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Comparative Bacterial Metagenomics of *Cnidoscolus aconitifolius* (Mill.) I. M. Johnston and Other Leafy Vegetables

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Authors' contributions

This work was carried out in collaboration between both authors. Authors EAA and NGO conceived and designed the research. Material preparation and data collection were performed by authors EAA and NGO. Data analysis was performed by author NGO. The first draft of manuscript was written by author NGO. Both authors commented on previous versions of the manuscript. Both authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: Vegetables provide a favourable habitat for diverse populations of microorganisms. Some vegetables, especially the ones used in salads are ready-to-eat food products and some phyllosphere bacteria might contribute to the prolonged presence of human food-borne pathogens in these vegetables.
Methodology: Phyllosphere bacteria associated with *Cnidoscolus aconitifolius* were evaluated using a culture-independent approach, Illumina MiSeq platform of 16S rRNA gene sequencing and then compared with publicly available data obtained from *Spinacia oleracea* (spinach) and *Lactuca sativa* (lettuce) on GenBank.
Results: The results from this study showed that the three vegetables harbor diverse bacterial organisms. Eighty-three (83) Operational Taxonomic Units (OTUs) assigned to five phyla were

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obtained from *C. aconitifolius* phyllosphere. The most predominant phyla across studied vegetables were: Proteobacteria (74.79%), Actinobacteria (8.69%) and Firmicutes (7.37%). Potential human pathogenic species such as *Bacillus* spp., *Enterococcus* spp., *Staphylococcus* spp., *Klebsiella* spp., and *Pseudomonas* spp. were also present in lettuce and spinach. Bacteria with potential for antibiotic production, anti-microbial and antibiotic resistant genes belong to the families Bacillaceae, Streptomycetaceae, Pseudomonaceae, Enterobacteriaceae, Staphylococcaceae, Enterococcaceae and Streptococcaceae. The most abundant taxa obtained from this study were *Pseudomonas*, *Erwinia*, *Brachybacterium*, *Megasphaera*, *Janthinobacterium*, *Sphingomonas* and *Lactobacillus*.

Conclusion: Our result successfully determined the relative abundance of potential human and plant pathogens in the leafy vegetables and also showed the bacterial community structure in the studied vegetables.

Keywords: Cnidoscolus aconitifolius; fungi; metagenomics; 16S rRNA gene; phyllosphere.

1. INTRODUCTION

Cnidoscolus aconitifolius (Mill.) I. M. Johnston commonly called "chaya" is an evergreen drought-resistant leafy perennial shrub that originated from the Maya region of Guatemala, Belize, and Southeast Mexico as a leafy vegetable during the pre-cambrian period [1]. Today, chaya is found growing in the wild and in cultivation in all the regions in Nigeria. The plant has both medicinal and nutritional values. It is believed to possess blood-building capacity. Azeez et al. [2] reported that ethanolic extract of chaya leaves increases motility of sperm cells and can be used for management of diabetes. According to Orji et al. [3], *C. aconitifolius* contains phenols which are well-known anti-cancer agents with high ability to fight against cancer. Chaya leaves have been reported by many authors to have anti-microbial and hepatoprotective properties, and also induces increase in white blood cells which are defensive against diseases [4,5,6].

Phyllosphere refers to the interior and exterior of the above-ground (aerial) parts of vascular plants [7]. The phyllosphere is colonized by diverse microorganisms that have the capacity to adapt to water or nutrient-limited environments, high temperatures and UV exposure [7,8]. Bacteria are known to colonize the phyllosphere to a greater extent than other microorganisms with the density of about 10^{26} cells g^{-1} leaf [9]. Microorganisms in the phyllosphere have been shown to play a vital role in maintaining the total development and health of their host plant [7,10,11]. The phyllosphere is also colonized by several bacteria that play significant roles in human health. The causative agent of bacterial leaf spot disease of lettuce, *Xanthomonas campestris* pv. *vitians*, co-existed positively with bacteria from the genus *Alkanindiges*, but antangonistic to species belonging to the genera, *Bacillus*, *Erwinia*, and *Pantoea* [12]. Certain strains of *Sphingomonas, Pseudomonas* and *Erwinia* have been recorded to confer protection on plants against some plant pathogens through certain mechanisms such as competition for limited nutrients in the phyllosphere [13,14]. Even though the phyllosphere is not an ideal habitat for human pathogens, *Salmonella and Escherichia coli* O157:H7 survive on plants in the field over long periods of time at low levels [15]**.**

There is accumulated evidence that plant species and season determine the composition of the microbiome in the phyllosphere [16,17] among other factors. Traditionally, culture-dependent methods have been used to isolate and identify microorganisms, but these methods have a lot of limitations as they are time-consuming and painstaking. Next-generation DNA sequencing which allows the analysis of numerous sequences in a single sequencing run brought about reduction in the cost of sequencing. Illumina next generation sequencing which is a second generation sequencing technology [18] involves an ultra-high-throughput sequencing of organisms and produces very high amounts of sequence data that are far greater than those obtained from the 'first-generation' molecular techniques (Sanger sequencing).

There is limited information on the microbial community of raw food and vegetables and also the assessment of potential human pathogens in these food produces, hence the need for this study. The objectives of this study are to (1) characterize the bacterial community of chaya leaves in order to identify the bacterial organisms that affect its quality and could be pathogenic to

the plant and to humans, (2) to compare the bacterial community of chaya with that of lettuce and spinach, and to determine the relative abundance of potential human and plant pathogens in the leafy vegetables.

2. MATERIALS AND METHODS

2.1 Sample Collection

Cnidoscolus aconitifolius leaves were obtained from Choba, Rivers State, Nigeria in April 2018. The coordinates of the sample collection location is 4.89^{o}N and 6.91^{o}E. The leaves were transferred to a zip bag and taken to the lab prior to DNA extraction.

2.2 DNA Extraction

Culture-independent approach was used for DNA extraction. DNA was extracted directly from *C. aconitifolius* leaves using Zymo Fungal/Bacterial DNA Extraction kit (Zymo Research Group, California, USA) with a slight modification as described in a previous study [19]. To analyze the total bacterial community on the leaves, 0.50g of leaves was used. This was transferred into a sterilized mortar containing 750 µl of Bashing bead buffer. The sample was homogenized using liquid nitrogen and then transferred into a micro-centrifuge tube (1.5 ml).

2.3 Illumina Sequencing and Data Processing

To assess the bacterial communities, the samples were analyzed with 300 bp paired-end read, Illumina MiSeq, at Inqaba Biotechnology Limited, South Africa. Genomic DNA were PCR amplified using forward primer 341F (5´-CCTACGGGNGGCWGCAG-3´) and reverse primer 785R (5´-GACTACHVGGGTATCTAATCC-3´). This primer pair is known to amplify nearly full length 16S rDNA gene sequence targeting variable regions; V3 and V4. The resulting amplicon was gel purified, end repaired and Illumina specific adapter sequence added to the 5´ end of each primer.

Raw sequences obtained were checked for PCR artifacts and low-quality reads and trimmed using *ngsShoRT* (next-generation sequencing Short Reads) trimmer as described by Chen et al. [20]. The sequences were trimmed on the basis of average quality score (<25). The 16 S rRNA gene sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME v.1.9.0) pipeline as described by Caporaso et al. [21]. After sorting, sequence reads containing less than 200 nucleotides and reads with 7% of homopolymers or more than 2% of ambiguities were excluded from the data used for the final analysis. The UCLUST

Fig. 1. *Cnidoscolus aconitifolius* plant

algorithm [22] was used to cluster sequences into operational taxonomic units (OTUs) at a 97% identity threshold. Each OTU sequence was represented by the most abundant read. The GREENGENES reference database was used for both open reference OTU picking and taxonomic assignment for the sequences. The OTU table was removed of singletons and rarefied to an even sampling depth (minimum sequence count) prior to diversity analysis. Also, sequences classified as chloroplast and mitochondria were removed from the OTU table. Alpha-diversity analysis based on observed OTUs, Shannon, Chao1 indices was done using the *vegan* R package [23]. PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) [24] was used to predict the functions of the bacterial communities associated with the vegetables, based on Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) Database. The predicted functions were subsequently collapsed into KEGG pathways and visualized using *STAMP* [25].

The sequence data for *Cnidoscolus aconitifolius* has been deposited in Sequence Read Archive (SRA) on GenBank as BioProject ID PRJNA592305. The sequence data of lettuce and spinach are available on the European Nucleotide Archive database under BioProject number PRJEB6233 and GenBank (Sequence Read Archive) under the BioProject number PRJNA517014 respectively.

3. RESULTS AND DISCUSSION

3.1 Bacterial Population among Samples

The results from this study showed a high bacterial diversity across the three vegetables analyzed. A total of 783,646.00 sequences was obtained from the three vegetables; *Cnidoscolus aconitifolius, Spinacia oleraceae* and *Lactuca sativa*. After the trimming and filtering of sequences, 13,555 OTUs from 664,774.00 quality reads were used for further analysis. Eleven thousand, six-hundred and eighty-five (11685) sequences clustered into eighty-three (83) OTUs at 97% similarity on GREENGENES database were obtained from *C. aconitifolius*. This was compared with those obtained from *S. oleracea and L. sativa*.

The alpha diversity indexes (Chao 1 and Shannon) showed that *S. oleraceae* had the highest richness in bacterial organisms and the highest diversity of bacteria species (Figs. 2-3).

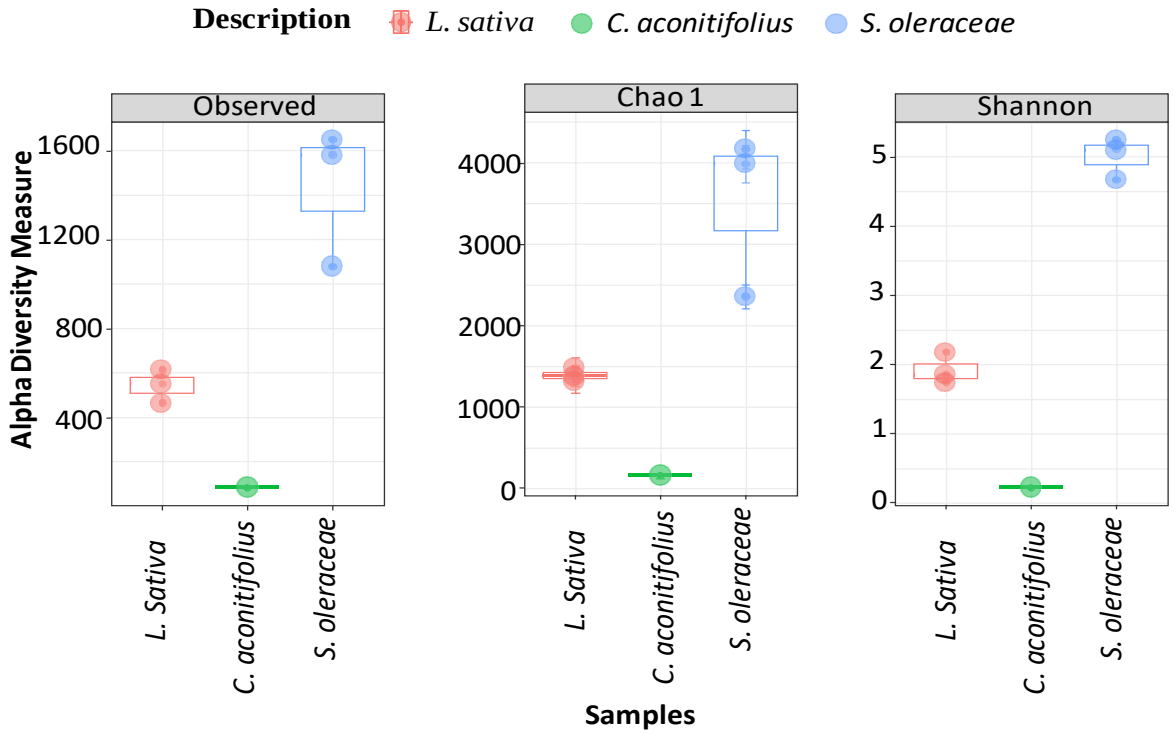

Fig. 2. Alpha diversity analysis of bacteria organisms obtained from chaya, lettuce and spinach leaves

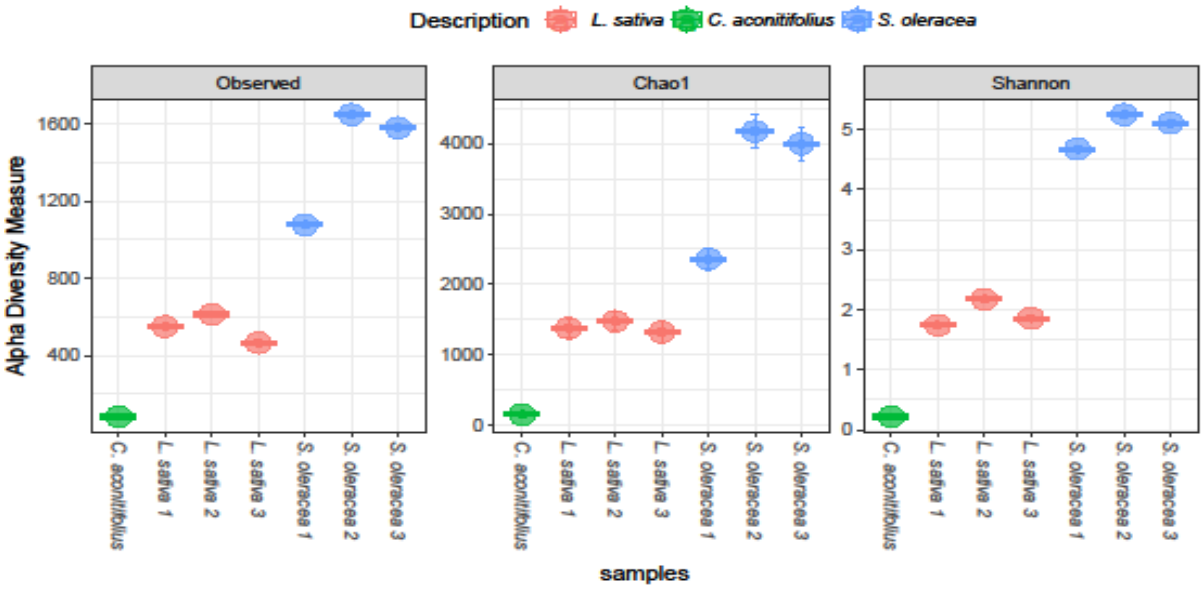

Fig. 3. Alpha diversity analysis of bacteria organisms obtained from each sample of chaya, lettuce and spinach leaves

3.2 Bacteria Phyla across the Three Vegetables

The most predominant phyla across the entire bacterial population of the three vegetables were Proteobacteria (74.79%), Actinobacteria (8.69%), Firmicutes (7.37%), Bacteroidetes (5.38%) and Fusobacteria (1.13%). The most dominant phyla in *C. aconitifolius* phyllosphere were Firmicutes (40.11%), Actinobacteria (19.77%), Proteobacteria (17.51%) and Bacteroidetes (7.91%). In *S. oleraceae*, Proteobacteria (92.70%) was however the most prevalent phylum, followed by Bacteroidetes (4.65%) and Firmicutes (1.93%) while in *L. sativa*, the most predominant phyla were Proteobacteria (75.96%), Actinobacteria (13.54%), Bacteroidetes (5.28%) and Firmicutes (1.88%). Acidobacteria occurred only in *L. sativa* while the phylum Fusobacteria was only observed in *C. aconitifolius*. Proteobacteria has been reported to dominate spinach and lettuce phyllosphere [17,26,27]. Firmicutes, Proteobacteria and Actinobacteria are found to usually dominate the phyllosphere of many plant species [11,12,28,29]. The three vegetables had almost similar taxonomic profiles (Fig. 4).

3.3 Bacteria Genera across the Three Vegetables

Pseudomonas was the most abundant genus obtained. Sequences assigned to the genus *Pseudomonas* obtained 35.84% of the total OTUs. This genus was however not obtained from *Cnidoscolus aconitifolius* but was in abundance on *Spinacia oleraceae* and *Lactuca sativa* (Fig. 2). The genus *Erwinia* was observed on all the vegetables with a relative abundance of 7.72%. *Brachybacterium*, Megasphaera, *Ochrobactrum* and *Brevibacterium* which obtained 4.52%, 3.39%, 2.83% and 2.82% of the total OTUs respectively were only observed in *C. aconitifolius*. *Janthinobacterium* (4.06%), *Sphingomonas* (4.82%) and *Acinetobacter* (2.19%) were present only on *S. oleraceae* and *L. sativa*. *Lactobacillus* obtained 3.96% of the total OTUs and was present in *S. oleraceae* and *C. aconitifolius*. *Sphingobacterium* which obtained 2.63% was only obtained from *C. aconitifolius* and *L. sativa* leaves.

The most predominant genera on *C. aconitifolius* were *Lactobacillus* (11.86%), *Sphingobacterium* (7.34%), *Brachybacterium* (4.52%), *Megasphaera* (3.39%), *Erwinia* (3.39%). Other genera and other unidentified bacteria obtained 69.5% of the total OTUs observed on *C. aconitifolius*. The most abundant genera on *S. oleraceae* were *Pseudomonas* (32.72%), *Erwinia* (14.56%), *Acinetobacter* (3.64%) and *Janthinobacterium* (3.38%), and others were assigned 45.7% of the OTUs. *Pseudomonas* (38.97%), *Sphingomonas* (7.91%), *Janthinobacterium* (4.24%), *Agrobacterium* (4.12%), *Arthrobacter* (3.23%), were the most predominant genera on *L. sativa*, and other genera were assigned 41.53% of the OTUs.

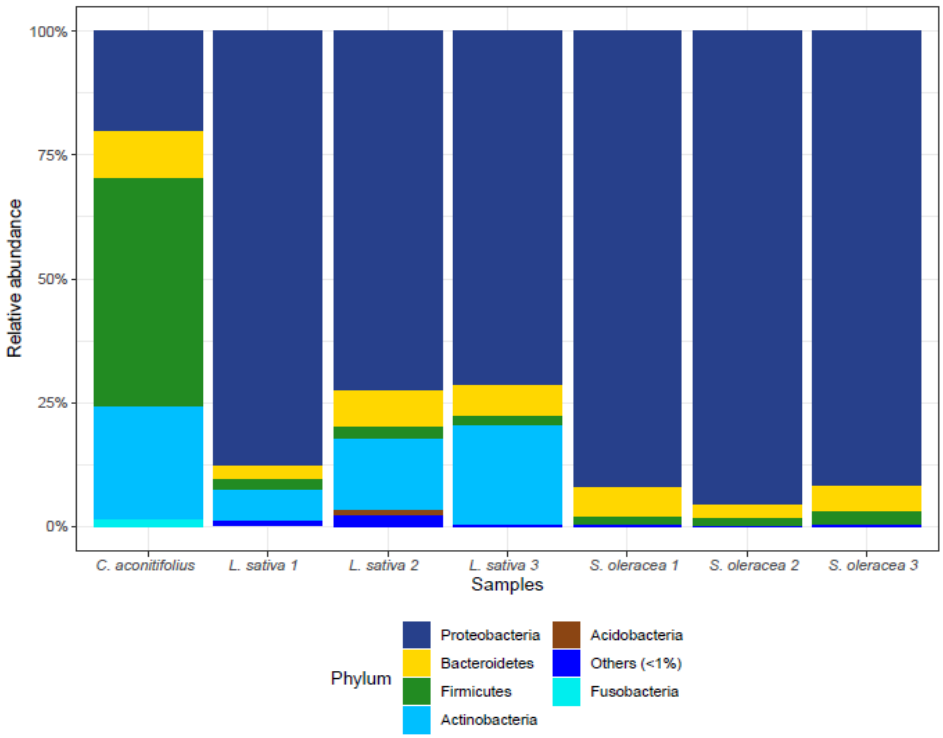

Fig. 4. Distribution of bacteria phyla across *Cnidoscolus aconitifolius*, *Spinacia oleraceae* and *Lactuca sativa* leaves

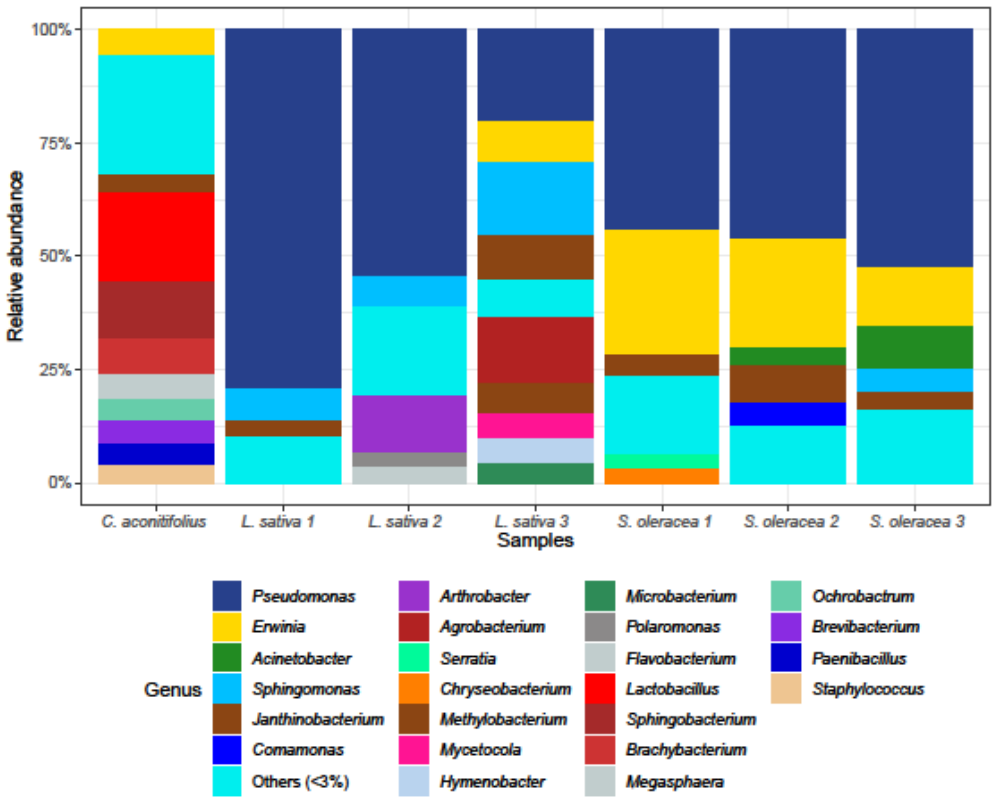

Fig. 5. Distribution of bacteria genera across *Cnidoscolus aconitifolius*, *Spinacia oleraceae* and *Lactuca sativa* leaves

Pseudomonas species exist as parasites and/or saprophytes, living in terrestrial habitats, including plants [30]. Symptoms observed on plants include: blight, galls, leaf spots and stem spots, cankers and soft rots [31]. There are about twenty-one plant pathogenic *Pseudomonas* species known with *P. syringae* being the most important of the species; having more than 50 pathovars [31]. *P. syringae*, *P*. *orientalis*, *P*. *viridiflava, P*. *lurida*, *P*. *simiae*, *P*. *monteilii*, *P*. *syringae* and *P*. *moraviensis* have been reported as phytopathogens [32,33,34]. Some *Pseudomonas* species are pathogenic to both human and plants [35,36]. *Pseudomonas aeruginosa* is both a human and animal pathogen and has been reported on dogs as the cause of urinary tract infections and otitis, on horses for endometritis disease and the cause of hemorrhagic pneumoniae in mink [37,38].

The rhizosphere of several plants is colonized by *Pseudomonas putida* strains in a symbiotic relationship. The strains are also found in fresh water and have the ability to metabolize biogenic and xenobiotic compounds [33,39,40]. Motility is important for attachment of *Pseudomonas* sp. to roots of plants as seen in the case of wheat roots under conditions of nutrient limitation [41]. *P. putida* is also a bio-control agent. Matilla et al. [33] demonstrated that *Pseudomonas putida* can protect *Arabidopsis thaliana* against the disease caused by *Pseudomonas syringae* pv. tomato. *Pseudomonas putida* strains have also been reported by Fernandez et al. [40] as opportunistic human pathogens. They showed a cytotoxic effect on cells of the human skin and damaged the skin epithelial layer. They severely damaged the epidermis and the underlying connective tissue on wounds in rat and were also harmful to an insect, *Chrysoperla carnea*.

Lactobacilli is composed of 152 species [42]. The species are the most important organisms used in human nutrition and food microbiology. *Lactobacillus* species may be useful or detrimental to plants and other products. *Lactobacillus plantarum* has been reported to act as a bio-control agent against some bacteria [43]. Paradh et al. [44]) detected *Lactobacillus* and *Megasphaera* as spoilage organisms in United Kingdom using multiplex PCR methodology.

The genus *Megasphaera* is a member of the Veillonellaceae family [45]. Species of this genus are usually found in the rumen and also in contaminated foods and sometimes in the liver, oral and vaginal samples [46]. *Megasphaera cerevisiae*, *Megasphaera paucivorans* and *Megasphaera sueciensis* are beer-spoilage species. *Megasphaera* species are important to human health, the environment, food, and production of renewable energy for the future [47].

Brachybacterium sp. has been isolated from human bloodstream as a causative pathogen of bloodstream infection [48]. *Brachybacterium* spp. have the ability to promote plant growth [49]. The genus *Erwinia* consists of well-known plant pathogenic species of economic importance causing severe damage to fruit crops [50]. Diseases caused by *Erwinia* species is one of the most devastating diseases of vegetables which results in the decay of the produce both in the field, on transit and in storage [51]. Doolotkeldieva and Bobusheva [52] reported that *Erwinia amylovora* caused fire blight on apple, pear and hawthorn trees in Kyrgyzstan and many other countries.

Sphingobacterium species rarely cause infectious diseases in humans though few cases have been reported. *Sphingobacterium spiritivorum, S. multivorum* and *S. thalpophilum* have been reported to be associated with various infections such as urinary tract infections, wound infections, peritonitis and abscesses [53]. *Sphingobacterium spiritivorum* caused bacteremia in an elderly man who was suffering from severe obstructive pulmonary disease [54].

Species of the genus *Janthinobacterium* are usually regarded as non-pathogenic microorganisms. The first isolation of *Janthinobacterium lividum* as a pathogenic bacterium infecting rainbow trout was reported in Korea [55]. The bacterium was also reported to be resistant to several antibiotics based on the antibiotic susceptibility tests. *J. lividum* was previously considered to be non-pathogenic to amphibians, and this may be the reason the organism has been used for treating fungal diseases associated with amphibians.

The genus *Acinetobacter* is made up of saprophytic microorganisms that are found almost everywhere. Though, different species of the genus occupy different habitats such as sewage, soil, foods, water, human and animals [56,57,58]. A member of the genus, *A. baumannii* has been isolated in various food items, such as milk, raw vegetables, fruits and dairy products [59]. *Acinetobacter* spp. are the major cause of

nosocomial infections. *A. baumannii* is resistant to a variety of antibiotics and has been reported in hospital settings to have led to enhanced nosocomial outbreaks linked with high death rates [60].

Sphingomonas spp. have been isolated from rice seed [61] and also found on the leaves of twenty-six (26) plant species; though a few are recognized as plant pathogens. Three species have been reported to be pathogenic to plants [62,63,64]. *Sphingomonas paucimobilis* has been reported to cause bacteraemia/ septicaemia as a result of contaminated solutions such as haemodialysis fluid and sterile drug solutions [65].

The genus *Agrobacterium* is a group of soil bacteria associated with plants. Many species in this genus are pathogenic to plants. Infections of wound sites by *Agrobacterium tumefaciens* are responsible for crown gall tumors on a variety of plants including some gymnosperms, monocots and most dicots. *A. rhizogenes* causes hairy root disease in plants and *A. vitis* causes necrotic lesions and tumors on grape vines. Regardless of the general perception that most *Agrobacterium* spp. cause diseases, *A. radiobacter*, which is mostly isolated from the soil, is not pathogenic to plants [66].

Arthrobacter spp. have been isolated from human clinical specimens. Some of these species have been reported to have exhibited antimicrobial susceptibility [67]. Some members of the genus *Arthrobacter* are known to degrade organic pollutants, and have a high potential as phyllosphere-based bioremediation agents as they can prevent pesticides from reaching the soil surface or groundwater [68].

Several factors influence the microbial composition of the phyllosphere. Redford et al. [69] reported differences in the bacterial communities of the phyllosphere strongly differ depending on the plant species. This may be due to differences in the physical characteristics of plants, variations in metabolites and symbiotic relationships between the host plant and other microbes [70]. Microbial populations on plant leaves have also been observed to be affected by management practices such as nitrogen fertilization and storage [27,28]. Nitrogen content has been reported to affect the microbial community structure in the phyllosphere of spinach (*Spinacia oleracea*) and rocket (*Diplotaxis tenuifolia*) [27]. In a study by Lopez-Velasco [28], there were changes in spinach phylloepiphytic bacteria communities following minimal processing and refrigerated storage. Also, farming practice and time has the potential to affect the bacteria diversity of vegetables as was obtained with spinach and lettuce in a study by Leff and Fierer [71]; and rocket salad (*Diplotaxis tenuifolia*) and lettuce (*Lactuca sativa*) in a study by Dees et al. [17] respectively.

All the vegetables were found to inhabit opportunistic human pathogens. Tatsika et al. [72] reported that household washing methods are inefficient in the removal of these pathogens. Newton et al. [7] suggested the manipulation of phyllosphere microbial communities through the application of agrochemicals or through plant breeding in order to ensure the safety of food products.

3.4 Functional Prediction of Genes Obtained

The functional analysis was focused on the detection of genes concerned with the biosynthesis of secondary metabolites such as those involved in antibiotics production, antibiotics resistance and growth regulation in plants. The biosynthesis of secondary metabolites was highly observed in the bacterial community of spinach, very low in lettuce and not observed at all in chaya. Therefore, the organisms responsible for the synthesis of these metabolites were present in large numbers in spinach, few in lettuce and absent in chaya leaves.

A total of 611753 genes and 179 genes linked to antibiotics production, antibiotics resistance, and growth regulation in plants were obtained from spinach and lettuce respectively. None of these genes were observed on chaya. In these pathways, twelve different KEGG numbers represented by 611932 genes were identified in our dataset (Table 1). A Heatmap showing the different KEGG pathways at the different levels is presented in Fig. 6.

Butirosin is an aminoglycoside antibiotic produced by *Bacillus circulans* [73] belonging to the family Bacillaceae. Butirosin synthesis was observed in the bacterial communities of spinach and lettuce. The aminoglycosides (including kanamycin, neomycin and gentamicin) are very effective agents for the treatment of complicated infections, especially those caused by methicillin-resistant *Staphylococcus aureus* (MRSA) [74].

Novobiocin, an aminocoumarin antibiotic is synthesized by the genus *Streptomyces* which belongs to the family Streptomycetaceae. *Streptomyces* species such as *S. spheroids* and *S. rishiriensis* synthesize novobiocin. Organisms associated with novobiocin synthesis were observed in spinach and lettuce phyllosphere. Novobiocin has been reported to have a synergistic effect with antitumor drugs [75]. The antibiotic, streptomycin is synthesized by the bacterium, *Streptomyces grieus* [76]. Streptomycin biosynthesis was observed in the bacterial communities of spinach and lettuce. *Streptomyces* spp. obtained from soil sample was found to possess antibacterial activity. The antibacterial agent from this isolate was very effective against some gram-negative bacteria but was less effective on gram-positive bacteria. The isolate was able to inhibit *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella typhi*, *Salmonella paratyphi*, *Staphylococcus aureus*, *Shigella sonnei*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *Escherichia coli*. The chemical feature of the antibacterial agent was similar to streptomycin [77].

Beta lactams resistance in bacteria is brought about by one or a combination of the following mechanisms; enzymatic degradation by β-lactamases, target modification of the PBPs (penicillin-binding proteins) leading to lack of β-lactam binding, and regulation of β-lactam entrance and efflux [78]. Gram-Positive Bacteria such as: *Pseudomonas aeruginosa* (Pseudomonaceae), *Enterobacter* sp. (Enterobacteriaceae), *Staphylococcus aureus* (Staphylococcaceae), *Enterococcus faecium* (Enterococcaceae), *Streptococcus pneumonia* (Streptococcaceae) and *Mycobacterium tuberculosis* (Mycobacteriaceae) have been reported to possess β-lactam resistance [79]. Bacteria organisms involved in β-Lactams resistance were obtained from both spinach and lettuce leaves in this study. *Pseudomonas* is one of the multi drug resistant saprophytic bacteria. The major *Pseudomonas* species involved in opportunistic human infections are *P. fluorescens, P. putrefaciens, P. cepacia, P. maltophilia, P. aeruginosa, P. putida* and *P. stutzeri*. *P. aeruginosa* plays a major role in nosocomial infections. It is resistant to antibiotics such as aminoglycosides and β-lactams [80]. Some of the identified genes and the antibiotic resistant mechanisms involved in the resistance of *P. aeruginosa* to drugs have been reported by Synder et al. [81] and Feliziani et al. [82].

Indole alkaloids, a secondary metabolite produced by plants are known to carry out antimicrobial activities [83]. These substances have also been reported to be synthesized by *Streptomyces* spp., *Pseudomonas aeruginosa* and cyanobacteria [84,85,86]. Genes that are involved in indole alkaloids biosynthesis were present only in the bacterial community of spinach in this study. Flavonoids are a large group of secondary metabolites synthesized by plants, used as supplements, medicinal agents and natural colorants. Flavonoids and isoflavonoids are believed to play essential roles in adaptation of legumes to their biological environments, as compounds involved in defensive mechanisms (phytoalexins) and also as chemicals that signal nitrogen fixation in a symbiotic relationship with rhizobia [87]. Microorganisms belonging to the family

Table 1/ KEGG pathways associated with biosynthesis of secondary metabolites detected in the phyllosphere of *Cnidoscolus aconitifolius*, *Spinacia oleraceae* and *Lactuca sativa* leaves

KEGG Ortholog no	KEGG Pathway	No of genes present		
		Spinach	Lettuce	Chaya
KO 00965	Betalain biosynthesis	2777	1	0
KO 00524	Butirosin, Kanamycin, Gentamicin and neomycin biosynthesis	26299	8	0
KO 00944	Flavone and flavonol biosynthesis	186	0	0
KO 00941	Flavonoid biosynthesis	11554	8	0
KO00901	Indole alkaloid biosynthesis	1064	0	0
KO 00941	Isoflavonoid biosynthesis	79	0	0
KO 00950	Isoquinoline alkaloid biosynthesis	71165	19	0
KO 00401	Novobiocin biosynthesis	122533	34	0
KO 00311	Penicillin and cephalosporin biosynthesis	49046	16	0
KO 00940	Phenylpropanoid biosynthesis	94585	19	0
KO 00521	Streptomycin biosynthesis	198866	62	0
KO 00550	Beta-Lactam resistance	33599	12	0

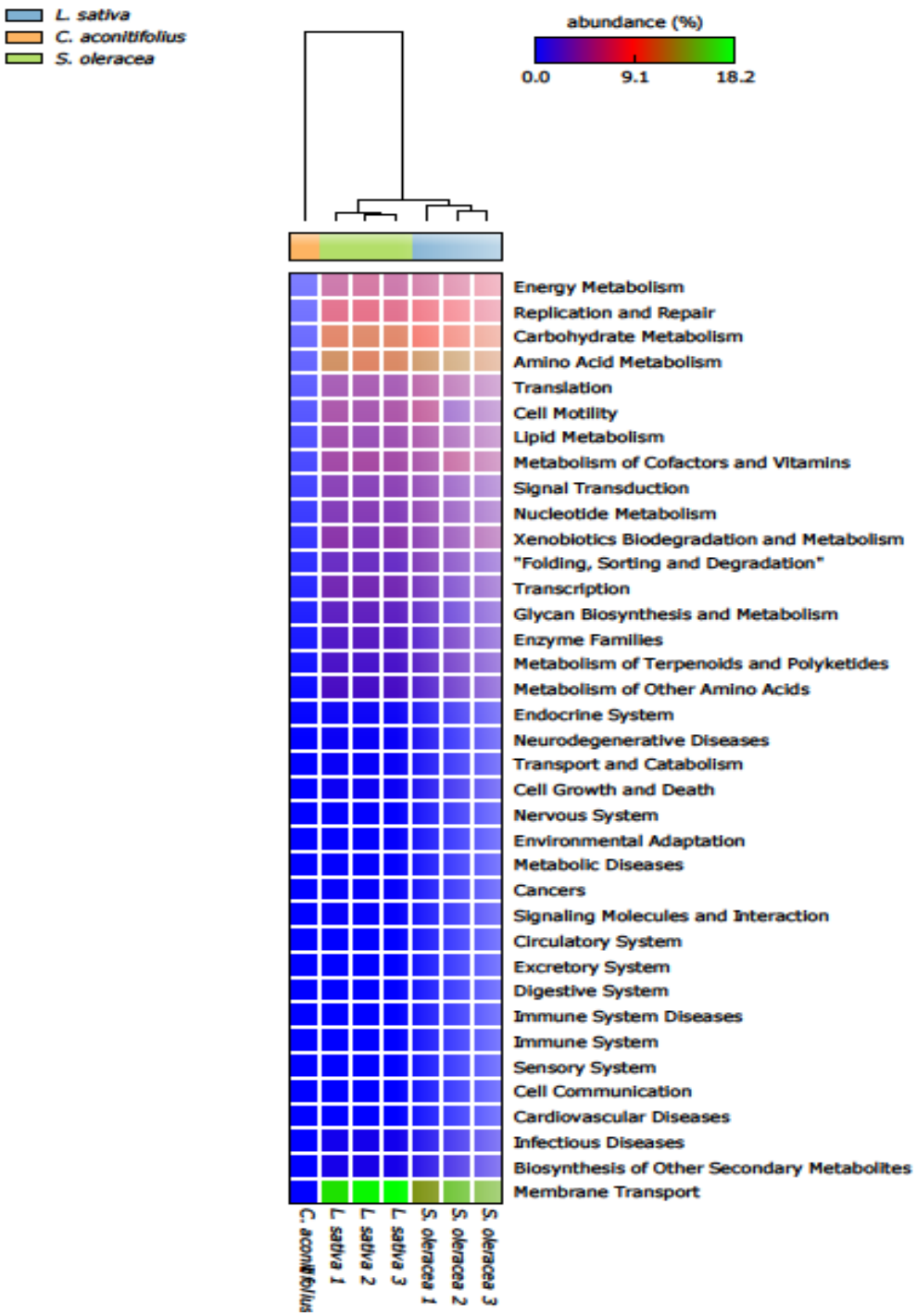

Fig. 6. Heatmap showing the different KEGG pathways at different levels detected in *Cnidoscolus aconitifolius*, *Spinacia oleraceae* and *Lactuca sativa* leaves

Enterobacteriaceae can be used as platforms (in form of recombinant microorganisms) that aid in the increased synthesis of secondary metabolites (such as: flavone, flavonoid, isoflavonoid, isoquinoline alkaloid and phenylpropanoid) in plants [88,89,90]. The utilization of recombinant yeast and bacteria in the production of various classes of flavonoid compounds such as: flavanones, anthocyanins, stilbenes, flavones and isoflavones was reported by Chemler et al. [89]. Flavonoid, isoquinoline alkaloid and phenylpropanoid biosynthesis were observed in both spinach and lettuce bacterial communities while isoflavonoid, flavone and flavonol

biosynthesis were observed only on spinach leaves in this study. Betalains are antioxidant pigments believed to be synthesized by only flowering plants belonging to the order, Caryophyllales. This belief was defeated by the discovery of a betalain-forming bacterium called *Gluconacetobacter diazotrophicus* [91]. Among the three vegetables studied here, genes responsible for betalains biosynthesis were highly present in spinach but was only one in lettuce.

4. CONCLUSION

Our results show that the microbial communities in vegetables are diverse and the relative abundance of potential human and plant pathogens in the vegetables was determined. The microbiome of the vegetables comprises of opportunistic human pathogens. Washing methods used locally at home cannot remove most of the organisms. In future studies, we hope to determine the level of contamination of these vegetables at which these organisms pose as a health risk especially for immune-compromised individuals. Also, an in-depth understanding of the interaction between microorganisms in the phyllosphere is necessary in order to develop better preventive or control strategies to reduce the population of pathogenic and ⁄ or spoilage bacteria in the phyllosphere. No food borne pathogenic organisms were found in the present study. The use of Illumina next generation sequencing of the 16S rRNA gene proved to be very reliable in the determination of the bacterial community structure in the three vegetables studied and also in the identification of human pathogens and organisms responsible for the spoilage of food products.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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