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Bacterial Contamination of Leaf Surfaces of Common Edible Plants in Ebonyi State, South East Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author AOC designed the study, drafted the manuscript and served as principal supervisor. Author NEI searched for the literatures and participated in manuscript writing. Author OCC collected the samples and served as the principal investigator. Authors NOF and AMV wrote the protocols. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study investigated the bacterial load and isolated the implicated bacteria on the leaf surfaces of *Alchornea cordifolia, Musa sapientum* and *Thaumatococcus danielli* in Ebonyi State, South Eastern Nigeria.
Study Design: The research was laboratory-based investigation.
Place and Duration of the Study: The study was carried out at the Department of Microbiology Laboratory, Ebonyi State University, Abakaliki, Nigeria between June 2014 and January 2015.

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Methodology: Thirty grams of each sample were washed with 100 ml of physiological saline and inoculated into petri dishes containing a prepared nutrient agar and incubated for 24 hrs at 37℃. The observed bacterial growth were sub-cultured and later subjected to Gram staining and some biochemical tests (indole test, catalase test, oxidase test, motility test, sugar fermentation test).
Results: *Staphylococcus* spp, *E. coli, Salmonella* spp*, Klebsiella* spp and *Citrobacter* spp were isolated with *Staphylococcus* spp having the highest percentage occurrence of 38.1% on the three leaf samples followed by *E. coli* 19.0%, *Citrobacter* spp 19.0%, *Salmonella* spp 14.3% and *Klebsiella* spp 9.5% having the least percentage occurrence. The contamination of these leaves with the isolated organisms may be due to poor handling. The highest count was observed from *T. danielli* which had a mean plate count of 2.27 $x10^2$ while the least plate count 1.98 $x10^2$ was from *A. cordifolia*. *M. sapientum,* however, showed a plate count of 2.08 $x10^2$.
Conclusion: These leaves were heavily contaminated with different bacteria isolates and could serve as vehicles for transmitting pathogenic agents to humans. It is recommended that attention should be channelled towards safeguarding the health of the populace by ensuring the hygienic nature of these leaves from their harvesting, distribution to usage since they are used raw.

Keywords: Alchornea cordifolia; Musa sapientum; Thaumatococcus danielli; contamination; bacteria.

1. INTRODUCTION

Plant leaves are often used as packaging materials for some native African delicacies such as Agidi, Okpa, Igbangwo, Ukpaka as locally called in Igbo language, east Nigeria. However, most of these leaves are used with little or no consideration to their level of contamination. The microbial communities of leaves are diverse and include many different genera of bacteria, filamentous fungi, yeasts, algae, and, less frequently, protozoa and nematodes. Filamentous fungi are considered transient inhabitants of leaf surfaces, being present predominantly as spores, whereas rapidly sporulating species and yeasts colonize this habitat more actively [1]. However, bacteria are by far the most abundant inhabitants of the phyllosphere. Additionally, plant species appear to influence the microbial carrying capacity of the leaf, since the total number of culturable bacteria recovered from broad-leaf plants such as cucumber and beans was significantly greater than that recovered from grasses or waxy broad-leaf plants [2].

Contamination sources of leaf surfaces include raw materials and contact with processing equipment. Leaves can become contaminated with human pathogens at multiple points along the farm-to-table production/supply chain [3]. The sources may be grouped into pre-harvest (focused on soil amendments, irrigation water,) and post-harvest (harvesting, handling and processing) [3]. Potential sources of pathogen contamination in pre-harvest include soil, wildlife faeces, soil amendments, agricultural water, reconstituted fungicide and insecticides, dust, wild or domestic animals, field workers, and harvesting equipment [3]. Pathogenic bacteria mainly associated with produce outbreaks such as *E. coli*, *Salmonella* and *L*. *monocytogenes* are known to be shed into the environment through animal hosts [4]. Research has demonstrated the long-term survival of enteric pathogens in agricultural waters, soil amendments and soil [5]. Research demonstrates that animal manure used as soil amendments may contain viruses, parasites or bacteria that represent a human health concern. Pellet forms of manure (e.g. chicken manure pellets) are widely used in soil amendment because it reduces offensive odour and facilitates storage and transport [6]. Not surprisingly, chicken litter can harbour a diverse range of pathogens (*Campylobacter*, *Clostridium*, *Salmonella* and *Staphylococcus*), and if not properly treated prior to field application, can result in crop contamination [7]. Irrigation is considered as one of the most important transmission modes of enteric human pathogens to leaves of plants [8].

Monitoring of presence or absence of human pathogens or testing of indicator microorganisms is done to determine the satisfactoriness of agricultural waters applied to crops [9]. In developing countries, untreated sewage water is often used for irrigation of plants. River water used for irrigation can also harbour microorganisms that cause human illness. Counts of faecal coliforms and *E. coli* in river water used in plant irrigation in South Africa were up to 1.6×10^6 cfu 100 ml^{-1} and 3.5×10^5 cfu 100 ml^{-1} respectively [10]. A study of pond and tidal creek water in Australia found water samples positive for genes suggesting the presence of *E. coli* O157, *Campylobcter jejuni* and *Salmonella* [11]. The risk of microbiological

contamination of the edible portion of a crop is directly impacted by the method of irrigation: surface furrow, subsurface drip or overhead sprinkler. Research confirms that sprinkle irrigation compared with furrow and subsurface drip increased the risk of crop contamination [12].

Cross-contamination of plant leaves can occur during harvest through contact with harvesting equipment, knives, workers' hands or gloves and containers such as bins, boxes and buckets [3]. Pathogens that may be present on hands can be transferred to leaves or food contact surfaces and harvested leaves can be contaminated as the result of improper handling during storage and transportation. Processing operations provide many opportunities for cross-contamination because cutting, washing and sanitizing, packaging, and storing are involved. Cutting of plant leaves releases exudates-containing nutrients that can aid in the growth of enteric pathogens [13]. Wash water of unsatisfactory microbial quality or not treated with a sanitizer can serve as a vehicle for dispersion of microorganisms. Washing was the primary vehicle for the homogenous spread of *Salmonella enteritidis* to fresh-cut leaves during processing. Unfavorable conditions during packaging and storage contribute to the growth and survival of spoilage and pathogenic microorganisms on leaves [14].

In recent years, there has been an increase in the number of reported cases of bacteria illness regarding the use of the leaves of some plants species in food wrapping [15]. This is as a result of high demand of this food especially when the components of these leaves are not cooked. The plants selected for this study are of great economic importance. The plants are widely spread in Ebonyi State and their leaves are used in wrapping local foods such as moi moi, okpa, agidi, African oil bean etc. It becomes necessary that their bacterial contamination be investigated in order to contribute information on the control of bacteria related infections. Therefore, this study investigated the bacterial contamination of the leaf surfaces of *Alchornea cordifolia, Musa sapientum* and *Thaumatococcus danielli* in Ebonyi State, Nigeria.

2. METHODOLOGY

2.1 Study Area

This study was carried out in Ebonyi State, South Eastern Nigeria. The vegetation of the area is a mixture of savanna and semi-tropical forest which is dense and remains green throughout the rainy season. Agriculture is the main stay of the economy and majority of the inhabitants of the area are peasant farmers and petty traders of low economic status.

2.2 Sampling Techniques

The leaves were collected from different areas in Ebonyi State. Leaves of *Alchornea cordifolia* (Christmas bush) were from Ebonyi Local Government Area, *Thaumatococcus danielli* (Miraculous fruit) from Abakaliki Local Government Area and *Musa sapientum* (Banana) from Ezza North Local Government Area all in Ebonyi State. These plant species were selected for study due to extensive use of their leaves for wrapping food stuffs. The samples were aseptically transferred into sterile polythene bags and conveyed to the Department of Applied Biology, Ebonyi State University for identification by a qualified taxonomist. The identification numbers of these plants include *Thaumatococcus daniellii*- EBSU-H- 0193, *Alchornea cordifolia* - EBSU- 0023 and *Musa sapientum*-EBSU-0054. They were thereafter taken to Department of Microbiology Laboratory Ebonyi State University for analysis.

2.2.1 Sample culture

Thirty grams of each leaf was washed with 100 ml of physiological saline and poured into three (3) different sterile containers. Each sample solution was well shaken to allow for homogeneity. Then 0.1 ml of each solution was aseptically and uniformly inoculated on a nutrient agar plate. The plates were labelled and incubated aerobically at 37℃ for 24 hours. The plates were then examined macroscopically and microscopically for bacterial growth according to [16]. Bacterial colonies were counted and multiplied by 100 to give an estimate of the number of bacteria present per milliliter of the sample [17]. The morphological characteristics of the colonies of the pure culture growing on the media were examined with reference to their sizes, pattern of their edge, margin, surface texture elevation, consistency and colour as described by [18].

2.2.2 Gram staining and identification of isolates

After incubation, plates which had growth were further examined for their cultural characteristics. Gram staining of discrete colonies was also done

using cloth crystal violet (primary stain), Lugol's iodine (mordant), acetone (differentiator) and carbon fusin (counter stain) and examined to determine the gram reaction and morphology of the isolates. The colonies were sub-cultured into nutrient agar plates and incubated for 24 hours at 37℃ for purification of the isolates.

2.2.3 Characterization

Characterization of the isolates was done by subjecting Gram positive colonies first to catalase test before subsequent biochemical tests were performed while gram negative colonies were first subjected to motility test before other identification tests were performed.

2.2.4 Catalase test

A drop of hydrogen peroxide was placed on a slide, and a colony of each isolate was added to it and observed for rapid formation of gas bubbles which is an indicator of a positive reaction and result was recorded.

2.2.5 Motility test

Motility test of the isolates was done using hanging drop preparation [19]. It was then mounted on a microscope slide and examined using X10 and X40 objectives lens respectively to determine the presence or absence of motility and the results recorded.

2.2.6 Oxidase test

Oxidase test was performed by placing filter paper in a petri dish and flooding it with Kovac's reagent. A sterilized wire loop was used to streak a colony of the isolates over the flooded filter paper and observed for the formation of deep purple color within 10 seconds as a positive result.

2.2.7 Spot indole test

A filter paper was placed in a petri dish and flooded with indole regent.

A sterilized wire loop was used to streak discrete colony of each isolate on the filter paper and was observed for rapid formation of a deep - blue colour within 10 seconds as a positive result.

The isolates were subsequently grouped into their respective genus and species by virtue of their biochemical reactions.

3. RESULTS

The biochemical profile of the isolates observed from the plant materials is shown in Table 1. The organisms isolated include *Staphylococcus* spp*., Citrobacter* spp*., Salmonella* spp*., Klebsiella* spp*. and E. coli.*

Table 2 shows the total plate counts in cfu/ml observed from different plate cultures. The highest count was observed from *T. danielli* which showed a mean plate count of 2.27 $x10^2$ while the least plate count was from *A. cordifolia* with a plate count of 1.98 $x10^2$. *M. sapientum* however showed a plate count of 2.08 $x10^2$.

The percentage occurrence of individual species is shown in Table 3. From the table, three bacterial species were isolated from *Alchornea cordifolia.* They are *Staphylococcus* spp*,* with the highest percentage occurrence of 58%, followed by *Citrobacter* spp 28% and *E. coli* having an occurrence of 14%. From *Musa sapientum Salmonella* spp had an occurrence of 44% while *Citrobacter* spp and *Staphylococcus* spp had an occurrence of 28% each.

Table 1. Biochemical profile of isolates

Sample no.	Test									
	Shape	**Gram**	**Cat**	**Oxi**	**Cit**	**Ind**	**Acid sugar fermentation**	**Gas production**	**Mot**	**Suspected Organisms**
1	Circle	+	+	-	-	-	A	-	-	*Staph.* spp
2	Circle	-	+	-	+	-	A	-	+	*Citrobacter.* spp
3	Round	-	+	-	-	-	A	G	-	*Salmonella* spp
4	Rod	-	+	-	+	-	A	-	-	*Klebsiella* spp
6	Rod	-	+	-	-	+	A	-	+	*E. Coli*

Key: + Positive - Negative A Acid

Table 2. Total plate count for samples

Sample no.	Bacterial Count (Cfu/g)		
	A. cordifolia	***M. sapientum***	***T. danielli***
1	2.5 x10^2	1.0 x10^2	2.0 x10^2
2	3.0 x10^2	3.0 x10^2	2.5 x10^2
3	1.0 x10^2	1.5 x10^2	3.1 x10^2
4	1.5 x10^2	2.0 x10^2	1.3 x10^2
5	2.9 x10^2	1.3 x10^2	2.1 x10^2
6	2.0 x10^2	2.7 x10^2	3.9 x10^2
7	1.0 x10^2	3.1 x10^2	1.0 x10^2
Total	13.9 x10^2	14.6 x10^2	15.9 x10^2
Means	**1.98 x10^2**	**2.08 x10^2**	**2.27 x10^2**

Table 3. Percentage occurrence of organisms on the leaves

Organisms	% Occurrence		
	A. cordifolia	***M. sapientum***	***T. danielli***
Staphylococcus spp	58	28	28
E. coli	14	-	44
Citrobacter spp	28	-	-
*Salmonell*a spp	-	44	-
Citrobacter spp	-	28	-
Klebsiella spp	-	-	28

Percentage occurrence of organisms from *Thaumatococcus danielli* showed *E. coli* (44%), *Klebsiella spp* 28% and *Staphylococcus* spp 28%.

Table 4 shows the total percentage frequency of occurrence of individual bacteria species. The table shows that *Staphylococcus* spp had the highest frequency of 8 out of the total 21 isolates identified, making a percentage occurrence of 38.1% while *Klebsiella* spp had a frequency of 2 out of the total 21 isolates and hence a percentage occurrence of 9.5%.

Table 4. Frequency of isolated organisms

Organisms	No. of positive	% Occurrence
Staphylococcus spp	8	38.1
Salmonella spp	3	14.3
E. coli	4	19.0
Klebsiella spp	2	9.5
Citrobacter	4	19.0
Total	21	100

4. DISCUSSION

Leaf samples of *Alcornea cordifolia*, *Musa sapientum* and *Thaumatococcus danielli* used in this research were analyzed for the presence of bacterial species of importance in food borne illnesses. The results showed that total bacterial plate count was high with 13.9 x10^2 cfu/ml, 14.6 x10^2 cfu/ml and 15.9 x10^2 cfu/ml for *Alchornea cordifolia*, *Musa sapientum* and *Thaumatococcus danielli* respectively. This finding is in accordance with the results of [10] who reported a high total bacteria plate count of 14.9x10^2 cfu/ml. This high level of contamination may be due to the fact that these leaves are not usually washed before use and even when they are washed, the cleanliness of the wash water is not guaranteed. The highest plate count recorded with *T. daniellii* could be due to the fact that it thrives in humid areas where most of the contaminated faecal materials are dumped during rainy season. In other words, contamination of the environment is a predisposing factor for the contamination of the plants.

Predominate organisms isolated from the leaves of *Alcornea cordifolia* includes, *Staphylococcus* spp, *E. coli* and *Citrobacter spp*. *Staphylococcus* spp. had the greatest percentage of occurrence (58%), which agrees with the report of [20] who reported a percentage occurrence of 55% for *Staphylococcal* contamination of *Alchornea cordifolia* leaf surface. Organisms isolated from the leaves of *Musa sapientum*, were *Salmonella* spp*, Citobacter* spp and *Staphyloccus* spp. The percentage occurrence of each of the organisms was as follows: *Salmonella* spp 44%, *Citrobacter* spp 28% and *Staphylococcus* spp 28%. This

finding corroborates those of [3] with a 49% occurrence of *Salmonella spp* contamination on the leaf surface of *Musa sapientum.* According to [21], this high occurrence of *Salmonella* on leaf surfaces might be as a result of contamination with animal and human feces which was probably used as manure. The high level of bacterial contamination observed in this particular plant leaves may be connected to the nature of environment where they are grown. This is because *M. sapientum* is usually grown in refuse dump sites around houses where there is high concentration of contaminants. On the leaves of *Thaumatococcus danielli, E. coli, Klebsiella* spp and *Staphylococcus* spp were isolated. *E. coli* had the highest percentage occurrence of 44%, *Klebsiella* spp 28% and *Staphylococcus* spp 28%.

An overview of the total percentage occurrence of individual organisms on all the three samples showed that *Staphylococcus* spp had the greatest occurrence of 38.1% followed by *E. coli* and *Citrobacter* spp with an occurrence of 19.0% each, *Salmonella* spp 14.3% and *Klebsiella* spp 9.5%. These high percentage occurrences of *Staphylococcus* spp and *E. coli* might be as a result of poor personal hygiene and unhygienic environment of the handlers and food processors and contamination from agricultural activities such as irrigation, application of manure and harvesting [21].

5. CONCLUSION AND RECOMMENDATIONS

The leaf surfaces of the three plants were observed to have been contaminate with different bacteria species. Among the leaves, *A. cordifolia* recorded the highest contamination while *Staphylococcus spp* was the most encountered isolate. Given the use of these leaves in the packaging of ready to eat foods in the study area, it is important to educate leaf handlers (Sellers and buyers) on the need for good hygiene and the use of portable water for the washing of the leaves before use in order to reduce microbial load to its barest minimum. Appropriate measures should also be taken to reduce, control or eliminate the potential introduction of human pathogens on the leaf surfaces during and after harvest. We also recommend that further investigations be carried out on these plant species using molecular methods.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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