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Antibiogram and Plasmid Profile of Foodborne Bacteria Isolates from Fermented Melon (*Cucumeropsis manii*) Seed

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ABSTRACT

Fermented melon (*Cucumeropsis manii*) seeds are used as condiment for cooking different delicacies in Nigeria. The microbiological quality and safety of fermented melon seed is critical for public health since it is a popular and commonly used condiment in soups. Nine bacteria isolates from fermented melon (*Cucumeropsis manii*) seed samples were used for this study. Morphological and physiological characterization identified the isolates as *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Lactobacillus* spp, *Escherichia coli*, *Proteus vulgaris*, *Serratia marcesceus*, *Enterobacter* spp and *Salmonella typhi*. The bacteria were evaluated for antibiotic sensitivity and plasmid analysis using Alkali-lysis method. Among the organisms, the most frequently occurring and predominant isolates were *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* with 22% occurrence followed by (*Lactobacillus* spp, *Proteus vulgaris*, *Serratia marcescens*, *Enterobacter* spp and *Salmonella typhi*) which have 11% occurrence. The antibiotic susceptibility testing revealed that all the isolates exhibited multiple drug resistance being resistant to two or more antibiotics. The antibiotic which was most effective in inhibiting the isolates were pefloxacin (PEF) and Amoxicillin (AM) with sensitivity of 100%. Whereas the least effective antibiotics were Zinacef (Z) and Rocephin (R) sensitivity of 44%. The most resistant isolate is *Enterobacter* spp. with 90% (9 out of 10) resistance of assayed antibiotics. The analyzed isolates were seen to possess significant level of $p<0.05$, with values ranging from 0.0±0.00 to 8.0±0.00. Furthermore, molecular studies revealed that, all the isolates contained plasmids. The result obtained in this study is of public health significance and concern as it highlights the need for systematic approach in the control of microbial contaminants in foods as this may be a potent source of antimicrobial drug-resistant microbial strains into the population.

Keywords: Fermented melon seed, foodborne bacteria, antibiotics resistance, public health, plasmid profile

Introduction

Fermented melon (*Cucumeropsis manii*) seeds are used as condiment for cooking different delicacies in Nigeria. The microbiological quality and safety of fermented melon seed is critical for public health since it is a popular and commonly used condiment in soups. Food-borne disease associated with contaminated food and food products has been a major public health challenge for years. Inadequate food handling practices such as weak or poor safety laws, poor sanitation exercise, and regulatory system enforcement, lack of enlightenment, and infection awareness are some of the major factors promoting food-borne diseases in developing countries (Gille *et al*., 2018).

Contamination of food with antibiotic-resistant bacteria has been a rising issue in the last 10 years and this is associated with different environmental and anthropogenic factors (Irapada *et al*., 2015). The antibiotic resistance determinants can be transferred to other bacteria that are significant to public health thus making the prevalence of antimicrobial resistance among food-borne pathogens on the increase (Cassani e*t al.*, 2019).

Plasmids are independent, circular, self-replicating extra-chromosomal DNA elements with characteristic copy numbers within the host. Properties encoded by plasmid include resistance to antibiotics, heavy metals, degradation of hydrocarbons, synthesis of bacteriocins and antibiotics, etc (Ranadheera *et al.*, 2017).

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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 (Bell *et al*., 2018). 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 (Cassani *et al*., 2019).

Antibiotic resistance occurs when bacteria change in response to the use of these medicines. Bacteria, not humans or animals, become antibiotic-resistant. These bacteria may infect humans and animals, and the infections they cause are harder to treat than those caused by non-resistant bacteria (Gulbandilar *et al*., 2017). Novel strategies to struggle antimicrobial multidrug resistance are required, and plasmid curing, and anti-plasmid strategies could reduce antimicrobial resistance genes frequency and sensitize bacteria to antibiotics (Adesulu-Dahunsi *et al*., 2018). Therefore, this study was to examine the plasmid profile of antibiotic resistant food-borne bacteria associated with traditionally fermented melon (*Cucumeropsis manii*) seed.

Materials and Methods

Sample collection

Tiny melon seeds were procured from local markets namely Swalli market (sample A), Opolo market (sample B), Tombia market (sample C) all in Yenagoa Metropolis. Three (3) dried melon seed samples of about 100g each samples were procured from each of the markets making a total of nine (9) samples. The tiny melon seeds were identified as melon seeds of (*Cucumeropsis manii*) by a plant taxonomist in the Department of Biological Sciences, Federal University Otuoke, Bayelsa State, Nigeria.

The melon seeds were cleaned to eliminate any foreign particles, dirt, or contaminants. The cleaning process involved rinsing the seeds thoroughly under running tap water and air-drying them in a well-ventilated area. Subsequently, the cleaned seeds were soaked in water for 24hrs, grounded and stored in clean, airtight containers to undergo fermentation.

Each sample was opened and emptied into a sterile beaker and carefully mixed with a sterile glass rod. One gram (1gm) of each sample was weighed out and added in respective test tubes and a 10-fold serial dilution was carried out in each of the samples (Cheesbough, 2005).

Inoculation of the isolate and incubation

An aliquot (100 µl) of each bacteria isolate 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 bacterial 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 (sugar fermentation, oxidase, methyl red, indole, catalase, coagulase, urease and citrate) (Cheesebrough, 2010).

Antibiotic sensitivity testing

Antimicrobial susceptibility of the isolates was tested using the modified Kirby-Bauer multi discs diffusion method (Bukhari, *et al*., 2004). Commercial antibiotic discs (Celtech Diagnostic) containing the antibiotics were applied on petri dishes containing Muller Hinton agar. The following antibiotics evaluated for efficacy against the isolates were present on the discs: 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). The petri dishes were incubated at 37°C for 18 to 24 hours to promote bacterial growth and they were examined after incubation. Using a ruler, the zones of inhibition around each disk was measured and recorded in millimeters.

The results were then classified as susceptible, intermediate, or resistant by comparing the zones of inhibition to a standard reference chart or interpretive criteria offered by the Clinical and Laboratory Standards Institute (CLSI) (Bukhari, *et al*., 2004).

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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 transferred into a pre-labelled eppendorf tube. The tubes were 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 resuspended with 50µl of TE buffer and stored at -20˚C for further use (Andrup *et al.*, 2008).

Agarose gel electrophoresis for the separation of DNA fragments

Agarose gel (0.8%) was prepared as described by Ogbulie and Nwakanma (2015). This was carried out by weighing and adding 0.8g of agarose powder into 100ml of 1×TBE buffer followed by heating in a microwave for 3-5mins to boil. The gel was allowed to cool to about 55°C, after which 10µl of ethidium bromide was added and gently mix by swirling. The gel was casted into electrophoresis tank with comb in place to obtain a gel thickness of about 4.5mm and allowed to stand for 20 minutes to solidify.

Thereafter, the comb was gently removed, and the tray placed in the electrophoretic tank, with TBE buffer prior to loading of extracted plasmid DNA molecule. 20µl of the extracted plasmid was mixed with 2.0µl of the loading dye and carefully loaded into the wells created by the comb alongside with marker in a different well to serve as ladder to extrapolate the molecular weight of the molecules. The electrodes were connected to the power pack ensuring that the negative terminal is at the well where the plasmid DNA samples are loaded. The gel was run at 75V until the loading dye has migrated to the end. The power supply was turned off and the electrodes were disconnected. The gel was removed from the gel tray and observed under UV-trans-illuminator. And the gel field volume fed for labelling and analysis of the molecular weight.

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 $p < 0.05$.

Results

Nine (9) bacteria were isolated from the fermented melon (*Cucumeropsis manii*) seed samples. The identified bacteria are; *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Lactobacillus* spp, *Escherichia coli*, *Proteus vulgaris*, *Serratia marcesceus*, *Enterobacter* spp and *Salmonella typhi*.

Among the organisms, the most occurring and predominant ones were *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli* with 22% of occurance followed by *Lactobacillus* spp, *Proteus vulgaris*, *Serratia marcesceus*, *Enterobacter* spp and *Salmonella typhi* with 11% occurrence.

The antibiotic susceptibility pattern of the isolates is presented in Table 1. The isolates showed some level of resistance to all assayed antibiotics. The antibiotic which was most effective in inhibiting the isolates were pefloxacin (PEF) and Amoxicillin (AM) which inhibited all the isolates with sensitivity of 100%. Whereas the least effective antibiotics were Zinacef (Z) and Rocephin (R) sensitivity of 44%.

The most resistant isolate is *Enterobacter* spp. which was resistant to 90% (9 out of 10) of assayed antibiotics. While the most sensitive organism is *E. coli* which was sensitive to 55.5% (7 out of 10) of the assayed antibiotics.

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Table1: Zones of inhibition (mm) of bacterial isolates from fermented melon (*Cucumeropsis manii*) seeds

Bacterial Isolate	PEF (10µg)	GN (10µg)	APX (30µg)	Z (20µg)	AM (30µg)	R (25µg)	CPX (30µg)	S (30µg)	SXT (30µg)	E (10µg)
S. aureus	28	0	0	0	0	0	20	20	0	16
Bacillus subtilis	20	15	15	0	15	0	20	20	20	15
S. epidermidis	20	18	18	0	18	20	20	20	20	20
Lactobacillus spp	20	0	0	0	13	0	0	16	0	18
Salmonella typhi	20	20	20	0	10	0	0	0	10	0
E. coli	30	0	0	0	15	0	0	0	20	0
Enterobacter spp	20	20	20	19	18	0	15	16	20	13
S. marcesceus	35	20	15	20	20	15	16	0	20	0
Proteus vulgaris	20	20	20	18	18	20	0	0	20	20

Key: PEF = Perfloxacin; Z = Zinacef; CPX = Ciprofloxacin; E = Erythromycin; GN = Gentamycin; AM= Amoxicillin; S=Streptomycin; APX=Ampiclox; R= Rocephin; SXT = Septrin; >20mm =Sensitive (S); 15-19mm =Intermediate (I); <14mm =Resistant (R).

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. The nine (9) isolates assayed, showed visible bands indicating the presence of plasmids.

The result shown on Table 2 reveals the difference in significance of the values gotten from the plasmid profiling of the analyzed samples and their base pairs. From which *Proteus vulgaris* has the highest value being 8.0±0.00, followed by 7.5±0.00 for *E. coli* and *S. aureus* having the least value of 0.0±0.00 which is clearly insignificant possess level of $p<0.05$, with values ranging from 0.0±0.00 to 8.0±0.00.

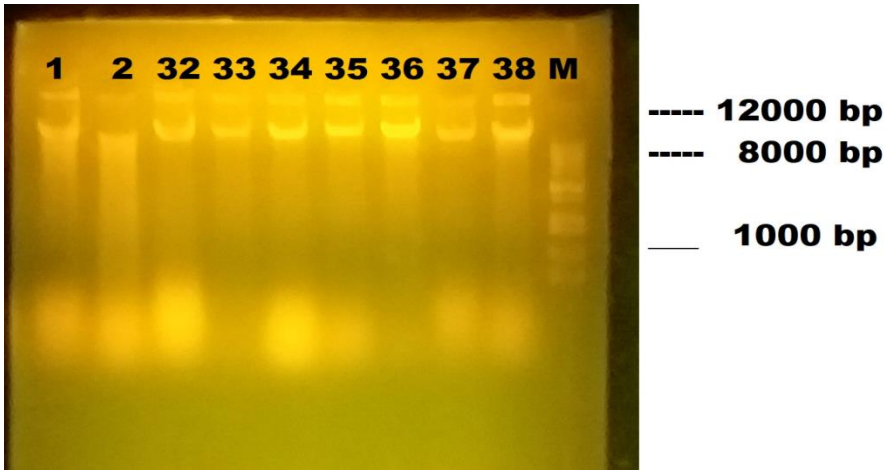

Plate 1: Plasmid profile pattern of bacterial isolates in 0.5% agarose gel

Plasmid profile photographic representation indicates the isolates and bands created by each isolate. Each well is represented in codes, while M is a 1Kb marker of standard molecular weight for calculation of the molecular weight of the plasmid DNA. Lane 1: *Proteus vulgaris*, - 2; *Salmonella typhi*, - 32; *E. coli*, - 33; *Enterobacter* spp, -34; *Serratia marcesceus*, -- 35; *Bacillus subtilis*, - 36; *S. aureus*, - 3: *S. epidermidis*, and - 38: *Lactobacillus* spp.

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Table 2: Statistical analysis of base-pair of bacteria isolated from fermented melon seed samples

Lane	Bacterial Isolate	Base pairs (10^2)
1	*Proteus vulgaris*	8.0±0.00
2	*Salmonella typhi*	7.0±0.00
32	*E. coli*	7.5±0.00
33	*Enterobacter* spp	00±0.00
34	*Serratia marcesceus*	7.0±0.00
35	*Bacillus subtilis*	6.0±0.00
36	*S. aureus*	00±0.00
37	*S. epidermidis*	7.0±0.00
38	*Lactobacillus* spp	7.0±0.00

Discussion

This present study has revealed that *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis* and *Lactobacillus* spp isolated from fermented melon (*Cucumeropsis manii*) seed were all sensitive to pefloxacin but resistant to zinacef which is in agreement with the report of Eboh *et al*., (2022) and the literature report of Ire and Eruteya (2017) on Antimicrobial susceptibility of spoilage bacteria of 'Atama' (*Heinsia crinata*) soup. *B. subtilis* is sensitive to perfloxacin, ciprofloxacin, septrin and streptomycin but resistant to rocephin and zinacef. Similarly, *S. epidermis* is sensitive to perfloxacin, ciprofloxacin, septrin streptomycin and rocephin but it is resistant to zinacef. Also, *S. aureus* is sensitive to perfloxacin, ciprofloxacin and septrin but resistant to streptomycin, rocephin and zinacef.

On the other hand, *Lactobacillus* spp is resistant to gentamycin, ampiclox, zinacef, amoxicillin, rocephin, ciprofloxacin and septrin while it is sensitive to only perfioxacin. *Escherichia coli, Salmonella typhi, Enterobacter spp, Serratia marcesceus* and *Proteus vulgaris* are sensitive to perfloxacin while almost all the organisms are resistant to zinacef (apart from *Enterobacter spp, Serratia marcesceus* and *Proteus vulgaris*). *Escherichia coli, Salmonella typhi,* and *Serratia marcesceus* are resistant to erythromycin and streptomycin while Proteus vulgaris is sensitive to streptomycin. Likewise, *Enterobacter spp, Serratia marcesceus* and *Proteus vulgaris* are sensitive to gentamycin (except *Escherichia coli, S. aureus, S. epidermidis, Bacillus subtilis* and *Lactobacillus* spp) and septrin affirming the report by Cassani *et al.*, (2019) and Oruntoyinbo *et al.*, (2016) that most antibiotics that *Escherichia coli* is resistant to may be sensitive and potent against organisms within the same family as the sensitivity of one organism in a family does not have total influence on the organisms within same terrain.

The result of the gel electrophoresis reveals the presence of plasmids in the bacterial isolates. This 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 (Talukder *et al*., 2021). There is evidence of horizontal gene transfer in diverse bacteria genera, including *Enterobacter* isolates containing plasmids that confer resistance to antibiotics to other bacteria (Vaidya, 2011). This is similar to studies conducted on *Serratia* sp. (Nmesirionye *et al*., 2022).

Additionally, the DNA carried on plasmids can be integrated into bacterial DNA, thus not only conferring the ability to resist antibiotics, but making the resistance genes fully inheritable (Talukder *et al*., 2021). Akter *et al* (2021) stated that plasmid can mediate antibiotic resistance by several mechanisms. Some of which is very popular among different gram negative bacteria. In his findings, all the *E. coli* strains showed different migration patterns on agarose gel electrophoresis. Similarly, *Salmonella enteritidis* and *S. typhi* were identified although they were highly heterogeneous but mostly limited to the top ten plasmid types of the pathogen. This supports the report of Alexey and Natalya (2021) in which most plasmids of *Salmonella enteritidis* and *S. typhi* were mostly cryptic and do not carry antimicrobial resistance genes. They further suggest that, despite the plasmid profile of the organism, it is good to know that the susceptibility to antibiotics in the pathogen is majorly developed via a specific means, especially for

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Proteus vulgaris, Staphylococcus aureus, Bacillus subtilis, Enterobacter spp, and *Serratia marcesceus*.

Furthermore, it is worthy to note that other bacteria reported in this study; such as *Streptococcus, Bacillus,* and *Lactobacillus,* has been documented in some literature showing the different resistance pattern to antibiotics (Afzaal *et al.*, 2019). This depicts the fact that there is no conflict of idea. For instance, in a study carried out by Tafida *et al*. (2013), the organisms identified has diverse rates of resistance to the different classes of antibiotics they were subjected to; and ranged from 5.6 to 100 % with resistance to streptomycin, trimethoprim or sulfamethoxazole, and amoxicillin.

The findings from the statistical analysis significantly reflect the report of Alexey and Natalya (2021) on "Antibiotic Resistance and Plasmid Profile Analysis of *Salmonella enteritidis* Isolated in Siberia" in which *Proteus vulgaris, Salmonella typhi, E. coli, Enterobacter* spp, and *Serratia marcesceus* were analyzed and seen to possess significant level of $p<0.05$, with values ranging from 5.0±0.02 to 6.0±0.01.

In conclusion, this present study has revealed the presence of some bacteria in fermented melon (*Cucumeropsis manii*) seed. the wide spread of antibiotic resistance and the various organisms that pose such threat to our society and public health. It shows the plasmid profiling of *Proteus vulgaris, Salmonella typhi, Escherichia coli, Enterobacter* spp, *Serratia marcesceus, Bacillus subtilis, Staphylococcus aureus, S. epidermidis,* and *Lactobacillus* spp whose plasmids can help mediate antibiotic resistance through different mechanisms that has been reported in literature. Findings from this study also reveals different migration pattern of organisms on agarose gel electrophoresis which further gives insights on their antibiotic resistant pattern and the possible means of curbing their virulence.

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