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Antibiotic Sensitivity and Plasmid Curing of Bacteria Isolates from Egusi (*Cucumeropsis Manii*) Cake (Usu)

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Abstract

This study was carried out to investigate the antibiotic sensitivity and plasmid curing of food borne bacteria isolated from egusi (Cucumeropsis manii) cake (Usu). Thirteen bacteria isolates from egusi cake samples were used which are staphylococcus aureus, staphylococcus, Escherichia coli, Proteus vulgaris, Enterobacter aerogenes, Proteus mirabilis, lactobacillus spp, bacillus pumilus and Enterobacteriacae. The plasmid curing was done using acridine orange (0.75mg/1ml). The efficacy of plasmid was assessed through agarose gel electrophoresis, and the impact on antibiotic susceptibility was evaluated using antibiotic susceptibility discs. From the antibiotic susceptibility test, all Escherichia coli strains were resistant to Nalidixic Acid and Ampicilin. 33.3% were sensitive to Septrin, 33.2% were resistant and 33.3% were intermediate. The result showed that gram negative bacteria are more sensitive with 38% of the isolates showing resistance, while 78% of gram positive were resistant. The results from this study showed that plasmids played a significant role in the observed resistance in the isolates as they all contained plasmids of sizes within 12,000bp. After curing, plasmids were successfully eliminated in some bacteria, restoring their susceptibility to antibiotics. This research highlights the effectiveness of plasmid curing in reducing antibiotic resistance in foodborne bacteria found in egusi cake, thereby enhancing food safety, and minimizing the transmission of resistance to consumers.

1.0. Introduction

Traditional Egusi cake is a popular Nigerian delicacy, which is prepared from melon seeds (*Cucumeropsis manii*) and other ingredients. However, like any food product, Egusi cake is susceptible to contamination by pathogenic bacteria during its preparation, handling, or packaging. Contamination of food with antibiotic-resistant has been rising issue in the last 10 years and this is associated with different environmental and anthropogenic factors [1]. The presence of antibiotic-resistant bacteria in traditional foods is a matter of great concern, as it poses challenges for effective treatment of foodborne infections and raises potential public health risks. Foodborne illnesses caused by pathogenic bacteria are a significant public health concern worldwide. Contaminated food products can serve as vehicles for the transmission of bacterial pathogens, leading to illness outbreaks and potentially severe consequences for affected individuals [2].

One critical aspect in addressing this issue is understanding the antibiotic sensitivity patterns of foodborne bacteria. Antibiotic sensitivity testing provides valuable insights into the susceptibility of bacterial strains to various antibiotics, guiding effective treatment strategies and informing food safety practices. [2]. The emergence and spread of antibiotic resistance in food borne bacteria is a complex issue influenced by various factors, including the misuse and overuse of antibiotics in humans. The presence of antibiotic-resistant bacteria in food products raises concerns about the potential transfer of resistance genes to human pathogens, limiting treatment options and increasing the risk of severe infections [3].

Plasmids are independent, circular, self-replicating extra-chromosomal DNA elements with characteristic copy numbers within the host. Various properties encoded by plasmid include resistance to antibiotics and heavy metals, degradation of hydrocarbons, synthesis of bacteriocins and antibiotics, etc [4]. They often carry additional genetic information, such as antibiotic resistance genes, which can be advantageous to bacteria in certain environments. However, plasmids can also contribute to the spread of antibiotic resistance and other undesirable traits [5]. Plamid profiling is a technique that helps with the identification of the potential of the spread of resistant genes. Thus, it is important for it to be examined as plasmids are a major mechanism for the spread of antibiotic resistant genes in bacteria population [6].

Therefore, it is essential to conduct comprehensive studies on antibiotic resistance in foodborne bacteria and plasmid curing in eliminating antibiotic resistance plasmids from the bacterial populations present in egusi cake samples.

2.0. Materials and Methods

2.1 Collection of Samples

Six raps of egusi cake (Usu) were purchased from three different points at two different markets (Ose market, Afia Nkpo), Onitsha, Anambra state. The sample was collected on the 27th of July, 2023. The samples were collected in clean polyethylene bags, Labeled and aseptically transported to the laboratory for analysis.

2.2 Sample Preparation

The collected Egusi cake samples were homogenized using a sterile mortar and pestle to create a uniform mixture under aseptic conditions. Serial dilutions of all the samples were carried out using stock solutions made from 1 g in 9 ml sterile distilled water of each sample. Ten-fold serial dilution of the samples was carried out as described by Cheesbrough [8]. Isolated bacteria was characterized and identified using colonial morphology, gram staining technique, biochemical test [9].

2.3. Bacterial isolates

Bacterial isolates from egusi cake were, *staphylococcus aureus*, *staphylococcus*, *Escherichia coli, Proteus vulgaris*, *Enterobacter aerogenes*, *Proteus mirabilis*, *lactobacillus* spp, *bacillus pumilus* and *Enterobacteriacae*.

2.4. Inoculation of the isolate and incubation

An aliquot (100 µl) of bacteria isolates were diluted to 10^{-4} and 10^{-6} of the 10-fold serial dilution were inoculated on Mannitol Salt agar (MSA) for *Staphylococcus* spp, Nutrient Agar (NA) a multipurpose culture media for different organisms, and Salmonella Shigella Agar (SSA). Using spread plate method. After which the plates were incubated in an inverted position for 24hrs at 37°C. Visible discrete colonies, were counted and expressed as colony forming units per gram (cfu/g). Sub-culturing the isolates was carried out and the isolates identified through morphological

description, Gram staining and biochemical test (oxidase, methyl red, indole, catalase, coagulase, urease and citrate) [10].

2.5. Antibiotic sensitivity testing

Antimicrobial susceptibility of the isolates was tested using the modified Kirby-Bauer multi discs diffusion method [11]. Commercial antibiotic discs (ROSCO) containing the antibiotics were applied on Muller Hinton agar. The antibiotics evaluated for efficacy against the isolates include; the following antibiotics were present on the discs (Celtech Diagnostic): PEF- Perfloxacin (10µg), Z- Zinacef (20µg), CPX- Ciprofloxacin (30µg), E- Erythromycin (10µg), GN- Gentamycin (10µg), AM-Amoxicillin (30µg), SXT Streptomycin (30µg), APX- Ampiclox (30µg), R- Rocephin (25µg) and S-Septrin (30µg).

2.6. Plasmid DNA extraction

The isolates were subjected to plasmid profile analysis using the modified Alkali-lysis method. An overnight culture of each bacteria isolate was prepared in 5ml of nutrient broth. The broth culture was properly mixed by vortexing, and 1.5ml was then transferred into a pre-labelled eppendorf tube. The tubes were then centrifuged for 4 minutes at 6500 rpm (revolutions per minute) to harvest the bacterial cells. The supernatant was gently decanted, leaving about 100µl of broth culture, which was then vortexed at high speed until the bacterial cell pellet became completely suspended. Alkali-lysis solution (350µl; 25mM Tris, 10mM EDTA, 0.1N NaOH, 0.5% SDS) was then added to lyse the bacterial cells. It was mixed by inversion for about 50 times until the solution became slimy, after which 150µl of 3.0M sodium acetate was added and again vortexed for about 10 seconds. It was further centrifuged at 6500 rpm for 15 minutes to pellet out cell debris and chromosomal DNA. The supernatant was then transferred into another labelled 1.5ml eppendorf tube, and 900µl of cold absolute ethanol was added. The solution was centrifuged at 6500 rpm for 10 minutes. The supernatant was discarded, and the white pellet containing the plasmid DNA was rinsed twice with 1000µl of 70% ethanol. The pellet was then air-dried. Thereafter, the pellet was re-suspended with 50µl of TE buffer and stored at -20˚C for further use [12].

2.6. Plasmid Curing

Curing of the plasmid was done to determine whether a plasmid encodes a trait that codes for antibiotics resistance or multiple antibiotic resistance using the modified method of [13]. Curing was done using the acridine orange (0.75 mg/1ml). The isolate that showed resistance to different antibiotics due to plasmid bands were subjected to plasmid curing. Acridine orange (100µl) of 0.75 mg/ml concentration, was added to 5ml of LB broth inoculated with the test isolates. The medium was incubated for 48 h in a shaker incubator. After incubation, cultures containing the highest concentration of acridine orange in which growth was clearly visible were diluted and spread on nutrient agar plates with appropriate antibiotics for susceptibility testing.

2.7. Statistical analysis

A one-way analysis of variance (ANOVA) test was used to compare the difference in the prevalence of isolates recovered from the various categories of samples with a significant level at $p < 0.05$.

3.0. Result and Discussion

A total of thirteen (13) bacterial Isolates from Egusi cake (Usu) were used for this study, which include, *Escherichia coli* (3), *Enterobacteriacae, Proteus vulgaris, Enterobacter aerogenes, Proteus mirabilia, Bacillus pumilus, Staphylococcus aureus* (2), *Staphylococcus epidermis* (2), and *Lactobacillus.* The results for antibiotics test performed are presented in Tables 1 and 2, below.

Table .1: Zone of inhibition (in mm) and Antibiotic Susceptibility Pattern of gram negative bacterial isolates for antibiotic susceptibility test before curing

SN	Isolates	OFX (mm)	PEF (mm)	CPX (mm)	AU (mm)	GN (mm)	S (mm)	CEP (mm)	NA (mm)	SXT (mm)	PN (mm)
1	*Eschrichia coli*	20 (S)	18 (I)	24 (S)	20 (S)	14 (I)	20 (S)	15 (I)	12 (R)	20 (S)	10 (R)
2	*Enterobacteriacae*	22 (S)	20 (I)	10 (R)	15 (R)	13 (I)	11 (R)	10 (R)	10 (R)	10 (R)	10 (R)
3	*Proteus vulgaris*	10 (R)	21 (S)	22 (S)	16 (R)	10 (R)	12 (I)	0 (R)	0 (R)	20 (S)	0 (R)
4	*Enterobacter aerogenes*	16 (S)	12 (R)	22 (S)	20 (S)	12 (I)	0 (R)	12 (I)	0 (R)	14 (I)	14 (I)
5	*Proteus mirabilis*	20 (S)	22 (S)	22 (S)	10 (R)	0 (R)	15 (R)	10 (R)	0 (R)	20 (S)	0 (R)
6	*Bacillus pumilus*	20 (S)	23 (S)	20 (S)	20 (S)	20 (S)	12 (I)	0 (R)	0 (R)	20 (S)	0 (R)
7	*Eschrichia coli*	18 (S)	0 (R)	21 (S)	17 (R)	17 (R)	0 (R)	0 (R)	0 (R)	14 (I)	10 (R)
8	*Eschrichia coli*	0 (R)	0 (R)	15 (R)	0 (R)	12 (R)	15 (R)	0 (R)	0 (R)	10 (R)	12 (R)

Key: SXT: Septrin (45µg), S: Zinnacef (5µg), OFX: Tarivid (10/10µg), PEF: Pefloxacin (30µg), GN: Gentamycin (5µg), AU: Augmentin (10µg), CPX: Ciproflaxacin (15µg). NA – Nalidixic Acid (5µg) and PN – Ampicilin . R = Resistant, S = Sensitive, I = Intermediate.

Table 2: Zone of inhibition (in mm) and Antibiotic Susceptibility Pattern of t of gram positive bacterial isoletes for antibiotic susceptibility test before curing

Isolates	PEF (10µg)	GN (10µg)	APX (30µg)	Z (20µg)	AM (30µg)	R (25µg)	CPX (30µg)	S (30µg)	SXT (30µg)	E (10µg)
S. aureus	15 (R)	10 (R)	0 (R)	15 (R)	15 (R)	8 (R)	24 (S)	10 (R)	0 (R)	0 (R)
S. epidermis	22 (S)	0 (R)	0 (R)	10 (R)	15 (R)	23 (S)	22 (S)	10 (R)	0 (R)	0 (R)
S. epidermis	24 (S)	12 (R)	0 (R)	15 (R)	12 (R)	10 (R)	0 (R)	15 (S)	0 (R)	0 (R)
Lactobacillus	10 (R)	0 (R)	0 (R)	10 (R)	15 (R)	12 (R)	24 (S)	10 (R)	0 (R)	0 (R)
S. aureus	22 (S)	0 (R)	10 (R)	12 (R)	10 (R)	14 (I)	22 (S)	10 (R)	10 (R)	0 (R)

Key: PEF- Perfloxacin Z- Zinacef CPX- Ciprofloxacin E- Erythromycin
GN- Gentamycin AM- Amoxicillin S- Streptomycin
APX- Ampiclox R- Rocephin SXT-Septrin

Resistance to multiple antibiotics was observed. Most of the gram negative isolates were sensitive to ciprofloxacin and resistance to augmentin, Erythromycin and Septrin. In a similar study, Udo *et al*. [14] reported the antimicrobial resistance profile of potential human pathogens isolated from fresh vegetable salad in Calabar, South-South Nigeria. From the study 33.3% *E coli* strains were resistant to PN and NA, while 33.3% were intermediate. 66.6% were resistant to CEP and Pefloxacin while 33.3% were sensitive and intermediate respectively. 100% of the five gram positive Isolates were resistant to Gentamycin, APX, Zinnacef, Amoxillin, Streptomycin, and Septrin, while 3 of the

5 Isolates were sensitive to Pefloxacin and 4 were sensitive to Ciproflaxacin. The results provide a clear warning about high-level resistance to commonly used antibiotics among bacterial isolates from egusi cake, which has not previously been observed or reported.

Table 3: Analysis of Variance (ANOVA) table for gram negative organisms

Sources of Variation	Sum of squares	Degrees of freedom	Mean squares	F(Variance ratio)	P-value
Between the groups	52.120	4	13.030	0.184	**0.945**
Within the groups	3182.200	45	70.716		
Total	**3234.320**	**49**			

Null hypothesis: All distributional means are the same.
Alternate hypothesis: At least one distributional mean is different.
Values in F-Fishers' table (0.05), FT (0.05, 4, 45) = 2.579, F calculated = 0.184, F tabulated = 2.579
Conclusion**:** F- calculated value is less than F- tabulated value, the null hypothesis is accepted. Therefore, all distributional means of the zones of inhibition for gram negative bacteria are the same. Table3 shows the Analysis of variance (ANOVA) for the zones of inhibition in gram negative bacteria which shows that the distributional means of the zones of inhibition are not different significantly since the F tabulated value (2.579) is greater than the F calculated value (0.184) at 0.05 level of significance.

Table **4: Analysis of Variance (ANOVA) table for gram positive organisms**

Sources of Variation	Sum of squares	Degrees of freedom	Mean squares	F (Variance ratio)	P-value
Between the groups	695.200	7	99.314	1.621	
Within the groups	4411.800	72	61.275		0.143
Total	5107.00	79			

Null hypothesis: All distributional means are the same.Alternate hypothesis: At least one distributional mean is different. Values in F-Fishers' table (0.05) FT (0.05, 7, 72) = 2.1397:F calculated = 0.143 :F tabulated = 2.13 Conclusion: F- calculated value is less than F- tabulated value, the null hypothesis is accepted. Therefore, all distributional means of the zones of inhibition for gram negative bacteria are the same. Table 4 also shows the Analysis of variance (ANOVA) for the zones of inhibition in gram positive bacteria which shows that the distributional means of the zones of inhibition are not different significantly since the F tabulated value (2.13) is greater than the F calculated value (1.621) at 0.05 level of significance.

3.1. Plasmid DNA present in isolates

Plasmid profiles of the bacteria isolates were carried out and visualized in a gel electrophoresis field as presented in figure 1. Plasmid profile showed that the isolates contained plasmid with the same

molecular weight and different sizes, and the presence of the plasmid can be the reason for multi-resistance to antibiotic observed in this work. A similar report by Kalantar *et al,* [15] showed the presence of plasmids of molecular sizes ranging from 1.4 kb to 4.5 kb among acute diarrhea-causing E. coli isolates showing resistance to some antibiotics and stated that these resistances are plasmid mediated. The movement of the sample bands are compared with the markers for calibrating the gel for the size and weight of the samples, and determining the sizes of the molecules studied and expressed in base pairs (bp) [16]. Plasmids are the major mechanism for the spread of antibiotic resistant genes in bacterial populations [17].

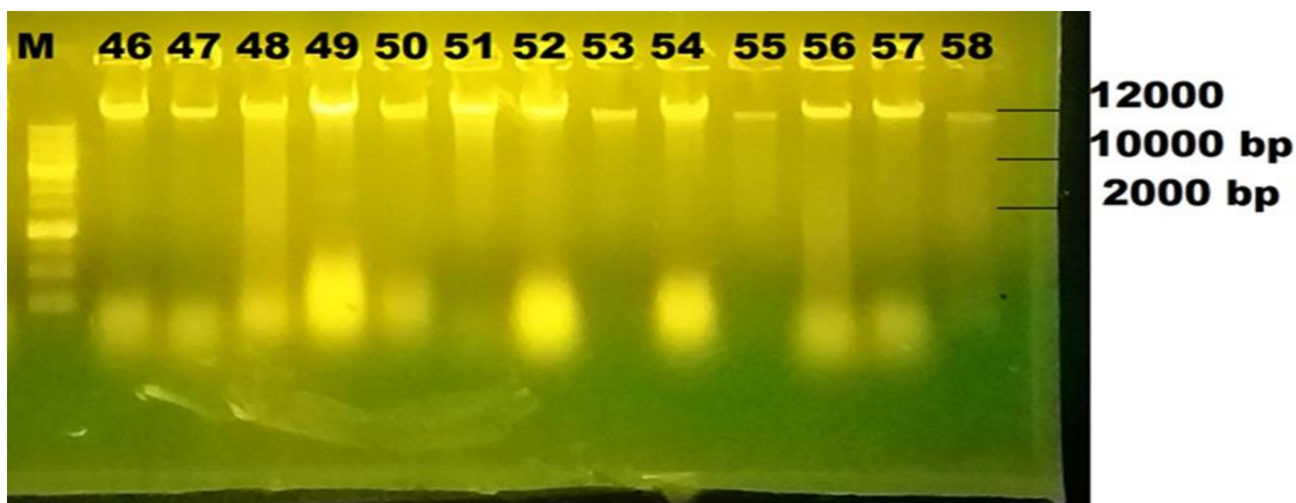

Fig 1: Agarose gel electrophoresis picture showing Plasmid profile of bacterial isolates from egusi cake samples (lanes 46-58)

46: -*Eschericha coli*
47: -*Staphylococcus aureus*
48: - *Pseudomonas epidermidis,*
49: - *Enterobacteriacae*,
50: -*Proteus vulgaris*,
51: -*Escherichia coli*,
52: -*Escherichia coli*,
53:-*Enterobacter aerogenes*
54: - *Proteus mirabilis*,
55: -*Bacillus pumilus*,
56: -*Lactobacillus spp*,
57: -*Staphylococcus aureus*,
58: -*Staphylococcus epididermis*
M = molecular weight markers

The results obtained in this gel electrophoresis indicates that, resistance to the assayed antibiotics may be mediated by plasmids, also highlighting a positive correlation between the presence of plasmids and bacterial resistance [18]. The chance that plasmid found in egusi cakes is worrisome and underscoring the sanitary status of the food handlers and probably cooking and packaging processors. Table 3 and 4. Showed the result of plasmid curing. After plasmid curing, antibiotic susceptibility testing using the initial antibiotics to which organisms were resistant following the similar procedure in antibiotic susceptibility testing. Post plasmid curing Results showed that most of the isolates remained resistant to tested antibiotics.

Table 5. Diameter of Zone of Inhibition (Mm) of the Antibiotics after plasmid curing (gram-negative bacteria isolates)

S/N	OFX (10µg)	PEF (10µg)	CPX (10µg)	AU (30µg)	GN (10µg)	S (30µg)	CEP (10µg)	NA (30µg)	SXT (30µg)	PN (30µg)	Isolate identity
1	20	20	22	20	16	20	18	20	20	17	*Escherichia. coli*
2	20	22	16	20	14	20	15	14	20	20	*Enterobacteriacae*
3	20	21	20	16	10	15	0	14	20	14	*Proteus vulgaris*
4	18	16	22	20	0	0	10	18	20	20	*Enterobacter aerogens*
5	20	22	22	20	20	16	18	0	20	0	*Bacillus pumillus*
6	20	20	22	10	0	20	16	0	20	0	*Proteus mirabilis*

KEYS:
OFX – Tarivid PEF – Reflacin CPX - Ciproflox
AU – Augumentin GN – gentamycin S - Streptomycin
CEP – Ceporex NA – Nalidixic Acid SXT - Septrin
PN – Ampicilin

Table 6.0 Diameter of Zone of Inhibition (Mm) of the Antibiotics after curing (gram-positive bacteria isolates)

S/N	Location codes	PEF (10µg)	GN (10µg)	APX (30µg)	Z (30µg)	AM (10µg)	R (25µg)	CPX (10µg)	S (30µg)	SXT (30µg)	E (30µg)	Isolate identity
1	EC1B	20	0	0	10	0	0	26	0	0	0	*Staphylococcus aureus*
2	EC1C	20	0	0	10	0	24	21	0	0	0	*Pseudomonas epidermidis*
3	EC2G	26	22	0	8	0	23	22	18	20	23	*Staphylococcus epidermis*
4	EC1E	0	0	0	10	0	24	24	16	20	12	*Proteus vulgaris*

KEYS: PEF – Pefloxacin GN – Gentamycin APX - Amiclox
Z – Zinnacef AM – Amoxacillin R - Rocephin
CPX – Ciprofoxacin S – Streptomycin SXT - Septrin
E – Erythromycin

There was a little reduction of resistance in some of the antibiotics while the resistance of the bacterial isolates to other antibiotics remained the same after curing as shown in Table 5 and 6. The resistance of the bacterial isolates to some of the antibiotics after curing may be attributed to the misuse and overuse of these antibiotics resulting in the emergence of antibiotics resistant strains. *S. aureus* was shown to exhibit a wide range of antibiotic resistance even after curing thus indicating the possibility of its genes for resistance as being chromosomal and not plasmid borne which in line with study of Ebakota , *et al.*,[19] who observed wide range of antibiotic resistance. Resistance to Amoxicillin was observed before and after curing. Amoxicillin is one of the most widely used antibiotics all over the world. The resistance of these isolates to Amoxicillin could be indicative that they possess β-lactamases capable to hydrolyzing amoxicillin in the presence of clavulanate.

At a concentration of 30µm, Septrin (SXT) exhibited the highest zone of inhibition after curing, measuring 20mm, against all the tested isolates. This result indicates that the antibiotic is effective in eliminating all the bacterial isolates in cases of infection, as they were found to be susceptible to all. Following Septrin, Ciproflox (CPX) at a concentration of 10µm showed susceptibility to *Escherichia coli, Proteus vulgaris, Enterobacter aerogens, Proteus mirabilis* and *Bacillus pumilus*, while inhibiting the growth of *Enterobacteriacae*. Isolate, *Enterobacteriaceae* was cured and became sensitive to all tested antibiotics except gentamycin, Nalidixic Acid and Ceporex which were resistance.

4.0. Conclusion and Recommendations

The present study revealed the high resistance of bacterial isolates obtained from egusi cake. All the isolates had plasmids and demonstrated high antibiotic resistance before and even after curing, indicating that they harbored several resistance genes. Most gram-negative isolates in this study were sensitive to Tarivid, Reflacin, Ciproflox and Augumentin after plasmid curing while for gram positive all the isolates were sensitive to only Ciprofoxacin. It is expected that these antibiotics can be the drug of choice for effective management of disease caused by such bacteria. The results of this study demonstrated that the plasmid is one of the important ways to spread resistance but chromosomal mutation by environmental selection might also responsible for resistance. In addition, the study also revealed that curing of the plasmid increased susceptibility (sensitivity) to the test antibiotics although, resistance was still observed.

Further research is needed to determine all major sources of antibiotic resistant food borne pathogens in egusi and product. Plasmid curing and plasmid profile procedure should be used and considered for antibiotics resistance to determine if resistance is plasmid mediated or chromosomally mediated. Also, molecular study on the plasmid-borne and chromosomal-borne gene associated with antibiotic resistance using wide range of egusi products samples should carry out.

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