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Microbial composition of guava (*Psidium guajava*), hibiscus (*Hibiscus-rosa sinensis*), mango (*Mangifera indica*) and pumpkin (*Telfairia occidentalis* Hook) phyllosphere

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Diverse groups of microorganisms colonize phyllosphere and carry out different but specific ecological functions. The phyllosphere of four different plants; guava (*Psidium guajava*), hibiscus (*Hibiscus-rosa sinensis*), mango (*Mangifera indica*) and pumpkin (*Telfairia occidentalis Hook*), each from four different sites Aluu Community Health Centre, Lulu Briggs Health Centre, University of Port Harcourt Abuja campus, and University of Port Harcourt Teaching Hospital were examined for bacterial and fungal growth using culture-dependent technique. A total of 32 bacterial and 13 fungal species covering seven and five genera respectively were isolated and characterized as *Bacillus*, *Enterobacter, Escherichia coli, Pseudomonas, Salmonella, Shigella* and *Staphylococcus; Aspergillus*, *Fusarium*, *Mucor*, *Penicillum* and *Rhizopus*. Among the epiphytes, bacteria were found to predominate. Statistically, plant type and sampling location were found to extremely influence observed microbial composition associated with each plant and site at P<0.0001. The microbial genera isolated from this study showed that, both human and plant pathogens can colonize plants' phyllosphere. Since most of the edible leafy vegetables have less waxy phyllosphere which permit microbial growth, it is necessary that they be washed and cooked properly before consumption to avoid ingestion of possible food pathogens that cause food-borne diseases.

Key words: Epiphytes, location, pathogens, plant type.

INTRODUCTION

Microorganisms are known to be ubiquitous, their growth and survival depend on complex sets of environmental and nutritional factors. The ubiquity of microorganisms is supported by Baas-Becking hypothesis that: "everything is everywhere but the environment selects" (de Wit and Bouvier, 2006). With reference to plants, there are three major microhabitats where microorganisms can inhabit depending on the one that most supports their growth (Montesino, 2003). These microhabitats are the aerial plant part (the phyllosphere), the zone of influence of root system (the rhizosphere) and the internal transport system (the endosphere). Microorganisms which inhabit

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such microhabitats are called epiphytes, rhizophytes and endophytes respectively (Montesino, 2003). Phyllosphere therefore is a microhabitat on the surface of plant's leaf where diverse group of microorganisms colonize and carry out their different and specific ecological function. The diversity of the microbial composition of phyllosphere include algae, bacteria, filamentous fungi, yeast and in rare cases nematodes and protozoans (Morris et al., 2002; Lindow and Brandl, 2003) with bacteria being the most abundant among the diverse groups of phyllosphere microorganisms (Inacio et al., 2002; Mukhtar et al., 2010). There are several sources to which microbial colonization of phyllosphere can be traced and they include animals, rain drops, wind, and other agents of cross and self pollination.

Suslow (2002), classified phyllosphere microorganisms into two major groups, transient epiphytes which show no limitation to multiplication and residual epiphytes which have the capacity to multiply in the absence of wounds. Microorganisms on the phyllosphere may occur individually but usually they occur as aggregate or biofilm-like structures containing bacteria, yeast and filamentous fungi (Bélanger and Avis, 2000; Jacques et al., 2005). Bacteria and fungi are known to be friends and foes of human, their specific role as friends on phyllosphere has little effect compared to the foes activities which they may impact on human upon consumption.

The study was aimed at determining the different bacteria and fungi associated with some plants' phyllosphere using culture-dependent technique and to compare the effect of sample sites on the extent of phyllosphere colonization by epiphytes.

MATERIALS AND METHODS

Sampling

The phyllosphere of four different plants namely guava (*Psidium guajava*), hibiscus (*Hibiscus-rosa sinensis*), mango (*Mangifera indica*) and pumpkin (*Telfairia occidentalis Hook*) each from four different sites, University of Port Harcourt Teaching Hospital (T), University of Port Harcourt Health Centre Lulu Briggs (L), Aluu Community Health Centre (A) and University of Port Harcourt Abuja campus (U). The plants' leaves used for the study were identified by Mrs. Josephine Agogbua at the Department of Plant Science and Biotechnology Herbarium, University of Port Harcourt, Nigeria. The leaves surfaces were aseptically swabbed. After the collection of the samples, 0.5 ml of diluent (phosphate buffer saline) was added to the swab container and transported in sterile polyethylene bag to the Microbiology Laboratory, Department of Microbiology, University of Port Harcourt where bacteriological and mycological analyses were carried out. In other to ensure the isolation of autochthonous epiphytes, the samples were collected in the morning three days after the last rain fall (Yashiro et al., 2011).

Isolation of bacteria and fungi

Appropriate aliquots of 5-fold serially diluted samples were aseptically transferred onto MacConkey agar, Mannitol salt agar, plate count agar and Salmonella-shigella agar plates (Yadav et al., 2010; Yashiro et al., 2011). To obtain discrete colonies and even distribution of colonies on the agar plates, the primary inocula were streaked in three directions after which the inoculated plates for bacterial isolation were incubated at 37°C for 48 h. Subculturing of the isolates was done to obtain pure colonies for biochemical tests. Discrete colonies of the bacteria to be identified were aseptically subcultured on nutrient agar medium. The various isolates were differentiated by their colonial morphologies (colour, edge, elevation, shape, size and texture) on the respective media after 24 h of incubation following the subculture. A tentative identification of bacterial isolates was made based on the results of various biochemical tests carried out (Cheesbrough, 2006). Fungal selective medium (Sabouraud dextrose agar, SDA) was prepared and supplemented with a known concentration of 0.05% (v/v) of chloramphenicol for fungal isolation. Following this, 1.0ml of the serially diluted samples were aseptically transferred onto the freshly prepared SDA, the plates were later incubated at 30°C for 7 days (Yadav et al., 2010; Yashiro et al*.*, 2011). Fungal identification was done under microscope using fungal atlas after KOH mount and lactophenol cotton blue staining (Malloch, 1997).

Statistical analysis

In order to determine the effects of sampling site and type of plant phyllosphere on the bacterial diversity observed, the data (frequency of isolation of plant-associated microbes) were subjected to statistical analysis using 2-way ANOVA and Bonferroni's multiple comparison test using GraphPad Prism software for Windows version 5.01.

RESULTS

Bacterial isolates from the phyllosphere samples

Culture-dependent technique was employed to study the diversity of microbial communities on the phyllosphere of four selected plants: *Hibiscus-rosa sinensis, Mangifera indica, Psidium guajava* and *Telfairia occidentalis* Hook. A total of 32 bacterial isolates covering seven genera were obtained and characterized as *Bacillus*, *Enterobacter, Escherichia, Pseudomonas, Salmonella, Shigella,* and *Staphylococcus.* It was observed that more bacteria (45.45%) were isolated from the phyllosphere of mango than other phyllosphere from the University of Port Harcourt. Also, no bacterium was isolated from the phyllosphere of guava from the same sample site (Table 1). More bacterial isolates (50%) were obtained from the phyllosphere of pumpkin from the University of Port Harcourt Teaching Hospital. In this site also, no bacterium was isolated from the phyllosphere of mango (Table 1). The bacteria isolated from the phyllosphere of pumpkin in Aluu Health Centre and Lulu Briggs Health Centre (50% and 42.89% respectively) predominated over the phyllosphere of other plants in both sites (Table 1). It was also observed that pumpkin had the highest frequency (43.75%) of bacterial isolates from the phyllosphere of the sampled plants, followed by hibiscus (25%), mango (18.75%) and guava (12.50%) (Figure 1). There was an even distribution of bacteria isolated from the phyllosphere of pumpkin and hibiscus, this contributed to them having the highest frequency of bacterial isolates,

Table 1. Bacterial isolates from different samples and sites.

Isolate code	Shape	Gram reaction	Oxidase	Indole	MR	VP	Citrate	Motility	Catalase	Slant	Butt	H_2S	Gas	Glucose	Lactose	Tentative Identity
UM1	R	-	+	-	-	-	+	+	+	A	A	-	+	+	+	*Pseudomonas* sp.
UM2	R	+	-	+	-	+	+	-	+	B	A	+	+	+	-	*Bacillus* sp.
UM3	R	-	-	+	+	-	+	+	+	B	A	+	+	+	+	*Salmonella* sp.
UM4	R	-	-	+	+	-	-	+	+	A	A	-	+	+	+	*Escherichia* sp.
UM5	C	+	-	-	-	-	+	-	+	A	A	-	-	+	+	*Staphylococcus* sp.
UH1	R	-	+	-	-	-	+	+	+	A	A	-	+	+	+	*Pseudomonas* sp.
UH2	C	+	-	-	-	-	+	-	+	A	A	-	-	+	+	*Staphylococcus* sp.
UP1	R	-	+	-	-	-	+	+	+	A	A	-	+	+	+	*Pseudomonas* sp.
UP2	R	+	-	+	-	+	+	-	+	B	A	+	+	+	-	*Bacillus* sp.
UP3	R	-	-	+	+	-	+	+	+	B	A	+	+	+	+	*Salmonella* sp.
UP4	R	-	-	+	+	-	-	+	+	A	A	-	+	+	+	*Escherichia* sp.
TGI	R	-	+	-	-	-	+	+	+	A	A	-	+	+	+	*Pseudomonas* sp.
TH1	R	-	+	-	-	-	+	+	+	A	A	-	+	+	+	*Pseudomonas* sp.
TH2	R	+	-	+	-	+	+	-	+	B	A	+	+	+	-	*Bacillus* sp.
TH3	C	+	-	-	-	-	+	-	+	A	A	-	-	+	+	*Staphylococcus* sp.
TP1	R	-	+	-	-	-	+	+	+	A	A	-	+	+	+	*Pseudomonas* sp.
TP2	R	-	-	-	+	-	-	-	-	B	A	-	-	+	-	*Shigella* sp.
TP3	R	-	-	-	-	+	+	+	+	A	A	-	+	+	+	*Enterobacter* sp.
TP4	C	+	-	-	-	-	+	-	+	A	A	-	-	+	+	*Staphylococcus* sp.
AG1	R	-	+	-	-	-	+	+	+	A	A	-	+	+	+	*Pseudomonas* sp.
AG2	R	-	-	+	+	-	-	+	+	A	A	-	+	+	+	*Escherichia* sp.
AH1	R	-	-	+	+	-	-	+	+	A	A	-	+	+	+	*Escherichia* sp.
AP1	R	+	-	+	-	+	+	-	+	B	A	+	+	+	-	*Bacillus* sp.
AP2	R	-	-	-	+	-	-	-	-	B	A	-	-	+	-	*Shigella* sp.
AP3	R	-	-	-	-	+	+	+	+	A	A	-	+	+	+	*Enterobacter* sp.
LM1	R	-	+	-	-	-	+	+	+	A	A	-	+	+	+	*Pseudomonas* sp.
LG1	R	-	+	-	-	-	+	+	+	A	A	-	+	+	+	*Pseudomonas* sp.
LH1	R	-	-	+	+	-	-	+	+	A	A	-	+	+	+	*Escherichia* sp.
LH2	C	+	-	-	-	-	+	-	+	A	A	-	-	+	+	*Staphylococcus* sp.
LP1	R	-	-	+	+	-	+	+	+	B	A	+	+	+	+	*Salmonella* sp.
LP2	R	-	-	-	+	-	-	-	-	B	A	-	-	+	-	*Shigella* sp.
LP3	R	-	-	+	+	-	-	+	+	A	A	-	+	+	+	*Escherichia* sp.

Key: positive (+); negative (-); rod (R); cocci (C); acid (A); alkaline (B). Isolate code: the first letters represent the samples sites (A, L, T, and U), the second letters represent the samples guava (G), hibiscus (H), mango (M) and pumpkin (P), the Arabic numerals represent the isolate number.

unlike the phyllosphere of mango and guava from the University Teaching Hospital and University campus sites in which no bacterium was isolated (Table 1).

Pseudomonas sp. (28.13%) and *Escherichia* sp. (18.18%) were found to be the predominant bacteria among the isolates, while *Enterobacter* sp. (6.25%) was found to have the least frequency of occurrence among the bacteria isolated (Figure 2). From the foregoing, it was observed that Gram negative bacteria (71.88%) predominated over Gram positive bacteria (28.13%). Sampling location significantly influenced the types of bacterial species while the type of plant phyllosphere sampled was an extremely significant factor that also selected specific bacteria found in such plants at P<0.0001. Multiple comparison tests using Bonferroni indicated that plants phyllosphere sampled in University of Port Harcourt Abuja campus when compared with other locations showed very significant bacterial diversity

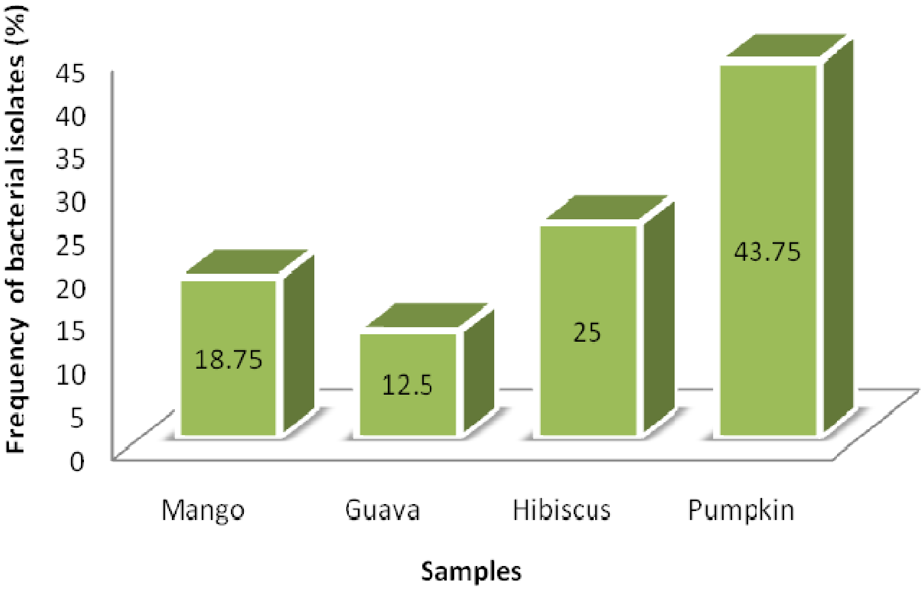

Figure 1. Frequency of bacterial isolates from different phyllosphere samples.

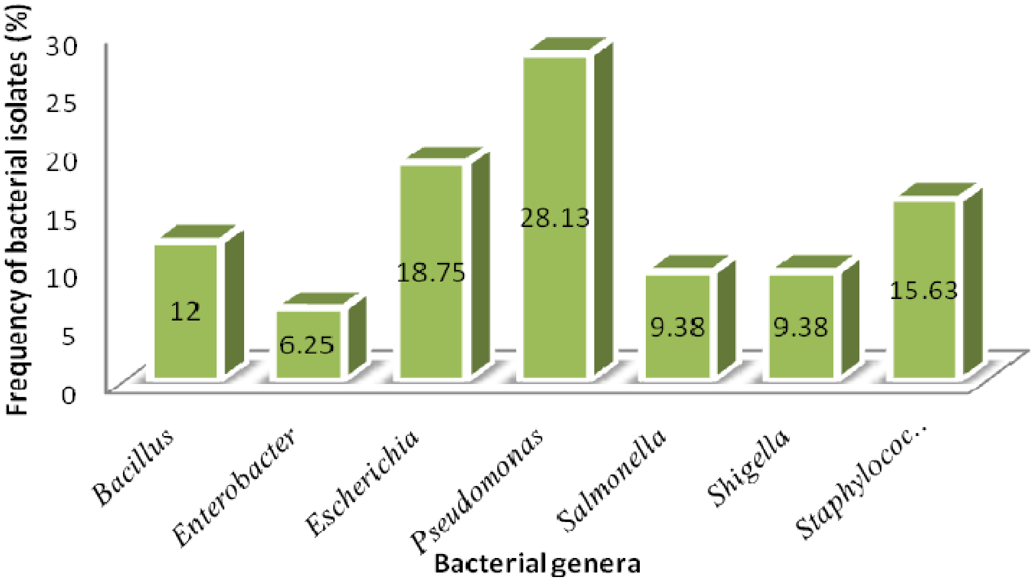

Figure 2. Frequency of bacterial genera from all the phyllosphere samples collected from the four sampled sites.

at $P<0.001$.

Fungal isolates from the phyllosphere samples

Thirteen fungal species covering five genera were also isolated and characterized as *Aspergillus* (30.77%), *Fusarium* (7.69%), *Mucor* (30.77%), *Penicillum* (15.38%) and *Rhizopus* (15.38%). It was also observed that *Aspergillus* and *Mucor* were the predominant fungi from the phyllosphere of the four different plants (Table 2 and Figure 4). Fungal spp. were isolated from all the phyllosphere of pumpkin in the different sampled sites (Table 2) but no fungus was isolated from the phyllosphere of hibiscus for all the sampled sites. Also, no fungal isolate was obtained from the phyllosphere of mango and guava from Teaching Hospital and Lulu Briggs Health Centre sites (Table 2 and Figure 3).

Table 2. Fungal isolates from different samples and sites.

Isolate code	Colony characteristics	Morphological characteristics	Tentative identity
Um1	yellow-brown colonies	Double spores forming entire vesicle and radiate head at the end of a long erect conidiophores	*Aspergillus* sp.
Um2	Green colonies	Coenocytic mass of mass branching hyphal growth with conidia lined at the tips of each branch of hyphae	*Penicillium* sp.
Ug1	White-grey	Long conidiophores having single and double spores that cover the entire vesicle.	*Mucor* sp.
Up1	White-brown	Long conidiophores having single double spore that cover the entire	*Aspergillus* sp.
Up2	Grey colonies	Single-celled conidia. Coenocytic mass of simple branching hyphal growth with condia lined at the tip of each branch of hyphae.	*Rhizopus* sp.
Tp1	White-brown	Non septate hyphae with branched long sporangiophores	*Mucor* sp
Tp2	Pink –orange	Macroconidia and microconidia with two hyaline each	*Fusarium* sp.
Am1	White-brown	Long conidiophores having single double spore which cover the entire vesicle	*Aspergillus* sp.
Ap1	yellow-brown colonies	Double spores forming entire vesicle and radiate head at the end of a long erect conidiophores	*Aspergillus* sp.
Ap2	Green colonies	Coenocytic mass of mass branching hyphal growth with conidia lined at the tips of each branch of hyphae	*Penicillium* sp.
Ap3	White-brown	Non septate hyphae with brached long sporangiophores	*Mucor* sp
Lp1	Grey colonies	Single-celled conidia. Coenocytic mass of simple branching hyphal growth with condia lined at the tip of each branch of hyphae.	*Rhizopus* sp.
Lp2	White-grey	Long conidiophores having single and double spores which cover the entire vesicle.	*Mucor* sp.

Isolate code: The first letters represent the samples sites (A, L, T, and U), the second letters represent the samples guava (G), hibiscus (H), mango (M) and pumpkin (P), the Arabic numerals represent the isolate number.

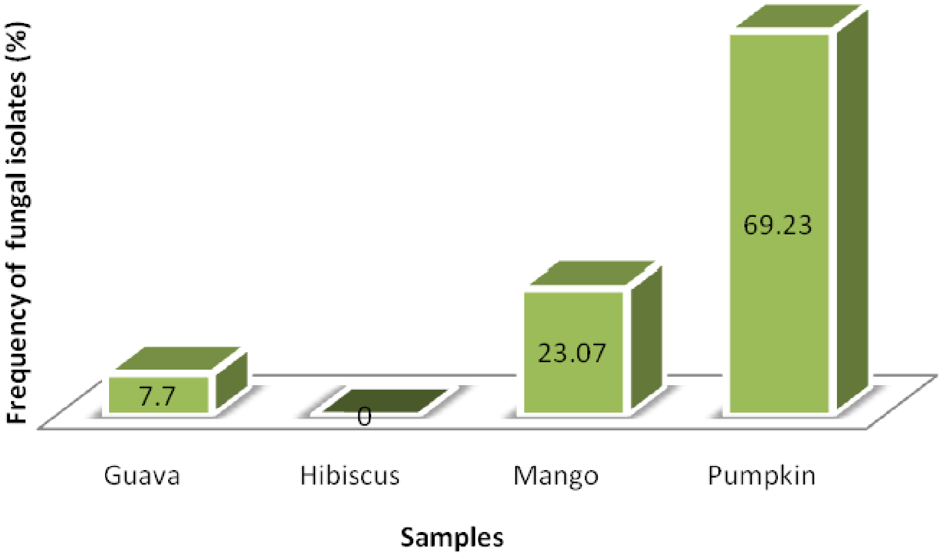

Figure 3. Frequency of fungal isolates from different phyllosphere samples.

Statistical analysis indicated that the type of plant phyllosphere and sampling location both greatly influenced the fungal diversity observed at P<0.0001. Post ANOVA test showed that plants phyllosphere in University of Port Harcourt Abuja campus as earlier observed had more fungal species when compared with other locations at P<0.001 (Figure 5).

DISCUSSION

The highest percentage (43.75%) of bacteria obtained

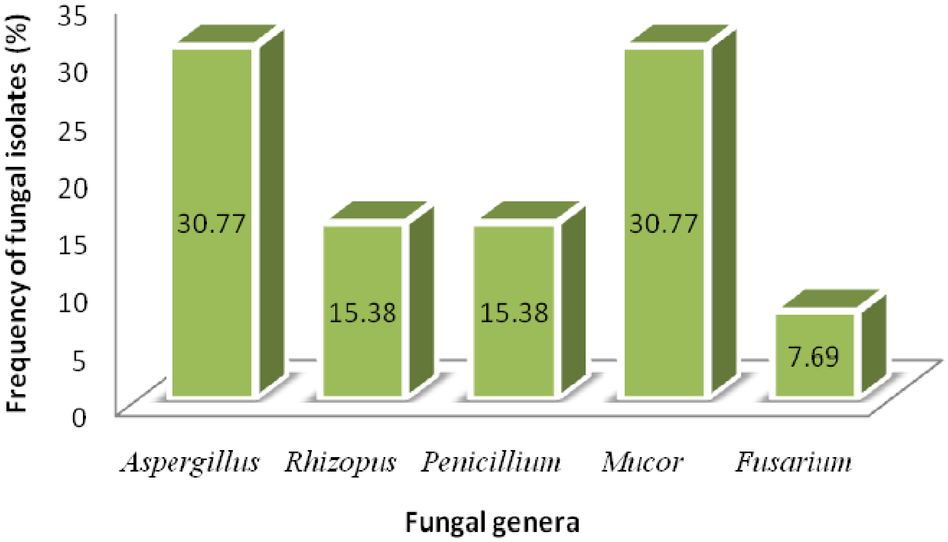

Figure 4. Frequency of fungal genera from all the phyllosphere samples collected from the four sampled sites.

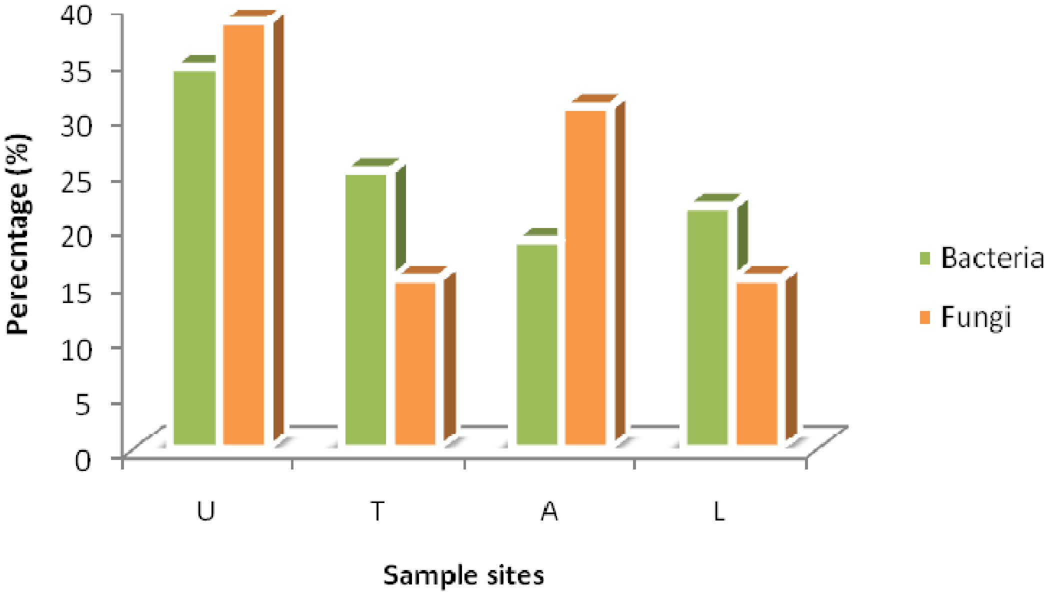

Figure 5. Percentage distribution of bacteria and fungi from the sample sites. U, University of Port Harcourt Abuja Campus; T, University of Port Harcourt Teaching Hospital; A, Aluu Community Health Centre and L, University of Port Harcourt Health Centre Lulu Briggs.

from the phyllosphere of pumpkin within the sampled sites was attributed to the plant species which appear to influence the microbial carrying capacity of the leaf. Pumpkin leaves are broad and less waxy; it is also located close to the ground surface compared to other leaves used in this study and tends to have more surface area for bacteria to thrive (Lindow and Brandl, 2003). Researchers have shown that, the total number of culturable bacteria recovered from broad-leaf plants were significantly greater than that recovered from grasses or waxy broad-leaf plant (Kinkel et al., 2000; Lindow and Brandl, 2003). The less frequency of bacteria obtained from hibiscus (25%), Mango (18.75%) and guava (12.5%) as compared to pumpkin was as a result of the waxy nature of the plant leaves (Kinkel et al., 2000; Lindow and Brandl, 2003). Waxy layer of plant leaf limit passive diffusion of nutrient and water vapour from the plant interior onto the surface and defines the hydrophobicity of the leaf (Lindow and Brandl, 2003). Thick waxy cuticles have been implicated to interfere with bacteria colonization of plants by limiting diffusion of nutrient and inhabiting the wetting of the leaf surface (Kinkel et al., 2000; Knoll and Schreiber 2000; Beattie, 2002; Beattie and Marcell, 2002; Lindow and Brandl, 2003). The predominance of *Pseudomonas* sp. (28.13%) over other bacteria isolated can be explained on the bases that, Pseudomonas spp. have the ability to modify their microbial habitat in order to increase nutrient availability on the phylloplane (Lindow and Brandl, 2003). Also, some phyllosphere bacteria have been shown to increase the permeability of the cuticle enhancing water and nutrient availability (Schreiber et al., 2005) while some are able to increase their nutrient availability by producing toxins which affect ion transport across plant cell plasma membrane (Hirano and Upper, 2000).

Finkel et al. (2012) observed that some plants such as *Tamarix* are able to provide an environment highly rich in organic carbon and sufficient moisture to support the growth of microorganisms on leaves even in highly desiccated area such as desert environment. It was also observed that bacteria isolated did not occur in uniform pattern across the leaves sampled, there ought to be a particular interaction and such interaction is said to determine the extent to which human pathogens are able to colonize and survive on plant tissue such as fresh edible produce (Beattie and Marcell, 2002; Whipps et al., 2008), was observed in the pumpkin used in this study. Researchers have also shown that production of Inole-3-acetic acid (IAA), pili, and flagella by phyllosphere bacteria facilitate quick colonization of the leaf surface by increasing the nutrient leakage, microbial fitness and easy attachment to the leaf surface (Brandl et al., 2001; Romantschuck et al., 2002; Lindow and Brandl, 2003). The highest frequency of fungal species isolated from pumpkin phyllosphere was also attributed to the nature of phyllosphere. Pumpkin as said earlier, have broad and less waxy leaves than other plant phyllosphere; this provided a good microhabitat for fungi to grow (Kinkel et al., 2000; Lindow and Brandl, 2003). Members of *Acremonium, Aspergillus, Penicillium* and *Mucor* have been isolated from phyllosphere of some plants (Inacio et al., 2002).

Several studies have shown that in older leaves phyllosphere, filamentous fungi usually occur as spores rather than active mycelia cells (Andrews and Harris, 2000; de Jager et al., 2001). Some plants' leaves are able to produce various volatile organic compounds into the boundary leaf, these compounds which include CO_2, acetone, terpenoids, aldehydes, alcohol, hydrocarbons, sesquiterpenoids, sulphides, and nitrogen containing compounds are known to be toxic to fungi and might have prevented and limited their growth on phyllosphere (Mechaber, 2002). These findings supported the results from other studies, which showed that pronounced interspecies variability in phyllosphere communities exit (Yang et al., 2001; Lambais et al., 2006; Whipps et al., 2008). Many studies have also shown that environmental conditions can have important effects on phyllosphere community structure (Lindow and Brandl, 2003). The variation in microbial load from the different sample sites was attributed to natural and human activities in the various sites. Sample site U was more populated and have more trees planted in the sites when compared to the other sample sites used in the study. Activities such as fumigation, antibiosis, antisepsis, disinfection and other medical related activities which prevent pests and microbial growth are usually carried out on routine basis in the sampled sites but sample site U. These activities and several others contributed to the less microbial load from the three hospital sites used in the study.

Conclusion

The findings from the study showed that phyllospheres are microhabitats, which support the growth of various groups of microorganisms including human pathogens with bacteria being the most abundant among the groups. Most of the edible leafy vegetables which are consumed by human have less waxy phyllosphere, which permit microbial growth; it is necessary that they may be washed and cooked properly before consumption to ensure healthy living.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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