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Characteristics and Antibiogram Studies of Bacteria Associated with Vegetables Stored In Raffia Baskets in Nigeria

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Authors' contributions

This work was done in collaboration among authors. Author MEI designed the study, performed the statistical analysis, wrote the protocol and manuscript while, author SID managed the analyses of the study and literature searches under the strict supervision of author DNO. All authors read and approved the final manuscript.

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ABSTRACT

Raffia baskets are used in rural communities to preserve fresh vegetables for a given period of time. This method of storage is aimed at extending the shelf life of the vegetables until they are used. Thus, this study was aimed at determining the microbial succession during storage and susceptibility pattern of bacterial isolates from stored leafy vegetables to various antibiotics. Five (5) leafy vegetables namely, Bitter leaf (*Vernomia anydalira)*, Water leaf (*Talinum triangulare)*, Fluted pumpkin leaf, (*Telfairia occidentalis),* Okazi leaf (*Gnetum africana)* and Scent leaf (*Ocimum gratissimum)* were obtained from the Nigerian Stored Products Research Institute (NSPRI) farm, Port Harcourt. These vegetables were stored in a raffia basket for fourteen (14) days while another set of the vegetables were left in the open air as control. This set up was monitored for changes to occur. Standard microbiological techniques were employed for the various analyses of the vegetable samples. Both cultural and molecular characterizations of the isolates were done. The disc diffusion method was used in determining the susceptibility pattern of the bacterial isolates.

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The results of the predominant microorganisms identified were of the genus; *Bacillus, Pseudomonas, Enterococcus, Enterobacter, Bordetella, Staphylococcus, Myroides, Escherichia, Serratia, Micrococcus and Acetobacter*. Also, *Bacillus* species occurred in all the vegetables while *Enterococcus faecalis, Acetobacter orientalis, Bordetella pertussis, Myroides xuanwuensis* and *Bacillus flexu*s were isolated on the third day of storage from the vegetables. The total heterotrophic bacterial counts ranged from 1.8 x10^6 cfu/g to 1.25 x10^7 cfu/g (bitter leaf), 9x10^5 cfu/g to 9.0 x10^6 cfu/g (Scent leaf), 7 x10^5 cfu/g to 1.88 x10^7 cfu/g (Okazi), 3.2x10^6 cfu/g to 1.05x10^7 cfu/g (Fluted pumpkin leaf) and 8.4 x10^6 cfu/g to 2.04 x10^7 cfu/g (water leaf). Antibiogram of bacterial isolates revealed that they were resistant to Augmentin and Ceftazidime according to Clinical Laboratory Standard Institute schemes. Combination of the antibiotics showed that most of the isolates were 100% susceptible to ciprofloxacin + erythromycin, and ciprofloxacin + ceftriaxone. The study revealed that for all the vegetables, those stored in the basket lasted longer and were fresher compared to those kept in the open space. This study revealed that the raffia baskets are suitable for storage and preservation of leafy vegetables, but microbial succession occurred as a result of increase in the period of storage leading to the proliferation of microorganisms. This could be from aerosols or by chance inoculation, through handling or materials used or other microorganisms which are inherent in the vegetables because of their contact with the soil or water used in the washing of the leaves.

Keywords: Bacterial isolates; raffia baskets; antibiotic susceptibility; leafy vegetables; NSPRI.

1. INTRODUCTION

Vegetables generally are good sources of nutrients such as minerals, oil, vitamins and carbohydrates [1]. Fresh vegetables contribute immensely in human nutrition as sources of vitamins (A, C, B6, thiamin, niacin, and thiamin), minerals and dietary fiber. Vegetables lower the risk of diseases such as cancer because of its composition as it contains flavonoids, carotenoids, polyphenols and phytonutrients [2]. Vegetables exist as either leaves or fruits. Commonly consumed leafy vegetables in Nigeria include; *Vernomia anydalira* (Bitter leaf), *Talinum triangulare* (Water leaf), *Telfairia occidentalis* (Fluted pumpkin leaf), *Gnetum africana* (Okazi leaf), *Ocimum gratissimum* (scent leaf), *Piper guineense* (Uziza leaf) and *Gongronema latifolium* (Utazi) while fruity vegetables include carrots, tomatoes, ginger, garlic etc [3]. They are used in preparing different delicacies such as soups and salads. Other vegetables include those of the *Alliaceae* family such as shallots, leek, garlic and chives and the cruciferous vegetables that belong to the *Brassicaceae* family which include cabbage, cauliflower, kale and broccoli and those that belong to *Cucurbitaceae* family include squash, pumpkin, cucumber, melon and bitter gourd. They are all rich in carotenoids and tocopherols [4]. They are more nutritive compared to cereals because of the mineral content [5].

Vegetable production has been on-going in Nigeria for a long time, providing sources of income for a teaming population especially in the rural/peasant farming communities. All fresh vegetables, like most horticultural produce are high in water content and are subject to desiccation (wilting or shrinking) and mechanical injury. They are also prone to bacterial and fungal attack followed by pathological breakdown during harvesting and storage [6]. Physiological processes associated with vegetable spoilage and quality losses include respiration rate, ethylene production, rate of compositional changes (which cause changes in color, texture, flavours and nutritive values), mechanical injuries, water stress, sprouting and rooting [7]. The rate of biological deterioration in vegetables depend on several environmental factors such as temperature, relative humidity (RH), air velocity, atmospheric composition (oxygen, carbon dioxide and ethylene) as well as handling. These spoilage factors impact on vegetable at post-harvest or during storage when they are not protected or subjected to conditions that are not good enough for safety of the products.

Vegetables spoilage can result to changes or hydrolysis or decomposition of matrices by microorganisms resulting in unpleasant disorder of the vegetable due to toxic substances released in the vegetable material resulting in pigmentation, degeneration and discolouration of the vegetable materials. These physical abnormalities can make the vegetables inedible and unacceptable to the public or in some cases may pose a public health risk to consumers due to the high microbial load or pathogens associated with spoiled vegetables. In Nigeria, vegetables are chewed raw or heated lightly in order to retain its organoleptic properties. This has in most cases been responsible for food–borne infections [8], because in the process of harvesting, disease-causing microorganisms may contaminate these vegetables through contact with organic wastes such as faecal matter, organic manure, sewages, untreated irrigation water or surface waters used as nutrients or composts to improve soil quality [9].

The water sprinkled on the vegetables to keep them fresh may also be a source of microbial contamination [10] depending on the source of the water used. Antinutritional factors such as hydrogen cyanide, phytates and oxalates are able to influence the metabolic processes needed for the increase and bioavailability of nutrients in vegetables [11]. Most vegetables are used as nutrients in flavoring soups and sauces. Vegetables have pleasant aroma when added to soups and sauces, also contribute to the protein and essential minerals. Subjecting them to heat in the form of cooking in soups and sauces increases their digestibility. Their presence or use in foods preparation introduces a variety in the flavors obtained from the same foodstuff and enhances nutritional value of the food. Therefore, vegetables have great potential as key protein and mineral sources which are basic ingredients for food supplementation.

In most rural areas and some urban centers, leafy vegetables are stored in covered baskets made with bamboo sticks and wrapped with jute bags. Most times the vegetables are exposed to dews overnight for preservation and when they are left exposed for long time, spoilage occurs. The wetting of the basket and evaporation from the basket gives a cooling effect to the inside of the basket making it conducive for survival of the leaves for a longer period of time compared with when they are left in the open [12]. This study therefore aims to determine the bacterial characteristics and antibiotic susceptibility of associated isolates from some selected leafy vegetables stored in raffia basket as a means of preservation. This artisanal method using rudimentary materials will enable consumers and farmers preserve their vegetables in local communities to avoid post- harvest losses.

2. MATERIALS AND METHODS

2.1 Collection of Vegetable

Five (5) leafy vegetables which include; *Vernomia anydalira* (Bitter leaf), *Talinum triangulare* (Water leaf), *Telfairia occidentalis* (Fluted pumpkin leaf), *Gnetum africana* (Okazi leaf), *Ocimum gratissimum* (scent leaf) were obtained from the Nigerian Stored Product Research Institute (NSPRI) farm in Port Harcourt. The vegetables were transported to the Microbiology laboratory for analyses.

2.2 Preparation of Vegetable Samples

The vegetables were prepared and preserved in raffia baskets; the baskets were sprinkled with about one liter of sterile water with the aid of a water sprinkler after 48 hours constantly until spoilage occured. This was done to keep the inner part cold without the vegetables being wet. This provides a humid environment for the leaves keeping them fresh for longer than when left in the open. The setups are illustrated below in Plates 1-15.

2.3 Microbiological Analysis

Ten grams (10 g) of each vegetable leaf was homogenized in 90 ml of sterilized normal saline solution in 200 ml conical flasks, after which the homogenized samples were diluted serially to achieve the 10^{-5} dilution [13].

2.4 Inoculation and Enumeration of Colonies on Plates

Aliquots (0.1 ml) of appropriate dilutions were transferred to plates of surface dried Nutrient agar (NA) media in duplicates and inoculated by spreading with flamed glass spreader and incubated at 37ºC for 24 hours. After incubation, colonies that appeared on the plates were counted and the mean expressed as cfu/g (or as $\log_{10}$/g) for the samples. This was carried out on each of the vegetables at two days interval until spoilage occurred. Counts for each sample were then calculated using the formula below [14].

THC (cfu/g) = Number of Colonies /Dilution (10-6 x Volume plated (0.1 ml)

2.5 Maintenance of Pure Cultures

Discrete bacterial colonies that developed on the Nutrient agar plates were sub-cultured using streaked plate method by transferring each colony of same morphology onto freshly prepared NA plates under strict aseptic conditions. This was incubated at 37ºC for 24 hours. The bacterial cultures were then maintained according to the method reported by Amadi, et al. [15] using 10% (v/v) glycerol suspension at -4ºC.

Plate 1. Day 1 bitter leaf

Plate 2. Day 3 bitter leaf

Plate 3. Day 6 bitter leaf (Spoiled)

Plate 4. Day 1 Stored pumpkin leaf

Plate 5. Day 3 Stored pumpkin leaf

Plate 6. Day 7 stored pumpkin leaf

Plate 7. Day 1 stored scent leaf

Plate 8. Day 3 stored scent leaf

Plate 9. Day 7 stored scent leaf

Plate 10. Day 1 stored okazi leaf

Plate 11. Day 3 stored okazi leaf

Plate 12. Day 7 stored okazi leaf

Plate 13. Day 1 stored water leaf

Plate 14. Day 3 stored water leaf

Plate 15. Day 7 stored water leaf

2.6 Biochemical Characterization and Identification of Isolates

The isolates were characterized based on their appearance on the culture media which include; shape, colour, wetness, and Gram's reaction. Biochemical tests such as; Indole production, motility, coagulase, Methyl Red, citrate utilization, sugar fermentation (sucrose, glucose, lactose, and maltose) were carried out based on the method of Cheesbrough [16]. To further confirm the bacterial isolates, molecular identification techniques were carried out. The molecular techniques were carried out as described by Wemedo and Robinson [17].

2.7 Preparation of Standard Bacterial Inoculum

Loop- full of 24-hour old pure culture of the test organisms from nutrient agar plates, were transferred into sterile diluents tubes and adjusted to 0.5 McFarland Turbidity Standard [16,17]. This was used as the standard inoculums.

2.8 Antimicrobial Susceptibility Testing

The antibiotic sensitivity of the bacterial isolates was determined using the disc diffusion method [18]. Multiple antibiotic sensitivity testing was also carried out using the method adopted by Ogbonna and Inana [19]. Standardized inoculums of 24-hour old cultures were spread on Mueller-Hinton agar plates using sterile swab sticks. The plates were dried at room temperature for 1 hour before placing the antibiotic discs at the centre. The plates were then incubated at 37ºC for 24 hours and the diameter of zone of inhibition was measured in millimetre. Organisms were classified as sensitive, intermediate or resistant, based on the Clinical and Laboratory Standard Institutes.

3. RESULTS AND DISCUSSION

3.1 Bacterial Counts

The results of total heterotrophic bacterial counts of the stored vegetable leaves from day one to the day that spoilage occurred are presented in Table 1. The result shows that water leaf had the highest bacterial load followed by pumpkin leaf, Okazi Leaf while bitter leaf had the lowest bacterial counts after sixteen days of storage. The total heterotrophic bacterial counts from day 1 to the day spoilage occurred in the five selected vegetable ranged from 1.8 $x10^6$ cfu/g to 1.25 $x10^7$ cfu/g (bitter leaf), 9 $x10^5$ cfu/g to 9.0 $x10^6$ cfu/g (Scent leaf), 7 $x10^5$ cfu/g to 1.88 $x10^7$ cfu/g (Okazi), 3.2 $x10^6$ cfu/g to 1.05 $x10^7$ cfu/g (Pumpkin leaf) and 8.4 $x10^6$ cfu/g to 2.04 $x10^7$ cfu/g (water leaf). The bacterial counts increased as the spoilage occurred with increase in the number of days respectively. For example, the highest microbial counts for pumpkin leaf was observed on day five which was the day spoilage began while for scent leaf spoilage occurred on day 7, bitter leaf was on day 5 while water leaf spoiled on day 5 and Okazi spoiled on day 12 (Figs. 1-5) respectively.

The results of total heterotrophic bacterial counts obtained in this study are in agreement with the counts reported by Pradnya and Patel [7]. The results obtained show that the bacterial counts increased as the storage in the baskets was extended to the day spoilage occurred. It was also observed that the control samples had a higher bacterial load and lasted for few days before spoilage occurred compared to the vegetables stored in the vegetable basket. The storage in this circumstance depends on chance inoculation from the environment. The storage conditions are inconsistent and were not optimized so the bacterial isolates could be distinguished as contaminants and could cause spoilage within a short period as against the vegetables preserved in a raffia basket [20].

The predominant bacterial genera identified were *Bacillus, Pseudomonas, Enterococcus, Enterobacter, Bordetella, Staphylococcus, Myroides, Escherichia, Serratia, Micrococcus and Acetobacter* (Table 2). Also, *Bacillus* sp occurred in all the vegetables during the period of the study. This organism is a normal flora of the soil environment, with the potential to remain in the environment for a longer time because of its ability to produce endospores [21]. However, *Enterococcus faecalis, Acetobacter orientalis, Bordetalla pertussis, Myroides xuawensis* and *Bacillus flexu*s were isolated in the stored vegetables from the third day, in most of the vegetables. The molecular characterization of the bacterial isolates showed that they have relatedness to the bacterial presented in the phylogenetic tree (Fig 6). This result is in agreement with the report of Mandrell, et al. [22] who reported similar bacterial isolates in their research. Bacteria, belonging to the genus *Bacillus* are spore formers and often heat-resistant. They are able to cause opportunistic infections such as endocarditis, bacteremia, wound abscesses etc [23,24]. *Myroides* are able to cause skin infections in patients with diabetes as well as urinary tract infections [25]. *Bordetella pertussis* is the causative agent of whooping cough while *Enterobacter* causes meningitis, pneumonia, bacteremia as well as urinary tract infection. *Enterococcus faecalis* are emerging as hospital pathogens and are mostly found in the gut. They are able to produce a cytolysin (streptolysin) that affects the gut as well as has been implicated in multi-drug resistance [26]. Hence it is important to improve the quality and processing by optimizing the process conditions or even manipulating process factors such as temperature, humidity, aeration and pH [20]. Against the background of the significance and benefits of this process in this study, it is desirable to improve the art of the storage conditions to improve the shelf life of vegetables. As of now, the process is still largely a traditional art and the characteristic features of preservation is lacking. This practice predisposes the process of preservation to contamination because the raffia baskets have tendencies to accumulate dust, although presumably provides warmth and humid environment.

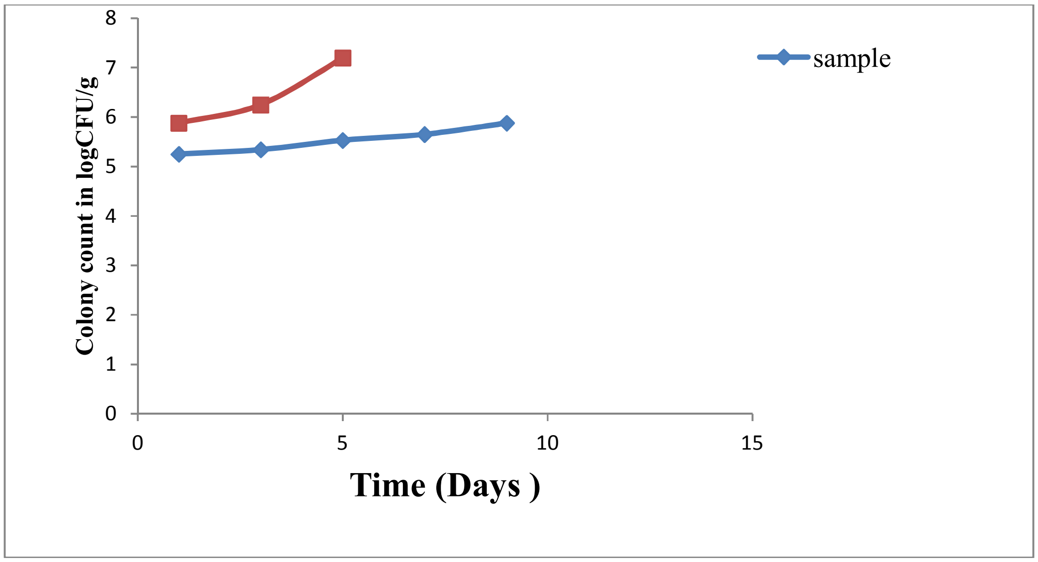

Fig. 1. Bacterial counts and shelf life for pumpkin leaf (*Teiferia occidentali*s)

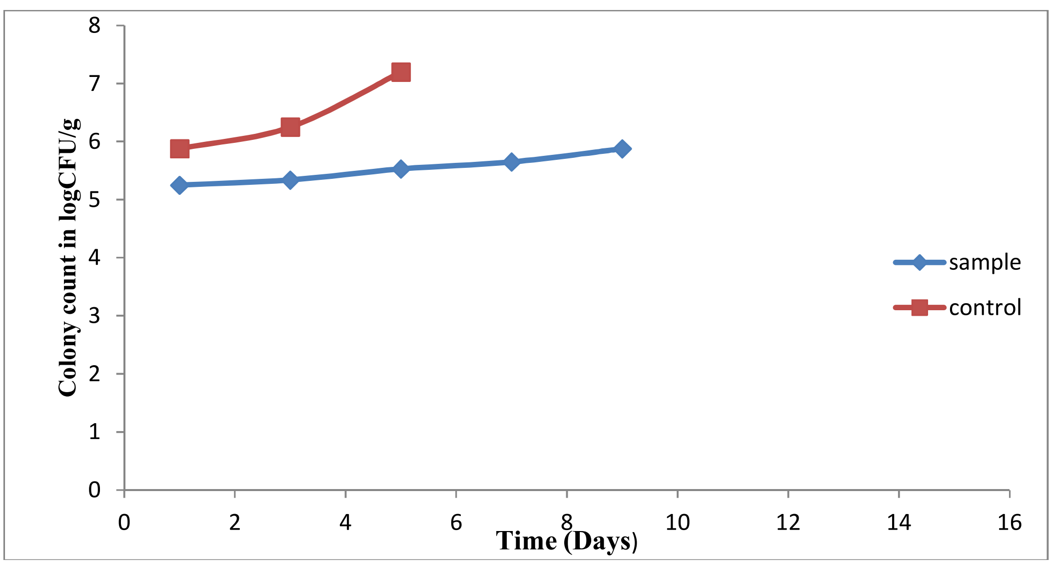

Fig. 2. Bacterial counts and shelf life for scent (*Ocimum gratissimum*)

Table 1. Total Heterotrophic Bacterial counts of the Vegetables (cfu/g)

	Pumkin leaf		Scent leaf		Water leaf		Okazi leaf		Bitter leaf	
	Sample	**control**	**Sample**	**control**	**Sample**	**control**	**Sample**	**Control**	**sample**	**control**
Day1	2.34×10^{6}	2.45×10^{6}	1.78×10^{5}	7.59×10^{5}	1.55×10^{6}	2.19×10^{5}	2.04×10^{6}	2.34×10^{6}	6.03×10^{5}	7.94×10^{5}
Day3	4.36×10^{6}	4.36×10^{6}	2.19×10^{5}	1.78×10^{6}	1.48×10^{7}	3.72×10^{8}	1.74×10^{6}	4.37×10^{6}	3.98×10^{5}	1.48×10^{6}
Day5	5.25×10^{6}	1.26×10^{8}	3.39×10^{5}	1.58×10^{7}	1.82×10^{7}	Sp	1.55×10^{6}	5.25×10^{7}	3.47×10^{6}	1.82×10^{7}
Day7	8.32×10^{6}	Sp	4.47×10^{5}	Sp	7.94×10^{7}	Sp	2.24×10^{6}	8.32×10^{7}	2.88×10^{6}	7.94×10^{7}
Day9	Sp	Sp	7.59×10^{5}	Sp	Sp	Sp	8.32×10^{6}	2.34×10^{8}	1.48×10^{7}	sp
Day12	Sp	Sp	Sp	Sp	Sp	Sp	1.38×10^{7}	Sp	4.37×10^{7}	sp
Day14	Sp	Sp	Sp	Sp	Sp	Sp	2.82×10^{7}	Sp	sp	sp
Day 16	Sp	Sp	Sp	Sp	Sp	Sp	sp	Sp	sp	sp

sp- spoilt

Table 2. Biochemical characteristics of isolates

					Tests							**Sugar fermentation**				
Colonial/cell characteristics	**Gram reaction**	**Coagulase**	**Catalase**	**Oxidase**	**Indole**	**MR**	**VP**	**Citrate**	**Motility**	**Sucrose**	**Mannitol**	**Lactose**	**Glucose**	**Galatose**	**Fructose**	**Probable organism**
Creamy, Round, Opaque, Entire Small, Elevated, Moist, rods	+ve	-	+	-	-	+	-	+	-	+	-	+	+	-	-	*Bacillus* sp.
Creamy, Circular, Flat, Opaque, Entire, Small, moist, rods	-ve	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*Acetobacter* sp.
Creamy, Circular, Flat, Opaque, Entire, Moist, rods	-ve	-	-	+	+	-	+	-	+	-	-	+	-	-	+	*Enterobacter* sp.
Greenish yellow, Smooth rods	-ve	-	+	+	-	-	-	+	+	-	+	-	+	-	+	*Pseudomonas* sp.
Red, Round, flat, Mucoid, Large, Translucent, Serrated rods	-ve	-	+	-	-	-	+	+	+	+	+	-	+	-	-	*Serratia* sp.
Golden yellow , Raised, Translucent, Moist cocci	+ve	-	+	-	-	+	+	+	-	+	+	+	+	+	+	*Staphylococcus* sp.
Smooth, Creamy, Raised, Translucent rods	-ve	-	+	-	+	+	-	-	+	+	+	+	+	+	-	*Escherichia coli*
Yellow, Round, Opaque-, Small	+ve	-	+	-	-	+	+	-	-	+	+	+	-	+	+	*Micrococcus* sp.
yellow, Round, Smooth rods	-ve	-	+	+	-	-	+	+	-		-	-		-	-	*Myroides* sp.
Small, Rough, Flat,rods	-ve	-	+	+				-	-							*Bordetella* sp.
Smooth, Creamy, Entire,cocci	+ve	-	-	-	-	-	+	-	-	+	+	+	+		+	*Enterococcus* sp.

Key: -ve = Gram negative, +ve =Gram positive, - = Negative, + = Positive

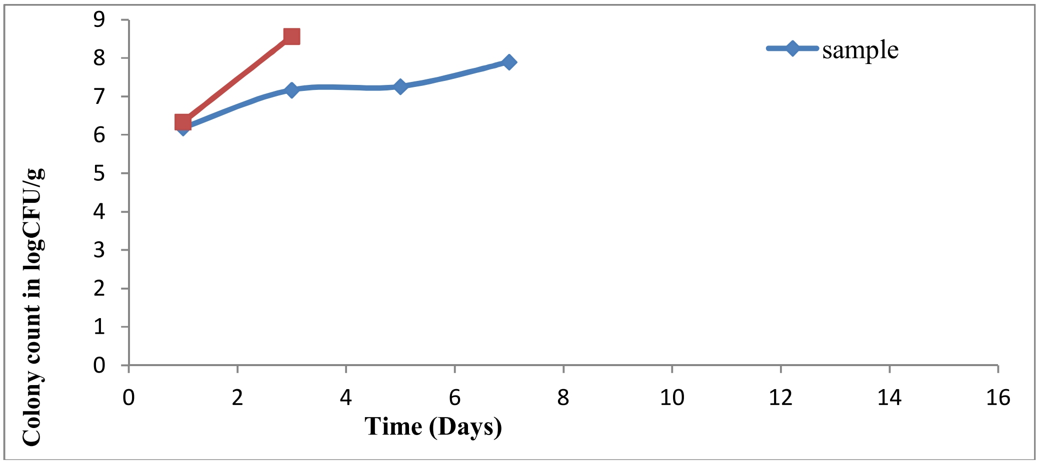

Fig. 3. Bacterial counts and shelf life for Waterleaf (*Talinum triangulare*)

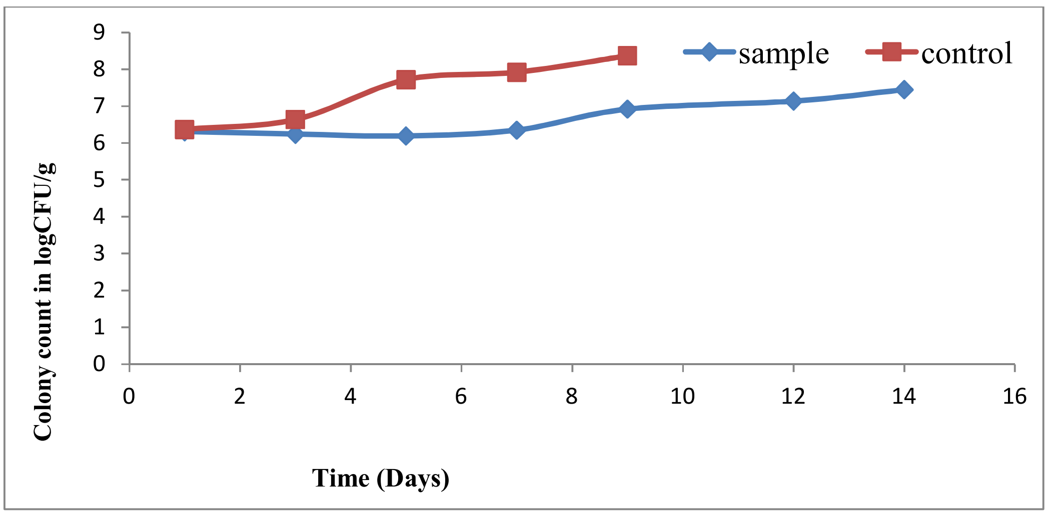

Fig. 4. Bacterial counts and shelf life for Okazi (*Gnetum africanum*)

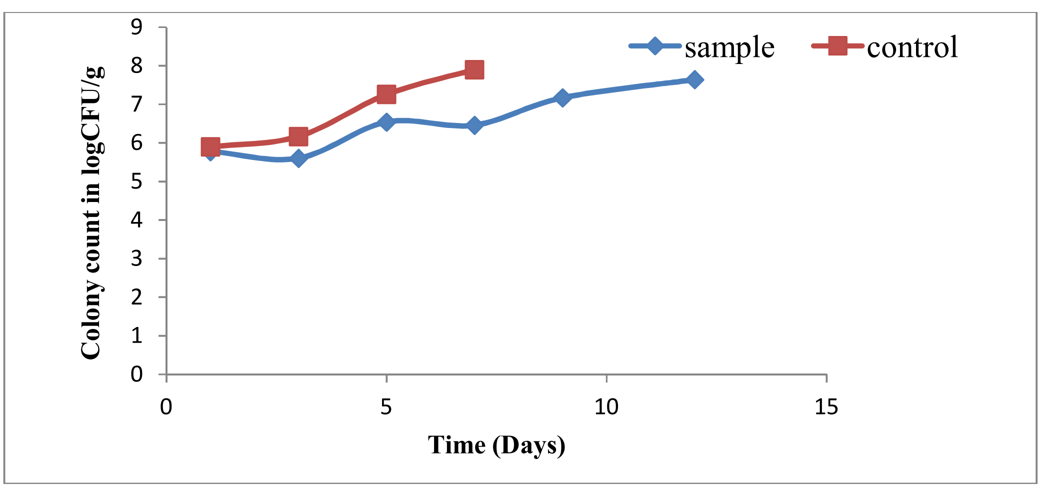

Fig. 5. Bacterial counts and shelf life for Bitterleaf (*Veronia amygdalina*)

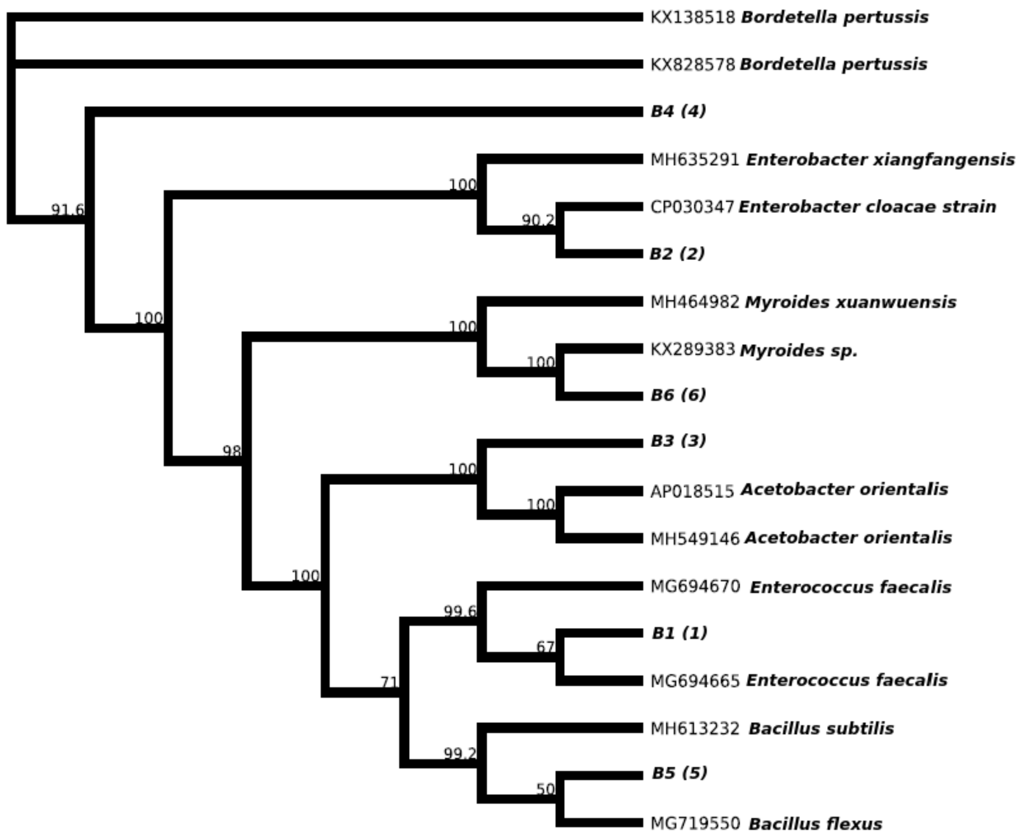

Fig. 6. Phylogenetic tree showing the evolutionary distance between the bacterial Isolates

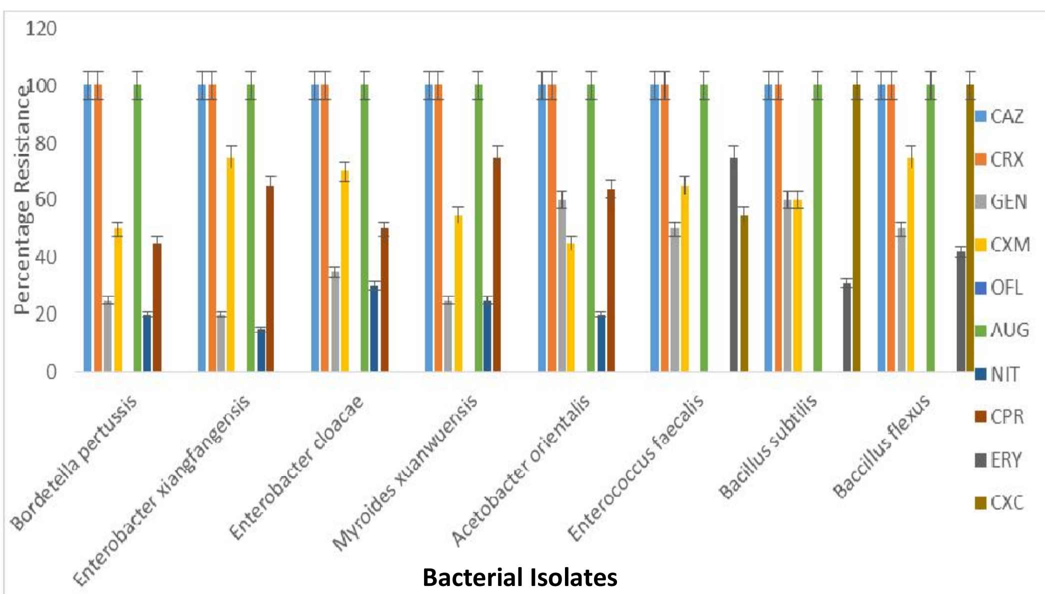

Fig. 7. Percentage resistance of the isolates to different antibiotics

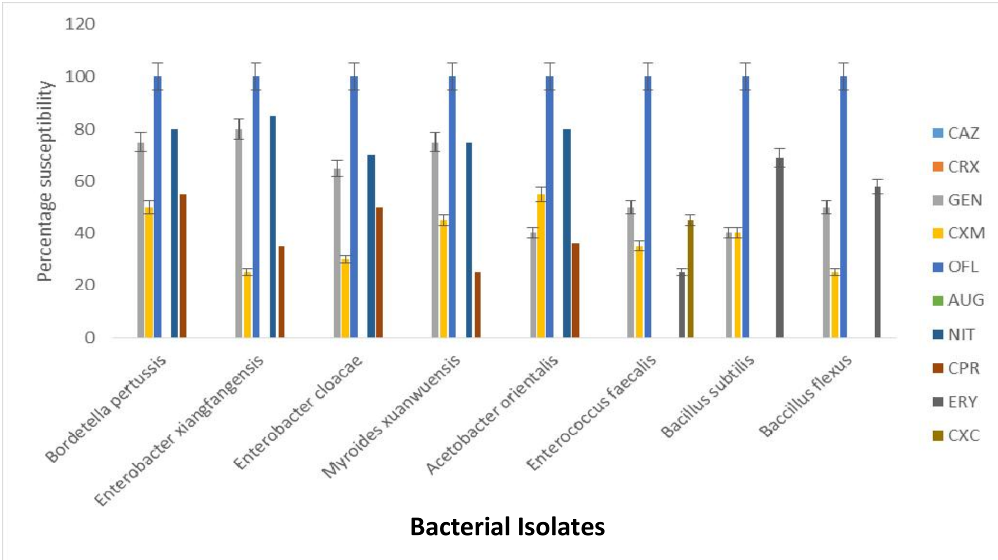

Fig. 8. Percentage susceptibility of the isolates to different antibiotics

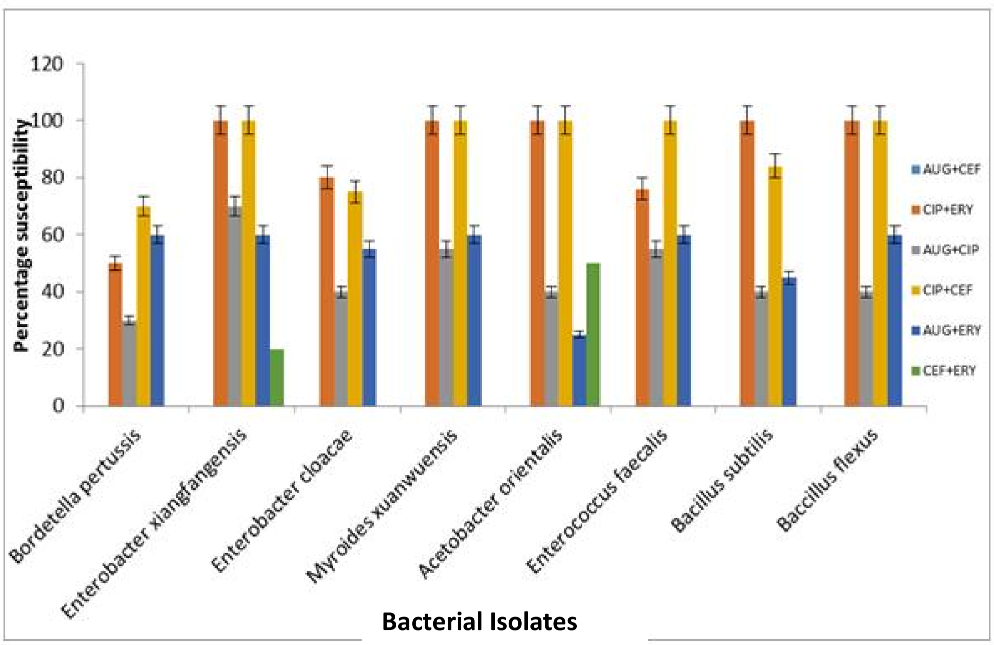

Fig. 9. Combined Antibiotic Sensitivity pattern of Isolates from the five selected Vegetables

All the bacterial species were susceptible to Ofloxacin, Ciprofloxacin + Erythromycin, Augmentin + Ciprofloxacin, and Ciprofloxacin+ Ceftriaxone while all were resistant to Augmentin and Ceftazidime. The antibiotic profile of the isolates and identified bacteria revealed that ciprofloxacin, ofloxacin, were more susceptible, hence, they were considered as drugs of choice for infections caused by bacteria isolated in this study. Comparatively, the results of antibiotic profile tests against the isolates and bacteria reported in this study is in agreement with observations by Wemedo and Robinson [17] who reported the resistance of some bacterial isolates against Augmentin. The resistance of the microbes to Augmentin and Ceftazidime may be due to a regular abuse of the drugs against the isolates *In vitro*.

Table 3. Bacterial succession during the period of storage in raffia baskets

Sample	Day 1	Day 3 to Day 15.
Pumpkin	*Bacillus* sp. *Micrococcus sp* *Escherichia coli*	*Acetobacter orientalis* *Bordetella pertussis* *Staphylococcus sp*
Water leaf	*Bacillus* spp. *Micrococcus* sp *Escherichia coli*	*Myroides xuanwuensis* *Enterobacter xiangfangensis*
Scent leaf	*Bacillus* sp *Micrococcus* sp	*Enterococcus faecalis* *Escherichia coli*
Okazi leaf	*Bacillus* sp *Pseudomonas* sp	*Myroides* sp *Bacillus flexus* *Bacillus subtilis* *Serratia* sp.
Bitter leaf	*Bacillus* sp *Staphylococcus* sp	*Pseudomonas* sp *Micrococcus* sp *Enterococcus faecalis*

4. CONCLUSION

The results of the bacterial counts obtained in this study revealed that, bacterial load of vegetables preserved in the Raffia baskets were lower than those kept in an open air and as spoilage began to occur, more bacteria were isolated and the bacterial load increased with increase in storage time. It was also observed that, the control samples had a higher bacterial load and lasted for fewer days before spoilage occurred compared with the vegetables stored in the vegetable basket that had fewer organisms and lasted longer. The antibiogram of the bacterial isolates revealed that all the bacterial species were susceptible to Ofloxacin, Ciprofloxacin + Erythromycin, Augmentin + Ciprofloxacin, and Ciprofloxacin+ Ceftriaxone while all were resistant to Augmentin and Ceftazidime.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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