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Nwaiwu O, Onyeaka H, Ibekwe VI, Okorondu SI, Nnokwe JC, *et al*. iThenticate report for: Molecular evolutionary phylogeny of 16S rRNA fragments from Ugba (Pentaclethra macrophylla) seeds. *Access Microbiology*. 2025. https://doi.org/10.1099/acmi.0.001060.v1.1

ACMI-S-25-00163.pdf

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WORD COUNT	7019	TIME SUBMITTED	03-JUN-2025 06:16PM
		PAPER ID	116570936

Access Microbiology

Molecular evolutionary phylogeny of 16S rRNA fragments from Ugba (Pentaclethra macrophylla) seeds

--Manuscript Draft--

Molecular evolutionary phylogeny of 16S rRNA fragments from *Ugba* (*Pentaclethra macrophylla*) seeds

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1.2 **Keywords:** Evolution, food safety, *Ugba*, *Pentaclethra macrophylla*, Ancestral sequences, phylogeny

1.3 **Data summary:** All data associated with this work are reported within the article and Sequences were deposited in GenBank under the accession numbers PQ865836 - 70.

1. Abstract

The evolutionary analysis of bacteria species harbouring 16S RNA sequences detected in the oil bean seeds of *Ugba* (*Pentaclethra macrophylla*) was carried out. The food product has a high socio-economic relevance to communities where they are consumed. However, reports from the media have shown that fatalities can sometimes occur after consumption, hence the need for a better understanding of bacteria associated with the product. Some of the species detected in this study like *Proteus mirabilis, Klebsiella pneumoniae* and *Enterococcus faecalis* can be food-borne and are well known for causing various diseases, which is a concern. Other species like *Kurthia gibsonii*, *Stenotrophomonas geniculata* and *Alcaligenes nematophilus* occur mainly in the environment and may have gotten into the sample in the field during or before harvest. The Phylogenetic analysis carried out showed that some strains of the same species resolved into different monophyletic groups suggesting species divergence or distinct evolutionary lineages. The species *Kurthia gibsonii*, was found to be the earliest ancestor following sequence ancestral analysis, which suggests that it was resident in the samples analysed before other bacteria. The *Ugba* seeds appear to harbour a diverse group of bacteria and will benefit from metagenomic investigations as well as studies of the mechanism of survival and succession to reveal the true nature of resident flora. This will help safeguard public health.

2. Impact statement

This study highlights that *Ugba* may cause fatalities and all stakeholders should join hands to unravel when it can change from a delicacy to a death agent. This study will help galvanise more action on understanding resident flora from farm to fork and initiate the introduction of modern food safety practices in its preparation, storage and sale. It is also hoped that this study will initiate studies on the biochemical constituents at harvest and point of sale and the effect of different processing methods on their toxicity. In the long run, it is hoped that fatalities will no longer be reported after consumption.

3. Introduction

The seeds of oil bean (*Pentaclethra macrophylla)* known as *Ugba* or *Ukpaka* in South Eastern Nigeria are consumed by many people in the whole of that region. It can be eaten on its own or used as a food condiment and can be used to make several food delicacies [1] The seed has a very high socio-economic and cultural importance. It is a regular delicacy served in traditional ceremonies and it generates income for traders. The preparation of the product has been described by other investigators [2,3]. Briefly, the seeds are boiled for hours after which spontaneous alkaline fermentation is carried out from half a day to a few days. The well-fermented seeds are then sliced into thin or thick slices depending on preference and then packed in dry banana leaves for sale. The taste and texture of the seeds may vary. Also, the preparation and finished product may depend on the history of a community. The variation in texture may be due to the tree from which the seeds are obtained. The genetic diversity of *Ugba* trees has been elucidated [4] using the *rbcl* gene and the data showed that there were variations in the guanine-cytosine (GC%) content of different tree accessions.

The seeds of *Ugba* are regarded to be beneficial as a probiotic and medicinal agent. The probiotic potential due to the microorganisms present in the product has been reported [5] in a review. In that report, two investigations using human subjects showed the beneficial effects of *Ugba* as a probiotic. In the first investigation [6], 20 people ate *Ugba* as the only fermented product for three weeks while the second one [7] used 165 subjects who ate the product for two weeks. In both studies, the recovered stools from participants showed an increased count of *Lactobacillus* species associated with probiotic qualities. In the past, the focus of probiotic organisms was on dairy products. *Ugba* is a non-dairy source and recently it has been noted that probiotic microorganisms from non-dairy food sources are becoming increasingly important [8]. *Ugba* is also believed to be rich in polyphenols and has medicinal qualities mainly from folklore and trials conducted with rats. The product has been reported to have bioactive compounds [9, 10], and it is believed to be active against sickle cell, high cholesterol and cancer [11]. Also, compounds present in seed pods may promote wound healing [12]. Furthermore, the seeds may have antioxidant potentials [13] and could mitigate memory loss [14].

Despite the nutritional and socioeconomic importance, a report [15] highlighted that there have been reported cases of fatalities after consumption of *Ugba*. The specific cause of death was not established in many instances since no post-mortem enquiries were carried out to establish if the fatalities were due to food-borne pathogens or chemical toxicants from the seeds. In over a century of consumption of the product, anecdotal evidence suggests that the processing steps are enough to break down toxicants and prevent the proliferation of foodborne pathogens. Hence, fatalities may be due to post-process contamination with microbes during packaging or incomplete fermentation that allowed the presence of toxicants.

In the literature, pathogens of food safety concern in *Ugba* have been reported after cultural methods were used, but the reports of molecular studies on bacteria that are associated with *Ugba* seeds are scarce. There are hardly any whole genome reports of organisms from the product or characterisation of 16S RNA sequences from bacteria in the last decade. The problem is not only with *Ugba* because several African fermented products are obtained from other oilseeds, but only a few have been investigated for their microbial content holistically [16]. Hence this investigation aimed to establish the phylogeny and ancestry of 16S RNA fragments found in *Ugba* seeds obtained from ten markets in South Eastern Nigeria. This may help in the surveillance of deleterious bacteria that occur in the product by regulatory authorities. It will equally be helpful for investigation during a food poisoning outbreak.

4. Methods

Sample and strain collection

Fermented sliced samples of *Ugba* were obtained from 10 different markets. Twenty-five grams of the food condiment were stomached with 225 of maximum recovery diluent (pH = 7.01). The pH of the mixture was taken and then incubated for 24 hours. A 100 μL was placed on Polymyxin Egg Yolk Mannitol Bromothymol Blue Agar (PEMBA) medium (Thermo Scientific™ Oxoid™) and spread out before being incubated at 37 degrees for 24 hours using sterile distilled water as control. Colonies that emerged were scraped off into Eppendorf tubes and suspended in DNA/RNA Shield (Zymo Research, USA).

DNA Extraction

Forty microlitres of the cell suspension were lysed with 120 μL of TE buffer containing lysozyme (final concentration, 0.1 mg/mL) and RNase A (ITW Reagents, Barcelona, Spain) (final concentration 0.1mg/mL), and incubated for 25 min at 37°C. Proteinase K (VWR Chemicals, Ohio, USA) (final concentration 0.1mg/mL) and SDS (Sigma-Aldrich, Missouri, USA) (final concentration 0.5% v/v) were added and then incubated for 5 min at 65°C after which genomic DNA obtained was purified using an equal volume of SPRI beads and resuspended in EB buffer (Qiagen, Germany). DNA quality was verified with the Quant-iT dsDNA HS kit (ThermoFisher Scientific) assay in an Eppendorf AF2200 plate reader (Eppendorf UK Ltd, United Kingdom).

Illumina sequencing

Genomic DNA libraries were prepared using the Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol. The input DNA was increased 2-fold, and PCR elongation time was set to 45 s. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Switzerland). Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina. Libraries were sequenced using Illumina sequencers (HiSeq/NovaSeq) using a 250bp paired-end protocol. Reads obtained were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cut-off of Q15 [17]. De novo assembly was performed on samples using SPAdes version 3.7 [18], and contigs were annotated using Prokka 1.11 [19]. The 16S rRNA gene sequences detected were used to perform a BLAST search [20] to identify sequences harbouring strains at the species level. Details of the 35 sequences obtained were deposited in GenBank under new accession numbers PQ865836 - PQ865870.

Evolutionary phylogeny and ancestral analysis

This was performed *in silico* with Molecular Evolutionary Genetic Analysis (MEGA 12) using default settings [21]. Sequences were imported into the MEGA software and aligned with the multiple sequence alignment (MUSCLE) program [22] . The evolutionary history and the ancestry were inferred using the maximum likelihood method.

5. Results and discussion

3.1 Bacteria harbouring detected 16S rRNA sequences

The pH of samples ranged from 5.57±0.02 to 7.59±0.06 and the sample (sample 3), which had the most fragments of different species had an acidic pH of 5.71. Overall 35 16S rRNA fragments found in the Ugba samples belonged to 13 species (Table 1). The genus *Proteus* was found in all samples. The predominant species was *Proteus mirabilis* with 19 sequences followed by *Klebsiella pneumoniae and Enterococcus faecalis* with four and two respectively. Two genera namely *Proteus* and *Enterococcus* had sequences from two or more species. The proteus species included *P. mirabilis* and *P. columbae* while that of *Enterococcus included E. faecalis, E. gallinarum E. casseliflavus* among other species (Table 1). Information from other Genbank submissions showed that bacteria harbouring the sequences studied have been isolated from a wide variety of sources in different countries around the world (Table 1). These sources include plants and animals and the main non-living components of ecological systems like water and soil.

The study was initially set up to study *Bacillus cereus* strains that are present in *Ugba* which is why PEMBA selective medium that is selective for the bacterium was used. When other colonies (e.g. swarms of *Proteus*) were observed to grow on it, sequencing was carried out on the mixed colonies that emerged. A common knowledge is that *P. mirabilis* has the propensity to swarm on a wide variety of media and interfere in the identification of other species in a sample. Hence, it may be beneficial to use a different compound to control swarming during cell growth [23].

The dominant *P. mirabilis has* been isolated from *Ugba* by others. Four out of the 13 species identified in this study have been identified by Okorie et al. [24] when they used cultural and molecular techniques to probe enteric pathogenes in *Ugba*. The species they identified among others include *P. mirabilis, E. casseliflavus, E. faecalis, and K. pneumoniae*. The genus *Acinetobacter* was isolated in our study and theirs. While they isolated *A. baumanii*, our investigation detected *A. bereziniae*. Similar to this study their molecular techniques did not detect any *Bacillus* species. Although *Bacillus* is believed to be prevalent in *Ugba*, it was possibly out-competed by *P. mirabilis* and other bacteria in the media used. If a non-selective media is used, various species of *Bacillus* would likely be detected.

Other bacteria that have been isolated by others include the species of *Staphylococcus, Micrococcus, Lactobacillus and other Enterobacteriaceae* [25] while another study [26] also isolated mainly *Bacillus* and Staphylococcus species. An analysis [27] of *Ugba* fermented in the laboratory yielded a lot of *Bacillus cereus*. The genus *Lysinibacillus* was detected in this study as well as our study. While their study found *L. xylanilyticus*, our study detected *L. sphaericus*. To the best of the knowledge of the authors, this is the first time that *L. sphaericus, A. bereziniae P. columbae, Alcaligenes, nematophilus. Chryseobacterium bernardetii, Stenotrophomonas geniculata, Enterococcus gallinarum, Mammaliicoccus sciuri, and Kurthia gibsonii* have been detected in *Ugba*.

Table 1: Organisms and strains isolated from different samples of Ugba in this study.

	Strain	Organism	Source and country of closest relative (Source: Genbank)
	Sample 1		
1	UGB1NG1	*Proteus mirabilis*	Animal waste; China
2	UGB1NG2	*Proteus mirabilis*	Gastropods under mangrove tree; India
3	UGB1NG3	*Proteus mirabilis*	Human faeces; United Kingdom
4	UGB1NG4	*Klebsiella pneumoniae*	Human blood; Taiwan

	Sample 2		
5	UGB2NG1	*Proteus mirabilis*	Human sputum; Czech Republic
6	UGB2NG2	*Proteus mirabilis*	Human sputum; China
7	UGB2NG3	*Alcaligenes nematophilus*	Industrial waste water; Switzerland
8	UGB2NG4	*Lysinibacillus sphaericus*	16S RNA; India
	Sample 3		
9	UGB3NG1	*Klebsiella pneumoniae*	Tracheal aspirate; Russia
10	UGB3NG2	*Klebsiella pneumoniae*	Pig manure; China
11	UGB3NG3	*Klebsiella pneumoniae*	Human gut; Spain
12	UGB3NG4	*Proteus mirabilis*	Duck intestine; China
13	UGB3NG5	*Chryseobacterium bernardetii*	Human DNA; United Kingdom
14	UGB3NG6	*Stenotrophomonas geniculata*	Asian citrus psyllid; United States
15	UGB3NG7	*Acinetobacter bereziniae*	Human urine; United Kingdom
	Sample 4		
16	UGB4NG1	*Proteus mirabilis*	Human sputum; China
	Sample 5		
17	UGB5NG1	*Proteus mirabilis*	Human sputum; China
18	UGB5NG2	*Proteus mirabilis*	Human sputum; China
19	UGB5NG3	*Proteus mirabilis*	Human sputum; China
20	UGB5NG4	*Mammaliicoccus sciuri*	Duck viscera; China
	Sample 6		
21	UGB6NG1	*Proteus mirabilis*	Clinical; China
22	UGB6NG2	*Proteus mirabilis*	Human sputum; China
23	UGB6NG3	*Proteus mirabilis*	Human sputum; China
24	UGB6NG4	*Enterococcus gallinarum*	Human faeces; China
	Sample 7		
25	UGB7NG1	*Proteus mirabilis*	Human DNA; Germany
26	UGB7NG2	*Proteus mirabilis*	Human DNA; Germany

27	UGB7NG3	*Proteus mirabilis*	Human sputum; Czech Republic
28	UGB7NG4	*Enterococcus casseliflavus*	Cloaca from *Gallus gallus*; Germany
	Sample 8		
29	UGB8NG1	*Proteus mirabilis*	Human sputum; China
30	UGB8NG2	*Proteus mirabilis*	Human sputum; China
31	UGB8NG3	*Kurthia gibsonii*	Water; China
	Sample 9		
32	UGB9NG1	*Proteus mirabilis*	Human sputum; China
	Sample 10		
33	UGB10NG1	*Enterococcus faecalis*	Pet food; Switzerland
34	UGB10NG2	*Enterococcus faecalis*	Fecal matter from *Gallus gallus* domesticus; Canada
35	UGB10NG3	*Proteus columbae*	16S RNA; China

5.1.1 Significance of bacteria harbouring detected sequences

Bacteria that occur in foods will not normally cause any health concerns if the numbers are controlled. Some may be part of the natural flora and may cause only spoilage whereas others get into the food through process contamination and may become pathogenic especially if the numbers increase. In this study, several clones of *P. mirabilis* were detected. When it is found in food, it suggests that the food was not prepared or stored properly and may be contaminated with faecal residues [28]. The bacteria has clinical significance because it has been associated with several infections in humans. These infections include appendicitis, foodborne and spontaneous gastroenteritis [29], urinary tract [30] and surgical site [31] infections. The other *Proteus* species (*P. columbae*) detected in the study was proposed just seven years ago [32] and the pathogenicity is yet to be fully determined.

K. pneumoniae is prevalent in meat and raw food [33] and has been found to cross-contaminate from market to market [34]. The bacteria is now a global concern because it can cause septicemia, urinary tract infections, pneumonia, meningitis, wound burn, surgical site infections, and liver abscesses in both the elderly and young people [35]. Multi-drug resistant strains have been found to overlap among isolates from food and humans [36]. Another species of clinical importance detected in this study is the genus *Enterococcus*. The species *E. faecalis* may occur in ready-to-eat food [37] and occurs in the gastrointestinal tract of animals. The bacterium is known for its antibiotic resistance and can be used as an indicator of faecal contamination. Several infections like peritonitis, endocarditis and bacteremia have been associated with the bacteria.

The properties of the other *Enterococcus species namely E. gallinarum E.* and *casseliflavus* have been reviewed. In the review [38], it was highlighted that both are vancomycin-resistant. Also, *E. gallinarum* may be found in fish, meat and cheese and even though it occasionally occurs in the gastrointestinal tract, it is not considered as an indicator of faecal contamination. Another highlight was that *E. casseliflavus* has been observed in forage plants and implicated with *E. gallinarum* in causing health infections in the urinary tract, bloodstream and surgical wounds.

The other species detected in the study do not cause infections on the scale of the aforementioned species. *A. nematophilus* is mainly an environmental contaminant and can cause gastroenteritis with the help of antibiotic resistance genes it harbours [39]. Also, *Acinetobacter bereziniae* is regarded as an opportunistic pathogen that possesses multi-drug resistance genes and it has been reported to cause infections in hospitals. The bacteria have been isolated from human milk [40]. Furthermore, *Chryseobacterium* species can cause nosocomial infections [41] while *Mammaliicoccus sciuri*, previously classified as an occasional pathogen, possesses resistance and virulence transfer genes [42]. The *bacteria Kurthia gibsonii* may be found in milk and meat products and can produce antimicrobial peptides [43] but information on its genomes is limited [44]. *Lysinibacillus sphaericus* is mainly found in the environment and has been used to control malaria and other tropical diseases [45] while *Stenotrophomonas geniculata* is an endophyte that can live in plant tissues and can be used to control postharvest fungi [46]. It is possible that the two bacteria mentioned found their way into *Ugba* from the field.

3.2 Evolutionary phylogeny of sequences

The only *P. columbae* detected and all the *P. mirabilis* strains arose from one node apart from strain UGB3NG4 which was found to be in a different clade (Fig.1). This indicates that the majority of the *P. mirabilis* detected may be the same clones. All the *K. pneumoniae* strains were in the same monophyletic group apart from strain UGB1NG4 while the two *E. faecalis* strains did not originate from the same node (Fig. 1). The strain UGB10NG1 formed a clade with other *Enterococcus* genera while UGB10NG2 fell under a monophyletic group with other bacteria. The pattern observed in *P. mirabilis K. pneumoniae* and *E. faecalis* suggests at least two distinct lineages or species divergence. The species *Chryseobacterium bernardetii* did not share any relationship with any other bacteria. When the overall phylogeny is considered, it appears that most of *P. mirabilis* got into the samples much later which suggests processing contamination.

The evolutionary phylogeny for different *Ugba* bacteria is yet to be reported. The phylogeny of the main Enterobacteriaceae bacteria detected in this study from other sources is well studied. A report [47] pointed out that *P. mirabilis* can be divided into three subspecies and that subspecies 1 represents 97% of all genomes and consists of related clonal groups. Another comprehensive analysis [48] of over 3700 genomes from 58 countries ascertained that there were 17 distinct clusters. Furthermore, a proposal [49] to use core genome multilocus sequence typing (cgMLST) as a method to identify clonal groups (CGs) in *P. mirabilis* found 205 which were influenced by regional differences. If whole genome sequences of the strains are obtained in future, it will help establish what their subspecies, clusters, and CGs are.

For *E. faecalis*, it has been pointed out [50] that there are two distinct lineages based on whole genome sequencing studies. These include lineage (clade A) which is hospital-associated and lineage (clade B) which is community-associated. Considering that the two strains detected in this study resolved into different clades, we posit that one may be lineage clade A and the other lineage clade B. Further work is required to establish the true lineages. The genetic diversity of other species in this study (*K. pneumoniae*) which showed multiple clones has also been studied by others. A study [51] clustered over 16000 genomes obtained from around the world and found that up to 60 clusters were established. The data helped to trace the global route of the evolution of *K. pneumoniae*. It was worrying that 52% of the strains traced harboured genes associated with carbapenem resistance. It would be interesting to investigate and establish what cluster, the four strains detected in this study belong to and ascertain if they are carbapenem-resistant.

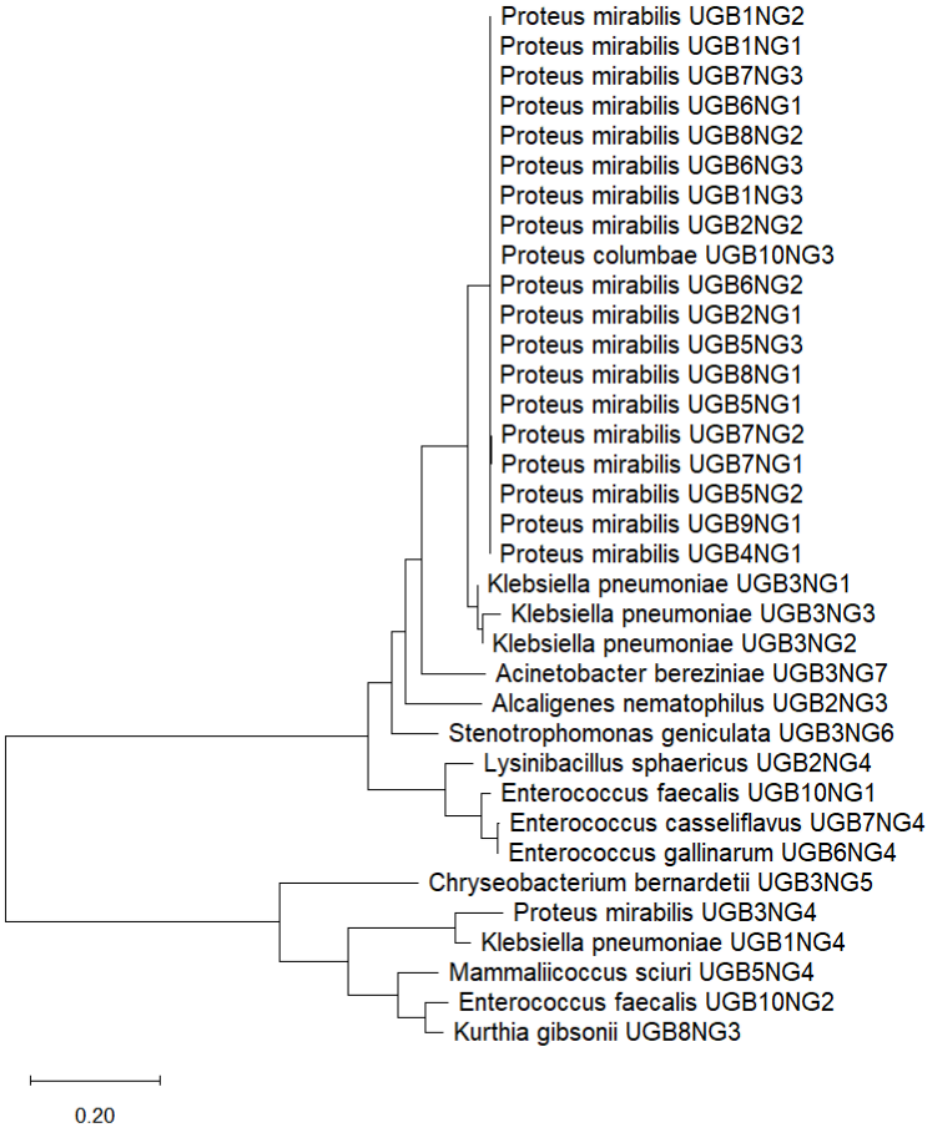

Figure 1. Evolutionary analysis by the Maximum likelihood method: The phylogeny was inferred using the Maximum Likelihood method model of nucleotide substitutions and the tree with the highest log-likelihood. The analytical procedure encompassed 35 taxons with 1,857 positions in the final dataset. Evolutionary analysis was conducted in MEGA12 [21]

3.3 Ancestry of sequences

The species *Kurthia gibsonii*, was found to be the earliest ancestor following ancestral sequence analysis and was followed by a *P. mirabilis* strain UGB7NG3 (Fig 2). This suggests that *P. mirabilis* may occur at various points in the processing of the product. *Kurthia gibsonii* occurs mainly in the environment and it likely got into the sample during harvest from the field as pointed out earlier. The *K. pneumoniae* strain UGB1NG4 had a longer branch length than other *K. pneumoniae* strains and this could mean that it occurred in Ugba samples first.

The point at which different bacteria got into the seeds is unknown, hence it may be beneficial to conduct a farm-to-fork study to determine species present when the seeds were on the field, at harvest, during processing and storage until it is finally packaged for sale. The ancestry and succession of bacteria species and the mechanisms that drive microbial succession in *Ugba* are yet to be well studied. A study [52] on succession of bacteria during fermentation of *Ugba* for three days found that up to 10 bacteria species were present at the beginning but only *Bacillus* and *Lactobacillus* species were present after 72 hours. The bacteria that were present at the beginning included *Staphylococcus aureus, Micrococcus, E. coli* and *Klebsiella* species. Other species were *Streptococcus, Pseudomonas, Proteus and Salmonella*.

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Food that is fermented is now described as "made through desired microbial growth and enzymatic conversions of food components" [53] and a lot of factors have been found to influence succession. A report highlighted [54] that differences in the assembly of microbiota may be influenced by climate conditions, geographical location, agricultural practices and processing after harvest. Despite these differences the microbial content and their primary metabolic functions in natural fermentations are consistent. Hence it can be concluded that the ancestral microbiome of *Ugba* in the last century has not changed much since the same flavour and texture after fermentation by microbes has remained unchanged for decades.

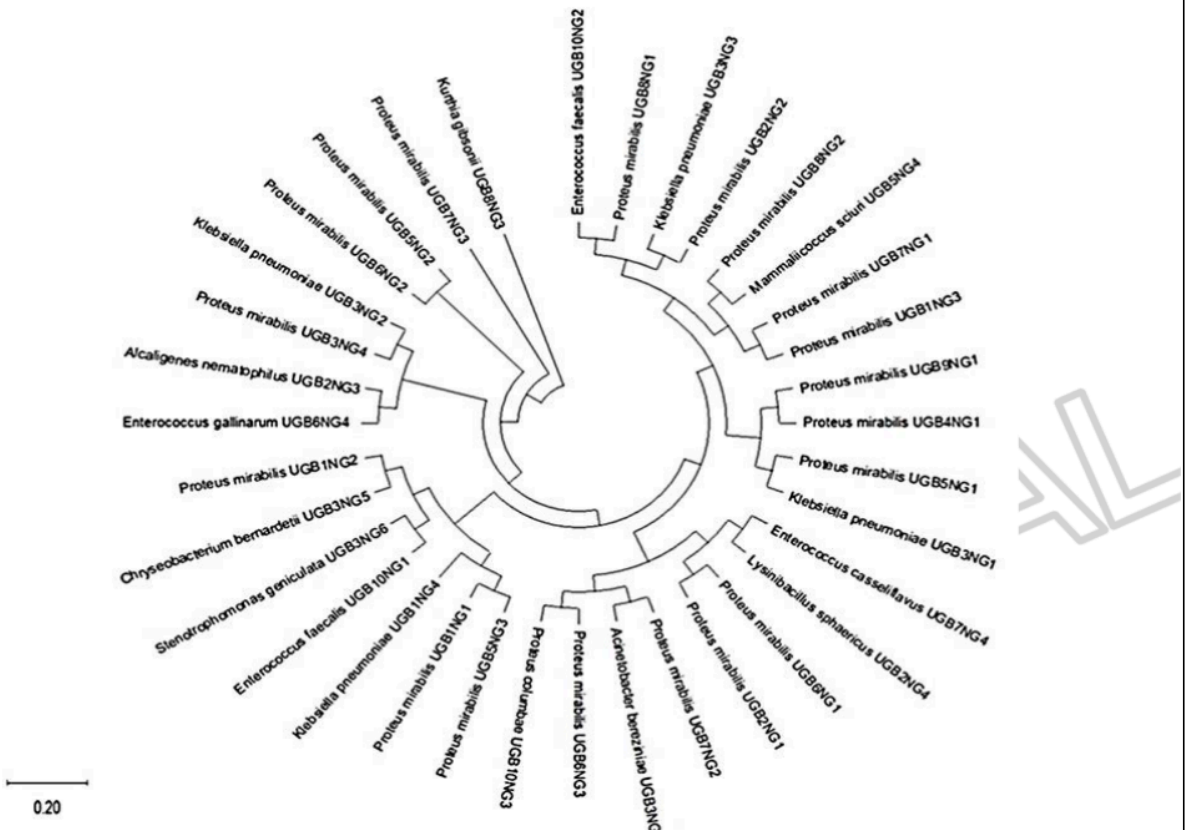

Figure 2 - Inferred Ancestral Sequences: Ancestral states were inferred using the Maximum Likelihood method. The tree shows a set of possible nucleotides (states) at each ancestral node based on their inferred likelihood. The analytical procedure encompassed 35 nucleotide sequences with 1,857 positions in the final dataset.

6. Conclusion

Bacteria of food safety concern were found to be present in fermented *Ugba* seeds and there may be distinct evolutionary lineages present in samples from different environment. Some of the species detected have been clinically proven to be pathogens, hence a pretreatment of the product to eliminate pathogenic bacteria before sale may help prevent food outbreaks. The ancestry of the sequences in the products studied showed that the species *Kurthia gibsonii*, was the earliest ancestor. Considering that species that have not been previously associated with the product were detected, more work is required to ascertain the microbial dynamics and their metabolic products in different environments. This will help to establish the *Ugba* fermentation aspects that are relevant to food safety.

7. Author contributions

ON, HO: Conception, planning, resources, writing, proofreading and editing

VII, SIO, JCN, KCE: Planning, resources, writing, proofreading and editing

PCC, IUO, NCE, NJA, INN, ECC, MMO: Resources, writing, proofreading and editing

7.1 Conflicts of interest

The authors declare that there are no conflicts of interest.

7.2 Funding information

The study was supported by the International Development Fund (IDF/2022) of Applied Microbiology International, United Kingdom.

7.3 Acknowledgements

The work is dedicated to the memory of Professor V.I. Ibekwe

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