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Microbial and Molecular Evaluation of Honey from Northern and Southern Guinea Savanah Zones of Niger State, Nigeria

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ABSTRACT

Honey is a natural sweet substance produced by honey bees, from nectar of plant flowers and honey dew. It has low water activity because of the great amount of sugar and antimicrobial compounds derived from the nectar of flowers. Despite all the honey antimicrobial properties, some species of microorganisms can survive and cause damage to honeybees or consumers. The study was aimed at evaluating the microbial content of honey from the Northern and Southern Guinea Savannah Zones of Niger State, Nigeria. The honey samples were collected from the two ecological zones based on purposive sampling. This study evaluated the quality of honey in terms of its microbial content. The total bacterial counts (TBC) in the Northern Guinea Savannah ranged from 4.03×10^{-3} to 4.65×10^{-3} cfu, while the Southern Guinea Savannah had a TBC range of 3.50×10^{-3} to 4.80×10^{-3} cfu. The isolated micro-organisms were Bacillus species and Staphylococcus species. Based on the parameters investigated, the quality of honey was good, falling within international recognized standard. Microbial counts in all samples were within the FDA-accepted limit of less than 15,000 CFU per gram, indicating low levels of contamination. The isolated bacteria, primarily from the genera Bacillus and Staphylococcus, were likely introduced through environmental factors or handling practices rather than being native to the honey. The absence of coliforms (TC) supports honey's natural antimicrobial properties. Phylogenetic analysis revealed genetic variation among the isolated species, showing evolutionary relationships and clustering into distinct groups. The findings reaffirm honey's antimicrobial qualities, while emphasizing the importance of proper handling to prevent contamination. Overall, the low microbial presence in the honey samples ensures their safety and marketability.

Keywords: *Guinea Savanah, Honey, Microbial, Niger State*
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INTRODUCTION

Honey is the naturally sweet substance produced by *Apis mellifera* bees that gather plant nectar, secretions from living plant parts, or excretion from insects that feed by sucking on plants (Ebrahimi *et al*, 2023). The bees gather, transform, and combine these elements with their unique substances, then proceed to dehydrate, and store them in honeycombs for the maturation process (Council, 2002; Singh 2022; Aminuwa *et al*., 2024a; Walker *et al*., 2022). It is processed by honeybee (*Apis mellifera*) (Pridal *et al*., 2023), a social insect known as the most economically valuable insect because of its honey production and pollinating activities (Adeyemi *et al*., 2021). Honeybees have been described as the most useful of all insects because of their ability to provide the basic services necessary for the survival of human and other forms of life (Khalifa *et al*., 2021; Brant *et al*., 2022). In contrast, other insects like mosquitoes, contribute to global mortality, causing over a million deaths annually (Aminuwa *et al*., 2018). Honey is well known for its health benefits, having antibacterial, antioxidant, anti-inflammatory, anti-mutagenic, anticarcinogenic, and, bacteriostatic qualities (Albaridi, 2019), and it also helps to cure wounds and sunburns (Samarghandian *et al*., 2017; Ahmed *et al*., 2018; Jia *et al*., 2020; Aminuwa *et al*., 2024b) In its composition, honey has minor compounds, like enzymes, minerals, amino acids, polyphenols, vitamins, and major compounds, such as monosaccharides (glucose and fructose) (De Silva *et al*., 2016) vary depending on its botanical origin (plant species visited by bees), seasonal differences, climatic conditions, geographical origin and applied processes (Alqarni *et al*., 2016; Matzen *et al*., 2018a, 2018b; Seraglio *et al*., 2019; Jia *et al*., 2020; Margaoan *et al*., 2021; Aminuwa *et al*., 2024a).

Honey can transfer many of the therapeutic qualities of plants, so it could be used as a way of delivering these qualities (Brudzynski, 2021). Therefore, honey is regarded as a vital natural resource that can be utilized in new treatments without causing the side effects that frequently go along with the use of synthetic chemical drugs (Ahmed *et al*., 2018; Szweda, 2017). Because honey can contain a variety of antimicrobial and prebiotic compounds, depending on the source of the flowers, it is regarded as a functional food that is good for both human and animal health (Grabowski & Klein, 2017), especially in favour of gut microbiota balanced composition (Mohan *et al*., 2017).

Honey can be used as a natural "sweetening agent" without further processing (Ibrahim *et al.,* 2021). Honey is considered as one of the sweetest natural foods in Nigeria and the world at large in terms of its nourishment and therapeutic properties (Ame *et al*., 2022). It can be used as food, for religious ceremonies, and as medicine for both humans and animals (Hossain *et al*., 2021). It also serves to feed animals and for sweetening drugs for children (George and Shuaib, 2009; Ibrahim *et al.,* 2021). The benefits obtained from the consumption of honey can be overshadowed by adulteration (Adadi and Obeng, 2017; Hidalgo *et al*., 2020). Adulteration of honey occurs by the addition of different materials. Addition of foreign substances such as molasses, starch solution, glucose, sucrose, water, and inverted sugar to honey has been reported (Damto, 2021). The addition of some foreign substances can microbially contaminate honey (Naila *et al.,* 2022). Good quality honey must be free from pathogenic microorganisms that cause enteric illnesses (Maitan *et al.,* 2021). The microorganisms that are found in honey are those that can tolerate high sugar content, acidity and antimicrobial properties of honey, they include certain yeast and spore- forming bacteria (Ebrahimi *et al*., 2022). Yeast present in honey could cause fermentation thereby resulting in formation of alcohol and carbon dioxide; the alcohol further gets oxidized into acetic acid in the presence of oxygen, thus causing sour taste (Agbagwa *et al*., 2011). Honey has several sources of microbial contamination; the primary source includes; pollen, the digestive tract of honeybees, dust, air, soil and nectar which are somewhat difficult to eliminate (Saklani and Kumar, 2021). On the other hand, secondary sources of microbial contamination due to honey handlers and processing are easier to control by the application of good processing practices (Machado *et al*., 2022). The major microbial contaminants of honey include molds and yeast as well as the spores of *Bacillus* and *Clostridium* species (Adenekan and Augustus, 2020), their counts being indicative of honeys' commercial quality and safety. Ayansola and Banjo (2012) reported the microbiological composition of samples of honey marketed in the six states of southwestern Nigeria was assessed. Seven species of heterotrophic fungi and four species of bacteria were isolated. The bacteria species were *Klebsiella edwardsii, K. pnuemoniae, Pseudomonas aeruginosa and Staphylococcus aureus.* The identified fungi were Cl*adosporium wernecki, C. herbarum, Cephalosporium sp; Mucor mucedo, Rhizopus rubrum, Trichophyton rubrum, and Scopulariopsis brevicaulis.* Two species of pathogenic bacteria were isolated from honey samples: *S. aureus* in Ogun State and *K. pneumoniae* in Ekiti State. Anaerobes have been reported as the predominant microflora, including *Clostridium botulinum*, which is responsible for infant botulism (European, Commission, 2002; Maikanov *et al.,* 2019; Rosli *et al.,* 2020). Several species of *Bacillus* have also been detected in honey (Pomastowski *et al.,* 2019; Ndukwe and Agbagwa, 2020; Balogun and Tunde, 2020). Other significant groups of microbes responsible for fermentation and spoilage of honey are yeasts and moulds (Kamal *et al.,* 2019; Wang *et al.,* 2019; Brudzynski, 2021).

Molecular techniques based on DNA have become the methods of choice since they tend to be quick, precise and more reliable (Romero *et al.,* 2019) to the extent of identifying bacteria and fungi found in the honey samples to species level. Bacteria and fungi present in honey can serve as the source of DNA, which can be used for the identification of their origin using DNA technology (Wirta *et al*., 2021).

There are reports on the healing effect on burns and wounds and some chemical and physical properties of Nigerian honey (Mokhtari *et al*., 2019; John-Isa *et al*., 2019; Nweze *et al*., 2020; Hossain *et al*., 2020; Jafar *et al*., 2020; Ajobiewe *et al*., 2022; Adeyemi *et al.,* 2023).

There are also enormous reports on the physicochemical, microbial and medicinal properties of honey from around the world (Nguyen *et al*., 2019; Zarei et al., 2019; Bobis *et al*., 2020, Berhanu *et al.,* 2022). In Nigeria, the consumption of honey as food has increased considerably in recent years. So far, there are few data reported on microbial content of honey produced in Niger State. Therefore, the present study was carried out to provide information on the microbial properties and characterization of bacteria found in the honey samples from the area.

MATERIALS AND METHODS

Microbiological analysis

Preparation of agar

The method of preparation of each agar was based on manufacturer's instruction. Microbial analysis was carried out using the method described by Harrigan and McCance, (1976). The viable cell count of the samples was determined by carrying out serial dilutions of the stock solution of each honey sample to obtain dilutions of $10^{-1}$to 10^{-4}. That is, 10 ml of each honey sample was measured and transferred aseptically into 90 ml of 0.1% sterile peptone water to form the stock solution. One milliliter of each stock solution was transferred into 9 ml of 0.1% sterile peptone water (10^{-1}) and this was serially diluted until 10^{-3} dilution was obtained. A solution of 0.1 ml of 10^{-3} dilutions was then aseptically transferred into sterile Plate CountAagar (PCA), Saboroud Dextrose Agar (SDA), Mackonkey Agar (MAC) and Mannitol Salt Agar (MSA) for bacterial and fungal counts. A sterile bent glass rod was used to spread the inocula (diluted sample) on the surface of the culture media. The inoculated plates were then incubated at 37°C for 24 h (Plate count agar). After the incubation period, the number of the bacteria colonies and fungal growth was counted using a colony counter (Stone, Staffordshire, ST15 OSA. USA model) and the results were recorded and expressed as colony forming unit per milliliter (Cfu/ml) of the sample using the equation below:

Cfu/ml = Total number of colonies counted ×dilution factor/Volume of inoculum, (Oshomah, 2017)

Characteristics of isolates

Discrete colonies were streaked into fresh Nutrient Agar to obtain pure culture slants of bacteria. The colonies to be identified were picked from the various media to obtain pure culture and maintained on slants (Nutrient Agar slants). Biochemical tests were also carried out on the different isolates.

Identification and biochemical tests following the procedure of Chessebrough, (2004)

The colonial morphology characteristics of the bacteria organisms on the different media were observed. The suspected isolates were then further characterized and identified based on their physiological and biochemical characteristics; Gram staining, Catalase, Citrate utilization, Indole, Methyl red-Voges Proskauer and Motility test.

Molecular characterization of bacteria

DNA extraction

The bacteria isolates were sub-cultured on nutrient agar, allowed to stand for 24 h in an incubator (37ºC). The isolates were then inoculated into Luria Broth (LB) agar to get a fresh culture and to enable bacteria isolated from honey to grow for the DNA extraction. Bacteria DNA (via LB medium) from honey samples were taken to the Center for Biotechnology Research laboratory ABU, Zaria and were extracted using quick- DNA^{Tm} mini-Prep Plus kit (Zymo Research Corporation, CA, USA) as described by the manufacturer. Briefly, 200 µl of each sample was transferred into 1.5 ml micro-centrifuge tube and 200 µl of Bio-fluid and Cell Buffer (Red) plus 20 µl of Proteinase K were added, mixed thoroughly and then incubated at 55ºC for 10 min. To the digested sample, one volume (420 µl) of the genomic binding buffer was added. The content was mixed thoroughly then transferred into a Zymo-Spin^{TM} IIC-XL Column in a collection tube and spurned at 12,000 rpm for 1 min. The collection tube was discarded with the flow through. 400 µl of DNA pre-wash was then added to the column in a new collection tube and centrifuged at 12,000 (rpm) for 1 min. The flow through was discarded and 700 µl of the g-DNA wash buffer was added, spurn at 12,000 (rpm) for 1 min; 200 µl wash buffer was added and spun to further wash the g-DNA in the Zymo-spin column.

Finally, to elude the g-DNA from the Zymo-spin column, the column was transferred into a clean micro centrifuge tube and 50 µl of the elution buffer was added and incubated at room temperature for 5 min. The tube was then transferred into the centrifuge machine spurn down to collect the pure g-DNA. The eluted DNA was checked for purity and quantification using Nano drop spectrophometer at 260 nm and 280 nm and was stored at -20ºC prior to Polymerase Chain Reaction (PCR).

Polymerase chain reaction

The DNA was amplified using PCR with primers designed

Table 1: Primer sequences used for Staphylococci Multiplex PCR Reactions of Honey Samples.

Species	Primer	Sequence	Amplicon (bp)
Staphylococcus species	TSTAG422	GGCCGTGTTGAACGTGGTCAAATCA	370
	TSTAG765	TIACCATTTCAGTACCTTCTGGTAA	
S. xylosus	XYLF	AACGCGCAACGTGATAAAATTAATG	539
	XYLR	AACGCGCAACAGCAATTACG	
S. saprophyticus	SAP 1	TCAAAAAGTTTTCTAAAAAATTTAC	221
	SAP 2	ACGGGCGTCCACAAAATCAATAGGA	
S. epidermidis	SE7051	ATCAAAAAGTTGGCGAACCTTTTCA	124
	SE705-2	CAAAAGAGCGTGGAGAAAAGTATCA	
S. aureus	SA4421	AATCTTTGTCGGTACACGATATTCTTCACG	108
	SA4422	CGTAATGAGATTTCAGTAGATAATACAACA	

Source: Park *et al.*, 2007.

Table 2: Primer Sequences for Bacilli Multiplex PCR Reactions of Honey Samples

Species	Primer	Sequence	Amplicon (bp)
B.cereus group	BCGSH1F	GTGCGAACCCAATGGGTCTTC	400
	BCGSH1R	CCTTGTTGTACCACTTGCTC	
B. anthracis	BASH2F	GGTAGATTAGCAGATTGCTCTTCAAAAGA	253
	BASH2R	ACGAGCTTTCTCAATATCAAAATCTCCGC	
B. thuringiensis	BTJH1F	GCTTACCAGGGAAATTGGCAG	299
	BTJHR	ATCAACGTCGGCGTCGG	
B. cereus	BCJHF	TCATGAAGAGCCTGTGTACG	475
	BCJH1R	CGACGTGTCAATTCACGCGC	
B. mycoides	BMSHF	TTTTAAGACTGCTCTAACACGTGTAA	604
	BMSHR	TTCAATAGCAAAATCCCCACCAAT	

Source: Martineau *et al.*, 2001.

by Park *et al.* (2007) and Martineus *et al.* (2001) as shown below in (Tables 1 and 2) respectively.

Staphylococci multiplex PCR amplification conditions and detection of the amplified products

The primers listed in (Table 1) were synthesized by MWG (Ebersberg, Germany). The *S. epidermidis*-specific primers Se705-1, Se705-2, the *S. aureus*-specific primers Sa442-1, Sa442-2, *S. saprophyticus*- specific primers Sap1, Sap2, *Staphylococcus*-specific primers Tstag765 and TstaG422 were designed by Martineau *et al.* (2001). The *S. xylosus*-specific primers XYL F and XYL R were designed by Morot-Bizot *et al.* (2003). Staphylococcal DNA used for the amplification was directly picked from LB agar plates. The 25 µl PCR mixture contained 0.5 µl of the primers XYL F and XYL R, 0.5 µl of the primers Sap1 and Sap2, 0.4 µl of the primers Se705-1and Se705-2, 0.4 µl of the primers Sa442-1 and Sa442-2, 0.2 µl of the primers Tstag765 and TstaG422, 0.5 µl of Deoxyribonucleoside Triphosphate (dNTPs), 1.5 µl MgCl2 and 0.5 µl of Taq DNA polymerase in 1X buffer according to the manufacturer's instructions. PCR was performed under the following conditions: 3 min at 94°C, then 40 cycles of 1 sec at 95°C, 30 s at 55°C, 30 s at 72°C and a final hold of 3 min at 72°C with PCR thermal cycler. Five microlitres of the PCR reaction mixture were analyzed by electrophoresis through a 2% high resolution agarose gel in 1 X TBE buffer. The sizes of the amplification products were estimated by comparison with a 100-bp molecular size ladder. Gels were stained with ethidium bromide and digitalized with the Gel documentation system (300p) Cleaver Scientific Nanopac.

Bacilli multiplex PCR amplification conditions and detection of the amplified products

Specific oligonucleotide primers were designed for *B. anthracis*, *B. thuringiensis, B. cereus*, *B. mycoides* and *B. cereus* group bacteria by Park *et al.*, (2007). The sequences of the primers used in this study are shown in Table 3.2. Multiplex PCR Conditions of the genomic DNA used in the PCR assays was extracted from Bacillus sp grown on lauria broth Agar Total genomic DNA (gDNA) of bacteria from honey samples were extracted using Quick-DNA^{TM} Miniprep Zymo Research kit according to the manufacturer's protocol. Extracted DNA purity and quantification was determined using a Nanodrop Spectrophotometer at a wavelength of 260 and 280 nm .The multiplex PCR was performed with a thermo cycler, the total reaction volume of 25 µl of the PCR mixture contained 1×PCR buffer (MgCl$_2$, 0.5µl dNTP, 0.5µl of the

Table 3: Microbial characteristics of honey samples obtained from northern and southern guinea savannah zone

Location	Sample	Staph ($\times 10^{-3}$ Cfu)	TBC ($\times 10^{-3}$Cfu)
Northern	Unprocessed	2.05±0.05	4.03±0.03
	Processed	2.50±0.50	4.65±2.65
	Retailer	4.50±1.32	4.50±0.99
	P-value	0.38	0.96
Southern	Unprocessed	3.05±1.95	3.50±0.50
	Processed	2.15±0.13	4.15±0.13
	Retailer	2.80±0.59	4.80±0.59
	P-value	0.79	0.79

Key: CFU=Colony Forming Unit
TBC=Total Bacterial Count

primers, 0.5µl template DNA, and 0.5µl Taq polymerase. The reaction parameters were an initial denaturation at 94°C for 5 min, 30 cycles of amplification with denaturation at 94°C for 30 sec, annealing at 63°C for 30 sec, an extension at 72°C for 30 sec, and final extension of the incompletely synthesized DNA at 72°C for 5 min. The PCR amplicons were analyzed by agarose gel electrophoresis.

Agarose gel electrophoresis

Five microlitres (0.5µl) of the PCR products were loaded onto 2.5% agarose gels containing 0.5µl ethidium bromide and subjected to electrophoresis for 30 min at 100 V in a 0.5×TAE buffer. The required amount of Agarose (0.8 g) was weighed and diluted in a 150 ml conical flask and 100 ml of 1X TAE buffer was added to prepare 2.5% solution. The solution was completely melted in a microwave oven (MX3 10TCSL) model for 3 min at 100ºC. The solution was allowed to cool to 60ºC and EZ-Vision®Bluelight Nucleic Acid Dye (AMRESCO, LLC) was added and mixed gently. The comb was positioned 1mm above the plate so that a complete well was formed when Agarose was added. The gel was allowed to set for 45 min at room temperature. Both the comb and rubber cork were removed carefully and the gel was mounted in the electrophoresis tank with TAE buffer. Five microliters of PCR product from each tube was mixed with 1 µl of 6X gel loading dye; loaded into the gel wells and then electrophoresed on 1% Agarose gel at 100Volt (V) for 25 min in 1X TAE buffer. A 100bp DNA Ladder was loaded and run along with the samples in the same gel. Finally, the separated products were visualized and documented on a Cleaver Scientific Nanopac (300p) gel documentation system.

Sequencing, alignment and phylogenetic analysis

The positive PCR products were sent to Inqaba Biotechnology Ltd. South Africa for purification and sequencing; the sequenced genes were subjected to Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) and data base were compared with references from GenBank. Phylogenetic analysis was conducted using maximum likelihood algorism according to Kumar *et al*. (2018).

Data and sequence analyses

The microbial content of the honey samples collected from the two vegetation zones. All analyses were carried out at 5% significance level using SPSS version 26. The obtained sequences were subjected to Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) and data base compared with references from GenBank and MEGA X was used to construct the phylogenetic tree.

RESULTS

Microbial analysis of honey

The staphylococcus counts were 2.05×10^{-3} cfu^{-1}, 2.50×10^{-3} cfu and 4.72 ×10 cfu for the unprocessed, processed and retailed samples for Northern Guinea Savannah zone while the mean value of 3.05×10^{-3}cfu, 2.15 $\times10^{-3}$ cfu and 2.80×10^{-3} cfu for the processed, processed and retailed samples from the Southern Guinea Savannah of Niger state respectively. The TBC in Northern Guinea Savannah zone were from range of 4.03- 4.6510^{-3} cfu and 3.50- 4.80 10^{-3} cfu for Southern Guinea Savannah Zone (Table 3).

Electrophoregram of PCR Amplicons Obtained from Honey Samples

Plate 1 showed Lane 1= {B.mycoide (604bp), B. cereus (475bp), *B. anthracis* (253bp)} Lane 2= *B. anthracis* (253bp), *B. cereus* (475bp), *B. thuringiensis* (299bp)} Lane 3=*B. anthracis (*253bp) Lane 4= (Negative control) Lane 5= {B. *thuringiensis* (299bp), *B. anthracis* (253bp)} Lane 6 and 7= *B. mycoides* (604 bp).n Plate 2 also showed Lane 4, (*S. xylosus (539*bp), *Staphylococcus*

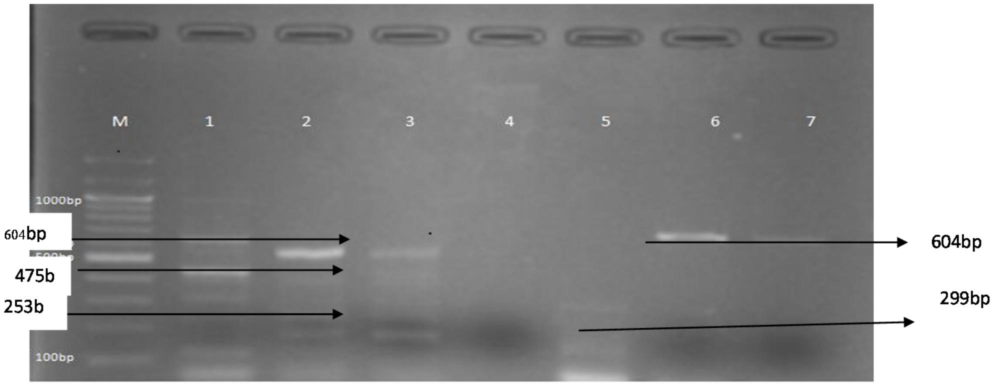


Plate 1: Electrophoregram of multiplex PCR Amplicons Obtained with the Strain of Gram-positve cocci Isolated from Honey Samples.KEY: Lane M= (100 base pair molecular size marker) Lane 1= {B.mycoide (604bp), B. cereus (475bp), *B. anthracis* (253bp)} Lane 2= *B. anthracis* (253bp), *B. cereus* (475bp), *B. thuringiensis* (299bp)} Lane 3= *B. anthracis (*253bp) Lane 4= (Negative control) Lane 5= {B. *thuringiensis* (299bp), *B. anthracis* (253bp)} Lane 6 and 7= *B. mycoides* (604 bp).

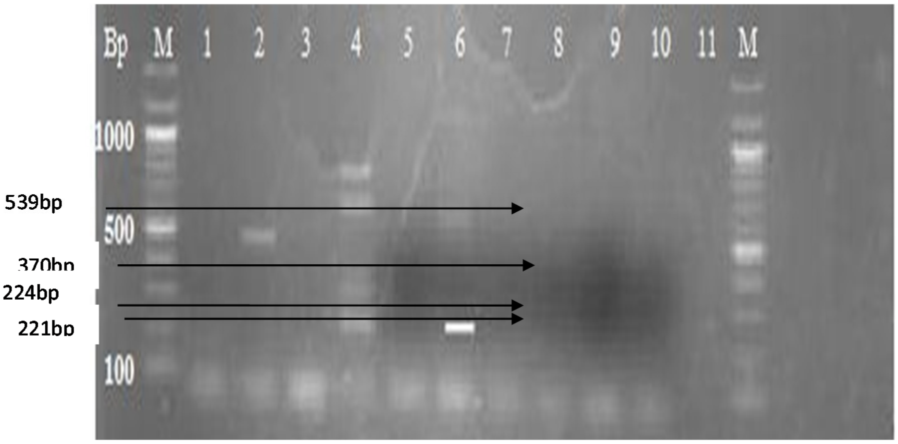
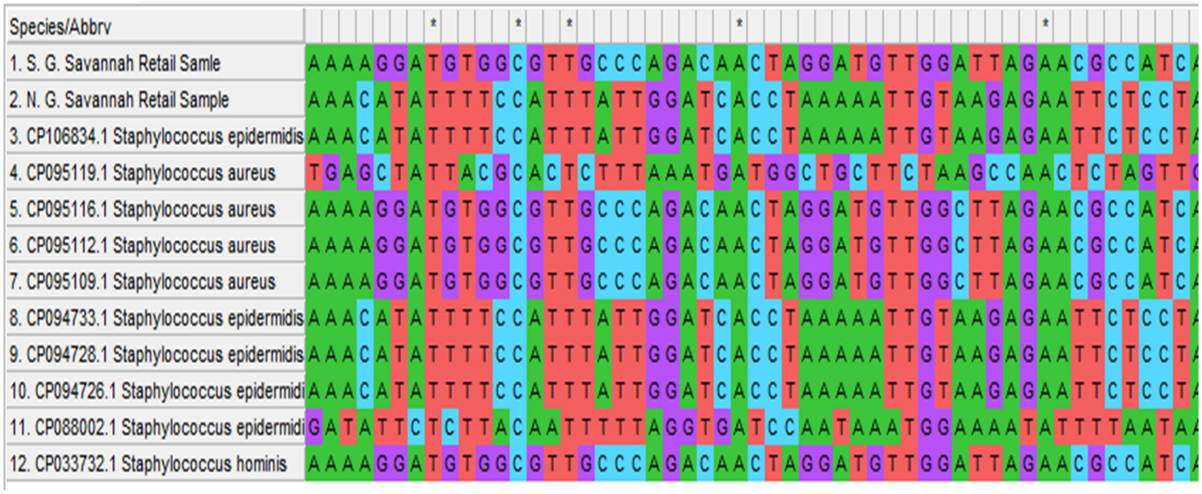


Plate 2: Electrophoregram of Multiplex PCR Amplicons Obtained with the strain of Gram-positve rod Isolated from Honey Samples.
Key: Lane M, (100 base pair Molecular DNA-Ladder); Lane 4, *S. xylosus* (539bp)*, Staphylococcus genus* (370bp), *S. saprophyticus* (221bp), *S. epidermis* (124bp); Lane 6, *S. saprophyticus* (221bp) and *S. epidermis* (124bp); Lane 11, (Nagative control).

*genus (370*bp), *S. saprophyticus (221*bp), *S. epidermis (124*bp); Lane 6, *S. saprophyticus* (221bp) and *S. epidermis* (124bp); Lane 11, (Nagative control).

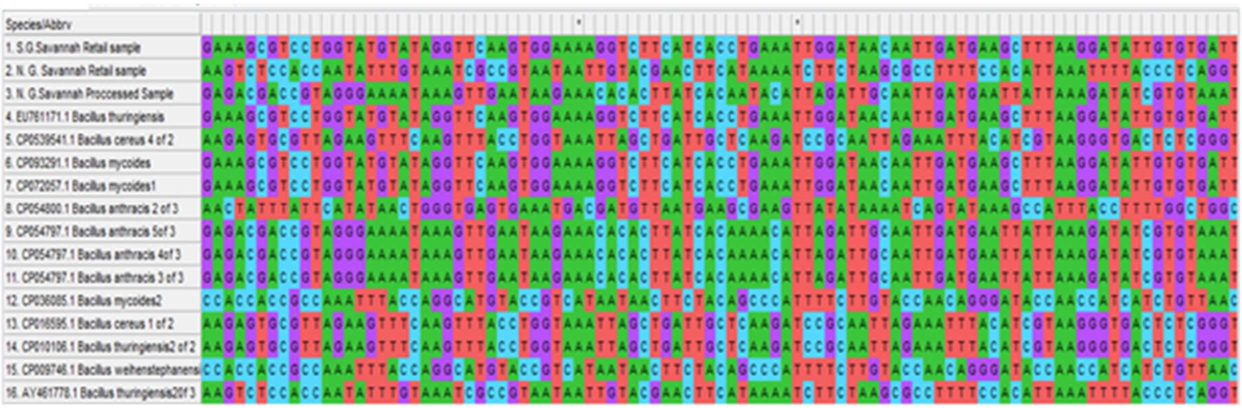
Multiple sequences alignment from staphylococcus species and bacillus species (Northern and Southern Guinea Savannah Zones)

Sequences number 1 and 2 are sequences from the study site. Sequences number 3-12 are bacterial sequences obtained from the GenBank (USA, Kenya, Germiny and Somalia): with accession number: CP033732.1, CP095119.1, CP095112.1, CP095109.1, CP088002.1, CP106834.1, CP094733.1, CP094728.1 and CP094726.1. Star (*) indicate the position of nucleotide alignment. Sequences number 1-3 are sequences from the study site (Figure 1). Sequences number 4-16 are bacterial sequences obtained from the GenBank (USA, Kenya Germany, Poland and South Korea): with accession number: CP016595.1,



Species/Abbrv	
1. S. G. Savannah Retail Samle	AAAAGGATGTGGCGTTGCCCAGACAACTAGGATGTTGGATTAGAACGCCATC
2. N. G. Savannah Retail Sample	AAACATATTTTCCATTTATTGGATCACCTAAAAATTGTAAGAGAATTCTCCT
3. CP106834.1 Staphylococcus epidermidis	AAACATATTTTCCATTTATTGGATCACCTAAAAATTGTAAGAGAATTCTCCT
4. CP095119.1 Staphylococcus aureus	TGAGCTATTACGCACTCTTTAAATGATGGCTGCTTCTAAGCCAACTCTAGTT
5. CP095116.1 Staphylococcus aureus	AAAAGGATGTGGCGTTGCCCAGACAACTAGGATGTTGGCTTAGAACGCCATC
6. CP095112.1 Staphylococcus aureus	AAAAGGATGTGGCGTTGCCCAGACAACTAGGATGTTGGCTTAGAACGCCATC
7. CP095109.1 Staphylococcus aureus	AAAAGGATGTGGCGTTGCCCAGACAACTAGGATGTTGGCTTAGAACGCCATC
8. CP094733.1 Staphylococcus epidermidis	AAACATATTTTCCATTTATTGGATCACCTAAAAATTGTAAGAGAATTCTCCT
9. CP094728.1 Staphylococcus epidermidis	AAACATATTTTCCATTTATTGGATCACCTAAAAATTGTAAGAGAATTCTCCT
10. CP094726.1 Staphylococcus epidermidi	AAACATATTTTCCATTTATTGGATCACCTAAAAATTGTAAGAGAATTCTCCT
11. CP088002.1 Staphylococcus epidermidi	GATATTCTCTTACAATTTTTAGGTGATCCAATAAATGGAAAATATTTTAATA
12. CP033732.1 Staphylococcus hominis	AAAAGGATGTGGCGTTGCCCAGACAACTAGGATGTTGGATTAGAACGCCATC

Figure 1: Multiple sequences alignment from *Staphylococci* species (Northern and Southern Guinea Savannah Zones).



Species/Abbrv
1. S.G.Savannah Retail sample
2. N. G. Savannah Retail sample
3. N. G.Savannah Proccessed Sample
4. EU761171.1 Bacillus thuringiensis
5. CP0539541.1 Bacillus cereus 4 of 2
6. CP093291.1 Bacillus mycoides
7. CP072057.1 Bacillus mycoides1
8. CP054800.1 Bacillus anthracis 2 of 3
9. CP054797.1 Bacillus anthracis 5of 3
10. CP054797.1 Bacillus anthracis 4of 3
11. CP054797.1 Bacillus anthracis 3 of 3
12. CP036085.1 Bacillus mycoides2
13. CP016595.1 Bacillus cereus 1 of 2
14. CP010106.1 Bacillus thuringiensis2 of 2
15. CP009746.1 Bacillus weihenstephanensi
16. AY461778.1 Bacillus thuringiensis20f 3

Figure 2: Multiple sequence alignment for *Bacilli* Bacteria (Northern and Southern Guinea Savannah Z ones).

CP010106.1, CP053954.1, CP0954816.1, CP054800.1, CP1009746.1, CP093291.1, CP072057.1, CP094726.1, CP036085.1, EU761171.1, AY461778.1 and AY461773.1. Star (*) indicate the position of nucleotide alignment (Figures 1 and 2).

Evolutionary relationships of taxa

The evolutionary history was inferred using UPGMA method (Sneath and Sokal, 1973) the optimal tree is shown. The percentage replication in which the association taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsentein, 1985). The evolutionary distances were computed using the maximum likelihood method (Tamura and Kumar, 2004), and are in units of the number of base substitutions per site. This analysis involved ten nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion options). Evolutionary analyses were conducted in MEGAX.

Phylogenetic analysis for Staphylococcus species

Using sequences retrieved from Genbank, the aligned *Staphylococcus epidermidis* sequence had 99% homology with the sequence of *S. epidermidis* satellite DNA (YIS751) and *S. epidermidis* (CP043801) satellite DNA respectively. *S. hominis* had 3690 homology with the sequence of *S. hominis* satellite DNA (cp033732), *S. epidermidis* had 46% (CP43847) and 99% homology with *S. aureus* (CP06799) as shown in (Figure 3).The analysis generated two related cluster. Cluster A composed of all sampled sequence that are similar to *S. epidermisdis* and cluster B composed of all sampled sequence that are similar to *S. hominis*.

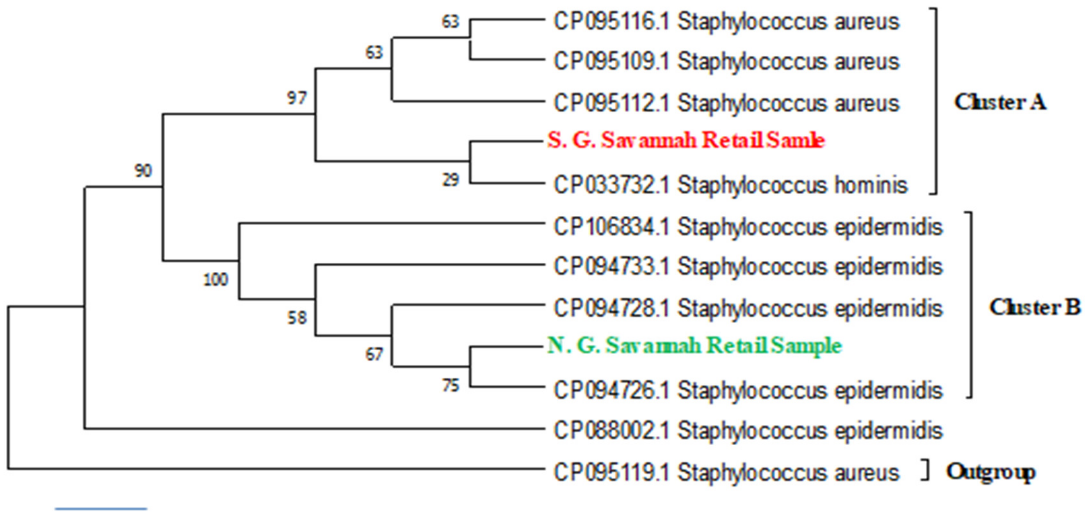


Figure 3: Evolutionary Analysis of Staphylococcus species found in Honey with the one in the GenBank.

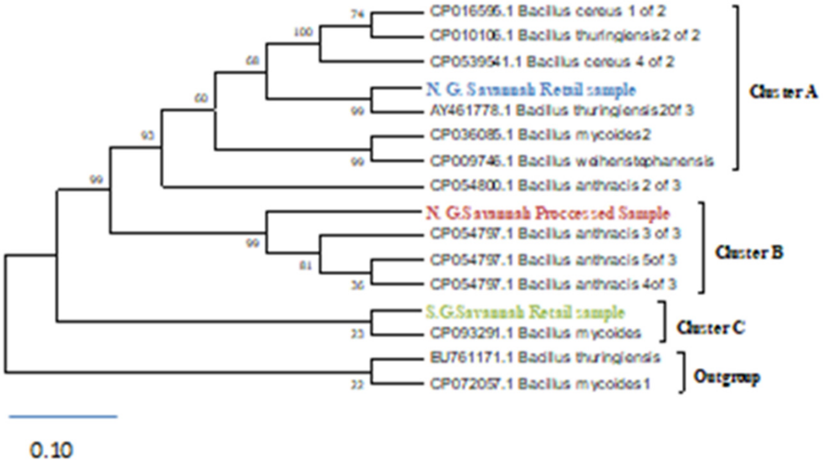


Figure 4: Evolutionary Analysis of Bacillus species found in Honey with the one in the GenBank

Phylogenetic analysis for bacilli species

Using sequence retrieved from GenBank, the aligned *Bacillus cereus* sequence had 87% homology with the sequence of *B. cereus* satellite DNA (CP1016595). *B. anthracis* had 99% homology with the sequence of *B. anthracis* DNA (CP054816) and *B. mycoides* DNA (EF210253) respectively. The isolate from Northern and Southern Guinea savanna zones shows location names. The analysis generated three related cluster. Cluster A composed of all sampled sequence that are similar to *B. cereus*, cluster B composed of all sampled sequence that are similar to *B. anthracis* and cluster C similar to *B. mycoides* respectively (Figure 4).

DISCUSSION

The results of the microbial counts in the different samples of honey obtained, showed that the honey samples had low levels of microbes and were all within the FDA accepted limit of CFU of not more than 15,000 CFU per gram. Adenekan *et al*. (2012) reported CFU of 1.0-6.4 x 10^{-3} cfu from Ogun state and Malika *et al*. (2005) reported counts of less than 10 cfu in Moroccan honey. The results of the selective media confirmed that the bacteria isolated from the honey samples belong to the genus *Staphylococci* and *Bacilli*. The low levels of microbes obtained from the honey samples are indications that honey has high anti-microbial activity.

Microbial contaminations occurring during and after the processing of honey, as opposed to the indigenous microflora of the honey itself, have the potential to lead to spoilage or the continued presence of certain bacteria (Adenekan and Augustus, 2020).

In this investigation, absence of Coliform Count (TC) could be explained by the evidence that honey is well preserved against bacteria such that these microorganisms would not survive unfavourable conditions which is in contrast with the work of Adenikan and Augustus, (2020) who reported low level TCC. The detected microbes (S*taphylococci)* are pathogens found as normal flora in man. *Baccilus* spp. is a spoilage organism in food due to its versatile metabolism and heat resistant spores; hence, might have originated from the plant host containing the nectars where the bees visited. This result is in agreement with work of Malika *et al*. (2005) who reported that honey collected from Morocco contained low level of microbial cells. Coliform count (TC) was not detected in honey samples obtained from the two zones in Niger State.

The application of molecular technique aided in identifying *Bacilli* and *Staphylococci* isolated from the honey. *Bacillus* and *Staphylococcus* species have been recognized as dominant in honey (Malika *et al*. (2005); Pomastowski *et al*. (2019) and Tsadila *et al*. (2021)). The occurrence of *Bacillus* species in honey ostensibly reflects its common presence in the soil, dust or contaminated water and invariably the contamination of pollinating bees (Alkassab *et al*., 2022). The presence of *Staphylococcus* sp in honey can lead to contamination due to improper hygienic practices while harvesting, handling and packaging of honey samples. In all, the low bacterial content gives confidence to honey safety and marketability.

The phylogenetic reconstructed tree showed Southern Guinea Savannah retailed sample formed a clade with CP033732.1S*staphylococcus hominis* isolated from the honey sample in cluster A, Making them a paraphyletic group to the sister terminals containing CP0951161.1 and CP0951811.1 (figure 3). On the other hand, Northern Guinea Savannah retailed sample in cluster B formed clade with CP094726.1 which is paraphyletic to CP106834.1, CP094730.1, CP094728.1 with a bootstrap support of 100 (Figure 4). This description is consistent with the report by Harison and Langale (2006) in their phylogeny reconstruction.

The evolutionary relationship among the three *Bacillus* species delimit them into three clusters (A, B and C) respectively. The Northern Guinea Savannah retail sample is seen to be in cluster A forming a clade with AY461778.1 (99 bootstrap) and is paraphyletic to the sister clade containing CP816596.1 and CP010106.1. Northern Guinea Savannah processed sample and CP54797.1 are paraphyletic with respect to the monophyletic group containing CP0547. The result showed that genetic variations existed within the population of *Staphylococcus* species and *Bacillus* species examined when aligned with some species of bacteria obtained from GenBank.

Conclusion

In conclusion, the results of this investigation showed that the honey samples tested had low microbial counts, all within the FDA-accepted limits. The absence of coliforms and the minimal presence of Bacillus and Staphylococcus species align with previous findings, indicating honey's natural antimicrobial properties. However, these bacteria may have originated from external contamination during or after processing, rather than being indigenous to honey. The presence of Bacillus, commonly found in soil and dust, and Staphylococcus, often linked to poor hygienic practices, highlights the importance of maintaining proper handling procedures. The phylogenetic analysis demonstrated genetic variation within Bacillus and Staphylococcus species, revealing evolutionary relationships and cluster formations. Despite the presence of these microbes, the low bacterial content ensures honey's safety and marketability, supporting its antimicrobial reputation.

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