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African Journal of Microbiology Research

Full Length Research Paper

Coronavirus disease 2019 (COVID-19) pandemic: What is the level of knowledge, attitude, and practice in Kandahar, Afghanistan?

Asmatullah Usmani[1]*, Abdul Qadeer Baseer[1,2], Bilal Ahmad Rahimi[3], Ajmal Jahid[1], Parwiz Niazi[1,4], Abdul Wahid Monib[1,5] and Wais Mohammad Lali[6]

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There is a rising concern for the rapid increase of COVID-19 confirmed cases in Kandahar province. From zero reported cases until 17th March 2020, then Kandahar saw a sudden rise in the cases by 16th May 2020. Decreased literacy rates, poor health education, lack of facilities, inconsistent government policies, and defying coronavirus safety advisory by the public have resulted in the rapid spread of COVID-19. The awareness and practices of the people towards the COVID-19 were significantly low. Therefore, the risk of coronavirus in Kandahar province is extremely high due to the aforementioned reasons. To overcome this virus, the local government must declare strict measures and provide the public with information about the severity and prevention mechanisms of this fatal disease. Mass and random diagnostic testing are required to track the actual infection rates, which can give a realistic picture of what is occurring. In this article, the current situation of COVID-19, available medical facilities, and public response to the ongoing pandemic in Kandahar, Afghanistan was highlighted.

Key words: COVID-19, Kandahar province, public response, curfew, random testing.

INTRODUCTION

Coronaviruses are a large group of single-stranded, non-segmented, positive-sense RNA viruses belonging to the family Coronaviridae (Cui et al., 2019; Wege and Ter Meulen, 1982). Members of this family cause respiratory and gastrointestinal infections in humans and animals (Perlman, 1998). In the past two decades, two well-known pathogenic strains of coronaviruses, e.g. SARS-CoV and MERS-CoV, have emerged in humans (Kuiken et al., 2003; Zaki et al., 2012). The newly appeared novel type coronavirus (2019-nCoV or SARS-COV-2), is the third infectious strain of the coronaviruses (Zhu et al., 2020) (Figure 1). Genomic sequence analysis of SARS-

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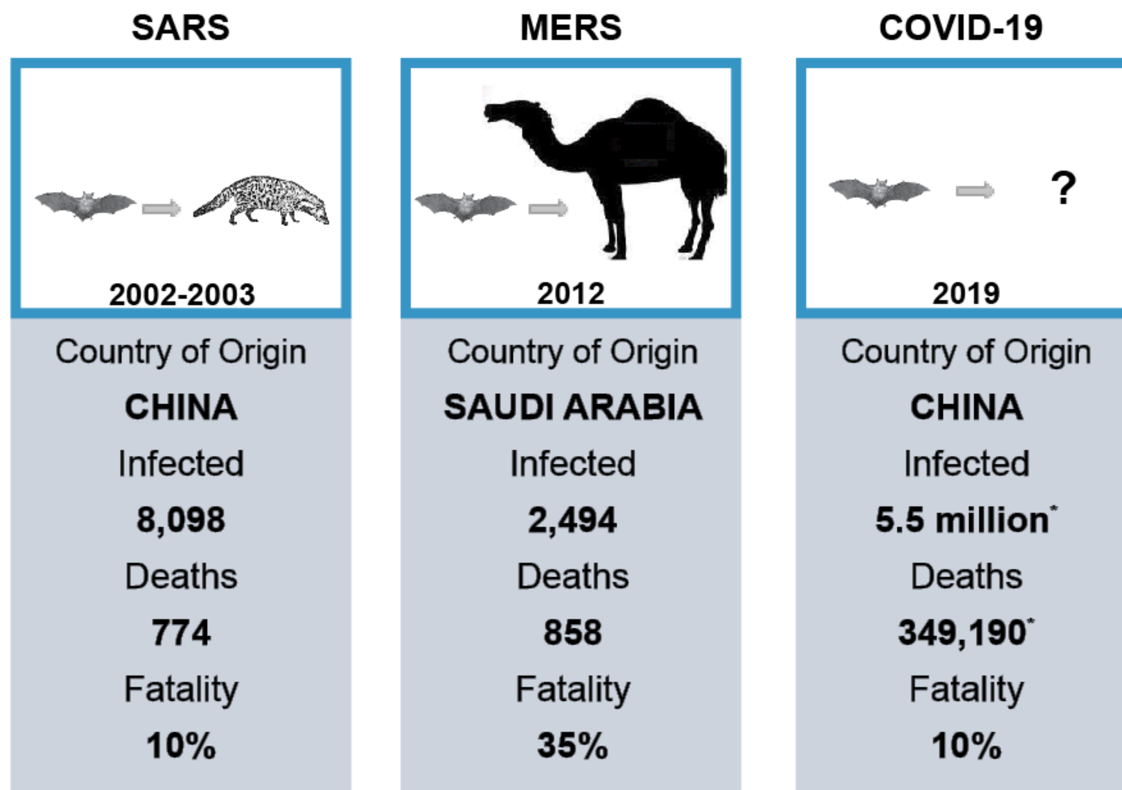

Figure 1. Epidemics of coronaviruses strains in recent history. (i) Severe acute respiratory syndrome (SARS-CoV), (ii) Middle east respiratory syndrome (MERS-CoV), (iii) SARS-COV-2. All these strains initially spilled out from bats and then through different intermediate hosts transmitted to humans. The intermediate host of SARS-COV-2 was not specified yet. *The number of infected people and deaths till 27th May 2020.
Source: Retrieved from Kuiken et al. (2003) and Zaki et al. (2012).

COV-2 coronavirus showed 80% identity to SARS and 50% to MERS (Lu et al., 2020; Ren et al., 2020). The novel coronavirus is likely to be harbored by bats (Andersen et al., 2020; Lu et al., 2020; Wan et al., 2020; Zhou et al., 2020) and transmitted to humans through an unknown intermediate animal in Wuhan, Hubei province, China in late December 2019 (Surveillances, 2020; Wang et al., 2020), and spread rapidly throughout the world causing a global pandemic. At the time of writing, over 5.5 million people have been infected, and 349,190 deaths have been reported worldwide (WHO, 2019). To date, no approved vaccine or antiviral drug has been found to fight against this disease. Prevention measures, including social distancing, self-isolation, lockdown, and quarantine are certain guidelines that have helped in the prevention of COVID-19 spread. The knowledge about SARS-COV-2 is evolving rapidly because scientists around the globe continue to study molecular, functional, and structural approaches in the light of international cooperation for the development of vaccines.

The COVID-19 pandemic reached Afghanistan on the 24th of February, 2020, when the first confirmed case was reported in Herat, a province that borders Iran, one of the major hubs of COVID-19 associated mortality. The pandemic has since spread throughout the 34 provinces of Afghanistan; however, three provinces (Kabul, Herat, and Kandahar) contain higher numbers of confirmed cases, respectively (Figure 2). We conducted this short study to pinpoint the current situation of COVID-19 and the behavior of the public to this pathogenic disease in Kandahar province, home to 3 million people and the second-largest city in the south of Afghanistan.

METHODOLOGY

Two types of study designs were used in this study, that is, retrospective study to collect the data of 636 COVID-19 confirmed cases. In contrast, a cross-sectional study was conducted to interview 250 randomly selected residents of Kandahar city. The initial data regarding COVID-19 cases in Kandahar province was obtained from the database of the office of National Disease Surveillance and Response (NDSR), Directorate of Public Health (DoPH), Kandahar Province, Afghanistan. Inclusion criteria were all the patients who were PCR positive for COVID-19. Patients with only positive rapid tests were excluded from the study. The response and practices of the public towards the novel COVID-19 were determined through a questionnaire method. In this part of the

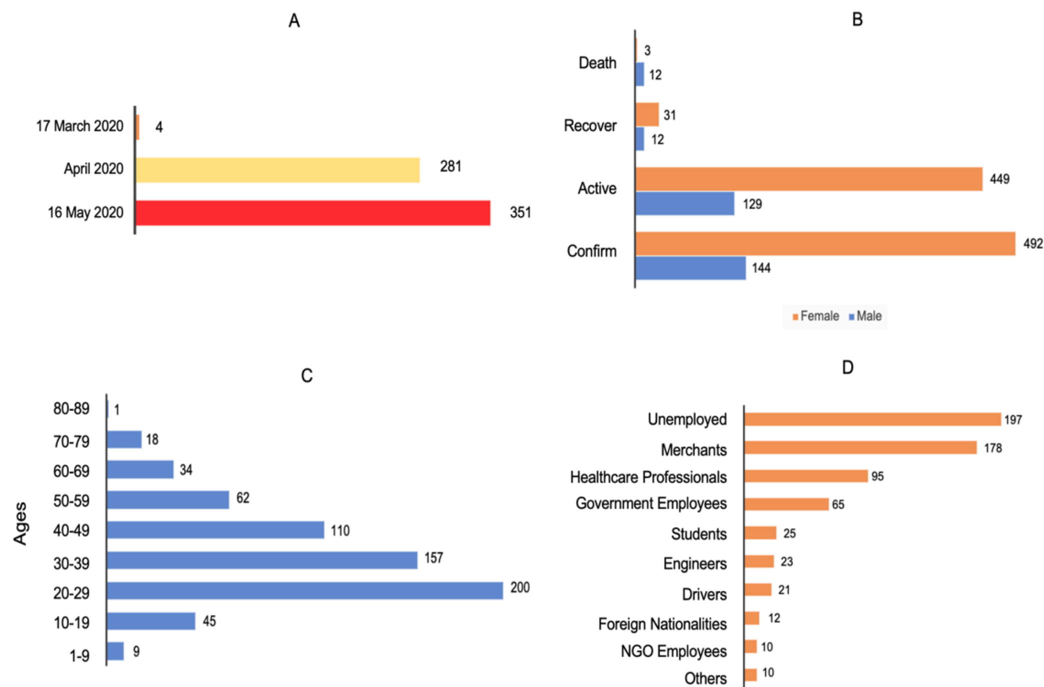

Figure 2. COVID-19 confirmed cases in Kandahar from 17 March 2020 to 16 May 2020. (A) The total number of confirmed cases per month. (B) Number of confirmed, active, recovered, and deaths of males and females. (C) Discrimination based on age and (D) Occupation.

study, inclusion criteria were residents of Kandahar city and willing to take part in this study. We collected the opinions of 250 randomly selected participants who volunteered to be interviewed. Consent was taken from all the participants before the interview. The surveillance system is not good in Afghanistan. Most of the cases are missed due to the limited number of tests available, as well as poor tracking of COVID-19 cases. The duration of the study was two months (17th March 2020 to 16th May 2020).

RESULTS AND DISCUSSION

Current situation of COVID-19 in Kandahar

The first positive COVID-19 case was reported in Kandahar province on 17 March 2020, when a doctor tested positive upon returning from Pakistan, which shares a border with Kandahar. Within a few weeks (16 May), the total confirmed numbers increased exponentially to 636. Included in this numbers, 95 were healthcare professionals (Doctors, Nurses, Technicians, and Midwives), reflecting that they have not followed standardized safety guidelines as stated in Jin et al. (2020). Since they are in close contact with the public, they might have inadvertently spread the virus to patients, other staff, and family members. Surprisingly, a total of 50 confirmed COVID-19 patients have vanished after the first diagnosis, which is a serious threat to the whole community as health authorities are unable to complete contact tracing. However, as of 16th May 2020, a total of 2,063 suspected samples had been tested, and 636 (36.80%) were positive, 492 (77.2%) were male, and 144 (22.8%) were female, 578 (90.9%) active cases, 43 (6.8%) recovered, and 15 (2.4%) deaths have recorded. The majority of those who tested positive were aged between 20 and 29 years. Additional details of COVID-19 patients are shown in Figure 2.

In response to cope with this virus, the local government did not react as quickly or with strong restrictions as the response observed in countries such as Italy or China. This can be attributed to low incomes, food insecurity, and the social structure in Kandahar, where many people in the community are already struggling with low wages and daily work. Once the pandemic was fueled across the country, therefore in the mid-April 2020, the local authorities took initial measures including: (i) Kandahar city was placed under partial lockdown but without any restrictions on entry and exit. During day time, a big portion of the city was in quarantine except for medicals, dairy products shops, and bakery. The markets and shops were scheduled to remain open from 12:00 to 6:00 am to try to reduce the number of crowds gathering at the same time. (ii) All educational institutions were suspended. (iii) Various campaigns were launched throughout the province to

increase awareness among the public. However, in the arrival of Muslims holy month (Ramadhan) on 22nd April 2020, the lockdown time was rescheduled from 5:00 am to 4:00 pm, so all the shops were opened in day hours, but during night time the city was in quarantine. With all these efforts, major lapses were seen at every step, ranging from inconsistent government policies, avoiding health advisory by public populations, and lack of awareness resulted in the rapid spread of the pandemic in Kandahar province. Based on such observations, it could be predicted that the number of confirmed cases will continue to rise rapidly in the coming weeks/months.

Medical facilities

The local government designated a newly built government hospital (Ainomina Hospital) with 350 bed capacity as the COVID-19 response facility where patients who were suspected or confirmed could be isolated; this is the only isolation and healing center in the province. The Ministry of Public Health along with other international health donor agencies such as International Committee of the Red Cross (ICRC), World Health Organization (WHO), UNICEF, and Afghan Red Crescent assisted in providing crucial supplies such as N95 masks, personal protective equipment (PPE), cleaning materials and other necessary health items to protect the healthcare professionals at the front line of pandemic battle.

Initially, Ainomina Hospital lacked COVID-19 diagnostic facilities; the suspected samples were sent to the Central Reference Laboratory at the Ministry of Public Health (MoPH) located in Kabul, the capital of Afghanistan. This whole process took almost a week to determine whether someone is infected or not. Fortunately, in early April 2020, the DoPH received diagnostic kits from MoPH, and Ainomina Hospital was given the kits to complete testing on patients who were suspected of COVID-19. Until 16th of May 2020, a total of 2,063 suspected people have visited this hospital for testing to confirm their diagnosis. The hospitalized patients were given supportive therapy, such as analgesics, antipyretics, and oxygen therapy. The recovered patients were discharged from the hospital once they were afebrile for three days and had tested negative twice for COVID-19. Infection prevention measures were put in place to ensure all the rooms where positive patients had been admitted were decontaminated with 0.5% chlorine.

Public response to COVID-19 outcome

The initial response in the community to the threat of COVID-19 was apathetic. Despite the risk of the health emergency, the public has been reluctant to cooperate with government calls and have violated or ignored the request for lockdown in the community to try to halt the transmission of the virus. The global slogan *“social distancing”* was wholly ignored in this part of the world. Throughout the province during the lockdown period, regardless of the time of day, a large-scale gathering of people continued with no concern around health safety measures such as shopping in crowded areas (Figure 3), attending large wedding parties, picnics, and funerals. Such activities for sure can exacerbate the spread of COVID-19. From our short and random interview with people, we found that the public ignored safety restrictions mainly for the following reasons: (i) the majority of the people interviewed had very little knowledge about COVID-19 due to poor educational literacy. They were unwilling to accept this pandemic as a serious risk to their health but rather consider it as a rumor. (ii) A portion of the participants-based response on religious beliefs where they think death is predetermined already, and they will die on specific time as written in destiny. Hence, there is no need for social distance or take any other health safety measures. (iii) We also found very few people in a cluster of our study participants who accepted COVID-19 as a potentially lethal virus and follow all the health and safety measures that are recommended (Figure 4).

CONCLUSIONS AND RECOMMENDATIONS

There are many reasons why the COVID-19 cases in Kandahar continue to rise rapidly; they include the community not maintaining social distancing or taking health measures such as using masks and gloves. Therefore, the local government and all other relevant authorities should be well prepared for the worst situation in the coming months. Although it is still not late, comprehensive measures like strict curfew to reduce person to person transmission of this virus are required to slow down the further spread of the disease. Efforts should be made against the spread of this pandemic, and there should be plans implemented with local authorities and international health donors under one platform. A strict curfew has shown to work in some countries, but the community should be educated on how to keep themselves safe to reduce transmission. However, if curfew cannot be implemented due to some reasons, at least other preventive measures, like wearing masks and gloves, should be made complimentary for the people. Public services’ offices, banks, hospitals, and all other places with mass gathering should be sprayed and provided with disinfectants regularly.

The diagnostic testing capacity was low; in the last three months, around 2,000 samples have been tested which is not enough for the population at risk among nearly 3 million residents of Kandahar, a large number of diagnostic kits and qualified technicians are required to conduct mass screening to track the actual infection rates

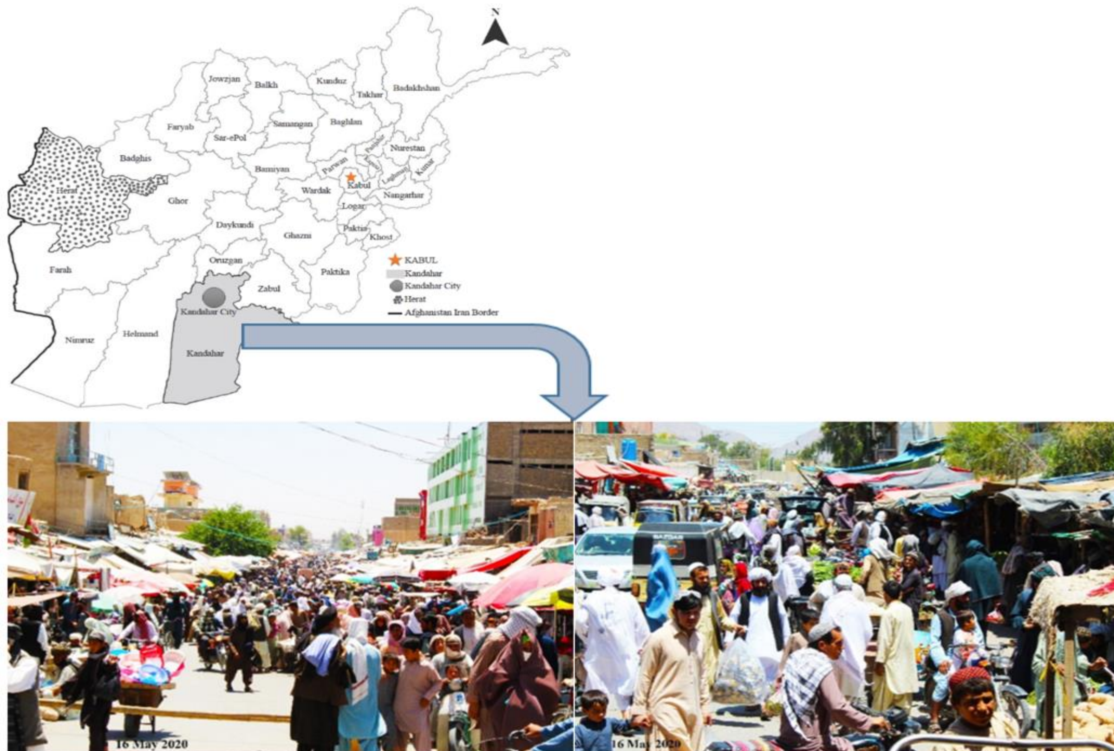

Figure 3. Administrative divisions of Afghanistan and the three provinces having a higher number of COVID-19 confirmed cases. Public community in a Bazar of Kandahar city. No health prevention measures are seen. The pictures were taken on 16 May 2020.

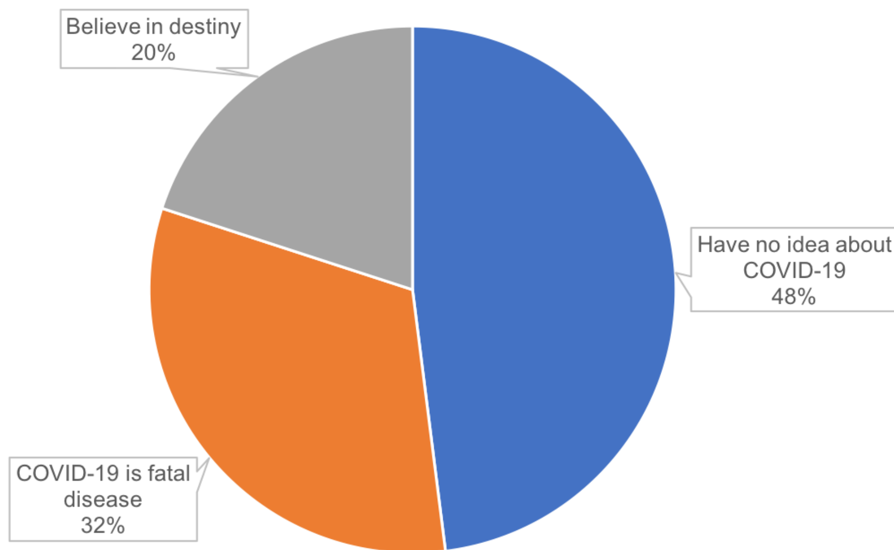

Figure 4. The summary of public opinions about COVID-19. A total of 250 people participated in this study. The number represents percentage.

front line of battle against COVID-19. For public awareness, the best option that government can use to avoid further disaster is to get the help of religious scholars since our society is very much religious-based and scholars have special influence among ordinary people. They can convey health alert messages better than anyone else throughout the province. Lastly, in the current study there were limitations in retrospective study

type and higher illiteracy rate of the majority of the interweaved individuals.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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The spatial distribution and diversity within bacterioplankton assemblages in four coastal sites along the southern points of the Atlantic Ocean were examined using the Illumina high-throughput that targets 16S rRNA genes to examine indigenous bacterial assemblages in the littoral zones along the coast of the ocean. Results of the study showed very similar bacterial representation between the coastal sites with majority of the sequences affiliated with members of the *Proteobacteria* (52 to 59%), *Bacteriodetes* (21 to 31%) followed by *Actinobacteria* (3 to 9.5%) and *Planctomycetes* (2.1 to 4.5%). The bacterioplankton assemblages at each site examined were quite diverse, with members of the Gammaproteobacteria found as the most abundant bacterial class among the four sites. However, clear differences were observed among the sites at the order level, with the *Chromatiales* the more dominant in the eastern coastal (CPTI) sites, while clades belonging to the *Flavobacteriales* and *Rhodobacterales* were more prevalent in the two western (CPTA) coastal sites. While the results of unweighted pair group method with arithmetic (UPGMA) clustering and principle coordinate (PCoA) revealed two spatially separate clusters among sites, canonical correspondence (CCA) analysis indicated that environmental variables such as temperature, pH and conductivity were probably the major influencers of bacterial occurrences at the coastal sites.

Key words: Bacterioplankton assemblages, ocean, 16S rRNA gene sequencing.

INTRODUCTION

The bacterioplankton assemblages in oceans have been described as comprising one of the largest and active microbial assemblages in the biosphere (Whitman et al.,1998; Salazar and Sunagawa, 2017), where they actively partake in the biogeochemical influxes and cycling of various nutrients and organic compounds (Azam and Malfatti, 2007; Falkowski et al., 2008, Zehr and Kudela, 2011). Contrasting findings have been previously reported regarding bacterial diversity and biogeographic distributions in marine systems, especially in tropical oceans (Pommier et al., 2007; Fuhrman et al., 2008; Milici et al., 2016). Milici et al. (2016) reported a

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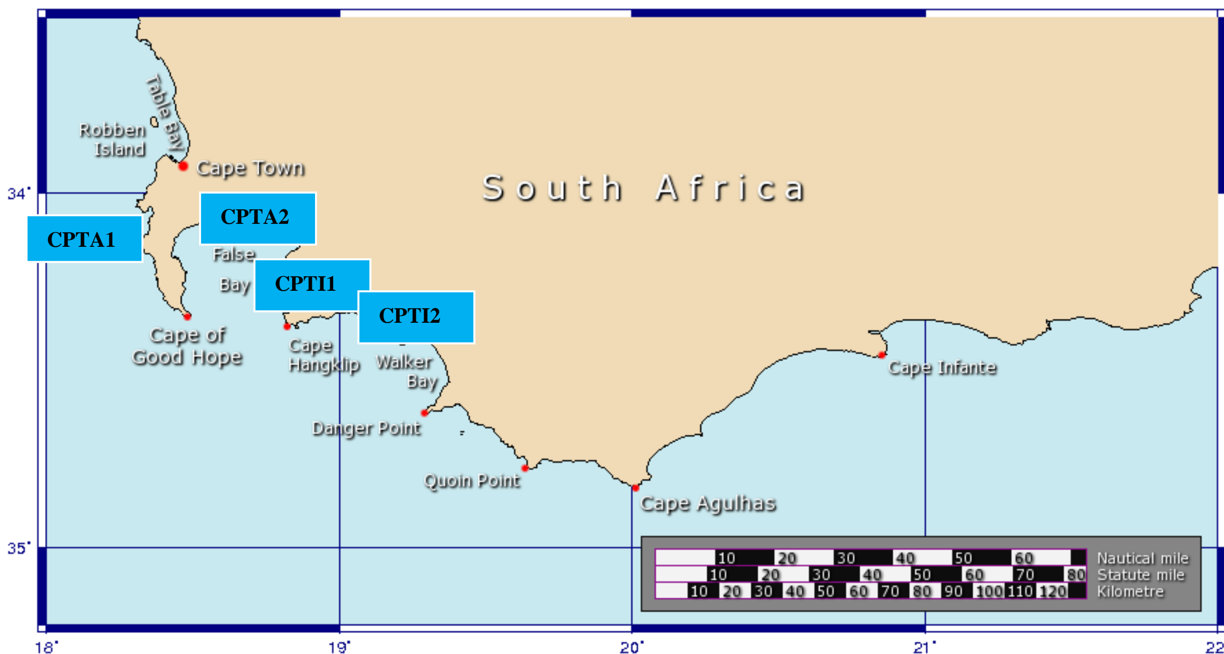


Figure 1. Map of study sites along the coastlines of the Atlantic and Indian Oceans in Cape Town, South Africa.

clear biogeographic pattern with a double inverted latitudinal gradient, with higher diversity in planktonic bacteria population in mid-latitudinal regions, and decreasing towards the equator in the Atlantic Ocean in their study. In contrast, Fuhrman et al. (2008) found a negative correlation between species richness and latitude both in the Northern and Southern hemisphere. Additionally, interesting observations regarding microbial provincialism and their discrete distributions in various marine habitats have been previously documented (Brown et al., 2009; Jeffries et al., 2015).

In order to better understand the structural compositions and diversity within bacterial assemblages in marine systems, this study was conducted on several coastal sites along the southern points of the Atlantic Ocean in Cape Town, South Africa by examining indigenous populations in the bacterioplankton communities using Illumina high-throughput sequencing approach that targets the 16S ribosomal RNA genes. The four sites that were selected for the study are located along the coastline within the metropolitan city of Cape Town, between the Cape of Good Hope and the Cape of Agulhas two touristy landmarks that are prone to various anthropogenic influences. The Atlantic Ocean is considered the second largest of the world's five oceans, second only to the Pacific Ocean, with a body of water located between Africa, Europe, the Arctic, America and the Southern Ocean. The southern parts of the Atlantic Ocean where this study was conducted is located around the metropolitan area of the city of Cape Town in South Africa, between the Cape of Good Hope (~34° 21´24.63" S, 18° 28´ 26.36" E) and Cape Agulhas (~34° 49´ 59.6" S, 20° 00´ 0" E), with mostly rocky headlands of coastlines in between these two landmarks that are about 90 miles apart (International Hydrographic Organization, 2002). The main aim of the study was to examine the taxonomic profiles of the microbial assemblages indigenous to these coastal marine sites as well as determine the influences of various environmental factors, such as temperature, pH and dissolved oxygen concentrations on the structural composition and diversity within the assemblages at the four sites.

MATERIALS AND METHODS

Sample collection and measurement of environmental variables

Samples were collected from the surface waters along the rocky headland coasts of the Atlantic Ocean in Cape Town, South Africa in September 2019. Specifically, water samples were collected from approximately 1 to 5 m depth into sterile falcon tubes from four separate sites along the ocean front close to the metropolitan city of Cape Town in South Africa (Figure 1). Collected samples were filtered through 0.2 µm pore-size polyethersulfone membrane filters and then stored frozen until nucleic acid extraction was performed. While on site, the water chemistry properties were also measured (Table 1) with probes for temperature, conductivity, pH, dissolved oxygen and oxidation-reduction potential using the YSI model 556 MPS multi-probe system (YSI Incorporated, USA).

DNA extraction and 16S rRNA gene pyrosequencing

Community DNA was extracted from the filters using FastDNA

Table 1. Environmental variables measured at the study sites.

Site	Latitude	Longitude	Temperature (°C)	Conductivity mS/cm	DO %	pH	ORP
CPTA1	34° 21' 25." S	18° 28' 26" E	14.80	48.45	127.60	8.92	-26.50
CPTA2	34° 21' 24." S	18° 28' 36" E	14.76	48.50	111	8.80	-16.50
CPTI1	34° 13' 36. " S	18° 28' 7." E	14.93	48.93	389.20	9.14	-23.60
CPTI2	34° 49' 59. " S	18° 28' 7." E	14.89	48.45	263.30	9.07	-20.50

SPIN Extraction kit (MP Biomedicals, Solon, OH, USA) and eluted in 50 µl of sterile deionized water according to the vendor's instructions. Determination of DNA quantity was then carried out with a NanoDrop Spectrophotometer (2% accuracy/range of purity, NanoDrop 2000, Thermo Scientific, Delaware, USA). The quality of extracted DNA was further assessed by amplifying with the 16S rRDA universal primer sets, 27F (5' AGA GTT GTA TCM TGG CTC AG 3') and 1492R (5'GGT TAC CTT GTT ACG ACT T3') as previously described in Olapade (2013, 2015).

The Illumina's 16S metagenomic sequencing library preparation protocol was used in generating amplicon libraries using universal primer pairs that consisted of an Illumina-specific overhang sequence and locus-specific sequence: 926wF_Illum: 5'-TCGTCGGCAGCGTCAGA TGTGTATAAGAGACAGAAACTYAAAKGAATTGRCGG and 1392R_Illum: 5'GTCTCGT GGGCTCGGAGATGTGTATAAGAGACAGACGGGCGGTGTGTRC. The pair of primers targets the V6-V8 hypervariable regions of 16S rRNA genes of all microbial groups (Jeffries et al., 2015).

Quality trimming and filtering of low-quality sequences

The raw pyrosequencing data was processes and analyzed using the open-source software program, Mothur (Mothur v. 1.36.1; http://www.mothur.org) as previously described (Schloss et al. 2009). Barcode and the fusion primers are trimmed before any of the bioinformatics commences. Sequences reads without a barcode or a primer region are dropped and not considered for further analysis. Low quality sequences that is, those less than 300 base pairs as well as those with less than average quality score (value of 25 or less) are filtered out and deleted (Zhang et al., 2012). Operational taxonomic units (OTUs) were constructed by comparing them to close relatives via global pairwise alignment (Altschul et al., 1997) to determine their close relatives using the BLASTN (blast.ncbi.nlm.nih.gov) system. Chimeras were detected in the sequences that were later omitted for further analysis by using the UCHIME version 4.1 program (Edgar et al., 2011).

Statistical and diversity analysis

The sequences were clustered into OTUs after setting 97% distance limit or cutoff similarity value (Tindall et al., 2009; Edgar et al., 2011) and then analyzed for species richness, Shannon Index, Simpson's (Reciprocal) Index of diversity, species evenness, ACE richness estimate and Chao-1 richness indicator (Chao, 1984, 1987; Chao and Lee, 1992; Schloss and Handelsman, 2006). In order to determine whether total diversity was covered by the numbers of sequences screened, Good's Library Coverage values were calculated as previously described (Good, 1953; Kemp and Aller, 2004). Alpha, beta and gamma diversity calculations were also carried out according to Whittaker (1972); in addition to rarefaction analysis that was performed to also determine the diversity of the clone libraries using the freeware program by CHUNLAB Bioinformatics Made Easy (CLcommunity version 3.30). Taxon exclusive (XOR) analysis was carried out based on the taxonomic assignment of sequencing read to reveal the sequences present in one library but absent in the others as described by Li and Godzik (2006). The UPGMA Fast UniFrac analysis was used to cluster the sequenced microbial communities based on phylogenetic relationship and abundance in order to generate a dendogram (Hamady et al., 2010), while the multi-dimensional UniFrac distance matrixes were then converted into vectors using the Principal coordinate analysis (PCoA) as described by Jolliffe (1989). Additionally, canonical correspondent analysis (CCA) was also used to analyze and examine which of the bacterial assemblages corresponds to the independent environmental variables that were measured at the study sites according to Ter Braak and Verdonschot (1995).

RESULTS

Environmental variables

The environmental variables measured at the four coastal sites examined along the ocean front included temperature, pH, dissolved oxygen (DO), conductivity and oxidation-reduction potential (ORP). Most of these variables were quite similar among the studied sites, with the exception of DO that was relatively higher in the two eastern sites (CPTI1 and CPTI2) closest to the Indian Ocean. Specifically, water temperature among the four coastal sites ranged from 14.76 to 14.89°C and slightly higher in the two easterly located sites, while pH was also in the range of 8.80 to 9.14 between the western and the eastern sites of the ocean, respectively (Table 1).

Community composition and diversity analysis

Based on the 16S ribosomal RNA gene sequencing, the relative abundance of bacterial taxa was determined at different taxonomic levels, and majority of the bacterial sequences (~99%) were ascribed to 29 different known bacterial phyla. Out of these 29 different phyla, bacterial members belonging to the *Proteobacteria, Bacteroidetes, Actinobacteria, Planctomycetes* and *Verrucomicrobia* were observed to have accounted for more than 90% of total community compositions among the sequences obtained from the four coastal sites (Figure 2A). Specifically, members of the *Proteobacteria* were the most numerically dominant phyla in the four sites,

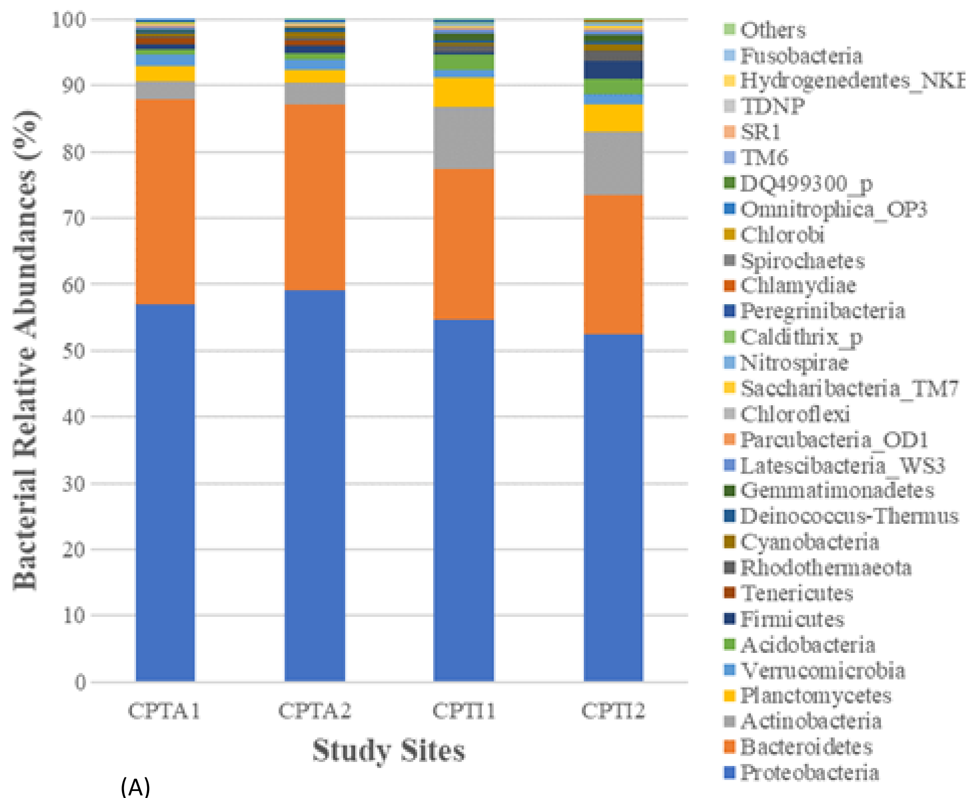


Figure 2A. Relative abundances of bacterial phylogenetic taxa at the phylum level.

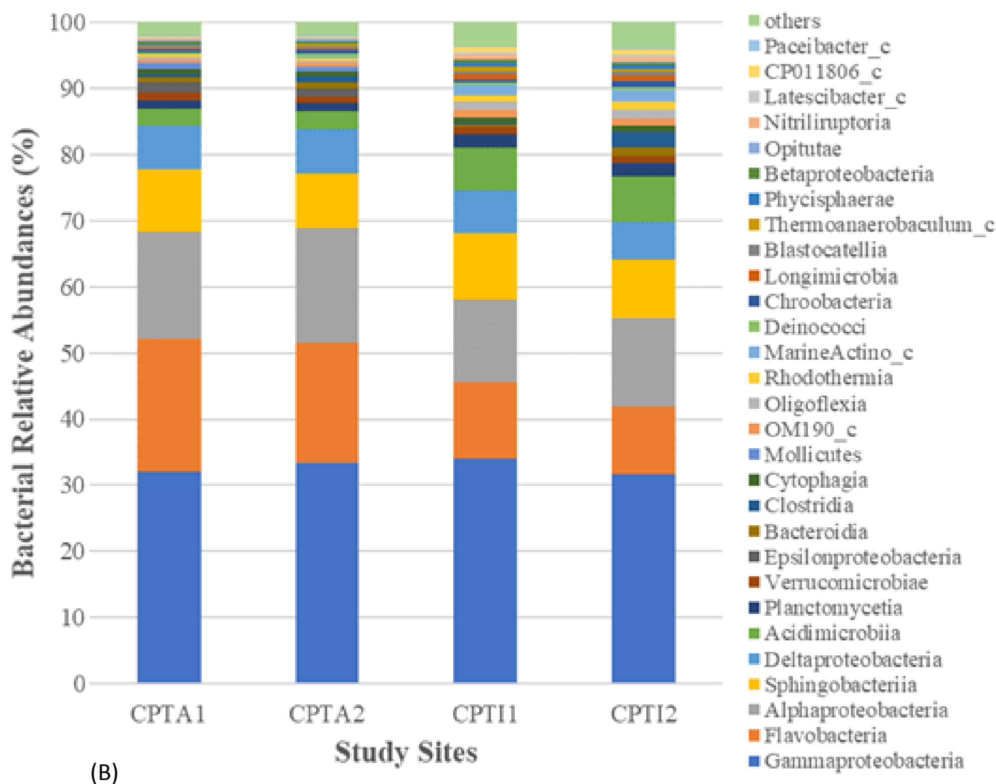


Figure 2B. Relative abundances of bacterial phylogenetic taxa at the class level.

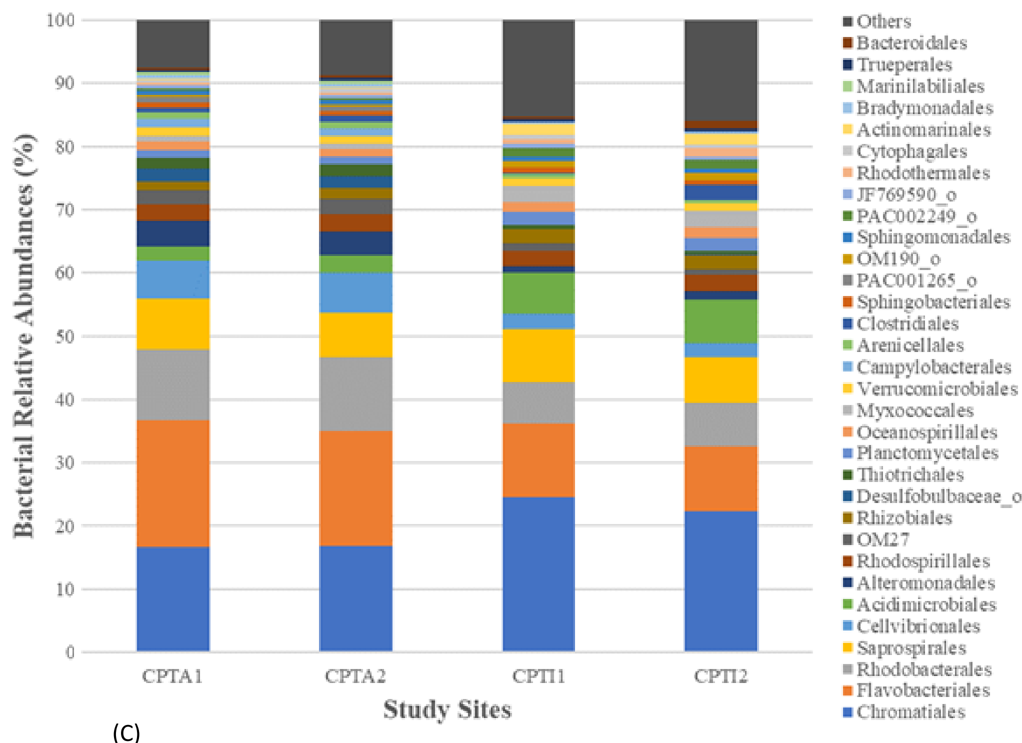


Figure 2C. Relative abundances of bacterial phylogenetic taxa at the order level.

Table 2. Community diversity analysis of the 16S ribosomal RNA gene sequences from the Bacterioplankton of the Atlantic and Indian Oceans in Cape Town, South Africa.

Sample	Valid reads	OTUs	Ace	Chao1	JackKnife	NPShannon	Shannon	Simpson	Good library coverage
CPTA1	225897	4712	4846.97	4761.60	5027	6.28	6.26	0.01	99.86
CPTA2	30087	1838	1948.57	1901.30	2055	6.18	6.11	0.01	99.28
CPTI1	44435	2942	3083.15	3010.00	3243	6.70	6.63	0.00	99.32
CPTI2	38470	2910	3087.23	2990.90	3254	6.82	6.73	0.00	99.10

accounting for between 52 and 59% of total bacterial sequences. The *Bacteroidetes* were in close second with between 21 to 31% sequence representations, followed by members of the *Actinobacteria* and *Planctomycetes* (between 3 to 9.50% and 2.10 to 4.50%, respectively). The next 25 bacterial phyla (including the members of the *Acidobacteria, Firmicutes, Tenericutes, Rhodothermaeota, Cyanobacteria, Deinoccocus-Thermus, Gemmatimonadetes*, among others) represented less than 10% of the total bacterial sequence abundance. Among the *Proteobacteria,* members of the *Gammaproteobacteria* were the predominant class, representing between 32 to 34%, followed closely by the *Alphaproteobacteria* with 13 to 14% representation among the four studied sites (Figure 2B). The *Chromatiales* were the dominant groups among the members of the *Gammaproteobacteria* at the order level, followed closely by the *Flavobacteriales* and the *Rhodobacterales*, both members of the *Flavobacteria* and *Alphaproteobacteria* classes, respectively (Figure 2B).

The Good Library Coverage analysis revealed that majority of the bacterial sequences was covered among the sites (Table 2). This result is also somewhat corroborated by the rarefaction curves that showed sufficient coverage in the numbers of the different bacterial phyla contained within three of the four assemblages examined at the coastal sites (Figure 3). The rarefaction curves revealed that three of the four microbial assemblages examined were tending towards saturation, with the exception of the CPTA1 site, that also had the highest Good Library Coverage of 99.86%. Diversity measures such as the Shannon diversity index

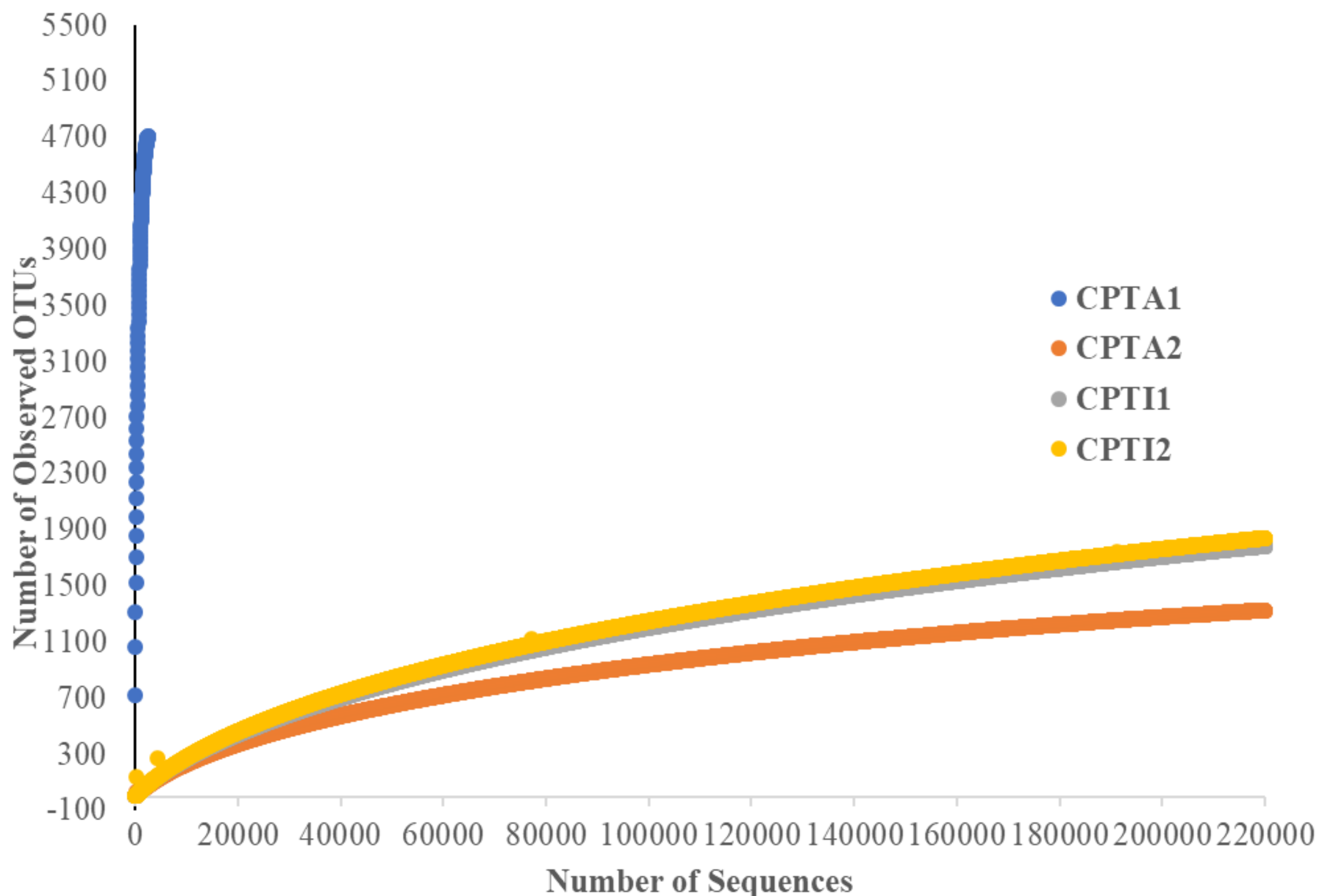


Figure 3. Rarefaction curves of OTUs based on 16S rRNA sequences from the bacterial assemblages from the study sites

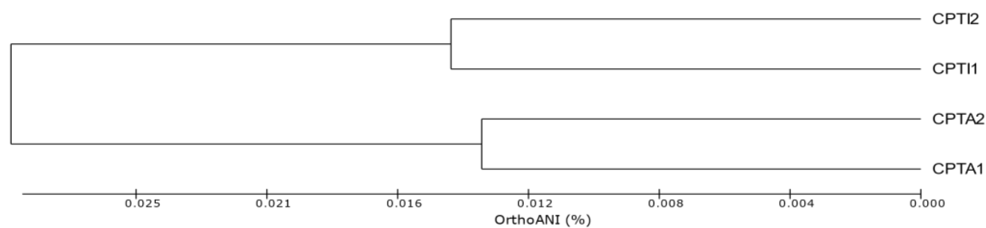
showed that bacterial diversity was comparatively higher in the two eastern coastal sites (CPTI1 and CPTI2) than in the two western Atlantic Ocean sites that were examined. Results of bacterial richness and species diversity based on ACE and Chao1 while not showing a distinct delineation among the four coastal sites, however revealed that the CPTA1 site had the highest diversity compared to the three other sites. This result is further validated by the results of the taxon exclusive analysis of the bacterial assemblages at the phylum level that showed disparate differences in bacterial diversity among the sites (Table 3). The bacterial assemblages found within the CPTA1 coastal site comprised of several bacterial phyla that were totally absent in the three other sites including the *Armatimonadetes, TM6, Lentisphaerae, BRC1, TDNP, SR1*, *Elusimicrobia, Fibrobacteres and WS6*. While the CPTA2 site comprised of *Deferribacteres* that were absent in the other sites, and phyla belonging to *Aminicenantes*, *GN04* and *Synergistetes* were exclusively found in CPTI1 and CPTI2, respectively.

Hierarchical clustering based on the Fast UniFrac distance matrix revealed that the bacterial sequences obtained from the two bacterioplankton assemblages in the western Atlantic Ocean sites were more similar, but were a bit distant from those in the easterly located assemblages (Figure 4). The PCoA that was also carried out to further explain the variations in bacterial community compositions between the four coastal sites also corroborated the results of the UPGMA clustering. Three axes were extracted that together explained 90.1% of the observed variance and showed that the bacterial assemblages within the two western sites (CPTA1 and CPTA2) clustered along the PC1 axis, while those from the eastern sites of the ocean (CPTI1 and CPTI2) clustered around the PC2 axis (Figure 5).

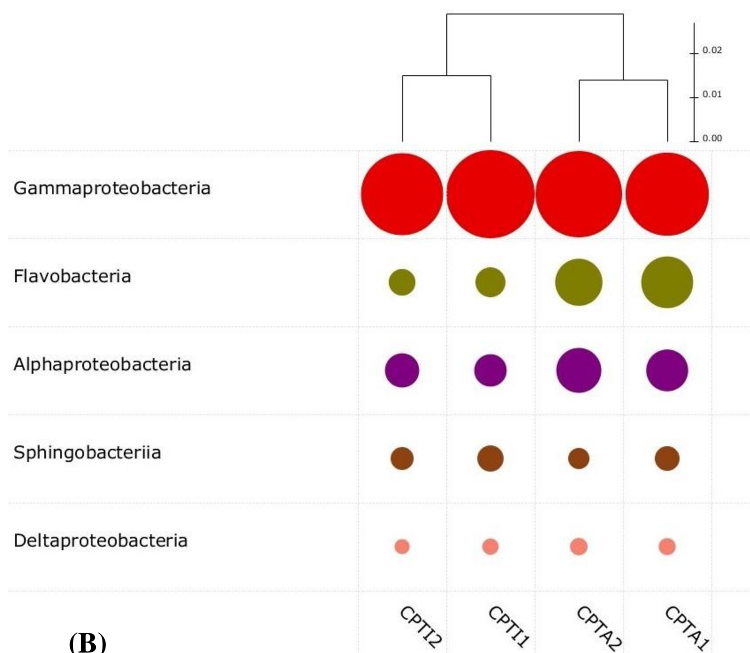
CCA was carried out to better understand bacterial distribution patterns along the coastal sites, especially regarding the spatial occurrences of the various environmental factors that were measured. Therefore, temperature, pH, conductivity, DO and ORP were included in the CCA analysis. The environmental variables in the two CCA axes (that is, CCA1 and CCA2) together explained more than 98.46% of total variations in the bacterial abundance distribution (Figure 6). Temperature, pH, conductivity and DO ($p < 0.01$) all contributed significantly to the total variance and were

Table 3. Result of taxon exclusive analysis at the phylum level to detect taxa that are present in one bacterioplankton assemblage but absent in the others based on 16S rRNA gene sequences/clones

Taxonomic group by phylum	Number of sequences/clones			
	CPTA1	CPTA2	CPTI1	CPTI2
Armatimonadetes	3	-	-	-
TM6	56	-	2	3
Lentisphaerae	6	-	17	7
BRC1	15	-	4	2
TDNP	40	-	-	-
SR1	47	-	-	-
Elusimicrobia	10	-	4	3
Fibrobacteres	13	-	-	-
WS6	5	-	-	-
Deferribacteres	-	3	-	-
GN04	-	-	4	-
Aminicenantes_OP8	-	-	1	-
Synergistetes	-	-	-	2
Bacteria_uc	2	-	-	-



(A)



(B)

Figure 4. UPGMA (Unweighted pair group method with arithmetic mean) dendogram (**A**) and heat map (**B**) showing the clustering of bacterial assemblages from the study sites.

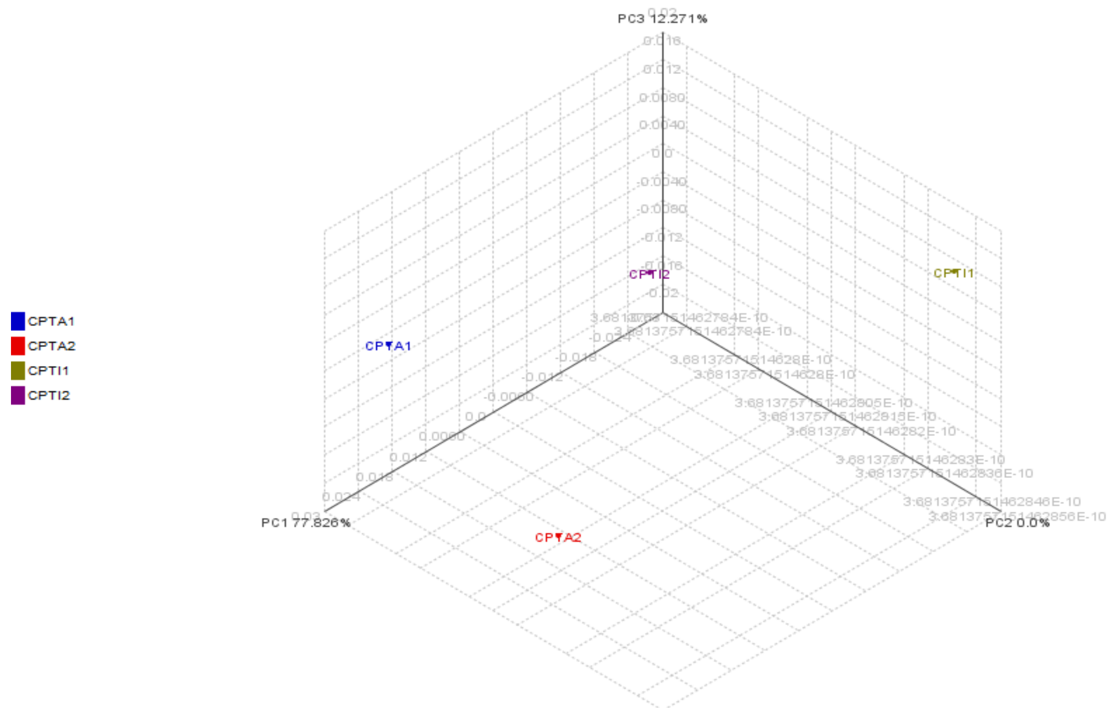


Figure 5. Three-dimensional principal coordinate analysis (PCoA) based on the Unifrac distance matrix of the bacterial assemblages for normalized OUT abundances within the study sites.

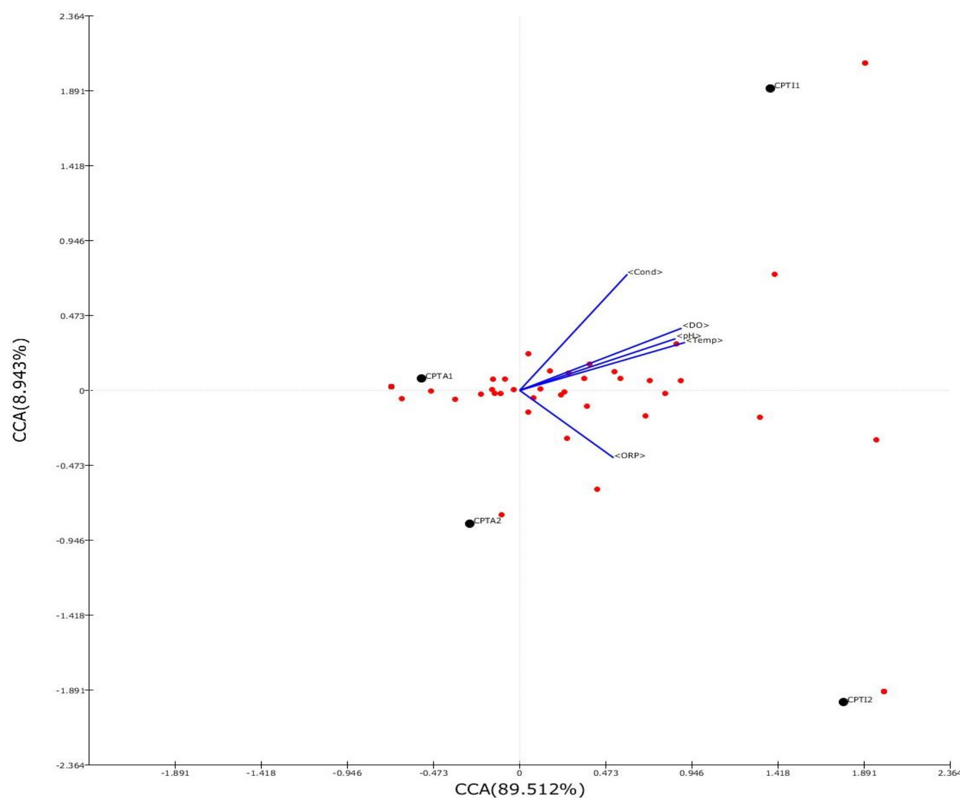


Figure 6. Canonical Correspondent analysis (CCA) of the bacterioplankton assemblages shown relationships with environmental variables within the study sites examined.

closely associated with the first and second CCA axes.

DISCUSSION

In this study, the 16S rRNA gene sequences obtained from 4 different coastal locations along the southernmost parts of the Atlantic Ocean in Cape Town, South Africa were analyzed in order to characterize the bacterial community structures in response to potential changes in environmental variables between these spatially different coastal marine sites. The 16S rRNA occurrences diverse phylogenetic groups within the assemblages revealed close similarity in the dominant taxa among the four coastal sites examined. Bacterial members at each of the sites were mostly dominated by the *Gammaproteobacteria* class followed by the *Flavobacteria.* The numerical dominance of members of the *Proteobacteria* and *Bacteroidetes* as observed in this study is consistent with previous studies that have also reported the high occurrences of these two bacterial phyla in various marine systems (Brown et al., 2009; Seo et al., 2017; Wang et al., 2018; Wu et al., 2019).

The relatively high occurrences of members of the *Gammaproteobacteria, Alphaproteobacteria* and *Flavobacteria* among the sequences in the four coastal sites examined in this study are fairly consistent with those reported for oceanic waters by previous studies (Kirchman, 2002; Rappe and Giovannoni, 2003; Schmidt et al., 1991; Raes et al., 2017; Wang et al., 2018; Wu et al., 2019). These bacterial groups are known to be major constituents of microbial assemblages in various marine systems (Kirchman, 2002; Rappe and Giovannoni, 2003), especially in coastal environments because of their propensity for the high availability of enhanced dissolved organic matter that are copiously produced by photosynthetically active autotrophs in this euphotic area of the ocean. More specifically, previous studies have revealed strong correlations between significant occurrences of these particular bacterial phyla and dissolved organic matters associated with phytoplankton productivity (Calson et al., 2009) and other environmental variables, including phosphate concentration (Morris et al., 2010; Seo et al., 2017), salinity and temperature (Milici et al., 2016; Wu et al., 2019) in coastal marine waters. For instance, Milici et al. (2016) particularly found bacterial diversity to change drastically with changing water temperature in the Atlantic Ocean, with the highest species diversity documented at between 15 and 20°C, but with significant reduction when water temperature was above 20°C. The results documented in this study seems to validate these particular previous observations, given that the water temperature of the four coastal sites examined here were on average around 15°C and that most of the diversity measures, especially as indicated by both ACE and Chao1 showed species richness among the microbial assemblages to be relatively high in all the coastal sites examined.

The CCA results of this study showed that combinations of the major environmental variables that were measured at the sites sufficiently explained the distribution of the different bacterial phyla elucidated among the coastal sites examined. Although other variables such as inorganic nutrients, including phosphate and nitrate concentrations were not included in the analysis conducted in this study, however, other studies have previously shown strong correlations between their concentrations and the spatial distributions and diversity of bacterial assemblages in marine waters (Raes et al., 2017; Seo et al., 2017). Raes et al. (2017) found strong correlations between total dissolved inorganic nitrogen, chlorophyll a, phytoplankton community structure and primary productivity with bacterial richness in their study on the surface waters of the eastern Indian Ocean. While, Seo et al. (2017) in their study on the coastal waters of the South Sea of Korea similarly reported significant influences of both phosphate and dissolved oxygen concentrations on the bacterial community compositions found in different stations along the coast of the sea.

The results obtained from this study that was designed to examine the occurrences, distribution and diversity of bacterial populations within the coastal bacterioplankton assemblages in the Atlantic Ocean around Cape Town, South Africa further validate and strongly corroborate several previous studies where various members of the heterotrophic bacterial populations, especially the *Gammaproteobacteria and Alphaproteobacteria* classes have also been observed dominant, especially within the coastal euphotic zones of marine environments (Wu et al., 2019). Conclusively, the multivariate analysis of the bacterial assemblages from the coastal sites in this study, revealed two spatially separate clusters among the sites, whereas environmental variables such as temperature, pH and conductivity were probably the major influencers of bacterial occurrences at the four coastal sites. Therefore, the results from this study further validate the interconnected of environmental variables and microbial assemblages as well as the potential influences of such factors on the ecology of bacterial populations in coastal aquatic environments.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Biochemical and molecular characterization of yeasts isolated from Nigerian traditional fermented food products

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Yeasts are very important in food production by affecting the quality and safety of different food products. Yeasts are commonly used in the production of beer, wine and bread; but many yeast species have been isolated from different African fermented foods. The present study aims to determine the biodiversity of yeasts isolated from selected Nigerian fermented foods. A combination of phenotypic and molecular tests were employed in the identification of yeasts. A total of 145 yeasts were isolated from six different food products. The yeasts belong to six genera namely *Saccharomyces, Candida, Cyberlindnera, Meyerozyma, Trichosporon and Galactomyces.* The most frequently encounter was *Saccharomyces cerevisiae* followed by *Candida glabrata*. The present study has confirmed the biodiversity of yeasts isolated from fermented food of Nigerian origin.

Key words: Phenotypic characterization, molecular, indigenous yeasts, Nigeria.

INTRODUCTION

In different parts of the world, humans consumed many types of fermented food products. Traditional knowledge is used in the processing of available plant and animal materials to produce fermented food (Achi, 2005). Fermentation is the chemical modification of foods by enzymes of living microorganisms. During the production of fermented foods, microorganisms that are present in the raw materials or added as starter culture change the raw materials both biochemically and organoleptically into edible products that are culturally acceptable to the maker and consumer.

In Nigeria different types of nutrient rich crops are processed by fermentation into foods or used as condiments (Iwuoha and Eke, 1996). Most of these food crops are not consumed without fermentation because of the presence of certain toxic components or antinutritional factors. When foods are fermented a general improvement takes place in the shelf life, texture, taste, aroma as well as nutritional value. The fermentation process is usually performed at the household level or at a small scale. The different types of traditional fermented food products in Nigeria include fufu, gari, ogi and nono.

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Fufu and gari are fermented cassava product while *ogi* is produced from different types of cereals such as maize, sorghum and millet (Omemu et al., 2007). Nono is a fermented milk product. The traditional fermented alcoholic beverages include the palm wine, burukutu and agadagidi. Palm wine is produced from the sugary sap of the African oil palm (*Elaeis guineensis*) and the raphia palm (*Raphia hookeri*) (Sanni and Lonner, 1993).

Burukutu is an alcoholic beverage produced from red sorghum (*Sorghum bicolor*) while agadagidi is produced from ripe plantain pulp (Sanni and Lonner, 1993). The production of these food products is by spontaneous fermentation and therefore different types of microbes are usually found. The microorganisms commonly found in Nigerian traditional fermented foods and alcoholic beverages are yeasts and lactic acid bacteria. However, yeasts are responsible for the alcoholic content of the beverages.

The fermentation of carbon sources by yeasts for production of alcoholic beverages is the oldest and most economically important of all biotechnologies. In order to maximise alcoholic yield and maintain sensory quality, it is necessary to select suitable yeast strains for alcoholic fermentation. The occurrences of yeasts in significant number ranging from 10^5 to 10^8 cfu/g in many traditional African fermented foods have been reported (Greppi et al., 2013). Most of the previous studies on identification of yeasts from Nigerian traditional fermented food products used cultural, physiological and biochemical tests for yeast strains identification (Sanni and Lonner, 1993; Omemu et al., 2007; Chilika et al., 2010; Umeh and Okafor, 2016 and Olowonibi, 2017). These methods of identification are laborious and most importantly often fails to identify strains correctly to the species level. DNA sequence based methods provide a rapid and accurate means of microbial identification and permit a certain level of phylogenetic classification of the species into genera or even families (El-Sharoud et al., 2009). The main objective of the current study is to identify yeasts isolated from some Nigerian traditional fermented food products.

MATERIALS AND METHODS

Sample collection

Ten samples each of traditional fermented food products like retted cassava (fufu) (FF), ogi-baba (OBB), burukutu (BKT), agadagidi (AGG) and palm wine (PAW) were collected from Ibadan Metropolis immediately after production in sterile containers and transported to the laboratory for immediate use in the isolation of yeasts. Agadagidi was prepared in the laboratory by simulating the traditional method.

Isolation of yeasts

The different food samples were used in the preparation of ten-fold serial dilutions using sterile distilled water. Then 0.1ml of the serial dilutions was spread on potato dextrose agar (PDA) that has been supplemented with 100 mg/ml of chloramphenicol to inhibit the growth of bacteria. The yeast cultures obtained were then purified by repeated streak-inoculation on PDA. The pure cultures obtained were stored under refrigeration until needed for further studies (Kurtzman et al., 2011).

Identification of yeast isolates

Microscopic examination of yeast isolates

The pure cultures of the yeasts were streaked onto thin sterile agar and the cells were examined with a Leitz Ortholux phase-contrast microscope (Leica Microsystems, Wetzlar, Germany) and the images were recorded electronically with a Sony XC-75CCD camera.

Phenotypic identification of yeast isolates

Physiological and biochemical tests were performed by fermentation and assimilation tests. Different carbon compounds such as hexoses, pentoses, polysaccharides, alcohols and organic acids were used. The nitrogen compounds include sodium nitrate, sodium nitrite, ethylamine, lysine and cadaverine (Kurtzman et al., 2011).The carbon compounds used in the study include glucose,sucrose,raffinose,melibiose,galactose,lactose,trehalose,maltose,melezitose,salicin,sorbose, rhamnose,xylose, L-arabinose, D-arabinose and ribose.The alcohol used in this test includes methanol, ethanol, 2-propanol, 1-butanol, glycerol, erythritol, ribitol, xylitol, galacitol, mannitol, glucitol and inositol. The yeasts were grouped together based on their phenotypic properties and representatives were selected for DNA sequencing.

Molecular identification.

DNA sequence analysis

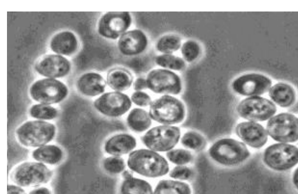
Whole cells grown on YM agar (1.0% glucose, 0.5% peptone, 0.3% malt extract, 0.3% yeast extract, 2.0% agar) for 1 to 3 days were suspended to a density of 1+ in water (Lachance et al., 1999). Ten microlitres of the suspension was incorporated into 20 µL amplification reactions. The D1 and D2 domains of the large subunit ribosomal RNA were amplified using primers NL1 (52-GCATATCAATAAGCGGAGGAAAAG) and NL4 (52-GGTCCGTGTTTCAAGACGG) (Kurtzman and Robnett, 1998). Amplification by the polymerase chain reaction was conducted following the instructions provided by the supplier of heat-activated *Taq* polymerase (*in vitrogen*), in the presence of 1.5mM $MgCl_2$ in an M.J Research PTS 200 cycler. The mixture was held for 2 min at 95°C and then subjected to 35 cycles at 94°C for 15 s, annealing temperature for 15 s, and 72°C for 40 s, with final extension for 5 min at 72°C. The annealing temperature varied from 55.7 to 53.7°C during the first 20 cycles and kept constant for the remainder. The amplified DNAs were concentrated and cleaned on Qiagen spin columns, and sequenced with ABI sequencer (Applied Biosystems) at the John P Robarts Research Institute, London, Ontario, Canada. The sequences were edited and aligned with the program Mega, version 5.0 and compared with published sequences (Kurtzman and Robnett, 1998).

RESULTS AND DISCUSSION

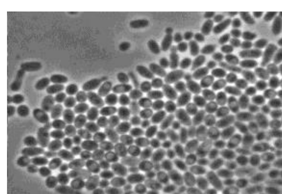
The yeasts isolated from differen fermented food products are presented in Table 1. *Saccharomyces*

Table 1. Selected Nigerian fermented foods and yeasts isolated from them.

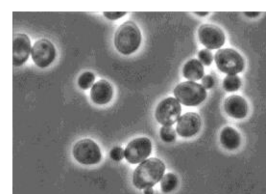
S/N	Yeast isolates	Fermented food products					
		Nono (NN)	Retted cassava (Fufu) (FF)	Ogi-baba (OBB)	Palm wine (PAW)	Burukutu (BKT)	Agadagidi (AGG)
1	*Saccharomyces cerevisiae*	0	20	12	13	12	6
2	*Candida glabrata*	19	0	1	0	14	0
3	*Meyerozyma guiliermondi*	0	0	0	4	0	0
4	*Candida parapsilosis*	4	0	0	0	0	0
5	*Candida tropicalis*	2	0	16	0	0	8
6	*Pichia fabianii*	0	1	0	5	0	3
7	*Pichia kudriavzevii*	1	0	0	0	0	0
8	*Galactomyces geotrichum*	0	0	2	0	1	0
9	*Trichosporon sp.*	1	0	0	0	0	0



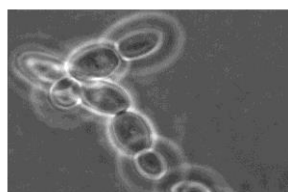
Saccharomyces cerevisiae FF01



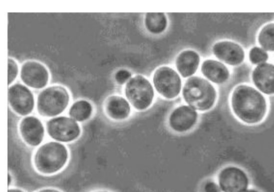
Saccharomyces cerevisiae AG08



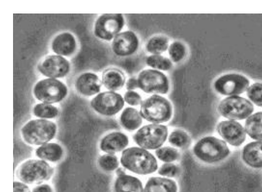
Saccharomyces cerevisiae BK07



Saccharomyces cerevisiae BK09



Saccharomyces cerevisiae BK16



Saccharomyces cerevisiae FF01

Plate 1. Phase contrast microscopy image of some *Saccharomyces cerevisiae* isolated from fermented foods and alcoholic beverages.

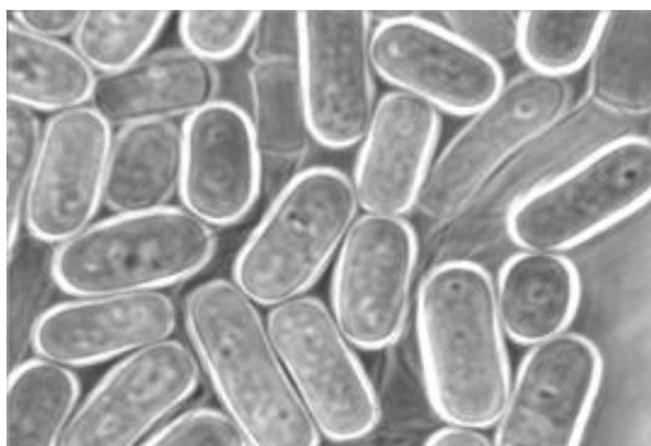


Plate 2. Phase contrast microscopy image of *Galactomyce geotrichum* OB 02A.

cerevisiae were isolated from all the food products analysed in this study with the exception of nono. *S. cerevisiae* is the most frequently isolated with highest number of occurrence. Other yeasts were isolated from one, two or three of the food products studied. *S. cerevisiae* have been isolated from different fermented food products such as palm wine, dolo, kpete-kpete, tchoukoutou and so on (Glover et al., 2009; Kayode et al., 2011; Ngoc et al., 2013; Djegui et al., 2015; Olowonibi, 2017).

The yeasts isolated in this work exhibited different cellular shapes. The majority had ovoid shapes as shown in Plate 1. The other shape exhibited by some is cylindrical as shown in Plate 2. The yeasts have been reported to exhibit different shapes such as oval and cylindrical as reported by Kurtzman et al. (2011).

The yeasts were identified by sequencing as species of

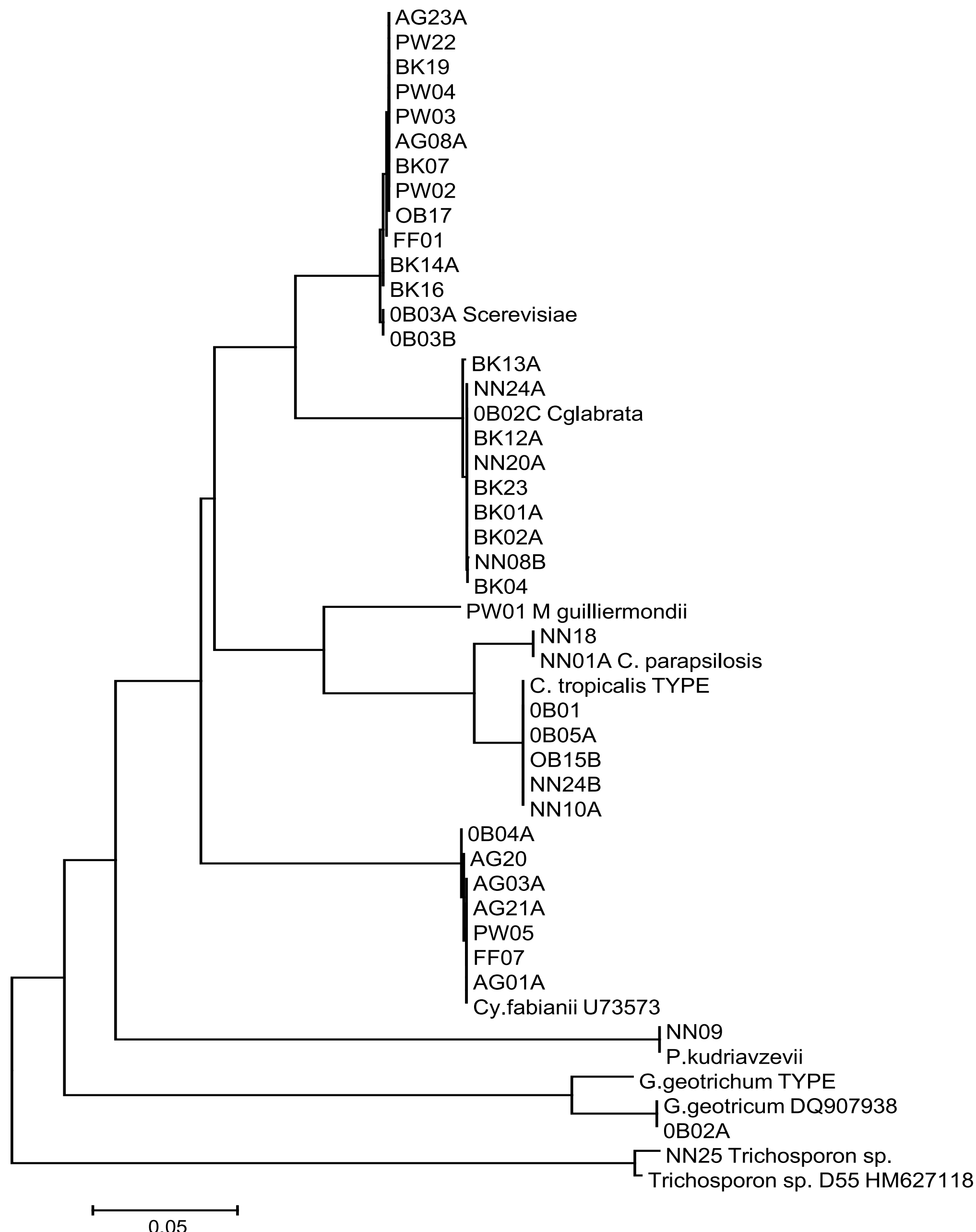


Figure 1. Dendogram showing phylogenetic relationship among the yeast isolates.

seven different genera (Figure 1) namely *Saccharomyces*, *Candida*, *Cyberlindnera, Pichia*, *Meyeromyces, Trichosporon* and *Galactomyces.* The species that were isolated are *S. cerevisiae.* Sanni and Lonner (1993) reported the isolation of eleven strains of *S. cerevisiae* from some Nigerian traditional alcoholic beverages like palm wine, burukutu, agadagidi and sekete. Also, Sefa-Dedeh et al. (1999) isolated eight strains of *S. cerevisiae* from traditional brewing of pito in Ghana.

Moreover, Greppi et al. (2013) reported on the diversity of yeasts in ogi, mawe, gowe and tchoukoutou in Benin Republic. *S. cerevisiae* was isolated from ogi and mawe. Apart from *S. cerevisiae* another group of yeast commonly encounter in Nigerian traditional fermented food are members of the genus *Candida*. *Candida glabrata* effectively a species of *Nakaseomyces* a relative of the genus *Saccharomyces* was also isolated. *Candida glabrata* has been isolated from Zimbabwean opaque beer among other yeasts (Misihairabgwi et al., 2015). *Candida tropicalis* was another yeasts isolated from the fermented food products. *C. tropicalis* was characterized from tchapalo, a traditional sorghum beer from

Coted'Ivoire.*Candida parapsilosis* was also isolated from the fermented food products. A study of yeasts in a small scale gari production in Nigeria gave rise to *M. guilliermondii, P kudriavzevii* (synonym *Candida krusei*) and two relatives of the genus *Pichia, C. inconspicua* and *C. rugopelliculosa* (Oguntoyinbo, 2008).

Pichia kudriavzevii was also isolated from the fermented food products. Occurrence of *Pichia kudriavzevii* in Ghanaian nunu was reported by Akabanda et al. (2013). *P. kudriavzevii* has also been isolated from Nigerian palm wine (Nwaiwu et al., 2016). *Pichia spp*. has also been reportedly isolated from Nigerian palm wine (Chilaka et al., 2010). *Satuurnispora* (*Pichia*) *saitoi* and *Wickerhamomyces anomala* (*Pichia anomala*) were isolated during fermentation of cassava for fufu production. *Meyerozyma* (*Pichia*) *caribbica* was isolated from local food crops in Nigeria (Ebabhi, 2013).

Cyberlindnera fabianii was also isolated from some of the fermented foods studied. *Cyberlindnera fabianii* have been isolated from the microbiota of fermented masau fruits in Zimbabwe (Irma et al., 2017). *Trichosporon* sp was also isolated from the fermented food studied. Pedersen et al. (2012) reported the occurrence of *Trichosporon asahii* from fura-a West African fermented cereal product. *Galactomyces geotrichum* was also isolated from the fermented food products. *Galactomyces geotrichum* has been listed as among the yeasts that occur in African fermented food products in a review by Johansen et al. (2019). Therefore, this present work has confirmed the biodiversity of yeasts that are associated with Nigerian traditional fermented food products. The next line of our research will be to investigate the technological applications of these yeasts.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Quality assessment of bacterial load present in drinking water in Woreta town, Ethiopia

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Access to adequate and safe water is a universal human need. Lack of safe and adequate water supply causes health risk and the situation is serious in developing countries The aim of this study was to determine the bacterial load, physicochemical quality of drinking water in wells, reservoir, taps and household storage containers water samples and to investigate the hygiene and sanitation practices of the consumers in Woreta Town. A cross sectional community based study was conducted from January to March 2016 in Woreta town. A total of 189 water samples were collected from wells, reservoir, private taps and household storage containers in three rounds for bacteriological, physicochemical quality of drinking water and the hygiene-sanitation practices of the consumers were assessed using interview. Bacterial load analysis of water samples revealed that well and reservoir water samples were 100%; 30 (100%) tap water samples and 30 (100%) household storage container water samples were contaminated with total coliforms and did not meet the recommended value of World Health Organization (0CFU/100 ml). Regarding thermotolerant coliforms, one well water sample, 21 (70%) tap water samples and 30 (100%) household water samples were contaminated. The bacteriological load was greater at the household storage container water samples. There was statistically significant difference in total coliform and thermotolerants coliforms among the water sources at $p < 0.01$. Proper management of water sources, appropriate disinfection of raw water sources with chlorine and promoting good hygiene and sanitation practices are recommended to deliver safe drinking water to the consumers of the study area.

Key words: Bacteriology, drinking water, household, hygiene, physicochemical, tap water.

INTRODUCTION

Outbreaks of waterborne diseases continue to occur throughout the world and the problem is especially serious in developing countries where there is lack of safe water for drinking and for sanitation. Access to safe water is a fundamental human need and, therefore, a basic human right (Mmuoegbulam et al., 2017). More than 80% of diseases in the world are attributed to unsafe drinking water or inadequate sanitation practices (Bedada et al., 2018). Globally, more than 1 billion people depend on perilous drinking water resources from rivers, lakes, and open wells. Several studies have confirmed that water-related microbial diseases not only remain leading

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causes of morbidity and mortality worldwide but that the spectrum of diseases is expanding and the incidence of many water-related microbial diseases are increasing (Duressa et al., 2019). Diarrhea remains a major killer in children and it is estimated that 80% of all illness in developing countries is related to water and sanitation; and that 15% of child deaths under the ages of 5 years in developing countries results from diarrheal diseases (Kassie and Hyelom, 2017; Malhotra et al., 2015). The human pathogens that present serious risk of diseases whenever present in drinking water include *Salmonella* species, *Shigella* species, *Yersinia enterocolitica, Campylobacter* species, various viruses such as Hepatitis A virus, Hepatitis E virus, Rotavirus and parasites like *Entamoeba histolytica,* and *Giardia lamblia* (Agbo et al., 2019).

Provision of safe household water includes conditions and practices of water collection, storage, handling and the choice of storage containers or vessels (Kassie and Hyelom, 2017). Water may become contaminated at any point between collection, storage, serving or handling in the house (Bedada et al., 2018). Microbial contamination of collected and stored household water is caused not only by the collection and use but unsanitary and inadequately protected (open, uncovered or poorly covered) water collection and storage containers. Unsanitary methods to dispense water from household storage vessels, including contaminated hands and dippers and inadequate cleaning of vessels, lead to accumulation of sediments and pathogens (Tambekar et al., 2008). The direct detection of pathogenic bacteria requires costly and time consuming procedures, and well-trained labor. These requirements lead to the concept of indicator organisms of fecal contamination (WHO, 2006b). Indicator bacteria are used to evaluate the potability of drinking water because it would be impossible to accurately enumerate all pathogenic organisms that are transmitted by water (Bedada et al., 2018). The use of indicator organisms, in particular the coliform, as a means of assessing the potential presence of waterborne pathogens has paramount importance in protecting public health. The presence of any coliform organism in drinking water is used as an indicator of fecal contamination since they are the most sensitive indicator bacteria for demonstrating contamination (Bedada et al., 2018; Nourani et al., 2007).

The World Health Organization (WHO) rated Ethiopia as having the lowest level of water supply coverage in sub-Saharan Africa, at 24%, and the second lowest sanitation coverage, at 15% (WHO, 2006a). The cause of this water quality problem is the backward socio-economic development which results in one of lowest standard of living, poor environmental conditions and low level of social services (UNWATER/WWAP, 2004). Ethiopia is one of the countries where only 52 and 28% of its population have access to safe water and sanitation coverage, respectively. For this reason, 60% to 80% of the population suffers from waterborne and water related diseases. According to Ministry of Water Resource (MoWR, 2007) of Ethiopia, these burden the country with enormous financial and social costs to take care of such a huge number of people suffering from these debilitating infections. Three-fourth of the health problems of children in Ethiopia are communicable diseases arising from the environment, especially water and sanitation. Forty six percent of under-five mortality is due to diarrhea. The Ministry of Health of Ethiopia (MoH, 2005) estimated that 6,000 children die each day from diarrhea and dehydration. In Amhara region, 90,000 children under 5 years of age die annually from diseases related water and sanitation (WHO, 2006a).

The drinking water supply of Woreta town is exposed to contamination at different points by a number of reasons. According to Woreta town Water Supply Office (WWSO, 2015), open defecation, agricultural activities around the water source, improper disposal of garbage in the field or streets, the poorly constructed pit latrines, construction of waste storage pits and latrines within a short distance from water distribution pipe line, unsanitary conditions of water storage containers, and personal hygiene problems are the major sources of pollutants of drinking water in the town. Appropriate treatment and sanitary survey are very important to protect and control the waterborne diseases (Eliku and Sulaiman, 2015). The currently accepted bacterial indicators (total coliforms, thermotolerant coliforms) and related physico-chemical parameters are very important to evaluate drinking water quality (WHO/UNICEF, 2010). So far no studies have been done in Woreta town on the bacteriological and physicochemical quality of drinking water in relation with hygienic practices of the consumers. The aim of the study was to determine the bacteriological and physicochemical quality of drinking water from source to point-of-consumption and to assess hygienic practices of consumers in Woreta town from January to March, 2016. The findings of this study will provide important baseline information about water quality for stake holders for further work and intervention.

MATERIALS AND METHODS

Study design and description of study area

A cross sectional study was conducted in Woreta town over a period of three months (January to March, 2016). It is located in North Western Ethiopia, Amhara National Regional State. The town is situated between 11º55´N latitude and 37º42´E longitude with an elevation of 1,828 meters above sea level. The topography of the town is characterized by plain and it has hot agroclimatic zone. The total population of the town in the four urban kebeles was 26,317 and the number of households was 5,550. The communities in the study area completely depend on ground water as its main source for drinking purpose. The communities of the town have access to tap water from two wells.

Ethical approval

The purpose and procedures of the study were explained to all participants and consent was obtained from all of them. The study was approved by the ethical clearance committees of Bahir Dar University.

Sampling procedures

From the total four kebeles found in the in Woreta town, 2 wells and 1 reservoir were found and all were included in the study. A representative sample of 30 taps and 30 households were selected randomly for household water handling practice of household storage container, bacteriological and physicochemical drinking water quality analysis.

Sample collection

A total of 189 water samples were collected aseptically from wells (n=2), reservoir (n=1), private taps (n=30) and household containers (n=30) in three rounds. For bacteriological water quality examination, water samples were collected in sterile glass bottles and transported to the Bahir Dar Town Water Supply Service Office water microbiology laboratory in a cold box containing ice freezer packs within 2 h collection. From each sampling point 250 ml of water sample was taken for analysis.

Bacteriological analysis

To examine bacteriological parameters, samples were analyzed using membrane filtration (MF) method (APHA, 1998). All samples were analyzed for the presence of total coliforms (TC) and thermotolerant coliforms (TTC). One hundred milliliter of water sample was filtered through a sterile cellulose membrane filter with a pore size of 0.45 μm. The membrane filter was transferred to a sterilized Petri dish containing absorbent pad soaked with membrane lauryl sulfate tryptose broth (Wagtech, England). The Petri dishes were incubated at 37ºC for 18-24 h for TC and 44.5ºC for 18-24 h for TTC. All yellow colonies were counted, recorded and the results were expressed in numbers of colony forming units (CFU) per 100 ml of water sample.

Physicochemical analyses

Physicochemical parameters were analyzed at the site of sample collection and in the laboratory. Temperature and pH were analyzed by using portable digital pH meter (Jenway model-370, England). Turbidity was analyzed by using portable microprocessor turbidity meter (H193703 ELE international, Hungary) within 1 h following the collection of samples, whereas free residual chlorine test was made for all chlorinated samples by using photometer 7100. The test was performed by using N, N-diethyl-1, and 4-phenlenediamine (DPD) chlorine tablets. Total dissolved solids and electrical conductivity were analysed by using portable digital conductivity meter (CC-401, Poland). Furthermore, consumers' hygiene-sanitation practices were assessed through interview. The interview questions and sanitary inspection forms were adapted from WHO and assessment of the conditions of household water containers was obtained through observation checklist (WHO, 2006).

Statistical analysis

Statistical analysis of bacteriological and physicochemical collected data was statistically analyzed by using SPSS version 20 and were compared with WHO guideline standards of drinking water quality and interpreted as safe, acceptable and polluted range. One-way analysis of variance (ANOVA) was done to test for differences among the parameters measured with respect to sampling sites. P value of ≤ 0.05 was considered to indicate statistical significance.

RESULTS

Bacteriological load of drinking water sources

Bacteriological analysis of drinking water samples taken from different sites of Woreta town is presented in Tables 1 and 2. Bacteriological quality of water samples from wells, reservoir, taps and household storage containers were carried out by using the TC and TTC. In the bacteriological water quality analyses of well water, all the water samples were contaminated and were in the range of 10-100 CFU/100 ml for TC. With regard to other bacteriological indicators, TTC, all water samples in well 3 were in the recommended value of WHO (0 CFU/100 ml) whereas in well 6 all water samples were in the range of 1.01-9.99 CFU/100 ml which was not in compliance with WHO guideline. The mean TC and TTC count in the well water samples was 47.17± 6.7/100 and 0.6 ± 0.2/100 ml, respectively. All reservoir water samples were contaminated with TC but were free from TTC. The mean TC and TTC counts were 105.3 ± 2 and 0.0 ± 0.0, respectively.

As shown in Table 1 and Figure 1, out of 30 tap water samples examined, 17 (56.7%) of them were in the range of 10-100 CFU/100 ml whereas 13 (43.3%) samples were in the range of >100 CFU/100 ml for TC counts which were at high and very high level of contamination, respectively. Regarding TTC, 18 (60%) and 4 (13.3%) samples were in the range of 1.01-9.99 CFU/100 ml and 0.01-1.0 CFU/100 ml, respectively. They were at medium and low level of contamination, respectively. Eight (26.7%) tap water samples were found 0 CFU/100 ml which were in the acceptable limit of WHO (0 CFU/100 ml). The mean TC and TTC count in the tap water samples were 116 ± 2.1/100 and 1.1 ± 0.2/100 ml, respectively.

Analysis of household water samples revealed that all the household storage container water samples had TC >100 CFU/100 ml and were above the recommended value of WHO. Regarding the TTC, among the total samples, 25 (83.3%) household container water samples and 5 (16.7%) household water sample had TTC from 1.01-9.99 and 0.01-1.0 CFU/100 ml, respectively. Therefore, the average TC and TTC counts for the 30 household water samples were beyond the recommended value of WHO which is 0 CFU/100 ml (Table 1). The mean TC and TTC count in the household storage container water samples were 422.17 ± 1.5/100

Table 1. Classification of tap and household storage container water according to the levels of bacteriological parameters.

Recommended level of parameters	Tap water (n=30)	Household water (n=30)
Total coliforms (CFU/100 ml)		
>100	13 (43.3%)	30 (100%)
10-100	17 (56.7%)	-
1.01-9.99	-	-
0.01-1.0	-	-
0	-	-
Total	**30 (100%)**	**30 (100%)**
Thermotolerant coliforms (CFU/100 ml)		
>100	-	-
10-100		-
1.01-9.99	18 (60%)	26 (86.7%)
0.01-1.0	4 (13.3%)	4 (13.3%)
0	8 (26.7%)	-
Total	**30 (100%)**	**30 (100%)**

0, safe water; 0.01-1.0, acceptable water; 1.01-9.99, polluted water; 10-100, dangerous water; > 100, very dangerous water.

Table 2. Mean and standard error of bacteriological counts of TC and TTC from wells, reservoir, taps and household water.

Water source/sites	Mean and S.E of TC counts/100 ml	Mean and S. E of TTC counts/100 ml
Well water	47.17 ± 6.7^{c}	0.6 ± 0.2^{b}
Reservoir water	105.3 ± 2.6^{b}	0.0 ± 0.0^{c}
Tap water	116 ± 2.1^{b}	1.1 ± 0.2^{b}
Household water	$422.17 \pm 1.5^{a*}$	$1.8 \pm 0.2^{a*}$
LSD (5%)	58.17	1.33

Mean values indicated with the same letters are not significant at $P<0.05$; * significant at $P<0.01$; LSD, List Significant Difference; S.E, Standard Error of the mean; TC, Total Coliform; TTC, Thermotolerant Coliform.

ml and 1.8 ± 0.2/100 ml, respectively. The results of analysis of variance (ANOVA) test showed that there was statistically significant difference in TC among the water source at $p < 0.01$.

Regardless of significance of difference the higher average value of TC was recorded at household water (422.17± 1.5) and the lowest value was recorded at well water (47.17± 6.7). Regardless of the TTC there was statistically significant difference among well, tap, reservoir and household water at $p < 0.01$. The higher average value of TTC was recorded for household water (2.0±0.1) and the lowest value was recorded for reservoir water (0.0±0.0) (Table 2).

Level of risk of contamination of water sources

To identify causes of contamination and the risks of future contamination of drinking water sources, sanitary inspection is important to show level of contamination (Tsega et al., 2013). In the case of risk classification (Table 3), all well water samples had medium sanitary risk scores for TTC count. Similarly, all reservoir water samples had low sanitary risk score for TTC. The overall risk-to-health status of tap water samples, 9 (30%) and 21 (70%) of tap water samples had low and medium risk score for TTC, respectively. In the case of household storage containers, 3 (10%) and 27 (90%) water samples had high and very high sanitary risk score for TTC, respectively. In the case of TTC, 8 (27%) and 22 (73%) household storage container water samples had low and medium sanitary risk score, respectively. The results of sanitary inspection support the presence of high bacteria counts in household storage container drinking water samples and indicated that the water has been faecally contaminated.

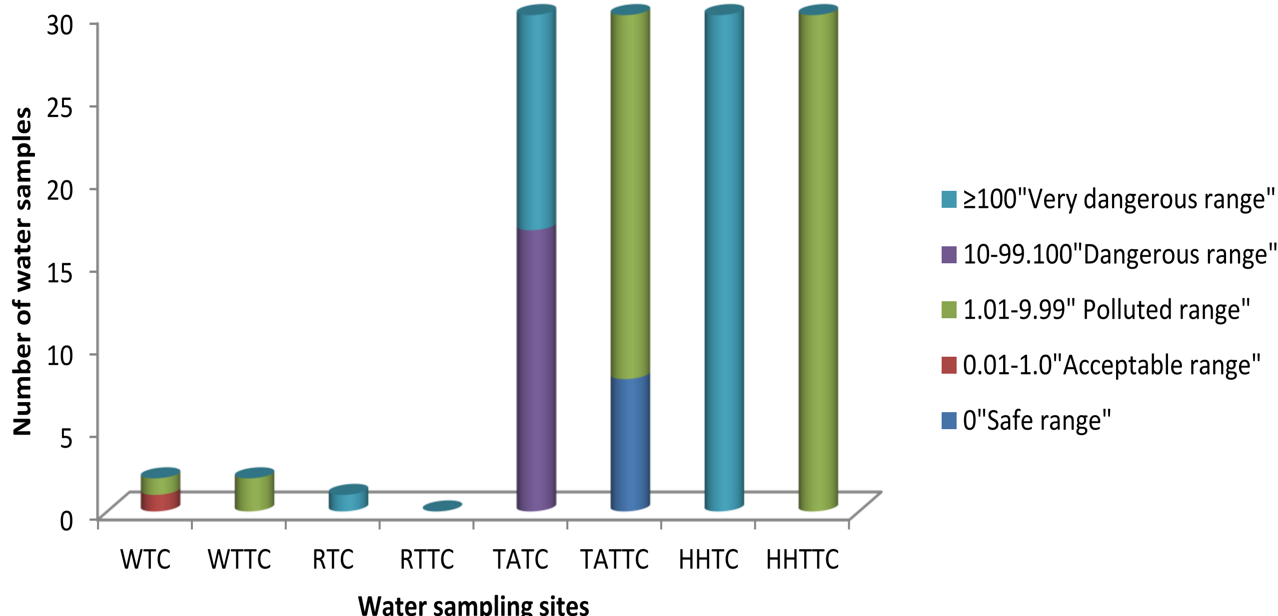

Figure 1. Ranges of TC and TTC bacteria from wells, reservoir, taps and household containers water. TCW, Total coliform at wells; TTCW, Thermotolerant coliforms at wells; TCR, Total coliform at reservoir; TTCR, Thermotolerant coliforms at reservoir; TCT, Total coliform at taps; TTCT, Thermotolerant coliforms at taps; TCHH, Total coliform at household storage containers; TTCHH, Thermotolerant coliforms at household storage containers.

Table 3. Levels of risk of contamination of 2 wells, 1reservoir, 30 taps and 30 household storage containers.

Risk category	Wells (n=2)		Reservoir (n=1)		Tap (n=30)		Household storage containers (n=30)	
	TC	TTC	TC	TTC	TC	TTC	TC	TTC
Very low	-	-	-	-	-	-	-	-
low	-							
Intermediate	-	2(100%)	1(100%)					
High	2(100%)	-	-	-	9(30%)	-	3(10%)	-
Very high	-							-

TC, Total coliforms; TTC, Thermotolerant coliforms

The physicochemical results of water sources

Physicochemical analysis of drinking water samples taken from different sites of Woreta town is presented in Tables 4 and 5. The water quality of wells, reservoir, taps and household storage containers were carried out by using the physicochemical parameters like temperature, pH, turbidity, total dissolved substance, electrical conductivity and residual free chlorine. All the measured parameters obtained from different water sampling sites were within the WHO standards of drinking water except temperature and residual free chlorine.

In the case of temperature analyses of wells, reservoir, taps and household storage containers water samples, all water samples had temperature above 20°C which were beyond the recommended value of WHO (<15°C). All water sources had pH value in the range of 6.5-8.0 which were in the recommended value of WHO (6.5-8.0). The result of turbidity of all water samples were in the range of 0.1-1.99 NTU which were in the recommended value of WHO (<5 NTU). The mean measurements of total dissolved substance (TDS) and electrical conductivity (EC) were between 309.58 - 382.50 mg/ l and 513.2 - 520.3 μs/cm, respectively. The mean value of residual free chlorine (RFC) in the reservoir, taps and household storage container water samples were between 0.02-0.10 mg/l which is less than the value recommended by WHO. The reasons might be due to irregular chlorination, presence of high bacterial load, high temperature and organic matter. The recommended WHO value of RFC in

Table 4. Classification of tap and household water according to the levels of physicochemical parameters.

Recommended level of parameters	Tap water (n=30)	Household (n=30)
Temperature (°C)		
>20	30 (100%)	30 (100%)
15.01-20	-	-
<15	-	-
Total	30 (100%)	30 (100%)
pH		
>8	-	-
6.5-8.0	30 (100%)	30 (100%)
Total	30 (100%)	30 (100%)
Turbidity (NTU)		
>5	-	-
2-5	-	5 (16.7%)
0.1-1.99	30 (100%)	25 (83.3%)
0	-	-
Total	30 (100%)	30 (100%)
Total dissolved solids(mg/l)		
>1200	-	-
900-1200	-	-
600-900	-	-
300-600	30 (100%)	30 (100%)
<300	-	-
Total	30 (100%)	30 (100%)
Electrical conductivity(µs/cm)		
>2000	-	-
1500-2000	-	-
1000-1500	-	-
500-1000	30 (100%)	30 (100%)
<500	-	-
Total	30 (100%)	30 (100%)
Free chlorine residual (mg/l) *		
>0.5	-	-
0.2-0.5	-	-
0.1-0.99	4 (13.3%)	-
0	26 (86.7%)	30 (100%)
Total	30 (100%)	30 (100%)

*Only for chlorinated tap and household storage container water samples.

drinking water was from 0.2-0.5 mg/l. Therefore, the water samples were safe for drinking purpose in terms of pH, TDS and EC except temperature and residual free chlorine. The results of ANOVA test showed that there was a statistically significant difference in free residual chlorine among reservoir, tap and household water samples at $p<0.05$. The highest RFC value was recorded at the reservoir (0.10 ± 0.01) and the lowest value was recorded at the household water samples (0.02 ±0.01). But there was no statistically significant difference in temperature, turbidity, pH, conductivity and total dissolved substance among wells, reservoir, taps and household

Table 5. Mean and standard error of physicochemical of T °, P^H, Turbidity, TDS, EC and RFC in different water sources.

Parameter	Well	Reservoir	Tap	Household	LSD (5%)
T ° (°C)	24.33±0.66[a]	24.60±0.83	23.27 ±0.37	23.77±0.23	NS
pH	7.8 ± 0.057	7.5 ±0.120	8.0 ± 0.290	8.1 ±0.176	NS
Turbidity (NTU)	0.72 ±0.174	1.39 ± 0.286	0.77 ± 0.144	1.07 ±0.353	NS
TDS (mg/l)	315.83±66.56	311.67±0.88	309.58±0.87	308.16±1.16	NS
EC (µs/cm)	519.8 ± 3.87	520.3 ±0.333	515.3 ± 1.34[a]	513.2 ±1.95	NS
RFC (mg/l)	-	0.10 ± 0.13[a *]	0.05 ± 0.01[b]	0.02 ±0.01[b]	0.08

Mean values indicated with the same letters are not significant at P<0.05; * significant at P<0.05; NS, Not significant; LSD, Least significant difference; T °, Temperature; pH, Hydrogen ion concentration; TDS, Total dissolved substance; EC, Electrical conductivity; RFC, Residual free chlorine.

Table 6. Results of hygiene- sanitation practices of consumers at the households.

Questions asked to the consumers	Responses	
	Yes	No
	No (%)	No (%)
Do you store water in a narrow mouth/opening container?	26 (86.7)	4 (13.3)
Do you wash your hand and container before water collection?	16 (53.3)	14 (46.7)
Do you clean your containers (vessels) every day to collect the tap water?	13 (43.3)	17 (56.7)
While you are collecting the tap water, is there a contact of hands with tap water?	13 (43.3)	17 (56.7)
Do you transport the collected tap water from tap to your house in a covered container?	17 (56.7)	13 (43.3)
Do water storage containers have a lid/cover material?	30 (100)	0 (0)
Is the drinking water that you take from the storage container has contact with your hands?	4 (13.3)	26 (86.7)
In your house, is drinking water stored in a separate container from water intended for other purposes?	3 (10)	27 (90)
Do you have latrine in your house?	30 (100)	0 (0)
After visiting toilet, do you wash your hands with soap or other chemicals?	7 (23.3)	23 (76.7)

storage container water samples (Table 5).

Hygiene-sanitation practices of consumers at household storage containers

The results of questionnaire survey on hygiene-sanitation practices of consumers at household are shown in Table 5. According to the respondents' responses, 26 (86.7%) of consumers collect their drinking water in a narrow mouth containers, 16 (53.3%) wash their hands and containers before water collection, 13 (43.3%) clean their containers (vessels) everyday, 17 (56.7%) collect water from tap without contact with their hands and 17 (56.7%) transport the collected tap water to house in a covered container. All respondents replied that they store water in a container which has cover. Four responded that they take water from their container with contact with their hands and three had separate water container for storing drinking water in the house. All respondents had latrines in their house and 7 (23.3%) answered that they wash their hands with water only after visiting toilet. As indicated in Table 6, using TC as bacteriological indicator to determine the overall risk to health status showed that majority of the household water containers were at high risk score. Using TTC, the majority household water containers were at intermediate risk score. Therefore, inappropriate cleaning of storage water containers, poor sanitation and poor hygienic practices of consumers in household water containers were the main factors for the contamination of stored water at home. Moreover, there was no strong tradition of hand washing with soap after defecation. This might be a because of chronic water shortage, lack of surplus cash to purchase soap and a general lack of awareness about the importance of hand washing.

DISCUSSION

Adequate, safe and accessible supply of drinking water is essential to sustain life. The World Health Organization recommends that drinking water intended for consumption be free from total TC and TTC, since the presence of

these indicator bacteria indicates a potential health risk for consumers (WHO, 2012). However, in this study the result showed that the average count of TC and TTC from wells, reservoir, taps and household storage containers water samples were above the recommended value of WHO (0 CFU/100 ml). In the case of bacteriological analysis of wells in this study all (100%) and 50% water samples were contaminated with TC and TTC, respectively. The same study done in Mali (Diakite et al., 2019) indicated 1.75% of the well water samples were contaminated by TC and TTC which was less than this study. The same study done in Fatrta Woreda, Ethiopia indicated 22 (91.7%) well water samples were not in compliance with WHO recommended values (0 CFU/100 ml) (Kassie and Hayelom, 2017). The possible contamination routes to wells might be agricultural activities, livestock grazing and sewage leakage. Bacteriological analyzes of TC and TTC from reservoir water samples indicated all water samples were contaminated with TC but in the case of TTC the water samples were incompliance with WHO standards (0CFU/100 ml). In a study done in Nepal (Panta et al., 2016), majority of water samples from reservoirs were contaminated with TC and TTC. In a study done in Adama town reported by Eliku and Sulaiman (2015), all reservoir water samples were in the acceptable limit of WHO (0 CFU/100 ml).

In this study, 30 (100%) and 22 (73.3%) of tap water samples were contaminated by TC and TTC, respectively. The result indicated the contamination level is higher in tap water samples than well and reservoir water samples. A study done in Jima (Yasin et al., 2015) reported 66.67 % of tap water samples were negative for TTC. A previous study reported in Nekemte town of Ethiopia (Duressa et al., 2019) showed 100% of the tap water samples did not meet the TC standard (0 CFU/100ml) set by WHO, whereas about 37% of the samples failed to meet safe water quality with regard to TTC. Another study conducted in Bahir Dar city of Ethiopia indicated that 44.8% of tap water samples had TC whereas 40% of the tap water samples had TTC (Tabor et al., 2011).

The bacteriological analysis of water at household storage containers in this study indicated 30 (100%) of water samples were contaminated with TC and TTC. The results also indicated that more number of TC and TTC were encountered in household storage containers compared to wells, a reservoir and tap water sample which was in line as that reported in Nyala city of South Darfur (Abdelrahman and Eltahir, 2011). Another study conducted in Bahir Dar indicated that 19 (54.2%) and 12 (34.2%) household storage containers water samples had TC count from 10-100 and 1.01-9.99 CFU/100 and 16 (45.7%), 14 (40%), 1 (2.8%) household storage containers water samples had TTC counts ranging from 10-100, 1.01-9.99, and 0.01-1.01 CFU/100 ml, respectively (Tabor et al., 2011). Another study conducted in Ethiopia showed that poor sanitation and poor hygiene in household were the main factors for the contamination of water during transportation and after storage at home (WHO/UNICEF, 2010). A similar study done in Northwest Ethiopia has reported a similar finding with this study (Tsega et al., 2013). The high load of TC and TTC counts in the household storage containers water samples are likely to reflect lower personal hygiene, poor sanitation and handling practice of the consumers in collection and storage.

The sanitary inspection results showed that all (100%) wells of this study had sanitary risk score high and medium for TC and TTC, respectively. A study in Farta Woreda of Ethiopia (Kassie and Hayelom, 2017) revealed 15 (50%) of protected water resources had high sanitary score for *E. coli* contamination. The sanitary inspection results indicated 30 (100%) of household storage containers of this study had sanitary risk score low and medium for TC and TTC, respectively, which was in agreement a study done in Farta Woreda Ethiopia (Kassie and Hayelom, 2017). Contamination of water sources at different points due to inadequate protection and poor hygienic practices of consumers may contribute for the deterioration of drinking water.

In this study, selected physicochemical quality of well, reservoir, taps and household storage container water samples were analysed to assess public health implications on the consumers. Temperature is one of the physico-chemical parameter used to assess the quality of drinking water. The results of the study showed all water samples from different sampling sites had a temperature >20°C which was in agreement with the report made in Jima town (Yasin et al., 2015) and Nekemte town of Ethiopia (Durassa et al., 2019). Similarly, earlier studies in Bahir Dar city (Tabor et al., 2011) reported a mean temperature of 23.8°C. Temperature could affect the quality of drinking water by reducing the solubility of gases and affecting the rate of chemical reaction (Yasin et al., 2015).

The pH of water samples from all water sources was within the range of 6.5-8 which is in the recommended standards of WHO (6.5-8). Though pH has no direct effect on the human health, all the biochemical reactions are sensitive to variation of pH (Nidhi, 2018; Gupta and Sunita, 2009). A similar study conducted in Abeokuta, Nigeria, showed that a pH value ranging 6.8-7.1 was recorded (Shittu, 2008). The turbidity of all water samples from wells, reservoir, taps and household storage containers were in the range of 0.1-5NTU and all of them were below WHO standards (<5NTU). For the results of other similar study in Nekemte town (Duressa et al., 2019) the turbidity of the tap water samples was in the range of 0.1–1.7 NTU. High level of suspended organic matter and microorganisms often cause high level of turbidity in drinking water. High level of turbidity

can stimulate the growth of bacteria and can protect pathogenic microorganisms from the effects of disinfectants (Yasin et al., 2015). The TDS measurements of water samples from wells, reservoir, taps and household container water samples were in the range of 300-600 mg/l. The TDS values of water samples from all sources were in the acceptable standard of 600 mg/l (WHO, 2006b). However, for the study done in Nekemte town (Duressa et al., 2019), the TDS measurements of the tap water samples were in the range of 37–46.5 mg/l which was below the maximum acceptable standard of 600 mg/l. The electrical conductivity (EC) measurements of water samples from different sampling points of this study were in the range of 500-1000 µs/cm. For the research conducted in Nekemte (Duressa et al*.,* 2019), the electrical conductivity (EC) measurements of water samples were found to be in the range of 58 –70 µs/cm which is much lower than for this study. In this study, all chlorinated water samples had a mean value of RFC 0.10 mg/ which was below the recommended limit of WHO (0.2 - 0.5 mg/l) which was in line with the report in Kote town (Roopavathi et al., 2016). The low level of residual free chlorine might be due to non-continuous disinfection process at the disinfection point, less concentration of RFC and the presence of high level of bacterial load and organic matter.

Conclusions

This study reveals that majority of drinking water samples were unsafe for consumption. There was an increase in bacterial indicator counts as the water moves from the wells to household level (point-of-consumption). The number of TC and TTC were not in compliance with the WHO guideline value 0 CFU/100 ml. Therefore, high counts of indicator organisms in majority water samples suggested the presence of pathogenic organisms that constitute a threat to anyone consuming these water sources. Sanitary inspection matrixes on health score of wells, reservoir, taps and household storage containers water samples indicated the majority were classified as high risk, while some were at medium risk. Insufficient and irregular chlorination, poor sanitation and hygiene practices have contributed to the higher level of bacterial contamination of water from wells to point-of-consumption. There should be a continuous chlorination of drinking water and awareness creation about hygiene and sanitation to maintain safe drinking water at household level.

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CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Differential traits of rhizobia associated to root-nodules of gum acacia (*Senegalia senegal)*, shittah tree (*Vachellia seyal*), pigeon pea (*Cajanus cajan* L) and cowpea (*Vigna unguiculata)*

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In the present study, rhizobia partners of gum acacia (Senegalia senegal L.), shittah tree (Vachellia seyal), pigeon pea (Cajanus cajan L.) and cowpea (Vigna unguiculata L.) were characterized for their specific traits to each of the three host plants. In total 12 soil samples were collected and used to trap root-nodule rhizobia. Averagely, 24 rhizobia strains were isolated, of which 3, 4, 6 and 11 were respectively from root-nodules of S. senegal, V. seyal, C. cajan and V. unguiculata. Isolates were morphologically rod-shaped and measured 2.5-3.2 mm in diameter. They were wet (22), wet/sticky (01) and wet/creamy (01) in appearance. Isolates (23) were convex, and flat (1) in elevation. They were all Gram-negative, and none form endospores. They indicated positive reactions to ammonia and indole acetic acid tests, but showed various reactions to catalase, phosphate solubilization, starch, triple sugars ion agar and urea tests. Nodulation and nitrogen fixation capacity were confirmed by either pink or brown internal coloration of nodules. Inoculation of hosts with isolates significantly ($p < 0.0001$) increased both the plant and nodule weights compared to that of control. This study has revealed that all the identified isolates possess are Rhizobium, belonging to the same cross-inoculation group, thus may be useful in increasing the symbiotic nitrogen fixation in the studied legumes. Theses findings provide the basis for further research on the phylogeny of rhizobia strains nodulating legumes, as well as their use as potential inoculants to improve production in semi-arid lands such as those of the northern Cameroon.

Key words: *Senegalia senegal*, *Vachellia seyal, Cajanus cajan, Vigna unguiculata, Rhizobium*, cross-nodulation, Northern-Cameroon.

INTRODUCTION

Legumes constitute a large group of flowering plants belonging to the Fabaceae or Leguminosae family (Lewis et al., 2005). Many crop legumes have the capacity to use two different sources of nitrogen, such as uptake of

mineral nitrogen present in the soil solution by root like every high plant, and biological of atmospheric nitrogen (N_2) fixation by rhizobia inhabiting their rhizosphere. These features make these crop legumes particularly attractive for agricultural and natural ecosystems, and hence, easily adaptable to infertile soils (Voisin et al., 2015; Giller et al., 2016). Legumes provide a stable and safe food for human, as well as fresh fodder for animals, because they constitute a source of nitrogen and micronutrients (Snapp et al., 2018).

Bacteria associated to crop legumes are collectively known as rhizobia. The term “rhizobia” was originally used to name bacteria belonging to the genus *Rhizobium*. Nowadays, other genera were later identified, such as *Bradyrhizobium* (Jordan, 1982), *Sinorhizobium* (Chen et al., 1988) and *Mesorhizobium* (Jarvis et al., 1997). Until recently, rhizobia were thought to belong exclusively to the Alphaproteobacteria, namely the order Rhizobiales, which also includes many species that are not legume microsymbionts. However, some unexpected findings from research on wild legumes microsymbionts has resulted in the identification of rhizobia from the Betaproteobacteria class (Moulin et al., 2001). They are commonly described as aerobic, rod-shaped, non-sporulating and Gram-negative soil-inhabiting bacteria containing nodulation and atmospheric N_2 fixation genes needed for the symbiosis (Giller et al., 2016). Rhizobia are also used as biofertilizers because of their ability to fix atmospheric nitrogen through their symbiotic association with crop legumes. Therefore, the mutualism between the two partners is a classic and reciprocal relationship in which, carbohydrates are provided to rhizobia by the host legume, while rhizobia in turn supply fixed-nitrogen to the host (Denison, 2000; West et al., 2002; Kiers et al., 2003). Rhizobia have also been reported to improve plant health or increase yield, and are usually referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al., 1980).

In order to know which root nodule bacteria are able to establish effective symbiosis with a particular legume, it is important to isolate and characterize them. To identify rhizobia inhabiting root nodules, the methods described by Vincent (1970) and Somasegaran and Hoben (1985) are often known to be easier to use in principle. Therefore, morphological, biochemical and symbiotic characterization on selective medium seem to be more adequate. Successful nodulation and N_2 fixation in the Legume-*Rhizobium* symbiosis require a close compatibility between the two partners and appropriate soil environment (Hirsch et al., 2003; Leibovitch et al., 2001; Zhang et al., 2002; Mishra et al., 2012). Although rhizobia from four crop legumes have been characterized and have shown efficiency in increasing the host plants yield in the Guinea-Savannah zone of Cameroon (Ngakou et al., 2009), nothing is known about the similarities/differences between rhizobia symbionts of food crop legumes and those of tree legumes such as acacia species. This study is focused on legume-*Rhizobium* relationships, specifically the morpho-physiological and biochemical characteristics of *Rhizobium* strains associated to some food (*Cajanus cajan, Vigna unguiculata*), and tree (*Senegalia senegal*, *Vachellia seyal*) crop legumes. In the present research, the characterization of rhizobia from root nodules of the above four crop legumes, and the evaluation of their nodulation efficiency through a cross-nodulation trial are discussed. This study could serve as preliminary databases for the further study relevant for biofertilizers research in the region.

MATERIALS AND METHODS

Study areas

Soils were collected from 2 different agro-ecolgical zones of Cameroon: the Sudano-Sahelian zone comprising the Far-North and North regions, characterized by a long dry season (November-June), with the annual rainfall between 500-1000 mm and annual average temperature of 28°C; and the Guinean Savannah of the Adamawa region, characterized by a long rainy season (April-October), a mixture of Woodland and grassy savannah as vegetation, an annual mean rainfall varying from 1227.9-1675.8 mm and annual average temperature of 22.93°C (Pamo, 2008).

Soil sampling

Soils were sampled at the beginning of the dry season in the rhizosphere of the four selected crop legumes (*S. senegal*, *V. seyal, C. cajan, V. unguiculata),* in order to optimize the chances of getting specific rhizobia of affiliated to these legumes. A total of 12 rhizospheric composite soil samples of 25 kg each (4 legumes × 3 regions) were collected at 20 cm depth and at 25 cm radius within each plant rhizosphere. On the site, each soil composite sample was sieved through a 2 mm pores sieve to discard stones or gravels, before they were separately filled into 20 sterile plastic bags of 1 kg each per rhizospheric soil.

Trapping experimental set up

For each soil from any of three regions (Ngaoundere, Garoua, Maroua), the trapping was carried out as pots experiment in a completely randomized block design (CRBD) system, in which the host plants (*S. senegal*, *V. seyal, C. cajan* and *V. unguiculata*) were considered as treatments, while the 20 plastic bags filled with 1 kg soil each were the replicates. For each host plant, seeds were surface sterilized by three successive immersions in 70% alcohol followed by three washings in sterile water. In each of the 1 kg plastic bags containing soils, rhizobia were trapped using four seeds per crop legume. Seeds of *S. senegal* and *V. seyal* were

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manually scarified before sowing, while *C. cajan* and *V. unguiculata* seeds were sown without any dormancy break off. After germination, plantlets were thinned to two to reduce competition between plantlets. The trial set was watered daily and maintained till the formation of effective root nodules (at 45 days after sowing).

Isolation and purification of *Rhizobium* isolates on Congo Red YEMA medium

At 45 days after sowing, plants were harvested by tearing the plastic bag, then the roots were delicately rinsed with tap water to clear roots from soil particles and to view root-nodules. Root-nodules were manually discarded from roots and introduced into labelled jars (name of plant and origin of soils) and transported to the laboratory. Petri dishes were cleaned with tap water, rinsed with distilled water and allowed to dry at room temperature. Cleaned dishes were sterilized in the dry oven at 105°C for 2 h. The Yeast Extract Mannitol Agar (YEMA) medium was prepared as described by Vincent (1970) with the following composition: 10 g mannitol; 0.2 g $MgSO_4$, $7H_2O$; 1 g yeast extract; 0.5 g KH_2PO_4/K_2HPO_4; 0.1 g NaCl; pH = 6.8. The medium was supplemented with 0.25% Congo red to restrict possible contaminants. Rhizobia were isolated from the fresh root nodules as described by Somasegaran and Hoben (1985). Hence, 15 efficient root nodules were randomly selected for each legume species and each soil type were immersed in 70% alcohol for 30 s, and then in 0.1-0.2% $HgCl_2$ for 5 min, before they were rinsed with distilled water to remove all traces of alcohol and $HgCl_2$. Sterilized nodules were ground in a Petri dish using a sterile carpel. Aliquot of the macerate was taken and aseptically struck onto the YEMA solid medium. Inoculated petri dishes were incubated at 28±°2C temperature. Three days after the first streak, different colonies grown separately were sub-cultured onto the YEMA-Congo Red solid medium under the same conditions, in order to screen all the single colonies. Three days after sub-culturing, the morpho-cultural characteristics of isolated colonies (number, elevation, surface, colour, aspect, edges) were described before the study of physiological and biochemical characteristics of isolates.

Morphological characterisation of isolates

Three days after incubation at 28±2°C on YEMA growth medium in the dark, different typical colonies were identified, counted and their morphological characters (shape, size, appearance, elevation, surface, colour and edge) evaluated as presented in Table 2 (CIAT, 1988; Somasegaran and Hoben, 1985, 1994).

Screening of *Rhizobium* strains

The method described by Vincent (1970) and Somasegaran and Hoben (1994) was used to screen *Rhizobium* strains through Congo red test, Bromothymol blue test, Gram staining and cell microscopy observation, while the method of Bernaerts and Deley (1963) was performed for the ketolactose test.

Physiological and biochemical characterization of isolates

The purified *Rhizobium* isolates were studied for their resistance to different stresses. Hence, they were streaked on media at different NaCl (1, 2, 3, 5 and 10%), pH (4, 7, 8, 9, 11) and also at different temperatures (28, 30, 35, 38 and 42°C) (Vincent, 1970; Somasegaran and Hoben, 1994).

Catalase test

All the isolates were struck on YEMA solid media and incubated at 28±2°C for 3 days. The test was performed by adding 2-3 drops of 3% hydrogen peroxide on different cultures of isolates. After incubation, the formation of gas bubbles indicates the presence (+) or the absence (-) of catalase (Graham and Parker, 1964).

Starch hydrolysis

Starch hydrolysis test consisted of adding 0.2% starch powder to nutrient agar medium plates per isolates. Labelled plates containing nutrient agar medium were incubated at 28±2°C for 3 days. Positive test indicated the isolates with competence to solubilize starch by producing the enzyme amylase. After incubation period, culture plates were flooded with Gram's iodine, and the presence (+) or absence (-) of halos around the bacterial colonies was recorded (De Oliveira et al., 2007).

Triple sugar ion agar test

Triple sugar iron agar medium (3 g/L beef extract, 3 g/L yeast extract, 15 g/l peptone, 5 g/L NaCl, 10 g/L lactose, 10 g/L sucrose, 1 g/m dextrose, 0.2g/l ferrous sulphate, 0.3g/l thiosulphate, 0.24 g/L phenol red, 15 g/L agar, pH 7.0) test was used to determine the ability of isolates to use varied carbohydrate sources as media for growth. The test was performed to distinguish among the different groups, which are able of fermenting glucose with the production of acid and hydrogen sulphide (Kliger, 1918).

Urea production

YEMA medium plate was prepared, consisting of a mixture of 2% urea and 0.012% Phenol Red (Jarvis et al., 1977). After sterilization by filtration and autoclaving, about 10 µl of each isolate type was struck on the prepared solid medium plates, and incubated at 28 ±2°C for 3 days. On the basis of colour shift of medium, which is initially red, the results were evaluated: as pink coloration indicating the hydrolysis (+) of urea to form ammonia and carbon dioxide in an alkaline environment. A yellowish coloration was a sign of a negative reaction (-) in an acidic environment.

Plant growth promoting traits

Ammonia production

The capacity of different isolates to synthetize ammonia in Peptone broth medium (1 g peptone, 0.5 g NaCl, 0.5 g potassium nitrate, and 1000 ml distilled water) was tested. A volume of 5 ml sterilized broth was dispersed in each labelled test tubes with a control, and incubated for 3 days. After incubation, 1ml of Nessler's reagent was added to each of the bacterial culture tubes, and the development of orange to brown colour indicatiing the presence (+) of ammonia was recorded (Joseph et al., 2007; Mahbouba et al., 2013).

Indole 3-acetic acid production

Qualitative analysis of indole acetic acid production by different isolates was carried out. Hence, culture tubes containing nutrient broth were prepared and autoclaved. About 50 µg/ml broth of filtered tryptophan solution was added to each tube. Culture tubes with different isolates, including the negative control were incubated at 28±2°C for 3 days. After addition of 4 ml of the reagent to 1 ml of

the supernatant, the solution was thoroughly mixed and incubated for 30 min for the development of pink colour which indicates IAA production (+) by isolates (Hartmann et al., 1983).

Phosphate solubilization

All the isolates were screened on Pikovaskaya agar medium (10 g glucose, 0.5 g yeast extract, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.2 g NaCl, 0.1 g $MgSO_4$ $7H_2O$, Trace $FeSO_4$, Trace $MnSO_4$ $7H_2O$, 5 g $Ca_3(PO_4)_2$, 18 g Agar, pH 7.0-7.2) in labelled sterile Petri plates as described by Sarker et al. (2014). Formation of clear zone around the growing isolated colony was the indication of phosphate solubilization (+) by the isolates, while the absence clear zone was the sign of no phosphate solubilization (-).

Cross-nodulation test and assessment of plant growth

Rhizobium colonies from each of the crop legumes (*S. senegal, V. seyal, C. cajan, V. unguiculata*) growing on solid medium were transferred into Erlenmeyer flasks containing 200 ml YEMA Congo Red liquid medium, where agar was reduced from 15 to 1.5 g/L. Rhizobia were allowed to grow to 10^6-10^8 cells/ml for 72 h under shaking conditions on a magnetic stirrer (Somasegaran and Hoben, 1994). A total of 18 inoculants were cultured with 1 inoculum (SGL), 3 inocula (VY1, VY2, mVY= VY1 and VY2 mixture), 5 inocula (CC1, CC2, CC3, CC4, mCC = CC1-4 mixture) and 8 inocula (VU1, VU2, VU3, VU4, VU5, VU6, VU7, mVU = VU1-7 mixture) produced from rhizobia isolated from *S. senegal, V. seyal, C. cajan* and *V. unguiculata* respectively, and 1 total mixture (mT). The *Rhizobium* isolates obtained were tested for their potential to induce the formation of efficient root nodules from the four studied crop legumes in a cross-nodulation study. To achieve this, sand was laid out in the autoclave at 121°C and filled in 1 kg sterile plastic bags. For each of the host plant (*S. senegal*, *V. seyal, C. cajan* and *V. unguiculata*), the plastic bags were disposed in a CRBD system with 19 treatments as Inocula (SGL, VY1, VY2, mVY, CC1, CC2, CC3, CC4, mCC, VU1, VU2, VU3, VU4, VU5, VU6, VU7, mVU, mT and NC = negative control), each of which was replicated in 6 plastic bags. Three surface sterilized seeds of each crop legume were sown in a plastic bag prior to inoculation with 2 ml of a specific inoculum. Plants were thinned to 1 after germination to avoid competition, and were daily watered with tap water (Somasegaran and Hoben, 1994).

Statistical analysis

The effects of different treatments were analysed by One-way ANOVA, using the plant weights and the nodule weights as variables. Means among treatments were compared with the least significant difference (LSD), using the STATGRAPHICS 5.0 programme.

RESULTS AND DISCUSSION

Morphological and cultural traits of the isolates

Three days after incubation at 28±2°C on YEMA medium, 24 colony types were identified and were represented by 12.5% *S. senegal,* 16.67% *V. seyal*, 25% *C. cajan* and 45.83% *V. unguiculata* respectively (Table 1).

All the isolates were round in shape, and reached a mean colony diameter size of 3.2 mm. Isolates appeared, either wet (22), creamy (01) or sticky (01). They were convex (23) or flat (01) in elevation. Colonies were either milky (10), white (03), yellowish (03), milky/translucent (02), white/turbid (02), pink (01), orange (01), translucent (01), and/or orange/yellowish (01) in colour. All the colonies were smooth in surface and regular in edge. Rhizobia isolates with similar characteristics from *Vigna radiata* L. were reported in India (Bhatt et al., 2013). Colony morphologies from 14 rhizobia isolates from *Crotolaria junceae* L. root nodules were also previously observed (Singha et al., 2015). Rhizobia with similar characteristics were reported from root nodules of four food legumes namely *Arachis hypogeae* L., *Glycine max* L., *Vigna subterranea* L. and *V. unguiculata* L. (Ngakou et al., 2009).

Based on these cultural characteristics, 14 distinct isolates representing 7.14, 14.29, 28.57 and 50% for *S. senegal, V. seyal*, *C. cajan* and *V. unguiculata* respectively, were considered for further studies. Isolates did not absorb Congo red at 28±2°C in the dark, indicating the absence of contaminants (Somasegaran and Hoben, 1994; Giller et al., 2016). All the isolates showed negative reaction to ketolactose test after incubation at 28±2°C for 3 days, indicating that they were all *Rhizobium* strains, but not *Agrobacteria* (Table 2). Isolates were tested Gram negative and were microscopically rod-shaped and pink in colour after treatment with iodine reagent, confirming that they belonging to the genus *Rhizobium*. Related results were obtained respectively from *Arachis hypogaea* L. and *Telfairia occidentalis* in Nigeria (Agah et al., 2016), from *Glycine max* L. in Zambia (Kapembwa et al., 2016), from *Mucuna pruriens* L. in Nepal (Paudyal and Gupat, 2017), from *V. mungo* L. and *V. radiata* L. in India (Tyagi et al., 2017). These isolates were also recognized as acid producers, since they shifted the green colour of Bromothymol blue to yellow when incubated at 28±2°C for 3 days (Table 2).

Physiological traits of the isolates

Physiological characterization of different rhizobia isolates was carried out based on their growth on YEMA medium at various salt concentrations, pH and at different incubation temperatures (Table 3). As the results, isolates grew well at 1-2% salt concentration, 28-35°C temperature range and at pH 7-8. A previous research has pointed out an optimum growth of root nodule bacteria from Mung bean at 1-2% salt, 28-37°C temperature, and at pH 7 (Bhatt et al., 2013). Optimal growth of most *Rhizobium* strains has been reported to occur at 25-30°C temperature and at pH 6-7 (O'Hara et al., 2016).

Biochemical traits of the isolates

Biochemical studies has revealed 64.29% of isolates with positive reaction to catalase test, 42.86% able to

Table 1. Colony traits in different soil types and host plants after 5 days of incubation in the dark.

Colony trait	Soil types	*S. senegal*	*V. seyal*	*C. cajan*	*V. unguiculata*
Number of colonies	Ma	1	2	2	3
	Ga	1	1	2	4
	Ng	1	1	2	4
Shape	Ma, Ga, Ng	3 Round	4 Round	6 Round	2 Double-round, 9 Round
Mean size (mm)	Ma	3	2.25	2.45	3.9
	Ga	3	3	2.65	2.45
	Ng	2.7	2.8	2.5	3.53
Appearance	Ma, Ga, Ng	3 Wet	4 Wet	5 Wet, 1 Sticky	10 Wet, 1 Creamy
Elevation	Ma, Ga, Ng	3 Convex	4 Convex	5 Convex, 1 Flat	11 Convex
Colour/texture	Ma, Ga Ng	3 M	3 M, 1 Y-O	3 M, 1 O, 1W, 1Y	2 M-T, 1M, 1P, 1T, 2 W-t, 2 W, 2 Y
Surface	Ma, Ga, Ng	Smooth	Smooth	Smooth	Smooth
Edge	Ma, Ga, Ng	Regular	Regular	Regular	Regular
Ti	Ma, Ga, Ng	3 days	3 days	3 days	3 days

Ga: Garoua, Ma: Maroua, Ng: Ngaoundere, Ti: time of incubation, M: milky, O: orangey, P: pink, T: translucent, t: turbid, W: white, Y: yellowish.

Table 2. Identification of isolates.

Host plants	Isolates	Distinctive criteria					
		CR	BTB	Ket	Cell colour	Cell shape	Gram stain
S. senegal	SGL	-	Y:AP	-	Pink	Rod	-
V. seyal	VY1	-	Y:AP	-	Pink	Rod	-
	VY2	-	Y:AP	-	Pink	Rod	-
C. cajan	CC1	-	Y:AP	-	Pink	Rod	-
	CC2	-	Y:AP	-	Pink	Rod	-
	CC3	-	Y:AP	-	Pink	Rod	-
	CC4	-	Y:AP	-	Pink	Rod	-
V. unguiculata	VU1	-	Y:AP	-	Pink	Rod	-
	VU2	-	Y:AP	-	Pink	Rod	-
	VU3	-	Y:AP	-	Pink	Rod	-
	VU4	-	Y:AP	-	Pink	Rod	-
	VU5	-	Y:AP	-	Pink	Rod	-
	VU6	-	Y:AP	-	Pink	Rod	-
	VU7	-	Y:AP	-	Pink	Rod	-

CR: Congo red, BTB: Bromothymol blue, Ket: Ketolactose, Y:AP: Yellow + Acid Production, (-): negative reaction.

solubilize starch, 71.43% positive to triple sugar iron test, 71.43% positive reaction to urease test, whereas all had very strong alpha-amylase (+++) activity (Table 4). Regarding the production of Plant Growth Promoting metabolites, 100% of isolates produced ammonia, 100% produced IAA, while only 28.57% were able to solubilize inorganic phosphate (Table 4). In a related study in Nigeria, Agah et al. (2016) reported 100% of isolates as producer of both ammonia and IAA. According to findings of Bhatt et al. (2013), none of the *Rhizobium* isolates from Mung Bean was revealed to produce IAA, neither was positive to urea tests, although other tests confirmed their characteristics as *Rhizobia*. Another result from *Crotolaria junceae* root nodules has reported 14 rhizobia isolates positive to catalase, starch and urea tests (Singha et al. 2015). The fact that some of the isolates from the present study were positive to TSI (sucrose, lactose and glucose) is in agreement with other results

Table 3. Effects of salinity (NaCl), pH and Temperature on growth of isolates.

Host plant species	Isolates	Factors and degrees of the tolerance of isolates														
		Concentration of NaCl (%)					pH				Temperature (°C)					
		1	2	3	5	10	4	7	8	9	11	28	30	35	38	42
S. senegal	SGL	++	+	-	-	-	-	+	+	+	±	+++	+++	+++	-	-
V. seyal	VY1	++	+	-	-	-	+	+	+	+	±	+++	+++	+++	++	-
	VY2	++	+	-	-	-	-	+	+	+	±	+	+++	+++	-	-
C. cajan	CC1	++	-	-	-	-	+	+	+	+	±	+++	+++	+++	-	-
	CC2	++	-	-	-	-	+	+	+	+	±	++	+++	+++	-	-
	CC3	++	+	+	+	-	+	+	+	+	±	+++	+++	+++	++	-
	CC4	++	+	-	-	-	+	+	+	+	±	+	+++	+++	-	-
V. unguiculata	VU1	++	+	+	-	-	+	+	+	+	±	+++	+++	+++	+	-
	VU2	++	+	+	-	-	+	+	+	+	±	+++	+++	+++	+++	+++
	VU3	++	+	+	+	-	+	+	+	+	±	+	+++	+++	-	-
	VU4	++	-	+	-	-	+	+	+	+	±	+++	+++	+++	++	-
	VU5	++	+	+	+	-	+	+	+	+	±	+	+++	+++	-	-
	VU6	++	+	-	-	-	+	+	+	+	±	+++	+++	+++	++	+
	VU7	++	+	-	+	-	-	+	+	+	±	-	+++	+++	-	-

(-): No growth, (±): poor growth, (+): Growth, (++): Good growth, (+++): Very good growth. 1%NaCl, pH7 and 30°C are used as positive controls for salinity, pH and temperature of incubation respectively.

Table 4. Effects of biochemical and Plant Growth Promoting trait tests on the growth of isolates.

Plant species	Isolate	Biochemical tests				Plant Growth Promoting Traits		
		Catalase	Starch	TSI	Urea	Ammonia	IAA	Phosphate
S. senegal	SGL	-	+++	+	+	+	++	-
V. seyal	VY1	+	+++	+	+	+	±	-
	VY2	+	+++	+	+	+++	+	+
C. cajan	CC1	-	+++	+	+	++	+	-
	CC2	+	-	-	+	++++	+++	+
	CC3	+	+++	-	-	++++	+++	+
	CC4	+	+++	+	+	+++	+	+
Vigna	VU1	-	-	+	-	+++	++++	-
	VU2	-	-	-	+	+++	+	-
	VU3	-	-	+	+	+++	++++	-
	VU4	+	-	+	-	+++	+++	-
	VU5	+	-	+	-	++++	++++	-
	VU6	+	-	-	+	+++	++	-
	VU7	+	-	+	+	+++	+++	-

IAA: Indole Acetic Acid, TSI: Triple Sugar Iron, (++++): excellent reaction, (+++): very strong reaction, (++): strong reaction, (+): fairly strong reaction, (±): weak reaction, (-): negative reaction.

indicating that 100% of isolates from root nodules of *S. senegal, V. seyal, C. cajan* and *V. unguiculata* (Saad, 2008), or from root nodules of *Hadysarum pallidum* Desf. (Rayane and Oussam, 2015), and were able to use carbohydrates as sole carbon sources. Moreover, 100% of isolates from root nodules of *Genista* and *Argyrolobium* genera were also shown to hydrolyse urea in Algeria (Dekak, 2010).

Table 5. Effects of cross-inoculation with rhizobia isolates on plant biomasses at 45 DAP.

Isolate	Plant biomasses (g)			
	S. senegal	***V. seyal***	***C. cajan***	***V. unguiculata***
NC	0.15±0.05[a]	0.17±0.06[a]	0.40±0.1[a]	0.86±0.47[a]
SGL	0.47±0.12[b]	0.68±0.08[bcdef]	0.75±0.52[ab]	2.23±0.20[bc]
VY1	0.73±0.25[cd]	0.91±0.12[fg]	1.88±0.16[f]	2.53±0.14[cde]
VY2	0.53±0.15[bc]	0.71±0.12[bcdefg]	1.54±0.13[def]	2.49±0.20[cde]
mVY	0.46±0.12[b]	0.57±0.06[bc]	1.17±0.31[bcd]	2.04±0.17[b]
CC1	0.64±0.05[bcd]	0.60±0.10[bcd]	1.28±0.48[cde]	2.68±0.24[ef]
CC2	0.45±0.50[b]	0.60±0.10[bcd]	1.33±0.35[cde]	3.15±0.22[g]
CC3	0.60±0.20[bcd]	0.53±0.32[bc]	1.27±0.31[cd]	2.49±0.10[cde]
CC4	0.62±0.18[bcd]	0.74±0.17[cdefg]	1.58±0.28[def]	2.70±0.26[ef]
mCC	0.68±0.24[bcd]	0.83±0.06[defg]	1.51±0.32[def]	2.02±0.13[b]
VU1	0.60±0.17[bcd]	0.88±0.24[fg]	1.78±0.17[ef]	2.54±0.13[cde]
VU2	0.62±0.18[bcd]	0.58±0.10[bcd]	1.18±0.25[bcd]	2.52±0.23[cde]
VU3	0.75±0.09[cd]	0.62±0.23[bcde]	1.50±0.26[def]	2.23±0.41[bcd]
VU4	0.58±0.13[bc]	0.53±0.23[bc]	1.18±0.32[bcd]	2.70±0.26[ef]
VU5	0.65±0.13[bcd]	0.78±0.18[cdefg]	1.44±0.17[def]	3.04±0.24[fg]
VU6	0.47±0.15[b]	0.48±0.08[b]	1.20±0.46[bcd]	2.60±0.10[de]
VU7	0.52±0.16[bc]	0.77±0.12[cdefg]	1.28±0.32[cde]	2.49±0.11[cde]
mVU	0.47±0.06[b]	0.86±0.12[efg]	1.26±0.31[cd]	2.79±0.01[efg]
mT	0.85±0.22[d]	0.95±0.13[g]	0.89±0.25[abc]	2.49±0.28[cde]
P-value	0.0031	0.0001	0.0003	< 0.0001

NC: Negative control, SE: Standard error. Data are mean plants biomasses of three replicates. For each column, values affected with the same uppercase letter are not significantly different by ANOVA; F-test = 3.51 at the indicated level of significance.

Responses of plants to cross-nodulation

The study of the effect of inoculation of one host plant with *Rhizobium* strains from another host has demonstrated that all rhizobia isolates respectively from *S. senegal* (p = 0.0031), *V. seyal* (p = 0.0001), *C. cajan* (p = 0.003) and *V. unguiculata* (p < 0.0001) had biomasses statistically higher than that of negative control. Inoculated plants of *A. senegal, A. seyal*, *C. cajan* and *V. unguiculata* with *Rhizobium* strains, mT, mVY1 and CC2 best contributed to improved plant biomasses (Table 5). Concerning nodulation, all *Rhizobium* isolates were able to form nodules on their host plant roots, except the control plants. There was a highly significant difference (p < 0.0001) between the nodule biomasses from all inoculated host plants and that of the control. Inoculation of plants with isolates SGL, mVY, mT and CC4 greatly increased the nodules biomass of *S. senegal, V. seyal*, *C. cajan* and *V. unguiculata* (Table 6). These results indicate that inoculating one host plant with rhizobia isolate from another under pot conditions positively and significantly impacted the treated host plant and nodule biomasses compared to values displayed by non-inoculated plants (Tables 5 and 6), suggesting that all the isolates were efficiently involved in biological nitrogen fixation with their four tested host legumes.

This is an advantage to the grower in terms of efficacy, reduce time and cost. The two tree legumes (*S. senegal* and *V. seyal*) had the plant and nodule biomasses consistently lower than those of the food crop legumes (*C. cajan* and *V. unguiculata*) at 45 DAP, which is obvious for the following reasons: i) growth of trees was slower than that of most herbaceous species; ii) even after scarification prior to sowing, *S. senegal* and *V. seyal*) seeds emerged after weeks, while the seed emergence of *C. cajan* and *V. unguiculata* occurred not more than after 4 DAP; iii) at 45 DAP, *C. cajan* and *V. unguiculata* already had huge biomasse, were even flowering and fruiting, while *S. senegal* and *V. seyal*) still had few leaves; iv) the nodule number and size of *C. cajan* and *V. unguiculata* was always higher than that of *S. senegal* and *V. seyal*), lining with the positive and significant correlation between the number, size and biomass of nodules reported by Ngakou et al. (2009).

These differences could probably be attributed to either the stage of plant development, or the plant sub-family, because, both *S. senegal* and *V. seyal* are perennial plants (Kew, 2016) which have a longer life cycle than that of *C. cajan* and *V. unguiculata,* which are semi-perennial and annual plants respectively (SY, 2001). Besides, responses of legumes to cross-inoculation with rhizobial inoculant may with legume species, a deeper root system exploring more soil mineral nitrogen contents

Table 6. Effects of cross-inoculation with rhizobia isolates on nodule biomasses at 45 DAP.

Isolates	Nodule biomasses (g)			
	S. senegal	***V. seyal***	***C. cajan***	***V. unguiculata***
NC	0.0±0.0[a]	0.0±0.0[a]	0.0± 0.0[a]	0.0±0.0[a]
AGL	0.35±0.03[g]	0.26±0.05[bcd]	0.68± 0.03[bc]	0.71±0.08[cd]
AY1	0.28±0.08[defg]	0.27±0.08[bcd]	0.69± 0.10[bc]	0.62± 0.06[bcd]
AY2	0.26±0.06[cdef]	0.18±0.02[b]	0.64± 0.05[bc]	0.69 ±0.09[bcd]
mAY	0.31±0.04[defg]	0.48±0.01[h]	0.64± 0.11[bc]	0.66±0.05[bcd]
CC1	0.27±0.04[cdef]	0.28±0.08[cde]	0.67± 0.07[bc]	0.71±0.08[bcd]
CC2	0.25±0.06[cde]	0.26±0.05[bcd]	0.65± 0.13[bc]	0.62 ±0.15[bcd]
CC3	0.30±0.07[efgh]	0.44±0.07[gh]	0.67± 0.04[bc]	0.73±0.09[cd]
CC4	0.27±0.05[cdef]	0.25±0.05[bcd]	0.63± 0.07[bc]	0.76±0.09[d]
mCC	0.16±0.04[b]	0.44±0.04[gh]	0.65± 0.04[bc]	0.72 ±0.08[cd]
VU1	0.02±0.01[a]	0.40±0.03[fgh]	0.60± 0.09[b]	0.64±0.07[bcd]
VU2	0.34±0.04[fg]	0.27±0.04[cde]	0.65± 0.06[bc]	0.72±0.08[cd]
VU3	0.26±0.06[cdef]	0.33±0.10[def]	0.66± 0.05[bc]	0.67±0.20[bcd]
VU4	0.25±0.05[cde]	0.23±0.04[bc]	0.66± 0.07[bc]	0.55±0.05[b]
VU5	0.30±0.06[defg]	0.31±0.06[cde]	0.66± 0.05[bc]	0.68± 0.10[bcd]
VU6	0.19±0.03[bc]	0.27±0.07[bcd]	0.62± 0.14[bc]	0.66 ± 0.09[bcd]
VU7	0.25±0.05[cd]	0.27±0.04[bcd]	0.68± 0.03[bc]	0.65±0.13[bcd]
mVU	0.30±0.03[defg]	0.36±0.03[efg]	0.66± 0.12[bc]	0.60±0.11[bcd]
mT	0.33±0.07[efg]	0.28±0.06[cde]	0.74± 0.12[c]	0.60±0.05[bc]
P-values	< 0.0001	< 0.0001	< 0.0001	< 0.0001

NC: Negative control, SE: Standard error. Data are mean plants biomasses of three replicates. For each column, values affected with the same uppercase letter are not significantly different by ANOVA; F-test = 3.51 at the indicated level of significance.

(Yates et al., 2016). Previous research have reported successful nodulation of *Vigna mungo* by all the rhizobia isolated from seven tree legumes, except rhizobia from *Abizia lebbeck* in response to inoculation under arid environment (Mahmood and Athar, 2008), in agreement with the obtained results from this atudy. Similarly, Argaw (2012) indicated that *Rhizobium leguminosarum* isolated from some Ethiopian soils was able to significantly increase plant performances through nodule numbers, nodule dry biomasses, plant dry biomasses and yields, just like Waheed et al. (2014) in a related research on pea crop in comparison to the negative control in Pakistan. Once again, this cross-inoculation experiment has confirmed the promiscuous status of rhizobia associated to food/tree crop legumes, similar to the same findings between four food crops legumes as pointed out by Gomoung et al. (2017).

Conclusion

This study has shown that all isolates possess rhizobial characteristics, and that a genetic compatibility exists between each *Rhizobium* strain and its host plant. These results suggest that all the isolated *Rhizobium* are members of the same cross-inoculation group. Hence, it would be advantageous for a farmer to use any of the inoculum to inoculate a food/tree crop legume instead of chemical fertilizer, in order to improve the soil fertility, crop yield, ecological health, and/or reduce the cost of fertilizer input. In the future, analytical knowledge on other parameters such as the Glucose Peptone-Agar test, use of other carbon source, casein test, antibiotic resistance and molecular characterization of strains could increase our understanding on inoculum production technology for the improvement of crop production.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Assessment of microbiological quality and drug resistance patterns of raw vegetables irrigated with Hasassa River, West Arsi Zone, Oromia Region, Ethiopia

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Irrigation scheme is among the major transmission modes of enteric human pathogens. Irrigation of vegetables with polluted water and untreated wastewater is a common practice worldwide. This practice is common in urban areas of low-income countries including Ethiopia, where there is increasing demands for fresh vegetables with poor wastewater treatment. The aim of the present study was to assess the microbial quality and prevalence of antimicrobial resistance of bacteria isolated from vegetables irrigated with Hasassa River, Southeastern Ethiopia. Irrigation water and vegetable samples (carrot, lettuce, and garlic) were collected from irrigation sites and analyzed for their bacteriological load and presence of any pathogenic microbes. The resistance patterns were detected following standard procedures. Appropriate serial dilutions of the suspension from 10^{-4}, 10^{-5} and 10^{-6} were spread-plated on a suitable solid media for counts of aerobic Mesophilic bacteria, Gram-negative Enterobacteriaceae and Staphylococci, and homogenized samples were heated at 80°C for 10 min in a water bath to count aerobic spore formers, respectively. The maximum overall mean counts of aerobic Mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, Staphylococci, and total coliform counts were 8.21, 6.58, 6,88, 6.88 log CFU ml^{-1} and >1100 MPN 100 ml^{-1}, respectively. The microflora of vegetable samples were dominated by *Bacillus* species (21.9%) followed by *Corynebacterium* species (12.5%), *Lactobacillus* species (12.5%). *Staphylococcus aureus* and *Salmonella* species were detected in 21.9 and 15.6% of the samples, respectively. The result of antimicrobial test showed that all the isolates 32 (100%) were resistant to Penicillin, 26 (81.3%) to Vancomycin, 23 (71.9%) to Ampicillin, 15 (46.9%) to Chloramphenicol, 15 (46.9%) to Erythromycin, and the least 3 (9.4%) to Perfloxacin. The present finding revealed that vegetables irrigated with Hasassa River appears to pose microbial contamination which may be transferred directly or indirectly during pre-harvest and post-harvest handling to fresh vegetables which potentially constitutes a health risk to consumers.

Key words: Antibiotic resistance, Hasassa River, irrigation water, vegetables.

INTRODUCTION

Despite the fact that fresh and minimally processed vegetables and fruits provide the most important human diet, consumption of fresh vegetables and fruits has been associated health risk for consumers. It has been

reported that disease outbreaks in recent years have been linked to lettuce, tomatoes, spinach, and seed sprouts particularly when freshly contaminated by foodborne pathogens (Jung et al., 2014). Surveillance of vegetables has indicated that these foods can be contaminated with various bacterial pathogens, including Shiga toxigenic *Escherichia coli* (STEC), *Staphylococcus aureus*, *Salmonella* species, *Shigella* species, *Listeria monocytogenes*, and *Campylobacter* species (Adane and Tsehayneh, 2017).

The sources of those pathogens and other microbes traverse the continuum from farm to plant and include contaminated agricultural water, soil amendments, contaminated harvesting equipment, field workers, processing plants and retail handling (Jung et al., 2014). As major component of agriculture, irrigation is considered as one of the most important transmission modes of enteric human pathogens to human through consumption of vegetables (Adane and Tsehayneh, 2017). It has been reported that irrigation of vegetables with polluted water and untreated wastewater is practiced worldwide. This practice is most common in the urban areas of low-income countries, which have no capacity to effectively treat wastewater and face increasing demands for fresh vegetables (Keraita et al., 2007). Keraita et al. (2007) also pinpointed that wastewater provides water and nutrients as important resources for irrigation, but has high levels of pathogens. The major pathogens associated with the use of highly polluted water are the fecal coliforms, *E. coli* and eggs of some helminthes such as *Ascaris lumbricoides*, *Trichuris trichiura* and others (Samuel et al., 2013).

Several studies reported the association of health risk and microbiological quality of irrigated vegetables using ponds or rivers. Studies in Jimma town, Western Ethiopia reported that the microflora of vegetables and irrigation river samples was dominated by *Bacillus* species (32.7%) followed by Enterobacteriaceae (25%) and Micrococcus (16%). Other pathogens such as *S. aureus* and *Salmonella* spp. were detected in 24.0 and 20.7% of the samples, respectively (Desta and Diriba, 2016). The study also suggested that water from the river that received both human and animal waste disposal poses a health risk due to contamination with all microorganisms of human and animal intestinal habitat.

It has been observed that Hasassa River is the primary source of water for a range of activities such as recreation, bathing, washing clothes, and household utensils, small-scale agricultural irrigation, car washing, and other uses. The deterioration of the quality of Hasassa River because of discharge of municipal wastes and urban runoff has been well observed. However, in Hasassa River, no study was reported on microbial quality of irrigated vegetables and irrigation water. Therefore, this study was initiated to assess the microbial quality of raw vegetables and their composition and prevalence of antimicrobial resistance patterns including multiple drug resistance (MDR) of the isolate bacteria identified from fresh vegetable irrigated with Hasassa River in west Arsi zone, Southeastern Ethiopia.

MATERIALS AND METHODS

Description of the study area

The study was conducted in Hasassa, West Arsi zone in Oromia Regional State of Southeastern Ethiopia. It is located at 7°10′N 39°10′E / 7.167°N 39.167°E (Figure 1). Hasassa River starts from the center of the town flowing to Melka Wakena Dam, which was built on Wabe Shebelle River. In Hasassa town and the surrounding areas, the river has been used for various domestic purposes including small-scale agricultural irrigation of vegetables and fruits.

Sample collection

Laboratory based cross sectional study design was used. A total number of 12 samples including three each vegetable (lettuce, garlic, and carrot) were randomly picked from both leaf and root aseptically cut into pieces by using 90% ethanol sterilized scissors. All vegetable samples were collected aseptically in sterilized plastic bags and transported to Adama Science and Technology University. The samples were processed for bacteriological analysis within 1 to 8 h.

Samples from irrigation water

Three irrigation water samples were collected from Hasassa River at different sites. A total of 0.75 L water samples were collected at the open surface flowing along the river course at the same time of the irrigation period. Samples were aseptically collected in sterile glass bottles maintained at 4°C in a cooler box and taken to the Laboratory of Microbiology, Department of Applied Biology, Adama Science and Technology University.

Sample processing

Each vegetable samples (unprocessed and large sized) were aseptically chopped into smaller pieces. A 25 g of subsample of each vegetable was aseptically weighed and vigorously shaken in 225 ml of sterile 0.1% (w/v) peptone water for 3 min to homogenize the samples. They were prepared to appropriate serial dilutions from 10^{-4}, 10^{-5} and 10^{-6}. They were spread-plated on a suitable solid media and incubated aerobically at 37°C for 24 to 48 h. Similar procedures were followed for water samples.

Bacterial counts

A volume of 0.1 ml aliquot of appropriate dilution was spread-plated in duplicates on pre-solidified Plate Count Agar, MacConkey Agar and Mannitol Salt Agar for counts of aerobic Mesophilic bacteria,

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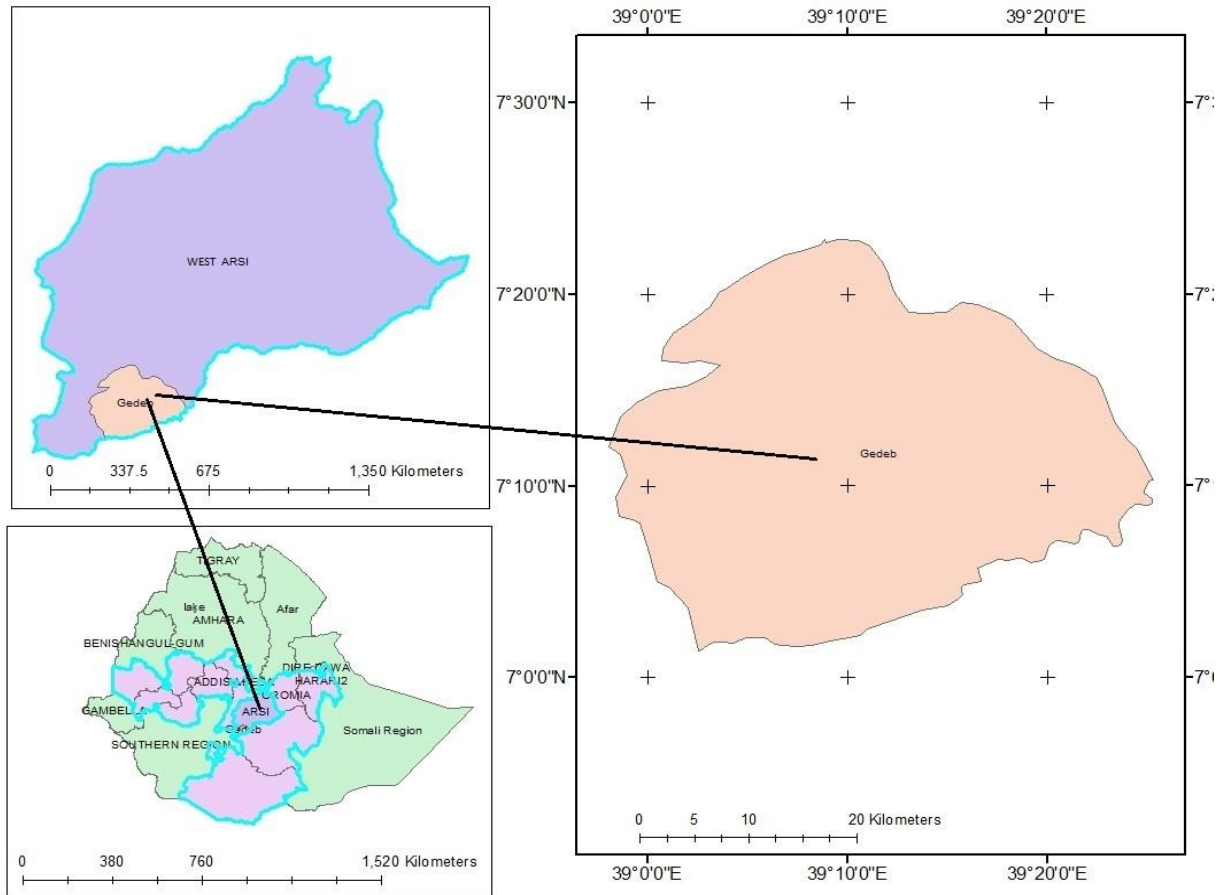


Figure 1. Map of study area with its longitude and latitude during April-July, 2018

Enterobacteriaceae and Staphylococci, respectively. Homogenized samples were heated at 80°C for 10 min in a water bath to count aerobic spore formers. The inoculated plates were incubated at 32 to 37°C for 24 to 48 h. The mean colony counts on each given dilution were used to estimate the total viable count for the samples in colony forming units (CFU ml^{-1}). Liquid sample to be tested was diluted serially and inoculated in lactose broth. The number of total coliforms was determined by counting the number of tubes giving positive reaction (both color change and gas production) compared with standard statistical tables and the results were expressed as MPN 100 $m^{-}l^{1}$.

Isolation and characterization of dominant microflora

After enumeration of aerobic Mesophilic bacteria, 21 colonies with distinct morphological characteristics of colonies such as color, size, and shape were randomly picked from countable plates and aseptically transferred into a tube containing 5 ml nutrient broth and incubated at 30°C for 24 to 48 h. The cultures were purified by repeated plating and pure cultures were temporarily preserved on nutrient agar slants at 4°C. An overnight activated culture of each isolates was further characterized by inoculating to the following standard tests such as cell morphology, endospore staining, Gram staining, motility, catalase, indole test, urease test, carbohydrate fermentation/utilization test, hydrogen sulfide production (H_2S), methyl red (MR) test, citrate utilization test, and oxidation fermentation (O/F) according to the protocol used by Oluyege et al. (2015) to differentiate into genus and family levels.

Isolation and identification of bacterial pathogens

For the identification of *S. aureus*, yellow colonies on Mannitol Salt Agar plates were aseptically picked and transferred into 5 ml nutrient broth and incubated at 37°C for 24 to 36 h for further purification. Then, a loop of culture from the nutrient broth was streaked on pre-solidified surface of nutrient agar supplemented with 0.75% sodium chloride and again incubated at 37°C for 24 to 36 h. Finally, the distinct colonies were characterized using the established microbiological methods such as Gram staining and preliminary biochemical tests (the catalase).

For isolation of *Salmonella* spp., 25 g or 25 ml of each sample was aseptically transferred into sterile flask, containing 225 ml buffered peptone water (BPW), and inoculated onto the *Salmonella-Shigella* (SS) agar and incubated at 35°C for 24 h. The presumptive *Salmonella* colonies were then sub-cultured by streaking onto the fresh SS agar and incubated for 24 h at 37°C. Since the selective media was used for the isolation, the presumptive *Salmonella* isolates were identified by two confirmatory biochemical tests, triple-sugar-iron (TSI) agar test. The presumptive *Salmonella* colonies were directly stabbed into the TSI agar slant and incubated with loosened caps for 24 h at 37°C. For the urease test, two loopful of pure and well-isolated *Salmonella* colonies were inoculated into the urea broth. The inoculated tubes were incubated with loosened caps for 48 h at 35°C in an incubator. The TSI agar was checked

Table 1. Bacterial counts from irrigation water and vegetable samples irrigated with Hasassa River during April-July, 2018

Samples type	Average of total viable bacteria in log of CFU mL^{-1} ± S.D				
	AMB	Enterobacteriaceae	ASF	Staphylococci	Total coliform MPN100 ml^{-1}
Carrot	7.62 ± 0.40	5.59 ± 0.11	6.58 ± 0.58	6.38 ± 0.7	>1100 ± 0
Lettuce	8.21 ± 0.12	6.58 ± 0.58	6.88 ± 0.49	6.88 ± 0.52	813 ± 496.5
Garlic	6.76 ± 0.55	5.95 ± 0.66	5.68 ± 0.45	6.12 ± 0.57	191 ± 84.9
Irrigation water	7.09 ± 0.16	6.05 ± 0.49	6.77 ± 0.53	6.40 ± 0.61	>1100 ± 0

AMB: Aerobic mesophilic bacteria; ASF: Aerobic spore formers.

for the production of hydrogen sulphide (H_2S) gas, while the urease test was checked for the degradation of urea.

Antimicrobial susceptibility testing

Once the bacteria were isolated and identified from each sample, the standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility profiles of the isolates. Bacterial inocula were prepared by suspending the freshly grown bacteria in 4 to 5 ml sterile nutrient broth and the turbidity was adjusted to that of a 0.5 McFarland standard prepared from adding 0.5 mL of 1.0% $BaCl_2$ to 99.5 mL of 1% H_2SO_4 solution. The antimicrobial susceptibility testing was performed using Mueller-Hinton agar medium against selected antibiotics, namely: Chloramphenicol 30 µg, Penicillin g 10 µg, Vancomycin 30 µg, Erythromycin 15 µg, Perfloxacin 5 µg, and Ampicillin 10 µg were obtained from pharmacy. The bacterial suspension was aseptically streaked uniformly on the entire agar surface using sterile cotton swab in three different directions by rotating the plates at 60° angles after each streaking. The Petri plates were left to dry for 20 min. Afterwards, the antibiotics containing discs were placed on the agar surface with sterile forceps and pressed carefully down to ensure contact. The plates were incubated aerobically at 35°C for 18 to 24 h. The plates were examined after incubation for diameters of zone of inhibition around the discs, which was measured using ruler (mm). The data obtained was interpreted with reference to CLSI (2013). The criteria used to select the antimicrobial agents tested in this study were based on availability and frequency of prescription of the drugs for the management of bacterial infection in Ethiopia. *E. coli* (ATCC25922) and *S. aureus* (ATCC6538) standard strains from Ethiopian Institute of Biodiversity were used as reference strains for quality control of the antibiotics used.

Data analysis

Bacterial counts were calculated as colony forming units per milliliter (CFU ml^{-1}) and converted into log10 values before computing means and standard deviations. Total coliform was expressed by MPN100 ml^{-1}. Then mean of bacterial counts and standard deviation was calculated using Microsoft Excel. The statistical analysis was performed by single factor (ANOVA) at $p<0.05$.

RESULTS

Bacterial counts

The mean bacterial counts (log CFU ml^{-1}) of aerobic Mesophilic bacteria, Enterobacteriaceae, aerobic spore formers, and Staphylococci were determined for the three vegetable and irrigation water samples. The average microbial counts of different vegetable samples of selected sites ranged from 5.59 to 8.21 log CFU ml^{-1} and MPN of total coliforms count and their overall mean in vegetables ranged from 191 to >1100 and in irrigation water total coliform was >1100 (Table 1). Mean counts of all bacterial groups including coliforms identified from the samples showed statistical significance at $p< 0.05$.

Analysis of microflora and the pathogens

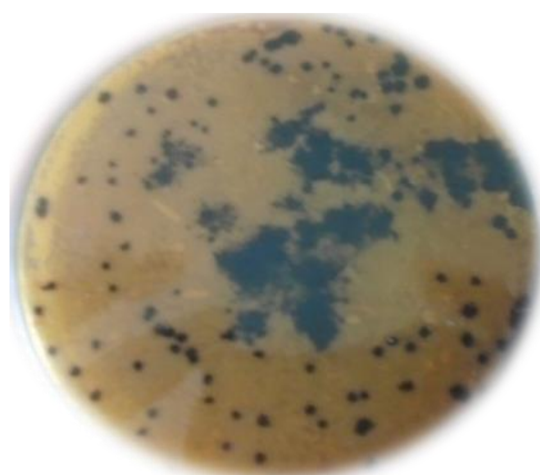
In the present study, eight genera of Gram positive and Gram-negative bacterial species were identified. Aerobic mesophilic bacterial flora identified from the water samples and different vegetables collected from the downstream of Hasassa River were dominated by *Bacillus* spp. (21.88%) followed by *Corynebacterium* species (12.5%) and *Lactobacillus* species (12.5%) and the least isolation was for *Neisseria* species (3.13%) (Table 2). Lettuce was the vegetable most contaminated, followed by the samples of carrot, irrigation water and garlic (Table 2).

S. aureus was observed fermenting Mannitol and producing a yellow zone surrounding colony on MSA plates from which isolated pure colonies were confirmed by salt tolerance and catalase test (Figure 2). Other colonies displayed typical *Salmonella* spp. morphological characteristics on *Salmonella-Shigella* agar, which were clearly with a black spot in the center due to H_2S gas production on *Salmonella-Shigella* agar (Figure 2). The presumptive colonies were scored to be *Salmonella* spp. after the colonies showed positive reaction to triple sugar iron test and negative to urease test.

S. aureus and *Salmonella* spp. isolated by special selective media accounted for a percentage of 21.88 and 15.6%, respectively, of the total isolates (Table 2). The distribution of these pathogens varied depending on the nature of vegetables. With regard to sample types, higher proportion (40.0%) of *Salmonella* spp. was encountered in lettuce while same proportion (28.6%) of *S. aureus* was identified from lettuce, garlic, and irrigation water, but relatively less proportion (14.2%) from carrot (Table 2).

Table 2. Prevalence of bacteria isolated from irrigation water and vegetables in Hasassa during April-July 2018.

Isolate	Sample					
	Irrigation water	Carrot	Garlic	Lettuce	Total isolates	Prevalence (%)
E. coli	1 (50)	1 (50)	-	-	2 (100)	6.25
Bacillus spp.	-	2 (31.7)	1 (14.3)	4 (57.1)	7 (100)	21.88
Corynebacterium spp.	2 (50)	1 (25)	1 (25)	-	4 (100)	12.5
Streptococcus spp.	1 (50)	1 (50)	-	-	2 (100)	6.25
Staphylococcus aureus	2 (28.6)	1 (14.2)	2 (28.6)	2 (28.6)	7 (100)	21.88
Salmonella spp.	1 (20)	1 (20)	1 (20)	2 (40)	5 (100)	15.6
Neisseria spp.	-	-	-	1 (100)	1 (100)	3.13
Lactobacillus spp.	-	1 (25)	1 (25)	2 (50)	4 (100)	12.5
Total	7 (21.88)	8 (25)	6 (18.75)	11 (34.37)	32 (100)	100



Black colonies on *Salmonella*-*Shigella* agar

Yellow colonies on MSA agar

Figure 2. Presumptive *Salmonella* spp. on SS agar and *Staphylococcus aureus* on mannitol salt Agar from Hasassa River and irrigated vegetables during April to July, 2018.

However, the prevalence of all bacterial microflora and pathogens was not significantly different at p<0.05 with respect to sample types.

Antimicrobial resistance patterns of bacterial isolates

The proportion of antibiotic resistance was observed for 32 isolates against six different antibiotics of which the highest 32 (100%) isolates were resistant to penicillin g 10 µg followed by Vancomycin 30 µg with 26 (81.25%) and the least 3 (9.4%) to Perfloxacin 5 µg (Table 3).

Multiple drug resistance patterns of bacterial isolates

Patterns of multiple drug resistance (MDR) among bacterial isolates of the present study varied from two to six antibiotics. The maximum MDR was recorded to all the six antibiotics under experiment, namely Vancomycin (Van), Chloramphenicol (Chlora), Penicillin (Penic), Erythromycin (Eryth) Ampicillin (Ampic) and Perfloxacilin (Perf), followed by all of the antibiotics except Perf (Table 4). The highest MDR patterns were observed in *Bacillus* spp., *S. aureus* and *Salmonella* spp. respectively, with combination of (Van, Ampic), (Penic, Ampic), and (Van, Peni) antibiotics (Table 4).

DISCUSSION

Microbial groups that belong to eight genera were isolated from the examined samples at varying percentages with Gram-negative flora accounting for 75 and 25%, respectively. The Gram-positive cells were represented by bacteria from the genera: *Bacillus* spp., *Lactobacillus* spp., *Corynebacterium* spp., *S. aureus,* and *Streptococcus* species, while the Gram-negative microflora constituted

Table 3. Proportion of antibiotic resistance among bacterial isolates identified from vegetable samples and irrigation water in Hasassa during April-July, 2018.

Identified isolate	Total (%)	Vancomycin 30 µg	Chloramphenicol 30 µg	Perfloxacin 5 µg	Penicillin g 10 µg	Ampicillin 10 µg	Erythromycin 15 µg
Bacillus spp.	7 (100)	5 (71.4)	2 (28.6)	-	7 (100)	4 (57.1)	1 (14.3)
E. coli	2 (100)	2 (100)	2 (100)	1 (50)	2 (100)	2 (100)	1 (50)
Corynebacterium spp.	4 (100)	3 (75)	1 (25)	1 (25)	4 (100)	3 (75)	-
Streptococcus spp.	2 (100)	1 (50)	2 (100)	-	2 (100)	2 (100)	-
Lactobacillus spp.	4 (100)	3 (75)	3 (75)	1 (25)	4 (100)	1 (25)	3 (75)
Neisseria spp.	1 (100)	1 (100)	1 (100)	-	1 (100)	1 (100)	1 (100)
Staphylococcus aureus	7 (100)	6 (85.71)	3 (42.86)	-	7 (100)	7 (100)	6 (85.71)
Salmonella spp.	5 (100)	5 (100)	1 (20)	-	5 (100)	3 (60)	3 (60)
Total	32 (100)	26 (81.25)	15 (46.9)	3 (9.4)	32 (100)	23 (71.9)	15 (46.9)

Table 4. Multiple drug resistance patterns in isolates identified from irrigation water and vegetables in Hasassa during April-July, 2018.

Isolate	No. of antibiotics	Antibiotic resistance pattern observed	No. of isolates that demonstrated resistance
Bacillus spp.	5	Van, Chlora, Peni, Eryth, Ampic	1 (7)
	4	Van, Chloram, Penic, Ampic	2 (7)
	3	Van, Peni, Ampic	4 (7)
	2	Van, Ampic	5 (7)
E. coli	6	Van, Chlora, Peni, Eryth, Ampic, Perf	1 (2)
	4	Van, Chlora, Penic, Ampic	2 (2)
	2	Van, Penic	2 (2)
Corynebacterium spp.	5	Van, Perf, Peni, Chlora, Ampic	1 (4)
	3	Ampi, Peni, Van	3 (4)
Streptococcus spp.	3	Van, Peni, Ampic	1 (3)
	2	Penic, Ampic	2 (3)
Lactobacillus spp.	6	Van,Chlora,Peni,Eryth,Ampic,Perf	1 (4)
	4	Van, Chlora, Peni, Eryth	3 (4)
Neisseria spp.	5	Van, Chlora, Peni, Eryth, Ampic	1 (1)
S. aureus	5	Van, Chlora, Peni, Eryth, Ampic	2 (7)
	4	Eryth, Peni, Van, Ampic	4 (7)
	2	Penic, Ampic	5 (7)
Salmonella spp.	5	Van, Chlora, Peni, Eryth, Ampic	1 (5)
	4	Eryth, Ampic, Van, Peni	3 (5)
	2	Van, Peni	5 (5)

Vancomycin =Van, Penicillin =Peni, Ampicillin =Ampic, Chloramphenicol = Chlora, Perfloxacilin = Perf, Erythromycin =Eryth.

members of *E. coli, Neisseria* spp., and *Salmonella* spp. This finding is partly similar to the previous reports by Ankita et al. (2014) in raw fruits and vegetables from India.

The predominant microflora of fresh vegetables in the present study was *Bacillus* spp. that occurred in all samples except in irrigation water, followed by *Corynebacterium* spp. and *Lactobacillus* spp. The predominance of *Bacillus* isolates in this study among the Gram-positive bacteria was in agreement with Desta and Diriba (2016). *Salmonella* spp. isolates were the dominant bacteria among Gram-negative isolates contrary to the previous reports by Biniam and Mogessie (2010) on lettuce and green pepper from Ethiopia which showed the dominance of *Pseudomonas* isolates.

Gram-positive bacteria could be higher effect in the spoilage of vegetables than Gram-negative bacteria. From wastewater-irrigated and manure-treated farmland of Nigeria samples, Oluyege et al. (2015) reported the predominant genera that include *Bacillus* spp. (33%), *S. aureus* and *Pseudomonas* species (15%). The presence of endospores that could make more resistant than the vegetative cells to harsh weather conditions and even to antimicrobial treatments may result in high percentage of *Bacillus* spp.

Corynebacterium spp. is inhabitants of the soil. Predominance of *Corynebacterium* spp. in the present study may come from the soil or from fecal-contaminated water used for irrigation and received sewage from diverse sources. Ankita et al. (2014) also reported dominant bacteria such as *Corynebacterium* spp., *Staphylococcus* spp., *Bacillus* spp., *Streptococcus* spp., *Lactobacillus* spp. and *Pseudomonas* spp. respectively, from raw fruits and vegetables. Oladele and Olakunle (2011) reported that the presence of *Bacillus subtilis*, *Serratia marcescens*, *Lactobacillus* spp. and *Proteus mirabilis* on the deteriorated spinach samples may be linked to the fact that these microbes are widely distributed in air, dusts and soils.

The prevalence of *S. aureus* in the current study was 21.9% lower as compared to 51.5% of the report of Halablab et al. (2011) from Lebanon. The presence of *S. aureus* (28.6%) in the irrigation water in this study was lower than those obtained by Ikpeme et al. (2011) (25 to 33%) from two rivers used for irrigation of vegetables in South Africa. On the other hand, the overall mean count of staphylococci from vegetable samples ranged from 6.12 to 6.88 log CFU ml^{-1}. This is higher than the bacterial count of 4.55 and 4.97 log CFU ml^{-1}, reports on lettuce and green pepper, respectively from super market in Addis Ababa, Ethiopia (Biniam and Mogessie, 2010). The relatively higher counts of staphylococci from the present study may be due to skin contact and environmental contamination. It has been reported that *S. aureus* is a dangerous pathogen with which surface of vegetables may be contaminated through human handling and other environmental factors and which is also able to survive for several weeks (Halablab et al., 2011). Human skin and nasal cavity are the main reservoir of staphylococci.

In the present study, the prevalence of *Salmonella* spp. in all vegetable samples was higher (15.6%) as compared to previous reports by Biniam and Mogessie (2010) which reported 10% in lettuce and green peppers. This difference might be attributed to the variation of the test techniques employed (pre-enrichment steps), the origin of sample or geographical differences and difference in management practice. The presence of *E. coli* can represent the existence of fecal pathogens like *Salmonella* and *Shigella*. Thus, it can be good indicator of poor sanitary conditions of sources of water used and the use of water with fecal contamination.

In the present study, another important point was antimicrobial resistance rate was high and this may cause a serious challenge to the management of common infections. The overall resistance rates among the bacteria isolates remarkably, demonstrated 100% resistance to Penicillin g, 81.25% to Vancomycin, 71.9% to Ampicillin, and 46.9% were resistant to Chloramphenicol and Erythromycin, while least resistance (9.4%) was observed for Perfloxacilin. Similar study reports from two Spanish Lakes showed 71% resistance of isolates to at least one antibiotic including Penicillin (68.9%), Erythromycin (31.1%) and Chloramphenicol (22.2%) (Maria, 2013). Similarly, Golly et al. (2016) reported that all the Gram-positive bacteria isolates showed 100% overall total resistance to Penicillin, Ampicillin and Perfloxacilin. The antibiotic resistance pattern obtained in this study is a serious challenge to public health because of the higher demanding for raw vegetables in different homes, societies and functions.

Twenty different multiple resistance patterns were observed when analyzed with the number of antibiotics (Table 4). Only two isolates (*E. coli* and *Lactobacillus* spp.) were resistant to all six antibiotics tested and six isolates were resistant to only five antibiotics. Relatively higher proportion 19 (55.9%) of isolates showed resistance to any of the two antibiotics and 14 (41.1%) of them were resistant to four antibiotics, 8 (23.53%) of them were resistant to Vancomycin, Penicillin and Ampicillin, respectively. Multi antibiotic resistance (MAR) bacteria which were isolated also showed some level of resistance to almost all antibiotics tested. Such multi antimicrobial resistance patterns clearly indicate vegetables were potential vehicle for microbial food poisoning as well as a source of infectious diseases that cannot be treated with commonly used antibiotics.

There have been reports that bacteria are able to gain antibiotic resistance via mechanisms like mutational changes or acquisition of resistance genes through horizontal gene transfer from other bacteria or phages in different environments (Vaz-Moreira et al., 2014; Munita and Arias, 2016). The resistance ability of the isolates can be transferred from to another, through the antibiotic resistance plasmids (Olsen et al., 2004). Long ago, bacteria resistant to multiple drugs were found mostly in hospitals, where antimicrobial agents were used most extensively, however, resistance is currently found

anywhere almost as frequently in the community. This is therefore to emphasize the need to be given to good hygienic practices, proper handling, storage and retail of fresh vegetables in a sanitized environment.

Concerning the level of contamination among the target vegetables, the highest bacterial load in lettuce samples probably due to the larger surface area exposed to irrigation water. This is in line with the work of Halablab et al. (2011) which reported that lettuce carried higher incidence of *E. coli* and *S. aureus* organisms (42.30 and 50%) than parsley samples (13.80 and 37.93%), and the higher microbial loads on lettuce samples than parsley counterpart may be due to the large surface area of the former leaves. Other study also showed that high bacteria counts could likely be associated with the morphology of leaves which have a broad and rather rough surface, indeed, in both vegetables their large surface made easily coming in contact with the ground and the irrigation water (Cinzia et al., 2015).

The overall mean aerobic mesophilic count observed in the present study ranged from 6.76 to 8.21 log CFU ml^{-1}, relatively higher than previous reports (6.94 to 8.06 log CFU ml^{-1}) from Ethiopia, by Desta and Diriba (2016). However, lower bacterial counts that ranged from 6 to 7 log CFU ml^{-1} were reported by Daniele et al. (2013).

The overall mean count of Enterobacteriaceae in the present study ranged from 5.56 to 6.88 log CFU ml^{-1}. This was lower than previous study conducted in Ethiopia by Desta and Diriba (2016) on lettuce, carrot and tomato which ranged from 6.09 to 7.10 log CFU g^{-1}, but higher than the microbial load of lettuce and green pepper 5.08 and 4.84 log CFU g^{-1}, respectively as reported by Biniam and Mogessie (2010) in Ethiopia. According to Gilbert et al. (2000) guideline recommended for fresh fruit and vegetables in London, overall mean counts log CFU g^{-1} of Enterobacteriaceae in carrot (7.10), cabbage (6.70), tomato (6.24), and lettuce (6.09) revealed unsatisfactory level (≥4 log CFU g^{-1}). Biniam and Mogessie (2010) suggested that the high level of Enterobacteriaceae in vegetables might indicate that the water used for irrigation could be heavily contaminated with fecal matter from sewerage effluent.

In case of aerobic spore formers, the overall mean counts ranged from 5.68 to 6.88 log CFU ml^{-1}. In all vegetables, the counts were higher compared to reports by Biniam and Mogessie (2010) where the counts ranged between 3.47 and 3.50 log CFU ml^{-1} in green pepper and lettuce, respectively, from Addis Ababa (Ethiopia).

The overall mean counts of total coliforms from vegetable samples in the present study were relatively lower except for carrot >1100 MPN 100 ml^{-1} than the report of Nipa et al. (2011), who observed >1100 MPN 100 ml^{-1} from salad vegetables. The observed difference in the counts could be attributed in part to the degree of original contamination, storage conditions, and the hygienic conditions of utensils and vegetables handlers. The total coliform counts from water samples in the present study were >1100 MPN 100 mL^{-1} which was higher than the WHO recommended standard. According to the standard, the fecal coliform level must not exceed 1000 counts 100 ml^{-1} for the safe use of wastewater for irrigation of vegetables. The presence of coliforms might be attributed to cattle faeces and excretion by farmers and others who use the farm environment as toilet.

Conclusion

The present study revealed the potential hazard of raw vegetables (lettuce, carrot and garlic) collected from Gedeb Asassa areas, which were irrigated by Hasassa River. This study was the first to evaluate the microbiological quality of vegetables grown by Hasassa River, where these vegetables harbored high microbial loads including aerobic mesophilic bacteria, coliforms, Enterobacteriaceae, aerobic spore formers, and staphylococci might be due to irrigation by untreated contaminated water in that area. The large number of aerobic mesophilic bacteria, indicator organisms (coliforms and *E. coli*) and pathogens (*S. aureus* and *Salmonella* spp.) detected in the vegetable samples revealed that the contamination of these foods by pathogenic microorganism might present a potential health hazard to consumers in the area. The *in vitro* assay result of the present study showed that irrigation water and samples (lettuce, carrot and garlic) contained bacteria with multiple antibiotic resistance patterns. This may be caused by high concentrations of microorganisms, nutrients, and antibiotics found in contaminated water makes it a favorable environment for bacterial growth and horizontal gene transfer of resistant genes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Leishmanicidal, cytotoxic, antimicrobial and enzymatic activities of *Diaporthe* species, a mangrove-isolated endophytic fungus

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This study evaluated the biotechnological potential of the endophytic fungus *Diaporthe* species, which is isolated from Brazilian mangroves. *In vitro* studies have investigated the antimicrobial activity of *Diaporthe* spp. against human pathogens, such as *Escherichia coli*, *Staphylococcus aureus*, *Salmonella enteritidis*, and *Candida albicans*, as well as phytopathogenic fungi, such as *Colletotrichum* species, *Ceratocystis paradoxa*, *Fusarium oxysporum*, *Phythophthora sojae* and *Rhizopus microspores*. Additionally, the enzyme production and leishmanicidal and cytotoxic activities of *Diaporthe* spp. were tested. Minimal inhibitory concentrations (MICs) of the crude extract of *Diaporthe* spp. 94 ranged from 756 to 949 µg.mL^{-1} (MIC_{50}) and from 3.094 to 7.082 µg.mL^{-1} (MIC90); the antagonism index (AI) ranged from 20 to 62% with type A interactions ("deadlock" with mycelial contact). An assay to detect enzyme production showed that this endophytic fungus produced the enzyme cellulase. The crude extract of *Diaporthe* spp. also demonstrated activity against the promastigote form of *Leishmania infantum chagasi*, exhibiting 90% cell death at 6.000 and 10.000 µg.mL^{-1} and 80% cell death at 4.000 µg.mL^{-1}. Cytotoxicity tests on cultured human skin fibroblasts (the HFF-1 cell line) indicated cell viability of between 73 and 55% at 4.000 to 10.000 µg.mL^{-1} at 24 h; between 69 and 56% at 4.000 to 8.000 µg.mL^{-1} for 48 h, and 59% at 4.000 to 6.000 µg.mL^{-1} for 72 h.

Key words: *Diaporthe* species, endophytic fungus, leishmanicidal activities, cytotoxic activities, antimicrobial activities, enzymatic activities.

INTRODUCTION

Mangroves are ecosystems located in transition areas between terrestrial and marine environments, which has forced them to adapt to such conditions as high salinity, tidal flooding, high temperatures, anaerobic soil, and large amounts of sludge. Thus, mangroves represent a useful ecosystem for identifying plants and microorganisms that have ecological, morphological, biological, and physiological adaptations and survive under these particular conditions (Jalgaonwala et al., 2011; Sebastianes et al., 2013). Endophytes are fungi or

bacteria that colonize plants from the inside in symbiotic relationships, causing no visible harm to the host (Strobel, 2018). However, depending on external factors, such as environmental conditions and the host, endophytes may switch between pathogenic and commensal or mutualistic lifestyles (Bacon et al., 2008; Fesel and Zuccaro, 2016). Since the identification of the first endophytic fungus, significant attention has been devoted to the identification of new bioactive compounds that can be synthesized by them (Sebastianes et al., 2012; Sebastianes et al., 2013; Strobel, 2018). This research provides an alternative to plant exploitation, thereby facilitating the conservation of flora diversity, which is increasingly under threat worldwide (Zheng et al., 2015; Gurgel et al., 2020), as well as reducing the market value of these biomolecules (Strobel and Daisy, 2003; Sebastianes et al., 2012; Bibi et al., 2020). The broad spectrum of biological activity exhibited by endophytic fungal isolates enables, among other things, the exploration of substances with potential for antimicrobial and enzyme production, which can be achieved on a large scale through fermentation and presents considerable potential for industrial applications (Dezam et al., 2017; Marques et al., 2018; Yan et al., 2018; Gurgel et al., 2020). Several studies have described endophytic fungi with antimicrobial activities (Ratnaweera et al., 2015; Bezerra et al., 2015; Campos et al., 2015; Chen et al., 2016, 2019; Khan et al., 2016; Deshmukh et al., 2020) and enzymatic activities in several plant species and mangroves from different regions (Bezerra et al., 2015; Martinho et al., 2019).

Secondary metabolites produced by endophytic fungi also have promising applications in phytopathogen control (Macías-Rubalcava and Sánchez-Fernández, 2017), as demonstrated by studies employing antagonism tests.

Endophytes may present activity against human parasites that are commonly related to neglected diseases that infect millions of people worldwide, especially in developing countries. Among these parasites, *Leishmania infantum chagasi*, the aetiological agent of visceral leishmaniasis, causes high morbidity and mortality (Maran et al., 2016).

The most common form of leishmaniasis is the cutaneous form. Cutaneous leishmaniasis is caused by several species of *Leishmania*, including *Leishmania braziliensis* and *Leishmania amazonensis*. However, this leishmaniasis is the least severe and lethal. A number of studies have demonstrated activities against this type of leishmaniasis (Santiago et al., 2011; Cota et al., 2018). To date, there have been no studies investigating the role played by related endophytic fungi from mangroves in producing secondary metabolites with leishmanicidal activity against *L. infantum chagasi*.

Fungi from marine environments grow under particular conditions that can contribute to the synthesis of new compounds (Sebastianes et al., 2012). In this context, endophytic fungi associated with plants that inhabit mangrove ecosystems may represent a promising source of secondary metabolites that have not been fully elucidated to date. The present study evaluated the *in vitro* antimicrobial, antagonistic and leishmanicidal activities, enzyme production, and cytotoxicity activities of secondary metabolites produced by the endophytic fungus *Diaporthe* species 94 strain isolated from Brazilian mangroves.

MATERIALS AND METHODS

Microorganisms

The endophytic fungus *Diaporthe* spp. FS-94(4) strain (https://www.ncbi.nlm.nih.gov/nuccore/HQ022906.1) was isolated from *Avicennia nitida* branches in mangrove areas of São Paulo State, Brazil (Sebastianes et al., 2013). The phytopathogens *Colletotrichum* species, *Ceratocystis paradoxa*, *Fusarium oxysporum*, *Phythophthora sojae*, and *Rhizopus microsporus* were selected for antagonistic assays. The human pathogen strains *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (ATCC 19196), *Staphylococcus aureus* (ATCC 6538) and *Candida albicans* (ATCC 10231) were selected from the American Type Culture Collection (ATCC) for antimicrobial assays. All microorganisms were deposited in the Microbiology and Biomolecules Laboratory (LaMiB), Microorganism Collection, Morphology and Pathology Department, Federal University of São Carlos, Brazil.

L. infantum chagasi (MHOM/BR/1972/LD), deposited in the collection of Inflammation and Infectious Diseases Laboratory (LIDI), Federal University of São Paulo, Brazil, and human skin fibroblasts from a cell line (HFF-1) deposited in the collection of the National Institute of Physics of Optics and Photonics Science and Technology, University of São Paulo, Brazil, were tested via cytotoxicity assays.

Culture conditions and obtaining crude extracts

To obtain crude extracts (CE), the *Diaporthe* spp. 94 strain was grown in Petri dishes containing medium potato dextrose agar (PDA) (KASVI^{TM}, Brazil) for 7 days at 28°C. Eight discs (5 mm diameter) of mycelia from these cultures were taken from the plates and inoculated into 500-mL Erlenmeyer flasks containing 200 mL of potato dextrose liquid medium (PD) (KASVI^{TM}, Brazil) followed by incubation for 7 days at 28°C in the absence of light and static conditions. The culture medium containing metabolites was separated from the mycelium by a 1-μm paper filter and subsequently utilized in biological assays.

Antimicrobial activity assays

Broth microdilution techniques were employed in 96-well

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microplates. The inoculum was standardized at 0.5 on the scale of McFarland (1.5 × 10^8) (McFarland, 1907) and carried out a dilution of 1:10 in Mueller Hinton (MH) broth (KASVI™, Brazil), obtaining a bacterial suspension of 1.0 × 10^7 colony forming units (CFU/mL). Serial dilutions of the CE were prepared in MH broth at concentrations of 10.000 to 1.000 μg.mL^{-1}. Penicillin and streptomycin (GIBCO, USA) were employed as antimicrobial agent standards at a concentration of 2.500 μg.mL^{-1} was added to 100 μL of bacterial cells adjusted to the concentration 0.5 on the McFarland scale and 100 μL of the crude extract of each concentration, obtaining a final volume of 200 μl per well. The microplates were incubated at 37°C for 24 h. After incubation, 20 μl of Alamar Blue® (Sigma-Aldrich, USA) was added to each well for a final concentration of 0.01%. Subsequently, the microplates were read at 570 nm in a fluorescence spectrophotometer (SPECTRAMAX M3).

Antagonism assays

A mycelial disc (5 mm in diameter) taken from a 7-day culture of the *Diaporthe* 94 (4) strain on PDA was placed at one edge of a 15-cm Petri dish containing PDA (KASVI™, Brazil). On the same day, an inoculum of each phytopathogen to be tested (5-mm-diameter mycelial disc) was added to another edge of the plate at a distance of 2 cm from the margin. The plates were incubated at 28°C and observed for 10 consecutive days. Plates inoculated with phytopathogens alone were employed as controls. The tests were performed in triplicate, and the analysis of endophytic-phytopathogen interactions was based on the scale of Badalyan et al. (2004) according to 3 types (A, B and C) and 4 subtypes (CA1, CA2, CB1, and CB2), where A means deadlock with mycelial contact, B means deadlock at a distance, C means replacement and overgrowth without initial deadlock, CA1 means partial and complete replacement after initial deadlock with mycelial contact, CA2 means complete replacement after initial deadlock, and CB1 and CB2 mean partial and complete replacement after initial deadlock at a distance, respectively. The antagonism index (AI) was calculated after 10 days of incubation using the formula of Campanile et al. (2007): AI = (RM – rm) / RM × 100, where rm is the ray of the colony towards the antagonist, and RM is the average of the three rays of the colony in the other directions.

Enzymatic activity

The *Diaporthe* 94 (4) strain was evaluated for enzymatic activity using a qualitative method. The following enzymes were analysed: amylase (Buzzini and Martini, 2002), cellulase (Teather and Wood, 1982), protease (Castro et al., 2014), lipase and esterase (Sierra, 1957). For these tests, the endophytic strain was previously grown on a 15-cm Petri dish containing PDA at 28°C for 8 to 12 days, and discs of mycelium (5 mm in diameter) were inoculated onto the centre of plates containing specific medium for each enzyme. The plates were incubated at 28°C, and the enzymatic activity was observed based on the presence or absence of halos surrounding mycelial discs. The tests were performed in triplicate.

Leishmanicidal activity

L. infantum chagasi in its promastigote form was cultured in 25-cm^2 T25 flasks (KASVI, BRA) containing medium 199 (SIGMA-ALDRICH, USA) supplemented with foetal bovine serum (FBS) (LGC, BRA), pen/strep antibiotics (GIBCO, USA) and L-glutamine (SIGMA-ALDRICH, USA). In the assay, each well contained 100 μL of a cell suspension previously adjusted to 1 × 10^5 and 100 μL of the CE diluted into four different concentrations: 10.000, 8.000, 6.000 and 4.000 μg.mL^{-1}, with 200 μL being the final volume of each well. Amphotericin B (GIBCO, USA) was used as a leishmanicidal agent standard at a concentration of 0.1 mM. The plates were incubated at 23°C for 24 and 48 h. For the cell viability test, the colorimetric Alamar Blue® (Sigma-Aldrich, USA) was employed. After each incubation period, 1.5 mM Alamar Blue® was added and incubated in the incubator, with CO_2 levels being maintained between 5 and 10%. Absorbance was read in a microplate reader (TP-READER, NM-THERMO PLATE) with a wavelength of 550 nm.

Cytotoxicity assay

For cytotoxicity tests, HFF1 cells were cultured in 75-cm^2 flasks (GREINER, USA) containing Dulbecco's Modified Eagle's Medium (DMEM) (SIGMA-ALDRICH, USA) supplemented with foetal bovine serum (FBS) (HYCLONE, USA), penicillin/streptomycin antibiotics (GIBCO, USA), L-glutamine, HEPES and bicarbonate (SIGMA-ALDRICH, USA). In 96-well plates (KASVI, BRA), 100 μL of medium was added containing cells at a concentration of 1×10^5, and 100 μL of EC at concentrations of 20.000, 10.000, 8.000, 6.000 and 4.000 μg.mL^{-1}, was added next, obtaining a final volume of 200 μL per well. After inoculation, cultures were kept in an incubator at 37°C and 5% CO_2 for 24, 48, and 72 h. For the assessment of cell viability, a colorimetric method was employed, with 3-(4,5-dimethyl thiazole 2-yl) 2,5-diphenyl bromide tetrazolium (MTT) (Sigma-Aldrich, USA) serving as the standard.

The absorbance was read in a microplate reader (Thermo Scientific®, USA) at a 570-nm wavelength.

Statistical analysis

Data were submitted to the Shapiro-Wilk normality test and Levene's test of homogeneity. Analysis of variance (ANOVA) and Tukey analysis were used to indicate the differences between the experimental groups. The values were considered to be significant when P-values ≤ 0.05 were obtained using GraphPad Prism software (Version 7.04 for Windows). Origin Pro version 9 software was employed to produce graphics and calculate the MIC_{50} and MIC_{90} (slope of linear regression) values.

RESULTS

Antimicrobial activity assays

The MIC_{50} and MIC_{90} were the minimum concentrations of CE required to inhibit 50 and 90%, respectively, of the pathogens tested. The data showed that CE was effective against the following pathogens: *E. coli*, MIC_{50} = 767 μg.mL^{-1} and MIC_{90} = 7.820 μg.mL^{-1}; *S. enteritidis*, MIC_{50} = 949 μg.mL^{-1} and MIC_{90} = 3.980 μg.mL^{-1}; and *S. aureus*, MIC_{50} = 756 μg.mL^{-1} and MIC_{90} = 3.940 μg.mL^{-1} (Table 1).

Antagonism assays

Diaporthe spp. 94 (4) strain inhibited the mycelial growth of *Colletotrichum* spp. by 33%, and the endophyte-phytopathogen interaction observed was of type CA1. For *F. oxysporum*, the mycelial inhibition was 50%, and the endophyte-phytopathogen interaction observed was of

Table 1. Antibacterial activity *in vitro* do CE of *Diaporthe* spp.

Pathogens	MIC_{50} (µg.mL^{-1})	MIC_{90} (µg.mL^{-1})
Staphylococcus aureus	756	3.940
Escherichia coli	767	7.820
Salmonella enteritidis	949	3.980
Candida albicans	-	-

Table 2. Index of antagonism and type of interaction between *Diaporthe* strain and phytopathogenic fungi.

Phytopathogens	IA (%)	Type of interaction
Colletotrichum spp.	36	CA1
Ceratocystis paradoxa	-	-
Fusarium oxyspora	50	CA1**
Phythophthora sojae	20	A*
Rhizopus microsporus	62	A

A* - Deadlock with mycelial contact; CA1** - partial replacement after initial deadlock with mycelial contact.

type CA1. For *P. sojae*, the mycelial inhibition was 20%, and the endophyte-phytopathogen interaction observed was of type A. Finally, for *R. microsporus*, the mycelia inhibition was 62%, and the endophyte-phytopathogen interaction observed was of type A. There was no activity against *C. paradoxa*. Type A interaction occurs when there is a deadlock with mycelial contact, and type CA1 interaction occurs when there is a partial replacement after the initial deadlock with mycelial contact (Table 2).

Enzymatic activity

Qualitative enzymatic assays showed that the endophytic *Diaporthe* spp. 94 (4) strain produced the enzyme cellulase. The assays did not indicate production of the enzymes amylase, protease, lipase or esterase.

Leishmanicidal assays

The CE produced by *Diaporthe* strain 94 (4) was tested against the promastigote forms of *L. infantum chagasi* in the periods of 24 and 48 h at 4.000, 6.000, 8.000 and 10.000 µg.mL^{-1}. In the 24-h period, the concentration of 4.000 µg.mL^{-1} had a mortality greater than 80%, and in the concentrations of 6.000, 8.000 and 10.000 µg.mL^{-1}, there was mortality of at least 95% of the cells. In the 48-h period, the concentration of 4.000 µg.mL^{-1} had a mortality greater than 75%, and in the concentrations of 6.000, 8.000 and 10.000 µg.mL^{-1}, there was mortality of at least 95% of the cells. (Figure 1). In the 24 and 48-h periods, the concentration of 4.000 µg.mL^{-1} was significantly different from the concentrations of 6.000, 8.000 and 10.000 µg.mL^{-1}; the concentrations of 6.000 and 8.000 µg.mL^{-1} were significantly different from the concentration of 10.000 µg.mL^{-1}.

Cytotoxicity

Assays for cytotoxicity showed cell viability above 60% at concentrations of 4.000 and 6.000 µg.mL^{-1}, above 50% at concentrations of 8.000 and 10.000 µg.mL^{-1}, and less than 10% at a concentration of 20.000 µg.mL^{-1} in the 24-h period. In the 48-h period, cell viability was above 60% at concentrations of 4.000 and 6.000 µg.mL^{-1}, above 50% at a concentration of 8.000 µg.mL^{-1}, above 40% at a concentration of 10.000 µg.mL^{-1}, and above 15% at a concentration of 20.000 µg.mL^{-1}. In the 72-h period, the cell viability was above 60% at a concentration of 4.000 µg.mL^{-1}, above 50% at a concentration of 6.000 µg.mL^{-1} and up to 20% at concentrations of 8.000, 10.000 and 20.000 µg.mL^{-1} (Figure 2). In the 24-h period, the concentrations of 4.000 and 6.000 µg.mL^{-1} were significantly different from the concentrations of 8.000, 10.000 and 20.000, and the concentration of 8.000 µg.mL^{-1} was significantly different from the concentration of 10.000 µg.mL^{-1}. In the 48-h period, the concentrations of 4.000, 6.000, 8.000 and 10.000 µg.mL^{-1} were significantly different from the concentration of 20.000, and the concentration of 8.000 µg.mL^{-1} was significantly different from the concentration of 10.000 µg.mL^{-1}. In the 72-h period, the concentrations of 4.000 and 6.000 µg.mL^{-1} were significantly different from the concentrations of 8.000, 10.000 and 20.000, and the

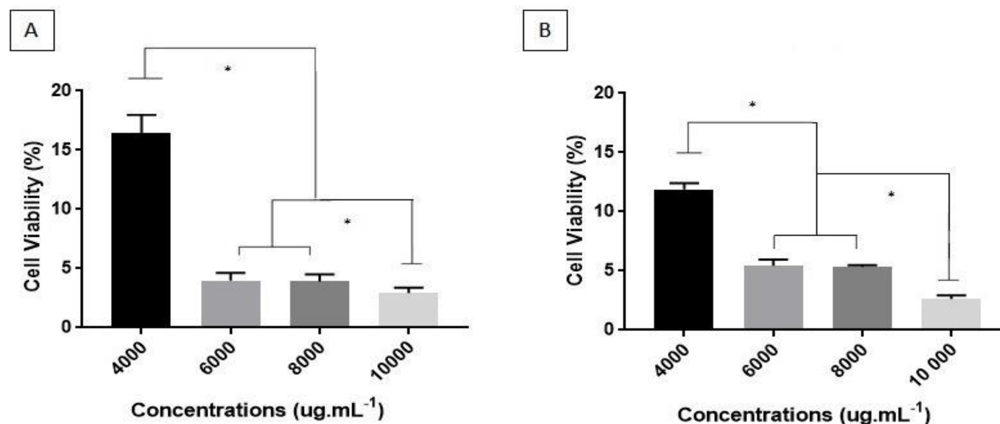

Figure 1. Evaluation of the viability of promastigotes of *Leishmania infantum chagasi* in different concentrations of CE from *Diaporthe* sp. strain through assay Alamar Blue®. (A) Viability of *L. infantum chagasi* in 24 hours. ®. (B) Viability of *L. infantum chagasi* in 48 h. It was observed a high-leishmaniasis activity at all concentrations and times tested and the concentrations of 6.000 to 10.000 µg.mL-1 different statistically from the concentration of 4.000 µg.mL-1. ANOVA followed by the Tukeys test were used to determine statistical significance (*p ≤ 0.05).

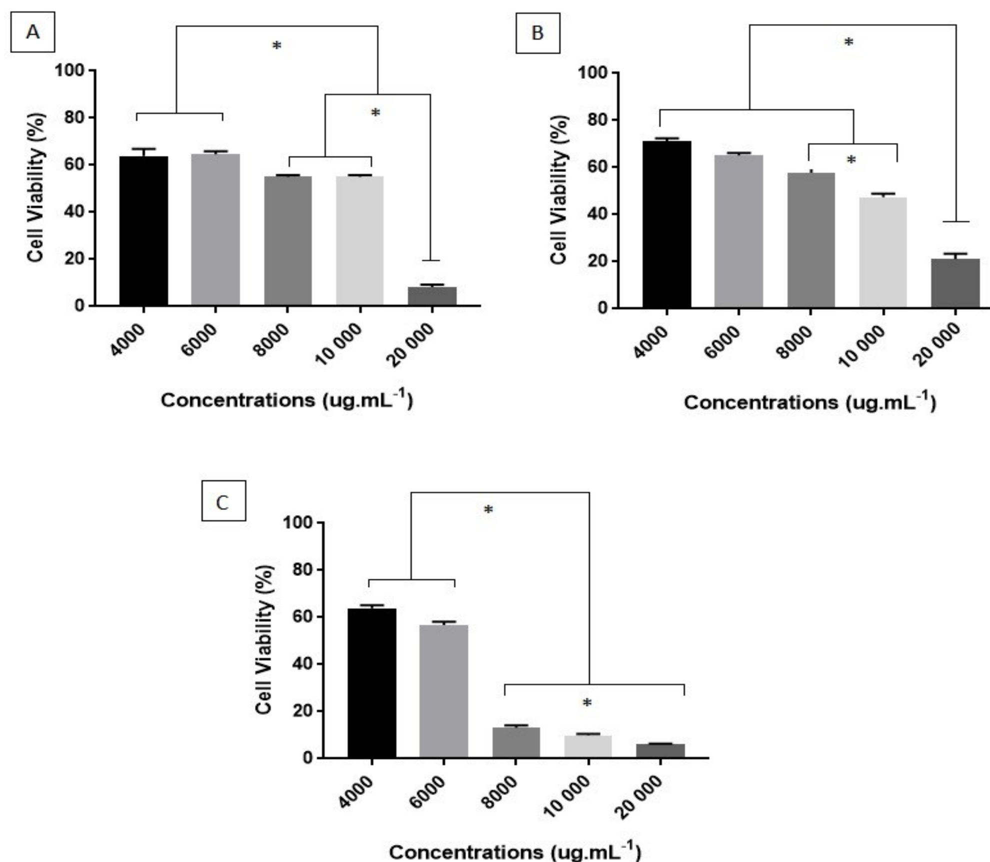

Figure 2. Evaluation of the cytotoxic activity of the CE of *Diaporthe* sp.. in the cell line HFF-1 (Fibroblasts). (A) Cell viability at 24 h, (B) Cell viability at 48 h and (C) cell viability at 72 h. The evaluation of the activity mitochondrial held through the test of MTT at different time to 4 concentrations of EB *Diaporthe* strain showed means above 60% in the concentrations of 4.000 and 6.000 µg.mL^{-1} the latter being different statistically from the concentrations of 8.000 and 10.000 µg.mL^{-1}. ANOVA followed by the Tukeys test were used to determine statistical significance (*p ≤ 0.05).

concentrations of 8.000, 10.000 and 20.000 µg.mL^{-1} were significantly different from each other.

DISCUSSION

Endophytic fungi isolated from mangrove ecosystems, including fungi belonging to the genus *Diaporthe*, have been described as rich sources of biologically active compounds with a variety of applications (Lin et al., 2005; Huang et al., 2008; Sebastianes et al., 2012; Brissow et al., 2017; de Medeiros et al., 2018; Huang et al., 2019), opening new avenues for biotechnological research. In this study, we evaluated the biotechnological potential of crude extract from *Diaporthe* spp. 94 isolates that were isolated from Brazilian mangroves for applications in the fields of medicine, agriculture and industry. Thus, evaluating the various effects of these compounds may enable researchers to identify an antimicrobial or antiparasitic effect to control different diseases.

The emergence of multidrug-resistant microorganisms has generated considerable attention and stimulated the search for new drugs. In this context, studies on the antimicrobial activity of endophytic mangrove-derived fungi against resistant human pathogens have been demonstrated (Prihanto et al., 2011; Sebastianes et al., 2012; Handayani et al., 2017; de Medeiros et al., 2018; Huang et al., 2019).

Bezerra et al. (2015) tested 32 endophytic fungi isolated from *Bauhinia forficata* against 10 pathogenic bacteria in disc diffusion assays. Of these 32 isolates, 11 presented antibacterial activity against one or more bacteria among *S. aureus* (UFPEDA02), *Streptococcus pyogenes* (UFPEDA07), *Mycobacterium smegmatis* (UFPEDA71), *Bacillus subtilis* (UFPEDA86), *Enterococcus faecalis* (UFPEDA138), *Salmonella* Typhi (UFPEDA478), *Pseudomonas aeruginosa* (UFPEDA735), *Enterobacter aerogenes* (UFPEDA739), *Proteus vulgaris* (UFPEDA740), and *E. coli* (UFPEDA224). Among the bacteria tested in common with this present study are *E. coli*, where six endophytic fungi presented antimicrobial action, *S. aureus* and the genus *Salmonella*, where for each, an endophytic fungus showed activity. Therefore, our results show promise, since the dose exhibiting effectiveness against pathogenic bacteria is ten times lower than the dose showing cytotoxicity in mammalian cells. Thus, our data suggest that this compound could be evaluated in a future study seeking new biomolecules with promising activity for future antibiotics.

Campos et al. (2015) isolated 82 endophytic fungi from the *Caesalpinia echinata* plant and tested their ethyl acetate extracts. Three extracts presented activity against *E. coli* and *S. aureus*, where MIC values varied between 32 and 64 µg/mL, and one presented activity against *Salmonella* bacteria, where MIC was 64 µg/mL. The MIC values were lower than those in the present study, probably due to the purification of the extract. However, the fact that our compound has a ten- or twelve-fold greater effect on fibroblast cytotoxicity makes it a promising biomolecule for future studies attempting to achieve microbial control.

Ratnaweera et al. (2015) isolated eight endophytic fungi of *Opuntia dillenii*. The eight strains showed antibacterial activity against at least one of the tested bacteria, including *E. coli* and *S. aureus*. Additionally, Khan et al. (2016) isolated the endophytic fungus *Cladosporium* species of *Rauwolfia serpentina*, and its extract exhibited antibacterial activity against *E. coli* and *S. aureus* among other bacteria. The endophytic *Diaporthe* presented promising antimicrobial activity against *S. aureus*, *E. coli* and *S. enteritidis*. When comparing our results with those of other authors, we must evaluate the antimicrobial effects and the dose-dependent effect in killing the studied bacteria. Our data suggest that this activity is dose-dependent and may favour the regulation of the dose used, given the evaluated antimicrobial effect. We suggest that in future tests, after producing these compounds in greater purity, we can amplify this effect and achieve greater bacterial control. Endophytic fungi provide novel opportunities to control phytopathogens (Campanile et al., 2007). The antagonistic activity displayed by *Diaporthe* spp. 94 in our study could be explained by their secreting diffusible antifungal compounds into the culture medium, thereby inhibiting mycelial growth of *Colletotrichum* spp., *F. oxysporum*, *P. sojae* and *R. microsporus*. This inhibition could also be due to competing for space and/or nutrient depletion in the medium in the dual-culture assay. Competitive interactions of type A (deadlock with mycelial contact) and type CA1 (partial replacement after initial deadlock with mycelial contact), which were observed in the antagonistic assays, indicate different modes of action of *Diaporthe* spp. on phytopathogens, supporting our hypotheses regarding the possible mechanisms of control employed by endophytes.

Lytic enzymes secreted by endophytes, including cellulases, are another important mechanism by which plant-associated fungi control pathogens (Tripathi et al., 2008). These enzymes can suppress phytopathogens from breaking down cell walls, thereby restricting their growth (Gao et al., 2010). In this study, *Diaporthe* spp. 94 secreted cellulase into the culture medium. This potential for extracellular enzyme production by endophytic fungi from mangroves has also been described by other studies (Maria et al., 2005; Thatoi et al., 2013; Bezerra et al., 2015; Maroldi et al., 2018). These microorganisms naturally degrade lignocellulosic plant biomass of sediments in mangrove environments (Behera et al., 2017) and thus also suggest other applications, such as the processing of fruit juices in the textile, food and paper industries (Gao et al., 2008).

Leishmaniasis is a neglected tropical disease, and its prevalence in the poorest populations is not a major stimulus for the pharmaceutical industry. Thus, the

search for new medicines to treat leishmaniasis has not been heavily funded. Therefore, contributing studies that may lead to the discovery of a compound or compounds with anti-leishmaniasis activity is of great importance for the control of these diseases. Similar works tested endophytic fungi isolated from plants from different Brazilian biomes, and they obtained leishmanicides that exhibited promise against other species of *Leishmania* that cause integumentary leishmaniasis, which is less deadly than visceral leishmaniasis (VL) (Marinho et al., 2005; Campos et al., 2008; Rosa et al., 2010; Brissow et al., 2017; Cota et al., 2018). Little is known about the anti-leishmaniasis activity of these isolates against the species that cause VL (*Leishmania donovani* and *L. infantum chagasi*). The results obtained for the control of *L. chagasi* demonstrate that the compound has leishmanicidal activity at a dose similar to the IC50 observed in fibroblasts. However, most of the drugs available and recommended to treat visceral leishmaniasis are highly toxic. Thus, in future research, we will evaluate *in vivo* whether the toxic effect is capable of compromising homeostasis in a murine model. Thus, we will be able to evaluate these data more globally. However, these results are promising for future studies seeking a new compound for the control of visceral leishmaniasis.

In Brazil, the aetiological agent of VL is *L. infantum chagasi*, our choice for the testing of inhibition. Our data demonstrated a high mortality rate at a dose of 4.000 $\mu g.mL^{-1}$ in different periods of exposure (24 and 48 h) (Figure 1). These data are promising compared with those found in the literature, where tests for less aggressive leishmaniasis were performed (Marinho et al., 2005; Campos et al., 2008; Santiago et al., 2011; Campos et al., 2015; Brissow et al., 2017).

The search for compounds with biological activity has great importance for research correlating new therapies for existing diseases. However, to continue the studies, it is of great relevance for these studies to demonstrate that for basal cells, the toxicity is considerably lower compared to the toxicity observed on the pathogenic microorganisms (Figure 2). Thus, we evaluated fibroblast cytotoxicity, an important model to establish these parameters. Thus, our study leads us to new bioprospects from these compounds, since the cytotoxicity in fibroblasts was considerably lower than that found in the pathogens studied. Thus, we suggest that these biomolecules are promising for future studies in which we are searching for new compounds with biological activity.

This study demonstrated the biotechnological potential of the endophytic fungus *Diaporthe* spp. 94 isolated from Brazilian mangroves. We described for the first time the leishmanicidal activity of the secondary metabolites of this fungus on the promastigote forms of *L. infantum chagasi* and the cytotoxic effects of metabolites on HFF-1 cells. Our results may provide a new biological source of novel drugs.

Conclusion

The results of this study indicate that the tested CE from *Diaporthe* spp. 94 has antimicrobial action and anti-leishmaniasis activity at the two lower concentrations and has low toxicity at those same concentrations. These results suggest a that is fungus has strong biotechnological potential for future applications, as it is important to identify new drugs and antimicrobials because the rapid resistance that microorganisms have acquired is not compatible with new drug production. It is also important to develop new leishmanicidal drugs with lower cost and toxicity and greater effectiveness.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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Molecular identification of bacterial isolates from the rhizospheres of four mangrove species in Kenya

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Mangrove ecosystems provide a unique ecological niche for diverse microbial communities. This study aimed to identify bacterial isolates from the rhizospheres of four mangrove species (*Sonneratia alba, Rhizophora mucronata, Ceriops tagal* and *Avicennia marina*) using the 16S rRNA gene analysis approach. Rhizospheric sediment samples of the mangroves were collected from Mida creek and Gazi bay, Kenya, using standard protocols. A total of 36 representative bacterial isolates were analyzed. The isolates were characterized using morphological and molecular characters. Pure gDNA was extracted from the isolates, polymerase chain reaction amplified and sequenced. The 16S rRNA gene sequences were BLASTN analyzed against the Genbank database; the closest taxonomically related bacterial sequences were retrieved and used for phylogenetic analysis using MEGA X software. Morphologically, the isolates differed in their cultural characteristic in color, shape, margin, elevation and gram reaction. Phylogenetic analysis classified the isolates into five genera, namely *Bacillus, Pseudomonas, Micrococcus, Microbacterium* and *Streptomyces* that belong to three different phyla (Firmicutes, Proteobacteria and Actinobacteria). The findings show that the underexplored tropical mangrove rhizospheres harbor useful diverse bacteria. Further analysis of the bioactive production potential of the isolates will give more insights into the types of bioactive compounds produced and their biotechnological potential.

Key words: 16S rRNA gene sequence, rhizosphere, mangrove sediments, marine bacteria, biotechnology.

INTRODUCTION

Mangroves occur in the intertidal zone of sheltered shores, lagoon, estuarine tidal stream, and swamps mudflats of the tropical and subtropical regions of the world (Sengupta et al., 2015). Mangrove ecosystems have unique conditions, including high salinity, high moisture, strong wind, high tides, anaerobic condition and

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muddy soils (Dissanayake and Chandrasekara, 2014). Mangrove forests are considered as one of the most prolific ecosystems in the world that have well established biological, cultural, and economic importance (Goessens et al., 2014). Besides, the mangrove ecosystems have significant ecological functions that include filtering and reducing dissolved and particulate nutrients, serving as a sink for carbon, nitrogen and phosphorus, as well as retaining heavy metals from adjacent land and fluvial imports (Sanders et al., 2014).

Mangrove ecosystems harbor a large number of microbial communities, including bacteria, fungi, archaea, protozoa, etc whose abundance and activities are controlled by various physical and chemical factors in this environment. Thus, mangrove ecosystems form an ecological niche for a wide spectrum of microbial diversity due to their unique geographical conditions (Xu et al., 2014). Diverse groups of bacteria including diazosthrophs, phosphate solubilizers, cellulose decomposers, nitrifiers and denitrifiers, sulphur oxidizers and iron oxidizers have been identified in mangrove ecosystems (Holguin et al., 2001). The dynamic conditions and complexity of the mangrove ecosystems have generated increasing interest among microbial ecologists who sought to understand these ecosystems better.

Furthermore, the phylogenetic and functional description of microbial diversity in the mangrove ecosystems have not been adequately addressed to the same extent as that of terrestrial environments (Saseeswari et al., 2016). In Kenya, microbial communities have widely been studied in the terrestrial environments (Makonde et al., 2015; Kambura et al., 2016a; Muwawa et al., 2016; Josiah et al., 2018; Kambura et al., 2016b; Salano et al., 2017; Kawaka et al., 2018; Muhonja et al., 2018a, b; Salano et al., 2018) and with less focus on the mangrove ecosystems (Jenoh et al., 2019; Ntabo et al., 2018). This is partly, due to skepticism regarding the existence of indigenous populations of mangrove microbial communities. It is known that microbial communities from the terrestrial environment produce resistant spores that are transported from land into the marine environment, where they can remain available but dormant for many years (Bull et al., 2000). Thus, it has been frequently assumed that microbial communities isolated from marine samples are of mere terrestrial origin (Bull et al., 2000).

Studies on microbial diversity, their distribution and functional roles in mangrove ecosystems are essential, since they would improve our understanding of their roles and interactions in such ecosystems (Kathiresan and Selvam, 2006). Microorganisms form an important component of mangrove ecosystems, and there is evidence that they are key to the biogeochemical productivity of the mangrove ecosystem (Zhang et al., 2017). Hence, there is the need to understand the bacterial species composition underlining mangrove ecosystems, especially within the rhizosphere of mangrove species, which still remains unclear. Previous studies on mangroves in Kenya have concentrated on floristic composition and distribution of mangrove species, economic utilization and regeneration strategies of the principal species (Mohamed et al., 2009). However, data on microbial community diversity is limited due to inadequate efforts spent in exploring the mangrove habitats for microbial diversity (Ntabo et al., 2018).

The 16S rRNA gene is approximately 1.5-kilobase pair DNA fragment with desirable properties and is the most commonly used molecular marker. The functional constancy of this gene assures it is a valid molecular chronometer, which is essential for a precise assessment of phylogenetic relatedness of organisms. This gene is present in all prokaryotic cells and has conserved and variable sequence regions evolving at very different rates. These characteristics allow the use of 16S rRNA in the assignment of close relationships at the genus (Clarridge, 2004; Srinivasan et al., 2015) and in some cases at the species level (Conlan et al., 2012; Fettweis et al., 2012). In addition, dedicated 16S databases (Cole et al., 2009; Pruesse et al., 2007) that include near full length sequences for a large number of strains and their taxonomic placements exist. Therefore, this study was designed to isolate and characterize bacterial species from the rhizospheres of four mangrove species (*Avicennia marina* (Forsk.) Vierh., *Ceriops tagal* (Perr.) C.B. Robinson., *Rhizophora mucronata* Poir. and *Sonneratia alba* Griff.) that are commonly found along the Kenyan Coastline by analyzing the 16S rRNA gene region.

MATERIALS AND METHODS

Ethical statement

The National Commission for Science, Technology and Innovation of Kenya (NACOSTI) approved this research study, National Environmental Management Authority of Kenya (NEMA) provided the access permit (for field sampling), Kenya Wild life Services (KWS), and Kenya Plant Health Inspectorate Services (KEPHIS) provided permits that facilitated the shipment of samples to Laval University, Canada. The field studies neither involved endangered nor protected species.

Study site

We investigated two mangrove sites (Mida Creek and Gazi Bay) in Kenya (Figure 1). Mida Creek, which lies in a planigraphic area of 32 km^2, is located in Kilifi County (3°21'S, 39°59'E), about 88 Km North of Mombasa and approximately 25 km South of Malindi town (Lang'at, 2008). The monthly temperature is between 23 and 27°C, rising to a maximum temperature of 34°C in the hottest months and a minimum temperature of 20°C in the coldest months; and total annual precipitation ranging between 1000 and 1600 mm (Lang'at, 2008). Gazi bay is located in Kwale County (4°44′S, 39°51′E), South Coast of Kenya, approximately 55 km from Mombasa. The Bay is sheltered from strong waves by the presence of the Chale peninsula to the East and a fringing coral reef to the

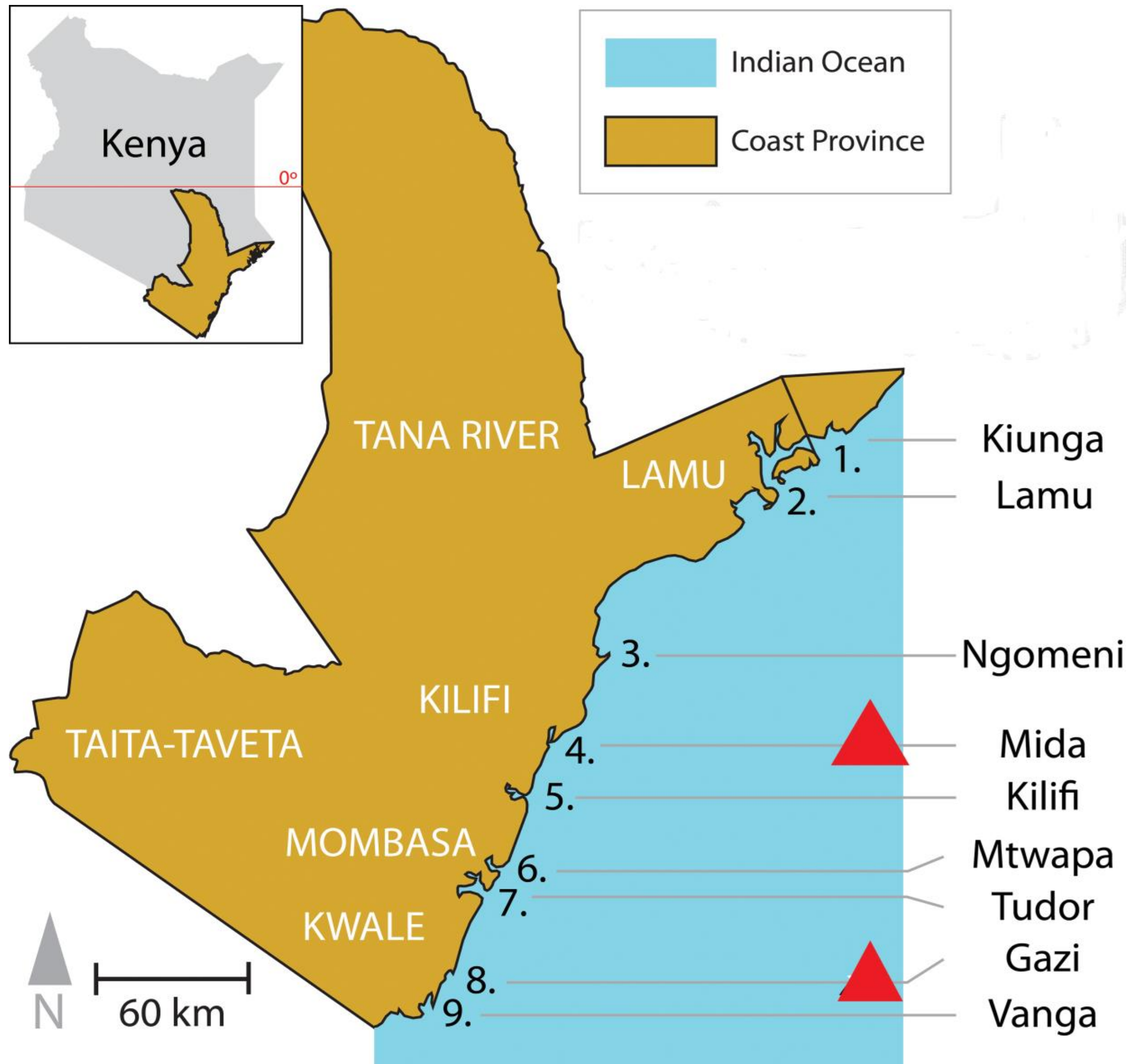

Figure 1. Map of Kenya highlighting the Kenyan coast region.
Source: Jenoh et al. (2016). Note: position numbers 4 and 8, represent the Mida creek and Gazi bay study site, respectively.

South. The climate is hot and humid, and the average annual temperature and humidity are about 28°C and up to 95%, respectively (Lang'at, 2008). Mangrove forests in Kenya often display the typical zonation pattern of mangroves in Eastern Africa: the seaward side is predominantly occupied by the *Sonneratia* and *Rhizophora* spp. (tall) assemblage, followed by *Rhizophora, Bruguiera* and *Ceriops* spp. in the middle zone and the *Avicennia, Lumnitzera* and *Xylocarpus* spp. complex with often dwarf *Avicennia* on the landward side (Dahdouh-Guebas et al., 2004; Matthijs et al., 1999).

Collection of samples

Sampling was conducted in May 2018, according to previously described methods (Wu et al., 2016). Four species of mangrove trees common to the two sites, namely *A. marina*, *C. tagal*, *R. mucronate*, and *S. alba* were identified by use of expertise from a plant taxonomist. Four mangrove trees of each species at intervals of 10 m were selected. For each species, the rhizosphere sediments (~100g) were sampled vertically along the base of the plant at depth (1-5 cm), using a standardized core sampler (Giannopoulos et al., 2019). A total of 32 samples (from 4 mangrove species x 4 replicates x 2 sites) were kept in sterile plastic bags. They were maintained in a dry iced box before they were transported and stored at -20°C prior to further analyses that were performed at the Laval University, Canada.

Physicochemical analysis of sediment samples

Nutrient analyses of soil samples for nitrogen, carbon, phosphorus, potassium, calcium, magnesium and sodium were conducted according to standard methods (Brupbacher et al., 1968). Determination of pH was done using the calcium chloride method at a ratio of 1:2 using a digital Corning pH meter 140 (Corning Life Sciences, Massachusetts, USA). The electrical conductivity was determined using the electrical conductivity meter type CDM 2d radiometer (Radiometer, Copenhagen, Denmark).

Isolation of bacteria from sediment samples

The sediment samples were pre-processed by air-drying at room temperature (27±1°C) for seven days and sieved with a 2.5mm sieve to remove larger particles such as stone and plant debris to obtain a consistent soil particle size for bacterial isolation. The isolation of bacteria from the sediment samples was performed by serial dilution method. About 0.1g of the sediment sample was suspended in 1ml sterile distilled water in a sterile 1.5ml Eppendorf tube and serial diluted to 10^{-3}. One hundred µl of the10^{-1}, 10^{-2}, and 10^{-3} suspensions were spread in triplicate onto three different types of isolation media which included Dextrose nitrate agar, ISP2 agar and Actinomycetes isolation agar. All media were prepared according to the manufacturer's instructions. All the plates were incubated at 28°C for 2-7 days. Follow up was made to observe any growth on the plates (Lee et al., 2014).

Morphological characterization of bacterial isolates

Colony morphologies of the bacterial isolates were described using standard microbiological criteria with special emphasis on pigmentation, shape, form, elevation and margin formation. Preliminary characterization by Gram staining was done of each of the isolates using the method described by (Bergey and Holt, 1994).

Molecular characterization of the bacterial isolates

Genomic DNA (gDNA) extraction and PCR amplification

Pure genomic DNA was extracted from pure bacterial culture using the GenElute Bacterial Genomic DNA extraction kit according to the manufacturer's instructions. The extracted DNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Waltham, MA, USA) and used as a template for the amplification of the 16S rRNA gene region. Nearly full-length 16S rRNA gene sequences was PCR-amplified using a universial bacterial primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR was carried out in a 25µl reaction and consisted of 2.5 µl 10x PCR buffer, 0.75µl $MgCl_2$, 0.5µldNTPs, 0.5µl of each primer, 0.2 µl platinum Taq (Invitrogen), 1µl of template DNA and 19.05µl of water. Amplification was performed with initial heating at 95°C for 30s followed by 30 cycles of denaturation at 95°C for 50s, annealing at 54°C for 50s and extension at 72°C for 1 min and a final extension period at 72°C for 5 min using MJ Research PTC-225 Peltier Thermal Cycler. Amplicons were confirmed by visualization on 1% ethidium bromide stained agarose gels under gel documentation chamber. The PCR products were sequenced directly using the Sanger sequencing platform at the Institute for Systems and Integrative Biology of Laval University, Canada.

Phylogenetic analysis

Sequences of the isolates were manually edited in chromas and checked for presence of artifacts or sequencing errors using Mallard software (Ashelford et al., 2006), an NCBI bioinformatic tool for detecting chimera sequences. A search for similar sequences using BLASTN (Altschul et al., 1990) was performed, and sequence alignment was performed using the CLUSTAL Omega program (http://www.clustal.org) against the nearest neighbours. A neighbor-joining tree of the aligned sequences was constructed (Saitou and Nei, 1987) using MEGA X software (Kumar et al., 2018). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). To obtain statistical support values for the branches, bootstrapping (Felsenstein, 1985) was conducted with 1000 replicates. All sites, including gaps in the sequence alignment, were excluded pairwise in the phylogenetic analysis. Using the resultant neighbor-joining tree, each isolate was assigned to the proper taxonomic group. The taxonomic assignment was confirmed at a 95% confidence level using the RDP Naïve Bayesian rRNA Classifier Version 2.11 on the RDP website (Wang et al., 2007).

Statistical analyses

Data from physicochemical parameters were analyzed using R v3.6.1 (Somanathan et al., 2004). A two-factor (sites and mangrove species differences) test of differences in physicochemical parameters was done by the non-parametric Kruskal-Wallis H test using the *agricolae* package implemented in R (de Mendiburu, 2020). Post hoc test for mean separations was based on Fisher's least significant difference. Results were expressed as the mean ± SD. A p-value of ≤ 0.05 was considered statistically significant. All experiments were performed independently at least three times.

RESULTS

Physicochemical analysis of sediment samples

The pH and calcium were significantly higher (Kruskal-Wallis, $p \leq 0.05$) in all mangrove plant species of Mida creek compared to Gazi bay, which had significantly higher EC and salinity values (Table 1). Apart from the rhizosphere sediment samples of *R. mucronata*, all other rhizosphere sediment samples of the mangrove species in Gazi bay had significantly lower ($p < 0.05$) physicochemical properties compared to the mangrove species in Mida creek. The physicochemical parameters that were higher in the rhizosphere of *R. mucronata* in Gazi bay included potassium, sodium, phosphorus, total carbon, nitrogen, salinity and electrical conductivity (Table 1).

Isolation and morphological characterization of bacteria

A total of 50 bacterial isolates were isolated from the rhizospheric sediments of the mangrove species. The isolates were able to grow within a period of between three and seven days. Morphologically, the isolates exhibited diverse colony characteristics differing in their form, elevation, color, margin, cell arrangement and the Gram reaction. Majority of the isolates were circular in form, cream in color, raised elevation and had entire margins. All the isolates were rod-shaped with the exception of two isolates (SAM110B1; SAG210B1) which were cocci. The gram reaction was also positive in all the isolates with the exception of one isolate (SAM210B1) (Table 2).

Molecular characterization of the bacterial isolates

A total of 36 representative bacterial isolates from the rhizospheric sediments of the four-mangrove species were picked based on their morphological characters and

Table 1. Characteristics of sediment samples from Mida creek and Gazi bay.

Parameter	Physicochemical							
	A. marina		*C. tagal*		*R. mucronata*		*S. alba*	
Site	**Gazi**	**Mida**	**Gazi**	**Mida**	**Gazi**	**Mida**	**Gazi**	**Mida**
Calcium (mg/kg)	325.75± 64.73	14085.5± 3686.55***	168.25± 27.76	79312.88±38869.51***	2670.62±544.23	62731.12±26956.45***	477± 147.65	52097± 18982.24***
Potassium (mg/kg)	663.12± 192.89	494±182.67	178.25± 40.78	464± 111.51***	1588.87±172.27***	597.375 ± 211.89	461.87± 138.12	594.75 ± 82.25*
Magnesium (mg/kg)	379.125± 76.07	841.75± 470.97*	115.12 ± 21.66	1776± 660.54***	1856.12±140.19*	1307.12 ± 665.24	406.87± 68.52	1166.62± 345.31***
Sodium (mg/kg)	2369±815.28	4162±3483.99	155.87±40.50	3933.87±1448.40***	8472.25±967.11***	3263.75±1446.15	1978±431.28	2948.37±471.96***
Phosphorus (mg/kg)	55±16.29	136.12±33.09***	40±2.87	164.5±61.01***	187.75±49.67**	98.87±54.49	55.125±14.06	99.75±36.32**
Total carbon (mg/kg)	0.82±0.41	1.90±0.57***	0.26±0.07	4.55±1.56***	7.40±0.85***	2.69±1.67	0.99±0.23	2.25±0.60***
Nitrogen (mg/kg)	0.04±0.02	0.16±0.03***	0.01±0.01	0.18±0.04***	0.37±0.06***	0.11±0.05	0.07±0.01	0.09±0.01*
Electrical conductivity (S/m)	5.78±0.95	4.56±1.39	3.37±1.31	6.51±2.98*	12.37±1.00***	4.05±1.36	4.88±0.88	5.22±1.34
pH	7.05±0.55	8.17±0.21***	6.21±0.10	7.93±0.07***	6.09±0.15	8.08±0.28***	6.08±0.08	7.91±0.16***
Salinity (mg/kg)	3.13±0.55	2.43±0.79	1.77±0.72	3.58±1.75*	7.08±0.61***	2.15±0.78	2.61±0.50	2.81±0.77

Values represent mean ± standard deviation. Superscripts beside values are significantly different measures ($p \leq 0.05$) based on Fisher's least significant difference. (Significance codes: 0 '***', 0.001 '**', 0.01 '*', 0.05).

further identified by analysis of their 16S ribosomal RNA gene sequences. About 44, 28, 19, and 8% of the bacterial isolates were recovered from the rhizospheric sediments of *A. marina, C. tagal, S. alba* and *R. mucronata,* respectively (Table 3). The isolates (with their accession numbers in parenthesis) in the inferred phylogenetic tree (Figure 2) were diverse and affiliated with known species from five genera (*Streptomyces*, *Microbacterium, Micrococcus, Pseudomonas* and *Bacillus*). Comparison of the newly isolated 16S rRNA gene sequences to known bacterial sequences in the Genbank database using BLASTN analysis indicated sequence similarities of between 98 and 100% (Table 3).

Affiliation of 16S rRNA gene sequences of the isolates

The inferred phylogenetic tree grouped the isolates into three main clusters belonging to the phyla *Firmicutes*, *Proteobacteria* and *Actinobacteria* (Figure 2). Most of the isolates (~55%) were affiliated with several known bacterial species (with >97% sequence identity) belonging to the phylum *Actinobacteria* (Table 3). About 42% of the total bacterial isolates had between 98 and 100% sequence identities with known members of the genus *Bacillus* and 3% of the total isolates had 100% sequence identity with *Pseudomonas stutzeri* [KM076597], which belong to the phylum *Proteobacteria* (Table 3). About 42% of the isolates formed another large cluster with known members from the genus *Bacillus.* Within this large *Bacillus* cluster, was a sub-cluster 2 (supported by a bootstrap value of 100%) that was represented by isolates CTM15B1 [MT2494405], CTM210B2 [MT249409], AVM210B6 [MT249397], CTM25B3 [MT249407], SAM15B1 [MT249418] and AVG210B1 [MT249388] and some known *Bacillus* species (*B. cereus* [MT020418], *B. cereus* [MT544972], *B. cereus* [MG491524], *B. mycoides* [MG598443] and *B. proteolyticus* [MT573794]) (Figure 2). Isolate SAM210B1 [MT249419] was obtained from the rhizospheric sediments of *S. alba* and had 100% sequence identity with *Pseudomonas stutzeri* [KM076597].

Methanoculleus thermophiles (AB065297) was used to root the tree. *Pseudomonas zhhaodongesis* [MH283851] formed a minor cluster supported with a bootstrap value of 100% (Figure 2). Isolates SAG210B1 [MT249414] and SAM110B1 [MT249420] together with closely related known bacterial species *(Micrococcus luteus* [MH142592], *Micrococcus aloeverae* [KX082870] and *Microbacterium paludicola* [NR_114939] formed a minor sub-cluster with a bootstrap value of 99% in the inferred phylogenetic tree (Figure 2). The genus Streptomyces was represented by the majority of the isolates (50%) that together with other closely related known species formed a single large cluster supported with a bootstrap value of 100% in the inferred

Table 2. Morphological characterization of bacterial isolates.

Isolate code	Form	Elevation	Color	Margin	Shape	Gram reaction
AVG25B1	Circular	Raised	Cream	Entire	Rod	+
AVG210B2	Circular	Raised	Cream	Entire	Rod	+
AVM15B3	Circular	Flat	Grey	Entire	Rod	+
AVM25B1	Circular	Raised	Cream	Entire	Rod	+
AVM25B2	Circular	Flat	Whitish	Entire	Rod	+
AVM210B2	Circular	Raised	Cream	Entire	Rod	+
AVM210B3	Circular	Flat	Cream	Entire	Rod	+
AVM210B4	Circular	Raised	Cream	Entire	Rod	+
AVM210B5	Circular	Raised	Cream	Entire	Rod	+
AVM210B6	Filamentous	Flat	Cream	Entire	Rod	+
AVM310B1	Filamentous	Flat	Cream	Entire	Rod	+
AVM410B1	Circular	Raised	Cream	Entire	Rod	+
AVM410B2	Circular	Raised	Cream	Entire	Rod	+
CTG25B1	Circular	Flat	Whitish	Entire	Rod	+
CTG210B3	Circular	Raised	Cream	Entire	Rod	+
CTM15B1	Irregular	Raised	Cream	Entire	Rod	+
CTM25B2	Irregular	Raised	Cream	Entire	Rod	+
CTM25B3	Irregular	Flat	Cream	Entire	Rod	+
CTM210B2	Irregular	Raised	Cream	Entire	Rod	+
RMG15B3	Circular	Raised	Cream	Entire	Rod	+
RMG15B4	Circular	Raised	Cream	Entire	Rod	+
RMM15B1	Circular	Raised	Cream	Entire	Rod	+
SAG210B1	Circular	Raised	Yellow	Entire	Cocci	+
SAG210B2	Circular	Raised	Cream	Entire	Rod	+
SAG45B3	Circular	Raised	Cream	Entire	Rod	+
SAM15B1	Circular	Raised	Cream	Entire	Rod	+
SAM210B1	Irregular	Flat	Brown	Entire	Rod	-
SAM110B1	Circular	Raised	Yellow	Entire	Cocci	+

phylogenetic tree (Figure 2 and Table 3). This large actinobacterial cluster had a sub-cluster 1, which was represented by isolates (AVM25B2 [MT249392], AVM25B1 [MT249391], AVM210B5 [MT249396], AVM410B1 [MT249399], AVM15B3 [MT249390], AVM210B4 [MT249395], AVM410B2 [MT249400], AVM210B2 [MT249393], CTG25B1 [MT249401] and RMG15B3 [MT249411] that had >97% sequence similarities with known species (*Streptomyces sanyensis* [NR_116599], *Streptomyces sp.* [MH613761], *Streptomyces sp*. [KX641393], and *Streptomyces sp.* [MK615854].

DISCUSSION

In this study, we isolated and identified rhizospheric bacterial communities from four mangrove species along the Kenyan coastline using the 16S ribosomal RNA approach. A total of 50 bacterial isolates were obtained from the rhizospheric sediment samples of the four-mangrove species. After morphotyping, a total of 36 bacterial isolates were further characterized. A close observation on the distribution of the identified bacterial isolates (36 isolates) among the mangrove species showed that most isolates (44% of the total isolates) were recovered from the rhizospheric sediments of *A. marina* in both Gazi bay and Mida creek (Table 3). This observation can be explained by the variation in nutrients and mangrove tree species in the two study sites. Nutrients such as calcium, magnesium, nitrogen, phosphorus, total carbon and pH (Table 1) may have influenced the bacterial diversity of *A. marina*. For example, most of the recovered bacteria from the rhizosphere of *A. marina* belonged to the genera *Streptomyces* and to a lesser extent the genus *Bacillus,* whose members were recovered the most in the rhizospheres of *C. tagal* and *S. alba* in Mida creek and Gazi bay. This demonstrates that the variation in nutrients and mangrove tree species may have contributed to the observed species distribution. Other studies in mangroves (Krüger et al., 2017; Wu et al., 2016) and

Table 3. Taxonomic affiliation and percentage sequence similarities of bacterial isolates with closest relatives from the Genbank database.

Isolate ID	Accession no.	Host mangrove species	Closest taxonomic affiliation	Isolation Source	% ID	Country
AVG25B1	MT249385	*Avicennia marina*	*Streptomyces* sp. strain JJ73 (KX352795)	Marine sediment	99	India
AVG25B2	MT249386	*Avicennia marina*	*Streptomyces* sp. strain JJ73 (KX352795)	Marine sediment	99	India
AVG25B3	MT249387	*Avicennia marina*	*Streptomyces monticola* (MG820052)	Soil	98	China
AVG210B1	MT249388	*Avicennia marina*	*Bacillus cereus* (MT020418)	Coral	99	India
AVG210B2	MT249389	*Avicennia marina*	*Bacillus paralicheniformis* (MT527538)	Oil contaminated Soil	100	China
AVM15B3	MT249390	*Avicennia marina*	*Streptomyces* sp. (MK615854)	Rhizosphere soil of *A. ilicifolius*	99	China
AVM25B1	MT249391	*Avicennia marina*	*Streptomyces* sp. (KX641393)	Coral	99	Colombia
AVM25B2	MT249392	*Avicennia marina*	*Streptomyces* sp. (MH613761)	Soil	99	India
AVM210B2	MT249393	*Avicennia marina*	*Streptomyces* sp. C10-9-1 (LC158575)	Mangrove sediment	99	Bangkok
AVM210B3	MT249394	*Avicennia marina*	*Bacillus subtilis* (MN960275)	*Calotropis procera* leaf	99	Egypt
AVM210B4	MT249395	*Avicennia marina*	*Streptomyces sanyensis* (NR_116599)	Mangrove sediment	99	China
AVM210B5	MT249396	*Avicennia marina*	*Streptomyces* sp. (MH613761)	Soil	99	India
AVM210B6	MT249397	*Avicennia marina*	*Bacillus cereus* (MG491524)	Salt marsh soil	100	India
AVM310B1	MT249398	*Avicennia marina*	*Bacillus licheniformis* (MT072145)	Soil	100	China
AVM410B1	MT249399	*Avicennia marina*	*Streptomyces* sp. (MK615854)	Rhizosphere soil of *A. ilicifolius*	99	China
AVM410B2	MT249400	*Avicennia marina*	*Streptomyces* sp. (MK850320)	Sea water	99	S.Korea
CTG25B1	MT249401	*Ceriops tagal*	*Streptomyces sanyensis* (NR_116599)	Mangrove sediment	99	China
CTG210B2	MT249402	*Ceriops tagal*	*Streptomyces* sp. (GQ924491)	Plant root	99	USA
CTG210B3	MT249403	*Ceriops tagal*	*Streptomyces diastatochromogenes (AB184503)*	N/A	99	Japan
CTG210B4	MT249404	*Ceriops tagal*	*Streptomyces miharaensis (GU166434)*	N/A	99	Korea
CTM15B1	MT249405	*Ceriops tagal*	*Bacillus proteolyticus (MT573794)*	N/A	100	China
CTM25B2	MT249406	*Ceriops tagal*	*Bacillus aryabhattai (MT538258)*	Sludges	100	Morocco
CTM25B3	MT249407	*Ceriops tagal*	*Bacillus cereus (MT020418)*	Coral	99	India
CTM210B1	MT249408	*Ceriops tagal*	*Bacillus aryabhattai (MT605509)*	N/A	100	China
CTM210B2	MT249409	*Ceriops tagal*	*Bacillus cereus (MK855405)*	Crocus sativus	99	India
CTM35B1	MT249410	*Ceriops tagal*	*Streptomyces spectabilis (NR_112467)*	N/A	98	Japan
RMG15B3	MT249411	*Rhizophora mucronata*	*Streptomyces sanyensis (NR_116599)*	Mangrove sediment	99	China
RMG15B4	MT249412	*Rhizophora mucronata*	*Bacillus megaterium (MT510154)*	Sediment	99	S. Korea
RMM15B1	MT249413	*Rhizophora mucronata*	*Bacillus endophyticus (MG988221)*	Panicum antidotale	99	Saudi Arabia
SAG210B1	MT249414	*Sonneratia alba*	*Microbacterium paludicola (NR_114939)*	Swamp forest	99	S. Korea
SAG210B2	MT249415	*Sonneratia alba*	*Streptomyces ferrugineus (NR_148288)*	Mangrove soil	99	Thailand
SAG35B1	MT249416	*Sonneratia alba*	*Bacillus megaterium (MN826585)*	Soil	99	Peru
SAG45B3	MT249417	*Sonneratia alba*	*Bacillus endophyticus (MT277424)*	Aedes aegypti	99	Sri Lanka
SAM15B1	MT249418	*Sonneratia alba*	*Bacillus mycoides (MG598443)*	Soil	100	India
SAM210B1	MT249419	*Sonneratia alba*	*Pseudomonas stutzeri (KM076597)*	Lonar soda lake	100	India
SAM110B1	MT249420	*Sonneratia alba*	*Micrococcus luteus (MH142592)*	Pine tree	99	S. Korea

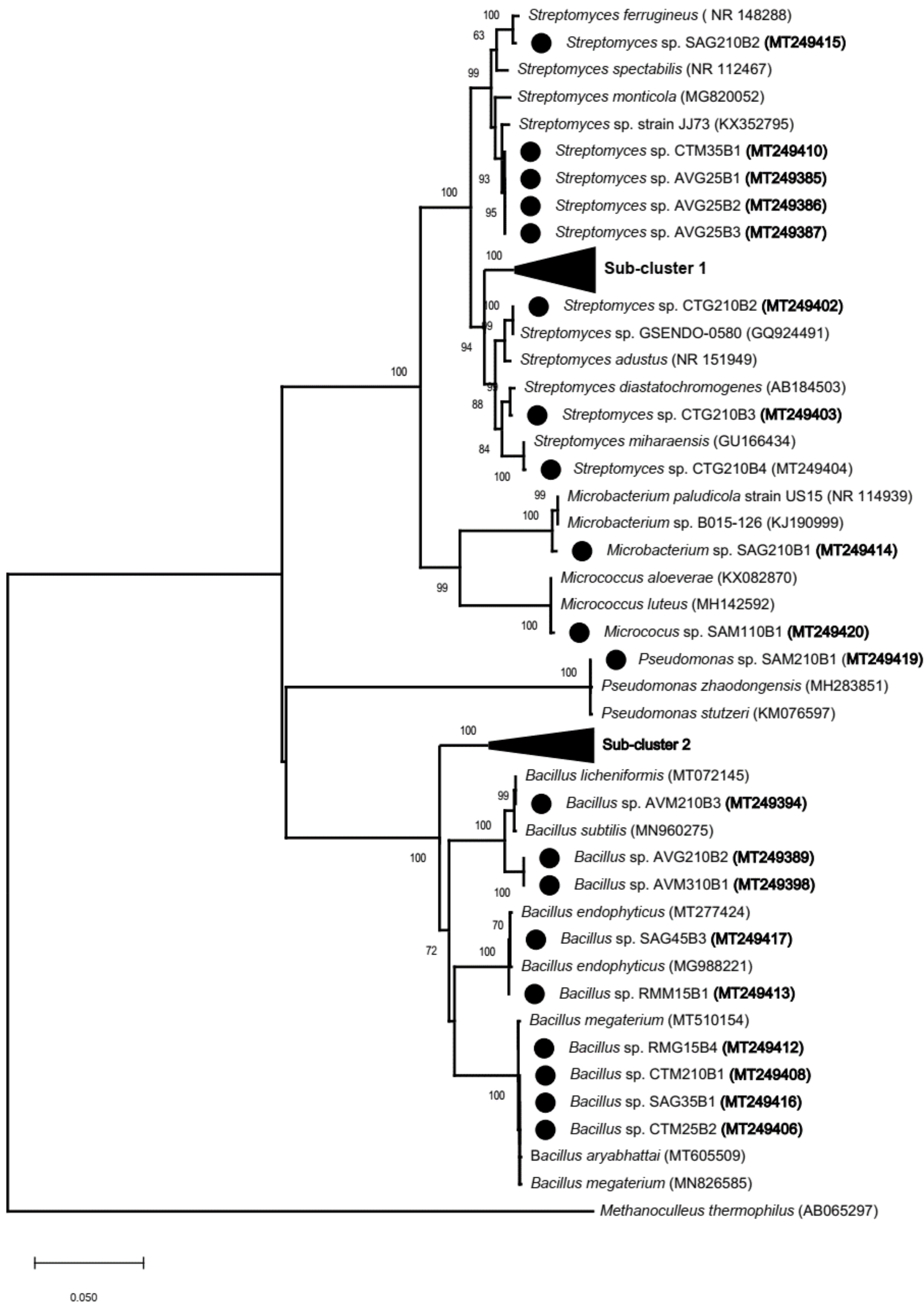

Figure 2. Evolutionary relationships between partial 16S rRNA gene sequences of the isolates and some selected known bacterial species.

terrestrial ecosystems (Mendes et al., 2013) have indicated that bacterial communities in the rhizosphere are influenced by plant species.

The results from morphological characterization indicated that the majority of the isolates were circular in form, cream in color, raised elevation, had entire margins and were gram positive. Morphological features have been widely used by most researchers for preliminary identification and placing of bacterial isolates into different morphotypes (Anna et al., 2018; Haldar and Nazareth, 2018; Saseeswari et al., 2016). Although useful, the information on morphological characters is insufficient to be used for final bacterial identification and, therefore, has to be supplemented with other data including the DNA relatedness studies, DNA-DNA hybridization, small subunit (SSU) sequences, cell wall composition and other characterization (Sarker et al., 2015).

In this study, the phylogenetic analysis of the 16S rRNA gene sequences of the isolates helped to identify and phylogenetically placed them into three phyla, namely *Firmicutes, Proteobacteria* and *Actinobacteria*. The phylum *Firmicutes* was the second most dominant and represented by members of the genus *Bacillus*. Several other studies have reported the occurrence of bacterial species from the genus *Bacillus* in mangrove habitats (Anna et al., 2018; Haldar and Nazareth, 2018; Mo et al., 2020). One of the reasons is that *Bacillus* species are easy to culture, and some can form endospores, whose primary function is to ensure their survival under harsh environmental conditions. *Bacillus* species are important for degradation of cellulose (Kurniawan et al., 2018) and phenolic compounds (Anna et al., 2018). They have also been reported to play a significant role in nitrogen fixation in the mangrove environment (Tam et al., 2017). Notably, members from this genus are considered beneficial to plant growth in the mangrove ecosystems as reported by Haldar and Nazareth (2018) who isolated phosphate solubilizing *Bacillus* species from mangrove soil. *Proteobacteria* was the least observed phyla in our study and was represented by one isolate of the genus *Pseudomonas*. This is consistent with the findings of Behera et al. (2014a) and Kurniawan et al. (2018) who reported the occurrence of *Pseudomonas* species in mangrove soils. *Pseudomonas* species are known to be key players in cellulose degradation and sulfur oxidation in the mangrove ecosystems (Behera et al., 2014b).

The phylum *Actinobacteria* was represented by the majority of the isolates since the media and protocol used for the isolation favored their recovery. Members from three genera (*Streptomyces*, *Mycobacterium* and *Micrococcus*) were obtained. Species from these genera are important for recycling biomaterials by humus formation and decomposition (Maldonado et al., 2005). In mangrove ecosystems, members of the phylum *Actinobacteria* are known to play important roles in mineralization of organic matter, control of mineral nutrients cycle and environmental protection (Pupin and Nahas, 2014). Literature indicates the importance of *Actinobacteria* from mangrove ecosystems owing to their economic value as a source of antibiotics (Mohan et al., 2014; Naik et al., 2013). The genera *Mycobacterium* and *Micrococcus* were the least identified among the *Actinobacteria*. Other studies have also reported the occurrence of these genera in the mangrove ecosystems (Behera et al., 2014a; Lee et al., 2014). Members from the genus *Streptomyces* were identified as the most dominant in our study. Species from this genus have been known to play key roles in soil ecology because of their ability to scavenge nutrients and, in particular, to hydrolyze a wide range of polysaccharides and other natural macromolecules (Barka et al., 2016). In addition, *Streptomyces* species have been useful to the pharmaceutical industry due to their enhanced capacity to produce secondary metabolites with diverse biological activities (Naik et al., 2013; Sengupta et al., 2015). Our results also concur with other studies (Priya et al., 2014; Malek et al., 2014) who also reported the occurrence of *Streptomyces* species in the mangrove sediments.

Bacteria are considered as potent and functional enzyme producer due to their high growth rate, availability of multi-enzyme complexes and stability at the harsh condition (Ladeira et al., 2015). Several studies have demonstrated diverse useful potential of bacteria isolated from mangrove ecosystems (Behera et al., 2014a; Kunasundari et al., 2017; Naresh et al., 2019; Soares Júnior et al., 2013). Our findings confirm that mangrove rhizospheres are a source of diverse bacterial communities that have been shown to produce secondary metabolites. For instance, most of the isolates recovered are from the genera that have been implicated in the production of different secondary metabolites including antimicrobial compounds and different enzymes in other studies (Naik et al., 2013; Pupin and Nahas, 2014; Behera et al., 2014a; Azman et al., 2015; Barka et al., 2016; Rasigraf et al., 2019; Wu et al., 2016). Further comprehensive studies focusing on isolation and screening of bacterial isolates for production of novel and or improved natural products is, therefore, recommended in order to give more insights on the types of antibacterial compounds produced and their effectiveness as antimicrobial agents.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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Isolation and identification of microorganisms in selected cosmetic products tester

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Components of cosmetics supply a rich medium for the growth of microorganisms. Customers usually like to try cosmetics before buying them. Repetitive use of the eye and makeup tester by different customers may become a potential vector of microbial pathogens. The major goal of this study was to evaluate microbial contamination of the cosmetics that are accessed as an unsealed make-up tester in the selected makeup shops located in Saudi Arabia. Sixty samples of cosmetic testers including mascara, lipstick, blushes, contour, powder, and brushes were collected from shopping malls. These samples were inoculated into different culture media including Blood agar, MacConkey agar, and Saboraud dextrose agar to cultivate fastidious bacteria, coliforms, and fungi, respectively. The microorganisms isolated and identified were: *Staphylococcus epidermidis* (57%), *Candida albicans* (27%), *Propionibacterium acnes* (12%), *Pseudomonas aeruginosa* (2%), and *Staphylococcus aureus* (2%). From the sample collected in this project, all bacteria species isolated are considered as skin normal flora except *P. acnes* and *S. aureus* that cause acne. The study has concluded that cosmetic testers from selected shopping malls are almost contaminated by bacteria and fungi; the risks generated by inappropriate usage and sharing cosmetics tester samples in public.

Key words: Microbial contamination, shared cosmetic testers, inappropriate usage, skin acne.

INTRODUCTION

Cosmetics have become an important part of everyday life and are widely used for beauty purposes, sun protection, and clearing extraneous matter (Onurdağ et al., 2010). Abu Shaqra and Groom (2012) assert that cosmetic products contain essential minerals and chemical compounds in water that provide a favorable medium for microbial growth (Dadashi and Dehghanzadeh, 2016). The majority of the people who use shared tools in the beauty shops are unaware of the fact that makeup can harbor variety of microorganisms that cause risk for exposure to potentially infectious microorganisms (Enemuor et al., 2013). It is believed that cosmetics can be associated with skin or eye infections which can be transmitted to and between clients if not handled properly (Detmer et al., 2010; Siegert, 2012).

Cosmetic testers in the beauty shops are often contaminated with microorganisms due to the sharing of makeup and repeated use of the same applicators as

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well as poor handling during the showcase of the product (Ibegbulam-Njoku and Chijioke-Osuji, 2016; Gamal et al., 2015). Risk of skin infection to consumers can occur as a result of use of skin products such as powder and cream, eye products like mascara and eyeliner (Dadashi and Dehghanzadeh, 2016), and hairdressing (Enemuor et al., 2013). Some pathogenic microorganisms including *Staphylococcus aureus* and *Pseudomonas aeruginosa* are detected in beauty products (Sreeparna et al., 2017; Detmer et al., 2010). *S. aureus* and *Staphylococcus epidermidis* were the most important bacteria that cause diseases to humans such as skin infection, boils, bullous impetigo, hair follicles, and scalded-skin syndrome in hairdressing and beauty salons in India and Nigeria (Sreeparna et al., 2017; Enemuor et al., 2013; Duggal et al., 2016). Another study showed that *Bacillus*, *Staphylococcus*, *Pseudomonas*, *Enterobacter*, *Escherichia coli*, and *Klebsiella* were the common bacteria isolated from cosmetic products (Bashir and Lambert, 2019).

Skincare products, hair preparations, and facial make-up were responsible for the majority of allergic contact dermatitis in some regions of the Middle East (Abu Shaqra and Al-Groom, 2012; Alswedi and Jaber, 2019). Herpes causes blisters on the lips and around the mouth from shared make-up tools. Lipsticks and powder brushes that touch these parts of the face can then spread the allergic contact dermatitis infection to other people (Belinda, 2015). Studies by Noor et al. (2015) have revealed that the most common bacteria such as *P. aeruginosa* and *Pseudomonas putida* presented on eye makeup include mascara and eyeliner. *P. aeruginosa* can cause irritation, conjunctivitis, pink eye, redness, and watery discharge, which could lead to irreversible blindness (Noor et al., 2015). Makeup brushes also have the potential to act as suitable homes for bacteria to thrive.

Although the microbial standards of cosmetics have been progressively improved by strict law; their contamination has been frequently reported and even in some cases, has generated serious problems for consumers (Wenli et al., 2019). Unfortunately, cosmetic contamination awareness and health risks are very poor among the users of all age groups. There is no established law, guidelines, and best practices for many public make-up testers. The main objective of this study was to assess the bacterial and fungal contamination from different cosmetic testers in six shopping malls and evaluate customers by using cross sectional study to examine if customers are conscious of the beauty products that are used.

MATERIAL AND METHODS

Study design: Survey study

A questionnaire survey model was designed and distributed to 60 randomly selected costumers who used cosmetic testers from 6 different beauty shops in the country of Saudi Arabia at Jeddah city. The ages of the costumers were between 20 and 40 years old at different education level. This survey was used to evaluate customers awareness about the health risks associated with the sharing of cosmetics.

Sampling

A total of 66 samples representing 6 different categories of shared cosmetics testers (10 lipsticks, 10 powders, 10 mascaras, 10 contours, 10 brushes, 10 blushes) as well as 6 sealed (new and unused) were taken from each group for controls. Different batch numbers of each category of testers samples were collected from 6 different cosmetics stores at shopping centers of Jeddah city in Saudi Arabia between January and March of 2019. The samples were collected with the approval of the stores using moistened sterile swabs, transferred into transport media. Then, the samples were carried to the laboratory and stored at room temperature for 24 h to be analyzed.

Media use and isolation process

Three different media were used (Blood agar, MacConkey agar and Saboraud dextrose agar) to cultivate fastidious bacteria, coliforms, and fungi, respectively. The culture media were prepared and instructions from the Micromaster, India manufacture were followed. Sixty swabs of shared cosmetics testers, six sealed samples were collected, and each sample was inoculated on 3 different solidified media mentioned earlier. The dishes were incubated for 24 h at 37°C while SDA plates for fungi identification were incubated at 28°C and observed daily for 7 days. All plates were observed for the growth of microbial colonies after incubation to identify colonial morphology. Gram stains were carried out to determine if bacteria were Gram-positive bacteria, Gram-negative bacteria, and yeast.

Biochemical tests

Biochemical tests fall under phenotypic methods for microbial isolation. Protocols for standard biochemical tests were followed and used to identify the bacteria and fungi in respect of the genus and the species level (Bergey and Holt, 2000). For Gram-positive cocci, we have used the following tests: catalase, coagulase, novobiocine, and bile-esculin. However, Kligler Iron Agar test, motility, indole test, and citrate biochemical tests were used to identify Gram-negative bacilli. Various *Candida* species can be detected by a germ test tube and sugar fermentation tests including glucose, maltose, and sucrose, which cause a color change as an indicator.

RESULTS

The cosmetic product tester's analysis showed that more than 70% of examined samples of lipsticks, powders, mascaras, contours, brushes, and blushes were contaminated with microorganisms.

The morphological characterization and biochemical tests showed that 73% of the samples were contaminated with bacteria in comparison to 27% of the samples contaminated of fungal growth (Table 1). The most

Table 1. Total number of microorganisms isolated from each cosmetic tester in beauty shops.

Cosmetic tester category	Name of bacteria	Number of samples with the microorganisms
Lipsticks	*Pseudomonasaeruginosa*	1
	Staphylococcus epidermidis	5
	Candida albicans	1
Powders	*Staphylococcus epidermidis*	4
	Candida albicans	3
	Propionibacterium acnes	1
Mascara	*Staphylococcus epidermidis*	1
	Candida albicans	1
Contours	*Staphylococcus epidermidis*	7
	Candida albicans	2
	Propionibacterium acnes	1
Brushes	*Staphylococcus epidermidis*	7
	Candida albicans	1
	Propionibacterium acnes	2
	Staphylococcus aureus	1
Blushes	*Staphylococcus epidermidis*	4
	Candida albicans	5
	Propionibacterium acnes	2
Total		49

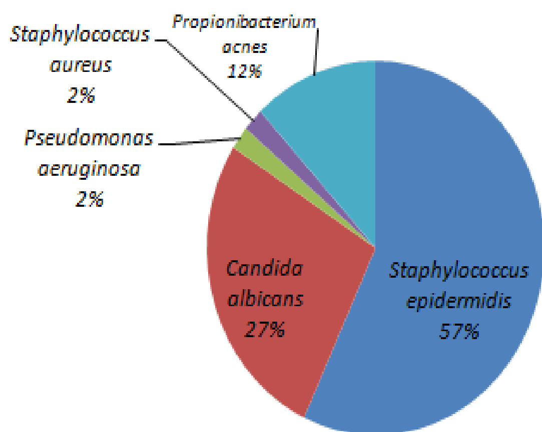

Figure 1. The percentage of each microbial growth isolated from different cosmetic testers.

predominant isolated bacteria were *S. epidermidis* with 57% of samples contaminated with this species. *P. aeruginosa* was the least frequently isolated bacteria with 2% only. *S. aureus* was only observed in brush samples with 2%.

Depending on the results of germ tube and sugar fermentation and results presented in Figure 1, it showed *Candida albicans* was the only isolated yeast with 27%. In addition, 12% of samples were contaminated with *Propionibacterium acnes* from most of the cosmetic testers: brushes, blushes, powders, and contours.

The written questionnaire that was collected from random customers showed that 52% of women believed that the cosmetics testers caused diseases, allergies, and irritation to the skin. The awareness rate of the bacteria that might appear in the cosmetics testers was high. Twenty four percent of women do not use cosmetics testers on their faces directly. Seventy six percent of women believed that the validity of cosmetics testers is affected by the use of products (Figure 2).

DISCUSSION

In this study, bacterial contaminations of the cosmetics tester material were more than those associated with fungi. The microorganism contamination rate in the contour, brushes, and blushes was higher than the other cosmetics because they are frequently in contact with air.

The use of non-sterile cosmetic testers can spread bacteria from one person to another and possibly can cause infection on the eye or skin. In this study, bacterial

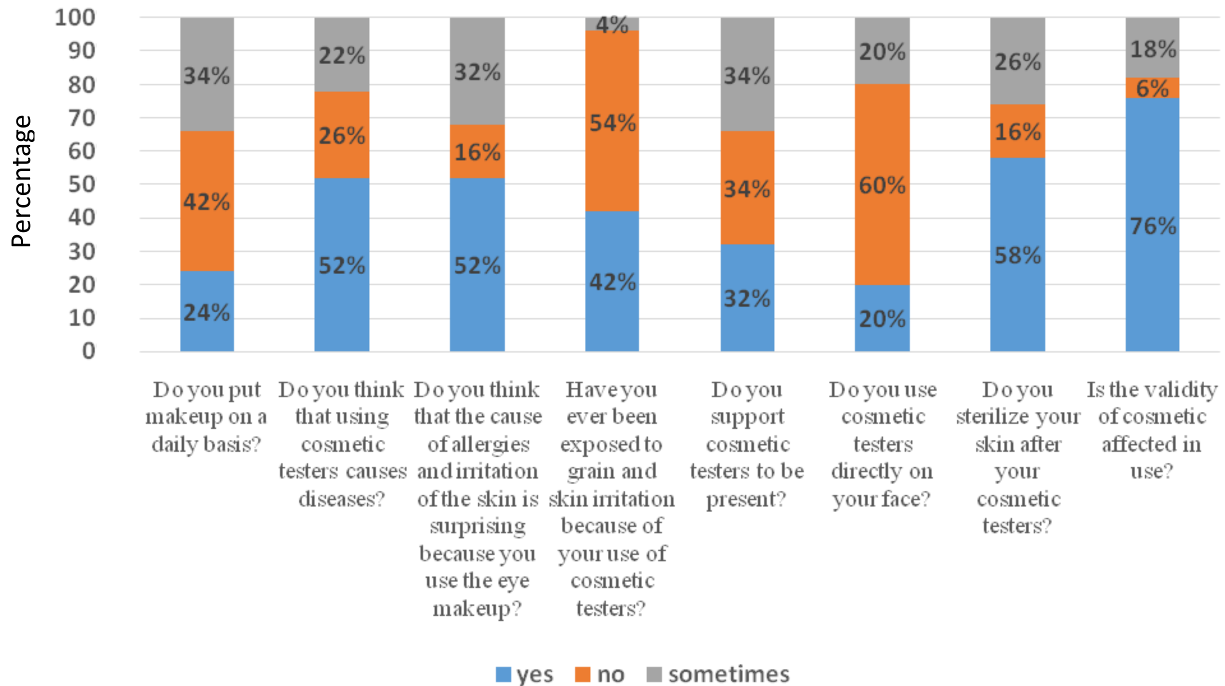

Figure 2. Survey collected from customers in the shopping malls to evaluate the awareness about the risk of using cosmetic testers.

contaminations were more than fungi. Our results showed 27% of shared cosmetic testers that were contaminated by fungus and yeast. In other studies, the fungus contamination ratio in cosmetics was low because beauty products have a protective material that prevents fungi growth (Dadashi and Dehghanzadeh, 2016). In the current study, a *S. epidermidis* was the most common species isolated from some different cosmetic testers. These species are considered to be a normal flora of the skin. However, *Streptococcus* species in the cosmetic testers were also identified and could be pathogenic organisms that occurred in some regions including the Middle East in which it could cause a skin infection such as acne and herpes (Gevers et al., 2012; Sohn, 2018; Abu Shaqra and Al-Groom, 2012; Alswedi and Shakeir, 2019). *P. acnes* were one of the bacteria that caused acne observed in tester samples (Scholz and Kilian, 2016). Furthermore, *S. aureus* was isolated and is among the most important bacteria that cause disease in humans including skin infections and abscesses (Al Kindi et al., 2019). These symptoms could occur due to the impairment of the human immune system, skin structure, and wounded epithelium (Al Kindi et al., 2019).

Based on the outcome of this study, it showed no microorganisms growth on the sealed cosmetic samples and possibly due to preservatives of the cosmetics in a sterile condition (Giacomel et al., 2013).

In the current study, the contamination rate in the contour, brushes, and blushes was higher than other testers and *C. albicans* was the only yeast observed in the cosmetic testers that induced skin infection. The preservatives ingredients in the formulation of cosmetics products might increase the contamination rate (Halla et al., 2018).

According to the results of the questionnaire, the study addressed the possibility of beauty shops acting as a source of infection among consumers. The survey results are interesting because it showed that customers tended to continue to use make-up beyond the contamination.

Public health organization recommended using cosmetic products tester correctly to decrease the health risks. Customers must use sterile tools and to avoid trying cosmetic testers on the face. It is necessary to use sealed makeup instead of testers. Moreover, dispense the makeup if it has been used for more than three months. If the individuals are educated on the risks of the dangers, they would help in improving their practices and by doing that, it decreases the spread of these contaminations. In the future, more microbial analysis needs to be examined.

Conclusion

Overall, this research was an informative view of the importance of not sharing cosmetic samples in public to prevent skin infections. In shopping malls, cosmetic testers almost contaminated by bacteria and fungi as a

study has been concluded. By sharing cosmetics in public, the possibility of infection can occur. Furthermore, scrutinizing this topic was practicable and intriguing.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Isolation, characterization and biotechnological potential of tropical culturable rhizospheric fungi from four mangrove species in Kenya

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Fungi play important roles, as both decomposers and plant symbionts in mangrove ecosystems. Their ability to survive extreme environmental conditions makes them potential rich sources for recovery of novel bioactive compounds. In this study, biotechnological potential of fungi recovered from the rhizospheres of four mangrove species (*Sonneratia alba, Rhizophora mucronata, Ceriops tagal* and *Avicennia marina*) was explored. Rhizospheric sediment samples of the mangroves were collected using standard protocols and different media used for isolation of fungi. A total of 33 fungal isolates were recovered and characterized based on morphological, physiological and Internal transcribed spacer (ITS) gene region analysis. The disk diffusion method was used to evaluate the antibacterial activity of the isolates. Morphologically, the isolates showed different characteristics with regard to color, margin and elevation. Physiologically, all the isolates were able to grow at different pH ranges, ranging from pH 4 to pH 12 and at different temperatures from 20 to 30°C and tolerated sodium chloride up to 7%. Phylogenetic analysis grouped the isolates into two phyla: *Ascomycota* and *Basidiomycota* affiliated to different genera; *Penicillium, Aspergillus, Talaromyces, Diatrypella, Thielavia, Hypocreales, Paracremonium, Geosmithia, Peniophora, Massarina* and *Fulvifomes*. A total of 17 representative isolates produced inhibition zones against three pathogenic bacterial strains. The findings demonstrate that the tropical mangroves rhizospheres are a rich source of fungi with antibacterial compounds and traits relevant for biotechnological application.

Key words: Antimicrobial activity, tropical marine fungi, Mangrove sediments, Internal transcribed spacer (ITS).

INTRODUCTION

Mangrove forests are considered a dynamic ecotone between terrestrial and marine habitats (Thatoi et al., 2013). They occupy about 181,000 km^2 worldwide (Jusoff, 2013). The mangrove ecosystems cover a

quarter of the world's tropical coastlines and are major drivers for transferring organic matter from land to oceans (Simões et al., 2015). They form a niche with a wide spectrum of microbial diversity with bacteria and fungi constituting 91% of the total biomass of mangrove ecosystems (Simões et al., 2015). Fungi are important soil component as both decomposers and plant symbionts (Liu et al., 2015). They play major roles in ecological and biogeochemical processes, contributing significantly to the degradation of mangrove-derived organic matter (Ghizelini et al., 2012). Similar to other ecosystems, fungi are believed to play an important role in carbon and nitrogen recycling in mangrove ecosystems (Reef et al., 2010). Other studies have suggested that mangrove fungi serve as a source of biologically active natural products (Wu et al., 2016).

Although a considerable number of fungi have been isolated from the mangrove ecosystems from time to time, they were first reported from mangrove roots in Australia (Cribb and Cribb, 1956). Since then, there is still a need for information on mangrove- associated fungi. This is important in order to gain new insight into the fungal communities in mangrove ecosystems, since most studies conducted in these ecosystems have mainly concentrated on bacterial diversity and less attention is given to fungal communities (Simões et al., 2015). Additionally, there is alarming increase of emerging and re-emerging diseases. This is due to newly discovered and constantly evolving pathogens. Considering the potential benefits of fungi, the search for natural biologically active compounds from fungi against pandemics and infectious agents is necessary.

The potential for the recovery of anticancer, antioxidant and antimicrobial compounds makes the prospecting for mangrove fungi important (Thatoi et al., 2013). Mangrove ecosystems are hotspots for morphologically and physiologically diverse fungal groups, considering that the species have adapted to the extreme environmental conditions (hypoxic, fluctuating pH, tidal pressures, high salinity, etc.). Anticancer / antioxidant biomolecules (Bhimba et al., 2012; Prathyusha et al., 2018), and novel enzymes with potential for biotechnological applications have been recovered from fungi isolated from mangroves (Wu et al., 2018). Though an impressive panoply of novel biomolecules have been recovered from mangrove associated fungi, much of the focus has been on endophytic fungi (Guerrero et al., 2018; Kuzhalvaymani et al., 2019), while free-living mangrove fungi remain highly underexplored. The recent discovery of five novel funicone derivatives from *Penicillium pinophilum* SCAU037 by He et al. (2019) demonstrates the biotechnological potential of free-living fungi adapted to mangrove sediments. Having in mind that plant species and geographical location can influence the diversity of microbes (Wu et al., 2016), this study was designed with an aim of isolating, characterizing and exploring the biotechnological potential of fungi from the rhizospheres of four mangrove species found in Mida creek (a polluted mangrove habitat) and Gazi Bay (a pristine mangrove site).

MATERIALS AND METHODS

Site description

This study investigated two mangrove sites (Mida creek and Gazi bay) in Kenya (Figure 1) for the presence of potential biotechnologically relevant fungal species. Gazi bay is located in Kwale County (04°44′S 039°51′E), on the South Coast of Kenya and approximately 55 km south of Mombasa. The Chale peninsula to the East and a fringing coral reef in the South protects the Bay from strong sea waves. The climate is mostly humid and hot and the average annual humidity and temperature are about 95% and 28°C respectively. The total annual precipitation ranges from 1000 to 1600 mm (Lang'at, 2008). Mida Creek is located in Kilifi County (03°34′S, 039°96′E), approximately 25 km South of Malindi town and about 88 km North of Mombasa (Lang'at, 2008). Monthly temperature ranges between 23 and 27°C, with monthly maximum at 34°C in the hottest months and minimum at 20°C in the coldest months. The total annual precipitation ranges from 1000 - 1600 mm (Lang'at, 2008).

Sample collection

Rhizospheric sediment samples were obtained from four mangrove species (*Sonneratia alba, Rhizophora mucronata, Ceriops tagal* and *Avicennia marina*) common to the two study sites. Sampling was done in May 2018 according to previously described methods (Wu et al., 2016). Four mangrove trees of each species at intervals of 10 m were selected. For each individual mangrove species, the rhizosphere sediments (~100g) were sampled vertically along the base of the plant at two depths (1-5 cm and 10-15cm), using a standardized core sampler (Giannopoulos et al., 2019). A total of 32 samples (4x4x2) per site were kept in sterile plastic bags, maintained on dry ice in a box before they were transported and stored at -20°C until further analyses was performed at the Laval University, Quebec City, QC, Canada.

Physicochemical analysis of sediment samples

Nutrient analyses of the soil samples for nitrogen, carbon, phosphorus, potassium, calcium, magnesium and sodium were conducted according to standard methods (Brupbacher et al., 1968). Determination of pH and electrical conductivity was done using the calcium chloride method at a ratio of 1:2 using a digital Corning pH meter 140 (Corning Life Sciences, Massachusetts, USA) and electrical conductivity meter type CDM 2d radiometer (Radiometer, Copenhagen, Denmark), respectively.

Sample pre-processing and isolation of fungi

The sediment samples were air dried at room temperature (27±1°C)

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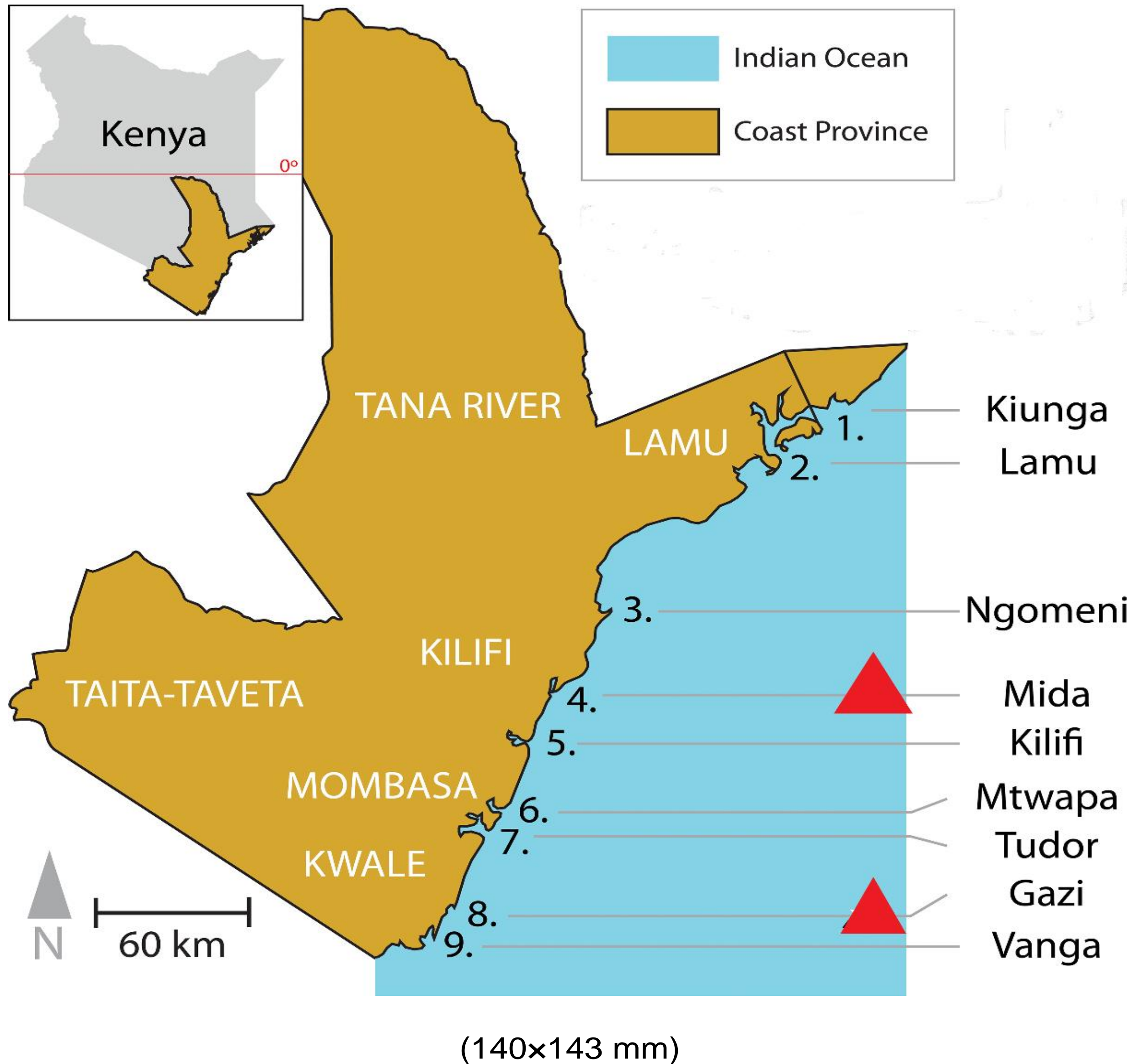


(140×143 mm)

Figure 1. Map of Kenya highlighting the Kenyan coast region. Position number 4 represents the Mida creek study site, and position number 8, represents the Gazi bay study site.
Source: Jenoh et al. (2016).

for seven days and sieved with a 2.5 mm sieve to remove larger particles such as stone and plant debris in order to obtain a consistent soil particle size for isolation using the soil dilution technique. The isolation of fungi from the sediment samples was performed by serial dilution method. About 0.1 g of the sediment sample was suspended in 1ml sterile distilled water in a sterile 1.5 ml Eppendorf tube and serially diluted to 10^{-3}. One hundred μL of the10^{-1}, 10^{-2}, and 10^{-3} suspensions were spread in triplicate onto isolation media. Dilutions were spread into three different types of isolation media, which included Potato dextrose agar (PDA), Czapek dox agar and Glucose, peptone, yeast extract agar (GPYA). All media were prepared according to the manufacturer's instructions and supplemented with streptomycin (100 µg ml^{-1}) to prevent any bacterial growth. All the plates were placed in a microbiological incubator at 28°C for 7 to 28 days. Daily follow up was done to observe any growth on the plates.

Morphological characterization of isolates

Morphological characterization of the fungal isolates was done by studying the colony characteristics of the fungal culture with regard to color on the upper and lower surface of the culture plate, margin and elevation (Kuzhalvaymani et al., 2019).

Physiological characterization of the fungal isolates

Growth behavior of the fungal isolates at different temperature, pH and sodium chloride concentration ranges were determined. For temperature, a 5 × 5 mm mycelia growth disc was inoculated on PDA media and incubated at 20, 30, 40 and 50°C for 7 days. The radial growth in mm for the colonies was measured in triplicate and recorded. For pH, the PDA media was adjusted to pH 4, 6, 8, 10

and 12 using 1M HCl and 1M NaOH solutions and poured into sterile plates. The agar plates were then inoculated with a 5× 5 mm mycelia growth slice of the isolates and incubated at 30°C for 7 days. Radial growth was measured (millimeters) and recorded. Sodium chloride tolerance test was done by supplementing the media with different concentrations of sodium chloride (5, 7, 9, 11 and 15%). For each case, a 5 × 5 mm mycelia growth slice was inoculated and plates incubated at 30° for 7 days before the radial growth was measured (mm) and recorded.

Extraction of secondary metabolites from the fungal isolates

Three pieces of 5×5 mm mycelia growth discs of selected fungal isolates were inoculated into 500-ml Erlenmeyer flasks containing 300 mL soybean casein broth and incubated at room temperature for fourteen days under stationary conditions. The broth culture was filtered to separate the filtrate and mycelia. The filtrate was then used for testing of antimicrobial activity.

Screening of fungal isolates for antimicrobial activity

Three standard pathogenic bacterial strains, a Gram-positive (*Staphylococcus aureus* ATCC 25923) and two Gram-negative (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) were used for the determination of antibacterial activity of extracts of the fungal isolates. The disk diffusion method (Balouiri et al., 2016) was used to evaluate the antibacterial activity of extracts from selected fungal isolates. Mueller-Hinton agar plates were inoculated with 100 mL of a suspension of bacteria (*S. aureus, E. coli, or P. aeruginosa*), adjusted to equal the turbidity of 0.5 McFarland standard. Inoculated plates were allowed to air dry. Paper disks (MAST, UK) were prepared by soaking sterile paper disks (Ø=6 mm) with prepared fungal extracts and dried before they were applied to the surface of the inoculated agar plates. Prepared plates were incubated at 30°C for 24h then the diameter of each inhibition zone was measured using a Vernier caliper (Mitutoyo, Tokyo, Japan). All the tests were done in triplicates.

Molecular characterization of the fungal isolates

Genomic DNA (gDNA) extraction and polymerase chain reaction (PCR) amplification

Genomic DNA was extracted from pure fungal cultures using the DNeasy Plant kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracted gDNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Fisher Waltham, MA, USA), as a template for the amplification of the ITS gene region. A set of ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') universal primers for fungi (White et al., 1990) were used. PCR was carried out in a 25 µl reaction volume; consisted of 2.5 µl 10× PCR buffer, 0.75 µl $MgCl_2$, 0.5 µldNTPs, 0.5 µl of each primer, 0.2 µl platinum Taq (Invitrogen), 1µl of template DNA and 19.05µl of water. Amplification was performed with initial heating at 95°C for 30s followed by 30 cycles of denaturation at 95°C for 40s, annealing at 54°C for 50 s and extension at 72°C for 1 min and a final extension period at 72°C for 5 min using MJ Research PTC-225 Peltier Thermal Cycler 9 (MJ Research, Nevada, U.S.A.). Amplicons were confirmed by visualization on 1% ethidium bromide stained agarose gels in a gel documentation chamber. The PCR products were sequenced directly using the Sanger sequencing platform at the Institute for Systems and Integrative Biology of Laval University, Canada. The sequences from this study have been deposited in GenBank (www.ncbi.nlm.nih.gov/Genbank/submit.html) under the accession numbers, MT543100 - MT543132.

Phylogenetic analysis

Sequences of the isolates were manually edited in chromas and checked for presence of artifacts or sequencing errors using Mallard software (Ashelford et al., 2006), an NCBI bioinformatic tool for detecting chimera sequences. A search for similar sequences using BLASTN (Altschul et al., 1990) was performed, and sequence alignment was performed using the CLUSTAL Omega program (http://www.clustal.org) against the nearest neighbors. A neighbor-joining tree of the aligned sequences was constructed (Saitou and Nei, 1987) using MEGA X software (Kumar et al., 2018). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004). To obtain statistical support values for the branches, bootstrapping (Felsenstein, 1985) was conducted with 1000 replicates. All sites, including gaps in the sequence alignment, were excluded pairwise in the phylogenetic analysis. Using the resultant neighbor-joining tree, each isolate was assigned to the proper taxonomic group. The taxonomic assignment was confirmed at a 95% confidence level using the RDP Naïve Bayesian rRNA Classifier Version 2.11 on the RDP website (Wang et al., 2007).

Statistical analyses

Data from physicochemical parameters was analysed using R v3.6.1 (Somanathan et al., 2004). A two-factor (sites and mangrove species differences) test of differences in physicochemical parameters was done by the non-parametric Kruskal-Wallis H test using the agricolae (de Mendiburu, 2020) R package. Post hoc test for mean separations was based on Fisher's least significant difference.

Data from physiological analysis and antimicrobial activity was analyzed using ANOVA followed by Tukey HSD post-hoc test using SigmaPlot version 14.0 (Systat Software, San Jose, CA). Results were expressed as the mean ± SD. A p-value of ≤ 0.05 was considered statistically significant. All experiments were performed independently at least three times.

RESULTS

Physicochemical analysis of sediment samples

The pH and calcium were significantly higher (Kruskal-Wallis, $p \leq 0.05$) in all mangrove plant species of Mida creek compared to Giza bay, which had significantly higher electrical conductivity (EC) and salinity values (Table 1). Besides the rhizosphere of *R. mucronata*, all other mangrove species rhizosphere in Gazi bay had significantly lower ($p < 0.05$) physicochemical properties compared to the species in Mida creek. The physicochemical parameters that were higher in the rhizosphere of *R. mucronata* in Gazi bay included potassium, sodium, phosphorus, total carbon, nitrogen, salinity and EC (Table 1).

Morphological characterization of isolates

A total of 33 fungal isolates were obtained from the

Table 1. Characteristics of mangrove sediment samples collected from Mida creek and Gazi bay.

Physicochemical parameters	*Avicennia marina*		*Ceriops tagal*		*Rhizophora mucronata*		*Sonneratia alba*	
Site	**Gazi**	**Mida**	**Gazi**	**Mida**	**Gazi**	**Mida**	**Gazi**	**Mida**
Calcium (mg/kg)	325.75± 64.73	14085.5± 3686.55***	168.25± 27.76	79312.88±38869.51***	2670.62±544.23	62731.12±26956.45***	477± 147.65	52097± 18982.24***
Potassium (mg/kg)	663.12± 192.89	494±182.67	178.25± 40.78	464± 111.51***	1588.87±172.27***	597.375 ± 211.89	461.87± 138.12	594.75 ± 82.25*
Magnesium (mg/kg)	379.125± 76.07	841.75± 470.97*	115.12 ± 21.66	1776± 660.54***	1856.12±140.19*	1307.12 ± 665.24	406.87± 68.52	1166.62± 345.31***
Sodium (mg/kg)	2369±815.28	4162±3483.99	155.87±40.50	3933.87±1448.40***	8472.25±967.11***	3263.75±1446.15	1978±431.28	2948.37±471.96***
Phosphorus (mg/kg)	55±16.29	136.12±33.09***	40±2.87	164.5±61.01***	187.75±49.67**	98.87±54.49	55.125±14.06	99.75±36.32**
Total carbon (mg/kg)	0.82±0.41	1.90±0.57***	0.26±0.07	4.55±1.56***	7.40±0.85***	2.69±1.67	0.99±0.23	2.25±0.60***
Nitrogen (mg/kg)	0.04±0.02	0.16±0.03***	0.01±0.01	0.18±0.04***	0.37±0.06***	0.11±0.05	0.07±0.01	0.09±0.01*
Electrical conductivity (S/m)	5.78±0.95	4.56±1.39	3.37±1.31	6.51±2.98*	12.37±1.00***	4.05±1.36	4.88±0.88	5.22±1.34
pH	7.05±0.55	8.17±0.21***	6.21±0.10	7.93±0.07***	6.09±0.15	8.08±0.28***	6.08±0.08	7.91±0.16***
Salinity (mg/kg)	3.13±0.55	2.43±0.79	1.77±0.72	3.58±1.75*	7.08±0.61***	2.15±0.78	2.61±0.50	2.81±0.77

Values represent mean and standard deviation. Superscripts beside values are significantly different measures (p ≤ 0.05) based on Fisher's least significant difference. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05).

mangrove sediments. Isolates were generally slow growers as they grew between 7-28 days. Morphological characterization was based on traditional macroscopic features of color, margin and elevation of the pure colonies. The isolates exhibited different colony characteristics with the majority of them having curled margins and raised elevation. The reverse color was mostly black, while the obverse was mainly grey.

Physiological characteristics of the isolates

All the isolates exhibited growth at different pH ranges, but individual isolates exhibited optimal growth at different pH ranges as shown in Figure 2a. For instance, isolates AVM110F1, CTG25F1, AVM110F2 and AVG410F1 had no significant growth at the tested pH ranges (p-value > 0.05). RMG110F1 had a significant growth at pH 4 and pH 10. Isolates RMG110F2 and RMG210F1 grew optimally at pH 8, while AVG35F1 grew optimally at pH 10 and pH 12. Isolate SAG35F1 had a significant growth at pH 6 and 12, while CTG45F1 grew significantly at pH 4, pH 6 and pH 10 (Figure 2a). Growth at different temperatures was only observed at 20 and 30°C for all the isolates (Figure 2b). No growth was observed at 40, 50 and 60°C; however, growth differences of the isolates were observed at both temperatures (20 and 30°C). There were insignificant growth changes at both temperatures for isolates CTG25F1, AVM110F2, RMG110F2 and AVG410F2. Isolates AVM110F1, RMG110F1, SAG35F1, RMG15F1, CTG45F1, RMG25F1 and SAG15F1 grew optimally at 20°C, while isolates RMG210F1, RMM15F1, AVG410F1 and AVM15F2 were observed to grow optimally at 30°C. At different NaCl concentrations, 29.4% of the tested isolates exhibited insignificant growth compared to 58.8% of the tested isolates, which had relatively growth changes with increase in NaCl concentration (Figure 2c). Isolates RMG110F2, RMM15F1, RMG15F1, RMG25F1 and SAG15F1 had significant growth at 5 and 7% NaCl (p-value ≤ 0.05).

Screening of isolates for antimicrobial activity

The result on antibacterial activity of the fungal isolates against some known bacterial strains (*E. coli*, *P. aureginosa* and *S. aureus*) is presented in Table 2. Extracts of 17 fungal isolates showed different inhibition zone diameters on all or any of the three pathogens. Some isolates exhibited significantly higher inhibition zone diameters (*P-value ≤ 0.05*) as indicated in Table 2. For instance, isolates RMG110F1, RMG210F1, AVG35F1, RMG35F1, AVG410F1, SAG35F1, RMG15F1, AVG410F2 and AVM15F2 produced significantly higher inhibition zone diameters against the tested bacterial strains. Notably, isolate AVG410F2 significantly inhibited the growth of all bacterial strains while isolates SAG35F1 and AVM15F2 had significantly higher inhibition zone

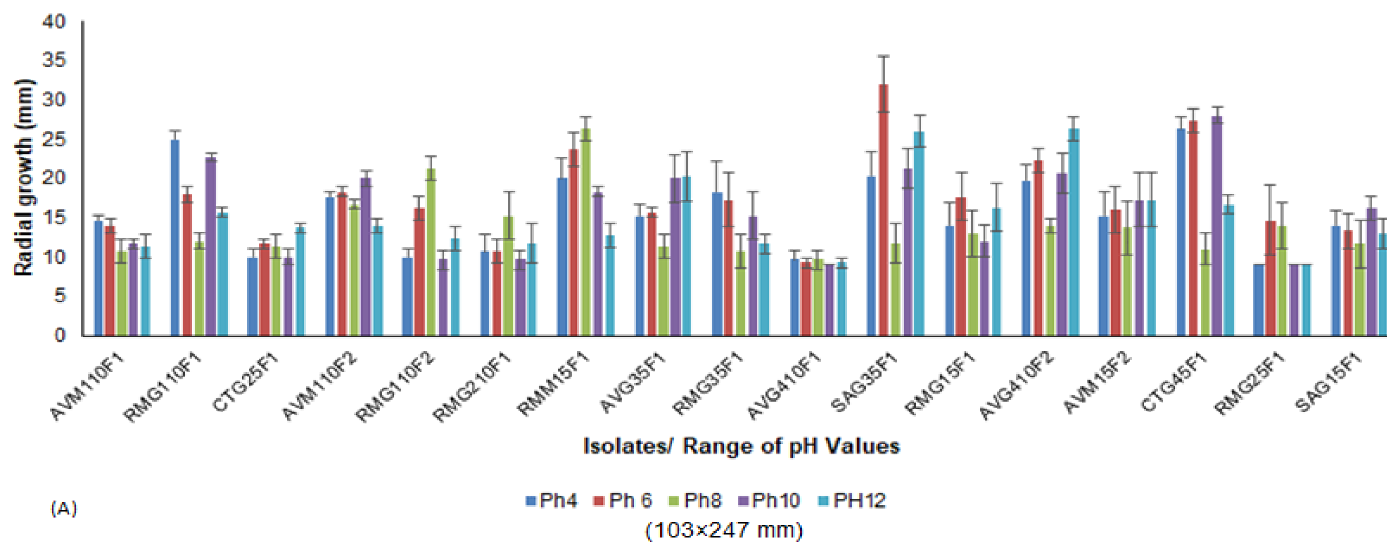


Figure 2a. Growth of isolates at different pH ranges.

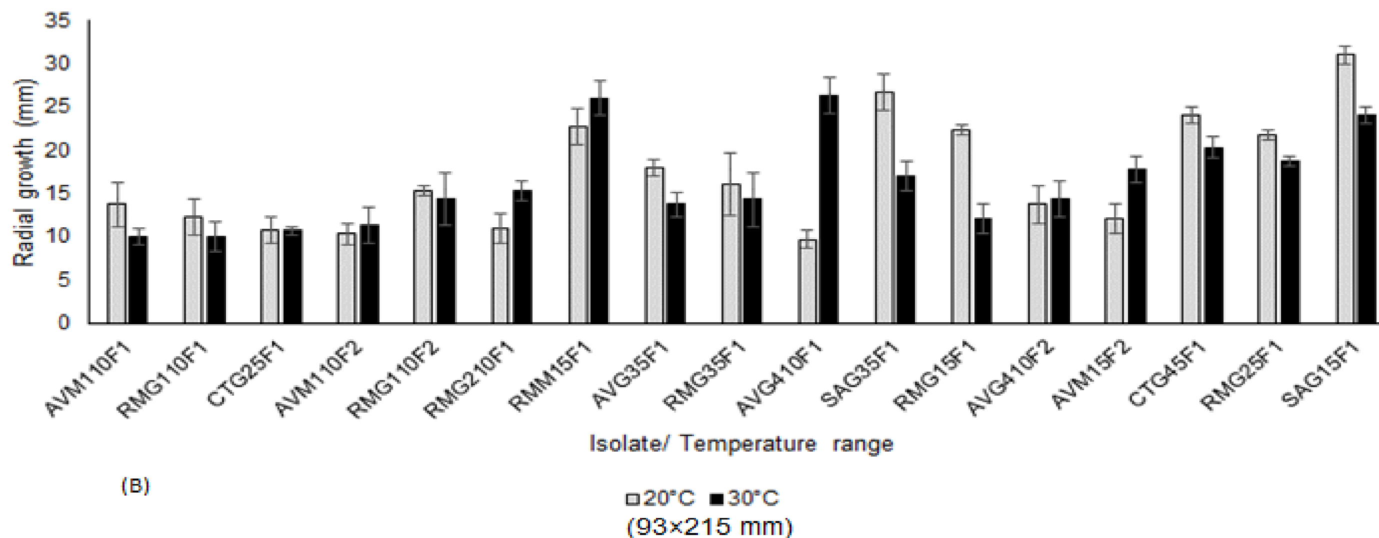


Figure 2b. Growth of isolates at different temperature ranges.

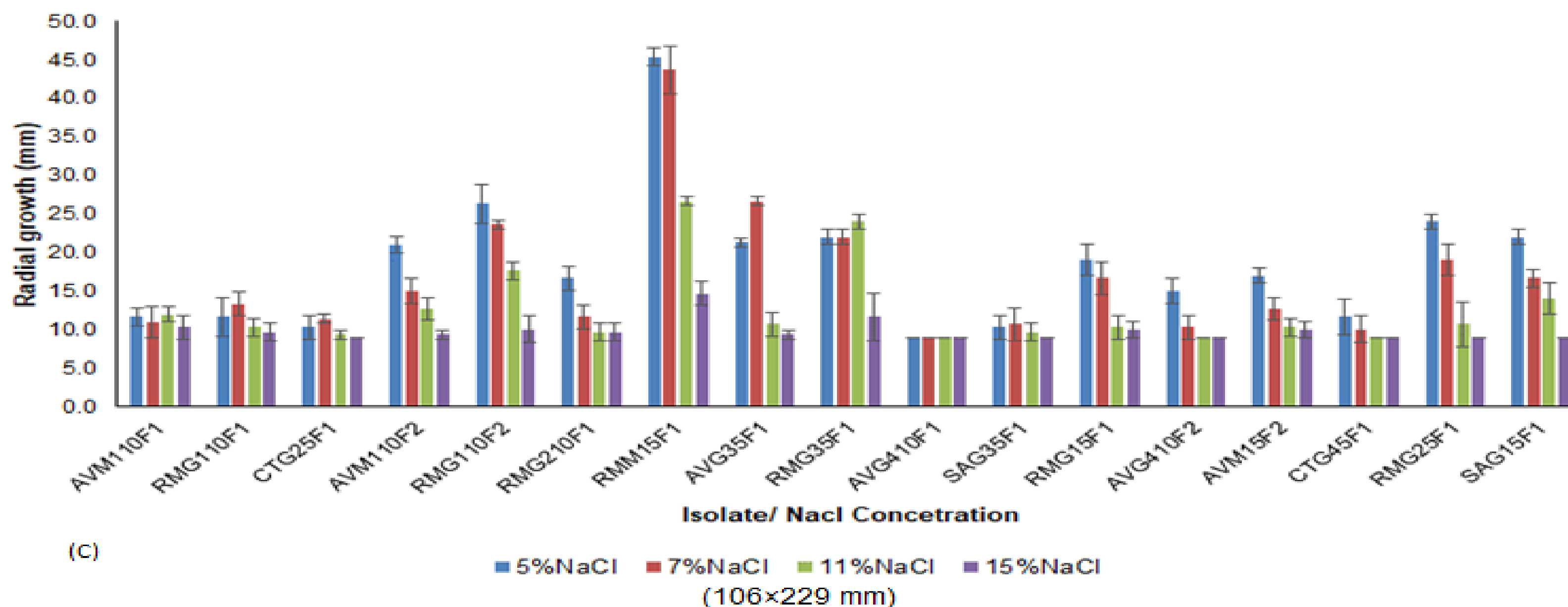


Figure 2c. Growth of isolates at different NaCl concentrations.

diameters on *E. coil* only (p-value ≤ 0.05).

Molecular characterization of the fungal isolates

A total of 33 isolates were recovered from the rhizospheric sediment samples of four-mangrove species. About 43, 30, 15 and 12% of the total isolates were obtained from the rhizospheric sediments of *Rhizophora mucronata, Avicennia marina, Sonneratia alba* and *Ceriops tagal,* respectively as shown in Table 3. The isolates (with their accession numbers in parenthesis) in the inferred phylogenetic trees (Figure 3a, b) were phylogenetically diverse and affiliated with known genera (*Penicillium, Aspergillus, Talaromyces, Diatrypella, Thielavia, Hypocreales, Paracremonium, Geosmithia, Peniophora, Massarina* and *Fulvifomes*) belonging to two fungal phyla: Ascomycota (yeast and sac fungi) and Basidiomycota (club fungi). Comparison of the newly isolated ITS gene sequences to known sequences in the GenBank database using Blastn analysis indicated sequences similarities of between 85 to 100% with known sequences (Table 3).

Affiliation of ITS gene sequences of the isolates

Most of the isolates (~97%) were affiliated with several known fungal species from the phylum Ascomycota with >85% sequence identity (Table 3; Figure 3a, b). Eight isolates (RMG15F1 [MT543114], RMG35F1 [MT543121], RMM15F4 [MT543124], AVG410F1 [MT543103], RMG25F1 [MT543118], AVM110F1 [MT543108], AVM15F1 [MT543105] and CTG25F1 [MT543110]) had between 85-100% sequence identities with known members of the genus *Talaromyces* that together formed a single sub-cluster supported with a bootstrap value of 99% (Table 3; Figure 3a, b). Out of the eight isolates, four (RMG15F1 [MT543114], RMG35F1 [MT543121], RMM15F4 [MT543124] and RMG25F1 [MT543118]) were isolated from the rhizospheric sediments of *R. mucronata,* while three isolates (AVG410F1 [MT543103], AVM15F1 [MT543105] and AVM110F1 [MT543108]) were obtained from the

Table 2. Antibacterial effect of fungal isolates against susceptible bacterial strains.

Sample ID	Accession No.	Isolate sp.	Antibacterial activity (Inhibition zone Ø in mm)		
			Escherichia coli	***Pseudomonas aureginosa***	***Staphylococcus aureus***
AVM110F1	MT543108	*Talaromyces* sp.	8.67±0.58	8.0±0.00*	8.00±00*
RMG110F1	MT543116	*Penicillium* sp.	10.67±1.53	11±1.00*	8.33±0.58*
CTG25F1	MT543110	*Talaromyces* sp.	9.00±1.00	10.33±1.15	9.33±0.58
AVM110F2	MT543109	*Aspergillus* sp.	8.00±1.00*	8.33±1.15*	7.67±1.15*
RMG110F2	MT543117	*Penicillium* sp.	8.33±0.58*	8.67±0.58	7.67±0.58*
RMG210F1	MT543119	*Penicillium* sp.	9.00±1.00	19±1.00*	20±1.00
RMM15F1	MT543122	*Geosmithia* sp.	10.00±1.00	8.33±0.58*	8.33±1.15*
AVG35F1	MT543101	*Aspergillus* sp.	8.33±0.58*	11.33±2.08*	11.67±1.53*
RMG35F1	MT543121	*Talaromyces* sp.	8.00±1.00*	12±1.00*	11±1.00*
AVG410F1	MT543103	*Talaromyces* sp.	9.00±0.00	11.67±0.58*	8.67±0.58
SAG35F1	MT543129	*Aspergillus* sp.	12.33±1.53*	8.67±0.58	8.33±0.58*
RMG15F1	MT543114	*Talaromyces* sp.	10.33±1.53	7.33±0.58*	14.33±1.15*
AVG410F2	MT543104	*Hypocreales* sp.	12.33±0.58*	14.67±2.31*	13±1.73*
AVM15F2	MT543106	*Fulvifomes* sp.	11.33±1.53*	7.67±1.15*	8.33±0.58*
CTG45F1	MT543112	*Hypocreales* sp.	10.33±1.53	7±0.00*	9±0.00
RMG25F1	MT543118	T*alaromyces* sp.	9.67±1.15	7.67±0.58*	8.67±0.58
SAG15F1	MT543128	*Phialemonium*sp.	9.67±2.08	8±1.00*	10±1.00

Values represent mean and standard deviation. Mean values with an asterisk (*) indicate significant difference (p ≤ 0.05). Sample ID's with prefix 'RM' denotes R. mucronata; 'AVG' denotes A. marina; 'SA' denotes S. alba; 'CT' denotes C. tagal. 'ID' denotes identity.

rhizospheric sediments of *A. marina*. Isolate CTG25F1 (MT543110) was obtained from the rhizospheric sediments of *C. tagal.* Six isolates (AVG35F2 [MT543102], RMG15F2 [MT543115], RMG110F2 [MT543117], RMG110F1 [MT543116], RMG210F1 [MT543119] and RMG210F2 [MT543120]) were affiliated with members of the genus *Penicillium* [with >98% sequence identities] (Table 3) and together formed a minor sub-cluster supported with a bootstrap value of 90% (Figure 3a, b). All the six isolates were obtained from the rhizospheric sediments of *R. mucronata* except isolate AVG35F2 [MT543102], which was isolated from the rhizospheric sediments of *A. marina.*

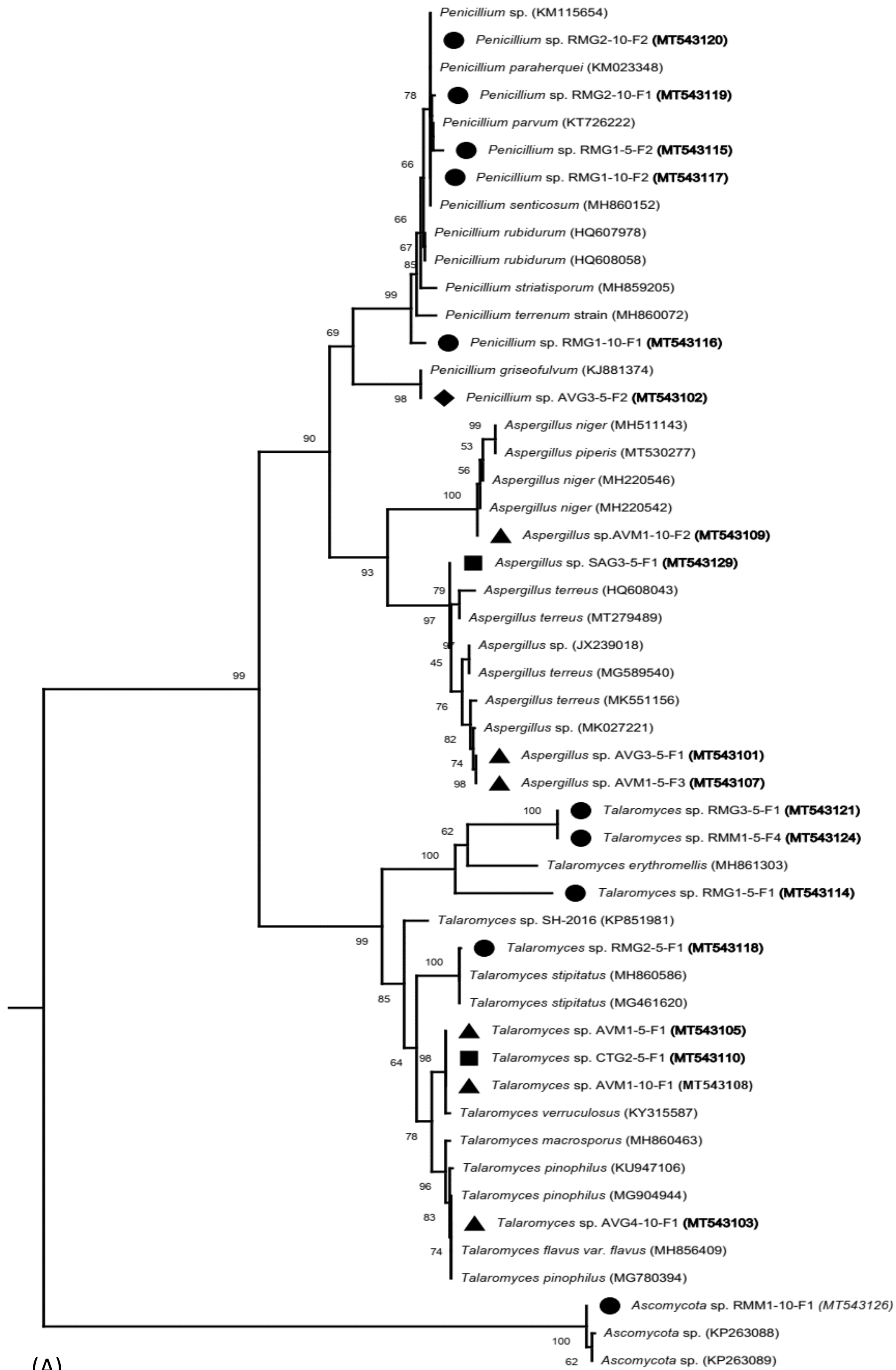
Four isolates (RMM15F5 [MT543125], RMM25F1 [MT543127], SAM15F1 [MT543130] and SAM15F3 [MT543131]) had 86% sequence affiliation with *Paracremonium* sp. 1 RJ2014 (MF782798) and together formed a minor sub-cluster supported with a bootstrap value of 97% (Table 3; Figure 3a, b). Two of these isolates (RMM15F5 [MT543125] and RMM25F1 [MT543127]) were obtained from the rhizospheric sediments of *R. mucronata,* while the other two were isolated from the rhizospheric sediments of *S. alba.* Other four isolates (CTG45F1 [MT543112], AVG410F2 [MT543104], RMM15F3 [MT543123] and CTG310F1 [MT543111] were >94% affiliated with sequences of members from the genus *Hypocreales* and formed a minor sub-cluster supported by a bootstrap value of 100% (Table 3; Figure 3a, b). Out of these four isolates, two (CTG310F1 [MT543111] and CTG45F1 [MT543112]) were isolated from the rhizospheric sediments of *C. tagal,* while AVG410F2 [MT543104] and RMM15F3 [MT543123] were obtained from the rhizospheric sediments of *A. marina* and *R. mucronata,* respectively.

Four other isolates (AVM110F2 [MT543109], AVM15F3 [MT543107], AVG35F1 [MT543101] and SAG35F1 [MT543129]) were affiliated with members of the genus *Aspergillus* [with >98% sequence similarities] (Table 3). These isolates formed a single sub-cluster with several other members of the genus *Aspergillus* as indicated in the inferred phylogenetic tree (Figure 3a, b). Three of these isolates (AVM110F2 [MT543109], AVM15F3 [MT543107] and AVG35F1 [MT543101]) were obtained from the rhizospheric sediments of *A. marina,* while SAG35F1 [MT543129] was isolated from the rhizospheric sediments of *S. alba.* Isolate CTM35F1 [MT543113] had a 99% sequence identity with *Massarina* sp. GX5-2C (FJ037767) and formed a separate minor sub-cluster supported by a bootstrap value of 85%. In addition, this sub-cluster has members of the *Ascomycota* species together with isolate RMM110F1 [MT543126], which was obtained from the rhizospheric sediments of *R. mucronata* (Table 3; Figure 3a & b). Isolates (AVM15F2 [MT543106], AVG210F1 [MT543100], SAG15F1 [MT543128], SAM35F1 [MT543132] and RMM15F1 [MT543122]) were affiliated with sequences from different species with between 95-100% sequence identities. These isolates also formed minor sub-clusters as shown in the phylogenetic tree (Figure 3a, b) and were obtained from the rhizospheric sediments of *A. marina, R.*

Table 3. Affiliation of fungal isolates with their closest taxonomic relatives.

Sample ID	Accession No.	Host mangrove species	Location	Closest taxonomic affiliation	Isolation Source	Country	% ID
AVG210F1	MT543100	*Avicennia marina*	Gazi bay	*Halocryptosphaeria bathurstensis* (MN061366)	*Avicennia marina*	India	98
AVG35F1	MT543101	*Avicennia marina*	Gazi bay	*Aspergillus terreus* (MK551156)	Soil	Nigeria	99
AVG410F2	MT543104	*Avicennia marina*	Gazi bay	*Hypocreales* sp. R348 (HQ649959)	*Dittrichia viscosa*	Spain: Alicante	100
AVM110F2	MT543109	*Avicennia marina*	Mida creek	*Aspergillus niger* (MH511143)	*Cyperus stolonifer*	India	98
AVM15F2	MT543106	*Avicennia marina*	Mida creek	*Fulvifomes* sp. SP-2012a (JX188000)	*Xylocarpus granatum*	Thailand	100
AVM15F3	MT543107	*Avicennia marina*	Mida creek	*Aspergillus* sp. (MK027221)	sugarcane straw	Brazil	98
CTG310F1	MT543111	*Ceriops tagal*	Gazi bay	*Hypocreales* sp. PSU-ES203 (JN116711)	*Enhalus acoroides*	Thailand	94
CTG45F1	MT543112	*Ceriops tagal*	Gazi bay	*Hypocreales* sp. PSU-ES203 (JN116711)	*Enhalus acoroides*	Thailand	94
CTM35F1	MT543113	*Ceriops tagal*	Mida creek	*Massarina* sp. GX5-2C (FJ037767)	Mangrove	China	99
RMM110F1	MT543126	*Rhizophora mucronata*	Mida creek	*Ascomycota* sp. (KP263088)	marine sponge	Panama	100
RMM15F1	MT543122	*Rhizophora mucronata*	Mida creek	*Geosmithia microcorthyli* (MG589551)	Mangrove twig	India	99
RMM15F3	MT543123	*Rhizophora mucronata*	Mida creek	*Hypocreales* sp. isolate MY10_3 (JO697536)	Sea Sponges	S. China	100
RMM15F5	MT543125	*Rhizophora mucronata*	Mida creek	*Paracremonium* sp. 1 RJ2014 (MF782798)	cavity of Picus viridis	Poland	86
RMM25F1	MT543127	*Rhizophora mucronata*	Mida creek	*Paracremonium* sp. 1 RJ2014 (MF782798)	cavity of Picus viridis	Poland	86
SAG35F1	MT543129	*Sonneratia alba*	Gazi bay	*Aspergillus terreus* (MT279489)	marine sediment	Viet Nam	100
SAM15F1	MT543130	*Sonneratia alba*	Mida creek	*Paracremonium* sp. 1 RJ2014 (MF782798)	cavity of Picus viridis	Poland	86
Sample ID	Accession No.	*Host mangrove species*	Location	*Closest taxonomic affiliation*	Isolation Source	Country	% ID
AVG35F2	MT543102	*Avicennia marina*	Gazi bay	*Penicillium griseofulvum (KJ881374)*	contaminated soil	India	100
AVG410F1	MT543103	*Avicennia marina*	Gazi bay	*Talaromyces pinophilus (MG904944)*	Rhizosphere soil	India	100
AVM110F1	MT543108	*Avicennia marina*	Mida creek	*Talaromyces verruculosus (KY315587)*	Cocos nucifera L	Canada	99
AVM15F1	MT543105	*Avicennia marina*	Mida creek	*Talaromyces verruculosus (KY315587)*	Cocos nucifera L	Canada	98
CTG25F1	MT543110	*Ceriops tagal*	Gazi bay	*Talaromyces verruculosus (KY315587)*	Cocos nucifera L	Canada	98
RMG110F1	MT543116	*Rhizophora mucronata*	Gazi bay	*Penicillium rubidurum (HQ608058)*	C. wheeleri nest	USA, Texas	98
RMG110F2	MT543117	*Rhizophora mucronata*	Gazi bay	*Penicillium parvum (MH859859)*	N/A	Mexico	100
RMG15F1	MT543114	*Rhizophora mucronata*	Gazi bay	*Talaromyces erythromellis (MH861303)*	N/A	Austrailia	86
RMG15F2	MT543115	*Rhizophora mucronata*	Gazi bay	*Penicillium parvum (KF706683)*	Deep sea sediment	China	98
RMG210F1	MT543119	*Rhizophora mucronata*	Gazi bay	*Penicillium senticosum (MH860152)*	N/A	S. Africa	99
RMG210F2	MT543120	*Rhizophora mucronata*	Gazi bay	*Penicillium senticosum (MH860152)*	N/A	S. Africa	100
RMG25F1	MT543118	*Rhizophora mucronata*	Gazi bay	*Talaromyces stipitatus (MG461620)*	Soil	India	100
RMG35F1	MT543121	*Rhizophora mucronata*	Gazi bay	*Talaromyces erythromellis (MH861303)*	N/A	Austrailia	87
RMM15F4	MT543124	*Rhizophora mucronata*	Mida creek	*Talaromyces erythromellis (MH861303)*	N/A	Austrailia	86
SAG15F1	MT543128	*Sonneratia alba*	Gazi bay	*Phialemonium sp. AUMC 5798 (JQ425380)*	Citrus (orange) leaf	Egypty	99
SAM15F3	MT543131	*Sonneratia alba*	Mida creek	*Paracremonium sp. 1 RJ2014 (MF782798)*	cavity of Picus viridis	Poland	86
SAM35F1	MT543132	*Sonneratia alba*	Mida creek	*Thielavia hyrcaniae (KY316381)*	Crop field soil	Korea	95

Sample ID's with prefix 'RM' denotes *Rhizophora mucronata;* 'AVG' denotes *Avicennia marina;* 'SA' denotes *Sonneratia alba*; 'CT' denotes *Ceriops tagal.* 'N/A' denotes not applicable; 'ID' denotes identity.



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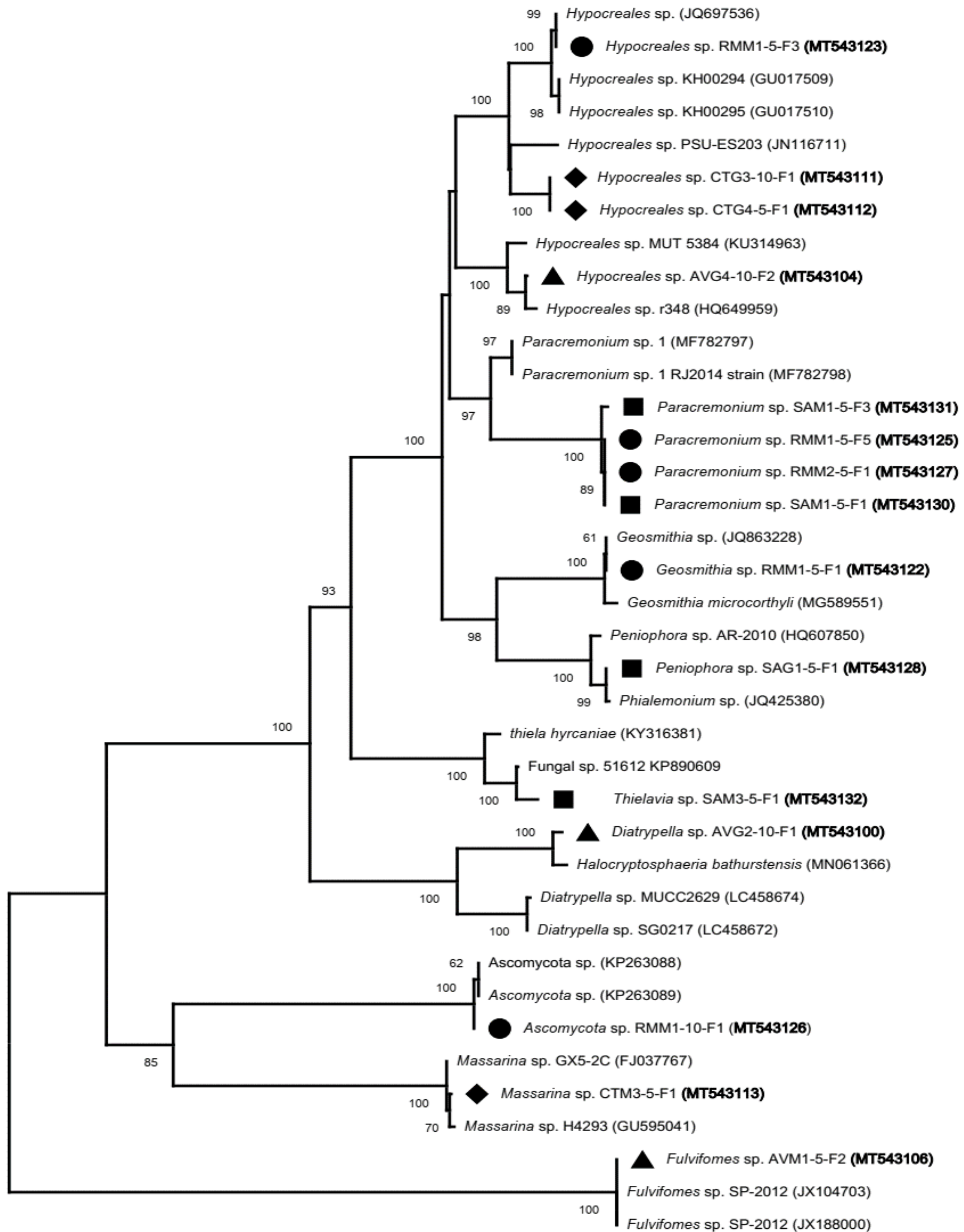

(193×138 mm)

Figure 3. Evolutionary relationships between partial ITS gene sequences of the isolates and some closest known fungal species. *Tuber maculatum* (FM205560) was used to root the phylogenetic tree.

mucronata and *S. alba* (Table 3).

DISCUSSION

Fungal biodiversity studies in mangroves are essential for exploring diversity from not only the biogeographical perspective but also with the view of bioactive secondary metabolites biosynthesis capability (Zhou et al., 2016). In this study, 33 fungal isolates were obtained from the rhizospheric sediments of four mangrove species *(R. mucronata, A. marina, S. alba* and *C. tagal*) that are common along the Kenyan Coastline. Close observation of the distribution of the fungal isolates among the mangrove species showed that most isolates (43% of the total isolates) were recovered from the rhizospheric sediments of *R. mucronata* in both Gazi bay and Mida creek (Table 3). This observation could be explained by the variation in nutrients within the mangrove zonation, where areas occupied by *R. mucronata* species are richer in nutrients than other areas. Nitrogen, phosphorus, potassium, calcium, sodium, carbon and magnesium were among the nutrients higher in the rhizosphere of *R. mucronata* in both Gazi bay and Mida creek (Table 1).

Phylogenetic analysis positioned the isolates into three major clusters that were represented by eleven genera (*Talaromyces, Aspergillus, Penicillium, Hypocreales, Paracremonium, Diatrypella, Thielavia, Geosmithia, Peniophora, Massarina* and *Fulvifomes*) belonging to two fungi divisions namely Ascomycota (accounted for ~ 97% of all the isolates) and Basidiomycota (accounted for ~3% of the total isolates). It is worthy to note that most of these genera are saprophytic fungi and may be implicated in biodegradation of litter in the mangrove ecosystem (Hýsek and Brožová, 2001). Majority of the isolates (24%) formed a single major cluster supported by a bootstrap value of 99% and were affiliated with members from the genus *Talaromyces* [with ≥86% sequence identity] (Table 3). Initially, there were few reports about the occurrence of this genus in the mangrove ecosystems since it was classified under the genus *Penicillium*. But following its formal separation from the genus *Penicillium*, most researchers have detected this genus in mangrove ecosystems (Lima et al., 2018; Venkatachalam et al., 2019). Members of the genus *Talaromyces* form part of the major fungi adapted to the extreme conditions in mangrove ecosystems and have been reported to be a source of biotechnologically relevant bioactive molecules (Jie et al., 2016).

Interestingly, about 30% of the isolates were closely related to members of the genera *Aspergillus* and *Penicillium* [with >97% sequence identity]. Members from these genera are widely distributed including in the marine ecosystem (Zhou et al., 2018; Venkatachalam et al., 2019). For instance, isolate SAG35F1 [MT543129] had 100% similarity with *Aspergillus terreus* (MT279489), which was isolated from marine sediment in Vietnam, while isolate RMG15F2 [MT543115] had 98% sequence identity with *Penicillium parvum* (KF706683), which was recovered from deep sea sediment in China (Table 3). Moreover, members from these genera have been isolated from mangrove ecosystems by other researchers (Al-Shibli et al., 2019; Kuzhalvaymani et al., 2019). Other classified fungi with appreciable frequency of isolation were members of the genera *Hypocreales* and *Paracremonium*. Zhou et al. (2018) reported the occurrence of species from these genera in the mangrove environment. Members from other genera (*Diatrypella, Thielavia, Geosmithia, Peniophora, Massarina* and *Fulvifomes*) formed the least abundant species among the isolates. It is noteworthy that fungal genera like *Geosmithia, Peniophora, Massarina* and *Fulvifomes* have previously been reported in mangrove ecosystems (Osorio et al., 2014; Ameen et al., 2016; Guerrero et al., 2018). Members of the genus *Diatrypella* are cosmopolitan in distribution, and occur on a broad range of dead or dying wood of angiosperms, but there are fewer reports on their occurrence in the mangrove environment (Shang et al., 2017).

The physiological analysis revealed that all the isolates were able to grow at different pH ranges (from low to high pH values [pH 4 to 12]), but some had growth preference for either slightly acidic or alkaline environment (Figure 2a). Some isolates (*Penicillium* sp. [MT543117], *Geosmithia* sp. [MT543122], *Talaromyces* sp. [MT543114], *Talaromyces* sp. [MT543118] and *Phialenonium* sp. [MT543128]) grew at significantly lower NaCl concentrations (5 and 7%NaCl) compared to others, indicating difference in salinity levels preference. This is attributed to the pH fluctuations and high salinity levels in mangrove ecosystems (Venkatachalam et al. (2019); thus, these fungal isolates are adapted to such conditions. A related study by Venkatachalam et al. (2019) revealed that marine-derived genera *Talaromyces* and *Penicillium,* were the most promising for the production of compounds such as pigmented secondary metabolites, which can be applied in cosmeceutical or food industries. These authors also observed that the production of the pigmented metabolites was regulated by the concentration of salt in the growth media. There were insignificant growth differences at both 20 and 30°C for some isolates, but others (Isolates AVM110F1, RMG110F1, SAG35F1, RMG15F1, CTG45F1, RMG25F1 and SAG15F1) grew optimally at 20°C; while isolates RMG210F1, RMM15F1, AVG410F1 and AVM15F2 were observed to grow optimally at 30°C. This indicates that these isolates preferred normal marine environmental temperature ranges above which (40 to 60°C) growth was not observed.

Moreover, seventeen fungal isolates showed antibacterial activity against the pathogenic bacterial strains (*E. coli, P. aeruginosa,* and *S. aureus*). Isolates such as *Penicillium* sp. [MT543116], *Penicillium* sp.

[MT543119], *Aspergillus* sp. [MT543101], *Talaromyces* sp. [MT543121], *Talaromyces* sp. [MT543103], *Thielavia* sp. [MT543132], *Penicillium* sp. [MT543115], *Hypocreales* sp. [MT543104] and *Fulvifomes* sp. [MT543106] exhibited significantly higher inhibition zone diameters against the bacterial strains (Table 2). This demonstrates that these fungal isolates have potential for formation of antimicrobial agents. Notably, isolate *Hypocreales* sp. [MT543104] significantly inhibited the growth of all tested bacterial strains, while isolates *Thielavia* sp. [MT543132] and *Fulvifomes* sp. [MT543106] had significantly higher inhibition zone for *E. coil* only ($P \leq 0.05$). This could mean that these isolates produce different types of antibacterial compounds, which are either effective against a narrow range of bacteria or a broad range of bacteria. One of the notions is that this is a defense mechanism attributed to the intense competition between microbial species, which evolved to secrete substances with high antibacterial activity inhibiting the growth of other strains (Zhou et al., 2016). A recent effort at exploring mangroves rhizospheric fungi associated with the roots of *Rhizophora stylosa*, on the Techeng Isle in China led to the recovery of 5 novel funicone derivates from *Penicillium pinophilum* (He et al., 2019). Other studies (Bhimba et al., 2012; Lima et al., 2018) have also demonstrated that species from these isolated fungal genera have antimicrobial activity, which is consistent with these findings.

Conclusion

This study demonstrated that the distribution of culturable fungi in the mangrove ecosystem of Gazi bay and Mida creek is influenced by both plant species and differences in environmental conditions. The recovery of 17 fungal isolates with antimicrobial activity against known bacterial pathogens implies that mangroves rhizospheres are also hotspots for the isolation of fungi with antibacterial potential. Further analysis on the antibacterial activity of the isolated fungi will give more insights on the types of antibacterial compounds produced and their effectiveness as antimicrobial agents.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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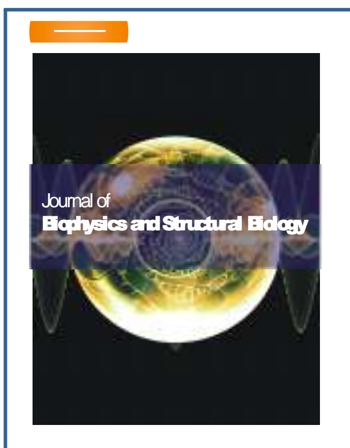

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