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Full Length Research Paper

Isolation and identification of cellulose-degrading endophytic bacteria from Tomoceridae (springtails)

Wei Deli[1,2], Miao Xiulian[1], Tian Yue[1], Du Jing[2] and Wang Meng[1*]

[1]School of Life Science, Liaocheng University, Liaocheng 252059, People's Republic of China.
[2]Department of Reproductive Medicine, Liaocheng People's Hospital, Liaocheng 252000, People's Republic of China.

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Springtails are considered as an important candidate bioindicator to assess soil quality spiked with trace metals, but little is known of their endophytic bacteria. In this study, a kind of Tomoceridae springtail was used, and a total of 45 effective sequences were obtained through the process of endophytic bacteria isolation, culture, polymerase chain reaction (PCR) amplification and sequencing. After NCBI-BLAST, the results showed that there were 20 bacterial colonies belonging to the genus *Staphylococcus*, 12 belonging to the genus *Bacillus*, 7 belonging to the genus *Paenibacillus*, 1 belonging to the genus *Exiguobacterium* and 5 belonging to *Acinetobacter lwoffii*. Furthermore, five bacterial strains from these five genera (named TomoRZH14, TomoRZH26, TomoRZH30, TomoRZH37, TomoRZH40) were selected for cellulose degradation analysis. The results showed that TomoRZH26 (*Bacillus* sp.) seemed to have a stronger cellulose degradation ability than those of the other four strains, while the three main components cellulase endo-β-glucanase, exo-β-glucanase and β-glucosidase in TomoRZH26 showed significantly higher enzymatic activity than in the other strains. Viscosity analysis also showed that the TomoRZH26 bacterium degraded relatively quickly in cellulase fermentation medium. In general, in this study, we preliminarily revealed several endophytic bacteria of Tomoceridae springtails and found that they had potentially strong cellulose degradation activity, which may be one of the important reasons behind springtail adaptation to this kind of soil ecological environment.

Key words: springtail, endophytic bacteria, cellulose.

INTRODUCTION

It is well known that endophytic bacteria play very important roles in host animal behavior and many flies with hyperactive locomotor behavior return to normal levels (Schretter et al., 2018). In bees, endophytic bacteria may also be associated with their social regulation (Kwong and Moran, 2016). In addition to host behaviors, endophytic bacteria may also affect insect development, lifespan, fecundity and so on (Gould et al., physiological processes. In *Drosophila melanogaster*, the commensal bacterium *Lactobacillus brevis* can help the 2018). In mammals, studies have demonstrated that specific endophytic bacteria can modulate the nutritional status, health, and disease susceptibility of the host (Huda et al., 2020). In general, endophytic bacteria play an important role in animal organisms, and to a certain extent, the microbiota has great similarity between some

*Corresponding author. E-mail: mengw1986@yeah.net.

kinds of insects and mammals. According to the impact on the host, there are mainly beneficial bacteria, harmful bacteria, and opportunistic pathogens. These endophytic bacteria have different effects on animal bodies.

Springtail is the general name of Collembola order animal species and plays very important roles in the process of soil ecological environment regulation. Springtails mainly feed on living plants, as well as fungi, animal debris, humus, bacteria and others, with a relatively miscellaneous feeding habit (Fujii et al., 2021). At the same time, they also have a certain resistance to heavy metals in the environment, such as nickel (Lin et al., 2019), mercury (Buch et al., 2016), and lead (Dai et al., 2020), or involve in certain related metabolic activities. A recent study showed that the gut bacteria of springtail had complex interactions within the gut community (Agamennone et al., 2018). In view of the complex feeding habits and diverse ecological functions of springtails, we believe that these characteristics are inseparable owing to the role of endophytic bacteria, although there is still a lack of in-depth research, especially in terms of cellulose degradation. These endophytic bacteria may play important roles in assisting the degradation of cellulose in soil and maintaining the normal energy flow of the ecological environment.

In this study, we used a kind of Tomoceridae springtail, which is a common species that lives in temperate forests, for endophytic bacterial analysis. To isolate these bacteria, we sterilized the surface of the springtails. After further culture and identification of these bacteria, we continued to analyze their ability to degrade cellulose. In summary, in this study, we wanted to know the general endophytic bacterial composition and their potential role in this kind of springtail. This study lays a foundation for further study of the physiology and ecology of bacteria and their hosts.

MATERIALS AND METHODS

Springtail acquisition and culturation

The springtails used in this study were of the family Tomoceridae and were collected from Wulian Mountain, Rizhao city of Shandong Province, China. The springtails were fed in a culture bottle containing gypsum/activated carbon matrix that was covered with a permeable transparent lid, cultured in a constant temperature incubator at 25°C, and fed with dry yeast every day.

Isolation and identification of endophytic bacteria

Ten springtail adults were placed in a 1.5 ml centrifuge tube, washed with sterile water, and then placed in 75% alcohol for body surface sterilization for 30 min. Then, springtails were washed twice with sterile water and ground with an electric pestle. After grinding, 150 µl sterile water was added to the grinding solution, which was then serially diluted to dilution factors of 10-1, 10-2, 10-3, 10-4 and 10-5. These dilutions were then spread evenly on Luria-Bertani (LB) plates and incubated at 30°C for 36 h. Then, single colonies were isolated and cultured at a speed of 200 r/min in LB liquid medium overnight. To eliminate the interference of epiphytic bacteria, the last time for springtails washed water was flatted on LB plate and cultured as the negetive control.

The bacteria were identified through Polymerase chain reaction (PCR) amplification and Sanger sequencing. PCR was carried out using 16S universal primer pairs F: AGAGTTTGATCCTGGCTCAG; R: GGTTACCTTGTTACGACTT(Gao et al., 2017). Each 50 µl amplification system included: Taq enzyme 0.25 µl, 10 × PCR buffer 5 µl, deoxynucleotide triphosphates (dNTP) 4 µl, bacteria solution 2 µl, upstream and downstream primers 1 µl. The reaction conditions were as follows: First step: 94°C 10min; second step: 35 cycles: 94°C 30 s, 58°C 30 s, 72°C 30 s; third step: 72°C 5 min. The amplified product was tested with 1% agarose gel, and the amplified single sample was selected for Sanger sequencing. The sequencing results were retrieved by the NCBI-BLAST online database, and the result with the highest consistency was selected as the candidate species.

CMC-Na plate experiment

The candidate cellulose-degrading bacteria were inoculated on the CMC-Na plate, which was used as the sole carbon source for culture. After 72 h of culture at a constant temperature of 30°C, 1 mg/ml Congo red was used for staining for 20 min, and 1 mol/L NaCl solution was used for decolorization for 30 min. A transparent hydrolytic ring was observed.

Determination of endophytic cellulase activity

Cellulase is a kind of complex enzyme with synergistic effects, mainly composed of endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase (Mihajlovski et al., 2016). Since the main product catalyzed by cellulase is glucose, in this study, an enzyme unit was defined as the enzyme activity that catalyzes the production of 1 g of glucose in one minute, and the content of glucose was determined by the 3,5-dinitrosalicylic acid (DNS) method.

Glucose standard curve

First, 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 ml were taken from a 1 g/L standard glucose solution and placed into 7 scale test tubes, and then distilled water was added to brighten the solutions to 2.0 ml. Then, 1.5 ml of DNS solution was added to the solution, mixed well and boiled for 5 min. After cooling to room temperature, distilled water was added to a final volume of 20 ml and mixed well. Next, 200 µl of solution was taken from each tube and placed into a 96-well plate. The absorbance at 540 nm was measured in a multifunctional EPOCH2 microplate reader (BioTek, USA). The standard curve was made according to the absorbance value of different glucose contents.

Determination of cellulase activity

The isolated bacteria were inoculated into the enzyme-producing fermentation medium containing 20 g/L CMC-Na, 2 g/L KH_2PO_4, and 14 g/L$(NH_4)_2SO_4$ and cultured for 7 days at 30°C and 150 r/min in a constant temperature oscillating incubator. The enzyme activity was measured every 24 h. The substrates used for endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase enzyme activity measurements were 1% CMC-Na solution, 1% microcrystalline cellulose solution and 1% salicin solution, respectively. The supernatant obtained from 1 ml of fermentation broth, which was centrifuged at 4°C and 6000 r/min for 10 min, was used as the crude enzyme solution. Then, 0.5 ml of citric acid buffer (pH 4.5)

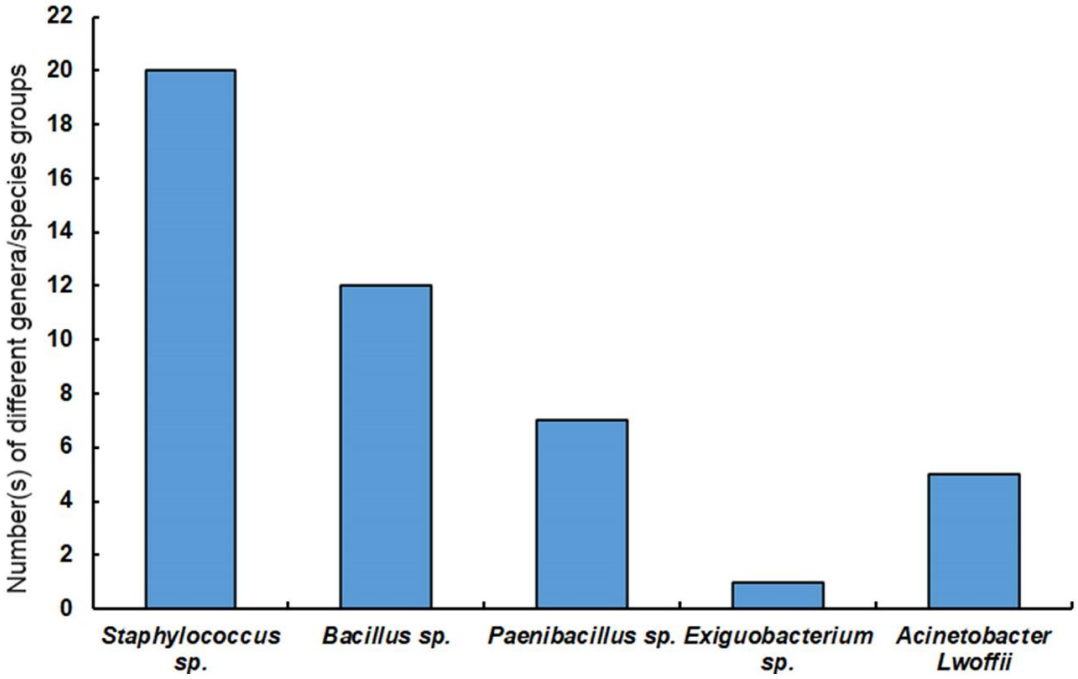

Figure 1. Number(s) of different genera/species groups of 45 endophytic bacteria isolated and selected in Tomoceridae springtails.

and 0.5 ml of crude enzyme solution were added into each 1 ml substrate solution. Next, the mixture was incubated in a metal block bath at 50°C for 30 min, and then 1.5 ml of DNS reagent was added to each tube. The reaction tube was then incubated in the metal bead bath at 100°C for 5 min. After quickly cooling to room temperature, the solutions were replenished with distilled water to 20 ml. After blending, the absorbance value at 540 nm was measured in a multifunctional micrometer. In the control group, no enzyme solution was added, and the amount of buffer solution was increased to 1.0 ml without reacting at 50°C.

RESULTS

Endophytic bacterial identification

In this study, we obtained 45 effective sequences by Sanger sequencing after endophytic bacterial isolation, cultivation and PCR amplification. These 45 sequences were input into the NCBI online database, and nucleic acid BLAST analysis was performed. The results are shown in Figure 1. Among the 45 sequences, 20 belonged to the genus *Staphylococcus*, 12 belonged to *Bacillus*, 7 belonged to *Paenibacillus*, 5 were identified as *Acinetobacter lwoffii*, and 1 belonged to *Exiguobacterium*. *Staphylococcus* was the most common springtail endophytic bacteria in this study.

Determination of cellulose degradation capacity and cellulase activity

We chose one strain of each genus to further study whether these bacteria had cellulose-degrading activity among which the sample TomoRZH14 belonged to Paenibacillus, TomoRZH26 belonged to *Bacillus*, TomoRZH37 belonged to *Exiguobacterium*, TomoRZH30 was *A. lwoffii*, and TomoRZH40 belonged to *Staphylococcus*. The five selected bacterial strains were inoculated on Sodium carboxymethyl cellulose (CMC-Na) plates and cultured at 30°C for 72 h. After Congo red staining and NaCl decolorization, the resulting transparent hydrolytic rings are shown in Figure 2. The results showed that TomoRZH26 had an obvious hydrolytic ring, followed by TomoRZH37 and TomoRZH30, which had smaller rings, while TomoRZH14 and TomoRZH40 had the smallest hydrolytic rings (Table 1).

The standard curve of glucose was generated with glucose content (mg/ml) as the abscissa and 540 nm absorption value as the ordinate. The equation of the generated curve is y = 0.2803 x - 0.0039, and the correlation coefficient R2 is 0.9994. The enzyme activity of endo-β-1,4-glucanase in the fermentation broth of these five bacteria was detected by the DNS method every 24 h for 7 days. The results are shown in Figure 3A. It is easy to see that the TomoRZH26 strain fermentation broth presented consistently high enzyme activity in the 7 days, and the enzyme activity of this strain was also the highest among the five strains. The highest enzyme activity was observed on the 4th day, reaching 8.3 U/ml. The other four strains, TomoRZH14, TomoRZH37, TomoRZH30 and TomoRZH40, had relatively low enzyme activities in the 7 days.

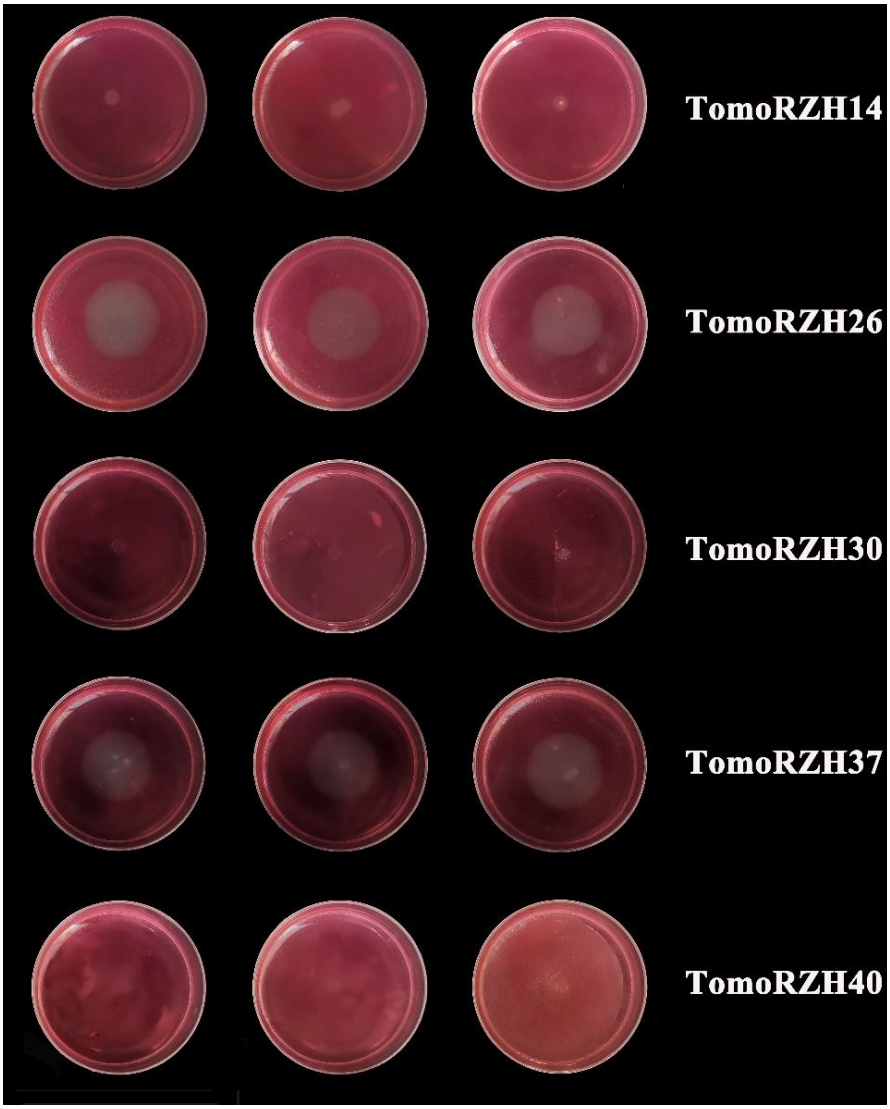

Figure 2. Hydrolytic zones of five springtails endophytic bacteria on CMC-Na plates.

Table 1. Hydrolytic zones of five springtails endophytic bacteria on CMC-Na plates.

Bacteria strains		TomoRZH14	TomoRZH26	TomoRZH30	TomoRZH37	TomoRZH40
Diameters of hydrolytic zones (D, mm)	Rep.1	6.07±0.12	21.47±0.55	6.13±0.12	19.00±0.50	7.03±0.26
	Rep.2	7.20±0.89	20.63±0.35	6.53±0.12	21.43±0.25	5.10±0.10
	Rep.3	5.43±0.16	18.7±0.23	6.46±0.06	18.70±0.26	6.87±0.12
Diameters of the colonyies (d, mm)	Rep.1	5.60±0.53	25.43±0.15	4.90±0.10	23.37±0.15	6.73±0.55
	Rep.2	7.38±1.14	24.50±0.50	5.33±0.28	25.27±0.25	5.67±0.58
	Rep.3	8.87±0.32	23.80±0.26	5.06±0.15	23.13±0.21	6.80±0.26
D/d	Total	1.01±0.08	1.21±0.05	0.80±0.02	1.22±0.03	1.022±0.08

Similar to endo-β-1,4-glucanase, as shown in Figure 3B, the TomoRZH26 strain fermentation broth presents a relatively higher exo-β-1,4-glucanase enzyme activity than those of the other four strains. However, the trends of enzyme activity for all five bacteria were roughly the same, with a lower level in the first two days than in the

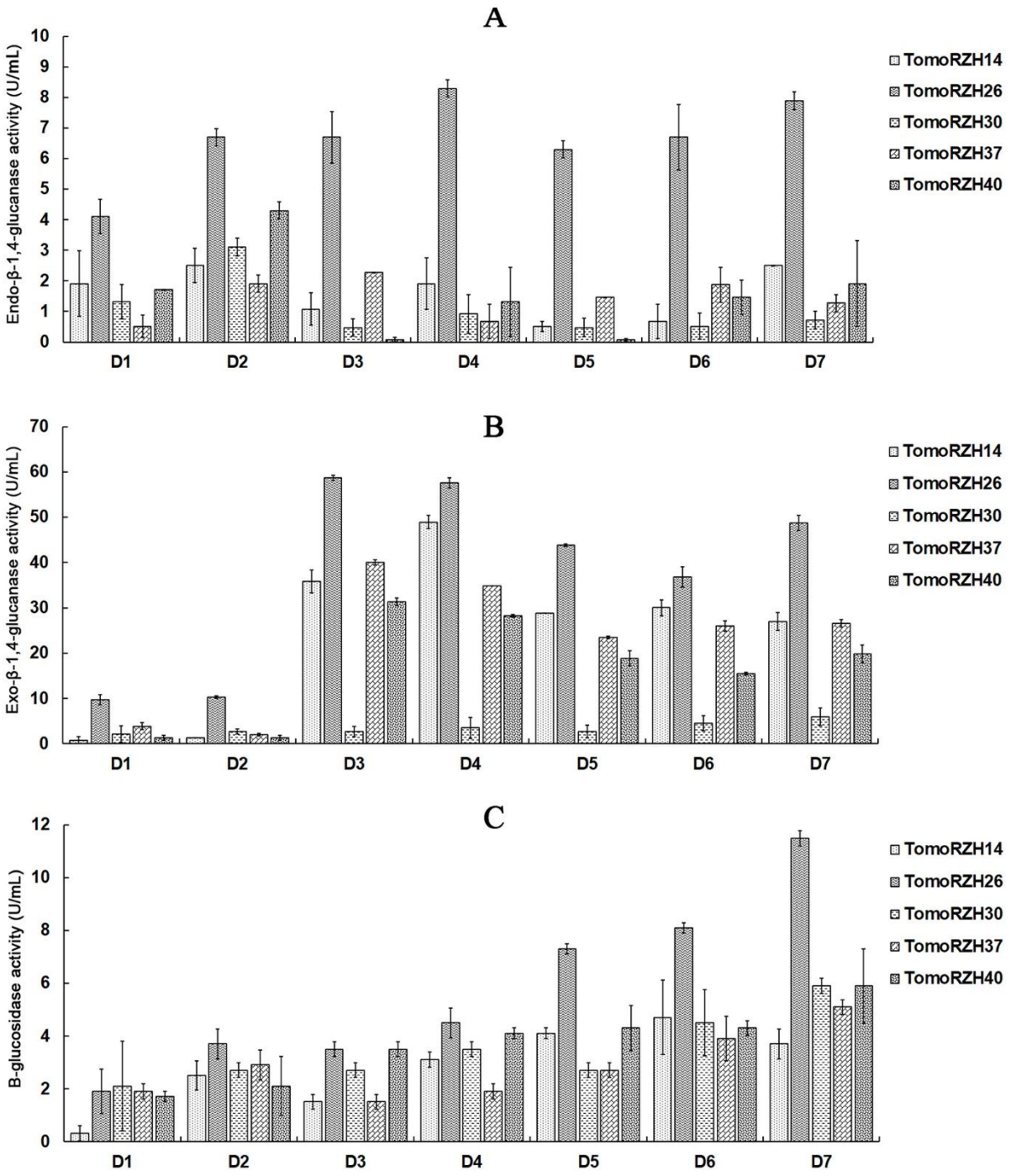

Figure 3. Cellulase activity identification of five springtails endophytic bacteria by DNS method. A) The enzyme activity of endo-β-1,4-glucanase; B) The enzyme activity of exo-β-1,4-glucanase; C) The enzyme activity of β-glucosidase.

proceeding five says. The enzyme activity of strains TomoRZH26, TomoRZH37, and TomoRZH40 reached the highest level on the 3rd day, which was 58.7, 40.0 and 31.4 U/ml, respectively, and strains TomoRZH14 and TomoRZH30 reached their highest levels of β-1,4-glucanase activity on the 4th day, which were 49.0 and 37.2 U/ml, respectively. In the following days, their enzyme activity decreased slightly. Therefore, these five bacteria all had certain enzyme activities of exo-β-1,4-glucanase and might be involved in some processes of cellulose degradation.

Unlike endo- and exo-β-1,4-glucanases, the enzyme activity of β-glucosidase continuously increased during the seven days of cultivation (Figure 3C). However, strain

TomoRZH26 also had higher enzyme activity than the other four strains after the 4th day. On the 7th day, the highest enzyme activity reached 11.5 U/ml. There was no significant difference in enzyme activity among the other four strains. On the 6th day, the enzyme activity of TomoRZH14 reached a maximum of 4.7 U/ml but decreased slightly on the 7th day.

DISCUSSION

Springtails are hexapod arthropods that live in soil. These have complex feeding habits and play important roles in the circulation of soil materials, soil development microstructure formation, soil physical and chemical property improvement and soil biome maintenance (Oliveira Filho et al., 2016). The ecological roles of springtails are likely to have a strong relationship with their endophytic bacteria. In this study, we isolated five different bacteria belonging to the genera *Staphylococcus*, *Bacillus*, *Paenibacillus*, and *Exiguobacterium* and the species *A. lwoffii*. The first four genera belong to the same order Bacillales and the phylum Firmicutes, while *A. lwoffii* belongs to the phylum Proteobacteria. The bacteria were not as abundant as expected because more endophytic bacteria were isolated from the springtail *Proisotoma ananevae*, and the isolated bacteria belonged to 6 genera and 3 phyla (Wang et al., 2018). The different living habits of these two different springtail species might be a reason for the difference in endophytic bacterial abundance; however, in this study, springtails were fed and cultured for a relatively long period of time, which might have caused the loss of some endophytic bacteria. As reported earlier, the phyla Firmicutes and Proteobacteria were also the most isolated bacteria from insect guts, which might be related to their coevolution process (Chen et al., 2016).

As reported earlier, many *Staphylococcus* species cannot cause disease and normally reside on the skin and mucous membranes of humans and other animals and are also a small component of the soil microbiome (Jacquemyn et al., 2013). Moreover, staphylococci were not commonly found to have effective cellulase activity, but cellulolytic compounds were occasionally isolated from soil-living small arthropods, such as termites (Pourramezan et al., 2012) and springtails in this study. However, the cellulase activity of staphylococci was found to be relatively low.

Bacillus species are well known in the food industry as troublesome spoilage organisms, two of which are parasitic pathogenic species, *B. anthracis* and *B. cereus*. Many *Bacillus* species can produce copious amounts of enzymes, such as α-amylase, subtilisin, surfactins, and mycosubtilins, which are used in various industries (Favaro et al., 2016). Additionally, some *Bacillus* species have been proven to have a significantly high cellulose degradation ability (Thomas et al., 2018; Cubas-Cano et al., 2020), which also varied in our study.

The other two genera, *Paenibacillus* and *Exiguobacterium*, also belong to the same order Caryophanales. Bacteria belonging to *Paenibacillus* have been detected in a variety of environments and vary with many effects in agriculture and horticulture and industrial and medical applications. Some *Paenibacillus* bacteria, such as *P. lautus* and *P. lactis*, with cellulolytic potential have been studied (Yadav and Dubey, 2018). Therefore, *Paenibacillus* bacteria also have a large potential application for cellulose degradation. *Exiguobacterium* bacteria have also been found in a variety of environments worldwide, and some species can grow within a wide range of pH values, tolerate high levels of UV radiation, and undergo heavy metal stress (Ordonez et al., 2013). A study showed that *Exiguobacterium* may have high β-glucosidase activity and would help with the fermentation of cellulose (Gao et al., 2015). In this study, the TomoRZH37 strain of *Exiguobacterium* exhibited relatively low β-glucosidase activity but with relatively high exo-β-1,4-glucanase enzyme activity, which need for further study.

The fifth type of bacterium found in this study was *A. lwoffii*, which is often considered a normal member of the human skin flora. However, most *Acinetobacter* bacteria are important soil organisms and contribute to aromatic compound mineralization. Some studies also found that the genus *Acinetobacter* bacteria could hydrolyze cellulose efficiently (Pourramezan et al., 2012; Karthika et al., 2020).

Overall, we isolated and identified five different genera of bacteria from Tomoceridae springtails. Although they exhibited different enzyme activities in the degradation of cellulose, these bacteria have potential application value in the degradation of cellulose. These bacteria may be a specific manifestation of springtails with extensive adaptation to the ecological environment. Therefore, more in-depth studies of the interaction between these bacteria and their springtail hosts will be needed in the future. Given the cellulose degradation activity of these bacteria, they also provide the possibility for large-scale biodegradation application for cellulose in industry.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Microbiological, nutritional and sensory evaluation of snack bars developed using Bambara groundnut (*Vigna subterranean* L.) and maize (*Zea mays*)

Ihuoma Ahaotu[1], Marvellous Okechukwu Ichendu[1] and Ndukwe Maduka[2*]

[1]Department of Microbiology, Faculty of Science, University of Port Harcourt, Rivers State, Nigeria.
[2]Department of Biological Sciences, College of Natural and Applied Sciences, Wellspring University, Benin City, Edo State, Nigeria.

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Over the years, snacking has become a regular habit for majority of the population with increasing need for food manufacturers to meet consumers' demand through product development. In this study, snack bars were prepared using maize and Bambara nuts mixed in the following ratios: A, 100% maize; B, 100% Bambara nuts; C, 50% maize: 50% Bambara nuts; D, 75% maize: 25% Bambara nuts; E, 75% Bambara nuts: 25% maize. The total heterotrophic bacterial count of samples A to E was within the limit stipulated by International Commission on Microbiological Specification for Food. The frequency of occurrence of bacterial isolates from the samples include *Bacillus* species (24%), *Staphylococcus* species (24%), *Lactobacillus* species (24%), *Escherichia coli* (19%) and *Serratia* species (9%), while the fungal isolates include *Aspergillus* (33%), *Penicillium* (27%), *Rhizopus* (20%) and *Saccharomyces* species (20%). The moisture, ash, carbohydrate, crude protein, fat and fiber content of the samples were within the range of 11.47±0.99-17.45±1.01, 1.09±0.07-2.00±0.15, 56.05±0.65-70.37±0.71, 6.32±0.36-15.00±0.22, 4.60±0.50-7.00±0.30 and 2.60±0.25-3.10±0.31%, respectively. There was a significant difference ($p<0.05$) in the proximate composition among the samples except for crude fiber. The calorie value range between 347.20-367.69 kcal and acceptability of the samples compared favourably with a commercialized snack bar.

Key words: Healthy snacking, natural sweetener, underutilized legumes, cereal bar, food product development.

INTRODUCTION

It has been the norm in different societies for people to eat three sizeable meals per day. In the past few decades, majority of the population have formed the habit of consuming smaller amounts of food and/or drink at short intervals between three standard meals (Chaplin and Smith, 2011). This feeding habit is referred to as snacking (Potter et al., 2018). Large population of inhabitants in cities and semi-urban areas experience daily hectic and busy lifestyle on which among others could be attributed to increasing job demands and

*Corresponding author. E-mail: maduks.mn@gmail.com. Tel: +234 803 089 8281.

dwindling economies. Consequently, workers are forced to spend long hours at work which encourages heavy snacking (Sharma et al., 2014). The manner at which an individual feel hungry and thirsty sensation before and after snacking depends on the size of snack consumed and its nutritional content and is usually not the same as with a standard meal (Chaplin and Smith, 2011; Boon et al., 2012). In a bid to lessen hunger by snacking, it is recommended that health-promoting foods containing vital nutrients should be consumed. According to Eke-Ejiofor and Okoye (2018), the habit of snacking irresponsibly could lead to weight gain and nutrition deficiency. Despite these assertions, the relationship between snacking and human health is still debatable (Potter et al., 2018). Ready-to-eat (RTE) foods which include wide varieties of snacks are predisposed to microbial contamination by diverse species of bacteria, fungi and parasites. Some viruses have also been implicated in contaminating RTE foods. Consumption of contaminated snacks could lead to microbial foodborne illness (Makinde et al., 2020).

Healthy snacking is gaining popularity among the people due to higher level of awareness for human health and nutrition (Ishak et al., 2021). Instead of relying heavily on snacks prepared using refined sugar, the use of natural sweeteners such as honey and date (*Phoenix dactylifera* L.) is preferable on health grounds (Nissar et al., 2017; Ibrahim et al., 2021). The use of honey as a sweetener is an age long practice (Ajibola et al., 2012). Honey is a well-known, sweet, aromatic and viscous liquid obtained from the nectars of plants by honeybees which store the product in hives (Hebbar et al., 2008). Honey has a high sugar and water content including vitamins, amino acids, minerals, trace compounds and enzymes. The use of honey as a natural sweetener in food product development contributes to nutritional, therapeutic and health benefits (Saha, 2015; Sharma et al., 2020).

The snack bar also known as the cereal bar is a popular snack prepared by compressing a mixture of cereals, nuts and dried fruits (Rush et al., 2016; Edima-Nyah et al., 2019). The shelf life of snack bars is moderate and does not require refrigeration (Ravindra and Sunil, 2018; Eyiz et al., 2020). A study carried out by Verma et al. (2018) reported that shelf life of sorghum based cereal bars was 60 days. Preparation of cereal bar involves the use of a wide range of ingredients such as walnut, almond nut, oats, dried raisins, coconut, sesame seed, honey and dried fig. These ingredients are combined in different proportions based on individual's choice and baked until it becomes crisp (Eke-Ejiofor and Okoye, 2018; Herawati et al., 2019). Many researchers have developed varieties of cereal bars using a wide range of nutrient dense ingredients (Jethwani et al., 2020; de Melo et al., 2020; Maia et al., 2021).

Fruit-based snack bars, wheat or soy-based bars, cereal snack bars, fruit and vegetable-based snack bars, vegetable snack bars and high-protein snack bars are various types of snack bars (Constantin and Istrati, 2018).

Initially, snack bars were meant for athletes to provide them energy. Due to high demand of snack bars by non-athletes, sales have been on the increase in many countries such as the US, UK, Germany and Brazil (Sharma et al., 2014; Carvalho and Conti-Silva, 2017; Dahri et al., 2017; Pinto et al., 2017). By 2025, it is expected that the cereal bar market globally will reach $16.9 billion (Lasta et al., 2021). This could be attributed to high level awareness about convenient, natural, nutritious and healthy food products. The snack bar is a source of nutrients which include fat, protein, fibers, minerals, vitamins, calories and carbohydrates (Ho et al., 2016; dos Prazeres et al., 2017; Constantin and Istrati, 2018). The nutritional composition of each ingredient used in preparing snack bars influences the nutrients and energy value of the product. Oftentimes, the dietary needs of consumers are taken into consideration during the stage of selecting ingredients to be used in preparing snack bars (Maia et al., 2021). According to Pinto et al. (2017), substituting meals with snack bars is effective in achieving weight loss. However, due to the level of available carbohydrates in cereal bars, the product is capable of increasing glycemic indexes (GI) which is not suitable for persons suffering from Type 2 Diabetes mellitus (Farago et al., 2021). The calorific value of Bambara groundnut and cereal grains are such that they can be used as ingredients in the production of snack bars (Igbabul et al., 2013).

Bambara groundnut (*Vigna subterranean* (L.) Verdc or *Voandzeia subterranea* (L.) Thouars) is generally regarded as an underutilized legume that grows abundantly in Africa (Orhevba and Mbamalu, 2017; Udeh et al., 2020; Khan et al., 2021). There has been a considerable increase in the utilization of Bambara groundnut in the past few years (Igbabul et al., 2013; Nwadi et al., 2020). Based on its nutritional composition, Bambara groundnut is a complete food. Surprisingly, it is also inexpensive. According to Anhwange and Atoo (2015), Bambara groundnut contains 6.35-7.78% moisture, 3.53-3.94% ash, 4.58-5.50% crude fiber, 18.25-20.44% protein, 5.82-6.31% lipid and 52.08-56.01% soluble carbohydrate. Accordingly, Bambara groundnut is a rich protein source (Orhevba and Mbamalu, 2017; Tan et al., 2020). It also contains minerals which include calcium, iron, potassium, and sodium in reasonable quantities. In Nigeria, many local dishes, well appreciated by people, are prepared using Bambara groundnut flour (Barimalaa et al., 2005; Ndidi et al., 2014).

Maize (*Zea mays* L.) is in the second position behind sorghum among all the cereals grown and consumed in Nigeria. Globally, maize is ranked third behind rice and wheat. Maize is referred as the 'queen of cereals' considering its maximum yield when compared with other cereals (Adeniyi and Ariwoola, 2019). Maize is the

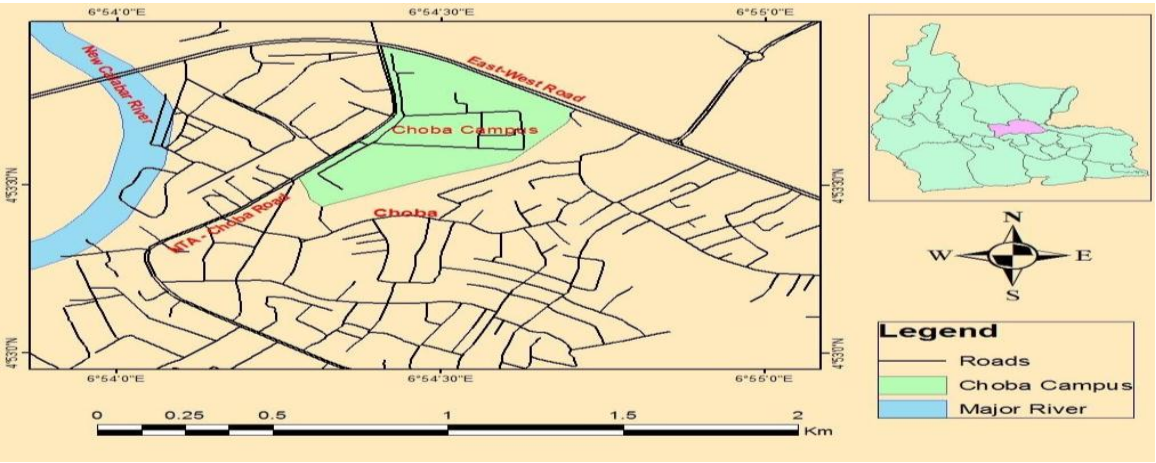

Figure 1. Choba Town, Obio-Akpor LGA, Rivers state, Nigeria.
Source: Geographic Information System Laboratory, Geography and Environmental Management, University of PortHarcourt, Choba, Nigeria.

highest source of energy in the national diets of 22 countries across the world which includes 16 African countries (Ahaotu et al., 2021). Cereals such as maize are rich sources of energy in diets. The quality of protein in cereals such as maize is poor because it contains little amount of amino acids known as lysine and tryptophan (N'Guessan et al., 2014; Moses and Makanjuola 2018). Maize is a staple food for inhabitants in sub-Saharan Africa. It is estimated that 30% of the total calorie intake of the people comes from maize. In different parts of Africa, many local foods are prepared using maize (Ndukwe et al., 2015; Ekpa et al., 2019). Food industries make use of maize flour to produce different products (Gwirtz and Garcia-Casal, 2013; Oladapo et al., 2017). A recent study carried out by Edima-Nyah et al. (2019) involved the use of African breadfruit (*Treculia africana*), maize (*Z. mays*), and coconut grits (*Cocos nucifera*) to prepare snack bars. Interestingly, these snack bars were nutritive and well acceptable by the sensory panelist. In a related study, Eke-Ejiofor and Okoye (2018) developed cereal bars using locally available cereals which include millet, guinea corn, yellow and white maize. The study revealed that these cereal bars possess better nutritional and sensory qualities than oat bar (control).

Introduction of new food products, such as energy or cereal bars, into the market takes into consideration the nutritional and sensory quality of the products (Srebernich et al., 2016). Food safety of such products is equally important. However, microbiological assessment of cereal bars developed by many researchers were not carried out nor reported. Munhoz et al. (2014) detected the presence of *Bacillus cereus* in snack bars containing bocaiuva. Snack bars are not popular in this part of the world. In Nigeria, most of the commercially available snack bars are imported and expensive. They usually contain high amount of refined sugar which might not be suitable for diabetic patients and the elderly. In the light of the aforementioned, this study is aimed at carrying out microbiological analysis, nutritional and sensory evaluation of a healthy snack bar developed using honey, Bambara groundnuts and maize.

MATERIALS AND METHODS

Yellow variety of maize grain, Bambara groundnut, fresh eggs from fowl and refined palm olein (branded) were purchased from traders at Choba market. A bottle of honey (local brand; sourced from hive), oat, iodized table salt, and liquid milk (branded; sourced from cow) were obtained from superstores along Choba-NTA road. Coconut (*C. nucifera*) was harvested from a coconut tree planted in Choba. Figure 1 shows the map of Choba town where all the materials were purchased. They were put inside a big sterile polythene bag and transported to Food and Industrial Microbiology Laboratory, University of Port Harcourt, Choba, Rivers State.

Preparation of snack bar

A composite of crushed maize and Bambara groundnut weighing 200 g was prepared in five different proportions (Table 1). The particle sizes vary from 1.2 to 3.8 mm. Each portion was mixed thoroughly in separate bowls. Dehusked coconut was grated manually. For each bowl, 5 tablespoons of coconut, 5 tablespoons of refined palm olein, 5 tablespoons of oat, 50 ml of liquid milk, 6 tablespoons of honey, one fresh egg and a pinch of salt were added and mixed thoroughly. After kneading, each of the portions was moulded into a flat thin shape and then baked in the oven for 25 min at 190°C. After baking, the samples were cut into bar shape and packaged. In Figure 2, the flow chart for the production of the snack bars is shown.

Serial dilution

A portion of each sample was ground into powder using laboratory blender (Usha Mixer Grinder, India) sterilized with 70 % ethanol.

Table 1. The proportion of maize and Bambara groundnut in the snack bar samples.

Trials	Maize (%)	Bambara groundnut (%)
A	100	0
B	0	100
C	50	50
D	75	25
E	25	75

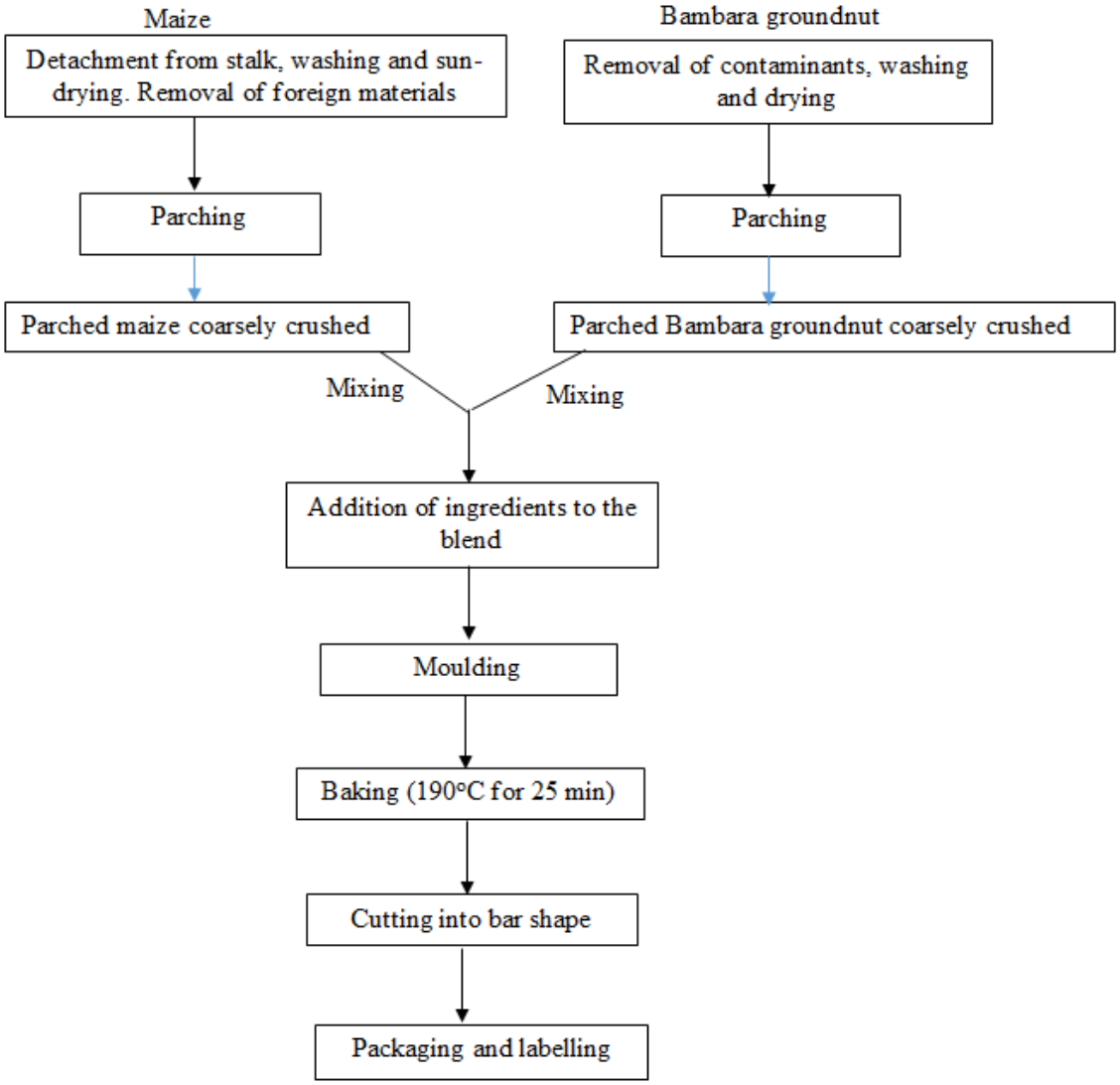

Figure 2. Flow chart for the production of snack bar.

Aseptically, 10 g of the sample was weighed using electronic balance (Metler MT-2000) and dispended into 90 ml sterile peptone water. It was mixed properly to form a suspension which serves as the stock solution of the samples. Ten-fold dilution was carried out by stepwise transfers to achieve higher dilutions with a sterile pipette for each transfer until dilution 10^{-4} was achieved.

Microbiological analysis

Total heterotrophic bacterial count

Aseptically, 1 ml of dilutions 10^{-1} and 10^{-2} was inoculated into Petri dishes containing sterile molten nutrient agar (NA) and Sabouraud

dextrose agar (SDA) prepared following manufacturer's instruction. The NA and SDA were autoclaved at 121°C for 15 min at 15 psi. The inoculated plates (NA) were incubated at 37°C for 24 h. The method described by Ekpakpale et al. (2021) with slight modification was used to enumerate the fungal population after incubating the SDA plates at 25°C for 96 h. All the culture plates were examined for microbial growth and the colonies which appeared were enumerated and recorded as colony forming units (CFU/ml).

The bacterial and fungal population of the samples was calculated using the formula:

$$\text{CFU/mL} = \text{No. of colonies} \times \frac{1}{\text{serial dilution}} \times \frac{1}{\text{dilution plated}}$$

Obtaining pure culture

Representative colonies from the NA and SDA culture plates were subcultured by repeated streaking in freshly prepared NA and SDA plates, respectively to obtain pure cultures. The NA plates were incubated at 37°C for 48 h. Similarly, the SDA plates were incubated at 25°C for 7 days as described by Ekpakpale et al. (2021). Further identification of the bacterial and fungal cultures was carried out. The pure culture of bacteria and fungi were inoculated into NA and SDA slants, respectively and stored in the refrigerator at 4°C until analyses is completed.

Identification of the bacterial isolates

Cultural characteristics of the isolates on the culture plates were examined. Gram reactions and cell morphology were examined from heat-fixed smears. Biochemical tests on the isolates which include indole, methyl red, citrate, catalase, oxidase, sugar fermentation, motility and triple sugar iron agar (TSIA) test for H_2S were carried out using the procedure described by Shoaib et al. (2020). Identification of all cultures was done using the procedure described by Holt et al. (1994) and Buchanan and Gibbons (1974).

Identification of the fungal isolates

Microscopically, the colonial characteristics and cell morphology of the fungal isolates were ascertained after staining with lactophenol cotton blue. A portion of fungal mycelium was teased out in a drop of lactophenol cotton blue on grease-free microscope slide and was examined under the microscope with low power high dry objective. The cultural and morphological characteristics of each fungal isolate were compared with earlier descriptions (Barnett and Hunter, 1972).

Proximate composition

The moisture, ash, fat, crude protein and fiber contents of the snack bars were determined using the AOAC (1995) methods. The difference method was used to determine the carbohydrate content.

Calorie value

The method described by Ho et al. (2016) was adopted in determining the calorie value of the snack bar samples. It involves multiplying the total crude protein, crude fat and carbohydrate content of each sample by the factor value (for each gram of carbohydrate and protein, what is obtained is 4 kcal and 1 g of crude fat provides 9 kcal of energy).

Energy = (crude protein × 4) + (carbohydrate × 4) + (crude fat × 9).

Sensory evaluation

Sensory evaluation of the snack bar samples was carried out by ten semi-trained panelists familiar with good quality snacks. The panelists were undergraduate students in the Department of Microbiology, University of Port Harcourt between the ages of 18 and 26 years. All the samples presented to them were coded with alphabets A - E. The panelists used 9-point Hedonic scale which range from 1 (dislike extremely) to 9 (like extremely) as a guide to evaluate the sensory attributes of each sample which include taste, color, aroma, appearance, mouthfeel and overall acceptability. Self-explanatory questionnaires were given to the sensory panelists to enter scores for the sensory parameters evaluated for each sample. Potable bottled water was provided for the panelists to rinse their mouth before and after evaluating each sample.

Statistical analysis

Data generated were subjected to one-way Analysis of Variance (ANOVA) with the aid of IBM SPSS Statistics version 23 software. It also determined significant differences at $p<0.05$. Duncan Multiple Range Test (DMRT) was used in separating the means.

RESULTS

Figure 3 shows the total heterotrophic bacterial count (THBC) of the snack bar samples. Among all the samples, the THBC of snack bar was prepared using 75% maize: 25% Bambara groundnut (4.43 $\log_{10}$ CFU/ml) and 100% maize (4.10 $\log_{10}$CFU/ml) were the highest and least values, respectively. Figure 4 depicts the total fungal count (TFC) of the snack bar samples. The TFC of snack bar was prepared using 100% maize (3.53 $\log_{10}$ CFU/ml) and 100% Bambara groundnut (3.68 $\log_{10}$ CFU/ml) had the least and highest values, respectively.

Plates 1 and 2 show the samples of snack bar developed in this study using maize and Bambara groundnut in different proportions. Table 2 shows the result of biochemical tests carried out on the bacterial isolates from snack bar samples. The isolates identified were *Bacillus* species, *Staphylococcus* species, *Lactobacillus* species, *Serratia* species and *Escherichia coli.* Table 3 shows the macroscopic and microscopic characteristics of the fungal isolates from snack bar samples. The fungal isolates were *Aspergillus*, *Rhizopus*, *Penicillium* and *Saccharomyces* species.

A total of twenty-one bacterial isolates were encountered in all the snack bars prepared using maize and Bambara groundnut in different proportions. The bacterial isolates encountered in the snack bar prepared using 50% maize: 50% Bambara groundnut include *Bacillus*, *Staphylococcus*, and *Lactobacillus* spp. The snack bar prepared using 100% maize and the sample prepared using 75% maize: 25% Bambara groundnut were contaminated with *Lactobacillus* spp., *Bacillus* spp., *Staphylococcus* spp. and *E. coli*. The bacterial genera isolated from snack bar prepared using 25% maize: 75%

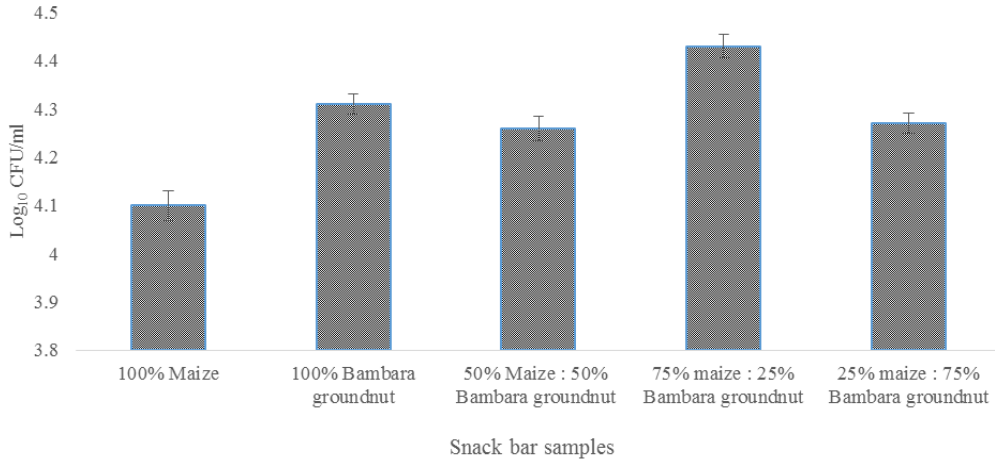

Figure 3. Total heterotrophic bacterial count of the snack bar samples.

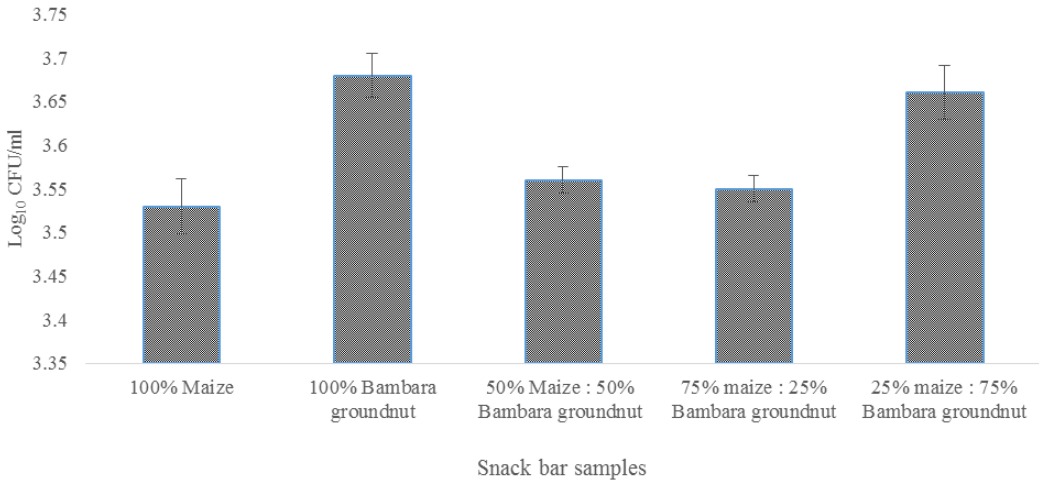

Figure 4. Total fungal count of the snack bar samples.

Plate 1. Snack bar samples before packaging.

Plate 2. Snack bar samples after packaging.

Table 2. Biochemical characteristics of bacteria isolated from the snack bar samples.

Isolate code	Gram reaction	Catalase	Oxidase	Citrate	Indole	Motility	Methyl Red	Voges Proskauer	Slant	Butt	Gas	H_2S	Glucose	Lactose	Probable organism
A1	+	-	-	+	-	-	+	-	A	A	+	+	A^+	+	*Lactobacillus* spp.
A2	+	+	-	+	-	+	-	+	B	A	-	-	+	-	*Bacillus* spp.
A3	+	+	-	+	-	-	-	+	A	A	-	-	+	+	*Staphylococcus* spp.
A4	-	+	-	-	+	+	+	-	A	A	+	-	+	+	*Escherichia coli*
B1	+	-	-	+	-	-	+	-	A	A	+	+	A^+	+	*Lactobacillus* spp.
B2	-	+	-	-	+	+	+	-	A	A	+	-	+	+	*Escherichia coli*
B3	+	+	-	+	-	+	-	+	B	A	-	-	+	-	*Bacillus* spp.
B4	+	+	-	+	-	-	-	+	A	A	-	-	+	+	*Staphylococcus* spp.
B5	-	-	-	+	-	+	-	+	B	A	-	-	+	-	*Serratia* spp.
C1	+	+	-	+	-	+	-	+	B	A	-	-	+	-	*Bacillus* spp.
C2	+	+	-	+	-	-	-	+	A	A	-	-	+	-	*Staphylococcus* spp.
C3	+	-	-	+	-	-	+	-	A	A	+	+	A^+	+	*Lactobacillus* spp.
D1	-	+	-	-	+	+	+	-	A	A	+	-	+	+	*Escherichia coli*
D2	+	-	-	+	-	-	+	-	A	A	+	+	+	+	*Lactobacillus* spp.
D3	+	+	-	+	-	+	-	+	B	A	-	-	+	-	*Bacillus* sp.
D4	+	+	-	+	-	-	-	+	A	A	-	-	+	+	*Staphylococcus* spp.
E1	-	-	-	+	-	+	-	+	B	A	-	-	+	-	*Serratia* spp.
E2	+	-	-	+	-	-	+	-	A	A	+	+	A^+	+	*Lactobacillus* spp.
E3	+	+	-	+	-	+	-	+	B	A	-	-	+	-	*Bacillus* spp.
E4	+	+	-	+	-	-	-	+	A	A	-	-	+	+	*Staphylococcus* spp.
E5	-	+	-	-	+	+	+	-	A	A	+	-	+	+	*Escherichia coli*

+, Positive; -, negative; A, acid production; B, alkaline production.

Bambara groundnut and the sample prepared using 100% Bambara groundnut include *Lactobacillus* spp., *Bacillus* spp., S*taphylococcus* spp., *E. coli* and *Serratia* spp. Among the bacterial isolates encountered in all the samples of snack bars prepared using maize and Bambara groundnut in different proportions, *Staphylococcus* (24%), *Lactobacillus* (24%) and *Bacillus* spp. (24%) had the highest frequency of occurrence, followed by *E. coli* (19%) and *Serratia* spp. (9%) had the least frequency of occurrence.

A total of fifteen fungal isolates were encountered in all the snack bars prepared using maize and Bambara groundnut in different proportions. *Aspergillus*, *Penicillium* and *Rhizopus* spp. were isolated from snack bars prepared using 100% maize and 25% maize: 75% Bambara groundnut. The fungal genera isolated from snack bars prepared using 50% maize: 50% Bambara groundnut and 75% maize: 25% Bambara groundnut include *Penicillium*, *Saccharomyces* and *Aspergillus* spp. The snack bar prepared using 100% Bambara groundnut was contaminated with *Aspergillus*, *Rhizopus* and *Saccharomyces* spp. Among the fungal isolates encountered in the snack bars prepared using maize and Bambara groundnut in different proportions, *Aspergillus* spp. (33%) recorded the highest frequency of occurrence, followed by *Penicillium* (27%), *Saccharomyces* and *Rhizopus* spp. each had the lowest frequency of occurrence (20%).

Table 4 shows the proximate composition of the snack bar samples. Considering each of the proximate parameters analyzed, protein and carbohydrate content showed significant difference ($p<0.05$) among all the samples of

Table 3. Macroscopic and microscopic characteristics of the fungal isolates

Organism	Macroscopic	Microscopic
Aspergillus spp.	Fastidious growth within 3-7 days, green colonies which appear velvety with flat rough-walled stripes and has cracked surface behind.	The phalides produced chains of round, rough conidia that appears pale green when viewed under the microscope.
Penicillium spp.	Between 3-7 days, there was a moderate growth. Flat surface, velvet-like texture with gray-green brush like clusters	The conidiophores appear branched with round to ovoid rough-walled chains.
Saccharomyces spp.	Colonies were white to cream in color, smooth, usually large, yeast-like cells that are roughly spherical in shape ranging from 3-10 mm in size.	They occur in clusters; round/ovoid in shape.
Rhizopus spp.	Fast growing mould with white mycelia and black sporangia which turns black with age.	Non-septate or sparsely septate broad hyphae, sporangiophores, and rhizoids

Table 4. Proximate composition of the snack bar samples.

Sample code	Moisture (%)	Ash (%)	CHO (%)	Crude Protein (%)	Crude fat (%)	Crude Fiber (%)
A	14.56±0.72^{ab}	1.09±0.07^{a}	70.37±0.71^{e}	6.32±0.36^{a}	4.60±0.50^{a}	3.06±0.21^{a}
B	17.45±1.01^{b}	1.90±0.17^{cd}	56.05±0.65^{a}	15.00±0.22^{e}	7.00±0.30^{c}	2.60±0.25^{a}
C	11.47±0.99^{a}	1.67±0.21^{bc}	64.94±0.51^{c}	12.29±0.43^{c}	6.53±0.55^{bc}	3.10±0.31^{a}
D	11.69±1.21^{a}	1.46±0.14^{b}	67.55±0.48^{d}	10.62±0.51^{b}	5.70±0.52^{b}	2.98±0.26^{a}
E	12.50±0.82^{a}	2.00±0.15^{d}	62.55±0.47^{b}	13.80±0.61^{d}	6.40±0.44^{bc}	2.75±0.37^{a}

Values show the means of triplicate analysis ±SD. Means with different superscript along the column are significantly different ($p<0.05$). The samples are composed of maize and Bambara groundnut in the following ratio: A, 100% maize; B, 100% Bambara groundnut; C, 50% maize : 50% Bambara groundnut; D, 75% maize : 25% Bambara groundnut; E, 25% maize : 75% Bambara groundnut.

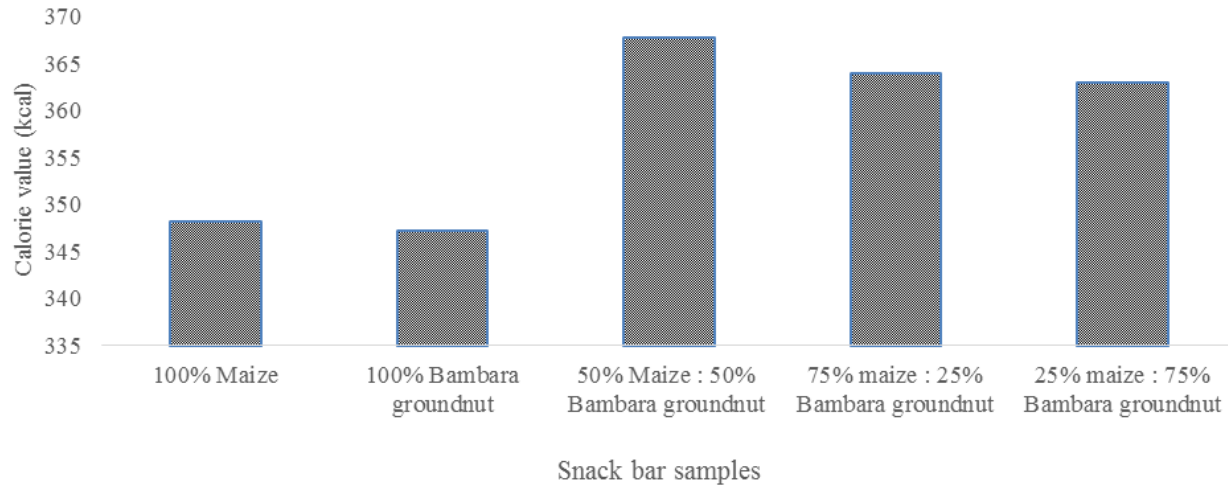

Figure 5. Calorie value of the snack bar samples.

snack bars. In contrast, there is no significant difference ($p>0.05$) in fiber content among all the snack bar samples.

The calorie value of the snack bar samples are presented in Figure 5. The snack bar prepared using 50% maize and 50% Bambara groundnut had the highest calorie value (367.69 kcal) whereas the sample which had the lowest calorie value (347.2 kcal) was prepared

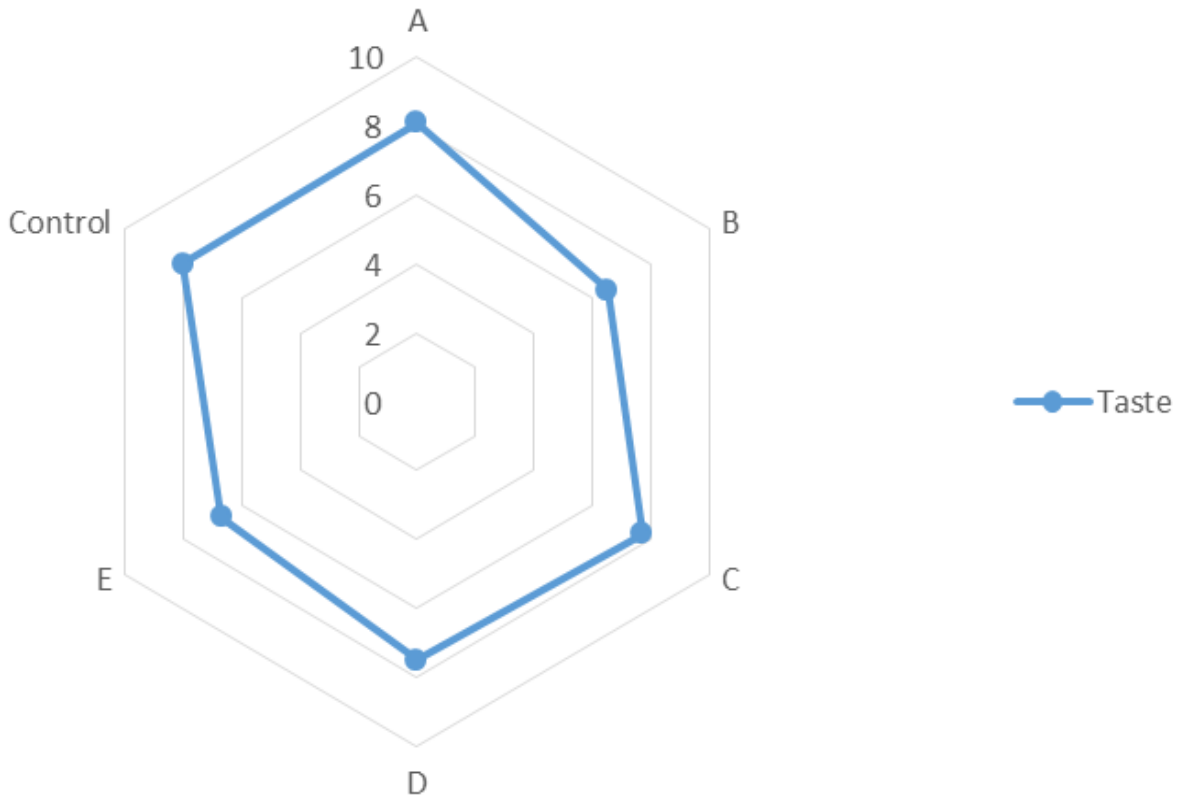

Figure 6. Taste of the snack bar samples. The samples are composed of maize and Bambara groundnut in the following ratio: A, 100% maize; B, 100% Bambara groundnut; C, 50% maize : 50% Bambara groundnut; D, 75% maize : 25% Bambara groundnut; E, 25% maize : 75% Bambara groundnut. Interpretation of the 9-point Hedonic scale: 9-Like extremely; 8-Like very much; 7-Like moderately; 6-Like slightly; 5-Neither liked nor disliked; 4-Disliked slightly; 3-Disliked moderately; 2-Disliked very much; 1-Disliked extremely.

using 100% Bambara groundnut. Worthy to note is that the snack bar prepared using 100% maize and the sample prepared using 100% Bambara groundnut had a little difference in their calorie content. Similarly, the calorie content of snack bar prepared using 75% maize: 25% Bambara groundnut is slightly different from the value recorded for snack bar prepared using 25% maize: 75% Bambara groundnut.

Figures 6 to 11 show the mean panelist score assigned to the snack bars evaluated based on their sensory attributes which ranged from 6.5-8.1 for taste, 7.1-8.0 for color, 6.4-8.0 for aroma, 7.0-8.0 for appearance, 7.1-8.0 for mouthfeel, and 7.4-8.2 for overall acceptability, respectively.

DISCUSSION

In this study, snack bars were prepared using maize and Bambara groundnut in different proportions. The total heterotrophic bacterial count (THBC) and total fungal count (TFC) of the snack bar samples is within the range of 4.10-4.43 $\log_{10}$ CFU/ml and 3.53-3.68 $\log_{10}$ CFU/ml, respectively. The snack bars were considered to be microbiologically safe for human consumption because the THBC of the samples were lower than 6 $\log_{10}$ CFU/ml which is the limit stipulated by International Commission on Microbiological Specification for Food (ICMSF) (Maduka et al., 2021). The total plate count of energy bars prepared by Bhavani et al. (2018) is within the range of 1.2-1.8 × 10^2 CFU/g whereas fungi was not detected in their product. In a related study that involved the production of snack bar using a blend of African breadfruit seed flour, maize flour and coconut grits, Edima-Nyah et al. (2019) also reported that the samples met the ICMSF specification.

Bacteria isolated from all the samples of snack bars include *Staphylococcus* spp., *Bacillus* spp., *Lactobacillus* spp., *Serratia* spp. and *E. coli.* Different cereals used as ingredients to prepare ready-to-eat (RTE) snack bars are possible sources of microbial contamination of the product (Los et al., 2018). At different stages of preparing snacks bars which include crushing of cereals and few other ingredients, mixing the ingredients, moulding, and cutting the snacks into bar shape and packaging, the products are predisposed to microbial contamination. In a

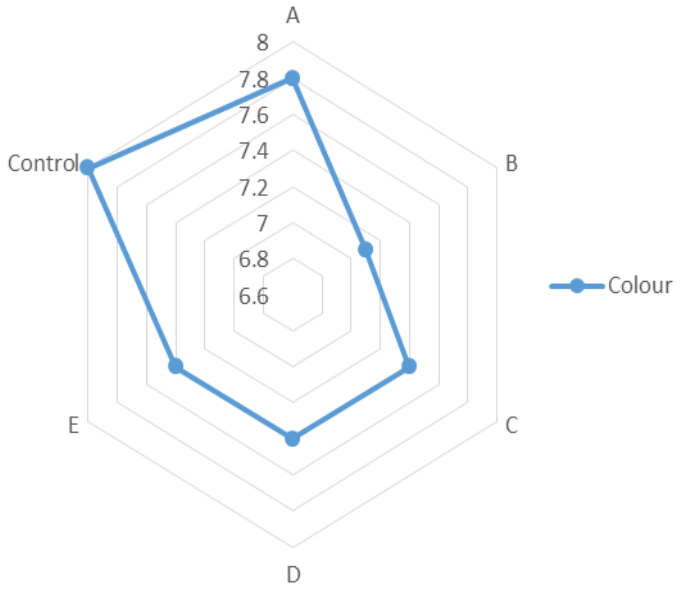

Figure 7. Colour of the snack bar samples. The samples are composed of maize and Bambara groundnut in the following ratio: A, 100% maize; B, 100 % Bambara groundnut; C, 50% maize : 50% Bambara groundnut; D, 75% maize : 25% Bambara groundnut; E, 25% maize: 75% Bambara groundnut. Interpretation of the 9-point Hedonic scale: 9-Like extremely; 8-Like very much; 7-Like moderately; 6-Like slightly; 5-Neither liked nor disliked; 4-Disliked slightly; 3-Disliked moderately; 2-Disliked very much; 1-Disliked extremely.

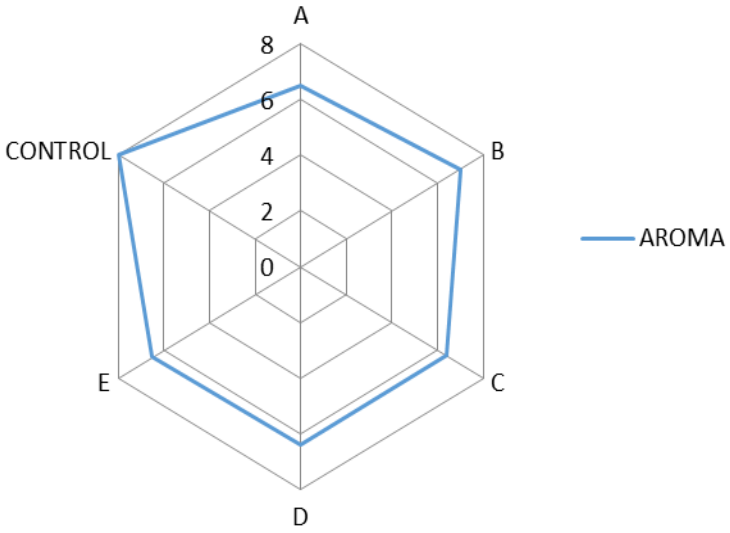

Figure 8. Aroma of the snack bar samples. The samples are composed of maize and Bambara groundnut in the following ratio: A, 100% maize; B, 100 % Bambara groundnut; C, 50% maize : 50% bambara groundnut; D, 75% maize : 25% Bambara groundnut; E, 25% maize : 75% Bambara groundnut. Interpretation of the 9-point Hedonic scale: 9-Like extremely; 8-Like very much; 7-Like moderately; 6-Like slightly; 5-Neither liked nor disliked; 4-Disliked slightly; 3-Disliked moderately; 2-Disliked very much; 1-Disliked extremely.

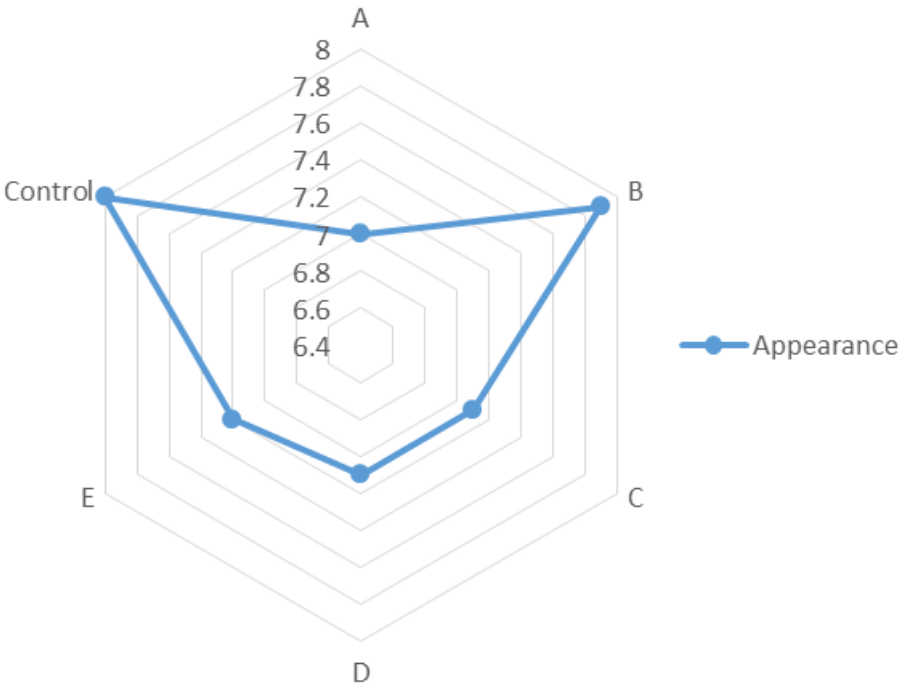

Figure 9. Appearance of the snack bar samples. The samples are composed of maize and Bambara groundnut in the following ratio: A, 100% maize; B, 100 % Bambara groundnut; C, 50% maize : 50% Bambara groundnut; D, 75% maize : 25% Bambara groundnut; E, 25% maize : 75% Bambara groundnut. Interpretation of the 9-point Hedonic scale: 9-Like extremely; 8-Like very much; 7-Like moderately; 6-Like slightly; 5-Neither liked nor disliked; 4-Disliked slightly; 3-Disliked moderately; 2-Disliked very much; 1-Disliked extremely.

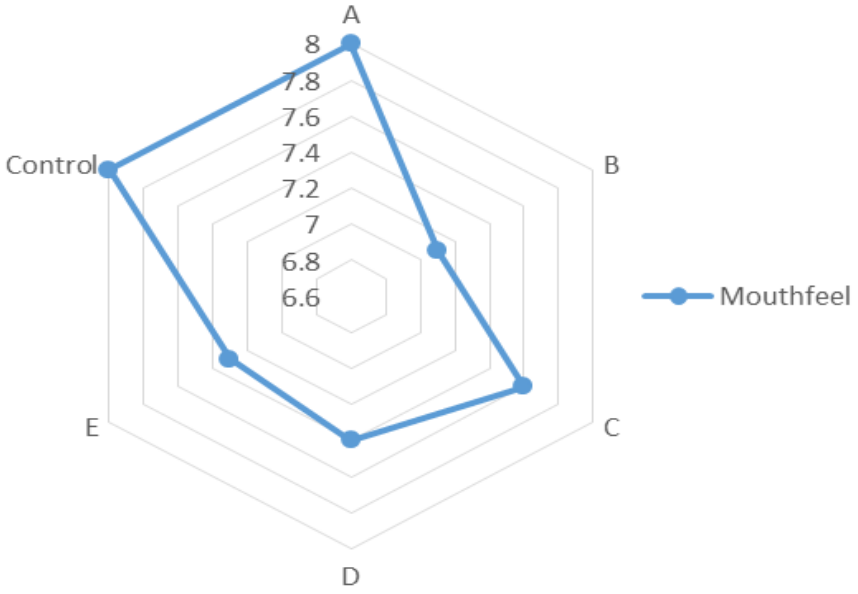

Figure 10. Mouthfeel of the snack bar samples. The samples are composed of maize and Bambara groundnut in the following ratio: A, 100% maize; B, 100 % Bambara groundnut; C, 50% maize : 50% Bambara groundnut; D, 75% maize : 25% Bambara groundnut; E, 25% maize : 75% Bambara groundnut. Interpretation of the 9-point Hedonic scale: 9-Like extremely; 8-Like very much; 7-Like moderately; 6-Like slightly; 5-Neither liked nor disliked; 4-Disliked slightly; 3-Disliked moderately; 2-Disliked very much; 1-Disliked extremely.

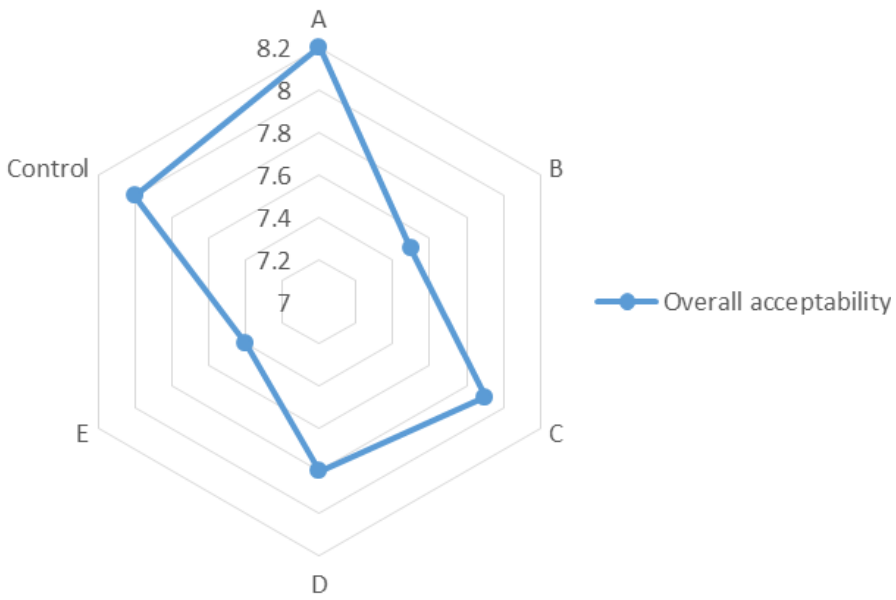

Figure 11. Overall acceptability of the snack bar samples. The samples are composed of maize and Bambara groundnut in the following ratio: A, 100% maize; B, 100 % Bambara groundnut; C, 50% maize : 50% Bambara groundnut; D, 75% maize : 25% Bambara groundnut; E, 25% maize : 75% Bambara groundnut. Interpretation of the 9-point Hedonic scale: 9-Like extremely; 8-Like very much; 7-Like moderately; 6-Like slightly; 5-Neither liked nor disliked; 4-Disliked slightly; 3-Disliked moderately; 2-Disliked very much; 1-Disliked extremely.

related study, Munhoz et al. (2014) reported that *Bacillus cereus* (< 10 MPN g^{-1}) was detected in cereal bars, but *Salmonella* spp. and coliforms were absent.

Excessive handling of ingredients used in preparing the snack bars with unwashed hands could be one of the sources of *E. coli* and *Staphylococcus* spp. in the samples. *Staphylococcus* spp. is commonly isolated from humans and animals especially their skin and mucus where the organism is part of the normal flora (Møretrø and Langsrud, 2017). It has been established that some cases of foodborne illness are caused by enterotoxigenic *Staphylococcus* strains and *E. coli* strains (Clarence et al., 2009). The presence of *E. coli* in food products is an indication that human and animal fecal contamination has occurred. The source of *Serratia* spp. in the snack bars could be from the environment. According to Møretrø and Langsrud (2017), *Serratia* spp. is commonly found in food processing plants, insects, vertebrate, water and soil. Due to spore forming ability of *Bacillus* spp., the microorganism which is ubiquitous in nature can survive harsh environmental condition. This could explain why *Bacillus* spp. is among the bacterial species which recorded the highest frequency of occurrence in the snack bar samples.

Antimicrobial activity of Bambara groundnut extract against *Klebsiella pneumonia* subsp. *pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* subsp. *aureus* ATCC 33591, *E. coli*, *B. cereus*, yeast (*Candida albicans*) and mold (*Aspergillus niger*) was reported by Klompong and Benjakul (2015). Given that some of these bacterial isolates were also isolated from the snack bars is an indication that antimicrobial properties of Bambara groundnut was less effective when it was used in preparing the snack bars. Since the steps involved in preparing the snack bars include baking at high temperature capable of destroying the antimicrobial properties of Bambara groundnut and killing all the microorganisms present in the snack bars, the bacteria and fungi found in and on the snack bars could be attributed to handling of the products after their preparation. It is likely that recontamination of the snack bars after baking occurred during cutting the snack into bar shape and packaging the product.

Fungi isolated from all the samples of snack bar include *Aspergillus*, *Saccharomyces*, *Penicillium* and *Rhizopus* spp. The sources of the fungal genera could be from soil and plants in the environment. The existence of some species of *Saccharomyces* only in nature, others as wild and domesticated strains have been described (Boynton and Greig, 2014). The yeast population in the snack bars could have a little influence in the sensory attributes of the samples. *Rhizopus* sp. is ubiquitous in the soil, excreta from animals and rotting vegetable. Both *Rhizopus* spp. and *Saccharomyces* spp. had the least frequency of occurrence (20%) among the fungal genera

isolated from the samples of snack bar. Some fermented products are produced using *Rhizopus* spp. (Gryganskyi et al., 2018). *Aspergillus* spp. is widely distributed in nature. This could have contributed to *Aspergillus* spp. (33%) being the fungal genera with the highest frequency of occurrence in the samples of snack bar. The presence of *Aspergillus* spp. in the snack bar samples is a concern to public health due to possibility of producing mycotoxins harmful to human health. Odetunde et al. (2021) reported that some species of *Aspergillus* isolated from Bambara nuts produced different amounts of mycotoxins which include Aflatoxins B_1, B_2, G_1 and G_2. Some species of *Aspergillus* and *Penicillium* are capable of causing food spoilage. The frequency of occurrence of *Penicillium* spp. in the samples of snack bar is 27%.

Moisture content is associated with the shelf life of food products. Ordinarily, food products with a low moisture content (low water activity) have an extended shelf life. The moisture content of the samples of the snack bars was between 11.47±0.99-17.45±1.01%. The moisture content of snack bars prepared using 100% maize and 100% Bambara groundnut are significantly different ($p<0.05$). In contrast, snack bars prepared using blends of maize and Bambara groundnuts were not significantly different ($p>0.05$). In a related study, Edima-Nyah et al. (2019) reported a lower moisture content in snack bars which range from 3.76 to 4.8% which is considerably lower than the results found in this study. Similarly, Eke-Ejiofor and Okoye (2018) reported that moisture content of cereal bars is within the range of 5.09-6.78%. Both products are expected to have a longer shelf life than the samples of snack bar developed in this study. According to Ire et al. (2020), digestion of food materials and some other physiological processes are enhanced by moisture which also helps in nutrient absorption from food. In the human body, water performs many important roles which include being a carrier for nutrients and waste products, among others (Jéquier and Constant, 2010).

The ash content of the snack bars was in the range of 1.09±0.07-2.00±0.15%. These results are in agreement with the ash content of cereal bars prepared by Eke-Ejiofor and Okoye (2018) which was within the range of 1.54 to 1.90%. There are significant differences ($p<0.05$) in ash content of the snack bars developed in this study. The values were lower than what was reported by Edima-Nyah et al. (2019) in a related study. According to these authors, the ash content of the snack bars are within the range of 2.83 to 4.57%. In order to estimate the amount of minerals in a food sample, the ash content of the sample is taking into consideration. Therefore, any food product reported to have with high ash content is expected to be rich in mineral elements. In humans, the consumption of diets that contain moderate amounts of mineral elements increases the speed at which metabolic processes occur. This brings about improvement in growth and development.

The protein content of all the snack bars in this study is within the range of 6.32±0.36 to 15.00±0.22%. With regards to protein content, all the snack bar samples except for Sample A is within the dietary reference intake's (DRI) acceptable macronutrient distribution range (AMDR) between 10 and 35% specifically for adults (Ire et al., 2020). In a related study, Edima-Nyah et al. (2019) reported that protein content of snack bars is within the range of 16.16 to 22.43%. The protein content of the snack bars is higher than the values reported in this study. This could be attributed to differences in the quantity and quality of the ingredients used in preparing the snack bars. The protein content of all the samples of the snack bars was significantly different ($p<0.05$). According to Kumar et al. (2017), proteins present in foods are required for body building and repair. Proteins are needed for the maintenance of body tissues. They also play a vital role in the synthesis of plasma proteins, hemoglobin, hormones, enzymes, coagulation factors and antibodies.

The crude fiber content of the snack bars in this study is within the range of 2.60±0.25 to 3.10±0.31%. The crude fiber content of the samples of snack bar is not significantly different ($p>0.05$). The values are lower than the result reported by Edima-Nyah et al. (2019) in a related study. The authors reported that crude fiber content of snack bars produced using blends of African seed flour, maize flour and coconut grits is within the range of 10.12 to 17.76%. Snack bar produced from a blend of 50% maize + 50% Bambara groundnut had the highest crude fiber content. To prevent constipation and other health maladies associated with inefficient waste removal from the body, regular consumption of diets rich in crude fiber is recommended. There are indications that consumption of vegetable fiber reduces the level of cholesterol in the body (Soliman, 2019). It could also reduce the risk of coronary heart diseases. The risk of developing hypertension, colon and breast cancer is also reduced by eating diets rich in vegetable fiber (Jenkins et al., 2001). Glucose tolerance is enhanced by vegetable fiber consumption which also increases insulin sensitivity (Edima-Nyah et al., 2019).

The fat content of all the samples of snack bars was within the range of 4.60±0.50 to 7.00±0.30% significantly different ($p<0.05$). Given the DRI's AMDR recommendation of 25 to 35% total fats for adults, all the samples of the snack bars did not meet the requirement. In a related study, Edima-Nyah et al. (2019) reported that crude fat content of snack bars range between 7.31 and 8.46%. Although the values are higher than what was reported in this study, it did not meet DRI's AMDR recommendations either. Fat is an energy source for humans necessary for growth and development. It also enables Vitamins A, D, E and K to be absorbed into the body. The amount of fat in food influences the taste and consistency (Ire et al., 2020). According to Prentice (2005), human beings obtain bulk of the food energy it requires from fat and carbohydrate. Relatively low crude

fat content of snack bars reported in this study is an indication that the bulk of the energy in the product comes from carbohydrate.

The carbohydrate content of the snack bars which was within the range of 56.05±0.65 to 70.37±0.71% and higher than the results reported by Edima-Nyah et al. (2019) in a related study. In contrast, the carbohydrate content of five samples of cereal bars prepared by Eke-Ejiofor and Okoye (2018) using rolled oat, yellow maize, white maize, millet and Guinea corn had a range of 36.6 to 41.4% which is lower than the result reported in this study. This could be attributed to the ingredients used in different proportions to prepare the snack bars. The carbohydrate content of all the snack bars are significantly different ($p<0.05$). Worthy to note is that carbohydrate content of some of the snack bar samples are within the recommended dietary reference intake's (DRI) acceptable macronutrient distribution range (AMDR) of 45 to 65% of energy obtained from carbohydrate for adults (Ire et al., 2020). According to Ho et al. (2016), post meal and diurnal glucose profiles in patients suffering from insulin resistance and type-2 diabetes might improve as a result of consuming snack bars characterized by high ratio of protein/carbohydrate.

All the samples of snack bar developed in this study had a calorie value within the range of 347.20 to 367.69 kcal. The snack bar prepared using 50% maize and 50% Bambara groundnut had the highest calorie value (367.69 kcal). In contrast, the snack bar (Sample B) prepared using 100% Bambara groundnut had the least calorie value (347.20 kcal). This could be because of high amount of Bambara groundnut which is rich in proteins used in preparing the snack bar (Sample B). Although protein is listed among the four principal classes of energy yielding macronutrients, it is not considered as a key supplier of dietary energy (Prentice, 2005). The calorie value of the snack bars is comparable with the result reported by Edima-Nyah et al. (2019). Their report stated that energy value of snack bars prepared using blends of African breadfruit seeds flour, maize flour and coconut grits is within the range of 336.12 to 369.71 Kcal/100 g.

According to Kim et al. (2009), the sensory characteristics of cereal snack bars go a long way to influence the acceptability of the product by consumers. The sensory report revealed that taste, mouthfeel and overall acceptability of snack bar prepared using 100% maize was liked very much by the panelist while other sensory attributes for all the samples of snack bar were either liked moderately or liked slightly. The panelist reported that appearance and color of all the samples of snack bar prepared using maize and Bambara groundnut in different proportion were liked moderately. All the sensory parameters of snack bars prepared using 100% Bambara groundnut were liked moderately by the panelist except taste which they liked slightly. Taking into consideration the sensory scores assigned to sensory attributes of Samples C and D, the panelist liked moderately all the sensory parameters of both samples with the exception of aroma which they liked slightly. All the sensory parameters of snack bar prepared using 25% maize and 75% Bambara groundnut were liked moderately by the panelist except aroma and taste which they liked slightly. Overall, the snack bar prepared using 100% maize was assigned the highest score for the sensory parameters. Therefore, it is the most preferred sample of snack bar developed in this study.

The sensory panelist very much liked all the sensory parameters of the commercialized snack bar which serve as control. The preference given to the control sample by the sensory panelist compared with snack bars prepared using maize and Bambara groundnut in different proportions could be as a result of the panelist being familiar with the commercialized snack bar. In a related study, Edima-Nyah et al. (2019) reported a slightly lower sensory score for appearance (5.00-8.33), aroma (5.73-7.76), taste (5.36-7.56) and overall acceptability (5.63-7.80) of snack bars compared with the sensory scores reported in this study. Eke-Ejiofor and Okoye (2018) also reported a slightly lower sensory scores for color (5.20-7.10), taste (5.60-7.10), aroma (5.60-6.10) and overall acceptability (5.63-7.80) of cereal bars compared with the result reported in this study. Differences in ingredients used in preparing the snack bars and preferences of the sensory panelists could have influenced the sensory scores reported by Eke-Ejiofor and Okoye (2018) and Edima-Nyah et al. (2019) which were lower than the result reported in this study.

Conclusion

The snack bars developed in this study are microbiologically safe for human consumption since the total heterotrophic bacterial count of the samples were below the limit stipulated by the ICMSF. Bacteria isolated from the samples were *Bacillus* spp., *Lactobacillus* spp., *Staphylococcus* spp., *Serratia* spp. and *Escherichia coli* while the fungal isolates were *Aspergillus*, *Penicillium*, *Rhizopus* and *Saccharomyces* spp. The moisture, ash, carbohydrate, crude protein, fat and fiber content of the samples were within the range of 11.47±0.99-17.45±1.01, 1.09±0.07-2.00±0.15, 56.05±0.65-70.37±0.71, 6.32±0.36-15.00±0.22, 4.60±0.50-7.00±0.30 and 2.60±0.25-3.10±0.31%, respectively. The taste, mouthfeel and overall acceptability of snack bar prepared using 100% maize was liked very much by the panelist while other sensory attributes for all the samples of snack bar prepared using maize and Bambara groundnuts in different proportions were either liked moderately or liked slightly. All the sensory parameters of the commercialized snack bar which serve as the control was liked very much by the panelist. Among the samples of snack bar developed in this study, the snack bar prepared using

100% maize was the most preferred sample based on sensorial characteristics. Nutritionally, the snack bar prepared using 50% maize and 50% Bambara groundnut had the highest calorie value; 100% Bambara groundnut had the highest protein content.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Impact of mycorrhization on transplanting stress and the juvenile growth of an Ivorian forest species *Guibourtia ehie* (Fabaceae, (A. Chev.) J. Leonard)

Don-Rodrigue Bi Rosin Voko*, Kouassi Clément Kouassi, Kra Athanase Kouassi and Ibrahim Konaté

Laboratoire d'Agrovalorisation, Département de Biochimie-Microbiologie, Université Jean Lorougon Guédé, Daloa, BP 150 Daloa, Côte d'Ivoire.

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Ivorian forest has been experiencing degradation for several decades despite reforestation efforts. The main cause of the failure of reforestation policies is an important mortality at the time of planting (transplanting stress). To remedy this problem, mycorrhization technology based on the beneficial effects of arbuscular mycorrhizal fungi (AMF) could provide a sustainable solution. The objective of this study was to evaluate the effects of AMF inocula (local and commercial inoculum) on the juvenile growth of a Côte d'Ivoire forest species (*Guibourtia ehie*). Vegetative growth parameters and mineral nutrition (N, P, K, and Ca) were evaluated. After 150 days of cultivation, the plants treated with the local inoculum had the highest mycorrhization frequencies (75%) and intensities (21.23%). Also for growth parameters (height, number of leaves, leaf area, and crown diameter) and for nitrogen and potassium contents, the plants treated with the local polyspecific inoculum had the highest values compared to the plants treated with the commercial inoculum and the control plants. Mycorrhization improved mineral nutrition as well as vegetative growth of *G. ehie* seedlings. The integration of mycorrhizal inoculation from local strains in reforestation policies could be a sustainable solution for the recolonization of degraded forests by endangered species.

Key words: *Guibourtia ehie*, inoculum, mycorrhization mineral nutrition, vegetative growth, Ivory Coast.

INTRODUCTION

Tropical forests harbour many forest species with high economic value (Parmentier et al., 2007; Schroeder et al., 2010; Slik et al., 2015). However, anthropogenic activities including agriculture and extractive activities have been responsible for the degradation of these ecosystems (Ghazoul and Sheil, 2010; Maystadt et al., 2020). The overexploitation of forest resources mainly leads to the degradation of the ecological characteristics of the ecosystems, the impoverishment of the vegetation cover and especially the extinction of important forest species such as *Guibourtia ehie* (Gone et al., 2013). Indeed, of the 43 local forest species commonly recorded,

*Corresponding author. E-mail: rosinrodrigue@gmail.com. Tel: +225 0708540750.

25 are considered by the IUCN as vulnerable, and at risk (Kouadio, 2012). *G. ehie* is a slow-growing plant (Oteng-Amoako and Essien, 2011) whose trees are susceptible of burning (Hall and Swaine, 1981). It is common in West African tropical forests (Keay, 1990), but now threatened by overexploitation because of its wood quality (Hawthorne and Jongkind, 2006; Tosso et al., 2017). In Côte d'Ivoire, reforestation efforts have been undertaken by the forest development company (SODEFOR) to recolonize environments with extinct or endangered species. However, very few forests have been reconstituted and the keystone species that were the pride of the Ivorian forests are continuing to disappear. The main reason for the failure of reforestation policies is significant mortality at transplantation crisis (Ouahmane et al., 2007). This mortality would be linked to the difficult adaptation of plant species to the new unfavorable ecological conditions of degraded ecosystems. To remedy this difficulty, mycorrhizal symbiosis could be a promising and sustainable alternative. Arbuscular mycorrhizal fungi could play an essential role in the adaptation and survival of plant species in adverse environments. These mycorrhizal fungi provide the host plant with better phosphate, nitrogen and water nutrition. In addition to its nutritional contribution, the symbiosis confers on plants a high resistance to abiotic stresses (Aroca et al., 2017) through the improvement of plant nutritional status (Colla et al., 2008), the dilution of the toxic effects of ions (Audet and Charest, 2006; Kapoor and Bhatnagar, 2007) and the modification of plant physiology (Kumar et al., 2010). In temperate regions, ectomycorrhizal fungi have improved the growth and mineral nutrition of trees (Smith and Read, 2008). They also allow trees to better resist certain root diseases and to better exploit water resources. In the tropics, the primordial role of endomycorrhizal fungi in reforestation has already been demonstrated in the Sahel. Mycorrhizal inoculation has allowed the successful reforestation of more than 5,104 ha out of 11,106 ha planned (Duponnois et al., 2010). Thus, the evaluation of the mycorrhizal status of forest species and the integration of this data in the realization of nurseries and then in the growth and development of seedlings in plantation could be a sustainable solution for the colonization of forests degraded by endangered species. In this study the main objective is to obtain by the mycorrhization technique balanced, vigorous, and healthy *G. ehie* seedlings able to withstand the stress of transplantation in degraded ecosystem conditions.

MATERIALS AND METHODS

Inoculum 1 characteristics

Inoculum 1 production

Inoculum 1 (Local inoculum) was produced by trapping AMFs in the soil of the INP-HB (Institut National Polytechnique Félix Houphouet-Boigny) forest. The trapping technique is a bioassay, which allows to obtain AMF propagules in quality and quantity to initiate inoculation tests (Morton et al., 1993). Cowpea, which has a 60- to 70-day cycle, was chosen as the host plant. Cowpea seeds disinfected with 12°-10% bleach and rinsed once for 2 min with sterile water were pre-germinated. Plants of the same size were selected and sown in 2-L plastic pots containing a mixture of 700 g of gardener's potting soil + sand (1v/1v) previously sterilized (110°C, 2 kg/cm^2, 3 h) and 150 g of forest soil serving as inoculum (2 plants per pot).

Arbuscular mycorrhizal fungal (AMF) spore identification in inoculum 1

After 3 months, the number of AMFs propagules (spores) from trap culture was established. Spores were extracted by wet-sieving and decanting (Gerdemann and Nicolson, 1963) using sieve with different sizes (45, 90, 125 and 500 µm) and the modified sucrose density gradient centrifugation method (Walker et al., 1982). For AMF spore's identification, healthy spores were mounted on glass microscope slides and stained with polyvinyl alcohollacto-glycerol (1 v/v PVLG) mixed with and without Melzer's reagent (Morton et al., 1993; Brundrett et al., 1994). Spores were cracked open to allow spore substructure characteristics under an optic microscope (EUROMEX Holland CSL/CKL) at a magnification of ×400. AMF spore morphotyping was based on Oehl et al. (2011) and the revision of *Glomeromycota* genera proposed by Redecker et al. (2013). The number of AMFs propagules (spores) in inoculum 1 (substrate in the pots) was estimated to be 700 spores per gram.

Inoculum 2 characteristics

Inoculum 2 is a commercial monospecific inoculum of *Glomus intraradices* produced by Myke Pro whose density has been estimated by the manufacturer at 3000 propagules/g.

Collection of G. ehie seedlings

G. ehie seedlings of about 10 cm high at the four-leaf stage were collected in Yamoussoukro (Côte d'Ivoire) in the arboriculture of the Institut National Polytechnique Félix Houphouët-Boigny (INPHB). This forest created in 1989 is full of essential forest species such as *Milicia excelsa* (Moraceae), *Mansonia altissima* (Sterculiaceae), *Pterygota marcrocarpa* (Sterculiaceae), *G. ehie* (Fabaceae), *Triplochiton scleroxylon* (Sterculiaceae), and *Terminalia ivorensis* (Combretaceae). To harvest a seedling, furrows were made around the seedling with a daba, then it was dug up with the clod of soil present on the roots. The roots were then cleaned of the soil clod and rinsed thoroughly with water to remove any surface microorganisms. On each plant, at the lateral roots, a sample of the finest roots likely to be colonized by native AMF were pulled out for a colonization check according to Trouvelot et al. (1986). Only seedlings with 0% colonization were retained for the study.

Inoculation process of G. ehie seedlings

Seedlings of the same size (about 10 cm high, about 0.185 mm in diameter) at the 4-leaf stage were selected for planting in 5-L plastic bags containing a mixture of 2000 g of sterilized potting soil (autoclaved at 110°C, 2 kg/cm^2, 3 h; characteristics: pH = 6.8; organic matter = 2.57%; total nitrogen = 0.16%; available phosphorus = 75 mg/kg; cation exchange capacity = 7.4 cmol.kg^{-1}) and 200 g of inoculum (1 plant/pot). The roots of the seedlings were placed in direct contact with the inoculum to optimize mycorrhizal

Table 1. Mycorrhization intensity and frequency.

Treatment	Mycorrhization intensity (%)	Mycorrhization frequency (%)
Control	00±0.00	00±0.00
Inoculum 1	21.23^{a}±4.37	75^{a}±3.77
Inoculum 2	5.40^{b}±1.77	14.5^{b}±2.30

Means within the same column followed by the same letter are not significantly different at the P = 0.05 level of probability based on Tukey's HSD statistics

colonization. The seedlings in the control bags were carried in 2000 g of sterilized potting soil + 200 g of sterilized substrate (autoclaved at 110°C, 2 kg/cm^2, 3 h). Each bag was watered with 500 ml of water every 3 days until the end of the experiment.

Experimental design

The experiment took place in the open area at the edge of the experimental forest of the INP-HB. The design is completely randomized and includes one (1) plant species (*G. ehie*), three (3) treatments and 30 replications. The treatment factor has three levels: Inoculum 1 (local inoculum), Inoculum 2 (commercial inoculum) and Control. A total number of 90 seedlings were used, 30 seedlings per treatment.

Assessment of root colonization

Fine roots were sampled at 150 days of cultivation with three replicates per treatment. Each treatment contained three plants. Roots were rinsed and cut into 1 cm fragments. These roots fragments were cleared by boiling in 10% (w/v) KOH and stained with 0.05% (v/v) trypan blue in lactoglycerol according to Phillips and Haymann (1970) method. Ten pieces of roots per plant were placed in glycerol (50%) between slide and coverslip (Kormanik and McGraw, 1982) and observed under an optical microscope. Root colonization was evaluated through two parameters: mycorrhization intensity and mycorrhization frequency. The mycorrhization intensity indicates the rate of mycorrhizal structures in a colonized root fragment. Mycorrhization frequency represents the percentage of root fragments with mycorrhizal structures out of the total number of fragments observed. The colonized roots were observed and evaluated according to Trouvelot et al. (1986).

Measurement of mineral nutrition parameters

After drying in an oven at 60°C for 5 days, the samples of aerial parts (leaves and stems) were reduced to fine powder by means of a mortar. Then the mineralization of the powders was performed in a muffle furnace at 500°C. The ashes were solubilized with HCl. The extracts obtained were then filtered on ash-free filter paper and made up to 50 ml with distilled water. The stock solutions obtained were stored in flasks and thus ready for the determination of mineral elements. Nitrogen was determined by the Kjeldahl method (Bremner, 1960) with mineralization in the presence of glucose to avoid nitrate losses and catalysts (SO_4K_2, SO_4Cu, and Selenium). Phosphorus was determined by phopho-vanado-molybdate colorimetry (Pansu and Gautheyrou, 2006); potassium and calcium by flame photometry after ion exchange; magnesium by the complexometric method.

Collection of growth data

Measurements were made on the first day of transplantation (D1), on the 30th day (D30), on the 60th day (D60), on the 90th day (D90), on the 120th day (D120) and on the 150th day (D150) corresponding to the number of days necessary to judge the resistance to transplantation stress. The data collected concerned the survival rate, the height of the seedlings, the diameter at the collar and the leaf area. The survival rate was determined according to the following formula:

Survival rate (%) = (Number of surviving plants)/(Number of replicates) × 100

Plant height was measured with a 30 cm ruler. The diameter at the neck of the plants was measured with a Vernier caliper. The number of leaves was obtained by counting. Total leaf area for each individual plant sampled per treatment was determined as follows: leaves were classified into "large" (L) and "small" (S) batches according to whether they had reached maximum growth or not. For each batch of leaves, a sample of 2 leaves was considered for the determination of the average leaf area using the MESURIUM software. The leaf area of each batch of leaves was obtained by multiplying the number of leaves by the corresponding average area. Thus, the total leaf area (SFT) is calculated from the following formula:

SFT = STG + STP with STG = Total leaf area of the "large" batch of leaves and STP = Total leaf area of the "small" batch of leaves.

Statistical analysis of the data

The data obtained in this study were processed by a single factor analysis of variance (ANOVA) with 3 modalities (Control: no inoculum, Inoculum 1: local inoculum; Inoculum 2: commercial inoculum). This analysis was performed by STATISTICA 7.1 software. Tukey's HSD test ($p \leq 0.05$) was used to identify which means actually differed when the analysis of variance revealed a significant difference. The Tukey HSD test also allowed for multiple comparisons of means to form homogeneous groups.

RESULTS

Mycorrhizal colonization rate of *G. ehie* roots at 150 days

At 150 days of cultivation, roots of plants treated with inoculum 1 showed higher mycorrhization intensities and frequencies than those treated with inoculum 2. Roots of control plants showed no mycorrhizal structure (Table 1).

Impact of mycorrhization on plant mineral nutrition

The average mineral element contents of aerial parts are

Table 2. Mineral contents of aerial parts of *Guibourtia ehie* seedlings.

Treatment	Nitrogen (%)	Phosphorus (ppm)	Potassium (cmol.kg^{-1})	Calcium (cmol.kg^{-1})	Magnesium (cmol.kg^{-1})
Control	1.68^{b}±0.15	0.22^{a}±0.05	0.81^{b}±0.15	1.46^{a}±0.25	0.4^{a}±0.22
Inoculum 1	2.25^{a}±0.21	0.23^{a}±0.03	1.89^{a}±0.07	1.45^{a}±0.23	0.4^{a}±0.27
Inoculum 2	2.01^{ab}±0.18	0.22^{a}±0.04	0.98^{b}±0.18	1.43^{a}±0.20	0.38^{a}±0.18

Means within the same column followed by the same letter are not significantly different at the P = 0.05 level of probability based on Tukey's HSD statistics

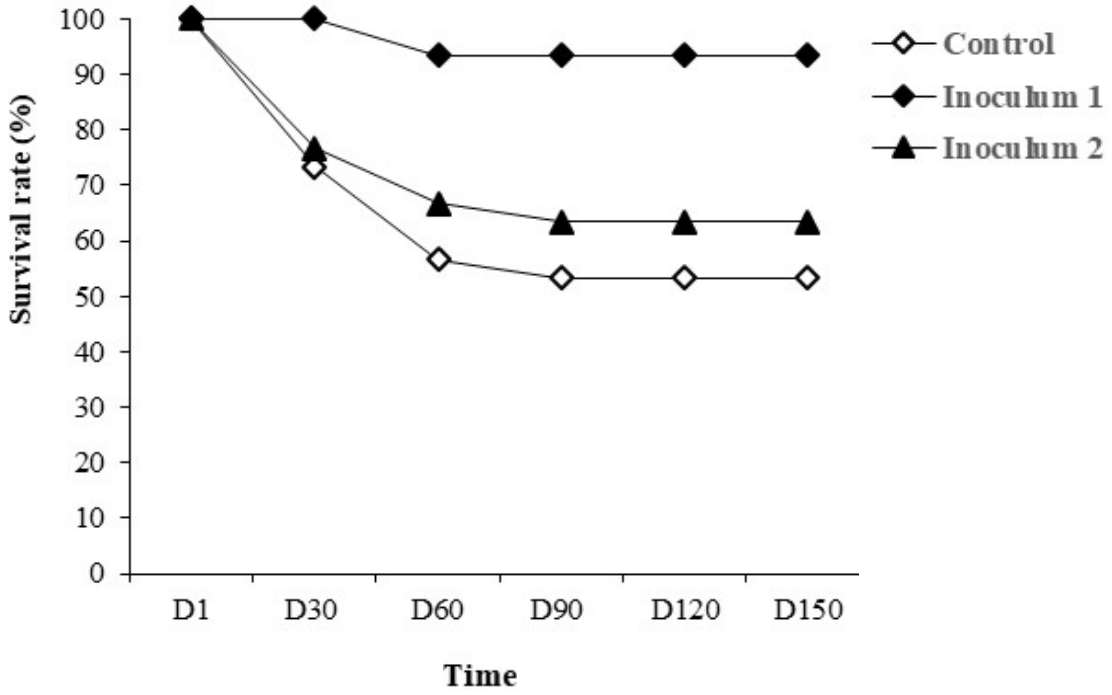

Figure 1. Survival rate of *Guibourtia ehei* seedlings as a function of treatments and growth duration.

presented in Table 2. The mineral nitrogen content of the leaves of plants treated with inoculum 1 was higher (2.52%) than those obtained with the control (1.68%). Similarly, the potassium content of the leaves of plants treated with inoculum 1 is higher (1.89 cmol.kg^{-1}) than those obtained with inoculum 2 (0.98 cmol.kg^{-1}) and the control (0.807 cmol/kg^{-1}). On the other hand, for phosphorus, calcium and magnesium contents, no significant difference was noted between the three different treatments with Tukey's HSD test (p≤ 0.05).

Adaptation of mycorrhizal seedlings to transplant stress

Survival of seedlings under transplanting stress

The survival rate of seedlings to transplantation stress was assessed from day 1 to day 150 (Figure 1). For seedlings treated with inoculum 1, the survival rate was 100% for the first 30 days of transplantation. From the 60th day, the survival rate decreased to 93.33% and then stabilized at this value until the 150th day. With the plants treated with inoculum 2, a continuous decrease from day 1 to day 90 of transplantation from 100 to 63.33% was observed, then this rate was maintained until day 150. In the case of plants that received no inoculum (controls), the survival rate dropped from 100 to 53.33% from day 1 to day 90 of transplantation, then the rate stabilized until day 150.

Impact of mycorrhization on the growth of G. ehie seedlings

The height of the plants as a function of time is as shown in Figure 2A. All plants have a continuous height growth from day 1 to day 150. The plants treated with inoculum 1

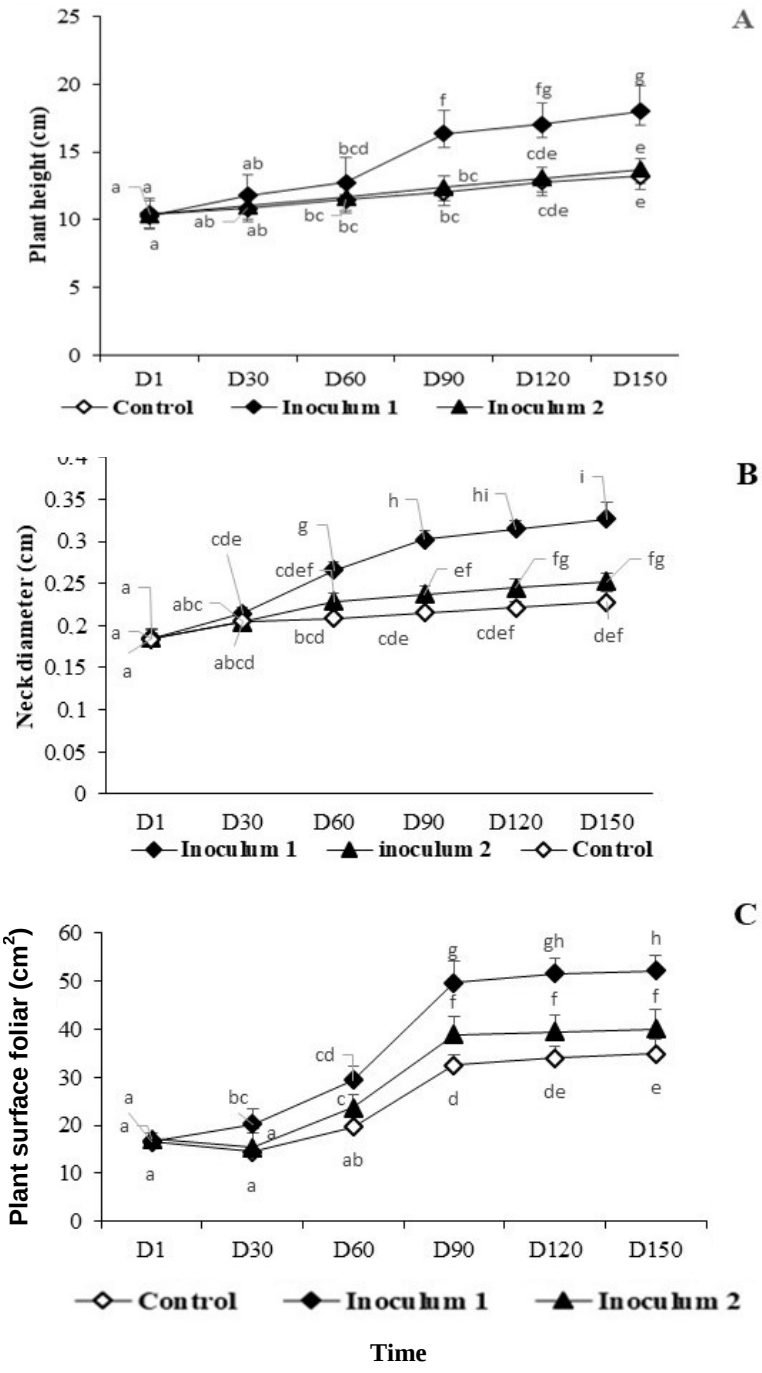

Figure 2. Evolution of growth parameters of *Guibourtia ehie* plants according to treatments and time. A. Average height; B. Average collar diameter; C. Average total leaf area. Means within the same column followed by the same letter are not significantly different at the P = 0.05 level of probability based on Tukey's HSD statistics.

show the greatest growth in height. Indeed, with inoculum 1 the growth of the plants is faster. However, no significant difference was observed between the height growth of the control plants and the plants treated with inoculum 2 until day 105.

Evolution of the diameter at the neck of the plants as a function of time was evaluated (Figure 2B). All curves show an increasing trend. However, the diameter of the plants treated with inoculum 1 evolved faster than those treated with inoculum 2. Also, the diameter of the plants

treated with inoculum 2 evolved faster than that of the control plants. Indeed, as early as 30 days after transplanting, a significant difference was observed between the diameter of the plants treated with inoculum 1 and the initial diameter of the seedlings at T0. It is from 60 days that a significant difference is noted between the diameter of the plants treated with inoculum 2 and the initial diameter at T0. It is only after 90 days that a significant difference is noted between the diameter of the control plants and the initial diameter at T0 of the control seedlings.

Evolution of the average total leaf area of *G. ehie* plants is as shown in Figure 2C. In control plants and inoculum 2, a decrease in leaf area was observed during the first 30 days followed by an increase in leaf area from day 30 to day 150. On the other hand, inoculum 1, the growth of leaf area is continuous from time T0 to 150 days. Also, the growth of the SFT of the plants treated with inoculum 1 is faster than that of the control plants and the plants treated with inoculum 2. As early as 60 days, there is a significant difference between the total leaf areas of the plants treated with inoculum 1 and those of the seedlings at T0. The growth of plants treated with inoculum 2 was faster than that of the control plants. However, it is from 90 days that a significant difference is observed between the plants treated with inoculum 2 and the seedlings at T0.

DISCUSSION

This study was conducted with the objective of evaluating the effect of mycorrhization on adaptation to transplanting stress, mineral nutrition and growth of young shoots of *G. ehie.* It is a proposal for sustainable solutions to the difficulties encountered by reforestation policies in a context of climate change and forest ecosystem degradation. This study shows that the mycorizogenic power of inoculum 1, that is, the local polyspecific inoculum, is more important on *G. ehie* seedlings than that of inoculum 2, that is, the exotic monospecific inoculum. The climatic and edaphic environment would influence the ability of arbuscular mycorrhizal fungi to colonize plant roots (Casazza et al., 2017; Melo et al., 2019). Thus, the exotic inoculum (inoculum 2) would have difficulty developing under local ecological conditions. The same conclusion has been drawn by several authors (Copeman et al., 1996; Berruti et al., 2016). In fact these authors proved that in general, local inocula colonize plants better than inocula of foreign origin. It was also obtained during this study that mycorrhization, especially with the local polyspecific inoculum, improved the nitrogen and potassium contents of the aerial parts on the other hand no improvement in phosphorus, magnesium and calcium contents was noted compared to the control. These results are contrary to some studies dealing with the impact of mycorrhization on plant mineral nutrition, which concluded that mycorrhization acted mainly by improving phosphate nutrition (Walder and van der Heijden, 2015; Shi et al., 2021). In contrast, most studies on tropical soils reach the same conclusion as ours. Namely, that mycorrhization has more effects on nitrogen and potassium nutrition than on phosphorus nutrition (Osonubi et al., 1995; Séry et al., 2016).

The mycorrhized seedlings had higher survival percentages than those that received no inoculum. That is, 63.33% survival with inoculum 2 and up to 93.33% survival with inoculum 1, compared to 53.33% with the control plants. The mycorrhized seedlings were more resistant to transplant stress despite the pre-transplant removal of the thinner roots. This important ability of mycorrhization to improve plant adaptation to stressful conditions or changing ecosystems has been cited several times (Smith and Read, 2008; Sinclair et al., 2014). Indeed these studies showed that mycorrhization improved plant adaptation to harsh conditions through its positive action on soil structure (Rillig and Steinberg, 2002; Zhang et al., 2017), inhibition of some soil pathogens (Elsen et al., 2003; Chen et al., 2018; Diagne et al., 2020) and mobilization of essential mineral elements.

Also, during this study, mycorrhization improved the overall growth parameters of *G. ehie* seedlings compared to control plants. Indeed, the increase in total leaf area of treated seedlings was greater than that of untreated seedlings. However, only inoculum 1 resulted in a greater growth in height and an increase in the diameter at the collar of the seedlings compared to the control plants. Overall, inoculum 1 appeared to perform better than inoculum 2 (N and K nutrition, transplant stress resistance, seedling height, and total leaf area). This can be explained by the greater root colonization with inoculum 1 compared to inoculum 2. Indeed, the benefits brought to the plant by mycorrhization are proportional to the root colonization rate (Campo et al., 2020). These results are consistent with the majority of studies that have compared the efficiency of local and exotic inocula. These studies have shown that native multispecific inocula have a better impact on plant nutrition and growth compared to exotic monospecific commercial inocula (Ortas and Ustuner, 2014; Kouadio et al., 2017).

Conclusion

Anthropogenic activities such as agriculture and overexploitation of species are at the origin of the disappearance of important forest species. The quality of the timber and the use of secondary metabolites in pharmaceutical and cosmetic industries make *G. ehie* a highly sought after plant. This study proved that mycorrhization could provide solutions to the reforestation difficulties of this plant. Indeed, adaptation

to transplanting stress, mineral nutrition and vegetative growth of *G. ehie* seedlings were improved by mycorrhization. However, for a better efficiency of the mycorrhization technology on reforestation and for a sustainable commercial exploitation of this plant, it will be necessary to develop an ecological engineering from local strains. This technology will consist of identification and selection of best strains for the production of efficient inoculums.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Characterization of culturable microbial community in oil contaminated soils in Greater Port Harcourt Area, Nigeria

Wanjala M. P.[1,5*], Odokuma L.[1], Etela I.[2], Ramkat R.[3] and Odogwu B. A.[4]

[1]Department of Microbiology, Faculty of Science, University of Port Harcourt, Nigeria.
[2]Department of Animal Science, Faculty of Agriculture, University of Port Harcourt, Nigeria.
[3]Center of Excellence in Phytochemicals, Textiles and Renewable Energy, Faculty of Science, Moi University, Eldoret, Kenya.
[4]Department of Plant Science and Biochemistry, Faculty of Science, University of Port Harcourt, Nigeria.
[5]Department of Physical and Biological Sciences, Faculty of Science, Bomet University College, Kenya.

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The study investigated microbial population dynamics in soils of Greater Port Harcourt Area, Nigeria. The study involved the quantification of petroleum pollutants and the enumeration and characterization of culturable fungi and bacteria. Gas Chromatograph FID method was used to quantify total petroleum hydrocarbons (TPH) in the soil while morphological and biochemical tests were used to characterize the microorganisms. The highest TPH was 9,759.37±883.36 ppm which exceeded the Department of Petroleum Resources' (DPRs') intervention value of 5,000 ppm. The percentage of hydrocarbon utilizing fungi (% HUF) ranged between 1.93±0.57 and 66.55±5.60 which were observed in sites A1 (Aluu) and CA (Agricultural control), respectively. The percentage of hydrocarbon utilizing bacteria (% HUB) ranged between 0.12±0.01 and 7.89±1.25 which was observed in sites A3 (Emuoha) and CU (Urban control), respectively. The most prevalent bacterial species includes *Bacillus subtilis, Micrococcus lylae, Staphylococcus aureus, Bacillus cereus* and *Alcaligen faecalis*. The most prevalent HUB isolate was *Bacillus subtilis.* The most prevalent fungal isolates were *Aspergillus niger, Aspergillus flavus* and *Candida torulopsis*. *A. niger* was the most prevalent among the HUF isolates which was identified in 12 sites. It was concluded that the study sites harboured TPH degrading microorganisms that are appropriate for bioremediation of TPH polluted sites.

Key words: Total petroleum hydrocarbons, soil pollution, biodiversity, fungi, bacteria.

INTRODUCTION

Rapid population growth and an immense industrial revolution, even though beneficial in the civilization of human living standards, have jeopardized the state of the environment (Zhao et al., 2017; Jacob et al., 2018; Liu et al., 2019) by introducing a variety of toxic substances such as total petroleum hydrocarbons (TPH), polycyclic

*Corresponding author. E-mail: piwanjala@gmail.com. Tel: +254725891794/+2348101149649.

aromatic hydrocarbons (PAHs), pesticides, heavy metals, synthetic pigments, and polychlorinated biphenyls (PCBs) (Bilal et al., 2017; Barrios-Estrada et al., 2018). In Nigeria, the Niger Delta region is a major centre of activities of oil mining and its' associated industrial sectors. This oil-rich region accounts for more than three quarters of Nigeria's total annual revenue. This industrial growth has led to environmental degradation and left vast footprints of hydrocarbons in the environment (Lindén and Pålsson, 2013). Exploration of oil in Nigeria started in the 1950s and large processing facilities were built to harvest this mineral resource. The eruption of oil spills is on the rise due to increased exploration and insufficient environmental management strategies and this has led to the accumulation of total petroleum hydrocarbons (TPH) over time in sensitive natural habitats. Such chemical spills have intensified, contaminating soils not just in industrial areas but also in the agricultural areas. The consequences of pollution have left undesirable environmental and socio-economic issues leading to loss of ecological resources, poverty and public health concerns (UNEP, 2011; Nkonya et al., 2016; Wali et al., 2019). Further, projections show that the global population may exceed 9 billion by 2050 and that agricultural production would have to rise by 70 to 100% to support the growing population (National Geographic Society, 2020). Yield improvements cannot be accomplished unless the ecosystem is controlled to protect the integrity of the soil ecosystem.

Microorganisms are ubiquitous and are an integral part of the environment since they play a vital role in maintaining processes including biogeochemical cycles in the ecosystem. Soil microorganisms' biogeography is essentially distinct from their counterparts found in animals and plants, and is thus still poorly understood (Whitman et al., 1998). It is vital to assess the impact of anthropogenic activities on the structure of the soil microorganism community in order to provide a basis for reference to the positive and negative impacts that may occur in soils. Microbial diversity of soil must be maintained at its optimum level in order to achieve long-term agricultural productivity. Also, knowledge of soil quality is important for the effective management of farms as it provides baseline data on strategies to maintain and improve soil fertility (Zhou et al., 2014). Soil microorganisms' metabolic activities are mainly driven by temperature and physicochemical parameters (Yang et al., 2020). Bacteria containing nirK, nirS, and nosZ-I genes often have a unique composition in farmland soils as compared to wetland soils, with nirK and nirS being particularly distinct from those containing nosZ-I (Bowen et al., 2020). A reduction in soil pH decreases the abundance of genes and changes the composition of nirK and nirS in agricultural and wetland soil, and raises the ratio of N_2O: (N_2+N_2O) in agricultural soils (Bowen et al., 2020). Agricultural practices have a significant influence on chemical and microbiological soil parameters affecting soil fertility (Bowen et al., 2020).

The patterns of microorganisms in soil polluted with petroleum products vary depending on the chemical composition of the soil and the type of petroleum products. *Escherichia coli, Pseudomonas* species*, Bacillus* species, *Proteus* species, and *Penicillin* species, have been identified to exist in soil contaminated with cyanide (Eze and Onyilide, 2015). The analysis shows the presence of microorganisms in soils contaminated with cyanide at a concentration of 3.0 mg/kg, showing that microorganisms can survive in cyanide contaminated habitats. Soil pollution affects the population and diversity of soil microorganisms. Microbial diversity is declining with an increase in contamination of the environment (Xie et al., 2016). The presence of low levels of microorganisms is related to increased intoxication of cadmium in soils (Xie et al., 2016). Exposure of microorganisms to the concentration of pollutants in soil is therefore causative to the development of adaptive characteristics among the various species found in contaminated soils. Acquisition of new genes that are responsible for resistance for toxicants is an option for microorganisms in the environment. Heavy metal contaminated soil in the marketplaces (Uyo, Umuabia, Sokoto and Oka) in Nigeria has been shown to influence the diversity and distribution of soil microorganisms (Akpoveta et al., 2010; Ogbemudia and Mbong, 2013; Eze et al., 2013; Imarhiagbe et al., 2017). Furthermore, the growth of microalgae (*Microcystis flos-aquae*) in crude oil contaminated media show an exponential growth and reduction of crude oil in the media, an indication of the potential of microorganisms for oil degradation in polluted environments (Ifeanyi and Ogbulie, 2016), and adaptation by shifts in microbial populations, species richness and diversity, thus the role played by microorganisms is diverse. The use of oil spills in Calabar Cross River State in Nigeria has been shown to influence the distribution of microorganisms in soil (Unimke et al., 2017). Some heterotrophic bacteria isolated from these soils included: *Pseudomonas* spp*., Bacillus* spp*., Klebsiella* species*, Proteus* spp*., Enterococcus faecalis* and *Flavobacterium* species (Unimke et al., 2017). The total hydrocarbon utilizing bacteria (THB) include *Bacillus* spp*.,* *Pseudomonas* spp., and *Micrococcus* species (Unimke et al., 2017). Highly prevalent genera were *Pseudomonas* spp., and *Bacillus* spp., indicating that oil degradation microbes are more abundant in oil contamination areas (Unimke et al., 2017). *Arthrobacter* species, strain YC-RL1, could use bisphenol A (BPA) as a carbon source to grow in contaminated soil (Ren et al., 2016). Sourced from soils that were contaminated with crude oil, *Planococcus maritimus* Isolate Y42 was able to use crude oil as its sole source of energy carbon (Yang et al., 2018). *Pseudomonas, Rhizobium, Rhodococcus, Sphingomonas, Enterobacter, Acinetobacter, Bacillus, Paenibacillus,* and *Variovorax* species were found in

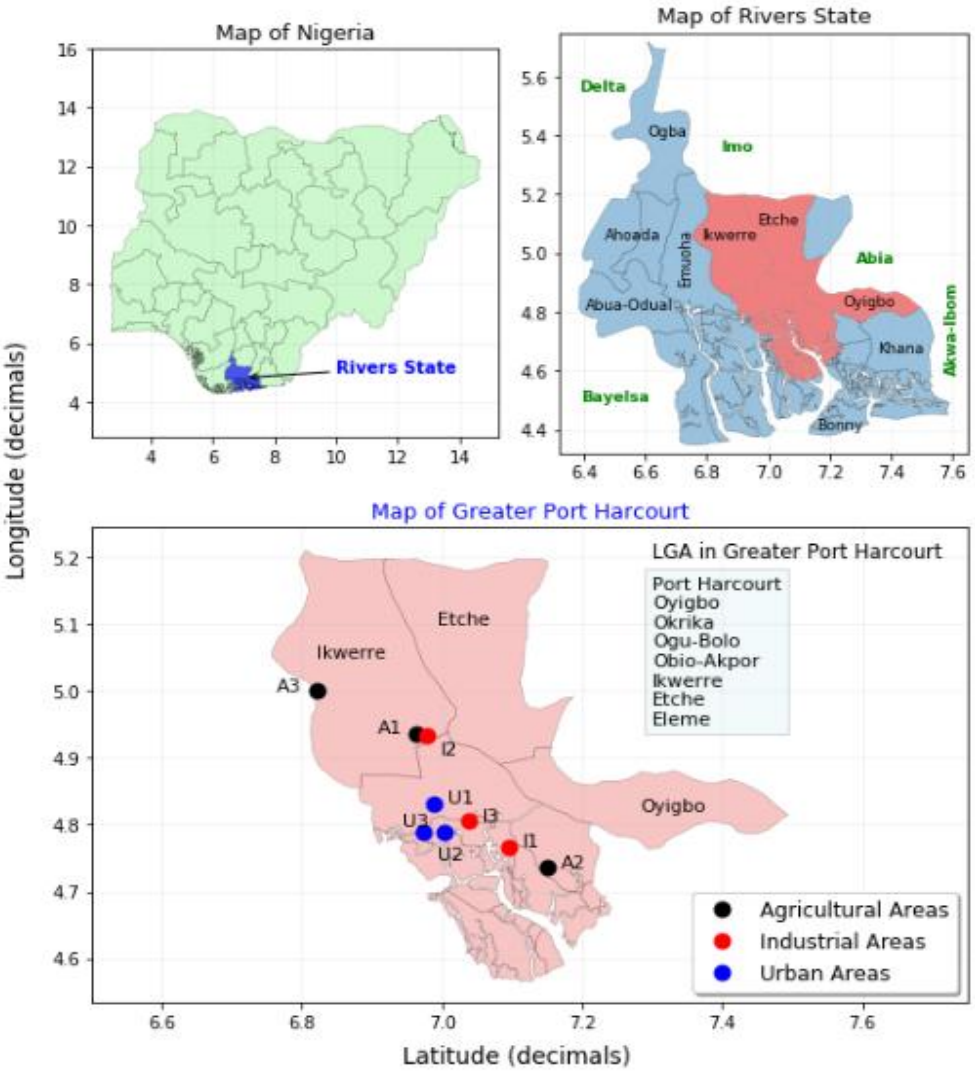

Figure 1. Location of sampling sites in selected areas in Greater Port Harcourt Area, Rivers State, Nigeria. The different dot colours show different economic activities (Black is agriculture, red is industry and blue is urban).

various petroleum contaminated soils and had high biodegradability on alkane mixtures with diverse lengths of the carbon chain ranging between C9 to C30 (Zheng et al., 2018). The microbial diversity in petroleum contaminated soils may be different in soils with similar or different types of contaminants, as most contaminated soils are also polluted by other industrial wastes and chemicals. These studies show that anthropological activities are a threat to soil ecosystem integrity and it is important to periodically monitor the concentration of pollutants in the soil and their effects on soil microorganisms.

The objective of this study was to determine microbial diversity in oil-contaminated soils in three broad sites; urban, industrial and agricultural. Morphological and biochemical experiments have been used to classify a variety of cultivable microbes. The findings of this study could be useful in the production of highly efficient isolates for bioremediation of soils contaminated with petroleum oil.

MATERIALS AND METHODS

Description of the study site

This study was conducted in nine selected test and three control sites in Port Harcourt, the Capital of Rivers State, Nigeria (Figure 1). The study sites were grouped into three areas, including urban (GRA phase 2, Diobu-Mile 1 and Mguoba), industrial (Eleme hosting NNPC Refinery, Agbada-SPDC-flow station) and agricultural (Aluu, Oquwi-Eleme, Emuoha-Eu). The study sites were characterized with different economic activities shown in Table 1.

Table 1. Codes and economic activities of study sites in Greater Port Harcourt Area, Nigeria.

Study site coding	Selected study areas	Coordinates N latitude E Longitude	Characteristic and main activities
Agricultural			
A1	Aluu	4° 56’ 11.160’ 6° 57’ 52.248	Flow station
A2	Eleme	4° 44’ 09.874’ 7° 08’ 58.494’	Village close to refinery
A3	Emuoha	5° 00’ 00.018’ 6° 49’ 13.032’	Flow station
CA	Control	5° 00’ 21.384’ 6° 49’ 00.000’	>1 km away from suspected areas
Industrial			
I1	Onne	4° 46’ 00.402’ 7° 05’ 43.092’	Hosts the NNPC Refinery
I2	Agbada	4° 56’ 03.444’ 6° 58’ 42.060’	Hosts SPDC- flow station in a rural setting
I3	Trans-Amadi	4° 48’ 20.455’ 7° 02’ 17.646’	Schlumberger/, Hallburton
CI	Control	4° 47’ 13.788’ 7° 07’ 44.620’	>1 km away from suspected areas
Urban			
U1	GRA Phase 2	4° 49’ 53.574’ 6° 59’ 45.552’	Inhabited areas Perecuma street
U2	Diobu-Mile 1	4° 47’ 20.382’ 7° 00’ 13.164’	Petroleum refinery
U3	gbuoba	4° 50’ 39.864’ 6° 58’ 20.232’	NTA
CU	Control	4° 49’ 17,040’ 6° 59’ 24.168’	>1 km away from suspected areas

Sampling

In the wet season (April to October 2018), composite samples were collected by random sampling from each of the three areas; urban, industrial, and agricultural. Five (5) samples were collected at random around each test field. The five individual samples were thoroughly mixed in a sterile jar by coning and quartering to achieve a homogeneous composite blend. A total of 12 composite samples; A1, A2, A3, I1, I2, I3 U1, U2 and U3 as test samples, and CA, CI and CU as control samples (Table 1), were collected simultaneously. The samples were obtained at a depth of 0 to 15 cm from the top of the soil using a regular auger three times during the rainy season. Homogenized composite samples (400 g) were then wrapped using a sterile wooden shovel into polyethylene bags. Samples were collected for microbial analysis using pre-sterilized materials to prevent sample contamination. The locations of the sampling sites were determined using the GPS and the measurements were recorded. Samples were taken to the

laboratory in an ice box for examination.

Laboratory analysis

Determination of total petroleum hydrocarbon (TPH) content of soil

The Hewlett Packard 5890 Series II Gas Chromatograph FID method was used. In this method, 1 g of well-mixed sample was weighed into Acetone rinsed beaker. Then, 1 g of anhydrous sodium sulphate was added to the soil sample and 5 ml of solvent (1:1 of dichloromethane and acetone) was added and stirred for 15 min using a magnetic stirrer and the ensuing mixture was poured into a round bottom flask. This was repeated once more by adding 5 ml of mixed solvent. It was stirred and permitted to stand/settle and then decanted into another round bottom flask. The solvent was concentrated with 1 ml hexane to exchange it and it was re-concentrated to 2 ml. The columns were eluted (washed off) with 10 ml n-hexane. 1 ml of the extract was pipetted into the column and 10 ml of n-hexane was used to collect the aliphatic components. The extract was concentrated to 1 ml and poured into a glass vial for Gas Chromatography.

Enumeration of total heterotrophic bacteria (THB)

Heterotrophic bacteria were enumerated by pour plate method (APHA, 1998). One gram of soil sample was weighed into 9 ml sterile diluent (0.85% NaCl) under aseptic condition (laminar bench floor). It was then homogenized using a laboratory vortex mixer (Model: 10101001, IP42) and serially diluted. Then 0.1 ml aliquot of the inoculum was collected using a sterile pipette, inoculated on Nutrient Agar (NA) medium. The inoculum was spread evenly using a sterile glass spreader stick. Plates were then incubated at 37°C for 24 h. Thereafter, colonies were counted and expressed as colony forming units (CFUs/mg of soil) value per gram of soil sample. Distinct colonies with different morphological patterns (color, size, shape, edge, elevation, surface and opacity) were picked and streaked or subculture on freshly prepared nutrient agar medium in order to obtain pure culture after 24 h of incubation at 37°C. The pure cultures were Gram stained for microscopic examination and were further subjected to biochemical tests.

Enumeration of hydrocarbon utilizing bacteria

Hydrocarbon utilizing bacteria (HUB) were enumerated by the pour plate method (APHA, 1998) method. 1 g of soil sample was weighed into a 9 ml sterile diluent (0.85% NaCl) under aseptic conditions. The sample was then homogenized using a laboratory vortex mixer (Model: 10101001, IP42) and serially diluted. Then 0.1 ml aliquot of the inoculum was inoculated on Mineral Salt Agar (MSA) medium containing g/l of $MgSO_4.7H_2O$ 0.42 g, KCl 0.29 g, K_2HPO_4 1.25 g, KH_2PO_4 0.83 g, $NaNO_3$ 0.42 g, NaCl 10 g and Agar Powder 18 g, using the spread technique. Sterile filter paper (Whatman 540) was soaked with crude oil and placed in the lid of petri dish. Plates were incubated in inverted position at room temperature for 5 days until there was observable growth. Thereafter, distinct colonies were purified by sub-culturing on a freshly prepared medium and incubated for 24 h, from which microscopic examination and biochemical tests.

Enumeration of total fungi

Total fungi were performed using a pour plate method (APHA, 1998). Under aseptic conditions, one gram of soil sample was weighed in a 9 ml sterile diluent (0.85 per cent NaCl). The sample was then homogenized using a vortex mixer (Model 10101001, IP42) and diluted in series using sterile pipettes. Thereafter, 0.1 ml of the inoculum aliquot was inoculated on Potato Dextrose Agar (PDA) mixed with an antibacterial reagent (Normocure™) to inhibit bacterial growth and allow only fungal growth. Then, the inoculated plates were incubated for 5 to 7 days at ambient temperature. To obtain colony forming unit per gram (CFU/g) of the soil, colonies were enumerated using a colony counter.

Enumeration of hydrocarbon utilizing fungi

Hydrocarbons utilizing fungi (HUF) were cultured using the pour plate method (APHA, 1998). Under aseptic conditions (laminar flow bench), 1 g of soil sample was weighed into a 9 ml sterile diluent (0.85% NaCl). The sample was then homogenized using a laboratory vortex mixer (Model: 10101001, IP42) and serially diluted using sterile pipettes. 0.1 ml aliquot of inoculum was then inoculated on Mineral Salt Agar (MSA) mixed with an antibacterial reagent (Normocure™) in order to inhibit the growth of bacteria and allow for only growth of fungi. Sterile filter paper (Whatman 540) was subsequently soaked with crude oil and put in the petri dish cover. At room temperature, the plates were then incubated in an inverted position for 5 to 7 days. Colonies were counted using a colony counter to get colony forming units per gram of soil. Cultural characteristics (colour and microscopic observations) of the isolates were then observed and purified by sub-culturing on freshly prepared medium and incubated again for 3 to 5 days. From the pure cultures, microscopic examination was done using lactophenol cotton blue stain and observed under ×400 magnification.

Determination of % hydrocarbon utilizing fungi and bacteria

Percent hydrocarbon utilizing fungi and bacteria were expressed as a fraction of the total heterotrophic viable count using the formula:

$$\%\ \text{HUF/HUB} = \frac{Hydrocarbon\ utilizing\ fungi/bacteria}{Total\ heterotrophic\ viable\ count} \times \frac{100}{1}$$

Characterization and identification of THB and TF fungi

The fungal and bacterial isolates were identified morphologically (color, size, shape, edge, elevation, surface and opacity). Further, bacterial isolates were identified biochemically and characterized according to the scheme of Bergey's manual of Determinative Bacteriology (Holt et al., 1994) [This was the locally available method and the characterization was limited to it] using standard procedures: Sucrose fermentation test, Indole test, Citrate utilization test, Catalase test, Oxidase test, Motility test, Methyl red test, Voges-Proskauer test, Triple sugar iron (TSI) agar test, Nitrate reduction test, Starch hydrolysis test, Glucose test and Lactose fermentation test.

RESULTS

Prevalence and characterization of microorganisms

Prevalence and diversity of THB in different sampling sites

Table 2 shows the ranks of prevalence, diversity of THB,

Table 2. Variation in prevalence of THB in soil from agricultural, industrial and urban areas in Greater Port Harcourt Area, Nigeria.

Identified microbe	Site of prevalence												P
Sampling site	**CA**	**A1**	**A2**	**A3**	**CI**	**I1**	**I2**	**I3**	**CU**	**U1**	**U2**	**U3**	
Bacillus subtilis	+	+	+	+	-	+	-	-	+	+	+	+	9
Micrococcus lylae	+	+	+	-	+	-	+	-	+	-	+	+	8
Staphylococcus aureus	+	+	-	+	-	+	-	-	+	+	+	+	8
Bacillus cereus	+	+	-	-	+	-	+	+	-	-	+	-	6
Alcaligen faecalis	-	+	+	+	+	-	+	-	+	-	-	-	6
Micrococcus kristinae	-	-	-	-	+	-	+	+	-	-	+	+	5
Pseudomonas aeruginosa	-	-	-	-	-	+	+	-	+	+	-	-	4
Bacillus thuringens	-	-	+	-	+	-	+	-	-	-	-	-	3
Micrococcus letus	+	-	-	+	-	+	-	-	-	-	-	-	3
Escherichia coli	-	-	-	-	-	-	-	+	-	+	-	-	2
Protues mirabilis	-	-	-	-	+	-	-	-	-	+	-	-	2
Flavobacterium breve	-	-	-	-	-	-	-	-	-	-	-	+	1
Serratia marscencens	-	-	-	-	-	-	-	-	-	+	-	-	1
Isolates per site	5	5	4	4	6	4	6	3	5	6	5	5	58

P stands for prevalence of species.

Table 3. Variation in prevalence of HUB in soil from agricultural, industrial and urbanized areas in parts of Rivers State, Nigeria.

Identified microbe	Site of prevalence												P
Sampling site	**CA**	**A1**	**A2**	**A3**	**CI**	**I1**	**I2**	**I3**	**CU**	**U1**	**U2**	**U3**	
Bacillus subtilis	-	+	+	+	+	-	-	+	+	+	+	+	9
Alcaligen faecalis	-	+	+	+	+	-	+	-	+	-	-	-	6
Bacillus cereus	+	+	-	-	-	+	+	-	-	-	-	-	3
Micrococcus kristinae	-	-	-	-	+	-	+	+	-	-	-	-	3
Micrococcus lylae	+	-	-	-	-	-	-	-	+	+	-	-	3
Bacillus thuringens	-	-	+	-	-	+	-	-	-	-	-	-	2
Flavobacterium breve	-	-	-	-	-	-	-	+	-	-	-	+	2
Pseudomonas aeruginosa	-	-	-	-	-	+	-	-	-	+	-	-	2
Staphylococcus aureus	-	-	-	+	-	-	-	-	-	-	+	-	2
Micrococcus letus	-	-	-	+	-	-	-	-	-	-	-	-	1
Isolates per site	2	3	3	4	3	3	3	3	2	3	2	2	33

P stands for prevalence of species.

microbial population and community composition in the study sites. *Bacillus subtilis* was most prevalent and was identified in 9 sites including CA (agricultural control), A1 (Aluu), A2 (Eleme), A3 (Emuoha), I1 (Onne), CU (urban control), U1 (GRA phase 2), U2 (Diobu-Mile 1), U3 (Mgbuoba). The least prevalent species was *Flavobacterium breve* which was isolated from samples from U3 (Mgbuoba). Agbada (I2) and industrial control (CI) sites showed the highest number (6) of isolates. There was variation in the number of isolates between control and contaminated sites.

Prevalence and diversity of HUB in sampling sites

Table 3 shows the ranks of prevalence and diversity of HUB in the sampling sites. The most prevalent isolate was *B. subtilis* which was prevalent in A1 (Aluu), A2 (Eleme), A3 (Emuoha), CI (industrial control), I3 (Trans-Amadi), CU (Urban control), U1 (GRA Phase 2), U2 (Diobu-Mile 1) and U3 (Mgbuoba). The highest diversity was observed in site A3 which had 4 isolates *B. subtilis, Alcaligen faecalis, Staphylococcus aureus* and *Micrococcus letus*. There was no difference in diversity

Table 4. Variation in prevalence of TF in soil from agricultural, industrial and urban areas in Greater Port Harcourt Area, Nigeria.

Identified microbe	Site of prevalence												P
Sampling sites	CA	A1	A2	A3	CI	I1	I2	I3	CU	U1	U2	U3	
Aspergillus niger	+	+	+	+	+	+	+	+	+	+	+	+	12
Aspergillus flavus	-	+	-	+	+	+	-	+	+	-	-	-	6
Candida torulopsis	-	+	-	-	+	-	-	+	-	+	+	-	5
Mucor mucedo.	-	+	+	-	-	-	-	+	+	+	-	-	5
Saccharomyces cerevisiae	-	-	+	-	-	+	+	-	+	+	-	-	5
Paecilomyces spp.	+	-	+	+	-	-	-	-	-	-	-	-	3
Chrysosporium spp.	+	-	-	-	-	-	-	-	-	-	-	+	2
Cladiosporium spp.	-	-	-	-	-	-	+	-	-	-	+	-	2
Geotrichium spp.	-	-	+	-	-	-	-	-	-	-	-	+	2
Penicillium expanum	-	-	-	-	+	+	-	-	-	-	-	-	2
Rhizopus stolonifer	-	-	-	-	-	-	-	-	-	-	+	-	1
Aspergillus fumigatus	+	-	-	-	-	-	-	-	-	-	-	-	1
Isolates per site	4	4	5	3	4	4	3	4	4	4	4	3	46

P stands for prevalence of species.

Table 5. Variation in Prevalence of HUF Isolates in Soil from Agricultural, Industrial and Urban areas in Greater Port Harcourt Area, Nigeria.

Identified microbe	Site of prevalence												P
Sampling sites	CA	A1	A2	A3	CI	I1	I2	I3	CU	U1	U2	U3	
Aspergillus niger	+	+	+	+	+	+	+	+	+	+	+	+	*12*
Aspergillus flavus	-	+	-	-	-	-	-	+	+	-	-	-	*3*
Paecilomyces spp.	+	-	+	+	-	-	-	-	-	-	-	-	*3*
Mucor mucedo.	-	-	+	-	-	-	-	-	+	-	-	-	*2*
Penicillium expanum	-	-	-	-	+	+	-	-	-	-	-	-	*2*
Cladiosporium spp.	-	-	-	-	-	-	+	-	-	-	+	-	*2*
Saccharomyces cerevisiae	-	-	-	-	-	+	+	-	-	-	-	-	*2*
Aspergillus fumigatus	+	-	-	-	-	-	-	-	-	-	-	-	*1*
Candida torulopsis	-	+	-	-	-	-	-	-	-	-	-	-	*1*
Chrysosporium spp.	-	-	-	+	-	-	-	-	-	-	-	-	*1*
Geotrichium spp.	-	-	-	-	-	-	-	-	-	-	-	+	*1*
Rhizopus stolonifer	-	-	-	-	-	-	-	-	-	-	-	-	*0*
Isolates per site	*3*	*3*	*3*	*3*	*2*	*3*	*3*	*2*	*3*	*1*	*2*	*2*	*30*

P stands for prevalence of species.

(3) between all the industrial and the control site; however, there was a difference in diversity between the controls and contaminated sites in urban and agricultural areas.

Prevalence and diversity of TF in sampling sites

Table 4 shows the ranks of prevalence and diversity of TF in the sampling sites. The most prevalent fungal isolate includes *Aspergillus niger, Aspergillus flavus* and *Candida torulopsis* which were prevalent in 12, 6 and 5 sites, respectively (Table 3). The highest diversity was observed in A2 (Eleme) with 5 isolates *A. niger, Mucor mucedo, Saccharomyces cerevisiae, Paecilomyces* species, and *Geotrichium* species (Table 3). The list diversity was observed in A3 (Emuoha), I1 (Onne) and U3 (Mgbuoba) with 3 isolates each.

Prevalence and diversity of HUF in different sampling locations

Table 5 shows the different hydrocarbon utilizing fungi (HUF) that were isolated from the soil samples. *A. niger*

Table 6. Variation of population of THB, HUB, %HUB, TF, HUF and %HUF in soil from agricultural, industrial and urban areas in Greater Port Harcourt Area, Nigeria (Wet season).

Code	THB	HUB	% HUB	TF	HUF	% HUF	TPH (ppm)
A1	$1.30\times10^{6}\pm4.36\times10^{5}$	$1.49\times10^{4}\pm2.31\times10^{2}$	1.22±0.34	$1.45\times10^{5}\pm8.08\times10^{3}$	$2.77\times10^{3}\pm6.81\times10^{2}$	1.93±0.57	3,307.27±125.51
A2	$7.07\times10^{7}\pm4.93\times10^{6}$	$6.83\times10^{5}\pm5.77\times10^{3}$	0.64±0.47	$3.27\times10^{4}\pm2.52\times10^{3}$	$3.70\times10^{3}\pm1.85\times10^{3}$	11.18±4.91	6,198.49±598.56
A3	$5.73\times10^{7}\pm1.15\times10^{6}$	$7.10\times10^{4}\pm4.36\times10^{3}$	0.12±0.01	$8.23\times10^{3}\pm2.52\times10^{2}$	$3.10\times10^{3}\pm2.65\times10^{2}$	37.63±2.62	5,253.17±1,014.27
CA	$6.17\times10^{7}\pm1.53\times10^{6}$	$1.73\times10^{4}\pm4.58\times10^{2}$	0.20±0.15	$6.18\times10^{3}\pm2.25\times10^{2}$	$4.10\times10^{3}\pm2.65\times10^{2}$	66.55±5.60	453.61±233.01
I1	$1.37\times10^{7}\pm1.10\times10^{6}$	$2.47\times10^{4}\pm1.33\times10^{3}$	0.14±0.08	$6.17\times10^{5}\pm4.04\times10^{5}$	$6.48\times10^{3}\pm1.04\times10^{2}$	29.99±42.10	6,543.74±1022.19
I2	$7.43\times10^{6}\pm4.51\times10^{5}$	$1.24\times10^{5}\pm7.51\times10^{3}$	1.16±0.87	$1.32\times10^{4}\pm1.06\times10^{3}$	$2.27\times10^{3}\pm5.03\times10^{2}$	17.39±5.38	4,151.89±688.19
I3	$3.37\times10^{7}\pm5.13\times10^{6}$	$3.97\times10^{4}\pm1.15\times10^{3}$	0.12±0.02	$7.50\times10^{4}\pm1.73\times10^{3}$	$6.67\times10^{3}\pm2.50\times10^{3}$	8.92±3.45	9,759.37±883.36
CI	$7.17\times10^{7}\pm2.52\times10^{6}$	$3.88\times10^{4}\pm3.46\times10^{2}$	0.27±0.23	$5.21\times10^{4}\pm8.08\times10^{2}$	$4.57\times10^{4}\pm3.70\times10^{4}$	6.74±5.96	173.45±18.75
U1	$4.57\times10^{7}\pm4.59\times10^{7}$	$3.67\times10^{3}\pm1.15\times10^{2}$	0.49±0.79	$1.44\times10^{4}\pm5.13\times10^{2}$	$3.40\times10^{3}\pm1.11\times10^{3}$	23.40±6.98	5,908.56±1,252.00
U2	$1.88\times10^{7}\pm8.27\times10^{6}$	$2.88\times10^{4}\pm1.06\times10^{3}$	0.48±0.46	$6.60\times10^{4}\pm2.00\times10^{3}$	$5.20\times10^{3}\pm4.00\times102$	7.87±0.85	2,810.28±530.53
U3	$1.96\times10^{7}\pm2.95\times10^{6}$	$5.27\times10^{4}\pm1.15\times10^{3}$	0.27±0.03	$6.23\times10^{4}\pm2.52\times10^{3}$	$3.45\times10^{4}\pm2.44\times10^{4}$	55.88±40.62	5,566.89±528.33
CU	$3.33\times10^{6}\pm4.16\times10^{5}$	$2.60\times10^{5}\pm2.59\times10^{4}$	7.89±1.25	$7.70\times10^{4}\pm4.00\times10^{3}$	$2.90\times10^{3}\pm2.00\times102$	16.52±22.47	467.05±401.73

Data are Mean ± standard deviations (Significant coefficients = 0.05).

was the most prevalent among the hydrocarbon utilizing fungal isolates which was identified in all the 12 test and control locations. There was no difference (3) in number of isolates between agricultural control site and contaminated sites. There was difference in number of isolates between urban and industrial control sites and contaminated sites (Table 5). The sites with the highest number of isolates were CA (agricultural control), A1 (Aluu), A2 (Eleme), A3 (Emuoha), I1 (Onne), I2 (Agbada) and CU (urban control).

Population and distribution of microorganisms

Population of THB, HUB, %HUB, TF, HUF and *%HUF*

The population and distribution of microorganisms was done in wet season. In the wet season, the % HUB ranged between 0.12±0.01 and 7.89±1.25 which was observed in sites A3 (Emuoha) and CU (urban control) respectively (Table 6). In the dry season, the %HUB ranged between 0.23±0.01 and 4.13±0.86 which were observed in sites A1 (Aluu) and CA (agricultural control) respectively. All sites had values of HUB below 10% in both the wet and the dry seasons. In the wet season, %HUF ranged between 1.93±0.57 and 66.55±5.60 which were observed in A1 (Aluu) and CA (agricultural control) respectively. In the dry season, %HUF ranged between 0.74±0.12 and 13.20±0.75 observed in locations A1 (Aluu) and CA (agricultural control), respectively (Table 6). All values of %HUF in the dry season were below the threshold value of 10%. The %HUF in A2 (Eleme), A3 (Emuoha), CA (agricultural control), CU (urban control), I1 (Onne), I2 (Agbada), U1 (GRA Phase 2) and U3 (Mgbuoba) were above the 10% threshold value in the wet season (Table 7).

DISCUSSION

There are differences in responses and distribution of fungi and bacteria in soils polluted with different contaminants. The difference in response of microorganisms to concentrations of different pollutants between fungi and bacteria can be attributed to difference in strategies of evasion or accommodation of pollutants in the environment (Zanardo et al., 2018). For example, similarity in bacteria richness and diversity in Pb contaminated sites while in non-contaminated sites show difference in fungal richness and diversity (Zanardo et al., 2018). The present study showed that %HUB was lower as related to %HUF. The discrepancy in distribution between fungi and bacterial richness and diversity can be attributed to their different strategies to avoid or tolerate high concentrations of pollutants (Zanardo et al., 2018). *B. subtilis* and *Alkaligen faecalis* were the

Table 7. Variation of population of THB, HUB, %HUB, TF, HUF and %HUF in soil from agricultural, industrial and urban areas in Greater Port Harcourt Area, Nigeria (Dry season).

Code	THB	HUB	% HUB	TF	HUF	% HUF	TPH (ppm)
A1	$6.87\times10^{6}\pm7.02\times10^{5}$	$1.57\times10^{4}\pm2.08\times10^{3}$	0.23±0.01	$2.93\times10^{4}\pm4.51\times10^{3}$	$2.20\times10^{2}\pm7.00\times10^{1}$	0.74±0.12	3,307.27±125.51
A2	$6.13\times10^{6}\pm1.10\times10^{6}$	$3.47\times10^{4}\pm1.53\times10^{3}$	0.58±0.12	$3.33\times10^{3}\pm1.53\times10^{2}$	$3.67\times10^{2}\pm3.51\times10^{1}$	11.00±0.87	6,198.49±598.56
A3	$6.50\times10^{6}\pm3.27\times10^{6}$	$2.20\times10^{4}\pm2.00\times10^{3}$	0.45±0.33	$4.70\times10^{3}\pm2.00\times10^{2}$	$2.77\times10^{2}\pm2.52\times10^{1}$	5.88±0.44	5,253.17±1,014.27
CA	$4.00\times10^{5}\pm3.61\times10^{4}$	$1.67\times10^{4}\pm4.73\times10^{3}$	4.13±0.86	$3.00\times10^{3}\pm2.00\times10^{2}$	$3.97\times10^{2}\pm4.73\times10^{1}$	13.20±0.75	453.61±233.01
I1	$6.37\times10^{6}\pm4.04\times10^{5}$	$2.47\times10^{4}\pm1.53\times10^{3}$	0.39±0.01	$4.20\times10^{4}\pm9.54\times10^{3}$	$5.07\times10^{2}\pm2.08\times10^{1}$	1.26±0.34	6,543.74±1022.19
I2	$6.17\times10^{6}\pm4.51\times10^{5}$	$2.07\times10^{4}\pm2.08\times10^{3}$	0.33±0.02	$5.30\times10^{3}\pm3.61\times10^{2}$	$2.27\times10^{2}\pm4.04\times10^{1}$	4.27±0.63	4,151.89±688.19
I3	$4.37\times10^{5}\pm2.52\times10^{4}$	$1.07\times10^{4}\pm1.15\times10^{3}$	2.44±0.17	$3.67\times10^{3}\pm4.04\times10^{2}$	$3.20\times10^{2}\pm2.65\times10^{1}$	8.75±0.44	9,759.37±883.36
CI	$6.80\times10^{6}\pm4.58\times10^{5}$	$2.33\times10^{4}\pm1.53\times10^{3}$	0.34±0.01	$5.23\times10^{3}\pm5.69\times10^{2}$	$3.80\times10^{2}\pm1.00\times10^{1}$	7.32±0.89	173.45±18.75
U1	$6.77\times10^{6}\pm9.87\times10^{5}$	$1.73\times10^{4}\pm1.53\times10^{3}$	0.26±0.02	$3.53\times10^{3}\pm3.06\times10^{2}$	$3.33\times10^{2}\pm2.52\times10^{1}$	9.51±1.50	5,908.56±1,252.00
U2	$7.73\times10^{6}\pm1.12\times10^{6}$	$3.43\times10^{4}\pm2.08\times10^{3}$	0.45±0.06	$5.60\times10^{3}\pm3.61\times10^{2}$	$3.60\times10^{2}\pm1.00\times10^{1}$	6.45±0.58	2,810.28±530.53
U3	$6.13\times10^{6}\pm9.45\times10^{5}$	$3.50\times10^{4}\pm4.58\times10^{3}$	0.59±0.15	$3.97\times10^{3}\pm3.06\times10^{2}$	$4.50\times10^{2}\pm5.29\times10^{1}$	11.33±0.72	5,566.89±528.33
CU	$7.00\times10^{6}\pm1.06\times106$	$2.13\times10^{4}\pm1.53\times10^{3}$	0.31±0.02	$4.67\times10^{3}\pm2.52\times10^{2}$	$3.13\times10^{2}\pm4.16\times10^{1}$	6.70±0.57	467.05±401.73

Data are Mean ± standard deviations (Significant coefficients = 0.05).

most prevalent HUB in the current study sites. The least prevalent HUB was *Micrococus letus* which was prevalent only in site A3 (Emuoha). The most prevalent HUF were *A. niger*, *A. flavus* and *Paecilomyces* species. The least prevalent isolates included *Aspergillus fumigatus, Candida torulopsis, Chrysosporium* species and *Geotrichium* species. Hydrocarbon utilizing microorganisms were diverse among all sites of study. Hydrocarbon utilizing bacteria are abundant in the environment but not limited to oil polluted sites (Okoh, 2006). However, this happens when there are optimal conditions as the observations showed a declined trend in populations of fungi and bacteria across the wet and dry seasons. High populations were favoured in the wet season as compared to the dry season. Presence of poisonous materials from pollution is a key slow down to microorganism activities and hence a cause to diverse microorganism species that have capacity to degrade hydrocarbons (Akoachere et al., 2008) and other pollutants in soil.

Atlas and Cerniglia (1999) identified *Pseudomonas* and *Bacillus* spp. as the dominant isolated bacterial genera in oil contaminated environment. *Micrococcus lylae* and *B. subtilis* were the utmost prevalent in 8 and 9 out of 12 study and control sites, respectively. *B. subtilis* has been identified in oil contaminated sites and has been attributed to bioremediation of the contaminated sites (Al-Dhabaan, 2019). Other bacterial species isolated from oil polluted sites (Atlas and Cerniglia, 1999) include, *Micrococcus, Flavobacterium, Enterococcus*, *Proteus*, and *Klebsiella* spp. Oil contaminated sites are dominated by Gram negative bacteria (Bartha, 2009; Agency for Toxic Substances and Disease Registry {ATSDR}, 2000; Singer and Finnerty, 2004). This was not the case in the present study, as Gram negative and Gram-positive bacteria were equally distributed among the study sites, where the dominant Gram-positive bacteria was *Bacillus* and *Micrococcus* while the dominant Gram-negative bacteria were *Pseudomonas* sp. *Pseudomonas aeruginosa* was isolated from two sites I1 (Onne) and U1 (GRA Phase 2) hence was less prevalent among the study sites. *P. aeruginosa* has been isolated in diesel polluted areas (Chikere et al., 2019). *Bacillus cereus* has also been associated with bioremediation of petroleum hydrocarbons (Al-Dhabaan, 2019). Additionally, *P. aeruginosa* is an efficient degrader of diesel. *Acinetobacter, Myroids, Pseudomonas*, and *Bacillus* are degraders in the spill sites of the long-chain hydrocarbon fraction (Chikere et al., 2019; Wang et al., 2019). There are variations in microbial community structure in the hydrocarbons polluted soils, where the community shows higher similarity between sites polluted with similar contaminants (Avidano et al., 2005). The presence of various chemical pollutants in the environment hardly affects bacterial density but rather the structure of the community (Avidano et al., 2005).

Characterizing soil chemical properties, metabolic fingerprinting, enzymatic activities, and bacterial community structure could be useful soil health assessment tools (Avidano et al., 2005).

CONCLUSION AND RECOMMENDATION

The study concludes that the most prevalent bacterial species includes *B. subtilis, Micrococcus lylae, S. aureus, B. cereus* and *A. faecalis*. The most prevalent HUB isolate was *Bacillus subtilis.* The most prevalent fungal isolates were *A. niger, A. flavus* and *C. torulopsis*. *A. niger* was the most prevalent among the HUF isolates which was identified in 12 contaminated and control sites. The findings conclude that the sites of study harboured TPH tolerant bacteria and fungi and are appropriate for the selection of microorganism's habitats for bioremediation of TPH polluted areas. Therefore, contamination of soils in the study areas negatively affected microbial populations, diversity and species richness. Microbes have adapted to tolerate the presence of petroleum hydrocarbons or can even use them for nourishment. Some implications are useful, such as the use of bacteria to clean up metal and/or hydrocarbon-contaminated sites. Overall, this study adds to our understanding of patterns of microbial succession in different anthropogenic activities where agricultural soils were the most affected. Further, this research contributes to our understanding of patterns of hydrocarbon use and microbial succession in different oil-polluted soils with various human activities (urban, industrial and agricultural).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antibiotic-producing bacteria isolated from some natural habitats in the Federal Capital Territory (FCT), Nigeria

Olasinbo Olumuyiwa Balogun[1*], Sylvanus Chukwudi Ugoh[2] and Peters Olawale Oladosu[3]

[1]Department of Clinical Laboratory Services, Institute of Human Virology, Nigeria Plot 252 Herbert Macaulay Way Central Business District, Abuja, Nigeria.
[2]Department of Microbiology, University of Abuja, Abuja, Nigeria.
[3]Department of Microbiology and Biotechnology, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria.

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High global prevalence of multidrug-resistant bacteria due to antibiotics misuse has prompted the need for novel antibacterial compounds to replace the failing antibiotics. This study investigated some natural habitats in Abuja, Nigeria, for antibiotics-producing bacteria. Thirty-six soil samples from termite mounds, river banks and rhizospheres of *Anacardium occidentale* L. (cashew tree), *Gmelina arborea* Roxb. ex Sm. (beechwood), *Ageratum conyzoides* L. (goat weed) including *Cymbopogon citratus* (DC) Stapf. (lemon grass) were cultured on nutrient media. Twelve potential antibiotic-producing isolates were identified by crowded plates method and characterized using Bergey's manual. The antimicrobial activities of the filtrates from the isolates against some pathogenic strains namely *Streptococcus pneumonia, Salmonella typhi, Escherichia coli* (ATCC 25922), *Proteus mirabilis, Staphylococcus aureus* (ATCC 25923), *Candida albicans, Bacillus subtilis* and *Pseudomonas aeruginosa* (ATCC 27853) were determined by agar-well diffusion method. Only 5 eventually inhibited at least 1 test microorganism; 4 showed activities against both Gram-positive and Gram-negative bacteria (broad spectrum) and 2 among the 4 also inhibited *Candida albicans*, while the remaining 1 inhibited only 1 Gram-positive bacterium (narrow spectrum). The 5 potent antibiotics-producers were *Bacillus* spp. In conclusion, some natural habitats in the FCT are important sources of antibiotic-producing bacteria. Their antimicrobial lead compounds could be extracted and developed locally for pharmaceutical applications.

Key words: Antibiotics-producing bacteria, *Bacillus* species, natural habitats.

INTRODUCTION

The discovery of antibiotics in the 20th century was a global success resulting in the improvement of treatment outcomes from infectious diseases thereby saving millions of lives (Shatzkes et al., 2017). However, this

*Corresponding author. E-mail: obalogun@ihvnigeria.org.

success is being threatened by antimicrobial resistant microorganisms as antibiotics are showing weaknesses to previously susceptible bacterial infections (Ventola, 2015).

Antibiotic resistant crises dated back to the introduction of antibiotics in the clinics and have continued with every new antibiotic developed for medical purposes (Rossolini, 2015). Every new class of antibiotics introduced also results in the evolution of resistant bacteria pathogens due to overuse, inappropriate prescription, extensive agricultural use and horizontal gene transfer which provide an evolutionary drive for bacteria to develop resistance (Nadeem et al., 2020).

The emergence of new infectious diseases including multidrug resistant pathogens is on the rise worldwide (Sautter and Halstead, 2018). The global morbidity and mortality cases as a result of the rise in antibiotic resistant bacteria are increasing as the effective treatment options decline (Rather et al., 2017). Antibiotic-resistant bacteria infections account for about 33,000 annual human death globally (Cassini et al., 2019), and estimated to rise to about 10 million by 2050 without check of which Africa will record 40% of cases (O'Neill, 2017). Developed countries like the United States records thousands of antibiotic-resistant microbial infections cases of which about 23,000 die because of limited treatment options (Rather et al., 2017). The World Health Organization (WHO) published the first global surveillance report on antibiotic resistance (ABR) in 2014 attributing multi-drug resistant (MDR) bacteria to be responsible for 45% of deaths in both Africa and South-East Asian (WHO, 2014).

Antimicrobial resistance constitutes a significant threat resulting in prolonged treatment, expensive therapy, morbidity, mortality, and economic loss to both the patient and nation (Ahmad and Khan, 2019). The clinical isolates like Methicillin Resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, enterococci especially vancomycin-resistant enterococci (VRE), and members of Family Enterobacteriaceae, like *Proteus sp*., *E. coli*, and *Klebsiella pneumoniae* have all displayed rapid antibiotic resistance and spread in the hospital domain (Basak et al., 2016).

Soil is the reservoir for novel antibiotic-producing microbes (Rafiq et al., 2018) and majority of the antibiotics in clinical use were discovered by screening for antimicrobial activities of microorganisms isolated from the soil (Rolain et al., 2016). The success recorded by soil microbes is attributable to their ability to produce antibiotics in the natural ecosystem as a mechanism of survival and dominance over other competing bacteria (Dwivedi and Sisodia, 2019).

The emergence of new diseases and reemergence of multidrug-resistant including pan drug-resistant pathogens that neutralizes the effectiveness of existing antibiotics has prompted the need for novel antibiotics (Rolain et al., 2016; Sautter and Halstead, 2018). Some new drugs are currently undergoing development; however, they fall below increasing medical needs. Furthermore, most of these drugs are derivatives of established classes of antimicrobials currently in use and therefore prone to existing bacterial resistance mechanisms (Chopra, 2013).

Consequently, the present study investigated biotechnologically important strains of antibiotic compounds-producing bacteria from soil samples in the Federal Capital Territory (FCT).

MATERIALS AND METHODS

Study area

The study area was in the FCT (Figure 1) which lies between 8° 15'N to 9° 12'N latitude and 6° 27'E to 7° 23'E longitude and located in the central region of Nigeria. The FCT has six area councils and occupies a landmass of approximately 713 km^2 (Okiemute et al., 2018). It is situated within the savannah region with moderate climatic conditions.

Study design and sample collection

Randomized experimental design was adopted for the collection of soil samples in the FCT. Six (6) soil replicates were collected from some natural habitat for analyses. Soil samples were collected from selected natural habitats in each of the 6 area councils in the FCT, North Central Nigeria. Between November 2 and December 14, 2019 a total of thirty-six (36) samples from six (6) soil replicates were collected from each selected natural habitat at the six (6) Local government Area Councils by randomized sampling. The six Area Councils include Abaji, Kuje, Abuja Municipal, Gwagwalada, Kwali and Bwari respectively. The samples were aseptically collected from 5 to 15 cm soil depth into labelled zip-lock bags using sterile garden trowel and transported in cold-chain (4 to 8°C) to the laboratory for analyses. One (1) natural habitat was mapped to each area council for the collection (Table 1). The habitats include termite mounds, rhizosphere of *Anacardium occidentale* L. (family: Anacardiaceae; popular name: cashew tree), rhizosphere of *Gmelina arborea* Roxb. ex Sm. (family: Lamiaceae; popular name: beechwood), rhizosphere of *Ageratum conyzoides* L. (family: Asteraceae; popular name: goat weed), rhizosphere *of Cymbopogon citratus* (DC) Stapf. (family: Poaceae; popular name: lemon grass) and river bank.

The plant species (of which the rhizospheres were examined) were also collected, identified and deposited at the Herbarium & Ethnobotany Unit of National Institute for Pharmaceutical Research and Development, Abuja, Nigeria (NIPRD).

Screening for potential antibiotic-producing microorganisms

Potential antibiotic-producing bacteria were isolated following the crowded plate technique according to Bavishi et al. (2017) with slight modifications. For this purpose, 1.0 g of each soil sample was weighed into 9.0 ml of sterile normal saline (0.85% sodium chloride solution) to obtain 1:10 dilution. The tubes were vortexed for 5 min and sediment was allowed to settle before subjecting the supernatant to serial dilutions up to $1:10^5$. By Spread plate technique, 0.1 ml of the inoculum from $1:10^2$ dilutions and $1:10^3$ dilutions were aseptically spread on nutrient agar (NA) plates (HiMedia, India). The inoculated agar plates had been seeded with

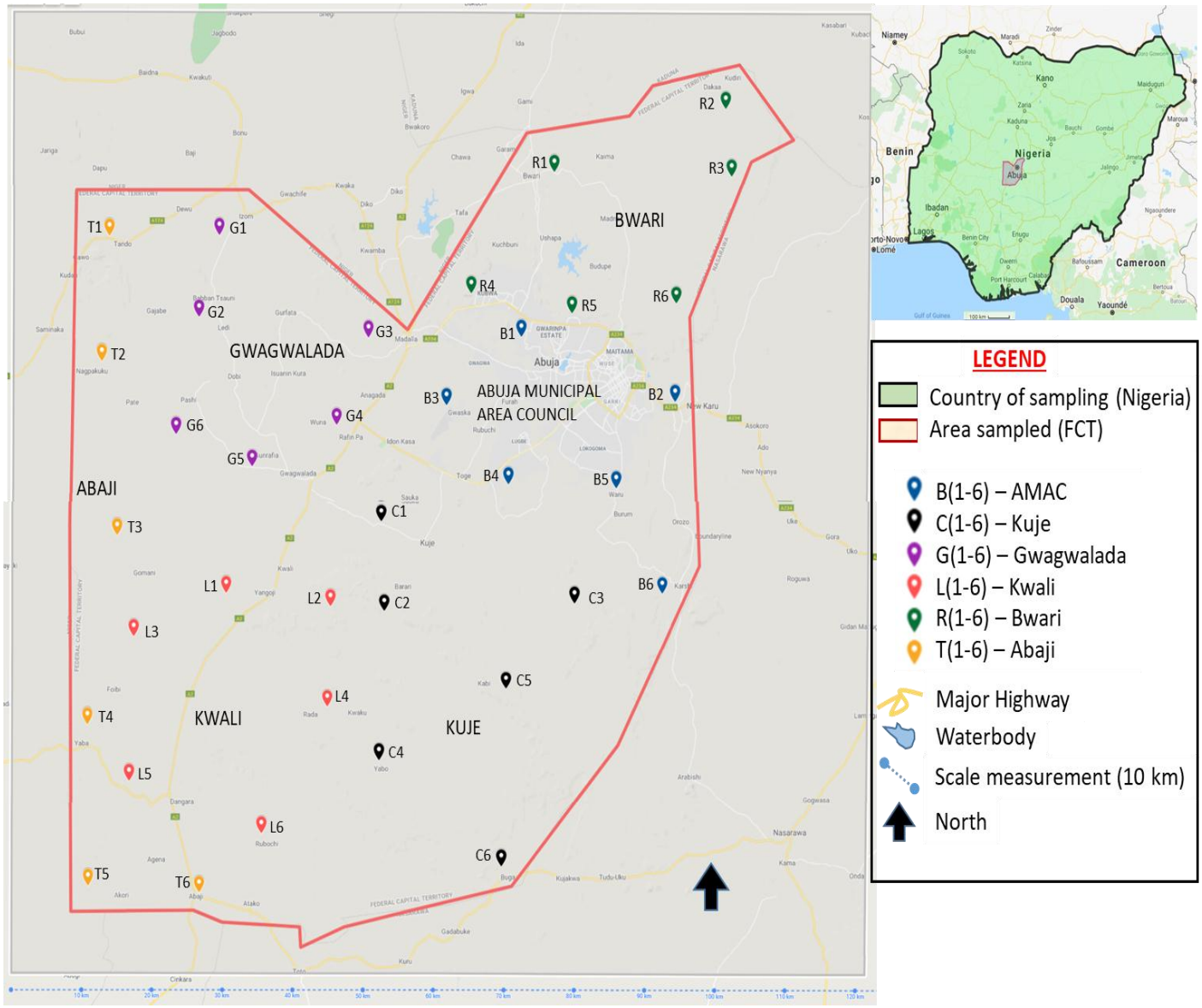

Figure 1. Map of FCT showing the study area.

nystatin (50 µg/ml) [Nanjing Sino Pharmaceutical] to inhibit fungal contaminant and were incubated at 37°C for 24 h in inverted positions. Culturing was performed in triplicate and colonies that displayed antagonism against other bacteria was identified by clear zones of inhibition around them were selected as potential antibiotic-producers. Selected colonies were sub-cultured on fresh NA plates and incubated at 37°C for 24 h to obtain pure cultures. The pure colonies were preserved on NA slants and stored in 4^{o}C until further use.

Characterization of identified isolates

Potential antibiotic-producers were characterized morphologically and biochemically and confirmed according to the Bergey's manual of systematic bacteriology 3 (Vos et al., 2011). The morphological characteristics of isolates were determined by performing tests like Gram's staining, endospore staining, motility test, growth in 6.5% NaCl at 37°C, growth in anaerobic condition and growth at 55°C. Biochemical tests include catalase test, methyl red, Voges-Proskauer test (VP), Indole, citrate, oxidase, nitrate reduction, starch hydrolysis. Carbohydrate fermentation including glucose, lactose, sucrose, mannitol and sorbitol were performed.

Test for antibiotic-producing potential of isolated bacteria

The organisms used for susceptibility testing were obtained from the Department of Microbiology and Biotechnology, NIPRD. The organisms were some American Type Culture Collection (ATCC) strains and some clinical isolates namely Gram-positives like *Bacillus subtilis, Staphylococcus aureus* (ATCC 25923), and *Streptococcus pneumoniae*; Gram-negative bacteria including *Proteus mirabilis, Pseudomonas aeruginosa* (ATCC 27853),

Table 1. Habitats and coordinates of the study area within the FCT, Nigeria.

S/N	Habitat	Sample region	Code	1	2	3	4	5	6
1	Termite mounds	Abaji	T	N9°13'5.268" E6°49'55.488"	N9° 4' 37.668" E6° 49' 19.380"	N8° 53' 4.272" E6° 50' 32.316"	N8° 40' 11.136" E6° 48' 13.860"	N8° 29' 11.868" E6° 48' 17.352"	N8° 28' 42.636" E6° 56' 54.240"
2	Rhizosphere of *Anacardium occidentale* L. (cashew tree)	Kuje	C	N8° 53' 59.784" E7° 11' 0.744"	N8° 47' 53.556" E7° 11' 13.560"	N8° 48' 25.488" E7° 25' 57.432"	N8° 42' 36.756" E7° 20' 38.940"	N8° 37' 45.336" E7° 10' 47.640"	N8° 30' 31.248" E7° 20' 16.224"
3	Rhizosphere of *Gmelina arborea* Roxb. ex Sm. (beechwood)	AMAC	B	N9° 6' 14.832" E7° 21' 54.540"	N9° 1' 51.204" E7° 33' 51.372"	N9° 1' 39.144" E7° 16' 6.024"	N8° 56' 18.636" E7° 20' 54.564"	N8° 56' 3.192" E7° 29' 15.252"	N8° 49' 4.404" E7° 32' 46.644"
4	Rhizosphere of *Ageratum conyzoides* L. (goat weed)	Gwagwalada	G	N8° 59' 42.072" E6° 55' 4.692"	N9° 7' 36.444" E6° 56' 52.296"	N9° 13' 8.256" E6° 58' 27.660"	N9° 6' 12.132" E7° 10' 1.416"	N9° 0' 17.676" E7° 7' 33.348"	N8° 57' 31.032" E7° 0' 59.364"
5	Rhizosphere *of Cymbopogon citratus* (DC) Stapf. (lemon grass)	Kwali	L	N8° 48' 13.140" E7° 7' 2.964"	N8° 49' 9.876" E6° 58' 59.340"	N8° 41' 22.704" E7° 6' 50.184"	N8° 46' 11.748" E6° 51' 49.464"	N8° 36' 20.052" E6° 51' 28.332"	N8° 32' 45.492" E7° 1' 43.104"
6	River bank	Bwari	R	N9° 21' 36.756" E7° 37' 47.064"	N9° 17' 5.100" E7° 38' 15.720"	N9° 17' 22.020" E7° 24' 29.268"	N9° 8' 30.228" E7° 33' 58.212"	N9° 9' 10.548" E7° 18' 2.160"	N9° 7' 51.132" E7° 25' 51.168"

Escherichia coli (ATCC 25922), *Salmonella typhi* and Yeast: *Candida albicans.* The identity of the supplied test microorganisms was further confirmed by morphological and biochemical tests.

Standardization of the test organisms

The microorganisms tested were standardized according to the Clinical and Laboratory Standards Institute (2016)**.** Gram positives and negatives tested bacteria from 24-hour culture plates were suspended in sterilized 2 ml of nutrient broth and incubated at 37°C for 2 h. The microbial suspension was adjusted to 0.5 McFarland turbidity standard equivalents to optical density of 0.08-0.13 for bacteria and 0.05 for yeast after measured at 600 nm in spectrophotometer (Jenway 6405 UV/VIS, UK). That corresponded to 1 x 10^8 colony-forming units per milliliter (CFU/ml) for the bacteria suspensions which was diluted 1:100 with nutrient broth to obtain 1 x 10^6 CFU/ml. The inoculum size for yeast (*C. albicans*) was 1 x 10^6 spore-forming unit/ml equivalents to 0.5 McFarland standards.

Susceptibility testing

Preliminary test for antibiotic-producing potential of the bacteria was carried out by agar-well diffusion technique as described by Rafiq et al. (2018) with slight modifications. For this purpose, pure culture from the potential antibiotic-producers were inoculated into sterile 10 ml Nutrient broth (NB) and kept in shaker incubator on 120 revolution/minute (rpm) at 37°C for 96 h. After incubation, the tubes were centrifuged at 6,000 rpm for 10 min to separate the partial cell-free supernatant from the cell pellet. The supernatant was finally filtered with 0.45 µm disposable membrane filter unit (Millipore brand, Massachusetts, USA) to obtain cell-free supernatant.

The standardized microbial suspension (10^6 CFU/ml) was swabbed with sterile cotton buds on Mueller-Hinton agar (MHA) **(**ThermoScientific, Massachusetts, USA)**.** Wells of 6mm diameter were aseptically bored on inoculated MHA plates, filled with 100 µl cell-free supernatants and allowed to diffuse at room temperature for 2 h. Sterile distilled water served as the negative control while standard antibiotics, 50 µg/ml of amoxicillin (GlaxoSmithKline) and antifungal, 50 µg/ml nystatin (Nanjing Sino Pharmaceutical) were the positive controls. The plates were incubated in the upright position at 37ºC for 24 h. The zones of inhibition (ZI) were observed after 24 h and the diameter of the zones were measured in millimeter (mm) using a Vernier caliper. The antibiotic-producing isolate was determined by the tested isolates inhibited after measuring the ZI displayed in the culture plates.

Molecular identification of the promising candidate isolates

The most potent antibiotic-producing isolates were further identified by molecular techniques as described by Pomastowski et al. (2019) with slight modifications. The five most active antibiotic producers (ASS02/01, AMP03/02, AMP03/05, AAH02 and AMR06/01) were cultured in tryptone-soy broth (TSB) (ThermoScientific, Massachusetts, USA) at 37ºC for 24 h. The genomic DNA was extracted using AxyPrep bacterial genomic DNA miniprep kit (Axygen Biosciences, California, USA) following the manufacturer's instructions and was checked for quality by using a NanoDrop device (ThermoScientific, Massachusetts, USA). The 16S rRNA gene fragment of the isolates were amplified by using the universal primers 27F(5′- AGAGTTTGATCCTGGCTCAG –3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) to yield the PCR products of approximately 1500 base pair. Polymerase Chain Reaction (PCR) was carried out in a GeneAmp 9700 PCR System thermal cycler (Applied Biosystem Inc., USA). The thermal cycling condition included a cycle of an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing of primers at 50°C for 30 s, extension at 72°C for 1.5 min and then a final extension at 72°C for 7 min. The amplified PCR products were checked on 1.5% agarose gel electrophoresis ran on

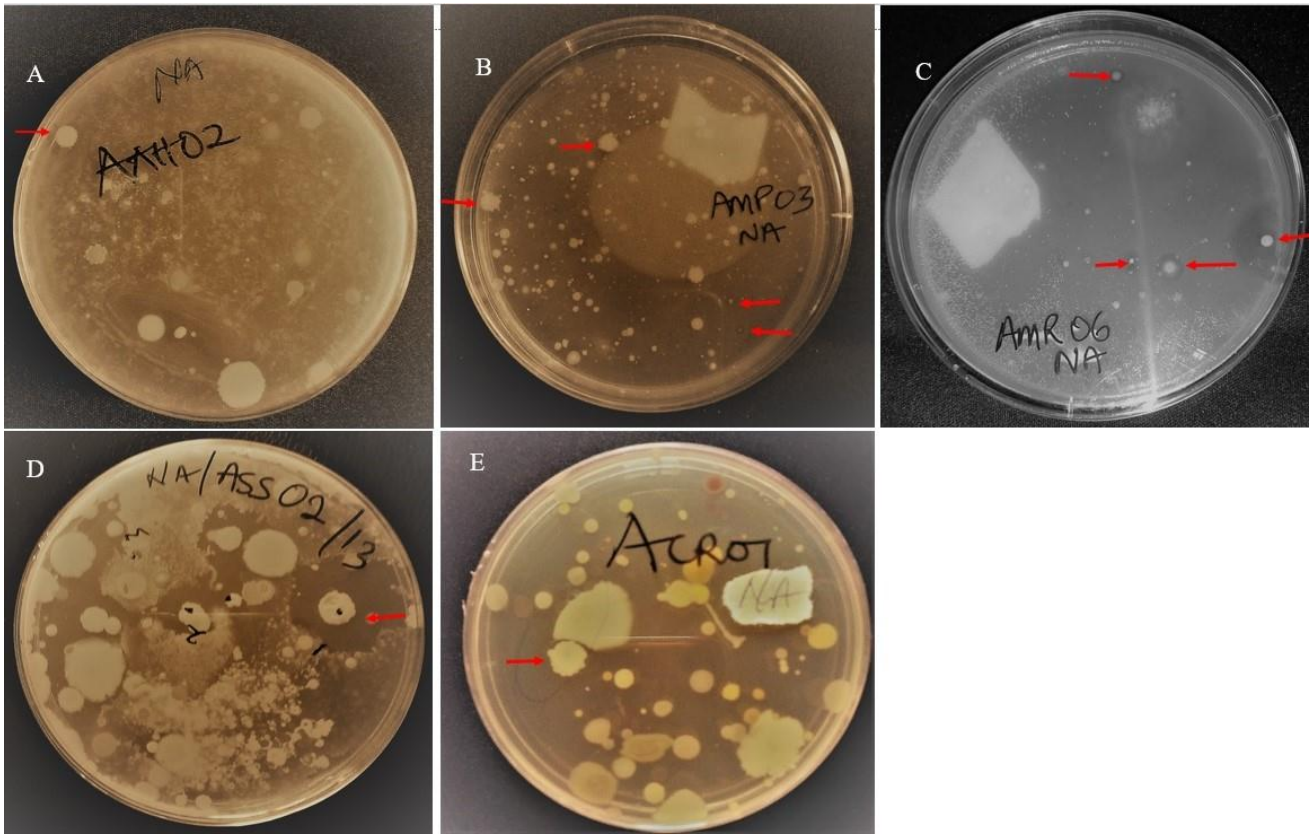

Figure 2. Inhibitory activity of the potential antibiotic-producing bacteria in Crowded plate for soil sample collected (A) from termite mound (B) from the rhizosphere of *Ageratum conyzoides* L.(goatweed) (C) from the rhizosphere of *Gmelina arborea* Roxb. ex Sm. (beechwood) (D) from the river bank (E) from the rhizosphere of *Anacardium occidentale* L. (cashew tree). Red arrows show the isolates with zones of inhibition.

a voltage of 120 V for 45 min. The DNA fragment was viewed under UV transilluminator and purified using QIAquick PCR purification kit (Qiagen, Germany) in preparation for sequencing. The sequencing was performed using ABI PRISM Big Dye Terminator cycle sequencer (Applied Biosystems, USA). The 16S rRNA gene sequences obtained were exported into the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information website (NCBI-http://www.ncbi.nlm.nih.gov) to identify matches with the annotated strains.

RESULTS

Isolation of antibiotic-producing microorganisms by crowded plate technique

Plates containing approximately 300 to 400 colonies with at least a colony showing halo around it were selected. A total of 12 bacteria colonies showing zones of inhibition following the crowded plate technique were recovered from soil samples from 5 of the 6 tested natural habitats (Figures 2A to E and Table 4). None of the isolates from samples from the rhizosphere *of Cymbopogon citratus* (DC) Stapf. (Lemon grass) showed zone of inhibition.

Termite mounds

Only 1 colony coded AAH02 displayed zone of inhibition of size 21 mm around it (Figure 2A).

Rhizosphere of Ageratum conyzoides L. (Goat weed)

Five colonies coded AMP03/01, AMP03/02, AMP03/03, AMP03/04 and AMP03/05 showed zones of inhibition of sizes 7, 7, 4, 3 and 8 mm respectively (Figure 2B).

Rhizosphere of Gmelina arborea Roxb. ex Sm. (Beechwood)

Three colonies coded AMR06/01, AMR06/02 and AMR 06/03 showed zones of inhibition of sizes 5, 16 and 6 mm around them respectively (Figure 2C).

River bank

Two colonies coded ASS02/01, ARS03/01 both showed zones of inhibition of sizes 22 and 16 mm respectively

(Figure 2D).

Rhizosphere of Anacardium occidentale L. (Cashew tree)

One colony coded ACR01 showed zone of inhibition of size 6 mm around it (Figure 2E).

Rhizosphere of Cymbopogon citratus (DC) Stapf. (Lemon grass)

None of the colonies displayed a zone of inhibition around it.

Characterization of potential antibiotic-producing isolates

The twelve potential antibiotic-producing bacteria isolated from diverse natural habitats were subjected to morphological and biochemical tests for identification as summarized in Table 2. The isolate coded AAH02 showed a whitish, rough, wrinkled and irregular form on a nutrient agar medium. The isolate was gram-positive rods with centrally located spores and showed positive reactions to motility, catalase, Voges-Proskauer (V-P), citrate, nitrate reduction, growth in 6.5% NaCl, growth in anaerobic condition, growth at 55^{o}C, starch hydrolysis, carbohydrate fermentation (glucose, lactose, sucrose, mannitol, and sorbitol). The isolate was negative to methyl-red test, indole and oxidase tests respectively. Considering the culture morphology and biochemical characteristics, bacteria isolate AAH02 was identified as *Bacillus licheniformis.*

Isolate coded ACR01 showed a yellow-greyish, umbonate, smooth, flat and irregular form on a nutrient agar medium. The isolate was gram-positive rods with sub-terminally located spores and showed positive reaction to motility, catalase, oxidase, nitrate reduction and growth at 55°C. However, the isolate displayed negative reaction to methyl red, V-P, indole, citrate, growth in 6.5% NaCl at 37°C, growth in anaerobic condition, starch hydrolysis and carbohydrate fermentation (glucose, lactose, sucrose, mannitol, and sorbitol). Based on the cultural characteristics and biochemical tests, the isolate was identified as *Bacillus brevis.*

Isolate coded AMR06/01 showed a greyish, wrinkled and irregular form on a nutrient agar medium. The isolate was gram-positive rods with centrally-located spores. It showed a positive reaction to motility, catalase, citrate, oxidase, nitrate reduction, growth in 6.5% NaCl at 37°C and starch hydrolysis; and weak positive reaction to carbohydrate fermentation (glucose, lactose, sucrose, mannitol, and sorbitol). However, the isolate showed a negative reaction to methyl red, V-P, indole, growth in anaerobic condition and growth at 55°C. Based on the cultural characteristics and biochemical tests, the isolate was identified as *Bacillus lentus.*

Isolate coded AMR06/02 was whitish, rough, wrinkle, and irregular form on a nutrient agar medium. The isolate was gram-positive rods with centrally-located spores. It showed a positive reaction to motility, catalase, V-P, citrate, nitrate reduction, growth in 6.5% NaCl at 37°C, growth in anaerobic condition, growth at 55°C and carbohydrate fermentation (glucose, lactose, sucrose, mannitol, and sorbitol). The isolate showed a negative reaction to methyl red, indole and oxidase. Considering the cultural characteristics and biochemical tests, the isolate was identified as *Bacillus licheniformis.*

Isolate coded AMR06/03 showed a greyish, smooth, flat and circular form on a nutrient agar medium. The isolate was gram-positive rods with centrally-located spores. It showed a positive reaction to motility, catalase, V-P, citrate, nitrate reduction, growth in 6.5% NaCl at 37°C, growth in anaerobic condition, starch hydrolysis, and some carbohydrate fermentation (glucose and sucrose). The isolate showed a negative reaction to methyl red, indole, oxidase, Growth at 55°C, and some carbohydrate fermentation (lactose, mannitol, and sorbitol). Given the cultural characteristics and biochemical tests results, the isolate was identified as *Bacillus cereus*.

Isolate coded AMP03/01 showed yellow-grey, umbonate, smooth, flat and irregular form on a nutrient agar medium. The isolate was gram-positive rods with sub-terminally located spores and showed positive reaction to motility, catalase, oxidase, nitrate reduction and growth at 55°C. However, the isolate displayed negative reaction to methyl red, V-P, indole, citrate, growth in 6.5% NaCl at 37°C, growth in anaerobic condition, starch hydrolysis and carbohydrate fermentation (glucose, lactose, sucrose, mannitol, and sorbitol). Based on the cultural characteristics and biochemical tests, AMP03/01 was identified as *B. brevis.*

Isolate coded AMP03/02 showed a greyish, smooth, flat and circular form on a nutrient agar medium. The isolate was gram-positive rods with centrally-located spores. It showed a positive reaction to motility, catalase, V-P, citrate, nitrate reduction, growth in 6.5% NaCl at 37°C, growth in anaerobic condition, starch hydrolysis, and some carbohydrate fermentation (glucose and sucrose). The isolate showed a negative reaction to methyl red, indole, oxidase, Growth at 55°C, and some carbohydrate fermentation (lactose, mannitol, and sorbitol). Given the cultural characteristics and biochemical tests results, the isolate was identified as *B. cereus*.

Isolate coded AMP03/03 showed a yellowish, smooth, convex, and circular form on a nutrient agar medium. The isolate was gram-positive cocci with no spores. Isolate showed positive catalase reaction, V-P, citrate, oxidase and growth in 6.5% NaCl at 37°C. The isolate showed a

Table 2. Morphological and biochemical characterization of the potential antibiotic-producing isolates

Test	AAH02	ACR01	AMR06/01	AMR06/02	AMR06/03	AMP03/01	AMP03/2	AMP03/03	AMP03/04	AMP03/05	ASS02/01	ARS03/01
Colony	White, Rough, Wrinkle, and Irregular	Yellow-grey, Umbonate, smooth, flat and Irregular	Grey, wrinkle, Irregular	White, Rough, Wrinkle, and Irregular	Grey, Smooth, Flat and Circular	Yellow-grey, Umbonate, smooth, flat and Irregular	Grey, Smooth, Flat and Circular	Yellow, smooth, convex, and Circular	Yellow-grey, Umbonate, smooth, flat and Irregular	Yellow-grey, Umbonate, smooth, flat and Irregular	White, Rough, Flat, and Irregular	Grey, Smooth, Flat and Circular
Gram staining	+ rods	+ rods	+ rods	+ rods	+ rods	+ rods	+ rods	+ cocci	+ rods	+ rods	+ rods	+ rods
Spore staining	+; central	+; sub-terminal	+; central	+; central	+; central	+; sub-terminal	+; central	-	+; sub-terminal	+; sub-terminal	+; central	+; central
Motility	+	+	+	+	+	+	+	-	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskaeur	+	-	-	+	+	-	+	+	-	-	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-	-
Citrate	+	-	+	+	+	-	+	+	-	-	+	+
Oxidase	-	+	+	-	-	+	-	+	+	+	-	-
Nitrate Reduction	+	+	+	+	+	+	+	-	+	+	+	+
Growth in 6.5% NaCl at 37°C	+	-	+	+	+	-	+	+	-	-	+	+
Growth in anaerobic condition	+	-	-	+	+	-	+	-	-	-	-	+
Growth at 55°C	+	+	-	+	-	+	-	-	+	+	+	-
Starch Hydrolysis	+	-	+	+	+	-	+	-	-	-	+	+
Glucose	+	-	±	+	+	-	+	-	-	-	+	+
Lactose	+	-	±	+	-	-	-	-	-	-	+	-
Sucrose	+	-	±	+	+	-	+	-	-	-	+	+
Mannitol	+	-	±	+	-	-	-	-	-	-	+	-
Sorbitol	+	-	±	+	-	-	-	-	-	-	+	-
CBI	*B. licheniformis*	*B. brevis*	*Bacillus lentus*	*Bacillus licheniformis*	*Bacillus cereus*	*Bacillus brevis*	*Bacillus cereus*	*Micrococcus luteus*	*Bacillus brevis*	*Bacillus brevis*	*Bacillus subtilis*	*Bacillus cereus*

+, Positive; -,negative; ±, weak positive; CBI, confirmed bacteria isolates.

negative reaction to motility, methyl red, indole, nitrate reduction, growth in anaerobic condition, growth at 55°C, starch hydrolysis, and carbohydrate fermentation (glucose, lactose, sucrose, mannitol, and sorbitol). Considering the cultural characteristics and biochemical tests results, the isolate was identified as *Micrococcus luteus.*

Isolate coded AMP03/04 showed yellow-grey, umbonate, smooth, flat and irregular form on a nutrient agar medium. The isolate was gram-positive rods with sub-terminally located spores and showed positive reaction to motility, catalase, oxidase, nitrate reduction and growth at 55^{o}C. However, the isolate displayed negative reaction to methyl red, V-P, indole, citrate, growth in 6.5% NaCl at 37°C, growth in anaerobic condition, starch hydrolysis and carbohydrate fermentation (glucose, lactose, sucrose, mannitol, and sorbitol). Given the cultural characteristics and biochemical tests, the isolate was identified as *B. brevis.*

Table 3. Summary of the susceptibility profile of potential antibiotic-producing bacteria against tested isolates.

Target organisms	Diameter of zones of Inhibition (mm) of the potential antibiotic- producing isolates											
	S01	S02	S03	S04	S05	S06	S09	S010	S011	S012	S013	S014
S. pneumoniae	0.0	0.0	0.0	0.0	10.0±0.4	0.0	0.0	0.0	11.4±0.4	0.0	0.0	0.0
S. typhi	0.0	9.4±0.4	0.0	0.0	0.0	11.1±0.4	0.0	0.0	10.2±0.2	0.0	0.0	0.0
E. coli (ATCC 25922)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.0±0.0	0.0	0.0	0.0
Proteus mirabilis	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S. aureus (ATCC 25923)	0.0	10.3±0.3	0.0	0.0	9.1±0.4	0.0	0.0	0.0	10.3±0.4	0.0	0.0	0.0
C. albicans	0.0	0.0	0.0	0.0	0.0	9.3±0.4	0.0	0.0	9.3±0.3	0.0	0.0	0.0
B. subtilis	0.0	12.2±0.4	0.0	0.0	10.0±0.4	12.1±0.4	0.0	0.0	9.5±0.4	9.3±0.4	0.0	0.0
P. aeruginosa(ATCC 27853)	0.0	10.1±0.4	0.0	0.0	0.0	9.4±0.6	0.0	0.0	8.4±0.4	0.0	0.0	0.0

Mean ± Standard Deviation (SD); S01= filtrate (cell-free supernatants) from AMP03/01; S02 from AMP03/02; S03 from AMP03/03; S04 from AMP03/04; S05 from AMP03/05; S06 from AAH02; S09 from ARS03/01; S10 from ACR01/01; S011 from ASS02/01; S012 from AMR06/01; S013 from AMR06/02; and S014 from AMR06/03.

Isolate coded AMP03/05 showed yellow-grey, umbonate, smooth, flat and irregular form on a nutrient agar medium. The isolate was gram-positive rods with sub-terminally located spores and showed positive reaction to motility, catalase, oxidase, nitrate reduction and growth at 55°C. However, the isolate displayed negative reaction to methyl red, V-P, indole, citrate, growth in 6.5% NaCl at 37°C, growth in anaerobic condition, starch hydrolysis and carbohydrate fermentation (glucose, lactose, sucrose, mannitol, and sorbitol). Based on the cultural characteristics and biochemical tests, the isolate was identified as *B. brevis.*

Isolate coded ASS02/01 showed a whitish, rough, flat, and circular form on a nutrient agar medium. The isolate was gram-positive rods with centrally located spores and showed positive reaction to motility, catalase, V-P, citrate, nitrate reduction, growth in 6.5% NaCl at 37°C, growth at 55°C, starch hydrolysis and carbohydrate fermentation (glucose, lactose, sucrose, mannitol, and sorbitol). It however displayed negative reaction to methyl-red, indole, oxidase, and growth in anaerobic condition. Considering the cultural characteristics and biochemical tests, the isolate was identified as *Bacillus subtilis.*

Isolate coded ARS03/01 showed a greyish, smooth, flat and circular form on a nutrient agar medium. The isolate was gram-positive rods with centrally-located spores. It showed a positive reaction to motility, catalase, V-P, citrate, nitrate reduction, growth in 6.5% NaCl at 37°C, growth in anaerobic condition, starch hydrolysis, and some carbohydrate fermentation (glucose and sucrose). The isolate showed a negative reaction to methyl red, indole, oxidase, growth at 55°C, and some carbohydrate fermentation (lactose, mannitol, and sorbitol). Given the cultural characteristics and biochemical tests results, the isolate was identified as *B. cereus*.

Susceptibility test for antibiotic-producing potential of the isolated bacteria

The preliminary test for potential antibiotic-producing bacteria was carried out by Agar-well diffusion technique (Table 3). Out of the 12 candidate bacteria isolate obtained from the crowded plates, 5 displayed inhibitory activities against at least 1 pathogenic microbe. Filtrates S01, S03, S04, S09, S10 S013, and S014 showed no activities against all the tested pathogens. Summary of the number of test pathogens inhibited by filtrates from the potential antibiotic-producing bacteria was also displayed in Table 4. Filtrate S02 showed activities of 12.2±0.4, 10.3±0.3, 10.1±0.4 and 9.4±0.4 mm against *B. subtilis, S. aureus, P. aeruginosa* and *S. typhi*, respectively; filtrate S05 showed activities of 10.0±0.4, 9.1±0.4 and 10.0±0.4 mm against *B. subtilis, S. aureus* and *S. pneumonia*, respectively; filtrate S06 showed activities of 12.1±0.4, 9.3±0.4, 9.4±0.6 and 11.1±0.4 mm against *B. subtilis, C. albicans*, *P. aeruginosa* and *S. typhi*, respectively. Filtrate S011 from ASS02/01 displayed the strongest activity as it inhibited seven of the eight (8) test pathogenic isolates as follows: 9.5±0.4, 9.3±0.3, 10.3±0.4, 11.4±0.4, 9.3±0.3, 8.4±0.4 and 10.2±0.2 mm activities against B. subtilis, *E. coli*, *S aureus*, *S. pneumoniae, C. albicans, P. aeruginosa*

Table 4. Summary of potential antibiotic-producing bacteria and the test isolates.

S/N	Specimen collection points	Habitats	Antagonistic isolate codes	Diameter of zone of Inhibition in crowded plates (mm)	Number of test microorganisms inhibited
1	Abaji	Termite mound	AAH02	21	4
2	Kuje	Rhizosphere of *Anacardium occidentale* L. (cashew tree)	ACR01	6	0
3	AMAC	Rhizosphere of *Gmelina arborea* Roxb. ex Sm. (beechwood)	AMR06/01	5	1
4	AMAC	Rhizosphere of *Gmelina arborea* Roxb. ex Sm. (beechwood)	AMR06/02	16	0
5	AMAC	Rhizosphere of *Gmelina arborea* Roxb. ex Sm. (beechwood)	AMR06/03	6	0
6	Gwagwalada	Rhizosphere of *Ageratum conyzoides* L. (goat weed)	AMP03/01	7	0
7`	Gwagwalada	Rhizosphere of *Ageratum conyzoides* L. (goat weed)	AMP03/02	7	4
8	Gwagwalada	Rhizosphere of *Ageratum conyzoides* L. (goat weed)	AMP03/03	4	0
9	Gwagwalada	Rhizosphere of *Ageratum conyzoides* L. (goat weed)	AMP03/04	3	0
10	Gwagwalada	Rhizosphere of *Ageratum conyzoides* L. (goat weed)	AMP03/05	8	3
11	Bwari	River bank	ASS02/01	22	7
12	Bwari	River bank	ARS03/01	16	0
13	Kwali	Rhizosphere *of Cymbopogon citratus* (DC) Stapf. (lemon grass)	No inhibitory bacteria	-	NA

AAH02: Cell-free supernatant (S) from *Bacillus licheniformis;* ACR01: S from *Bacillus brevis*; AMR06/01: S from *Bacillus lentus;* AMR06/02: S from *Bacillus licheniformis;* AMR06/03: S from *Bacillus cereus;* AMP03/01: S from *Bacillus brevis;* AMP03/02: S from *Bacillus cereus;* AMP03/03: S from *Micrococcus luteus;* AMP03/04: S from *Bacillus brevis;* AMP03/05: S from *Bacillus brevis;* ASS02/01: S from *Bacillus subtilis;* ARS03/01: S from *Bacillus cereus;* NA=Not applicable.

and *S. typhi* respectively. Filtrate S012 showed activities of 9.3±0.4 mm against only *B. subtilis*.

None of the filtrates from the potential antibiotic-producing strains displayed inhibitory activities against Proteus mirabilis.

Molecular identification of the promising candidate isolates

The PCR result was analyzed by using agarose gel electrophoresis as summarized in Figure 3. The observed bands validated the amplification of 16S rDNA fragments. The amplicons obtained from the genomic DNAs of the five candidate isolates were approximately 1500 base pair (bp). The homology search for the sequences in the BLAST revealed that isolates ASS02/01, AAH02, AMP03/02, AMP03/05 and AMR06/01 had closest similarity to *B. subtilis* subsp. subtilis 168, *B. licheniformis* strain SCDB 34, *B. cereus* strain A1, *B. brevis* strain NCTC2611, and *B. lentus* strain NCTC4824 respectively. Table 5 illustrated the summary of the molecular result.

DISCUSSION

Isolation and screening for antibiotic-producing bacteria are vital to the global effort in curbing the challenges from antimicrobial-resistant pathogens. The conventional antimicrobials are showing weaknesses against the plethora of previously susceptible microbes including the emerging and re-emerging drug-resistant pathogens. To this end, screening natural habitats of soil origin for lead compounds from candidate antimicrobial-producing microorganisms in order to replace the failing antimicrobials is crucial considering the successes previously recorded (Elkholy et al., 2019; Makut and Owolewa, 2011; Rajivgandhi et al., 2019). In this study, some natural habitats such as termite mounds, river bank and plant rhizospheres were investigated for potential antibiotic-producing bacteria in the FCT. Twelve potential antibiotics-producers were isolated and were subjected to morphological and biochemical tests for identification (Table 2).

The potential antibiotics producers were identified and confirmed according to the Bergey's manual (Vos et al., 2011). Isolate coded AAH02 isolated from termite mound sample was identified as *B*. *licheniformis*. Isolate ACR01 isolated from sample from the rhizosphere of *Anacardium*

occidentale L.(cashew tree) was identified as *B. brevis*. Isolate AMR06/01 isolated from the rhizosphere of *Gmelina arborea* Roxb. ex Sm. (beechwood) was identified as *B. lentus*. Isolate coded AMR06/02 isolated from the rhizosphere of *G. arborea* Roxb. ex Sm. (beechwood) was identified as *B. licheniformis*. Isolate coded AMR06/03 isolated from the rhizosphere of *G. arborea* Roxb. ex Sm. (beechwood) was identified as *B. cereus*. Isolate coded AMP03/01 isolated from the rhizosphere of *A. conyzoides* L. (goat weed) was identified as *B. brevis*.

Isolate coded AMP03/02 isolated from the rhizosphere of *A. conyzoides* L. (goat weed) was identified as *B. cereus*. Isolate coded AMP03/03 isolated from the rhizosphere of *A. conyzoides* L. (goat weed) was identified as *M. luteus.* Isolate coded AMP03/04 isolated from the rhizosphere of *A. conyzoides* L. (goat weed) was identified as *B. brevis.* Isolate coded AMP03/05 isolated from the rhizosphere of *A. conyzoides* L. (goat weed) was identified as *B. brevis.* Isolate coded ASS02/01 isolated from the river bank was identified as *B. subtilis.* Isolate coded ARS03/01 isolated from the river bank was identified as *B. cereus*.

Eleven among the twelve isolated potential antibiotic-producers were *Bacillus* species while one was *M. luteus.* This is similar to the study by Abdulkadir and Waliyu (2012) where the majority of antibiotic-producers screened from soil were *Bacillus* spp. (*B. luteus, B. alvei and B. pumillus*) including one *Micrococcus spp.* (*M. roseus*).

Of the twelve potential antibiotic-producing isolates, ten were rhizospheric bacteria, one from the termite mound and another 1 was from the river bank. Among these 12 isolates, only five inhibited the test microorganisms of which four showed activities against both Gram-positive and Gram-negative bacteria (broad spectrum of activities), while the remaining 1 inhibited only 1 Gram-positive bacterium (narrow spectrum of activities). Two among the 4 with broad spectrum of activities also showed activities against *Candida albicans* (yeast).

The 5 antibiotics-producing isolates belong to the genus *Bacillus*. *Bacillus* species are predominant in several habitats including soil and possess between 5 to 8% secondary metabolite-producing genes (Fira et al., 2018). The five organisms include: *Bacillus cereus* isolated from the rhizosphere of *Ageratum conyzoides* L. (goat weed). *Bacillus cereus* (AMP03/02 filtrate) showed inhibitory activities against *B. subtilis (*12.2±0.4 mm)*, S. aureus (*10.3±0.3 mm)*, P. aeruginosa* (10.1±0.4 mm) and *S. typhi* (9.4±0.4 mm) respectively. This is similar to the study by Yilmaz et al. (2006) where *Bacillus cereus* M15 strain isolated from soil showed inhibitory activities against both Gram-positive and Gram-negative pathogenic isolates.

Bacillus brevis was also isolated from the rhizosphere of *Ageratum conyzoides* L. (goat weed). *Bacillus brevis* (AMP03/05 filtrate) showed activities against *B. subtilis (10.0±0.4 mm), S. aureus (9.1±0.4 mm) and S. pneumonia*e (10.0±0.4 mm) respectively. Ghai et al. (2007) isolated *B. brevis* (M116 and T122 strains) from soil and both isolates showed antibacterial activities against both gram-positive and gram-negative pathogenic bacteria.

Bacillus licheniformis isolated from termite mound. *B. licheniformis* (AAH02 filtrate) showed activities against *B. subtilis* (12.1±0.4 mm), *C. albicans* (9.3±0.4 mm), *P. aeruginosa* (9.4±0.6 mm) and *S. typhi* (11.1±0.4 mm) respectively. This is similar to the study by Al-Turk et al. (2020) where *B. licheniformis* isolated from the soil displayed inhibitory activities against some Gram-positive and Gram-negative bacteria.

Bacillus subtilis isolated from river bank and *B. lentus* from the rhizosphere of *G. arborea* Roxb. ex Sm. (Beechwood)*. B. lentus* (AMR06/01 filtrate) inhibited only *B. subtilis (*9.3±0.4 mm) among the tested pathogens. A similar study by Abdulkadir and Waliyu (2012) also showed that *B. lentus* isolated from the soil displayed antibacterial activity against a gram-positive pathogenic strain.

Among the 5 antibiotic-producers, *Bacillus subtilis* (ASS02/01 filtrate) displayed the strongest inhibitory ability against the test pathogenic strains. It showed antimicrobial activities against seven (7) of the eight (8) test isolates namely *Streptococcus pneumonia* (11.4±0.4 mm), *Salmonella typhi* (10.2±0.2 mm), *Escherichia coli* (11.0±0.0 mm), *Staphylococcus aureus* (10.3±0.4 mm)*, Bacillus subtilis* (9.5±0.4 mm)*, Pseudomonas aeruginosa* (8.4±0.4 mm) and *Candida albicans* (9.3±0.3 mm)*.* This is contrary to the study by Kiesewalter et al. (2020) that reported that the environmental strain of *B. subtilis* P5_B1, a non-ribosomal peptides producer, showed a weak impact on the soil bacteria community. Non-ribosomal peptides antibiotics are among the secondary metabolites produced by *B. subtilis* that function by targeting bacteria protein synthesis, lysis of fungal membrane and enzyme inhibition (Kiesewalter et al., 2020; Wang et al., 2015).

The antimicrobial activities displayed by the Bacillus isolates is in agreement with the study by Fira et al. (2018) in which case multiple strains of *Bacillus* species exhibited strong antibacterial and antifungal activities *in vivo* and *in vitro*. *Bacillus cereus, Bacillus brevis, Bacillus licheniformis* and *Bacillus subtilis* all displayed broad-spectrum of activities while *Bacillus lentus* showed a narrow spectrum of activities.

The other seven (7) isolates that displayed no inhibitory activities (0.0 mm) against test organisms includes *B. brevis, M. luteus and B. brevis,* all of which were isolated from the rhizospheres of *A. conyzoides* L. (goat weed). Others include *B. cereus* isolated from the river bank*, B. brevis* isolated from the rhizosphere of *Anacardium occidentale* L. (cashew tree)*, B. licheniformis* isolated from the rhizosphere of *G. arborea* Roxb. ex Sm. (beechwood) and *B. cereus* also from the rhizosphere of *G. arborea* Roxb. ex Sm. (beechwood). Their inability to inhibit test pathogenic isolates may be due to the minimal

Table 5. Summary of molecular identification of the five antibiotic-producing isolates.

Sample ID	Source	Matches in the BLAST	Query covered	E-value	Percentage Identity	Accession number
ASS02/01	River bank	*Bacillus subtilis* subsp. *subtilis* 168	97	0.0	100	NC_000964.3
AAH02	Termite mound	*Bacillus licheniformis* strain SCDB 34	98	0.0	95.5	NZ_CP014793.1
AMP03/02	Rhizosphere[a]	*Bacillus cereus* strain A1	99	0.0	99.2	NZ_CP015727.1
AMP03/05	Rhizosphere[a]	*Bacillus brevis* strain NCTC2611	100	0.0	99.4	NZ_LR134338.1
AMR06/01	Rhizosphere[b]	*Bacillus lentus* strain NCTC4824	96	0.0	100	NZ_LS483476.1

[a]Of *Ageratum conyzoides* L. (goat weed); [b] Of *Gmelina arborea* Roxb. ex Sm. (beechwood)

competition from other microbes in their natural habitats, resulting in their indisposition to producing potent antimicrobial molecules.

P. mirabilis showed resistance to all the 12 potential antibiotic-producing isolates. This may be associated with the presence of transferrable resistant genes and mobile genetic elements common with multi-drug resistant *P. mirabilis* (Alabi et al., 2017).

Our study also demonstrates the uncertainty in the use of zones of inhibition obtained by the antibiotic-producing microorganisms in the crowded plates as predictor of the number of inhibitory test pathogenic strains (Table 4). For example, the filtrate from ASS02/01 which showed a zone of inhibition (ZI) 22 mm from crowded plate inhibited seven test microbes. However, AAH02 with ZI of 21 mm from the crowded plate inhibited only four (4) test isolates as similar to AMP03/02 with ZI 7 mm from the crowded plates. AMP03/05 with ZI 8 mm inhibited three (3) test isolates, AMR06/01 with ZI 5 mm inhibited only one (1) of the test microbes (Table 5). However, isolates ACR01, AMR06/02, AMR06/03, AMP03/01, AMP03/03, AMP03/04, ARS03/01 with ZI 6, 16, 6, 7, 4, 3 and 16 mm respectively in the crowded plates displayed no inhibition against all the test microbes. The inconsistencies could be as a result of the variable sensitivity levels displayed by the test pathogens, including the strengths of resistance already familiar with by the antibiotics-producing bacteria from other competing bacteria in their natural ecosystem.

Our findings suggest a noteworthy episode in the ecological niche. For example, the filtrate from *B. licheniformis* (AAH02) isolated from the termite mound showed broad spectrum of activities against some of the tested pathogenic strains. Termites are social insects that cohabitate in the termitarium. Among the mechanisms for survival adopted by termites is the release of antimicrobial compounds into the termitarium to inhibit pathogen microbes (Cole et al., 2021; He et al., 2018). Therefore, the persistent exposure of the isolate in this study to the antimicrobial metabolites in the termite mound may have primed the organism into antimicrobial-producing capabilities.

The antimicrobial activities displayed by *B. cereus* (AMP03/02), *B. brevis* (AMP03/05) and *B. lentus* (AMR06/01) isolated from the rhizospheres of some plants corroborate with some other ecological findings. Plants adopt several strategies to compete favorably within the resource-limited environ. Studies have shown that plants release bioactive compounds into the rhizospheres to modulate the soil environ in their favor, and also to attract rhizosphere microbial community required for their fitness in the ecosystem (Hu et al., 2018; Zhalnina et al., 2018). Some of the released metabolites attract antibiotic-producing microorganisms needed as weapons against existing plant pathogens (Babalola et al., 2021; Hu et al., 2018; Schulz-Bohm et al., 2018).

Conclusion

The outcome of this study indicates that some natural habitats in the FCT are important sources of antibiotic-producing bacteria. The 5 bacteria with filtrates showing inhibitory activities against tested pathogenic isolates, namely *B. cereus, B. brevis, B. licheniformis, B. subtilis* and *B. lentus* are potential candidates for antibiotic production. The strongest among the isolated antibiotic-producing isolates, *B. subtilis* displayed broad spectrum of antimicrobial activity haven inhibited 7 out of the 8 test isolates.

Recommendation

Further investigations like liquid chromatography mass spectrometry analysis, nuclear magnetic

resonance spectroscopy or fourier transform infrared spectroscopy (FTIR) will be needed to elucidate the potential antibiotic compounds in the candidate isolates. Development and purification
of the broad-spectrum antimicrobial active compounds from the antibiotic-producing isolates will assist in narrowing the gap of new antimicrobial agents. The discovery of indigenous candidate isolates with antimicrobial capabilities in the FCT has indicated that antimicrobial lead compounds can be sourced locally.

CONFLICT OF INTERESTS

The authors have no conflict of interests.

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