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Anti-bacterial Susceptibility and Biofilm-Forming Ability of Foodborne Pathogens Isolated from Minimally Processed Fruits and Vegetables Obtained from Markets in Southeastern Nigeria

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ABSTRACT

The resistance of foodborne pathogens to antibiotics and their ability to form biofilms poses a serious public health burden globally. This study investigated the antibacterial susceptibility pattern and biofilm-forming ability of bacterial isolates from surfaces of minimally processed fruits and vegetables from markets in Southeastern Nigeria. Randomly selected samples of fresh minimally processed watermelon, cucumber, and garden egg were collected and evaluated by standard plate techniques. Pure cultures were identified macroscopically, microscopically, and using biochemical analysis. The antibiotic susceptibility studies were conducted using the Kirby-Bauer disk diffusion method on eleven antibiotic discs. The biofilm screening was conducted using Congo Red Agar medium and scanning electron microscopy (SEM) was to determine morphological features of biofilm formers. Characterization revealed seventeen probable species of pathogenic bacteria. Antibiotic susceptibility results revealed MTX50 having the least antibacterial activity with percentage susceptibility of 0 and AMP10, the most effective antibiotic on all isolates with percentage susceptibility of 70.41.*Salmonella* sp. had a mean IZD of 18.67 mm to PEF5 and according to CLSI guidelines, it is said to be resistant to PEF5.All isolates were strong biofilm formers except four which were non- and moderate biofilm formers. SEM micrograph revealed organisms enclosed in an extracellular matrix. The detection of these opportunistic pathogens capable of forming biofilms in freshly minimally processed fruits and vegetable poses a serious risk for consumers because of their bacteria colonization indication ratio and resistance patterns which varied in response to the various antibiotics used. Biofilm formers indicate resistant pathogens as they formed extracellular polymeric matrices.

Keywords: Antibacterial susceptibility, Microbial contamination, Biofilm formation, Food-borne pathogens, Vegetables, Fruits.

Introduction

Minimally processed vegetables and fruits refer to food products or items that require little or no further processing prior to consumption. They are fresh-cut, partially-processed, vended, or ready-to-eat food items devoid of any form of addictives.[1] They can be processed following traditional or industrial techniques but this minimal processing technique does not influence or alter their nutritional quality and sensory characteristics. These food items are indispensable due to their high nutritive value. [2] The safety of these food products is crucial because they are usually consumed raw and consumers are usually enticed by them as they are already packed for convenience purposes.[3] Minimally processed fruits and vegetables have the advantage of ensuring convenience due to their easy and quick preparation, less severe processing methods, retaining quality and freshness of products, and maintaining the products' nutritive and sensory attributes. [4] Because they are consumed in their fresh state, they could therefore be a vehicle to several pathogens. [5] These pathogenic microbes colonize the outer surfaces of minimally processed fruits and vegetables and consumption of such contaminated fruits and vegetables can lead to food-borne illnesses.

Globally, numerous cases of foodborne diseases have been linked to the consumption of contaminated foods including fruits and vegetables which have minimally processed. [6] Foodborne diseases are significant contributors to public health burden. Its outbreak caused by microbial pathogens impacts heavily on public health, not only through illness but also through the costs linked with approaches taken to reduce its impacts on human populations. In today's world, there is an immense spread of foodborne illnesses across countries and continents due to foodborne pathogens. [7] WHO estimates globally that almost 1 in 10 people fall ill after consumption of contaminated food and about 420,000 die annually leading to healthy life years loss of 33 million. In Africa, over 91 million people are affected by foodborne related illnesses and in developing countries; about 2.2 million children die annually of foodborne induced diarrheal. [8] Several factors could contribute to an increased risk of foodborne diseases such as consumption of foods as ready-to-eat, partially-processed, or minimally processed.[2] Innumerable reports of foodborne disease outbreaks some of which have been stated in the history of foodborne disease outbreaks have identified cross-contamination during handling

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as the major cause of illness. The greatest challenge has been that proper personal hygiene and environmental sanitation are not met by food product handlers. This has been reported by a variety of studies that brought attention to approaches and opportunities for improvement of hygiene and sanitation practices in the commercial food production service environment.[9] Consumers of food and food products at home may adopt many unsafe handling practices but commercial outlets are obvious settings where these practices have an important health impact as hygienic practices are highly ignored and microbes thrive on raw surfaces of exposed food products.[10,11]

There are more drug-resistant microbes mainly in biofilm forms that colonize the surfaces of fruits and vegetables. [12] Jamal et al. reported that 99.9% of all microorganisms can form biofilm on a wide range of surfaces, [13] including diverse kinds of microorganisms, ranging from the prokaryotes such as Gram-positive and Gram-negative bacteria, archaea, cyanobacteria, eukaryotes, and microalgae.[14-18] Living as a biofilm offers advantages of protection from harmful environmental conditions to member cells such as abrupt changes in pH values, UV radiation, temperature changes, draining and desiccation, e.t.c.[19] Various microorganisms exist as biofilms but bacteria biofilms are most studied.[20] Bacterial biofilms also colonize inert food processing or medical equipment forming dense, multi colonies of the different layers of organisms on the food contact surfaces, in the mouth, or the lining of the intestine.[21] They are significantly more tolerable of antibiotics compared to the free-floating organisms and they have an increased potential to absorb and nullify the activity of antimicrobial agents resulting in an extended treatment.[22] Bacterial biofilms result in continuous contaminations and diseases due to their increased resistance to antimicrobial agents as well as opposing phagocytosis, antibodies, and other immune systems.[23] Antimicrobial chemotherapy reverses all symptoms caused by free-floating planktonic microbial cells released from the biofilm but fails to destroy the biofilms. [24] Despite the availability and innovations in the advancement of antimicrobial agents, infections caused by bacteria biofilms have remained a healthcare challenge as they remain resistant to most antibiotics. The bacterial biofilm forms a non-permeable barrier that protects the activity of bacteria for the antimicrobial agents to penetrate. Biofilm forming capability has been reported in several large bacterial species.[25] The morphology of biofilm is usually scanned using a scanning electron microscope. SEM, a powerful technique used to investigate the colonization of bacteria on living and non-living surfaces, provides ultra-structural details on the interactions of bacteria with one another and with the surfaces.[26] Useful information on morphology, interactions, and location of bacteria within the biofilm, as well as biofilm formation processes, are always provided by SEM. Microbial colonization of fresh and minimally processed vegetables and fruits by handlers could be true for Nsukka a small city in Enugu state, Southeastern Nigeria where there are no monitored and certified malls or markets where these fresh food products are sold. Lack of monitoring by appropriate regulatory agencies might result in foodborne infections which show resistance to conventional antibiotics used to treat them because these pathogenic microbes form biofilms on the surfaces of minimally processed fruits and vegetables owing to poor sanitation. The presence of these bacteria that are resistant to antibiotics poses serious health risk to the consumer resulting in foodborne disease.[27]

Although studies on the colonization of food and food processing equipment by bacteria forming biofilms have been conducted in Nigeria,[28] the ability of bacteria isolated from minimally processed fresh fruits and vegetables to form biofilms and antibiotic susceptibility pattern of such bacterial isolates have not been examined. Therefore, this study was aimed at evaluating the anti-bacterial susceptibility pattern and biofilm-forming ability of foodborne pathogens isolated from surfaces of fresh minimally processed fruits and vegetables obtained in Nsukka, Southeastern Nigeria.

Materials and Methods

Materials

Minimally processed fruit and vegetable samples were obtained from Ogige market, Ikpa market, Orie-Orba market, Orie Igbo-Eze market, and Eke-Ede Oballa market of rural and urban markets in Nsukka, southeastern Nigeria. Other materials used include MacConkey agar, nutrient broth, nutrient agar, Simmon citrate medium, eosin methylene blue agar, mannitol salt agar, sulfide indole motility medium, urea medium, starch agar medium, peptone water powder(Oxoid, England),oxidase reagent (Oxoid, England), Kovac's reagent (Oxoid, England) distilled water (Lion water, University of Nigeria, Nsukka, Nigeria), normal saline (Juhel Pharmaceuticals, Nigeria), lactose, sucrose, mannitol, and glucose powder (Ace International LIp, Greater Kailash, New Delhi), and antibiotic discs (Oxoid, England).

Methods

Collection and transportation of samples

A total of fifteen (15) samples, three (3) each of fresh minimally processed fruits and vegetables namely: garden egg (*Solanum melongena* L.), watermelon (*Citrullus lanatus* Thunb.), and cucumber (*Cucumis sativus* L.) were collected in sterile polythene bags from each major market around July, 2019 in Nsukka and transported in cold box temperature to the laboratory.

Preparation of culture media

The culture media for isolation were prepared following standard microbiological practices in batches according to the manufacturer's specifications for a total of fifteen (15) samples. [29]

Isolation and total viable plate count of bacteria

The modified method of Dashwood *et al.*[30] and Balali *et al.*[31] was adopted with slight modifications. A total of 10 g of the chopped surfaces of each minimally processed fruit and vegetable samples sliced using sterile blade were washed in sterile 90 mls saline from which 1 mL was transferred to the first test tube containing 9 mls of sterile distilled water as diluent. This was repeated for all samples set and diluted to 10^{10}. From all dilutions, 1 ml each was dispensed and spread aseptically on all pre-sterilized media in triplicate and incubated for 24 - 48 h at 37^{o}C to allow colony formation. At the end of the incubation, discrete colonies were counted where possible, multiplied by the dilution factor, and expressed as the colony-forming units per gram (cfu/g).[32]

Identification and characterization of selected bacterial isolates

Colonies were presumptively identified by cultural, morphological, spore staining, and Gram staining characteristics. Pure bacterial cultures were obtained by sub-culturing distinct colonies onto freshly prepared media plates. The isolates were confirmed by carrying out biochemical characterization including catalase test, coagulase test, starch hydrolysis test, Simmon's citrate utilization test, carbohydrate utilization test, indole, urease, and oxidase test according to standard methods.[33]The bacterial isolates were further sub-cultured on agar slants and stored in the refrigerator.

Antibiotic susceptibility test

The susceptibility of bacterial isolates to antibiotics was conducted using the Kirby-Bauer disk diffusion method.[34]A suspension of each test organism was prepared by inoculating 2-3 distinct colonies into 5 ml of sterile water, using a sterile loop. Each suspension was thoroughly mixed and adjusted to 10^{6}, using 0.5 McFarland Standard. The resulting suspension was applied to the surfaces of overdried Mueller Hinton agar and spread evenly. The inoculated plates were incubated at 37 °C for 20 min for acclimatization and growth of the inoculum. Aseptically, each antibiotic discs were lightly but firmly pressed onto the surface of the plates, using sterile forceps, and placed equidistant to each other. The plates were refrigerated after application of the discs at 4 °C for 30 min to ensure adequate diffusion of antibiotics. The test was carried out in triplicate. All plates were incubated at 37 °C for 24 h. The inhibition zone diameters of each isolate were measured in mm and interpreted following the Clinical Laboratory Standards Institute (CLSI, 2018) and European Committee on Antimicrobial Susceptibility Testing (EUCAST version 10.0, 2020) breakpoint tables. The antibiotics tested included: ampicillin (AMP) (10 µg), cloxacillin (OB) (5 µg), amoxicillin/clavulanic acid (AMC) (30 µg), cefuroxime (CXM) (30 µg), ceftriaxone (CRO) (30 µg), ciprofloxacin (CIP) (5 µg), pefloxacin (PEF) (5 µg), azithromycin (AZM) (15 µg), erythromycin (E) (15 µg), metronidazole (MTZ) (50

µg) and sulfamethoxazole/trimethoprim (SXT) (25 µg) (Oxoid, England). The organisms were categorized as resistant or susceptible based on the Clinical Laboratory Standards Institute (2018) and EUCAST recommended breakpoint tables version 10.0, 2020.

Biofilm screening and morphological confirmation of biofilms
Biofilm screening was conducted to detect biofilm production by each identified bacteria using the Congo Red Agar (CRA) method with brain heart infusion agar.[35]The physical and structural changes of biofilm-forming bacteria were examined by SEM (model PhenomProX, by phenom-world Eindhoven, The Netherlands) analysis for morphological confirmation.

Statistical analysis
Descriptive statistics such as percentages, pie chart, bar charts, and inferential statistics (one-way analysis of variance, ANOVA) at a 5 % significant level were used for analysis. Heatmap was used for visualizing the clustering of multivariate data where coloring gives an overview of the numeric differences and the analysis was performed with ClustVis web tool.[36]

Results and Discussions

This work deals with the anti-bacterial susceptibility pattern and biofilm-forming ability of foodborne pathogens isolated from minimally processed fruits and vegetables obtained from markets in Southeastern Nigeria. The total viable bacteria present in the minimally processed fruits and vegetable ranged, from 8.0×10^6 to 1.0×10^6cfu/g, with watermelon sample having the highest viable bacterial count (5.5×10^6, 3.4×10^6, 8.0×10^6, 3.4×10^6, 7.7×10^6 cfu/g) and cucumber sample having the lowest count (1.4×10^6, 1.0×10^6, 3.5×10^6, 2.3×10^6, 1.5×10^6 cfu/g) from all the markets (Table 1). The total viable plate count is an indication of the microbiological quality of any food product. In this study, all minimally processed fruit and vegetable samples examined, irrespective of the produce market, had mean contamination levels of $> 1 \times 10^5$cfu/g. World Health Organization (WHO) and Food and Agricultural Organization (FAO) recommends that standard values for microbial colonization should not exceed 10^5cells/ml for total aerobic bacteria, 10^3cells/ml for enteric bacteria, and salmonella and *E.coli* should totally be absent.[37] The bacteria colonization ratio of the samples exceeded the standard recommendations and has also included salmonella and *E.coli*. Following the above recommendations, our findings suggests that all examined fresh minimally processed fruit and vegetable samples harbor unwholesome products hence are unsatisfactory for human consumption. Similar results on microbial contamination of fresh minimally processed fruits
and vegetables have been reported both in Nigeria and other countries.[38-45]Cultural and biochemical characterization of isolated bacteria from all minimally processed fruits and vegetables revealed
the presence of seventeen (17) probable bacteria, namely: *Yersinia* sp.(n=1), *Serratia marcescens* (n=1),*Listeria* sp. (n=1), *Salmonella* sp. (n=1), *Pseudomonas* sp.(n=1), *Klebsiella* sp. (n=2), *Bacillus cereus* (n=2), *Clostridium* sp.(n=2), *Streptococcus* sp. (n=2), *Lactobacillus* sp. (n=2), *Proteus* sp. (n=2) *Citrobacter* sp. (n=3),*E. coli* (n=3),*Corynebacterium* sp. (n=3), other *Staphylococcus* sp.such as *Staphylococcus epidermidis*(n=7), *Bacillus subtilis* (n=8), and *Staphylococcus aureus* (n=17) (Figure 1).
Percentage occurrences of bacterial isolates revealed *Staphylococcus aureus* (40%) as highest followed by *Bacillus* sp.(21%), *Escherichia coli* (18%), *Staphylococcus epidermidis*, *Corynebacterium* sp. and *Citrobacter* sp. (17%), *Lactobacillus* sp. and *Proteus* sp. (12%), *Yersinia* sp., *Serratia marcescens*, *Listeria* sp., and *Pseudomonas* sp.(6%), *Klebsiella* sp., *Streptococcus* sp., *Bacillus cereus* and *Clostridium* sp.(5%), and *Salmonella* sp. (2%) was the least in occurrence (Figure 2). The presence of these organisms in food products reflects existence of favorable conditions for the multiplication of microorganisms. Similar studies carried out on minimally processed fruits and vegetables identified *Staphylococcus aureus* as the most abundant bacterial strain, followed by *Bacillus* sp. and *Salmonella* sp. having the least percentage abundance.[46,47]All types of food and food products have the potential to harbor pathogens including bacteria, viruses, and parasites all of which are of global health concern. The presence of these bacteria species is of public health concern owing to numerous life-threatening infections associated with them.[39] This present study conforms to findings from all similar studies. These microbes have been identified by numerous researchers as causes of foodborne diseases.[42]*E. coli* O157:H7, *Salmonella* sp., *Bacillus* sp. and *Listeria monocytogenes* have been identified as foodborne pathogens often present on the surface of fresh produce and may cause public health problems.[48]It has been reported that some of these identified bacteria can produce toxins causing a wide variety of infections. An example is the *Staphylococcus aureus* which had the highest percentage occurrence of 40%. This indicates a serious public health concern as *Staphylococcus aureus* has been clinically implicated in food poisoning, boils, impetigo e.t.c.[49] The United States Food and Drug Administration reported that its presence or its enterotoxins in processed foods or on food processing equipment is generally an indication of poor sanitation and the agency emphasized that *S. aureus* including other species of *Staphylococcus* have been identified as the causative agent in many cases of food poisoning/infection outbreaks globally.[50] Globally, *Staphylococcus aureus* has been identified as an important pathogen in foodborne disease. Minimally processed fruits and vegetables are often contaminated with enterotoxigenic strains of this bacterium resulting in public health burden.The presence of *Bacillus* and *Clostridium* species is an indication of serious health concerns because they have a spore-forming ability. Endospores of their members are able to contaminate fruits and vegetables especially during processing and packaging under conditions for spore germination.[51]Genera of Bacillus have been associated with foodborne disease outbreaks as they are widely found in the soil where these fruits and vegetables are cultivated. *B. cereus* has been associated with diarrheal-type food poisoning on the consumption of contaminated fruits and vegetables. Species of Clostridium such as *C. perfringens* have also been greatly associated with food poisoning.[52]

Table 1: The various sampling locations of minimally processed fruits and vegetables with the total viable counts of microbial contaminants

S/No	Sampling locations	Bacterial count (cfu/g)		
		Cucumber (C)	Water melon (W)	Garden egg (G)
1.	Ede-oballa (E)	1.5×10^6	7.7×10^6	3.0×10^6
2.	Ogige (O)	1.0×10^6*	3.4×10^6	1.7×10^6
3.	Ikpa (I)	1.4×10^6	5.5×10^6	1.6×10^6
4.	OrieOrba (OR)	3.5×10^6	8.0×10^6*	7.6×10^6
5.	Orie Igbo-Eze (IG)	2.3×10^6	3.4×10^6	2.5×10^6

Key: CFU= colony forming unit

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The isolation of *Escherichia coli* in the minimally processed samples analyzed is indicative of fecal contamination of such samples. As part of the normal flora of the human intestines, *Escherichia coli* has been linked to urinary tract infection as it has been identified as a urinary tract pathogen. [40]*Klebsiella*sp., a ubiquitous opportunistic pathogen has been isolated from many sources such as soil, water, raw fruits, and vegetables. The presence of *Klebsiella*sp. as an opportunistic pathogen and Streptococcus have been identified to inhabit the upper respiratory tract and could cause diseases associated with food. *Salmonella* sp., a non-lactose fermenter has been isolated from raw fruits and vegetables in many countries of the world.[50]The isolation of *Pseudomonas* sp. from some fruit and vegetable samples is also of a public health concern as it accounts for 10 % and 20 % of hospital-acquired infections. The isolation of Yesinia and Proteus species are indicative of foodborne diseases in host with impaired resistance.[49]Generally, contamination of these samples with bacteria could arise from inappropriate processing by vendors that ignore simple hygienic standards. Therefore, the presence of these pathogenic bacteria poses a public health challenge as most of them are associated with foodborne diseases. Each antibiogram result was expressed as susceptible, intermediate, or resistant according to the criteria of the Clinical Laboratory Standards Institute 2018 (CLSI) and European Committee on Antimicrobial Susceptibility Testing version 10.0, 2020 (EUCAST). Among the eleven selected antibiotics used, metronidazole 50 µg (MTX50) had the least effect on the 17 identified bacteria with an F-ratio of 961.00 at a 5% significant level (Table 2). This can also be seen in Figure 3 showing a single bar chart of the inhibition zone diameters of all identified bacteria to metronidazole (MTZ 50 µg). No inhibition zone diameter was noticed with all the isolates except *Streptococcus* sp. having an inhibition zone diameter of 10.8 ± 0.58 mm. Thus, according to this study, MTX50 proved to be the least effective drug against all identified bacteria with low significance at $p \leq 0.05$. This implies that it had the least antibacterial activity on the different bacteria isolates followed by the CIP5 with an F-ratio of 100.877, E15 (69.522), CRO30 (62.471), AZM15 (56.095), OB5 (55.625), SXT25 (50.061), AMC30 (49.285), CXM30 (32.204), PEF5 (30.264) and AMP10 (2.427) (Table 2).

Ampicillin 10 µg (AMP10) proved its antibacterial effectiveness against all seventeen identified bacteria compared to all other antibiotics used (Figure 4).

Therefore, AMP10 can be said to be the most effective drug inhibiting the seventeen (17) probable bacteria. From Table 3, the percentage susceptibility of all probable bacteria was calculated as follows: 6.67 % of all probable bacteria were sensitive to CIP5, 8.32% were sensitive to E15, 11.90% were sensitive to CRO30, 13.51% were sensitive to AZM15, 15.06% were sensitive to OB5, 25.38% were sensitive to SXT25, 55.80% were sensitive to AMC30, 58.57% were sensitive to CXM30, 62.53% were sensitive to PEF5, and 70.41% were sensitive to AMP10.

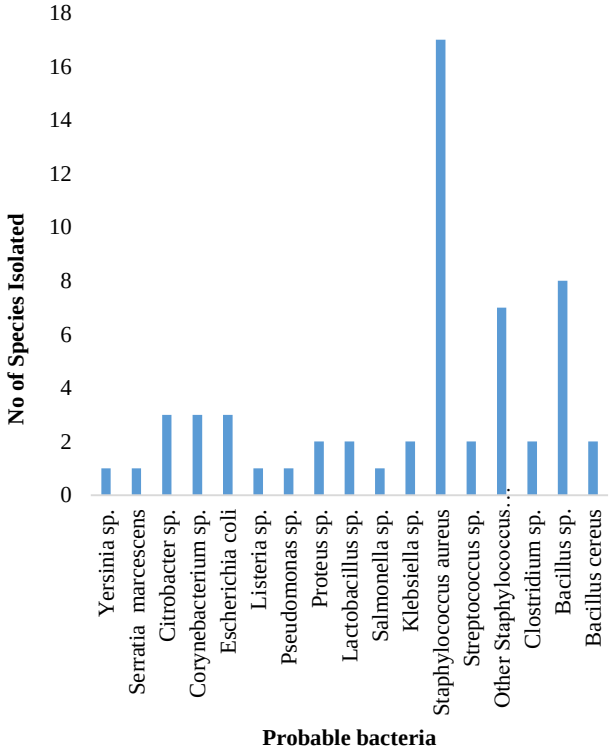

Figure 1: Number of each isolated probable bacteria species.

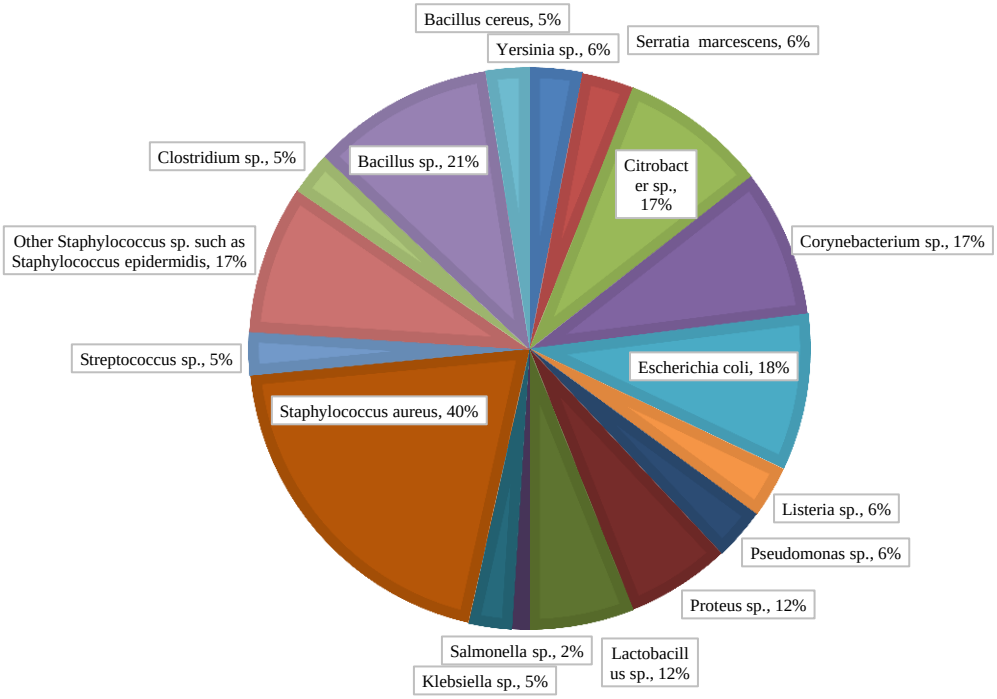

Figure 2: A simple pie chart showing the percentage abundance of all bacteria isolates.

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Table 2: The One-way Anova table showing the effect of the different antibiotics on all bacterial isolates at $p \leq 0.05$

ANOVA						
Variates		**Sum of Squares**	**Df**	**Mean Square**	**F**	**Sig.**
CIP5	Between Groups	1708.980	16	106.811	100.877	.000
	Within Groups	36.000	34	1.059		
	Total	1744.980	50			
MTX50	Between Groups	301.490	16	18.843	961.000	.000
	Within Groups	.667	34	.020		
	Total	302.157	50			
CXM30	Between Groups	1060.824	16	66.301	32.204	.000
	Within Groups	70.000	34	2.059		
	Total	1130.824	50			
AZM15	Between Groups	1337.490	16	83.593	56.095	.000
	Within Groups	50.667	34	1.490		
	Total	1388.157	50			
OB5	Between Groups	2181.373	16	136.336	55.625	.000
	Within Groups	83.333	34	2.451		
	Total	2264.706	50			
PEF5	Between Groups	664.627	16	41.539	30.264	.000
	Within Groups	46.667	34	1.373		
	Total	711.294	50			
AMC30	Between Groups	1035.961	16	64.748	49.285	.000
	Within Groups	44.667	34	1.314		
	Total	1080.627	50			
SXT25	Between Groups	706.745	16	44.172	50.061	.000
	Within Groups	30.000	34	.882		
	Total	736.745	50			
E15	Between Groups	1221.412	16	76.338	69.522	.000
	Within Groups	37.333	34	1.098		
	Total	1258.745	50			
AMP10	Between Groups	254.314	16	15.895	2.427	.015
	Within Groups	222.667	34	6.549		
	Total	476.980	50			
CRO30	Between Groups	1332.706	16	83.294	62.471	.000
	Within Groups	45.333	34	1.333		
	Total	1378.039	50			

From the study, ampicillin proved to be the most effective antibiotic against all seventeen probable bacteria identified. Metronidazole 50 µg proved to be the least drug of choice for all probable bacteria.

Heatmap analysis was also used to estimate whether pre-defined groups form separate or overlapping clusters. The brighter (blue to white) coloration showed a negative correlation (low sensitivity) while darker (white to brown) showed a positive correlation (higher sensitivity) (Figure 5). Similarly, heatmap analysis also revealed that MTX50 showed no or low sensitivity to all the probable organisms. This agrees with the simple bar chart for MTX50 (Figure 3) where no inhibition zone diameter was observed for metronidazole except for *Streptococcus* sp. which was also resistant with low sensitivity. The heatmap result also agrees with the statement that ampicillin is the most effective against all identified bacteria because all the maps showed a positive correlation indicating higher sensitivity.

In biofilm screening **(**Table 4), the presence of black colonies with a dry crystalline consistency showed a color change from red to black indicating the ability of the inoculated bacteria to form biofilm. *Citrobacter* and *Bacillus cereus* with red colonies are non-biofilm formers, *Listeria* sp. and *Streptococcus* sp. were moderate biofilm formers with faint black colors, while all other bacteria strains gave black colonies indicating strong biofilm formers. Figure 6 showed agar plates of black colonies (strong biofilm formers) and red colonies (non-biofilm formers). The biofilm-forming ability of bacteria species isolated from minimally processed fresh fruits and vegetables has been reported to account for the virulence action of pathogenic strain because of their resistant nature to antibacterial agents.The formation of extracellular polymeric substances acts as a protective barrier in biofilms against antimicrobial agents allowing them to cause diseases.[53]

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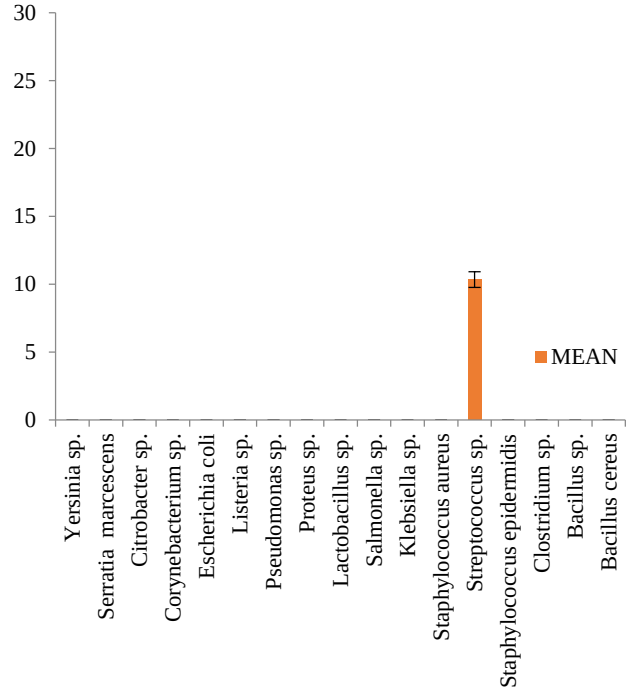

Figure 3: A single bar chart showing the inhibition zone diameters in mm of all identified bacterial isolates to Metronidazole 50 µg.

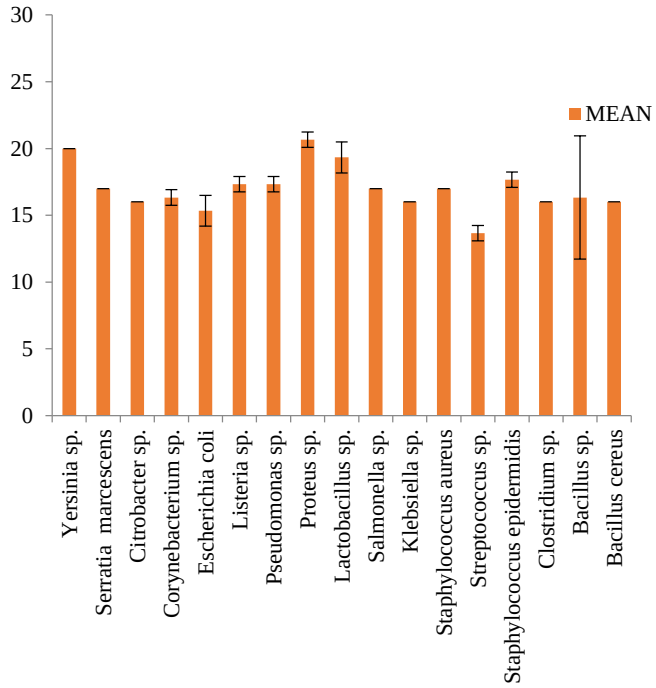

Figure 4: A single bar chart showing the inhibition zone diameters in mm of all identified bacterial isolates to Ampicillin (AMP) 10 µg.

Table 3: The antibiotic susceptibility test of the antibiotic discs against seventeen identified bacteria showing their inhibition zone diameters and percentage susceptibility in millimeter.

S/No	PROBABLE BACTERIA	SUSCEPTIBILTY PROFILES										
		CIP 5 (mm)	MTX 50 (mm)	CXM 30 (mm)	AZM 15 (mm)	OB 5 (mm)	PEF 5 (mm)	AMC 30 (mm)	SXT 25 (mm)	E 15 (mm)	AMP 10 (mm)	CRO 30 (mm)
1.	*Yersinia sp.*	16	0	15.67	14	15	19.33	16.33	15.33	8.33	20	11
2.	*Serratia marcescens*	9.67	0	11.67	24.33	14.33	12.33	11.33	24.33	11	17	24.33
3.	*Citrobacter sp.*	15.67	0	13	19.67	0	15.33	17.33	17.33	19	16	20.67
4.	*Corynebacterium sp.*	20	0	15.67	20	0	18	20	17	20.67	16.33	18
5.	*Escherichia coli*	19.33	0	13.67	12	17	12.33	12	10.33	15	15.33	21
6.	*Listeria sp.*	14	0	10.33	20	17.67	18.33	20	16	16	17.33	11
7.	*Pseudomonas sp.*	14.67	0	16	17	20	19	10	20.33	12.67	17.33	19
8.	*Proteus sp.*	15.67	0	10.67	24.33	11.33	20.67	10.33	11.33	19.33	20.67	11
9.	*Lactobacillus sp.*	27.33	0	20.33	17	13.67	20	12.33	16	12	19.33	11
10.	*Salmonella sp.*	25.33	0	20	25	13.67	18.67	26.33	20	0	17	20
11.	*Klebsiella sp.*	31.67	0	25	22.33	0	23.33	10	9.67	10.33	16	10
12.	*Staphylococcus aureus*	24.33	0	22.33	12	18.33	18.33	12.67	20	10	17	18.67
13.	*Streptococcus sp.*	12	10.33	19.33	12.67	10	20.33	20.33	19	21.67	13.67	20
14.	Other *Staphylococcus sp.* such as *Staphylococcus epidermidis*	15	0	13.33	17.67	21.33	19	19.33	18	20	17.67	18
15.	*Clostridium sp.*	24.67	0	20.67	10.67	17	14	12.33	14.33	11.33	16	21.33
16.	*Bacillus sp.*	21.33	0	17.33	14	11	11	16	19	9	16.33	11
17.	*Bacillus cereus*	21.67	0	25	28	11.67	11	16	18.33	8	16	26.22
	Percentage (%) susceptible	6.67	0	58.57	13.51	15.06	62.53	55.80	25.38	8.32	70.41	11.90

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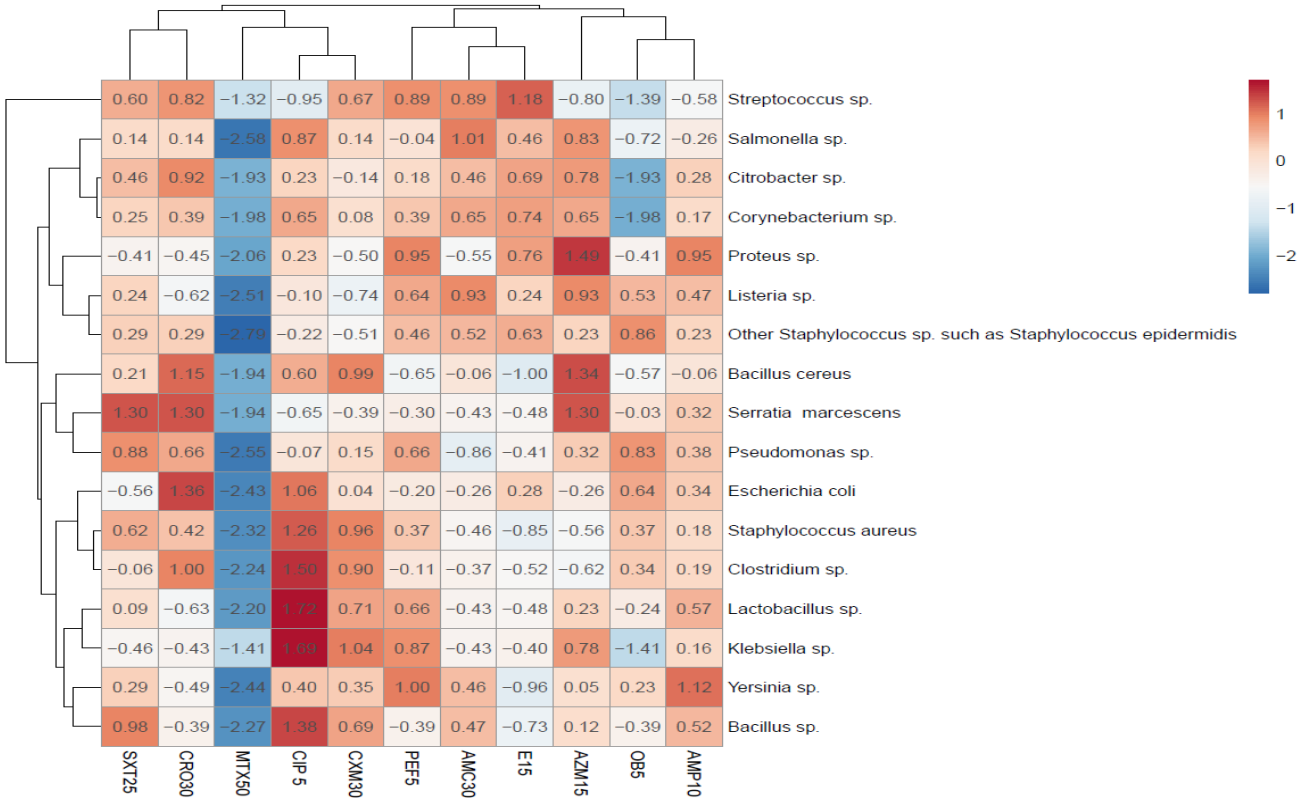

Figure 5: Heatmapantibiotic susceptibility test of eleven antibiotics against seventeen identified bacterial isolates.

Table 4: Biofilm screening result of identified bacteria.

S/NO	Probable Bacteria Species	Biofilm Forming Ability
1.	*Yersinia sp.*	Black colonies
2.	*Serratia marcescens*	Black colonies
3.	*Citrobacter sp.*	Red colonies
4.	*Corynebacterium sp.*	Black colonies
5.	*Escherichia coli*	Black colonies
6.	*Listeria sp.*	Faint Black colonies
7.	*Pseudomonas sp.*	Black colonies
8.	*Proteus sp.*	Black colonies
9.	*Lactobacillus sp.*	Black colonies
10.	*Salmonella sp.*	Black colonies
11.	*Klebsiella sp.*	Black colonies
12.	*Staphylococcus aureus*	Black colonies
13.	*Streptococcus sp.*	Faint Black colonies
14.	Other *Staphylococcus sp.* such as *Staphylococcus epidermidis*	Black colonies
15.	*Clostridium sp.*	Black colonies
16.	*Bacillus sp.*	Black colonies
17.	*Bacillus cereus*	Red colonies

KEY: Black colonies = strong biofilm formers,Faint black colonies = moderate biofilm former, Red colonies = non-biofilm formers

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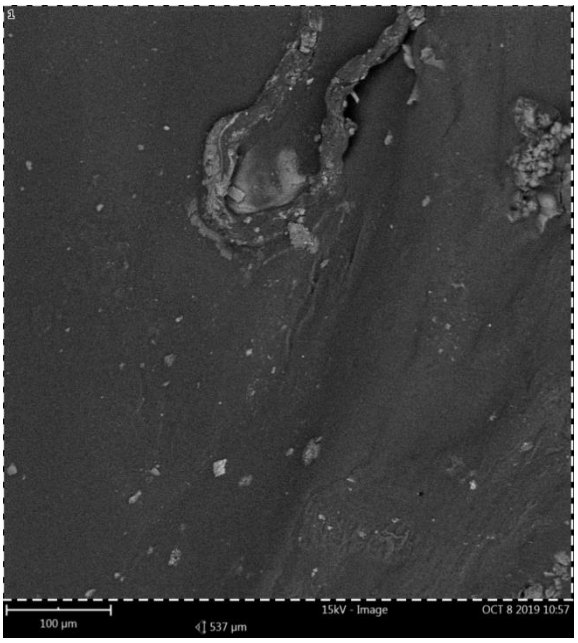
Figure 6: An agar plate of a biofilm former (black colonies) and non-biofilm former (red colonies).

A study conducted using congo red agar plate method on fresh fruits and vegetables revealed the ability of *E. coli* and *Salmonella* to form biofilm. From the results, 48.5% of *E. coli* and 50% of *Salmonella* sp were strong biofilms. These strong biofilm formers attribute to potentially virulent capacities of *E. coli* and *Salmonella sp* from the surface of fruits and vegetables.[54] Studies on the presence of biofilms on surfaces of fruits and vegetables have been reported on CR plates though it is not known to what extent these biofilms reduce food safety by harboring food-borne pathogens.[55,56]

Strong biofilm formers were subjected to SEM analysis for morphological confirmation. The scanning electron micrograph of the watermelon sample surface (WIG_3) (probably *Serratiamarcescens*) revealed a short rod-shaped, spherically-shaped, singly dispersed organism enclosed in a self-produced extracellular matrix with a scale size bar of 537 µm.



Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
6	C	Carbon	57.07	45.47
8	O	Oxygen	23.08	24.49
17	Cl	Chlorine	3.64	8.56
7	N	Nitrogen	8.87	8.24
11	Na	Sodium	3.76	5.74
15	P	Phosphorus	1.05	2.17
19	K	Potassium	0.49	1.28
20	Ca	Calcium	0.43	1.15
14	Si	Silicon	0.44	0.82
13	Al	Aluminium	0.44	0.79
12	Mg	Magnesium	0.45	0.72
16	S	Sulfur	0.27	0.57
22	Ti	Titanium	0.00	0.00
30	Zn	Zinc	0.00	0.00

FOV: 537 µm, Mode: 15kV - Image, Detector: BSD Full, Time: OCT 8 2019 10:57

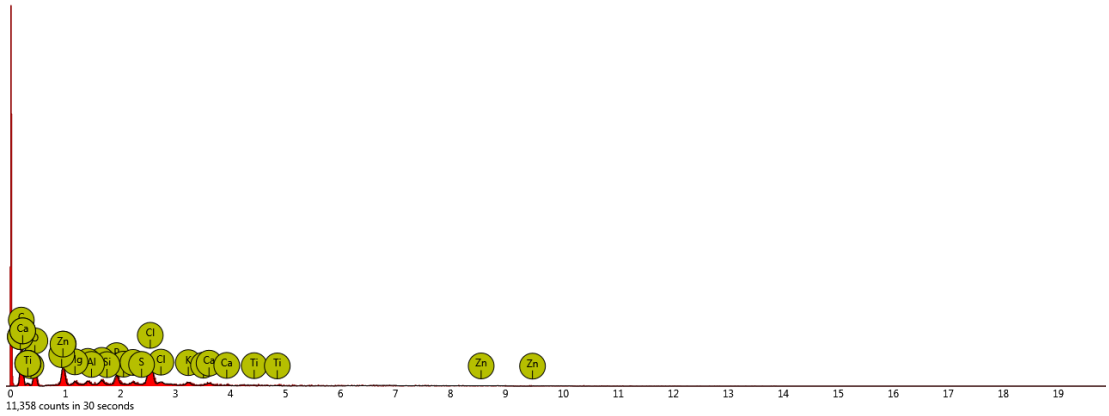


Figure 7: SEM monograph of WIG_3

KEY: WIG_3 = water melon sample from Orie Igbo-eze market, the subscript numbers stands for different dilutions from which each isolate was obtained

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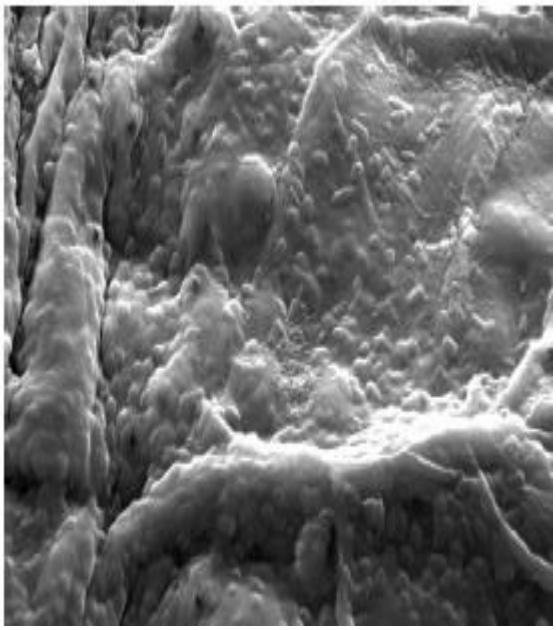

Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
6	C	Carbon	59.36	46.78
8	O	Oxygen	25.08	26.33
17	Cl	Chlorine	3.68	8.56
11	Na	Sodium	3.36	5.06
7	N	Nitrogen	4.92	4.52
30	Zn	Zinc	0.54	2.31
15	P	Phosphorus	0.58	1.18
13	Al	Aluminium	0.52	0.93
19	K	Potassium	0.35	0.90
14	Si	Silicon	0.46	0.84
16	S	Sulfur	0.36	0.76
20	Ca	Calcium	0.28	0.73
22	Ti	Titanium	0.18	0.58
12	Mg	Magnesium	0.33	0.53

FOV: 537 µm, Mode: 15kV - Image, Detector: BSD Full, Time: OCT 8 2019 10:44

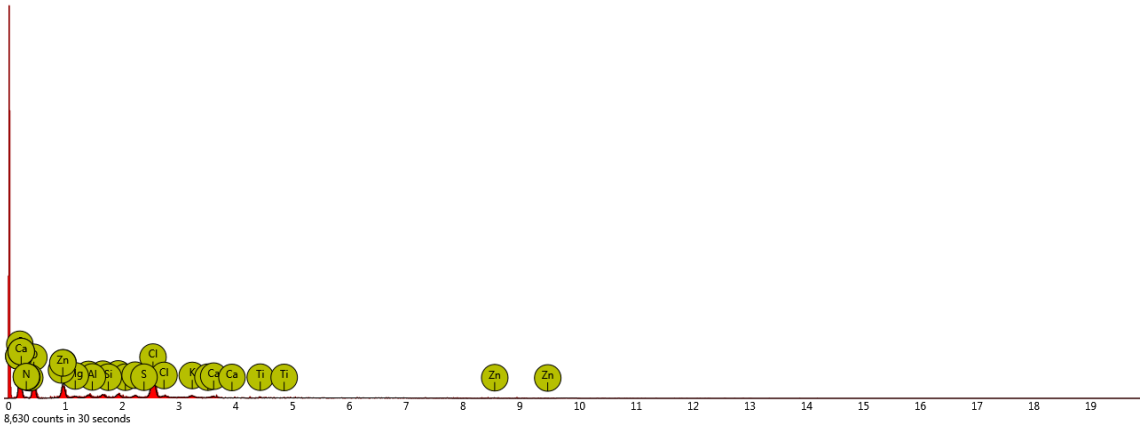

Figure 8: SEM monograph of GIG_1

KEY: GIG_1= garden egg sample from Orie Igbo-eze market, the subscript numbers stands for different dilutions from which each isolate was obtained

The SEM micrographs revealed biofilms in the form of an extensive aggregation of microbial cells on the surfaces of fruits and vegetables (Figures 7 and 8). Reports have shown that many bacteria are capable of forming distinct biofilms on surfaces of fruits and vegetables, including *E. coli*, *Bacillus*, *Salmonella*, *Listeria*, *Campylobacter*, and *Shigella*.[57] Comparing the antibiotic susceptibility and the biofilm screening result, resistant bacteria strains have been identified posing a greater challenge to the treatment of foodborne illnesses caused by such bacteria because according to FDA, new and emerging microorganisms and toxins, the increase in antibiotic resistance, increasing food contamination e.t.c promote foodborne illnesses. One of the major causes of antibiotic resistance is the ability of organisms to form biofilms. It can be concluded that these biofilms would not be eradicated by antimicrobial agents when ingested via these minimally processed fruits and vegetables.

Conclusion

The finding from this study has revealed the bacteria species colonizing surfaces of minimally processed fruits and vegetables where their resistance to antibiotics used in the treatment of foodborne diseases and their ability to form biofilms is a threat to global health care. The detection of resistant bacteria species on the surfaces of fresh minimally processed fruits, and biofilm-forming ability poses serious public health risks as studies have revealed them as causes of foodborne diseases. This can be life-threatening as biofilms are resistant to antimicrobial agents. Biofilm formation renders bacterial cells less susceptible to anti-bacterial agents. The antibiotic susceptibility test conducted also revealed bacteria resistance against most of the antibiotics used. Therefore, there is a need for urgent public sensitization and strict measures instituted by appropriate regulatory bodies to oversee the activities of fruit and vegetable sellers and handlers in the study area and other commercial outlets where fruits and vegetables are sold in Nigeria. It calls for simple hygienic procedures to be employed because hygienic handling of minimally processed fruits and vegetables is essential for the maintenance of food safety to avoid public health risks of foodborne diseases. However, the identification of bacteria isolates phenotypically is not exhaustive and is no longer the mainstay; therefore, molecular studies for both bacteria identification and biofilm formation are recommended.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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