3 X 10^4 CFU/mL was obtained in sample W, which is similar to the value (2.1 x 10^4 CFU/mL)

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obtained by Afolabi *et al.* (2017). This value (4.25 x 10^7 CFU/mL), however, is lower than that reported by Uzeh *et al.* (2006). The significant coliform growth seen in the yoghurt could indicate poor hygienic conditions due to direct contact with faeces or contamination at retail and storage facilities. The presence of coliform growth higher than the allowable limit is a serious concern as it indicates contamination and a lack of hygiene following processing. Yoghurt is not expected to contain coliforms due to the high temperature, quick pasteurization, and efficient cleaning and hygienic processes used (Kawo *et al.,* 2006). Improper food handling practices caused a significant number of illnesses and majority of the outbreaks were caused by poor food and beverage handling practices (Ehiri and Morris, 1996; Flint *et al*., 2005). The handlers' hands, noses, and skin are all entry points for the pathogens, as well as their faeces (Samuel and Ifeanyi, 2016. Taiwo *et al.,* 2018). Personal observations during samples collection revealed that majority of peddlers use ineffective handling techniques, exposing food and beverages to potentially dangerous situations such as cross contamination and unfavorable temperature. This has been equally reported by Taiwo *et al*. (2018).

We reported *P. aeruginosa* (25%) and *S. aureus* (25%) as the most prevalent bacteria in the assayed samples. According to Samuel and Ifeanyi (2016), *P. aeruginosa* had the lowest frequency of incidence whereas *S. aureus* was the most detected bacterium. However, Tohoyessou *et al*. (2021) described *E. coli* strains as the most dominant isolate in yoghurt samples from Benin republic while *P. aeruginosa* was the most dominant followed by *K. pneumoniae* among the Gram-negative bacteria in this study. *E. coli* was identified in 7% of the yoghurt samples. *E. coli* contamination of milk products constitutes a public health hazard (Disassa *et al*., 2017). The presence of various strains of *E. coli* is a strong indicator of dairy product contamination and faecal pollution, both of which cause gastroenteritis and food poisoning in people (Galal *et al*., 2013).

Fungal species was present in 63.3% of the yoghurt samples with *Aspergillus niger* being the most occurring species (54.2%). Samuel and Ifeanyi (2016) reported *Aspergillus flavus* as the most dominant fungal isolate in yoghurt. The highest levels of fungal species could be linked to industries' poor processing methods, equipment, environment and preservation methods (Keta *et al*., 2019). As a result, yoghurt producing industries must improve the quality of their products by improving their processing methods, sanitary surroundings, personnel and storage methods (Samuel and Ifeanyi, 2016).

One of the biggest threats to modern development, food security, and global health is antibiotic resistance. As antibiotics used to treat infections lose their efficacy due to overuse and abuse, a growing number of infections are becoming increasingly difficult to treat (WHO, 2020). In a study conducted by Sultana *et al*. (2021), there was high resistance of *E. coli* to penicillin (100%) and gentamicin (60%), while *E. coli* equally showed the highest resistance to levofloxacin (100%) and Gentamicin (100%) in this current study. Emergence of multidrug resistance facilitates the spread of antibiotic resistance through plasmid encoded genes to other bacteria; these bacteria become resistant to many antibiotics at once. According to the findings by Badasa *et al.* (2018), *E. coli* had the highest percentage (92.5%) of multidrug resistance strains while all *E. coli, K. oxytoca, Shigella* and *Acinetobacter* species in this study were multidrug resistant.

This study reported 16% of Gram-negative bacteria as positive by phenotypic method which was higher than that observed by Tepeli and Zorba (2018) that 10.9% of Enterobacteriaceae

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isolates were positive for ESBL by phenotypic method. However, ESBL-positive *Klebsiella* spp. were found in 34.9% of the *Klebsiella* spp. isolated from raw milk and cheese samples, according to Gundogan and Yakar (2007). Additionally, ESBL-positive Shiga toxin-producing *E. coli* (STEC) O157:H7 isolates were identified in 65.5% of milk and milk products in Egypt (Ahmed and Shimamoto, 2015). In this study, only bla_{SHV} was detected in two *P. aeruginosa* isolates while Tohoyessou *et al*. (2021) found the bla_{TEM}, bla_{CTX-M}, and bla_{SHV} genes in fermented milk product isolates, with the bla_{TEM} gene being the most prevalent. However, bla_{TEM} and bla_{CTX-M} genes were not detected in any of the Gram-negative bacterial isolates in this study.

CONCLUSION

Multidrug-resistant Gram-negative bacteria were found in high numbers in this study. The yoghurt samples contained viable bacterial cells, including ESBL-producing pathogenic strains that were resistant to multiple antibiotics and are also known cause a variety of health issues, including gastrointestinal problems. As a result, importance of good handling and storage facilities must be emphasized. Also, appropriate monitoring and quality standards among manufacturers and healthcare staff are required in order to reduce the likelihood of food-borne diseases and intoxications from intake of contaminated yoghurt.

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