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Full Length Research Article

OCCURRENCE OF ESBLS-PRODUCING *ENTEROBACTERIACEAE* IN LOCAL READY-TO-EAT MEAT PRODUCTS AND THEIR SUSCEPTIBILITIES TO *CASSIA TORA* AND *FICUS THONINGII* LEAF EXTRACTS

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ABSTRACT
The emergence and acquisition of antimicrobial resistance by pathogenic strains of bacteria pose a very serious global public health problem. Extended-spectrum beta lactamases (ESBLs) production by some members of *Enterobacteriaceae* represent one of the most important mechanisms of antimicrobial resistance, particularly due to increased morbidity, mortality, longer hospital stays, and higher healthcare costs associated with infections caused by these organisms. Using standard microbiological methods, this study isolated 206 *Enterobacteriaceae* isolates from 400 samples of local ready-to-eat (RTE) meat products, including *Tsire, Balangu, Kilishi* and *Danbun*, representing 51.5 % isolation rate. Among the *Enterobacteriaceae* isolates, 33 (16.02 %) were confirmed be ESBLs-producers constituting *E. coli* isolates, *K. pneumonia, Proteus, Salmonella, Cirobacter* and *Enterobacter* spp. Antibacterial susceptibility assay revealed both plants leaf extracts have significant activity against the ESBLs-producing bacteria, with *F. thoningii* ethanolic extract exhibiting higher activity with MIC ranging from 6.25 mg/ml to 25 mg/ml than that of *C. tora* (MIC: 12.5 to 100 mg/ml). The results obtained from this study suggest that leaf extract from *F. thoningii* and *C. tora* could serve as potential sources of antibacterial agents for treatment of ESBLs-producing bacterial infections.

Keywords: Extended-spectrum β-lactamases (ESBLs); *Enterobacteriaceae;* Ready-to-eat meat; Antibacterial activity; *Cassia tora*; *Ficus thoningii*.

INTRODUCTION

Antimicrobial resistance is a serious global public health problem that continues to threaten the effectiveness of infectious disease treatments. This is attributed to the misuse and overuse of antibiotics in healthcare, agriculture and environmental settings, which generate selective pressure that derives microbial pathogens to evolve into resistant strains (Rani & Saharawat, 2024). Alternatively, susceptible strains may acquire resistance genes from the environment through horizontal gene transfer. Among the various mechanisms of antibiotic resistance, the production of extended-spectrum β-lactamases (ESBLs) has become of particular concern due to their increasing prevalence and resistance to multiple antibiotics (Chong, Shimoda, & Shimono, 2018). Bacteria producing these enzymes confer resistance to a broad range of β-lactam antibiotics, including third-generation cephalosporins and monobactams. Infections caused by ESBLs-producing bacteria are associated with increased morbidity and mortality, longer hospital stays, and higher healthcare costs (Rodríguez-Baño & Pascual, 2008).

Antimicrobial resistance poses a significant threat to food safety, which adversely impacts sustainable development goals (Founou, Founou, & Essack, 2021; Gongal, 2022) Antibiotic-resistant bacteria spread to food primarily through insects such as cockroaches and houseflies that carry the contamination from environments, and through direct contact with contaminated surfaces or food handlers during processing (Zurek & Ghosh, 2014). Ready-to-eat (RTE) meat products pose potential risks to consumers due to microbiological contamination as they undergo minimal or no heat treatment that would eliminate pathogenic microorganisms, thus constituting potential reservoirs for antibiotic-resistant bacteria. In Nigeria, traditional meat products such as *Kilishi* (sun-dried seasoned meat strips), *Tsire* (spiced grilled meat), *Dambun nama* (shredded dried meat), and *Balangu* (roasted meat) are widely consumed RTE foods that are processed under varying hygienic conditions and often stored at ambient temperatures, that can support bacterial growth. Additionally, Post-processing contamination, particularly with ESBLs-producing bacteria, further aggravates the safety concern for such RTE meat products.

Several studies have reported the presence of ESBLs-producing bacteria in various foods across different geographical regions. A systematic review documented moderate to high occurrence of ESBLs-producing *E. coli* in European food producing animals, with prevalence ranging from 1 % to 96 % depending on species (Kuhnke, Werner, & Kreienbrock, 2020). In Vietnam, 68.4 % of food samples from livestock and fishery products were reported to be contaminated with multidrug-resistant ESBLs-producing *E. coli* (Le *et al*., 2015). A hospital-based study in Switzerland revealed 92 % of ESBLs-producing *Enterobacteriaceae* contamination in raw chicken, but not in other food types or cooked chicken, while 6.5 % of food handlers were carriers. Similar studies found ESBLs-producing bacteria in 43.9 % and 53.1 % of raw chicken samples in Germany (Kola *et al*., 2012) and Spain (Vitas, Naik, Pérez-Etayo, & González, 2018), respectively. Systematic reviews and meta-analysis study reported a general ESBLs-prevalence rate of 34.6 % in Nigeria (Tanko, Bolaji, Olayinka, & Olayinka, 2020). However,

limited data exist regarding the prevalence of ESBLs-producing bacteria in local Nigerian RTE meat products, despite their widespread consumption and potential role in disseminating antibiotic resistance.

The ever-rising problem of antimicrobial resistance has generated a lot of interest in the search for alternative antimicrobial agents, particularly from natural sources. Medicinal plants have been used in traditional medicine systems for centuries and represent a vast source of bioactive compounds with numerous antimicrobial properties. Nigeria's rich biodiversity provides essential resources for traditional medicine, including numerous plants with documented ethnomedicinal uses (Adamu *et al*., 2024; Akunna, Lucyann, & Saalu, 2023). Plants such *Ficus thonningii* and *Cassia tora* have been widely used in traditional African and Indian medicine, respectively. *F. thonningii* is employed to treat various conditions including urinary tract infections, diarrhoea, and respiratory illnesses (Dangarembizi, Erlwanger, Moyo, & Chivandi, 2013). Similarly, *C. tora*, was reported to be rich in anthraquinone glycosides and has diverse pharmacological activities including hepatoprotective, anti-inflammatory, and antidiabetic effects (Choudhary & Gulia, 2011). Although, integration of such ethnomedically relevant plants into mainstream healthcare is increasing, there exist challenges related to treatment specificity, safety, dosage, regulation, and sustainability.

Given the limited data on ESBLs-producing bacteria in Nigerian traditional RTE meat products and the understudied antimicrobial potential of indigenous medicinal plants, this study used standard microbial isolation techniques, phytochemical extraction methods and antimicrobial susceptibility assays to determine the occurrence of ESBLs-producing bacteria in *Tsire*, *Kilishi*, *Balangu*, and *Dambun nama;* and assess their susceptibility to the leaf extract of *F. thonningii* and *C. tora.*

MATERIALS AND METHODS

Meat Sample Collection Characterisation

Using the aseptic technique, a total of 400 RTE meat samples, 100 for each of *Tsire, Kilishi, Balangu,* and *Dambun nama* were collected within Kaduna metropolis, Kaduna, Nigeria. At the laboratory, the samples were homogenised by blending 25 g in 225 ml peptone water, allowed to stay overnight and then cultured for 24-48 hrs in MacConkey agar. Distinct colonies from culture plates were sub-cultured to obtain pure cultures. The colonial characteristics of the pure cultures were observed; and using standard methods, other properties such as Gram's reaction, and various biochemical characteristics as defined by Bergey's manual of determinative bacteriology (Bergey, 1994), isolates were identified.

ESBLs Screening and Confirmation

The isolates were screened for ESBLs-production phenotypically, by disc diffusion method based on Clinical and Laboratory Standards Institute (CLSI) antimicrobial susceptibility testing standards (CLSI, 2020). The isolates were subjected to an antibiotic susceptibility test on Mueller Hinton ager, against three (3) of the following third generation cephalosporins: ceftriaxone (30 µg), cefotaxime (30 µg) and ceftazidime (30 µg). Isolates that showed resistance to at least one of the tested antibiotics with growth inhibition zone diameter of ≤22 mm for ceftazidime, ≤25 mm for ceftriaxone and ≤27 mm for cefotaxime were selected for ESBLs-production confirmatory test.

The ESBLs-production was confirmed by the double disc synergy test (DDST) as described by NCLS (2020). The test was performed on Mueller Hinton agar with a 30 µg disk of cefotaxime (and / or ceftriaxone, and / or ceftazidime) and a disk of amoxicillin-clavulanic acid (Augmentin) (containing 10 µg of clavulanate) placed at a distance of 30 mm (centre to centre) (Drieux, Brossier, Sougakoff, & Jarlier, 2008). Isolates that showed clear enhancement of the inhibition zone of cefotaxime in front of the clavulanate-containing disk were considered as ESBL producer. This characteristic shape-zone for confirmed ESBLs-producers is often referred to as 'champagne-cork' or 'keyhole'

Collection and Identification of Plant Materials

The *C. tora, F. thoningii,* were identified within Kaduna metropolis and their leaves were plucked accordingly. The leaves identities were further authenticated at the herbarium of the Biological Science Department, Kaduna State University, Kaduna, Nigeria. The leaves were then air-dried and ground into fine powdery form.

Phytochemical Extraction and Screening

The plants leaves phytochemicals were extracted in ethanol and aqueous solvents, using maceration method as described in (Padhi & Panda, 2015). An amount of 50 g of the powdered leaf was dissolved in 200 ml of solvents at 28 ^{O}C and allowed to stay for 2 days, after which it was filtered through Whiteman No 2. filter paper. The extraction was repeated three times and the solvent was evaporated and removed under vacuum using rotary evaporator at about 45 ^{O}C to generate dry crude extract. The presence of phytochemical compounds were detected as described in (Egbe, Garba, Adamu, & Aliyu, 2022).

Antibacterial Activity Assay of the Extracts against ESBL-producing Isolates

The antibacterial activity assay of the plants extracts were carried out using agar well diffusion (agar cup) method as described by (Padhi & Panda, 2015). Overnight Mueller Hinton broth culture of the ESBLs-producing isolates were standardised with 0.5 % McFarland standard solution and then evenly seeded on Mueller Hinton agar. Using a sterile borer, wells of about 6 mm diameter and 2.5 mm depth were dug on the agar plates. Each well was filled with 40 µl of 30 mg/ml extract dissolved in DMSO. Respective solvents without extract were used as standard control and a standard antibiotic, 10 µg/ml meropenem as a reference control. The plates were incubated for 24 hours, after which zones of inhibition were measured. Growth inhibition zone of ≥ 8 mm was regarded as positive activity. However, in instances where no inhibition was observed, the extract concentration was extended up 50 mg/ml and rechecked for activity.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC was determined using broth microdilution technique in 96 well microtiter plate and 2,3,5-triphenyltetrazolium chloride (TTC) as indicator. crude extracts were added to the column B wells of the plate and serially diluted across the columns with 100 µl of the 0.5 McFarland adjusted activated culture in Mueller Hinton broth to obtain concentrations of 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 mg/ml as described in (Panda, 2014). However, meropenem was used as control with concentrations ranging from 10-0.16 µg/ml.

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The column A wells were filled with equal volume of the indicator and the corresponding extract as baselines. The plate was sealed with foil paper and incubated for 24 hours at 37 ^{o}C in an incubator shaker (130 rpm) and observed for growth of the bacteria. A visible colour change to pink indicates growth of bacteria. The MIC value of the extract was taken as the lowest concentration that showed no growth for individual test bacteria.

MBC was determined as described in (Padhi and Panda 2015). Samples of 10 mL of the broth from wells of the 96- well microtiter plate exhibiting MIC and from control wells were taken aseptically and inoculated on MH agar plates as a spot inoculum under the laminar flow hood. The plates were then sealed and incubated at 37 ^{o}C for 24 hours and observed for growth. Absence of bacterial growth indicated the MBC for the respective bacteria. However, if growth was not observed at MIC, a higher concertation is examined similarly until a concertation is reached that recorded no growth.

RESULTS AND DISCUSSION

Occurrence of *Enterobacteriaceae* in Ready-to-Eat Meat Products

This study investigated the occurrence of *Enterobacteriaceae* in four local RTE meat products commonly consumed in Kaduna metropolis, Nigeria. A total of 206 *Enterobacteriaceae* isolates were recovered from 400 samples, representing an overall occurrence of 51.5 % (Table 1). This high incidence rate indicates significant bacterial contamination in these local meat products, which poses potential public health risks to consumers.

Among the RTE meat products assessed, *Kilishi* showed the highest presence of *Enterobacteriaceae* with 75 isolates, followed by *Tsire* with 70 isolates, *Balangu* with 40 isolates, and *Dambun Nama* with 21 isolates. The higher contamination rates in *Tsire* and *Kilishi* may be attributed to their processing methods and handling practices during sale. *Tsire* for instance, is normally wrapped in used paper and served with fresh vegetables and cut into small pieces by the handler. These provide direct contact of the product with difference surfaces, which could serve as additional contamination source. Other possible reasons the higher contamination in *Tsire* and *Kilish*i is storage conditions.

E. coli was the most frequent isolated organism, accounting for 67 isolates (16.75% of total samples), with the highest occurrence in *Kilishi* (28 isolates) and *Tsire* (20 isolates). This finding is particularly concerning given that *E. coli* is an indicator of faecal contamination and poor hygiene practices. The mere presence of *E. coli* in these RTE products indicates cross-contamination with faecal matter during handling, or poor storage conditions. It noteworthy to highlight the absence of Salmonella spp. in *Dambun Nama* samples, suggesting better processing or storage practices for this product. The isolates of *Enterobacteriaceae* found to be associated with local RTE meat product in this study are consistent with those isolated in a study of bacteriological quality of stick meat (*tsire*) sold in Garko local government area of Kano State, Nigeria (Dahiru & Maigari, 2021)..

Table 1: Occurrence of *Enterobacteriaceae* in Ready-to-Eat Products

Isolated Organisms	*Tsire (n=100)*	*Kilishi (n=100)*	*Balangu (n=100)*	*Dambun Nama (n=100)*	Total	% Occurrence *(n=400)*
E. coli	20	28	12	7	67	16.75
Klebsiella pneumoniae	16	12	8	5	41	10.25
Proteus spp.	10	8	8	3	29	7.25
Salmonella spp.	5	8	4	0	17	4.25
Citrobacter spp.	12	7	5	4	28	7.00
Enterobacter spp	7	12	3	2	24	6.00
Total	70	75	40	21	206	51.5

Occurrence of ESBLs in Ready-to-Eat Meat Products

Screening and confirmation of the 206 *Enterobacteriaceae* isolates for ESBLs-production showed 33 isolates (16.02 %) to be ESBLs-producers (Table 2 and 3). This represents a significant public health concern, giving that ESBLs-producing bacteria are resistant to multiple antibiotics including penicillins, first, second and third generation cephalosporins as well as monobactams, thus causing infections that are difficult to treat. A previous study carried out in 2016 at the same location reported a 14.84 % occurrence rate of ESBL producers among bacteria isolates from similar RTE meat products (Yusha & Umar, 2016). The current findings suggest an increase in ESBL occurrence over the nine years, with the incidence rising to 16.02 %. Although this increase appears small (1.18 %), it is troubling particularly due to the serious clinical implications of infections caused by these multidrug-resistant bacteria. This increase indicates that current strategies employed to control this antibiotic resistance mechanism are ineffective or inadequate in this setting.

The occurrence of the ESBLs-producing bacteria varied among the different meat products. *Tsire* showed the highest percentage of ESBL producers constituting 36.67 % of its isolates. The other RTE products *Dambun Nama*, *Balangu*, and *Kilishi*, had 19.05 %, 17.5 % and 14.67 % occurrence rates, respectively. Among the bacterial species, *E. coli* showed the highest ESBL occurrence rate with 19 ESBLs-positive isolates representing 28.36 % of all *E. coli* isolates, followed by *K. pneumoniae* (8 isolates, 19.51%). This pattern is consistent with global trends where *E. coli* is the most common ESBLs-producer, followed by *K. pneumoniae* and then other member of *Enterobacteriacea* (Coque, Baquero, & Cantón, 2008; Nakai *et al*., 2016; Storberg, 2014). The presence of ESBLs-producing *Salmonella* spp., though limited to one isolate, is concerning due to the pathogenic nature of this organism.

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Table 2: Occurrence of ESBLs in Local Ready-to-Eat Meat Products

RTE Meat Product	Isolated Organisms	No Isolated	Screened ESPLs	Conformed ESBLs	% Occurrence
Tsire	*E. coli*	20	12	6	30.00
	Klebsiella pneumoniae	16	7	3	18.75
	Proteus spp.	10	3	1	10.00
	Salmonella spp.	5	1	0	0.00
	Citrobacter spp.	12	5	1	8.33
	Enterobacter spp	7	0	0	0.00
	Total	**70**	**28**	**11**	**36.67**
Kilishi	*E. coli*	28	15	8	28.57
	Klebsiella pneumoniae	12	5	2	16.67
	Proteus spp.	8	3	0	0.00
	Salmonella spp.	8	2	0	0.00
	Citrobacter spp.	7	0	0	0.00
	Enterobacter spp	12	4	1	8.33
	Total	**75**	**29**	**11**	**14.67**
Balangu	*E. coli*	12	7	3	25
	Klebsiella pneumoniae	8	3	2	25
	Proteus spp.	8	3	1	12.5
	Salmonella spp.	4	2	1	25
	Citrobacter spp.	5	0	0	0
	Enterobacter spp	3	0	0	0
	Total	**40**	**15**	**7**	**17.50**
Danbun Nama	*E. coli*	7	4	2	28.57
	Klebsiella pneumoniae	5	4	1	20.00
	Proteus spp.	3	1	1	33.33
	Salmonella spp.	0	0	0	0.00
	Citrobacter spp.	4	2	0	0.00
	Enterobacter spp	2	1	0	0.00
	Total	**21**	**12**	**4**	**19.05**

Table 3: Distribution of ESBLs across *Enterobacteriaceae* isolates of RTE Meat Products

Isolated Organisms	No. of Isolates	Confirmed ESBLs					
		Tsire	*Kilishi*	*Balangu*	*Dambun Nama*	Total	% Occurrence
E. coli	67	6	8	3	2	19	28.36
Klebsiella pneumoniae	41	3	2	2	1	8	19.51
Proteus spp.	29	1	0	1	1	3	10.34
Salmonella spp.	17	0	0	1	0	1	5.88
Citrobacter spp.	28	1	0	0	0	1	3.57
Enterobacter spp	24	0	1	0	0	1	4.17
Total	206	11	11	7	4	33	16.02

Phytochemical Analysis of Plant Extracts

The phytochemical compounds detected in *C. tora* and *F. thoningii* are summarised in Table 4. The phytochemical screening of leaf extracts of both plants showed the presence of various bioactive compounds with previously established antimicrobial properties. Both plants contained tannins, alkaloids, glycosides, steroids, and anthraquinones in both ethanolic and aqueous extracts. These compounds were established to have antimicrobial activities (Deeni & Hussain, 1994) and may contribute to the observed antibacterial effects in this study.

F. thoningii extracts showed a wider range of bioactive compounds, including saponins and terpenoids in both extract types, and phenols in the ethanolic extract. The presence of saponins is particularly significant as these compounds can disrupt bacterial cell membranes. The variation in phytochemical composition between the two plants and extraction methods may explain the differences in their antimicrobial activities.

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Table 4: Phytochemical Composition of *C. tora* and *F. thoningii* Leaf Extracts

Phytochemicals	*C. tora*		*F. thoningii*	
	Ethanolic Extract	Aqueous Extract	Ethanolic Extract	Aqueous Extract
Tannins	+	+	+	+
Saponins	-	-	+	+
Flavonoids	+	-	+	-
Alkaloids	+	+	+	+
Glycosides	+	+	+	+
steroids	+	+	+	+
Terpenoides	+	-	+	+
Anthraquinones	+	+	+	+
Phenols	-	-	+	-

Antibacterial Activity of the Plant Extracts

The antimicrobial susceptibility testing revealed that both plant extracts demonstrated significant activity against the ESBLs-producing isolates, with varying degrees (Tables 5 and 6). The ethanolic extracts generally showed higher antibacterial activity compared to aqueous extracts, which is consistent with the better extraction of lipophilic antimicrobial compounds in organic solvents.

F. thoningii ethanolic extract demonstrated the most potent antimicrobial activity, with MIC values ranging from 6.25 mg/ml against *E. coli* to 25 mg/ml against *Salmonella* spp. and *Citrobacter* spp. Moderate activity was observed in aqueous extract of *F. thoningii* with MIC values between 25 to100 mg/ml across different bacterial species. *C. tora* extracts presented variable activity, with the ethanolic extract being more effective (MIC: 12.5 to 100 mg/ml) than the aqueous extract (MIC: 50 to >100 mg/ml). The superior activity of *F. thoningii* may be attributed to its richer phytochemical profile, including the presence of saponins and phenolic compounds.

The minimum bactericidal concentrations (MBC) were generally 2-4 times higher than the MIC values, suggesting bacteriostatic rather than bactericidal activity at lower concentrations. This pattern is typical for plant extracts and suggests that higher concentrations may be required for complete bacterial killing.

Table 5: Minimum Inhibitory Concentrations (MIC) of *C. tora* and *F. thoningii* Leaf Extracts

Isolated Organisms	No. of Isolates tested	Minimum Inhibitory Concentration (mg/ml)				
		C. tora		*F. thoningii*		Control
		Ethanolic Extract	Aqueous Extract	Ethanolic Extract	Aqueous Extract	Meropenem (µg/ml)
E. coli	13	50.00	100.00	12.50	25	0.63
E. coli	6	50.00	100.00	6.25	25	0.63
Klebsiella pneumoniae	8	25.00	100.00	12.5	50	1.25
Proteus spp.	3	25.00	50.00	12.5	50	0.63
Salmonella spp.	1	100.00	>100.00	25	100	0.63
Citrobacter spp.	1	12.50	50.00	25	50	0.31
Enterobacter spp	1	25.00	50.00	12.5	50	1.25

Table 6: Minimum Bactericidal Concentrations (MBC) of *C. tora* and *F. thoningii* Leaf Extracts

Isolated Organisms	No. of Isolates tested	Minimum Bactericidal Concentration (mg/ml)				
		C. tora		*F. thoningii*		Control
		Ethanolic Extract	Aqueous Extract	Ethanolic Extract	Aqueous Extract	Meropenem (µg/ml)
E. coli	13	100.00	>100.00	25.00	50	1.25
E. coli	6	100.00	>100.00	12.50	50	1.25
Klebsiella pneumoniae	8	50.00	>100.00	25.00	100	2.50
Proteus spp.	3	50.00	100.00	25.00	100	1.25
Salmonella spp.	1	>100.00	>100.00	50.00	>100	1.25
Citrobacter spp.	1	25.00	100.00	50.00	100	0.63
Enterobacter spp	1	50.00	50.00	25.00	100	2.50

Conclusion

In general, this study demonstrated the high contamination rate (51.5 %) of local RTE meat products with *Enterobacteriaceae,* including a significant occurrence of ESBLs-producers (16.02 %). This prompt the need for urgent control strategies, particularly due to the serious medical implications posed the infections caused by

these resistant strains of bacteria. On the other hand, the antibacterial assessments of *F. thoningii* and *C. tora* demonstrated the leaf ethanolic extracts of the plant to have significant activity against the ESBLs-producing bacteria with MIC of 6.25- 25 mg/ml and 12.5-100 mg/ml, respectively. Thus, suggesting their potentials as natural sources for development of antibacterial agent against ESBLs-producing bacteria.

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