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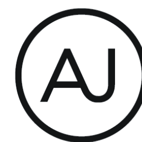

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African Journal of Microbiology Research

Full Length Research Paper

Evaluation of the microbiological quality of "mabokés" smothered fish and, proteolytic activity of bacteria of the genus *Bacillus* in Brazzaville, Congo

Cyr Jonas Morabandza[1], Tarcisse Baloki Ngoulou[2*], Valentin Dibangou[2], Saphia Jemylah Galekoua Ndjiwa Empilo[1], Armel Faly Soloka Mabika[2], Christian Ngo-Itsouhou[2], Rachel Moyen[2] and Gabriel Ahombo[2]

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In order to assess the health risks related to the consumption of smothered fish "mabokés" sold in the markets of Brazzaville, samples of smothered fish sold in three markets in Brazzaville were analyzed for their microbiological quality. The different samples were cultured for isolation on solid media using conventional microbiological methods. The bacteria isolated were identified on the basis of cultural, morphological and biochemical characteristics. At the end of this analysis, 79 bacteria were isolated, of which 29 (36.70%) at site 2, 25 (31.65%) at site 1 and 25 (31.65%) at site 3. Of the 79 strains, 46 (58.22%) were Gram-positive bacteria and 33 (41.78%) were Gram-negative bacteria. These strains consisted of 34 (43.03%) bacteria of the genus *Bacillus*, 12 (15.18%) bacteria of the genus *Staphylococcus* and 33 (41.79%) *Enterobacteria* including 15 bacteria of the genus *Shigella*. The enumeration results show that the average value of the total flora varies from $2.90.10^{8}$±$1.5.10^{7}$ CFU/ml (Site 2) to $5.55.10^{9}$±2.10^{7}CFU/ml (Site 3), that of *Bacillus* between $1.30.10^{7}$±$1.41.10^{6}$ CFU/ml (Site 2) and 7.85. 10^{7}± $1.02.10^{6}$ CFU/ml (Site 3), for *Staphylococcus* between $1.44.10^{6}$±2.02. 10^{6}CFU/ml (Site 1) and $6.17.10^{6}$±$8.25.10^{6}$CFU/ml (Site 2). The average value of *Enterobacteriaceae* ranges from 70.10^{4}±$2.4.10^{4}$ CFU/ml (Site 2) to $9.15.10^{5}$±$1.29.10^{4}$ CFU/mL (Site 3) and from 0 (Site 3) to $5.65.10^{4}$±$7.85.10^{4}$ CFU/ml (Site 1) for *Shigella*. Proteolytic enzyme production was observed in 9 out of 12 selected strains. Although these smothered fish have strains with proteolytic activity, the presence of *Staphylococcus* and *Enterobacteriaceae* make this food unfit for consumption.

Key words: Microbiological quality, fish, smothered, proteolytic activity.

INTRODUCTION

Fish is an important nutritional supplement in a diet low in protein, vitamins and minerals (Degnon et al., 2012). Its worldwide consumption is increasing significantly. In 2016, out of the 171 million tons of fish produced in the

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world, 151 million tons were used for human consumption, 88% compared to 67% in the 1960s (FAO, 2008). To date, more than 400 million Africans regularly consume fish in all its forms (Worldfish, 2017). Congo, because of its hydrographic density, has different species of fish that contribute to the economic livelihoods of the population (Kimbatsa, 2016). The diversity of cultures in the world has resulted in a wealth of recipes and methods of fish preparation. In Congo, in rural areas as well as in urban areas including Brazzaville, fresh fish is eaten in stews, sauces, barbeques, fried in oil, grilled and stewed. Summers called smothered fish "mabokés" are very popular and eaten a lot. In addition to households, fish stews are prepared and sold in markets, on public roads, in restaurants and drinking establishments, and at fish auctions by itinerant women. Any food product intended for consumption and offered for sale must protect consumers from infection or contamination and be of good quality (Assogba et al., 2018). The presence and multiplication of microorganisms makes this type of food unsuitable and therefore presents a health risk that can cause gastroenteritis for the consumer. To date, no microbiological studies have been conducted on this product. This work is part of an effort to assess the microbiological quality of the smothered fish "mabokés" sold in some markets in Brazzaville in order to evaluate the sanitary conditions related to the consumption of these products and to determine the proteolytic activity of some strains of *Bacillus*.

MATERIALS AND METHODS

Sample collection, culture conditions, enumeration

Samples of stewed fish commonly known as "mabokes" in Congo Brazzaville, wrapped in leaves and stewed were purchased from three markets in Brazzaville. Three samples were purchased per market or site. At the time of purchase, the samples were placed in ice box containing ice bread and sent to the laboratory for analysis. The targeted sites were:

Site 1: Moukondo market located more or less in the center of the city,
Site 2: Total market located south of the city of Brazzaville,
Site 3: Moungali market located in the center of the city.

In a test tube containing 9 ml of distilled water, 1 ml of fish soup was added as the stock solution from which the decimal dilutions were made. The inoculations were carried out on Petri dishes in six different culture media including: PCA for the total count of microorganisms, Mannitol salt agar for the enumeration of bacteria of the genus *Staphylococcus*, *Salmonella* -*Shigella* Agar for the enumeration of *Salmonella* and *Shigella* bacteria, Methylene eosin blue for the enumeration of *enterobacteria*, Mossel Agar for the enumeration of bacteria of the genus *Bacillus* and Chloramphenicol Sabauraud for the enumeration of yeasts.

After the counting of appeared the colonies, the enumeration was carried out according to the formula (AFNOR, 1997).

$$CFU = \frac{N}{VD}$$

CFU/ml = Colony Forming Units, N = number of colonies, V = used volume of plating, D = dilution

Colony purification and isolation

Purification is one of the most important steps in strain isolation. It was carried out in nutrient agar. Each colony was seeded separately with streaks until distinct and homogeneous colony was obtained. To ensure the purity of the strains, microscopic observation was performed. The characterization of the isolates was started by the application of classical microbiology techniques, based on the search for a certain number of characters (cell morphology by the fresh status under the optical microscope, the type of Gram) (Mabika et al., 2020). Only *Bacillus* isolated and purified on Mossel Agar were stored at 4°C in cryotubes containing the appropriate broth (Luria-Bertani) with 20% glycerol added.

Proteolytic potential of some *Bacillus* strains

To test the production of the caseinolytic enzyme effect of twelve (12) strains of *Bacillus*, we used the techniques modified by Puri et al. (2002). The cells were cultured in Luria Bertani (LB) medium under agitation at 37°C, for 48 h. 1 ml of the culture was introduced into a 1.5 ml eppendorf tube and centrifuged at 6000 rpm for 5 min in a micro centrifuge. The supernatant was recovered. 2 ml of culture was used to measure optical density with the spectrophotometer at a wavelength of 600 nm (Nguimbi and Wu, 2002). In a 250-ml Erlenmeyer flask containing 100 ml of 0.1N PBS, we dissolved 1 g of agarose and heated it until completely dissolved. After cooling the mixture to 55-60°C, we added 10 ml skim milk and homogenized the mixture. It was poured into Petri dishes; after solidification, wells were prepared in the gel. 50 μl supernatant was placed in each well from the centrifugation of the culture. The plates were placed in the oven at 37°C for 12 h. Observation of a clear translucent zone indicates that the strain produces a proteolytic enzyme with a caseinolytic effect (caseinolytic protease) (Nguimbi et al., 2014; Mabika et al., 2017).

RESULTS

Bacterial isolation

A total of 79 bacteria were isolated from smothered fish. Of these strains, 29 (36.70%) were isolated from site 2 (total market), 25 (31.65%) from site 1 (Moukondo market) and 25 (31.65%) from site 3 (Moungali market) (Figure 1).

Identification

Of the 79 strains, 46 (58.22%) were Gram-positive bacteria and 33 (41.78%) were Gram-negative bacteria (Figure 2). These strains consisted of 34 (43.03%) bacteria of the genus *Bacillus*, 12 (15.18%) bacteria of the genus *Staphylococcus* and 33 (41.79%) *Enterobacteria* including 15 bacteria of the genus *Shigella* (Figure 3). Table 1 shows that *staphylococcus* was mostly isolated from Market 2 samples. *Shigella* spp. were not isolated from the samples from site 3, although higher total flora and *Bacillus* were observed. Yeasts were not isolated from the samples.

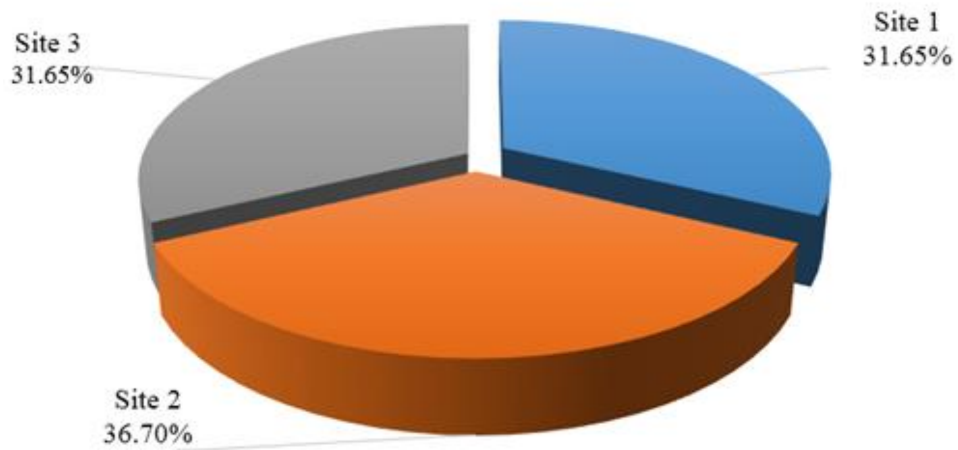

Figure 1. Isolation of bacteria numbers. Site 1: Moukondo market, Site 2: Total market, Site 3: Moungali market.

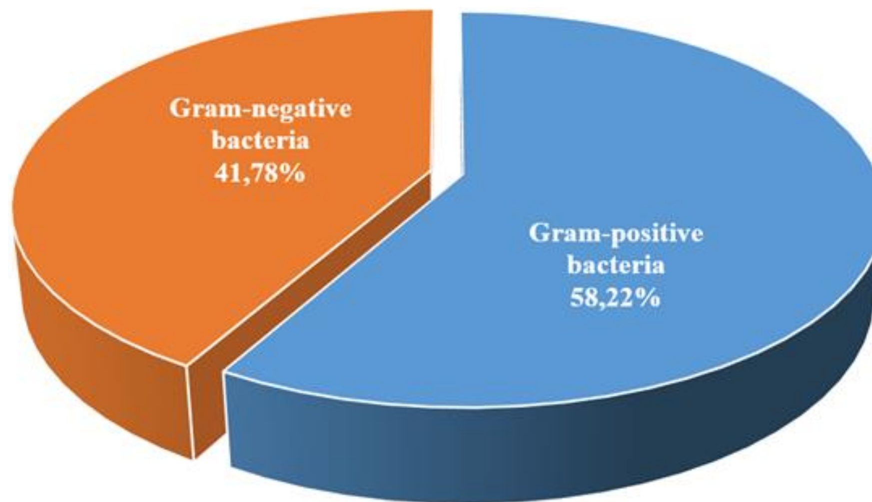

Figure 2. Repartition of isolated bacteria.

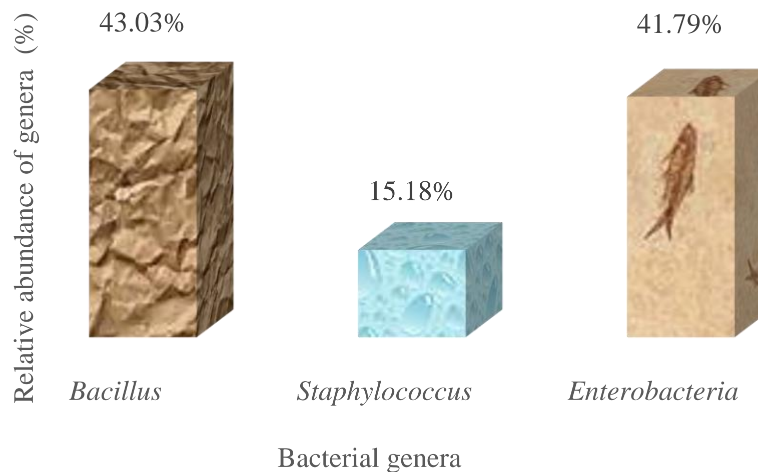

Figure 3. Relative abundance of strains by genera.

Table 1. Average values and standard deviations of microorganisms in CFU/ml.

Microorganisms	Average values ± Standard deviations		
	Site 1	Site 2	Site 3
Total flora	$1.61.10^9 \pm 2.10^8$	$2.90.10^8 \pm 1.510^7$	$5.55.10^9 \pm 2.10^7$
Genus *Bacillus*	$6.07.10^7 \pm 5.5.10^6$	$1.30.10^7 \pm 1.41.10^6$	$7.85.10^7 \pm 1.02.10^6$
Genus *Staphylococcus*	$1.44.10^6 \pm 2.02.10^6$	$6.17.10^6 \pm 8.25.10^6$	$3.45.10^6 \pm 1.06.\ 10^6$
Genus *Enterobacteriaceae*	$9.15.10^5 \pm 1.29.10^4$	$1.70.10^4 \pm 2.4.10^4$	$2.73.10^4 \pm 1.65.10^4$
Shigella spp	$5.65.10^4 \pm 7.85.10^4$	$1.75.10^4 \pm 2.47.10^3$	00

Site 1: Moukondo market, Site 2: Total market, Site 3: Moungali market.

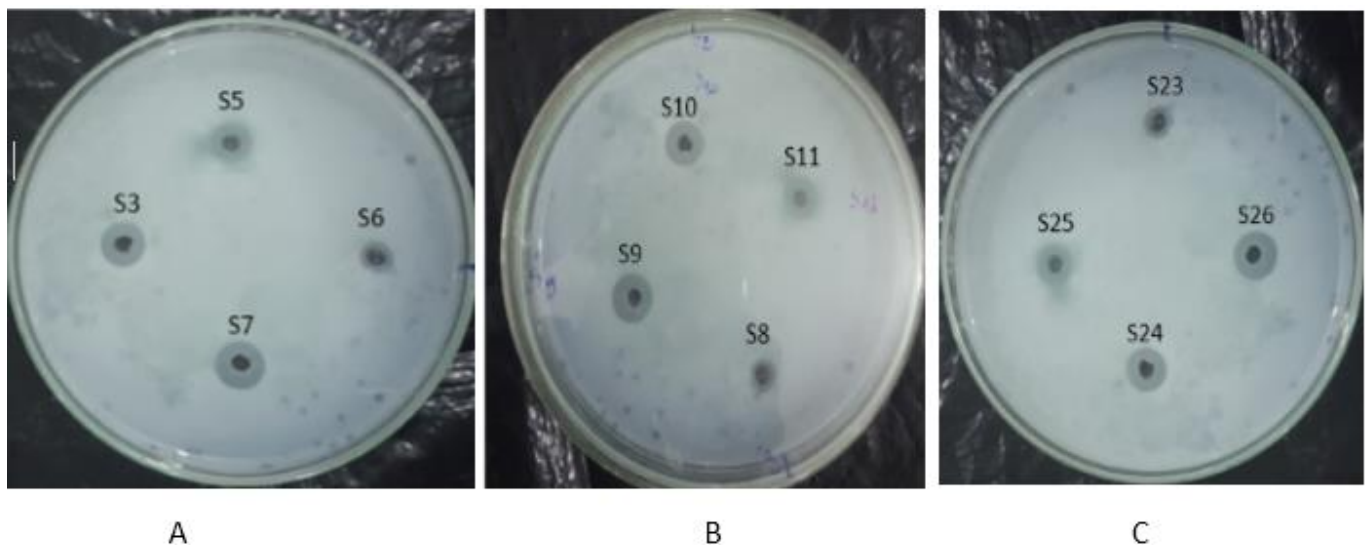

Figure 4. Proteolysis halos of the different strains. A: Site 1 strains; B: Site 2 strains and C: Site 3 strains.

Production of proteolytic enzymes

Detection of the production of a proteolytic enzyme

Figure 4 shows the stains produced by proteolytic enzymes. The smallest clear zones were observed in strains 6, 8 and 23. In the other strains, the diameter of the clear zone was variable.

Evaluation of proteolytic enzyme production

Here, the evaluation of proteolytic enzyme production is done simultaneously with growth, which is expressed as optical density (OD) and enzyme production in clear halo diameter. The values of the optical densities vary from strain to strain and range from 0.623 to 0.914. Strains 5.6, 11 and 26 showed the best growth. With the exception of strains 6, 8 and 23, the production of proteolytic enzymes was observed for the other selected strains. Enzyme production was very high for strains 4, 5, 24 and 26 (Figure 5).

DISCUSSION

The main objective of this study is to assess the microbiological quality of the smothered fish "mabokés" sold in some markets in Brazzaville in order to evaluate the sanitary conditions related to the consumption of these products and to determine the proteolytic production of some *Bacillus* strains. During this study, the average value of the microorganism varies from one site to another. The enumeration results show that the average value of the total flora varies from $2.90.10^8 \pm 1.5.10^7$ CFU/mL (Site 2) to $5.55.10^9 \pm 2.10^7$CFU/mL (Site 3), that of *Bacillus* between $1.30.10^7 \pm 1.41.10^6$ CFU/mL (Site 2) and $7.85.\ 10^7 \pm 1.02.10^6$ CFU/ml (Site 3), for *Staphylococcus* between $1.44.10^6 \pm 2.02.10^6$ CFU/ml (Site 1) and $6.17.10^6 \pm 8.25.10^6$ CFU/ml (Site 2). The average value of *Enterobacteriaceae* ranges from $70.10^4 \pm 2.4.10^4$ CFU/ml (Site 2) to $9.15.10^5 \pm 1.29.10^4$CFU/ml (Site 3) and from 0 (Site 3) to $5.65.10^4 \pm 7.85.10^4$CFU/ml (Site 1) for *Shigella*. Yeasts were absent in all samples.

Variations in the microbial load from one sample to

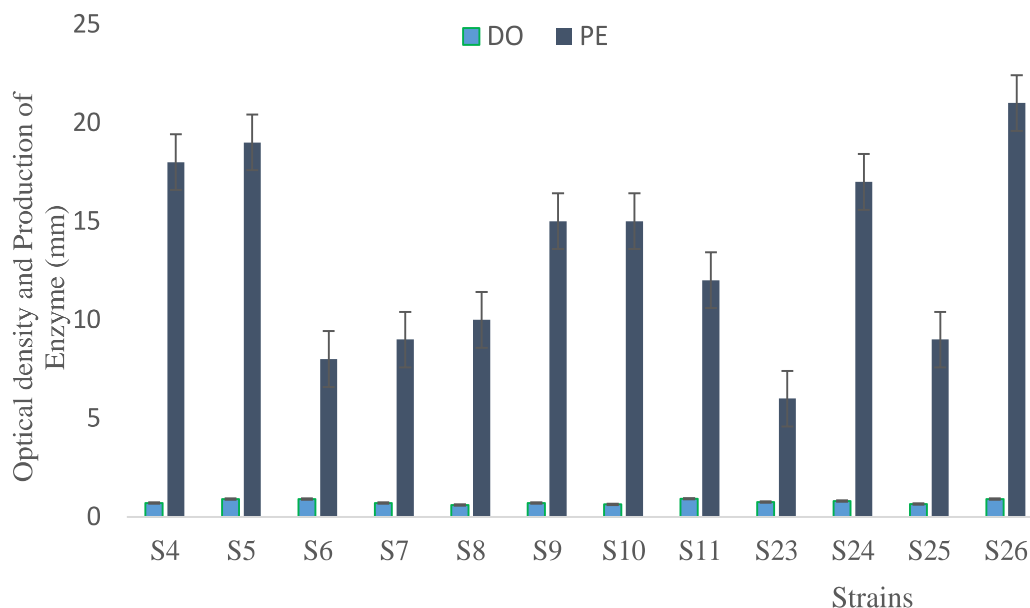

Figure 5. Optical density and proteolytic enzyme production of *Bacillus* strains.

another could be due to preparation and cooking conditions that often express a lack of hygiene that encourages various types of contamination. The presence of microorganisms indicates the alteration of the product in the different samples (Abotchi, 2010). The results on the total flora are similar to those reported in Senegal by Thiam (1993) ($3.4.10^8$ CFU/g) on the microbiological and chemical quality of braised-dried fish (kétiakh) sold on the Dakar market.

A lower average was reported by Watanabe (1974) (2.610^6 CFU/g), working on the technology and hygiene of salt-dried fish processing methods in Africa with special reference to Ghana, Senegal and Zambia. The work carried out by Mokemiabeka et al. (2016) in Congo on a fermented food revealed a total flora variable from one sample to less (68.10^5 CFU/mL) than that reported. Mabika et al. (2020), working on stewed squash sold in markets in Brazzaville, reported lower total flora loads ranging from $1.73.10^7$ CFU/g for the Moukondo Market to $9.43.10^7$ CFU/g (Tsieme Market). *Bacillus* loads ranged from 3.5. 10^6 CFU/g for the total market to 8.96. 10^6 CFU/g for the Tsieme Market. This charge is much lower than the one reported in this work. The presence of *Bacillus* could be explained by the fact that *Bacillus* sporulate and resist cooking temperatures (Ehon et al., 2015). This ability allows this genus to resist even antibiotics and thus opens the way to other more severe pathologies although, the *Bacillus* genus would contribute to the reduction of odours as demonstrated by some work on a mixed population of lactic acid bacteria and *Bacillus* in the microflora of cassava retting (Achi, 2006).

The count of yeasts and moulds in a product is a key indicator of its sanitary quality before it is placed on the market. It is often well-known species that cause undesirable changes in products (Laredj and Waffa, 2017). The absence of yeasts in the samples analyzed from the three sites indicates the freshness of the food. These results are contrary to the results of work on the microbiological quality of smoked fish, which demonstrated the presence of fungal flora (Abotchi, 2010). These results obtained, suggest that cooking as well as the closed environment in which the smothered fish is found, not being exposed to the outside environment, no fungal development is possible. These results also suggest that the batches analyzed were certainly prepared and disposed of on the same day they were acquired. Cleaning with water of the cassava leaves used for packaging of smothered fish could effectively contribute to the improvement of the microbiological quality of this food. The proteolytic (caseinolytic) test shows that 9 out of 12 selected strains showed better degradation of milk casein (Figure 4). This activity was demonstrated in Bacillus strains isolated from Ntoba mbodi and squashes in the Republic of Congo by Mabika et al. (2017, 2018, 2020). It is important to note the presence of proteolytic activity in strains isolated from fish in the smothered state. The genus Bacillus is recognized among bacteria that secrete bioactive substances such as proteases (Nguimbi and Wu, 2002), bacteriocins (Barboza et al., 2009; Mokemiabeka et al., 2016). These results on caseinolytic enzyme production in strains 4, 5, 24 and 26 with diameters between 15 and 21 mm are consistent with those reported by Adinarayana et al. (2003); Nihan and Elif (2011); Ehon et al. (2015); Mabika et al. (2017); Mabika et al. (2018); Ngo-Itsouhou et al. (2019) and Mabika et al. (2020).

Conclusion

This study made it possible to assess the microbiological quality of the smothered fish "mabokés" sold in some markets in Brazzaville in order to evaluate the sanitary conditions related to the consumption of these products and to determine the proteolytic activity of some *Bacillus* strains. Gram postive bacteria were mostly isolated from choking poisons. These include bacteria of the genus *Bacillus*, *Staphylococcus*. Gram-negative bacteria were *Enterobacteria*. The presence of these bacteria in this food indicates that the food is unfit for consumption. It would therefore be advisable to reduce consumption or even stop eating these smothered fish to avoid being exposed to foodborne diseases. On the other hand, the proteolytic test revealed strains of *Bacillus* with a high capacity to produce the caseinolytic enzyme.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of micro-fungi associated with leaf spot of *Allanblackia floribunda* Oliv. in Southern Nigeria

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Fruit of *Allanblackia floribunda* is an active ingredient in the pharmaceutical industry. Fruit production in the species is hindered by incidence of pathogenic fungi leading to economic loss. This study was conducted to investigate micro-fungi species associated with leaf spot of *A. floribunda.* Field surveys were carried out in natural stands containing matured *A. floribunda* trees located at Owu Ikija, Ogun State (6.80°N, 4.03°E) and Benin, Edo State (6.29°N and 5.58°E) in Southern Nigeria. Diseased leaf samples were collected during wet and dry seasons. Pure cultures of fungal isolates obtained from the leaf samples were examined to determine their cultural and morphological characteristics. Percentage incidence of micro-fungi in each location was estimated. Leaves of healthy seedlings were sprayed with 10^4 conidial/ml spore concentration of fungal isolates to determine their pathogenicity. Fourteen fungal species were isolated from leaves of *A. floribunda* across the two sites. *Aspergillus spp., Macrophomina phaseolina, Penicillium* species, *Pestalotia palmarum, Rhizopus nigricans* and *Trichoderma pseudokoningii* were isolated from both sites during both seasons. *Fusarium oxysporum, Lasiodiplodia theobromae, Penicillium javanicum* and *Pythium aphanidermatum* were present at Owu Ikija while *Colletotrichum capsici, C. coccodes* and *Curvularia lunata* were present at Benin with fungal incidence of 12.5, 12.5 and 4.17%, respectively. *P. palmarum* had modal fungal incidence (35.29%) at Benin followed by *T. pseudokoningii* at Owu-Ikija with frequency value of 18.75 and 17.54%, respectively. *P. palmarum* was the most prevalent out of all micro-fungi species associated with *A. floribunda* in all locations. Pathogenicity test was negative for all tested isolates, variety of micro-fungi are associated with *A. floribunda.*

Key words: *Allanblackia floribunda,* fruit, fungal isolates, leaf samples, pathogenicity.

INTRODUCTION

Micro-organisms such as bacteria, fungi, viruses, and nematodes are integral parts of the forest ecosystems. They play important roles in every sphere of our human lives contributing to processes that involve food production, medicine, industrial development bioremediation and agriculture (Miles and Chang, 2004; Krzywinski et al., 2009). Plant development and health are affected by different pathogens at various stages in their life cycle and the combination of these agents make up the disease complex. Plant fungi are the predominant

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pathogens responsible for tree diseases and thus have changed tree population diversity and ecosystem dynamics (Casadevall, 2007; Pujari et al., 2015). The devastating effect of these diseases on important forest trees has resulted in reduction in their quality, economic and aesthetic values as well as food production. *Pseudocercospora ranjita* was associated with leaf spot of *Gmelina arborea* (Wingfield and Robison, 2004); *Fomes lignosus* and *Phellinus noxious* (Corner) G. Cunn are the causative agents of root rot of *Tectona grandis* (Momoh, 1976; Moh'd Farid et al., 2009), while *Cryphonectria parasitica* (Murr) causing chestnut blight and *Ceratocystic ulmi* (Buism.) are the causal organism of Dutch elm diseases (USDA-APHIS, 2012). These fungi cause diseases in forest trees leaving discouraging results.

However, the seriousness of these diseases is often based on evaluation of the lethal effects of the diseases. For example, in the United States, the chestnut, which was once a major hardwood timber species is reportedly reduced to a less valuable bush species by chestnut blight (Manion, 1991) which is a result of the devastating fungus.

Allanblackia floribunda Oliv. is an evergreen, multi-purpose indigenous fruit tree with great potential as a source of alternate income to farmers and communities in tropical Africa (Munjuga et al., 2008). The fruits contain seeds that have large proportion of edible fat. The fat has a high melting point which solidifies at room temperature but thaws in the mouth which makes it a major raw material in food and pharmaceutical industries because it does not require further modification. The tree species is also used as a timber product and for medicinal purposes (Pye-Smith, 2009). However, fruit production in species such as *A. floribunda* is sometimes limited by the incidence of pathogens such as fungi leading to poor yield and economic loss. Also, there is dearth of information on micro-fungi associated with *A. floribunda*. Therefore, the study isolated micro-fungi species associated with *A. floribunda* leaves.

MATERIALS AND METHODS

Study area

Field surveys were carried out at *A. floribunda* stands located at Owu Ikija, Ogun State (6.80°N, 4.03°E) and Benin, Edo State (6.29°N and 5.58°E) in Southern Nigeria. There are two seasons in the study area: rainy (March to November) and dry seasons (December to February). The average annual rainfall ranges from 1300 to 1600 mm while average annual temperature ranges from 26.5 to 28.9°C (FRIN, 2018).

Sample collection

Leaf samples with typical leaf spot symptoms were collected during the dry and rainy seasons. These samples were purposively selected. Prior to collection, each tree was examined thoroughly for signs and symptoms of diseases: necrotic symptoms on the leaves such as spot, blight, scorch and other symptoms associated with the leaves. At Owu-IKija, 25 disease trees were sampled while 35 were sampled at Benin. Samples were taken from diseased trees with at least two diseased trees in each sample plot. Collected leaf samples were kept in sterile sampling bags and taken for laboratory analysis at the Plant Pathology laboratory of Forestry Research Institute of Nigeria for isolation of associated organisms.

Isolation of associated fungi

Leaf samples were cut into 2 mm × 2 mm sizes, surface-sterilized in 1% sodium hypochlorite and rinsed in 5 changes of sterile distilled water. Cut sections were obtained from the boundary area between infected and healthy tissues. They were blot-dried and aseptically placed on PDA growth medium. The plates were replicated three times and incubated at 29±2°C. The plates were examined daily for fungal growth.

Identification of fungal isolates

The isolates were purified through sub-culture of fungal growth. The cultures were examined to determine their cultural and morphological characteristics. The isolates were identified as soon as sporulation was observed as their structures are best viewed at this period. Wet mounts of each isolate were prepared on slides and stained with lactophenol cotton blue. The mounts were then observed using the Olympus BX51M reflected light optical microscope. Identifications were carried out based on the cultural and morphological characteristics of the isolates using Standard Manual of Fungi as reference (Barnett and Hunter, 1998; CMI, 1972).

Determination of frequency of occurrence of isolates

The number of times each fungus was isolated from the diseased leaf samples was expressed as a percentage of all fungi isolated (Ilondu, 2011).

$$\text{Frequency of occurrence} = \frac{\text{Total no of times fungus was isolated}}{\text{Total no of fungi isolated}} \times 100 \quad (1)$$

Pathogenicity test

Seven fungal pathogens namely *Colletotrichum capsici, Colletotrichum coccodes, Curvularia lunata, Fusarium oxysporum, Macrophomina phaseolina, Lasiodiplodia theobromae* and *Pestalotia palmarum* isolated from *A. floribunda* leaves were inoculated into healthy leaves in order to establish the actual causal organism of the disease and to satisfy Koch's postulate. Two-year old *A. floribunda* seedlings were inoculated with inoculum suspension of pathogenic isolates in a screen house. Twelve seedlings were used for each isolate with three replicates. All leaves in each replicate were inoculated with spore solution of each fungal isolate using a high pressure hand sprayer till run-off Inoculum suspension was prepared by addition of distilled water to sporulating culture of the isolates. With the aid of a sterile inoculation loop, the culture was gently scraped into a beaker to

Table 1. Seasonal variation of fungi species associated with leaves of *Allanblackia floribunda* at Owu-Ikija and Benin, Nigeria.

Organism	Ikija		Benin	
	Rainy season	Dry season	Rainy season	Dry season
Aspergillus niger.	+	+	+	+
Aspergillus flavus	+	+	+	+
Colletotrichum capsici	-	-	+	-
Colletotrichum coccodes	-	-	+	+
Curvularia lunata	-	-	-	+
Fusarium oxysporum	+	+	-	-
Macrophomina phaseolina	+	+	+	+
Lasiodiplodia theobromae	+	+	-	-
Penicillium italicum	+	+	+	+
Penicillium javanicum	+	+	-	-
Pestalotia palmarum	+	+	+	+
Pythium aphanidermatum	+	-	-	-
Rhizopus nigricans	+	+	+	+
Trichoderma pseudokoningii	+	+	+	+

+ Present, - Absent.

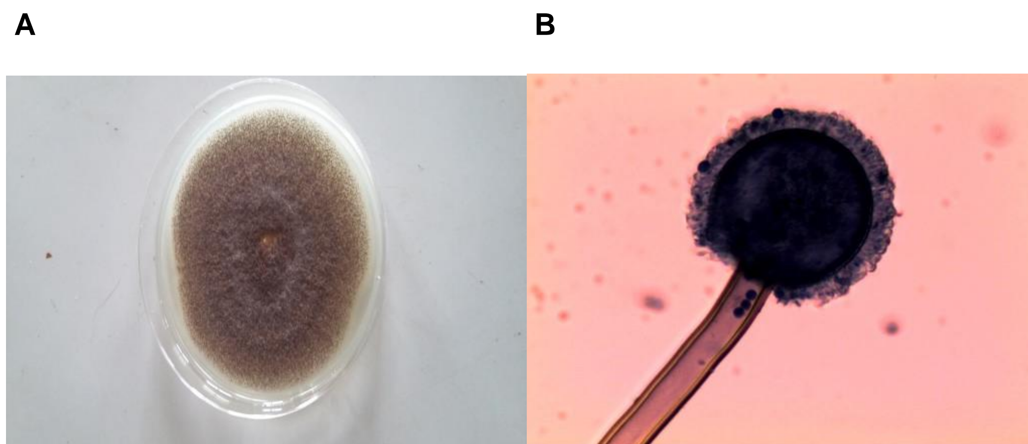

Figure 1. *A. niger* associated with leaves of *Allanblackia floribunda* Oliv. A: Colony growth. B: Micrograph.

dislodge spores from the aerial mycelium. This was repeatedly done to obtain enough quantity of inoculum suspension. The suspension was adjusted with sterile distilled water (1×10^4 spore/ml) after which two drops of tween 20 detergents (polyoxyethylene sorbitan mono-oleat) was added to reduce surface tension before the suspension was sprayed on leaves of the healthy seedlings.

Inoculated leaves were incubated for 48 h and then leaf spot disease symptom development was monitored. Control (that is, treatment without pathogen) was spray inoculated with sterile distilled water. Artificially inoculated leaves were taken back to the laboratory after 6 months for re-isolation of fungi.

RESULTS AND DISCUSSION

Fourteen fungi species were isolated from leaves of *A. floribunda* across the two sites (Table 1 and Figures 1 to 9). Micro-fungi species such as *Aspergillus niger, Aspergillus flavus, Macrophomina phaseolina, Penicillium italicum*, *P. palmarum, Rhizopus nigricans* and *Trichoderma pseudokoningii* were present at both study sites during the two seasons. Some of these organisms have been established to cause diseases in several forest species. For example, leaf blight of *Terminalia catappa* was caused by *Fusarium solani* (Rai and Mamatha, 2005); leaf spots of Aloe vera (*Aloe barbadensis* Miller) caused by *Fusarium* species (Avasthi et al., 2018) and leaf spot of *Harungana madagascariensis* was caused by *Pestalotia harongae* (Nsolomo and Venn, 1994). The occurrences of these pathogens always have adverse effect on the host tree

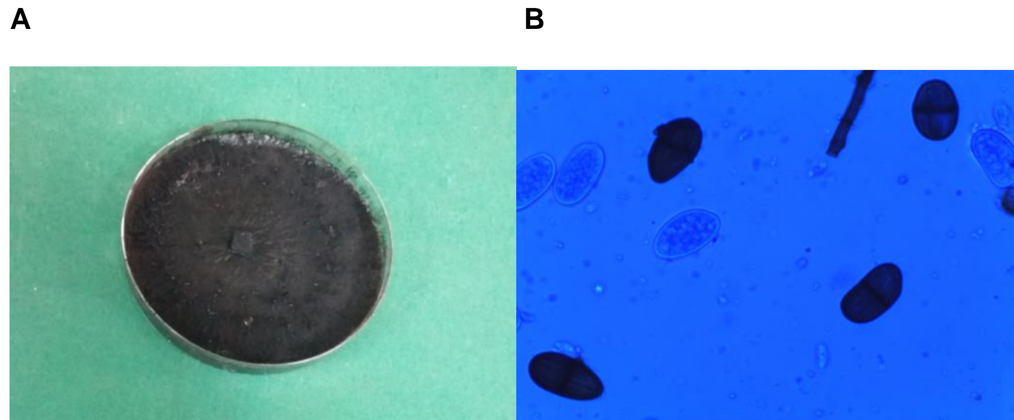

Figure 2. *Lasiodiplodia theobromae* associated with leaves of *Allanblackia floribunda* Oliv. A: Colony growth. B: Micrograph.

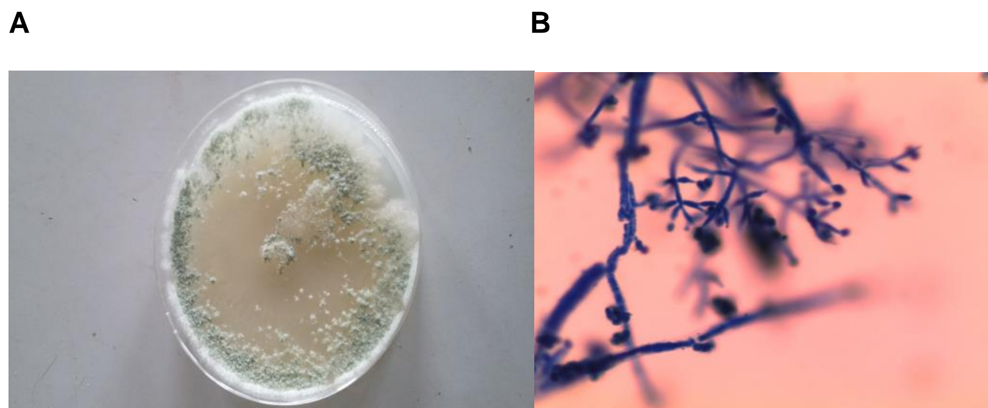

Figure 3. *Trichoderma pseudokoningii* associated with leaves of *A. floribunda* Oliv. A: Colony growth; B: Micrograph.

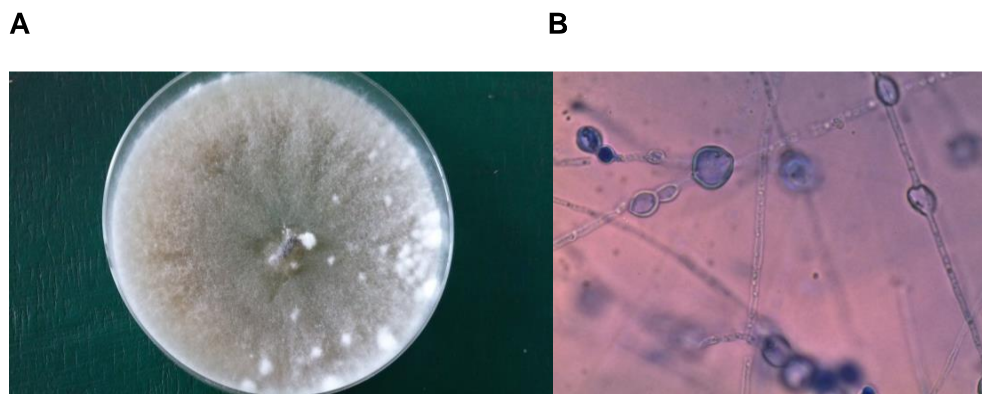

Figure 4. *Pythium aphanidermatum* associated with leaves of *A. floribunda* Oliv. A: Colony growth. B: Micrograph.

species.

A similar finding was reported by Ukoima et al. (2013) while assessing the pathogens associated with seedlings of *T. grandis*. The study identified *A. niger, Sclerotium*

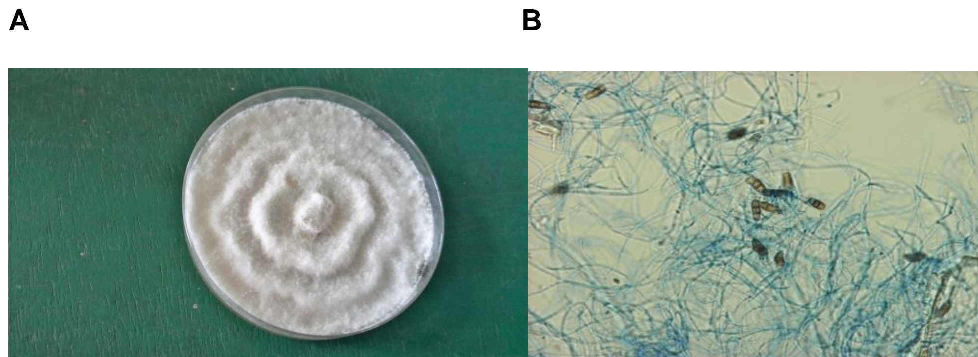

Figure 5. *Pestalotia palmarum* associated with leaves of *Allanblackia floribunda* Oliv. A: Colony growth; B: Micrograph.

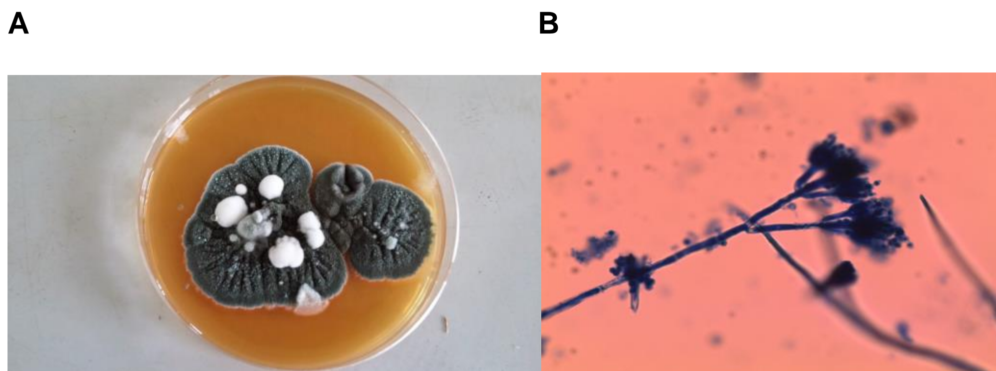

Figure 6. *Penicillium italicum* associated with leaves of *A. floribunda* Oliv. A: Colony growth; B: Micrograph.

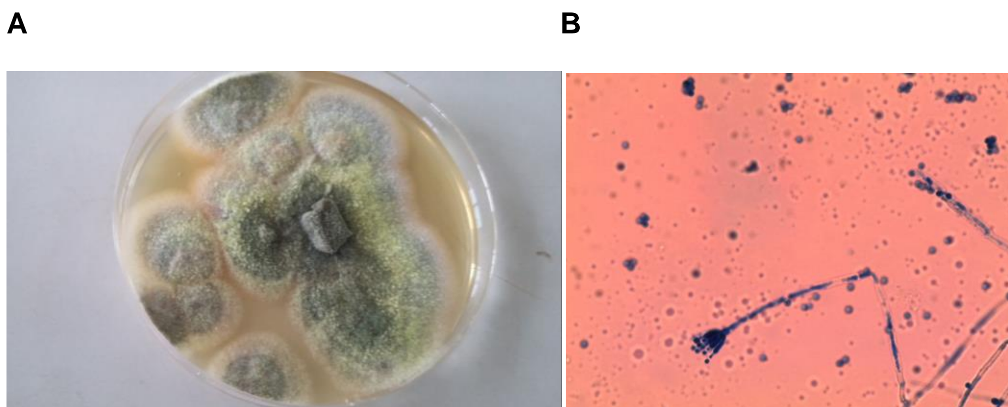

Figure 7. *Penicillium javanicum* associated with leaves of *A. floribunda* Oliv. A: colony growth. B: Micrograph.

rolfsii, A. flavus, Pythium debaryanum, Armillaria mallea, F. oxysporum, Rhizopus stolonifer, *Penicillium* species and *Serratia* species in all study sites evaluated in Akwa Ibom State.

P. palmarum was isolated in all study sites with high incidence of occurrence. These species are reported to be common in rainforest zone of Africa and are frequently associated with leaves of woody plants (Langenheim et

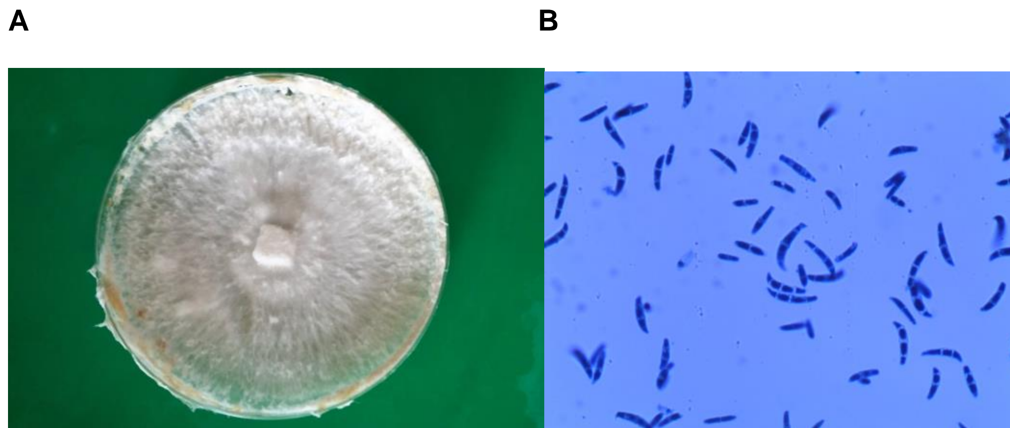

Figure 8. *Fusarium oxysporum* associated with leaves of *A. floribunda* Oliv. A: colony growth. B: Micrograph.

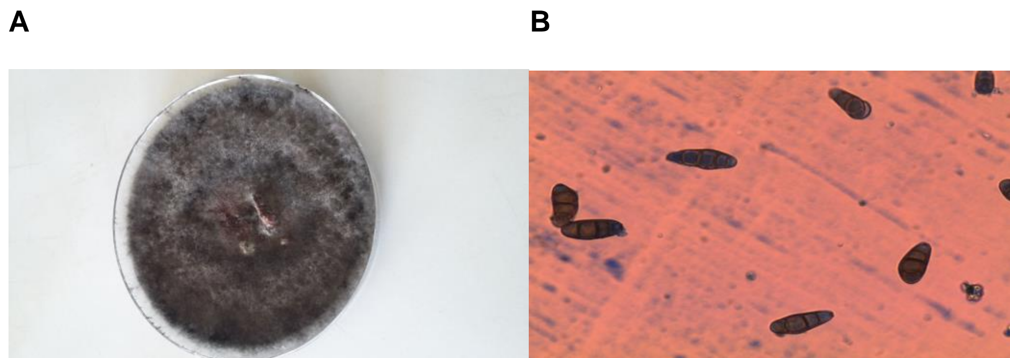

Figure 9. *Curvularia lunata* associated with leaves of *A. floribunda Oliv.* A: Colony growth. B: Micrograph.

al., 1981; Fail and Langenheim, 1990). The highest percentage incidence was recorded during the dry season (35.29%) at Benin followed by *T. pseudokoningii* (18.75%) during the dry and raining season at Owu-Ikija (Table 2). *A. niger* also recorded relatively high incidence (17.64%) of occurrence at Benin during the dry season.

C. capsici, C. coccodes and *C. lunata* were also associated with *A. floribunda* though totally absent during both seasons at Owu-Ikija while *F. oxysporum, L. theobromae* and *Pythium aphanidermatum* were absolutely absent at Benin. The variation of these organisms could be attributed to biotic and abiotic components. The presence of two species of *Colletotrichum* in this study is in accordance with Freeman (2008) who reported that *Colletotrichum* species are broad range of pathogens, with many species affecting a single host and a single species infecting a diverse number of hosts. Bagwari et al. (2014) reported *C. lunata* associated with leaf spots of *Populus deltoids.* Other reports stated that the pathogen was associated with leaf blight of rice (Zhong et al., 2016).

A. niger, M. phaseolina, *Penicillium* spp., *P. palmarum, R. nigricans* and *T. pseudokoningii* were present at different percentage frequency at both Ikija and Benin. This result indicates that under favorable conditions several pathogens can co-exist to attack susceptible hosts like *A. floribunda* leaves.

Consequently, pathogenicity test result revealed that *C. capsici*, *C. coccodes, C. lunata, F. oxysporum, M. phaseolina, L. theobromae* and *P. palmarum* were not pathogenic on *A. floribunda* leaves. Many workers have also reported non pathogenicity of these organisms on various plant species (Arrhenius and Langenheim, 1986; Sanchez Hernadez et al., 1998; Slippers and Wingfield, 2007; Dania et al., 2010; Bagwari et al., 2014). These organisms may not be pathogenic under artificial conditions (seedling inoculation) because potted plants receive different levels of fertilization and moisture (Wingfield, 1996), this finding also corroborate previous results that, greenhouse trials have a moderate to weak correlation with those obtained from the field.

In addition, the biology of the specific pathogen may

Table 2. Incidence of diseases of fungi associated with leaves of *Allanblackia floribunda* during rainy and dry seasons at Owu-Ikija and Benin, Nigeria.

Organism	Owu-Ikija		Benin	
	Rainy season	Dry season	Rainy season	Dry season
Aspergillus niger	7.02	12.5	12.5	17.64
Aspergillus flavus	5.26	8.33	12.5	-
Colletotrichum capsici	-	-	12.5	-
Colletotrichum coccodes	-	-	12.5	11.76
Curvularia lunata	-	-	4.17	5.88
Fusarium oxysporum	8.77	4.16	-	-
Macrophomina phaseolina	8.77	10.42	12.5	11.76
Lasiodiplodia theobromae	8.77	12.5		
Penicillium italicum	10.43	8.33	8.33	5.88
Penicillium javanicum	3.51	4.17	-	-
Pestalotia palmarum	15.79	10.42	16.67	35.29
Pythium aphanidermatum	3.51	-	-	-
Rhizopus nigricans	10.53	10.42	4.17	5.88
Trichoderma pseudokoningii	17.54	18.75	4.17	5.88

influence the result. On the other hand, the tree species may be resistant as a result of production of antimicrobial molecules (phytoalexins) which is triggered immediately after attack by the pathogenic fungi (Bowyer et al., 1995), hence, prevent the disease spread. However, the disease-diversity hypothesis states that high species or high genetic diversity in a community confers disease resistance (Heybroek, 1982; Burdon, 2001). Positive or neutral ecosystem functioning effects on pathogen richness might also occur if additional plant species are important for completing the pathogens life cycles (Cheatham et al., 2009; Mundt et al., 2011). The incidence of microfungi such as *Pestalotia*, *Macrophomina, Colletotrichum, Cercospora, Fusarium, Penicillium,* and *Aspergillus* associated with leaves of *A. floribunda* may lead to reduction and loss of productivity. Although the associated organisms were not pathogenic on this important plant, sustainable management strategy should be adopted in order to forestall devastating effect of the associated organisms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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A comparison of antibacterial efficacy of some household detergents available in Makkah, Saudi Arabia, against 16 ATCC bacterial strains and clinical isolates

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The emergence and spread of multidrug-resistant microorganisms are threats to public health due to its related increasing rates of morbidity and mortality in the world. Routine disinfection practices do not eliminate many of these resistant species and may contribute to the development in their resistance. Therefore, the current study evaluates the effectiveness of some popular detergents labelled as antibacterial and available at local market. Fifteen different brands of detergents were tested against thirteen American-type culture collection (ATCC) strains; five species related to Gram-positive bacteria, eight species to Gram-negative bacteria and three clinical isolates, (*Escherichia coli, Klebsiella pneumonia* and Methicillin resistant *Staphylococcus aureus* (MRSA) by measuring their efficacy by agar well diffusion technique and contact time assay. Dilution levels of detergents exhibited different zones of inhibition against the tested bacterial strains. The initial dilutions of detergents (DAC, SUNOVA, CLOROX, Drummer, GENTO, Dettol with pine, Dettol, Clorox Original, EMLAG and 3 M) showed inhibition zones ranging from 10 mm to >40 mm on Gram positive bacteria. Dettol, Clorox Original, EMLAG and 3 M showed inhibition zones ranging from 11 to 20 mm on limited numbers of Gram-negative bacteria at the first dilutions of detergents. The results showed that, Dettol, Clorox and 3 M were the strongest detergents compared to the other detergents included in this study. A contact-time assay showed a positive relationship between exposure time and efficacy of detergent against the tested bacterial strains.

Key words: Detergents, multidrug-resistant microorganisms, nosocomial infection, Makkah.

INTRODUCTION

Detergents are chemicals contain a lipophilic and a hydrophilic component that have the potential to prevent the growth of or destroy microorganisms on the surface of inanimate objects, including potentially pathogenic organisms, and played an important role in controlling the spread of infectious diseases (Rutala and Weber, 2013).

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Disinfectant is chemical or physical agent that destroys pathogenic microorganisms but might not kill microbial spores and the disinfection process was defined by the British Institute as reducing the levels of harmful organisms to harmless while not killing all microorganisms (Thomas et al., 2005). Multidrug-resistant bacteria are the major cause of nosocomial infections (NI), these infections are a public health issue throughout the world (Wellington et al., 2013; Savard et al., 2013). These organisms can be transmitted from the hospital environment and health care workers to patients (Siegel et al., 2007; Alfa et al., 2015; Otter et al., 2013).

Some studies confirmed that patients who stay in hospitals transmit these bacteria to the facilities, which leads to the possibility of the transmission of these microbes to other patients later admitted to these rooms (Donskey, 2013; Eckstein et al., 2007; Ontario Agency for Health Protection and Promotion, 2012). Proper disinfection techniques by broad spectrum biocidal compounds can prevent nosocomial infections (Kundrapu et al., 2012). Some studies have concluded that daily disinfection of medical equipment that used by patients and their accommodation places by effective disinfectants limits the levels of contamination by these organisms (Boyce, 2007; Theraud et al., 2004; John, 2016).

Scientific methods of sterilization and disinfection are necessary for controlling the prevalence and spread of hospital-acquired infections (Zoutman et al., 2011). Therefore, failures to follow these methods may lead to many hospital-acquired infections contributing to increased morbidity and mortality in hospitals (Strassle, et al., 2012). The selection of effective disinfectants for health-care facilities is a critical factor, and so must be based on scientific analysis to ensure the efficacy of the disinfectants against microbes that cause the emergence and spread of hospital-acquired infections (Tacconelli et al., 2014; Kampf et al., 2014).

The disinfection process can be affected by several factors such as temperature, acidity, concentration of the disinfectant and contact time during the disinfection process (Ferreira et al., 2015). Pathogenic organisms vary in their degrees of response to different detergents, as they constantly acquire resistance to different formulas. The efficacy of disinfectants against targeted pathogens that are selected for the disinfection process must be studied and analyzed (Santos-Junior et al., 2018). It is necessary to evaluate the effectiveness of detergents against pathogens before use and not rely on the information provided by the manufacturing companies to ensure the optimal efficacy (Kawamura-Sato et al., 2008; Boyce and Pitted, 2002).

Many health care workers are not properly informed on how to choose appropriate disinfectants and use phenolic disinfectants, which have a generally low effectiveness in the disinfection process. This leads to an uncontrolled increase in infections among patients in hospitals (BSG, 2003; Alfa et al., 2010; Manitoba, 2007; Shang et al., 2015). The aim of the current study is to evaluate the effectiveness of a number of some common brands of detergents sold in Makkah, Saudi Arabia used in hospital disinfection against pathogenic bacteria which cause infection among patients, as well as determine the required time to eliminate these pathogens by disinfectants.

MATERIALS AND METHODS

Disinfectants

Fifteen different brands of detergents available in Makkah labeled as "99.9 bacterial reduction" or "antibacterial" were included in this study. The following detergents were chosen: DAC, SUNOVA, UDO, CLOROX, DRUMMER, GENTO, DETTOL with pine, SAFE, FLASH, DETTOL, BAHAR, FLAG, CLOROX Original, EMLAG and 3M. Thirteen detergents were diluted to different five concentrations in sterile water (10, 20, 40, 80 and 100%), the first dilution was the manufacturer-recommended and two of them were used without dilution in accordance with the instructions of use as shown in Table 1.

Tested bacterial strains

Thirteen ATCC strains were obtained from the Microbiology Department at the Medical College of Umm Al Qura University, Makkah, Saudi Arabia. Five of the species were related to Gram-positive bacteria: *Enterococcus faecalis* ATCC 29212, Vancomycin resistant *Enterococcus faecalis* (VRE) ATCC 51299, *Staphylococcus aureus* ATCC 25923, Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300, and *Staphylococcus epidermidis* ATCC 12228. Eight species were related to Gram-negative bacteria: *Salmonella typhimurium* ATCC700720, *K. pneumonia* (ESBL) ATCC 14028, *K. pneumonia* (CRE) ATCC 700603, *Acinetobacter baumannii* ATCC 1605, *Shigella sonnei* ATCC 25931, *Pseudomonas aeruginosa* ATCC 15442, *Proteus mirabilis* ATCC 29906 and *Escherichia coli* ATCC 35218. Three clinical isolates, (*E. coli, K. pneumonia* and *S. aureus* MRSA) were obtained from a diagnostic microbiology laboratory at a tertiary care hospital in Makkah, Saudi Arabia.

Culture media

Muller-Hinton agar medium was used to determine the susceptibility of tested bacterial strains to the selected detergents by the agar well diffusion method. Muller-Hinton broth media was used for the kill-time assay.

Preparation of standard inoculums

Organisms were cultured on a Muller-Hinton agar media at 37°C for 24 h. A single colony was obtained from a strain tested using a sterile loop and inoculated in 3 ml of Muller-Hinton broth to obtain a homogenous suspension. Turbidity was standardized to 0.5 McFarland using calibrated VITEK 2 DENSICHEK.

Well diffusion method

A diffusion assay on Mueller-Hinton agar plates was performed as

Table 1. Active ingredients and different concentrations for the tested detergents.

Tested product	Active ingredient (s)	Dilution levels (ml)				
		D1	D2	D3	D4	D5
DAC	Alkyl benzyl dimenthyl ammonium chloride <5%	0.6 /10	1.2 /10	2.4/10	4.8 /10	Absolute
SUNOVA	Alkyl benzyl dimenthyl ammonium chloride <5%	0.1/10	0.2/10	0.4/10	0.8/10	Absolute
U.DO	Not available	1.2 /10	2.4 /10	4.8 /10	9.6 /10	Absolute
CLOROX	6.0% sodium hypochlorite	1 /10	2 /10	4/10	8/10	Absolute
Drummer	Cloroxylenol 0.485% w/w	0.5 /10	1 /10	2 /10	4 /10	Absolute
GENTO	Quaternary ammonium compound	1.2/10	2.4/10	4.8/10	9.6/10	Absolute
Dettol with pine	Chloroxylenol 4.8% w/w	0.6 /10	1.2 /10	2.4 /10	4.8/10	Absolute
SAFE	Chloroxylenol	0.3 /10	0.6 /10	1.2 /10	2.4 /10	Absolute
Flash	Hydrogen chloride			Absolute		
Dettol	Chloroxylenol 4.85% w/w	1 /10	2/10	4/10	8/10	Absolute
BAHAR	<5% Cationic Surfactant	0.4 /10	0.8 /10	1.6 /10	3.2 /10	Absolute
FLAG	Triton 2-5 %			Absolute		
Clorox original	6.0% sodium hypochlorite	1 /10	2 /10	4 /10	8 /10	Absolute
EMLAG	Not available	1 /10	2 /10	4 /10	8/10	Absolute
3 M.	Not available	1 /10	2 /10	4/10	8/10	Absolute

D1= First Dilution (manufacturer-recommendation); D2= Second Dilution; D3= Third Dilution; D4= Forth Dilution; D5= Five Dilution.

described by the Clinical and Laboratory Standards Institute (CLSI) (Hindler and Keiser, 1994; Jorgensen, 1993). The bacterial strain was suspended in a Mueller-Hinton broth and adjusted to 0.5 McFarland turbidity. A sterile swab was dipped into the bacterial suspension and swabbed on the Muller and Hinton agar plates. Plates were allowed to dry for 10 minutes before being punched with a 6 mm diameter sterile sharp glass rod. After that, wells were filled with 100 µl from different detergents and one well was filled with 100 µl of dimethyl sulfoxide (DMSO) as a negative control. Vancomycin 30 µg disc and Amikacin 30 µg disc diffusion solutions were used as positive controls for Gram-positive bacteria and Gram-negative bacteria respectively, then the plates were incubated at 37°C for 24 h and examined for inhibition zones. These steps were repeated with the second, third, fourth and fifth dilutions for each detergent. Inhibition zones around the wells indicated effectiveness of detergents against the tested bacterial strains. Absences of inhibition zones indicated ineffectiveness of detergents against tested strains. Results are shown in Table 2.

Time kill assay

A contact time assay was performed as described by Clinical and Laboratory Standards Institute (CLSI M26-A, 1998) depending on the turbidity of the bacterial growth in broth culture media (CLSI, 1998). Tested bacterial strains were suspended in Mueller Hinton broth and adjusted at 0.5 McFarland. 500 µl had been removed and added to 9.5 ml of the first dilution of the detergent solution at room temperature. At the end of the first minute from addition, 10 µl of the suspension was removed and added to 5 ml Mueller-Hinton broth tube. This was repeated after two, three, four, and five minutes from zero time. A Mueller-Hinton broth tube without the addition of detergent was used as a negative control. This step was repeated with the second, third, fourth and fifth dilutions of detergents. The inoculated tubes were labeled and incubated at 37°C for 24 h. After the incubation period, the tubes were classified as turbid (indicating growth) or clear (indicating no growth), corresponding to resistance or sensitivity to detergents as shown in Table 3.

RESULTS

Fifteen different brands of household detergents were used, two of them without dilutions and the others with five concentrations each, for determining their efficacies against 16 bacterial strains (13 ATCC species and three clinical isolates) using two methods: Well diffusion assay and contact time assay. Results of the well diffusion method with the five concentrations were recorded according with a color-coding system with green indicating the first dilution, brown indicating the second dilution, blue indicating the third dilution, yellow indicating the fourth dilution, and pink indicating the undiluted detergents as shown in Table 2. Diluted and undiluted concentrations of detergents showed different zones of inhibition on the tested bacterial strains are shown in Table 2. Initial dilutions of detergents DAC, SUNOVA, CLOROX, Drummer, GENTO, Dettol with pine, Dettol, Clorox Original, EMLAG and 3M showed inhibition zones ranging from 10 mm (Drummer) to >40 mm (Clorox original) on Gram-positive bacteria, while other detergents (SAFE, FLASH, BAHAR, FLAG and UDO) showed varying inhibition areas on Gram-positive bacteria at the highest concentrations. Initial dilutions of detergents Dettol, Clorox Original, EMLAG and 3M showed inhibition zones ranging from 11 mm (EMLAG) to 22 mm (Dettol) on limited numbers of Gram-negative bacteria, while detergents SUNOVA, UDO, Drummer, SAFE, Flash, BAHAR and FLAG exhibited varying efficacy on Gram- negative bacteria at the highest concentrations. *K. pneumonia* (ESBL) and *Shigella sonnei* strains were resistant to any concentrations of Drummer and BAHAR detergents. *Acinetobacter*

Table 2. Results of investigation of the efficacy of detergents against bacterial strains (13 ATCC species and three clinical isolates) by agar well diffusion assay.

Tested strains	Diameter of inhibition zone (mm) of five dilutions of tested detergents														
	DAC	SUNOVA	U.DO	CLOROX	Drummer	GENTO	Dettol with pine	SAFE	Flash	Dettol	BAHAR	FLAG	Clorox original	EMLAG	3 M
Enterococcus faecalis ATCC (mm)	16[a]	**16**[a]	18	**18**[a]	**10**[a]	18[a]	16[a]	15	18	14	14	20[b]	15[a]	20[a]	22[a]
Enterococcus faecalis (VRE) ATCC (mm)	**18**[a]	**15**[a]	12	**20**[a]	**12**[a]	15[a]	20[a]	18	22	18	20	20[b]	15[a]	16[a]	22[a]
Staphylococcus aureus ATCC (mm)	**21**[a]	**16**[a]	**14**	**20**[a]	**20**[a]	20[a]	20[a]	14[a]	24	20	24	25[b]	16[a]	20[a]	22[a]
Staphylococcus aureus (MRSA) ATCC (mm)	**22**[a]	**16**[a]	14	**21**[a]	**20**[a]	22[a]	20[a]	11[a]	26	20	10	20[b]	18[a]	20[a]	22[a]
Staphylococcus epidermidis ATCC (mm)	**20**[a]	**18**[a]	16[c]	**22**[a]	**16**[a]	22[a]	25[a]	12[a]	30	25	24	30[b]	>40[a]	15[a]	14[a]
Salmonella typhimurium ATCC (mm)	**12**[a]	16[c]	14[c]	**14**	**16**	16	12	22[b]	22	20	15	20[b]	16[a]	14[a]	15[a]
Klebsiella pneumonia (ESBL) ATCC (mm)	16	**21**[b]	16[c]	14	R	12	14	20[b]	27	12	R	18[b]	18[a]	15[a]	17[a]
Klebsiella pneumonia (CRE) ATCC (mm)	12	**20**[b]	14[c]	**12**	14	12	16	20[b]	28	12	R	18[b]	20[a]	15[a]	14[a]
Acinetobacter baumannii (CRE) ATCC (mm)	16	**20**[b]	**17**	16	R	14	12	22[b]	25	22	16	18[b]	15[a]	15[a]	13[a]
Shigella sonnei ATCC (mm)	**18**	**15**[b]	**16**	13	R[b]	14	18	10	20	14	R	14[b]	18[a]	11[a]	11[a]
pseudomonas aeruginosa ATCC (mm)	**12**	**22**[b]	**24**	16	R[b]	**16**[b]	13[c]	20[b]	20	R	R	24[b]	18[a]	12[a]	R[a]
Proteus mirabilis ATCC (mm)	**18**	**12**[b]	R	14	R[b]	**16**[b]	14[c]	20[b]	R[b]	12[a]	16	14[b]	R[b]	R[b]	11[a]
E. coli ATCC (mm)	12	13[c]	15[c]	**14**	R[b]	14	13	20[b]	25	12[a]	12	18[b]	16[a]	20[a]	15[a]
Klebsiella pneumonia (C.I. MDR) (mm)	18	**20**	16[c]	**11**	R[b]	18	16	22	26	20[a]	22	18[b]	16[a]	12[a]	13[a]
E. coil C.I. MDR*)* (mm)	**12**[a]	**18**[b]	15[c]	**14**	R[b]	16	14	14	14	15[a]	20	16[b]	15[a]	R[b]	16[a]
MRSA (C.I.MDR) (mm)	**21**[a]	**32**[b]	**12**	**24**	**12**	20	22	15	25	22[a]	15	24[b]	20[a]	20[a]	22[a]
Efficacy of D1 (%)[a]	64	38	13	69	38	81	69	31	0	94	19	0	94	88	94
Efficacy of D2 (%)[e]	64	38	19	69	38	81	69	**44**	0	94	31	0	94	88	94
Efficacy of D3(%)[d]	70	38	31	75	44	88	81	**44**	0	94	37	0	94	88	94
Efficacy of D4(%)[c]	82	50	75	88	44	88	94	**44**	0	94	50	0	94	88	94
Efficacy of D5 (%)[b]	100	100	94	100	50	100	100	100	100	100	75	100	94	88	94
First dilution resistance (%)[f]	36	62	87	31	62	19	31	69	100	**6**	81	100	6	12	6

R= Resistant, (C.I.MDR) = Clinical isolate multidrug resistant, mm= millimeter (measuring unit of diameter of inhibition zone).[a], Indicates the first dilution (pink colour); [e], indicates the second dilution (brown colour); [d], indicates the third dilution (blue colur); [c], indicates the fourth dilution (yellow colour); [b] indicates undiluted detergents (blue colour). [f], First dilution resistance (%) (red).

baumannii, *E. coli* and *K. pneumonia* (clinical isolate) strains did not respond to any concentrations of Drummer. Dettol, 3 M and Clorox Original were the most effective on the majority of tested bacterial strains, except *P. aeruginosa,* which did not respond to Dettol nor 3 M at any concentrations.

Table 3 depicts the results of kill-time assays of detergents on bacterial strains. It was observed that detergents DAC, CLOROX, GENTO, Dettol with pine, Dettol and 3 M were most effective on Gram-positive bacteria during the first minute of the first dilution. SUNOVA, Drummer, Clorox original and EMLAG showed efficacy on Gram-positive bacteria from the first minute until the fifth minute of the first dilution. Gram-positive bacteria did not response to the detergent Flash from the first minute up to the fifth minute of exposure. FLAG only to fifth dilutions was effective at the first. Most of Gram-negative bacteria exhibited limited responses to some of the tested detergents at the first minute of exposure to the first dilutions. Dettol with pin, Clorox and 3 M detergents were observed to be highly effective

Table 3. Results of contact time assay of five dilutions of detergents against 13 ATCC strains and 3 strains clinical bacterial isolates (MDR) within five minutes.

Tested strains	Contact time (1-5 min) of five dilutions														
	DAC	SUNOVA	U.DO	CLOROX	Drummer	GENTO	Dettol with pine	SAFE	Flash	Dettol	BAHAR	FLAG	Clorox Original	EMLAG	3 M
Enterococcus faecalis ATCC	1[a]	1[a]	>5[g]	1[a]	>5	1[a]	1[a]	>5[g]	>5[d]	1[a]	>5[g]	1[d]	1[a]	1[e]	1[a]
Enterococcus faecalis (VRE) ATCC	1[a]	>5[a]	>5[f]	1[a]	>5	1[a]	1[a]	>5[g]	>5[d]	1[a]	1[g]	1[d]	>5[a]	1[e]	1[a]
Staphylococcus aureus ATCC	1[a]	1[a]	5	1[a]	5	1[a]	1[a]	1	5[d]	1[a]	1[c]	1[d]	1[a]	1[e]	1[a]
Staphylococcus aureus (MRSA) ATCC	1[a]	1[a]	>5[f]	1[a]	>5	1[a]	1[a]	1	>5[d]	1[a]	2	1[d]	4[a]	2[e]	1[a]
Staphylococcus epidermidis ATCC	1[a]	1[a]	>5[c]	1[a]	5	1[a]	1[a]	1	1[d]	5[a]	1[g]	1[d]	1[a]	1[e]	1[a]
Salmonella typhimurium ATCC	1[a]	>5[c]	>5[c]	1[c]	3[d]	1[a]	1[a]	1[d]	1[d]	1[a]	1[c]	1[d]	1[a]	1[e]	1[a]
Klebsiella pneumonia (ESBL) ATCC	1[f]	1[d]	>5[c]	1[a]	>5[d]	4[a]	1[f]	>5[d]	1[d]	3[a]	>5[d]	1[d]	>5[a]	1[e]	1[a]
Klebsiella pneumonia (CRE) ATCC	1[a]	1[d]	5[c]	1[a]	>5[f]	1[a]	1[f]	1[d]	1[d]	>5[a]	>5[d]	1[d]	1[a]	1[e]	1[a]
Acinetobacter baumannii (CRE) ATCC	1[c]	1[d]	>5	1[f]	3[d]	>5	2[d]	1[d]	1[d]	1[a]	1[c]	1[d]	1[a]	1[e]	1[a]
Shigella sonnei ATCC	1[d]	4[d]	>5	1	>5[d]	>5[f]	1[d]	1[a]	1[d]	>5[a]	>5[d]	1[d]	1[a]	1[e]	1[a]
Pseudomonas aeruginosa ATCC	1[d]	1[d]	>5	1	>5[d]	>5[d]	1[c]	1[d]	1[d]	5	>5[d]	1[d]	1[a]	1[e]	>5[d]
Proteus mirabilis ATCC	1[d]	1[d]	>5	1[c]	1[d]	5[d]	2[c]	2[d]	>5[d]	1[a]	>5[d]	1	>5[d]	>5[d]	1[a]
E. coli ATCC	1[a]	1	>5[c]	1[a]	>5[d]	1[a]	1[a]	1[d]	1[d]	1[a]	1[d]	1[d d]	1[a]	1[a]	1[a]
Klebsiella pneumonia (C.I. MDR)	1[c]	1[a]	>5[c]	>5[a]	1[d]	1[a]	1[a]	1[d]	1[d]	1[a]	1[d]	1[d]	4[a]	1[a]	1[a]
E. coil (C.I. MDR)	1[a]	1[d]	>5[c]	1[a]	>5[d]	1[a]	1[a]	1[d]	1[d]	1[a]	1[d]	1[d]	4[a]	>5[d]	1[a]
MRSA(C.I.MDR)	1[a]	1[d]	>5[a]	1[a]	>5[a]	1[a]	1[a]	1[a]	>5[d]	1[a]	1[f]	1[d]	1[a]	1[a]	1[a]

1- 5 min = required contact time of the detergents against tested organisms with the five-dilutions; [a](Green color) = First Dilution; [g](Brown color) = Second Dilution; [f](Blue color) = Third Dilution; [c](Yellow color) = Fourth Dilution; [d](Pink color) = Five Dilution.

against the majority of the tested bacterial strains at the first minute of contact with the first dilution except *P. aeruginosa* and *P. mirabilis* which exhibited resistance at all dilution levels from the first minute until the fifth minute of exposure time.

DISCUSSION

Standard hygiene measures are necessary to prevent the transmission of many antibiotic-resistant bacteria among hospital patients. The cleaning and disinfection of target surfaces is essential for limiting risks associated with these organisms (Reitzel et al., 2009; Savard et al., 2013; Buescher et al., 2016; Magiorakos et al., 2013; Willey et al., 2008). The procurement of detergents without a scientific basis as well as a prevalence of incorrect methods of disinfection in health-care facilities may contribute to the development of microbial resistance. Phenolic products, which are highly toxic, are commonly used at health-care facilities in many countries due to their low cost. Ideal detergents should have a broad spectrum of effects on microorganisms and minimal toxicity (Pierson, 2009; Kaliyadan et al., 2014). None of the recommended dilutions of the tested detergents were found to be effective against the majority of the tested bacterial strains, except Dettol and 3 M, which expressed efficacy patterns when applied to the majority of tested bacteria at the manufacturer-recommended dilutions with the exception of *P. aeruginosa,* which showed resistance to all dilutions. and Clorox Original and EMLAG, which expressed efficacy against all tested bacteria except *P. mirabilis* which exhibited high resistances to all dilutions. Only certain detergents of popular brands were observed to inhibit the growth of Gram-positive species and limited Gram-negative bacteria in this study. The highest efficacy of the used detergents against the tested bacterial strains at the first dilution was 94%, with only three detergents (Dettol, Clorox and 3 M) out of the fifteen detergents tested observed to be effective when applied to sixteen bacterial species, while there was no efficacy for both FLAG and Flash on all tested bacterial strains

at the first dilution. Gram-positive bacteria were less resistant to successive dilutions of tested detergents. Some of the Gram-negative bacterial strains exhibited high resistances to the tested successive dilutions, with some of them exhibiting resistance to all dilutions, as depicted in Table 3. Gram-positive bacteria exhibited the highest response to the first dilution of Dettol, Clorox, DAC, Gentoo and 3 M at the first minute of contact time assay, with the lowest response recorded with exposure to Drummer. Gram-negative strains exhibited the greatest response to Dettol, Clorox and 3 M and the lowest response to Gentoo at the first minute of the first dilution. These results indicated that Dettol, Clorox Original and 3 M are the most effective antimicrobial agents among the detergents included against tested strains in this study, which supports the results of previous studies (Jain et al., 2016; Olasehinde et al., 2008; Sickbert-Bennett et al., 2005; Bockmühl et al., 2019; McDonnell and Russell, 1999). This study has highlighted two important factors for increasing detergent efficiency against pathogenic bacteria that should be considered appropriate concentration and exposure time. The results of this study indicated that an exposure time of microorganisms to disinfectants of at least five minutes could increase their effectiveness.

The usage of higher concentrations of disinfectants associated with increased toxicity is not recommended. Results also indicated that manufacturer instructions for the usage of detergents should not be relied upon. Rather, rigorous testing should be carried out to determine their effectiveness on pathogens before usage to avoid the spread and resistance of microorganisms. Therefore, it is highly recommended to evaluate new detergents before procuring them for hospitals and also to periodically verify their effectiveness (Peter and Scott; 2019; Rutala and Weber, 2014; Ngu et al., 2015). It is also recommended to select detergents that are most effective for eliminating the microorganisms that cause nosocomial infections, apply them at the appropriate concentrations and recommended contact times to ensure their efficacy and use them periodically to ensure that the spread and resistance of these microorganisms are reduced. Further research is needed to study the appropriateness, safety and efficacy of detergents against the nosocomial infections.

Conclusion

The efficacy of detergents against pathogenic microorganisms is an important factor for controlling the spread of microbial infections and disease transmission. Most of the recommended dilutions of detergents included in this study were effective against all bacterial strains tested. Only certain brands of detergents were able to suppress the growth of gram-positive bacteria and certain gram-negative species tested in this study. It was observed that Dettol, Clorox and 3M were the strongest detergents compared to the other detergents included in this study. A contact-time assay showed a positive relationship between exposure time and efficacy of detergent against the tested bacterial strains. It is recommended to evaluate new detergents before applying them in hospitals and to verify their use periodically to ensure their effectiveness for reducing rates of nosocomial infection. Accordingly, we recommend using detergents which have high efficacies against most bacterial strains and avoiding the usage of detergents that have poor efficacies in order to prevent increasing bacterial resistance in health care units.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Antibiotic resistance and genotypic detection of extended spectrum beta-lactamase producing pathogens in catheter associated urinary tract infection at a teaching facility in Kumasi, Ghana

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This study determines the antibiotic resistance pattern of extended spectrum beta-lactamase (ESBL) producing pathogens responsible for catheter associated urinary tract infection (CAUTI) and the genes associated with the resistance. The study used 35 ESBL-producing pathogens isolated from urine and biofilm found in CAUTI from June 2018 to November 2018. Pathogens were confirmed phenotypically for ESBL production with cefpodoxime combination disc kits. Antibiotic resistance was tested using the Kirby-Baurer disc diffusion method on Muller Hinton Agar. ESBL genotypes were identified with PCR. Urine isolates showed higher frequency of resistance against ciprofloxacin (94.11%), cefuroxime and ceftazidime (82.35%) and with no recorded resistance against Eertepenem (0.0%). The average resistance of the biofilm isolates ranged from 0.0% (Ertepenem) to 88.89% (Cefuroxime, Cefpodoxime, Ciprofloxacin and Trimethoprim). All the targeted genes were identified with CTX-M (40%) being the most dominant among them. The ESBL-producing pathogens showed zero resistance against Ertepenem. Ciprofloxacin and other Cephems commonly used in CAUTI treatment were shown to be less effective. The high resistance is as a result of the bacterial cells present in the biofilm with *Klebsiella pneumonia* exhibiting more resistance than the ESBL-producing *E. coli* and CTX-M-1 was identified as the most prevalent gene among the identified genotypes. Ertepenem should therefore be recommended for treatment of Catheter associated urinary tract infection.

Key words: Catheter, CAUTI, extended spectrum beta-lactamase (ESBL), urine, biofilm, resistance, microorganisms.

INTRODUCTION

The incidence of Catheter Associated Urinary Tract Infection (CAUTI) is inevitable having in mind that there is about 10% daily infection rate when catheter is present *in situ* (Hartley and Valley, 2014; Hooton et al., 2010). This infection can lead to more serious complications such as sepsis and endocarditis, and it is estimated that over 13000 deaths each year are associated with healthcare-associated UTIs in the United States of America (Parida

and Mishra, 2013). The microorganisms responsible for this infection are mostly the micro flora from the patient, healthcare attendants and the environment. These organisms are able to form biofilm when they attach themselves on these solid surfaces for extended periods of time. Microorganisms usually encountered in CAUTI include *Escherichia coli, Klebsiella pneumonia*, other enterobacteriaceae and *Staphylococcus* species (Albu et al., 2018; Percival et al., 2018). *E. coli* and *K. pneumonia* are the leading cause of CAUTI with increasing resistant rates (Köves et al., 2017; Percival et al., 2018).

These microorganisms have the ability to hydrolyze both beta lactam antibiotics (3rd generation cephalosporins, penicillins and cephamycins) and non-beta lactam antibiotics such as tetracycline, chloramphenicol, aminoglycosides, quinolones as well as vancomycin (Shaikh et al., 2015). This may be attributed to the overuse of antibiotics for infection treatments, the ability of the microbes to grow as biofilms, genetic mutations and pathogens sharing resistance genes with each other (Albu et al., 2018). The extended spectrum beta-lactamase (ESBL) enzymes are classified into four (A, B, C and D) variants based on their amino acid sequences. The most common class, A β-lactamases, encountered in *K. pneumonia* and *E. coli* are the SHV (Sulphydryl variable), TEM (Temoniera) and CTX-M (cefotaximase). Both SHV and TEM are plasmid mediated, degrading penicillins and first generation cephalosporins. These enzymes are however inhibited by clavulanic acid but susceptible to third generation cephalosporins (Paterson et al., 2001). CTX-M (cefotaximase), is another class A ESBL enzyme which preferentially hydrolyzes cefotaxime as compared to ceftazidime and it is organized in major groups as CTX-M- (1, 2, 8, 9 and 25). Reid et al. (2018) reports that CTX-M is increasing and spreading faster and therefore becoming more prevalent than TEM and SHV. Oxacillinase (OXA), a class D ESBL with the ability to hydrolyze oxacillin, is mostly responsible for carbapenems resistance but it is not as common as TEM, SHV and CTX-M (Dallenne et al., 2010).

Several studies described specific genotypes in the ESBL producing pathogens causing resistance to some antibiotics globally (Reid et al., 2018; Sana et al., 2011; Bajpai et al., 2017; Lewis et al., 2007). In India TEM has been reported to be frequent among urine isolates (Bajpai et al., 2017) whilst in Europe, United States and Africa, CTX-M is mostly detected (Reid et al., 2018; Lewis et al., 2007; Zeynudin et al., 2018). In Ghana the prevalence of ESBL reported from the major teaching hospitals ranges from 37.96% to 57.8% with TEM and CTX-M variants being prevalent among *E. coli* and *K. pneumonia* (Mensah et al., 2016; Agyekum et al., 2016). This is of major concern, since both pathogens are the predominant cause of health and community related infections. Even though some studies have investigated ESBL genes causing antibiotic resistance in Ghana (Agyekum et al., 2016; Feglo and Adu, 2016; Hackman et al., 2014), there is no data on antibiotic resistance of ESBL producing pathogens related to CAUTI in Ghana. This study investigates the resistance pattern of ESBL-producing pathogens causing CAUTI and the genes associated with such resistance using the polymerase chain reaction method as a novel way to bridge the gap by providing baseline surveillance on CAUTI, it prevalence and antimicrobial resistance pattern at Komfo Anokye Teaching Hospital (KATH) in Ghana.

MATERIALS AND METHODS

Study site

Urine and biofilm samples were collected from 105 catheterized patients at the urology unit of Komfo Anokye Teaching Hospital (KATH), Kumasi from June 2018 to November 2018. The hospital is a 1200 bed capacity facility located in the heart of Kumasi, the capital town of the Ashanti region. It is the second largest referral/teaching hospital in the country. It serves 12 out of the 16 administrative regions of the country and neighboring countries like Burkina Faso, Togo and Ivory Coast. This is due to the hospital's location and the road network of the country.

Sampling

All samples tested positive for CAUTI. Criteria for CAUTI were based on catheterization duration, bacteria load and patients' symptoms as described by CDC/IDSA. All samples that met CAUTI definition due to ESBL producing *E. coli* and *K. pneumonia* were used for further tests.

The ESBL producing isolates stored in brain heat infusion and glycerol broth were sub cultured on nutrient agar (Oxoid, UK) and incubated at 37°C for 24 h. The biofilms from the catheters were pre enriched with 15 ml of maximum recovery diluent and shaken for 2 min to extract biofilm from the catheter lumen into the diluent and incubated at 37°C for about 6 h before inoculation. Morphological appearance, Gram staining and other standard biochemical methods were used to confirm the identity of the ESBL isolates. ESBL detection was phenotypically confirmed with the Cefpodoxime combination disc kits (Oxiod, UK) using the disc diffusion method on Muller-Hinton Agar (MHA) (Oxiod, UK) as described by CLSI (2020). Zone of diameter ≥5 mm observed in the combined disc when compared to the single disc was considered positive ESBL production. Quality control was done using *K. pneumonia* ATCC 700603 and *E. coli* ATCC 25922 organisms.

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Table 1. bla TEM, bla SHV, bla OXA primer sequences and the cyclic conditions used in PCR.

Gene	Primer sequence	PCR Cyclic conditions	Amplicon size (bp)
M	F: CATTTCCGTGTCGCCCTTATTC R: CGTTCATCCATAGTTGCCTGAC		800
SHV	F: AGCCGCTTGAGCAAATTAAAC R: ATCCCGCAGATAAATCACCAC	5 min at 94°C and 30 cycles of amplification consisting of 1min at 94°C, 1 min at 57°C and 1 min at 72°C,with 10 min at 72°C for the final extension	718
OXA	F: GGCACCAGATTCAACTTTCAAG R: GACCCCAAGTTTCCTGTAAGTG		564

Primer source: Dallenne et al. (2010).

Table 2. bla CTX-M reaction volumes sequences used in PCR master mix.

Gene	Primer sequence	Reaction volume (µl)	PCR Cyclic conditions	Amplicon size (bp)
CTX-M-1	F: TTAGGAARTGTGCCGC R: CGATATCGTTGGTGGT	2 1		688
CTX-M-2	F: CGTTAACGGCACGATG R: CGATATCGTTGGTGGT	1 1	5 min at 94°C and 30 cycles of amplification consisting of 1min at 94°C, 1 min at 55°C and 1min at 72°C,with 10 min at 72°C for the final extension	404
CTX-M-9	F: TCAAGCCTGCCGATCT R: TGATTCTCGCCGCTGA	2 2		561

Primer source: Dallenne et al. (2010).

Antibiotic resistance test

The antibiotic resistance test was done on the ESBL-producing isolates using the disc diffusion method on MHA with the following antibiotics; Ertapenem (10 µg), Cefuroxime (30 µg), Cefpodoxime (30 µg), Ceftazidime (30 µg), Fosfomycin (50 µg), Ciprofloxacin (5 µg), Cefazolin (30 µg), Cefoxitin (30 µg), Trimethoprim (5 µg) and Nitrofurantoin (300 µg). Zones of inhibition around the discs were measured and compared with the CLSI break points for interpretation.

DNA extraction

Using the boiling method of DNA extraction, two to three bacteria colonies from nutrient agar were inoculated in 200 µl nuclease free water. The suspension was placed in a 100°C heat block for 10 minand centrifuged for 10 min at a speed of 14000 rpm. The supernatant was pipetted into a new tube for the PCR analysis.

Gene identification using multiplex PCR

Multiplex PCR was carried out with bla TEM, bla SHV and bla OXA primers in a single tube. The master mix prepared was in 50 µl reaction volume that contained 25 µl of Emerald premix (2×), 7.5 µl primer mix, 15.5 µl of nuclease free water and 2 µl of the DNA template. DNA amplification was done in a thermo cycler. Table 1 shows the cycling conditions and primer sequence.

A separate multiplex PCR was carried out for bla CTX-M-1, CTX-M-2 and CTX-M-9 genes. The multiplex reaction was performed in 50 µl reactions containing; 25 µl of 2×multiplex PCR buffer with Mg^{2+} and dNTP plus, 0.25 µl multiplex PCR enzyme mix, 13.75 µl of nuclease free water, 2 µl of DNA template and primers listed in Table 2 with their reaction volumes, sequences, target gene, cyclic condition and product size. Multiplex PCR was run on 2% agarose gel and 1×TAE buffer at 100 V for 30 min. Gel bands were viewed using a gel transluminator (Reid et al., 2018).

Data analysis

Data was entered into a Microsoft excel and analyzed using graph Pad Prism 5. Study variables were subjected to descriptive analysis such as frequencies and percentages. Chi-square goodness of fit examine was run with *chisq.test* function in the stats package in R programme (version 4.0.0; R Core Team, 2020) to assess the difference in resistance of *E. coli* and *K. pneumonia* under each antibiotic. A value of $p < 0.05$ was regarded as statistically significant.

Ethical approval

Ethical clearance was obtained from the committee on Human Research, Publication and Ethics of the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Written informed consent was obtained from all study participants. Infected patients

Table 3. Resistance pattern of ESBL pathogens isolated from urine samples.

Antibiotics (µg)	Total (n=17)	*E. coli* (n=9)	*K. pneumonia* (n=8)	*p-value*
Ertapenem 10	0(0)	0(0)	0(0)	
Cefuroxime 30	14(82.35%)	7(77.78%)	7(87.5%)	1
Cefpodoxime 30	13(76.47%)	6(66.67%)	7(87.5%)	0.782
Ceftazidime30	14(82.35%)	7(77. 78%)	7(87.5%)	1
Fosfomycin 50	3(17.64%)	2(22.22)	1(12.5%)	NA
Ciprofloxacin 5	16(94.11%)	8(88.89%)	8(100%)	1
Cefazolin 30	13(76.47%)	7(77.78%)	6(75%)	0.782
Cefoxitin 30	6(35.29%)	3(33.34%)	3(37.5%)	1
Trimethoprim 5	10(58.82%)	6(66.67%)	4(50%)	0.527
Nitrofurantoin 300	7(41.18%)	3(33.34%)	4(50%)	NA

NA: Omitted from chi-square analysis because expected counts are less than five.

Table 4. Resistance pattern of ESBL pathogens isolated from biofilm samples.

Antibiotic (µg)	Total (n=18)	*E. coli* (n=12)	*K. pneumonia* (n=6)	*p-value*
Ertapenem 10	0(0)	0(0)	0(0)	
Cefuroxime 30	16(88.89%)	10(83.33%)	6(100%)	0.001
Cefpodoxime 30	16(88.89%)	10(83.33%)	6(100%)	0.000
Ceftazidime30	14(77.78%)	9(75%)	5(83.33%)	0.000
Fosfomycin 50	14(77.78%)	8(66.67%)	6(100%)	0.002
Ciprofloxacin 5	16(88.89%)	12(100%)	4(66.67%)	0.000
Cefazolin 30	15(83.33%)	9(75%)	6(100%)	0.000
Cefoxitin 30	7(38.88%)	5(41.67%)	2(33.33%)	0.008
Trimethoprim 5	16(88.89%)	10(83.33%)	6(100%)	0.000
Nitrofurantoin 300	8(44.44%)	2(16.67%)	6(100%)	0.005

were treated based on their laboratory result.

RESULTS

Antibiotic resistance of ESBL- producing pathogens isolated from urine specimen

The ESBL producing pathogens isolated from the urine show high resistance to Ciprofloxacin (94.11%) followed by the four Cephems/beta-lactam antibiotics with 82.35% for Cefuroxime and Ceftazidime and 76.47% for Cefpodoxime and Cefazolin. Comparing the resistance between *K. pneumonia* and *E. coli*, two of the antibiotics (Fosfomycin and Trimethoprim) were found to be more resistant against *E. coli* than *K. pneumonia.* However, Cefpodoxime ($p>0.05$) was not significantly different from *K. pneumonia* (Table 3). For Ertepenem no resistance was found for the ESBL-pathogens. On average lower resistance was recorded for Fosfomycin (17.64%), Cefoxitin (35.49%) and Nitrofurantoin (41.18%).

Antibiotic resistance of ESBL- producing pathogens isolated from biofilm specimen

For the biofilm isolates, the average resistance numbers ranged between 38.88% (Cefoxitin) and 88.89% (Cefuroxime, Cefpodoxime, Ciprofloxacin and Trimethoprim). There was statistical difference between *E. coli* and *K. pneumonia* for all tested drugs except for Ertepenem. *E. coli* was significantly more resistant than *K. pneumonia* to Ciprofloxacin (0.000) and Cefoxitin (0.008). Apart from Cefoxitin and Ertepenem, all the *K. pneumonia* were resistant to the rest of the antibiotics. The resistance pattern as presented in Table 4 shows that *K. pneumonia* was more resistant than *E. coli.* However, none of the ESBL-pathogen from the biofilm recorded resistance against Ertepenem.

PCR detection of ESBL genes

Thirty-five ESBLs were analyzed using the multiplex PCR

Table 5. Distribution of ESBL genotypes (no., %) among isolates.

None	Single gene	Double genes					More than two genes	
All genes absent	CTX-M	SHV + OXA	TEM + CTX-M	SHV + TEM	SHV + CTX-M	OXA + CTX-M	TEM + SHV + CTX-M	SHV + OXA + CTX-M
8(22.86)	14(40)	3(8.57)	1(2.86)	1(2.86)	2(5.71)	1(2.86)	2(5.71)	1(2.86)

to identify specific resistance genes such as TEM, SHV, OXA and CTX-M. The CTX-M genes were further analyzed to identify CTX-M-1, CTX-M-2 and CTX-M-9 subgroups. More than half of the resistance genes identified were CTX-M, followed by SHV, OXA and the TEM. The 23 isolates identified to contain CTX-M genes were sub grouped into three by multiplex PCR analysis. These isolates had at least one of the sub groups analyzed. CTX-M-1 was most prevalent 14 (60.87%) followed by 8 (34.78%) for CTX-M-9 and 1 (4.35%) for CTX-M-2. The total prevalence of resistance genes identified in the isolates is 77.14%. CTX-M was only identified in 40% of the isolates, 5 double genes were identified in 28.57% isolates (SHV+ OXA, SHV + TEM, SHV + CTX –M, TEM + CTX-M, OXA+ CTX –M), and three combined genes were identified in 8.57% isolates (TEM + SHV + CTX-M, SHV + OXA + CTX-M). None of the resistant genes was detected in 22.86% of the isolates. Table 5 shows that, at least one of the resistance genes was identified in each of the isolates. However, isolates that had two or three resistance genes mostly recorded CTX-M and SHV as part of the combined resistance genes.

DISCUSSION

ESBL-producing pathogens isolated from urine

Resistance of ESBL-producing *K. pneumonia* and *E. coli* to ciprofloxacin in this study is similar to what was reported by Hyun et al. (2015) who recorded about 80% resistance among ESBL-producing *K. pneumonia* and *E. coli*. The resistance rate of *E. coli* for this study was also similar to those of earlier reported study (Albu et al., 2018). It was also established that, four out of the five tested Cephems/beta-lactam antibiotics were resistant confirming a previous study in Ghana (Agyekum et al., 2016) and in France (Martin et al., 2016). High resistance against fluroquinolones and β-lactam in this present study could be due to excessive use of such antibiotics in the UTI therapy. This is of major concern since this group of antibiotics are frequently prescribed and consumed in our setting (Afriyie et al., 2018). On the other hand, none of the isolates recorded a resistance to Ertepenem which is in agreement with other studies in Ghana indicating no Carbapenem resistance (Agyekum et al., 2016; Hackman et al., 2014). This affirms the fact that Ertepenem and other Carbapenems are not prescribed routinely in Ghana according to the guidelines of the country's standard health treatment even though Carbapenem resistance has recently been recorded in other parts of Africa (Okoche et al., 2015).

Other drugs with low resistance recorded in this study were Fosfomycin, Cefoxitin and Nitrofurantoin which is in conformity to other similar studies (Sana et al., 2011; Hyun et al., 2015). This finding could be associated with less accessibility and high price of these drugs in the local market as suggested by Albu et al. (2018) and Feglo and Adu (2016) when they identified *K. pneumonia* as more resistance than *E. coli,* while a study in Korea reported otherwise (Hyun et al., 2015).

ESBL-producing pathogens isolated from biofilm

The ESBL-producing *K. pneumonia* isolated from biofilm showed resistance to all Nitrofurans, Fosfomycins, Fluoroquinolones, Folate pathway and all but one Cephems which is in accordance with a study by Vuotto et al. (2017) in which ESBL uropathogens were resistant to similar classes of antibiotic. Our results are also supported by a study that suggested that genes responsible for antibiotic resistance correlates with genes involved in biofilm production (Subramanian et al., 2012). In other studies there was 95% tendency of ESBL-producing *E. coli* to form biofilm than for non ESBL-producing *E. coli.* This might explain why *E. coli* showed high resistance to all antibiotics except the Carbapenem (Ertepenem) (Neupane et al., 2016; Subramanian et al., 2012). Biofilm protects bacterial cells against the effect of antibiotica resulting in treatment failure also for new and powerful drugs (Verderosa et al., 2019). Another treatment difficulty may occur with the ability of the bacteria to produce several ESBL enzymes simultaneously.

That the ESBL-producing pathogens resistance is not limited to Cephem/beta-lactam class of antibiotics indicates the ability for cross-resistance to other classes of antibiotics as has been previously observed in some studies in Ghana, limiting the therapeutic option for complicated infections (Feglo and Adu 2016; Hackman et al., 2014). The resistance of ESBL-producing pathogens isolated from biofilm to Ciprofloxacin is consistent with

earlier studies (Subramanian, 2012; Neupane et al., 2016). However, Ertepenem, Cefoxitin and Nitrofurantoin were effective against the biofilm producing pathogens, which is in agreement with earlier findings in Nepal and India (Neupane et al., 2016; Shanmugam et al., 2017). The biofilm isolates were more resistant than the urine isolates. The *K. pneumonia* higher resistance pattern compared to *E. coli* is in agreement with some earlier studies (Albu et al., 2018; Feglo and Adu, 2016) but contrary to the findings of Hyun et al. (2015). Even though Ertepenem was active for both biofilm and urine isolates the results contradicts that of Vuotto et al. (2017).

Molecular detection of ESBL genes

Resistance genes in the Enterobacteriaceae were specifically identified using the multiplex PCR, with r the high prevalence rate of 53.48% to CTX-M. This result is in accordance with a studies in Leicestershire, UK (Reid et al., 2018), Ghana (Agyekum et al., 2016) and Nigeria (Nuhu et al., 2020) but in contrast to a similar study in India (Bajpai et al., 2017) were TEM was the predominate gene i. These notwithstanding, other studies in Ghana and elsewhere outline CTX-M-15 as the dominant genotype in the CTX-M group (Lewis et al., 2007; Agyekum et al., 2016; Eibach et al., 2018; Reid et al., 2018; Hackman et al., 2014). It is worth noting that CTX-M-1 are prevalent in domestic animals like cats and dogs (Ghazanfar et al., 2019) as well as in both local and imported poultry products (Eibach et al., 2018). The CTX-M-1 prevalence found in this study could be attributed to the common lifestyle of many Ghanaian sharing their compound with dogs and cats as food or pet coupled with high consumption of poultry products reported to contain the CTX-M-1 genotype (Kwadzo et al., 2015). About 60% of the isolates had multi-ESBL genotypes with some of their combinations being (CTX +TEM, SHV + TEM and CTX+TEM +SHV) which is consistent with a previous study at the same facility (Feglo and Adu 2016). Of all the seven multi-ESBL genotypes identified, CTX-M and SHV were mostly present in each combination. This gives credence to CTX-M and SHV as key components to antibiotic resistance.

Conclusion

It is clear that CAUTI caused by ESBL-producing *E. coli* and *K. pneumonia* are highly resistant against commonly used antibiotics. Since isolates from the urine also show resistance to many of the antibiotics that were surveyed, this high resistance may partially be credited to the bacteria cells in the biofilm with *K. pneumonia* exhibiting more resistance than the ESBL-producing *E. coli.* Ciprofloxacin and other Cephems commonly used in CAUTI treatment were less effective. However, Ertepenem which recorded no bacteria resistance to the biofilm isolates, could be considered as the best drug of choice for CAUTI treatment. The one resistant genotype identified in about 80% of the ESBL-pathogens was CTX-M-1 and it is the most prevalent gene among the identified genotypes. Ertepenem and other Carbapenems should be considered for treatment of CAUTI since they also eliminate biofilm pathogens.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Water quality index (WQI) and bacteriological assessment of some selected hand-dug wells in the Adenta municipality, Ghana

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Water quality was assessed from 11 hand dug wells in the Adentan Municipality using Water Quality Index (WQI) and bacteriological load as indicators. The sampling was conducted during the months of July to September, 2019. A total of 33 samples were taken from 11 hand dug wells at monthly intervals. Weighted Average Water Quality Index (WAWQI) was used to compute parameters which include temperature, pH, conductivity, total dissolved solids, turbidity, total solids, biological oxygen demand, salinity and total alkalinity. Indicator fecal coliforms were also enumerated. All samples showed presence of *E. coli* and *Salmonella* in the dug wells with AD_2 having the highest *E. coli* count of $1.32x10^3$cfu/100 ml. AD_{11} had the least count of both *E. coli* and *Salmonella* ($1.28x10^2$ cfu/100 ml and $1.68x10^1$ cfu/100 ml) respectively. The highest WQI value was recorded for AD_7 and the lowest was recorded for AD_9. 36.4% of the wells were graded as "A" whilst the remaining 63.6% were graded "B". The combined results of WQI and bacteriological assessment shown that the water quality determined only by WQI (physico-chemical) parameters cannot reflect the true water quality status.

Key words: Water quality index, bacteriology, weighted average water quality index, indicator.

INTRODUCTION

Water for domestic activities should be potable to guarantee good public health using water-quality index and bacteriological assessment as effective tools for assessment allowing for reporting of information of the quality of the water to citizens and policy makers (Atulegwu and Njoku, 2004). Ground water is generally considered 'safe' and requires no treatment due to the natural filtering action (Abila et al., 2012), however, it is susceptible to contamination from sanitary hazards and fecal matter in close proximity to the wells (Adelana and MacDonald, 2008). It is estimated that about 100 million people in rural areas in Sub Saharan Africa rely on ground water for domestic purposes. A report by the Ghana Water Company (GWCL, 2019) indicated that

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20% of urban communities are without access to public water services and depend on other sources like ground water for survival. UN figures on Sustainable Development Goal 6 reveal that millions die yearly from diseases connected to insufficient supply and quality of water.

The rapid increase in water demand has placed a great stress on available water resources through both poor waste management and over exploitation (Ramakrishnaiah et al., 2011). As a result, groundwater quality is deteriorating due to among others disposal of massive industrial effluents and mining activities, as reported for different parts of India (Rodell et al., 2009; MacDonald et al., 2015). The quality of water from shallow aquifers is easily compromised when sited close to septic systems in homes and communities (Lutterodt et al., 2018). This is more prevalent in peri-urban areas of Sub Saharan Africa where septic systems are rife (Lapworth et al., 2017). Improving the water supply remains a challenge and many countries have implemented water quality protection measures and monitoring regimens (Astel et al., 2006; Behmel et al., 2016; Romero 2016) including multivariate statistical methods (Singh et al., 2005), modeling techniques (Huang et al., 2016), and methods based on multi-metric indices (Wu et al., 2012).

The Adenta Municipality in Ghana has had a water crisis for many years as a result of a malfunctioning water treatment plant. In 2014, the Kpone water treatment plant was expanded to a capacity of 40 million gallons per day (GWCL, 2014) allowing the Adenta community to have access to quality water. However, some localities like in New Adenta are not connected to the Ghana Water Company Limited (GWCL). Many households in New Adenta have resorted to hand dug wells as a source of water.

Visual inspection of these wells show sub-standard construction, proximity to contaminant sources like septic tanks, toilets and others, raising doubts about the quality of the water. The World Health organization (WHO, 2004) recommends that, shallow wells and boreholes ought to be situated at minimum distance of 30 and 17 m from latrines and septic tanks respectively (WHO, 2004; Chukwurah, 2001). This is not the case with dug wells of New Adenta closer than the 17 m distance recommended, coupled with the low water table of the dug wells.

Statistics from the Adentan Municipal Health Directorate on Water Borne Diseases show high incidence of Typhoid fever and Diarrheal cases. Frequency of typhoid fever increased from 4793 cases in 2016 to 7147 reported cases in 2018 representing 49.1% increase over a two-year period. Within that same period diarrhoeal cases increased from 2290 to 3176 representing a 38.7% increase. The suitability of water from hand dug wells have not been analyzed for either bacteriological or physicochemical quality together with the water quality index in the Adenta municipality. The aim of this study was to assess the quality of some hand dug wells of the Adentan Municipality using both the water quality index and bacteriological load as a novel way to determine water quality for our municipality.

MATERIALS AND METHODS

Study area

Adentan municipality is located on latitude 5′ 43″N and longitude 0′ 09″W, and is 10 km to the North-East of Accra, Ghana. The total land area of the municipality is 123 km^2 and is bound by Kpong Katamanso to the North, Ledzokuku Krowor and Accra Metro to the South, Tema Metro to the East and La Nkwantanang Madina to the West. The municipality is divided into four sub-districts; Gbentenaa, Koose, Nii Ashale Botwe and Sutsurunaa.

Sampling and analysis

Sampling was based on the sanitary inspections that were conducted during the months of July to September, 2019 from 11 different sampling locations around the Adentan Municipality. The risk assessment was based on five factors: relative position of septic systems (whether downhill or uphill), closeness to septic tanks, protection mechanisms and appropriate receptacles for fetching water. A risk matrix with appropriate colour coding (not shown) was used to rank the wells based on these risk factors. Preferences were given to wells available for communal use and for drinking purposes. A total of 33 samples were taken from 11 hand dug wells (triplicate from each well) at monthly intervals and coded as follows AD_1, AD_2, AD_3, AD_4, AD_5, AD_6, AD_7, AD_8, AD_9, AD_{10}, and AD_{11} (Table 1) and analyzed within 24 h.

All chemicals and reagents were of analytical grade. Samples were collected in 500 ml polythene bottles having double stopper. Prior to the collection, the clean sample bottles were rinsed thoroughly with the sample water to be collected. The physical and chemical parameters analyzed included temperature, pH, TSS, TDS, conductivity, salinity, turbidity, DO, BOD and total alkalinity. Total coliforms, Fecal coliforms, *E. coli* and *Salmonella* present in the samples were enumerated using lactose and lauryl tryptose broths for fecal coliforms and *E. coli*, while pre-enrichment broth selenite cystine was used for *Salmonella*.

Water quality index

The Overall WQI (OWQI) for surface water and groundwater for drinking purposes was developed by Singh et al. (2015) and Stigter et al. (2006). The present study used the Weighted Average Water Quality Index (WAWQI) for ten water quality parameters to compute WQI. The parameters are temperature, pH, conductivity, total dissolved solids, turbidity, total solids, biological oxygen demand, salinity and total alkalinity. The water quality index was calculated using quality rating scale and assigning weight values to the selected parameters (Dinius, 1987). The following weighted average aggregation functions were employed for this purpose: Where W_i = weight of the *i*th variable and Q_i is the quality score rating. These scores are further converted to a common scale based on their relative importance to impact the quality of water. These sub-indices functions are developed based on the water quality standards and their concentrations to meet in particular range. For this purpose, mathematical expressions were fitted for each parameter to obtain the sub-index equations (WAWQI =

Table 1. Sample location and respective GPS coordinate at Adenta used for water sampling.

Well ID	Coordinate	Sampling location
AD_1	5.704-0.182	Well near WASS JHS
AD_2	5.703-0.179	Well opposite New Adenta Royal House Chapel
AD_3	5.708-0.77	Well at Adenta Transformer bus stop
AD_4	5.709-0.177	Well near Rotana
AD_5	5.708-0.182	New Adenta well close to Busy Tots
AD_6	5.709-0.172	Well at container quarters
AD_7	5.707-0.182	Well at BTS station
AD_8	5.710-0.179	Well near Okataban Methodist Church
AD_9	5.709-0177	New Adenta Well near Taxi rank
AD_{10}	5.709-0.178	New Site Well near Medi-moses
AD_{11}	5.705-0.181	Well near Last stop Mosque

Source: Field work (2019).

Table 2. Grading scheme for WAWQI.

Category	Range	Description
I	0 - 25	Excellent (A)
II	26 - 50	Good (B)
III	51 - 75	Poor (C)
IV	76 - 100	Very Poor (D)
V	>100	Unsuitable for drinking (E)

Source: Tyagi et al. (2013); Brown et al. (1970).

$\sum QiWi/\sum Wi$). Based on the status of water quality data, the index value ranges from 0 to 100 and is classified into five categories: excellent (0-25), good (26-50), poor (51-75) very poor (76-100) and unsuitable for drinking (>100). The status of water corresponding to different WQI values is presented in Table 2. If the index is high, it indicates that some of the water quality parameters are beyond permissible ranges and suitable measures are needed to improve the quality of water.

RESULTS

Sanitary inspection and risk assessment

A standardized inspection checklist originally developed by British Geological survey and previously used by Lutterodt et al. (2018) was adapted for risk inspection and assessment. Results of Sanitary inspection and Risk assessment of the 11 dug wells showed that AD_9 and AD_{10} were found to have risk of extreme contaminations, AD_4 and AD_8 moderate contaminations, AD_1 and AD_3 high contaminations and the remaining five wells (AD_2, AD_5, AD_6, AD_7, AD_{11}) had very high contaminations.

Bacteriological analysis

Table 3 shows the mean count and the range of *E. coli* and *Salmonella* count in the dug wells. The highest E. *coli* count was 1.32×10^3 cfu/100 ml in well AD_2 whilst the least count was recorded in AD_{11} with a count of 1.28×10^2 cfu/100 ml. Similarly, the highest and the least count of *Salmonella* were 1.89×10^2 cfu/100 ml and 1.68×10^1 cfu/100 ml for wells AD_1 and AD_{11} respectively. The *E. coli* counts in all the dug wells were consistently higher than the *Salmonella* count. The results indicate non-conformity with the WHO (WHO, 2011) and GSA (GSA, 2009) guidelines of 0 CFU/100 ml.

Physico-chemical analysis

The temperatures recorded for the dug wells were within the range of 28.1±0.6 to 31.7±2.2°C. The maximum temperature was recorded at AD_9 with AD_6 recording the minimum. The pH values were within the range of 6.4±0.5 to 7.1±0.3 with AD_1 recording the maximum. Conductivity and Total Dissolved Solids (TDS) were within the range of 742±247.6 uS/cm to 5529±521.7 uS/cm and 433±53.5 mg/l to 3328.6±116.0 mg/l respectively (Tables 4 to 7). AD_7 and AD_{11} in both cases recorded the maximum and minimum values respectively. The turbidity recorded for the wells were 2.333±0.5NTU to 6.1±0.9 NTU with AD_8 and AD_9 recording the maximum and minimum respectively. The TSS values range between 12.3±3.5 to

Table 3. Geometric mean count and range of *Escherichia coli* and Salmonella in the samples.

Sample	Bacteria Isolate	Geometric mean (cfu/100 ml)	Range (cfu/100 ml)	WHO/GSA (cfu/100 ml)
AD_1	*E. coli*	7.84 x 10^2	3.70 x 10^2 - 1.30 x 10^2	0
	Samonella	1.89 x 10^2	1.10 x 10^2 - 6.15 x 10^2	0
AD_2	*E. coli*	1.32 x 10^3	9.40 x 10^2 - 2.00 x 10^3	0
	Samonella	8.17 x 10^1	5.80 x 10^1 - 9.70 x 10^1	0
AD_3	*E. coli*	5.98 x 10^2	5.00 x 10^2 - 6.80 x 10^2	0
	Samonella	7.54 x1 0^1	4.80 x 10^1 - 9.50 x 10^1	0
AD_4	*E. coli*	3.61 x 10^2	3.10 x 10^2 - 4.00 x 10^2	0
	Samonella	7.19 x 10^1	4.30 x 10^1 - 9.30 x 10^1	0
AD_5	*E. coli*	2.55 x 10^2	9.20 x 10^1 - 4.50 x 10^2	0
	Samonella	2.81 x 10^1	2.30 x 10^1 - 4.20 x 10^1	0
AD_6	*E. coli*	1.00 x 10^2	9.50 x 10^1 - 1.10 x 10^2	0
	Samonella	1.88 x 10^1	1.20 x 10^1 - 3.10 x 10^1	0
AD_7	*E. coli*	6.16 x 10^1	2.60 x 10^1 - 1.10 x 10^2	0
	Samonella	7.51 x 10^1	6.20 x 10^1 - 1.05 x 10^2	0
AD_8	*E. coli*	1.00 x 10^2	9.40 x 10^1 - 1.08 x 10^2	0
	samonella	5.22 x 10^1	3.00 x 10^1 - 9.90 x 10^1	0
AD_9	*E. coli*	3.52 x 10^2	3.10 x 10^2 - 4.01 x 10^2	0
	Samonella	4.92 x 10^1	2.70 x 10^1 - 9.60 x 10^1	0
AD_{10}	*E. coli*	5.06 x 10^2	4.80 x 10^2 - 5.39 x 10^2	0
	Samonella	4.50 x 10^1	2.30 x 10^1 - 9.20 x 10^2	0
AD_{11}	*E. coli*	1.28 x 10^2	9.30 x 10^1 - 2.40 x 10^2	0
	Samonella	1.68 x 10^1	9.00 x 10^1 - 2.30 x 10^1	0

WHO, World Health Organisation; GSA, Ghana Standard Authority.

29.3±11.0. The maximum total suspended solids (TSS) was recorded at well AD_9 whilst the minimum TSS was recorded at the Well at AD_3. The Dissolved Oxygen (DO) recorded for the dug wells were within the range of 3.6±0.2 to 6.6±0.5 with AD_9 and AD_{11} recording maximum and minimum values respectively. AD7 recorded the highest Biological Oxygen Demand (BOD) of 1.0±0.5 (Tables 4 to 7). The salinity recorded for the dug wells were within the range of 1.1±0.2 to 2.7±0.5. The maximum salinity was recorded at AD_1 whilst the minimum salinity was recorded at the Well at AD_3. Total alkalinity recorded for the dug wells were within the range of 25.6±4.1 to 393.3±230.0. The maximum alkalinity was recorded at well at AD_6 whilst the minimum alkalinity was recorded at AD_9.

Water quality index

The computed WQI for the eleven 11 dug wells are summarized in Tables 4 to 7. The WQI values of the wells were in the range from 17.87 to 37.64. With respect to the WAWQI grading, the smaller the WQI value, the better the grading of water. The highest WQI value was recorded at AD_7 whilst the least WQI value was recorded at AD_9. 36.4% of the wells could be graded as "A" whilst the remaining 63.6% falls within the "B" grade (Tables 4 to 7). None of the samples were of poor, very poor or not suitable for drinking grade.

DISCUSSION

The risk ranking and the score assigned to each of the dug wells did not correlate with actual contamination levels in the samples (Oluwasanya, 2013). For instance, wells AD_9 and AD_{10} which were ranked as having potential for extreme contamination had lower *E. coli* count compared to AD_1 and AD_3. This lack of correlation was also observed with *Salmonella* counts and may be

Table 4. Water quality status and WQI values from sampling sites AD_1, AD_2 and AD_3.

Water quality parameter (unit)	Sampling sites								
	AD_1			AD_2			AD_3		
	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean
Temperature (°C)	30.5	28.5	29.43	29.2	28.1	28.57	29.7	28.4	29.17
pH	7.5	6.8	7.17	6.7	6.2	6.5	6.9	6	6.37
EC (µS/cm)	5039	2510	3987	4270	2998	3443	5943	3140	4151
TDS (mg/L)	3300	1517	2681	2360	1942	2172	2931	1810	2313
Turbidity (NTU)	6.2	2.4	4.1	3.8	1.5	2.7	3.4	2.1	2.5
TSS (mg/L)	40	18	29.3	23	11	17.6	16	9	12.3
DO (mg/L)	5.3	3.2	4.3	4.3	3.6	3.9	5.1	3.2	4.1
BOD (mg/L)	2.2	1.2	1.7	2.6	1.4	2.2	2.4	0.9	1.7
Salinity (PSU)	3.1	1.3	1.9	1.4	0.9	1.1	2.7	2.3	2.4
Alkalinity (mg/L)	166	110	135	36	25	30	281	142	200
WAWQI		32.05			29.97			29.26	
WQ status		Good (B)			Good (B)			Good (B)	

WAWQI, Weighted average water quality index; WQ, water quality; EC, electrical conductivity.

Table 5. Water quality status and WQI values from sampling sites AD_4, AD_5 and AD_6.

Water quality parameters (unit)	Sampling sites								
	AD_4			AD_5			AD_6		
	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean
Temperature (°C)	29.2	28.9	29.07	28.8	28.6	28.7	28.7	27.5	28.1
pH	7.2	6.3	6.73	7.3	6.5	6.8	6.8	6.3	6.5
EC (µS/cm)	2760	2115	2399	4684	3241	3904	4486	1153	2336
TDS (mg/L)	1557	1440	1486	2530	2240	2411	2609	750	1406
Turbidity (NTU)	2.9	1.9	2.3	5.7	2.8	4.1	4.9	2.1	3.4
TSS (mg/L)	18	14	16	23	18	20	25	19	21
DO (mg/L)	5.2	4.9	5.1	5.2	4.1	4.6	5.2	3.5	4.4
BOD (mg/L)	1.5	1.2	1.3	2.2	1.4	1.8	2.6	0.7	1.9
Salinity (PSU)	1.5	1.1	1.2	2.4	1.8	2.1	2.2	1.6	1.8
Alkalinity (mg/L)	240	103	153	453	130	264	653	215	393
WAWQI		22.39			30.42			26.08	
WQ status		Excellent (A)			Good (B)			Good (B)	

WAWQI, Weighted average water quality index; WQ, water quality; EC, electrical conductivity.

due to lack of protective systems in the construction of the wells or failure to adhere to standard practices (Nkansah et al., 2010a). Lutterodt et al. (2018) also reported incoherence of risk score to correlate with actual contamination levels in dug well and boreholes at Dodowa in Ghana.

Physico-chemical properties

Many of the dug wells had temperatures slightly higher than the recommended range of 22 to 29 (WHO, 2011). The relatively high temperatures recorded may be due to major ions from water/rock interaction from dissolved amounts of carbonates like calcite or sulphides like pyrite, as both dissolution reactions are highly exothermic (Corbella and Ayora, 2003). Nkansah et al. (2010b) attributes high water temperature to either microbial activity or depth at which water is sampled since worm tends to float in stratified waters.

The positive correlation of temperature with DO (r=0.8), weak positive correlation with Salinity (r=0.3) and a weak negative correlation with electrical conductivity (r=-0.1) may be due to the fact that temperature affects chemical parameters like dissolved oxygen, electrical conductivity and salinity (Chapman, 1996). Yang et al. (2007) reports

Table 6. Water quality status and WQI values from sampling sites AD_7, AD_8 and AD_9.

Water quality parameter (unit)	Sampling sites								
	AD_7			AD_8			AD_9		
	Max.	Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean
Temperature (°C)	29.6	29.4	29.4	29.3	28.6	29	34.3	30.2	31.73
pH	7.4	6.8	7	6.7	6.2	6.4	6.9	6.4	6.6
EC (µS/cm)	6125	5152	5529	3672	2300	2920	1509	923	1179
TDS (mg/L)	3460	3240	3328	2148	1679	1929	982	726	799
Turbidity (NTU)	3.8	2.3	3.1	6.9	5.1	6.1	2.3	1.1	1.7
TSS (mg/L)	27	18	22	46	21	33	23	13	16
DO (mg/L)	5.3	3.9	4.5	4.3	3.2	3.9	7.2	6.2	6.6
BOD (mg/L)	4.6	2.6	3.5	1.8	1.3	1.5	2	1.5	1.8
Salinity (PSU)	3.3	2.2	2.7	1.6	1.3	1.4	2.4	1.4	2
Alkalinity (mg/L)	256	131	193	70	48	55	29	21	25
WAWQI	37.63			33.77			17.87		
WQ status	Good (B)			Good (B)			Excellent (A)		

WAWQI, Weighted average water quality index; WQ, water quality; EC, electrical conductivity.

Table 7. Water quality status and WQI values from sampling sites AD_{10} and AD_{11}.

Water quality parameters (unit)	Sampling sites					
	AD_{10}			AD_{11}		
	Max.	Min.	Mean	Max.	Min.	Mean
Temperature (°C)	29.2	27.7	28.5	28.7	28.5	28.5
pH	7.1	6.4	6.7	6.8	6.4	6.6
EC (µS/cm)	1050	980	1003	975	482	742
TDS (mg/L)	1310	1061	1173	493	390	433
Turbidity (NTU)	1310	1.6	2.4	4.1	2.2	3.2
TSS (mg/L)	3.8	12	16	21	12	15
DO (mg/L)	20	3.3	4.6	3.8	3.4	3.6
BOD (mg/L)	5.4	0.5	1	2.6	0.2	1.6
Salinity (PSU)	1.6	0.9	1.2	2.3	0.9	1.8
Alkalinity (mg/L)	36	30	33	124	43	72
WAWQI	20.86			23.94		
WQ status	Excellent (A)			Excellent (A)		

WAWQI, Weighted average water quality index; WQ, water quality; EC, electrical conductivity.

that climatic conditions and chemical, biological, and microbial processes are effective in determining oxygen variation in water. Except wells AD_3 and AD_8 which were mildly acidic (6.36 and 6.43 respectively), the compliance level of pH was very high (82%). Addo et al. (2016) reported that acidic geology of an area may contribute significantly to the lower pH, below the permissible limit, as observed in the study. Chapman (1996) reported the formation of weak carbonic acid which dissolves carbon dioxide to react with H_2O to release hydrogen ions (H^+) to lower the pH of the water.

There was a 73% non-compliance in terms of the measured conductivity which present a health threat to consumers as a result of the very high amount of dissolved salts in the water samples. The linear relationship between conductivity and TDS of all the samples (r=0.97) may be attributed to the direct proportionality between conductivity and TDS. The turbidity and TSS recorded in this study were all within the recommended range of WHO (2011) for drinking water. Compliance was 91 and 100% for all the analyzed water samples, respectively. This is supported by Amoako et al. (2011) who reported similar values of TSS for ground water samples. All the samples recorded a 0% BOD compliance level as recommended by World Health Organization (WHO, 2011) and thereby rendering them unsafe for consumers without prior treatment. There was a weak negative correlation with BOD (r=-0.03). There

was a 100% salinity compliance rate for all the water samples as the recorded values were below the recommended threshold of less than 200 mg/l. Similarly, the compliance rate of total alkalinity was 100% as they all fell below the WHO recommended value of less than 500 mg/l.

Bacteriological indicators

The results of the bacteriological analysis revealed that all the 11 samples recorded *E. coli* and *Salmonella* spp. counts which exceed the WHO standard limit for drinking water which is 0 cfu/100 ml. This represents 0% compliance and is a public health concern in this municipality. Tekpor et al. (2017) reported similar findings of high levels of bacteriological contamination of dug wells at Atebubu in Ghana whilst Akple et al. (2011) stated that location of dug wells and unsanitary features poses greater influence on contamination levels compared with the WHO standard. This may be due to the sitting of dug wells in proximity to septic systems and unsanitary conditions which might have rendered the wells susceptible to microbial contamination. Cairncross and Cliff (1987) indicated that soakage pits and pit latrines can extend their influence on ground-water quality up to 10 m or more as groundwater flow is either lateral or vertical. Even though the presence of coliforms is a source of worry, Binnie et al. (2002) and Griffith et al. (2003) reported that coliform bacteria are widely found in nature and do not necessarily indicate fecal pollution.

Water quality index

According to the computed WQI, the overall water quality status in the Adentan municipality was "excellent" for AD_4, AD_9, AD_{10} and AD_{11} whilst the rest (63.6%) were of "good" threshold. These results which were within the range of 17.8 to 37.6 indicated that the water quality in the municipality meet the WAWQI standard for drinking water even though *E. coli* and *Salmonella* spp. were found in the water. Sener et al. (2017) and Bordalo et al. (2006) have all reported that even though single-factor methods (for example the major parameter that influence water quality) provide valuable information, multiple parameters can benefit water quality evaluation, and such methods have been increasingly adopted in various studies. Evtimova and Donohue (2016) recently showed that water level is an important parameter regulating the structure and function of natural lake ecosystems. This may be one of the reasons why all the samples recorded either excellent or good water quality status.

Conclusion

Eleven different parameters were used to assess the suitability of hand dug wells for drinking purposes in the Adentan municipality in Ghana. Even though the computed WQI shows that 36.4 and 63.6% of water sample were of excellent or good water category, there was bacterial contamination in all the samples. The combined result of WQI and bacteriological assessment show that, water quality determined only by physico-chemical parameters cannot reflect the true water quality status. Therefore, to improve the water quality in the municipality, local management agencies should pay attention to the bacteriological quality of the water samples.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Metabolomics analysis of carotenoid production from *Proteiniphilum acetatigenes* PSB-W based on LC-MS

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Metabolomics based on LC-MS was used for the analysis of the fermentation of *Proteiniphilum acetatigenes* PSB-W treated by different culture time and different light intensity. 26 putative metabolites were detected in samples treated by different culture time. Most of the components were produced in the metabolic activity in order to participate in the whole life process of bacteria. Compared with samples treated with light intensity, a total of 37 metabolites were detected and analyzed. Only 10 compounds were detected in 1000 Lux group, which is significantly less than 1500 Lux group. The metabolite data of light groups were passed through principal component analysis (PCA) and differential metabolites were screened for each comparison group by using the Volcano Plot model. The differential metabolites were annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and 7 differential metabolites related to the carotenoid synthesis enrichment pathway were screened. The results showed that carotenoids synthesis may occur during the stationary phase of bacterial growth. The precursor of carotenoid synthesis of photosynthetic bacteria is isopentenyl diphosphate (IPP) synthesized by acetyl coenzyme A under the catalysis of HMG CoA reductase. It is preliminarily clarified that it is the accumulation mechanism of bacterial carotenoid biosynthesis and provides a new clue for the comprehensive study of the synthesis and regulation mechanism of carotenoids.

Key words: Carotenoids, metabolomics analysis, *Proteiniphilum acetatigenes* PSB-W.

INTRODUCTION

Photosynthetic bacteria (PSB) are a group of phototrophic anoxygenic prokaryotic organisms that are distributed widely in nature. PSB contains rich pigments such as bacteriochlorophyll and carotenoids (Oudin et al., 2007). The remarkable physiological functions of carotenoids include the following: they act as antioxidants to quench singlet oxygen and capture oxygen free radicals, are nutritional supplements, food pigments and feed colorants in industry (Hirschberg, 2001). Most commercial carotenoids are obtained by chemical synthesis, but the growing demand for natural additives has spurred several metabolic engineering approaches to increase carotenoids production in plants and microorganisms (Carrera et al., 2007). About 80 different carotenoids are synthesized by photosynthetic bacteria. All the carotenoids in photosynthetic bacteria are bound

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to light-harvesting or reaction center complexes (Zhou et al., 2015). As early as 1970s, the chemical structure of carotenoids was elucidated, and their biosynthetic pathways were researched demonstratively and clearly (Viveiros et al., 2000). Structurally carotenoids are tetraterpenes derived from the 5-carbon unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (Eisenreich et al., 2001). There are two pathways for the synthesis of these carotenoids: the mevalonic acid (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway. The MEP pathway uses glyceraldehy 3-phosphate and pyruvate as initial substrates (Sandmann, 1994). In recent years, people have been devoted to the cloning and analysis of genes related to carotenoid biosynthesis pathway in microorganisms and plants; in particular, huge progress has been made in modifying plant carotenoid biosynthesis pathway through genetic engineering. Although some intermediates, mechanisms, enzymes and genes of this pathway have been identified, many details of this widely distributed pathway have not been identified (Viveiros et al., 2000).

The synthesis of carotenoids in PSB is a highly coordinated biological process. Its metabolic pathways are complex, and can easily be interrupted by other metabolites. The external environmental factors such as the intensity of light and temperature also greatly affect the accumulation of pigments (Guan et al., 2015). Therefore, the study of bacterial carotenoid biosynthetic pathways is very important.

Metabonomics provides a comprehensive description of all metabolite levels in organisms and has been increasingly used to characterize microbial characteristics, such as cell stress response, high yield mechanisms, and microbial complex interactions. The differences in the fermentation processes of microorganisms at the metabolic level can provide unique insights into the study of metabolic pathways (Okuda et al., 2008). Large differences in the type and quantity of metabolites are often key limiting factors for microbial fermentation. However, there are very few reports on carotenoids synthesized by bacteria around the world. In this study, metabolomics based on LC-MS technology was used to detect the metabolites of the fermentation of PSB-W under different fermentation times and different light intensity treatments. Through comparative metabolomics analysis, the key metabolites for carotenoid synthesis were found, and the carotenoid synthesis mechanism was initially established in combination with the KEGG database, providing a basis for further research on carotenoid metabolism engineering.

MATERIALS AND METHODS

Bacterial strain and growth conditions

A photosynthetic bacterium *P. acetatigenes* PSB-W was isolated from a water purifying agent stored in the Microbiology Laboratory of Shanxi Normal University. The PSB-W cells were cultured on a basic medium consisting of (in g/L) NH_4Cl (1.5), K_2HPO (0.5), $NaHCO_3$ (2.0) and yeast extract (3.5). The initial pH value was adjusted to 6.8-7.2. Cells were activated under the condition of incubation at 30°C and 60-W tungsten lamp illumination of 1500 Lux for 4 days.

Sample preparation

Duration of fermentation

The activated strain from above was transferred to another basic medium for fermentation to accomplish a concentration of 10% inoculum; it was cultured in light-anaerobic conditions at different times. Fermentation broth was taken at the 48, 60, 72, 96 h as the analysis and experimental objects. The light intensity was kept at 1500 Lux.

Light intensity

The activated strain was transferred to the fermentation medium (10% inoculum amount). It was placed in incubators with light intensities of 1000 Lux and 1500 Lux respectively for anaerobic culture for 4 days to obtain the fermentation liquid as samples. There were three replications at each light intensity.

Metabolites extraction

Samples were thawed at 4°C on ice and then extracted with extraction solvent (methanol: acetonitrile, 1:1, V:V), followed by vortexing for 30 s, ultrasound treatment for 5min, and incubated for 1h at -20℃ to precipitate proteins. These were centrifuged at 12000 rpm for 15 min at 4°C; then the supernatant was collected and the pellet was discarded. The supernatant was dried in a vacuum concentrator without heating, and solvent extraction (acetonitrile: water, 1:1, V:V) reconstitution was added. It was vortexed for 30 s and sonicated for 10 min, and centrifuged for 15 min at 12000 rpm. The supernatant was transferred for the UHPLC-QTOF-MS analysis.

LC-MS/MS analysis

LC-MS/MS analyses were performed using an UHPLC system (1290, Agilent Technologies) with a UPLC BEH Amide column (1.7 μm 2.1*100 mm, Waters) coupled to Triple TOF 6600 (Q-TOF, AB Sciex). The mobile phase consisted of 25 mM NH_4Ac and 25 mM NH_4OH in water (A) and acetonitrile (B) was carried with elution gradient delivered at 0.5 mL min^{-1}. The injection volumes were 1 μL. The Triple TOF mass spectrometer was used for its ability to acquire MS/MS spectra on an information-dependent basis (IDA) during an LC/MS experiment. In this mode, the acquisition software was Analyst TF 1.7, AB Sciex, collision energy (CE) 30 V, ion source gas as 60 Psi, source temperature 600°C, Ion Spray Voltage Floating (ISVF) 5000 V or -4000 V in positive or negative modes respectively.

Data processing and statistical analysis

MS raw data (.wiff) files were converted to the mzXML format using ProteoWizard, and processed by R package XCMS (version 3.2). R package CAMERA was used for peak annotation after XCMS data processing. In-house MS2 database was applied in metabolite

Table 1. Metabolite composition of fermentation broth at different culture time.

Number	Compound name	Molecular formula	Relative value				
			0 h	48 h	60 h	72 h	96 h
1	2,3-dimethylcyclopent-2-enone	$C_7H_{10}O$	√	√	√	↓	↓
2	Tridecane	$C_{13}H_{28}$	√	√	√	√	√
3	Disulfide-bis(1-methylpropyl)	$C_8H_{18}S_2$	√	√	√	√	√
4	Methyl undecanoate	$C_{13}H_{26}O_2$	√	√	√	√	√
5	1,1-dibutoxybutane	$C_{12}H_{26}O_2$	√	√	√	√	√
6	2-ethylhexylfluorenyl fumarate	$C_{21}H_{38}O_4$	√	√	√	↓	↓
7	L-glutamic acid	$C_5H_9NO_4$	-	↑	↑	↑	-
8	Aspartic acid	$C_4H_7NO_4$	-	↑	↑	↑	-
9	Imidazol-4-one-5-propionic acid	$C_6H_8N_2O_3$	-	↑	↑	-	-
10	2-hydroxyacetone phosphate	$C_3H_6PO_3$	-	↑	↑	-	-
11	N-tetracosane	$C_{24}H_{50}$	-	↑	↑	-	-
12	Alanine	$C_3H_7NO_2$	-	↑	↑	-	-
13	Proline	$C_5H_{11}NO_2$	-	↑	↑	√	√
14	Butenedioic acid	$C_3H_4O_4$	-	-	-	↑	-
15	D-ribose	$C_5H_{10}O_5$	-	-	↑	↑	-
16	Acetic acid	$C_3H_4O_2$	-	-	↑	-	-
17	Diisobutyl adipate	$C_{14}H_{26}O_4$	-	-	↑	↑	√
18	N-icosane	$C_{20}H_{42}$	-	-	-	↑	-
19	3,5-dimethoxyphenol	$C_{18}H_{38}$	-	-	-	↑	-
20	Nineteen	$C_{19}H_{38}$	-	-	-	↑	-
21	Pentadecanoic acid	$C_{15}H_{30}O_2$	-	-	↑	↑	-
22	1-heptanol	$C_7H_{16}O$	-	-	-	↑	↑
23	Octadecyl 2-ethylhexanoate	$C_{26}H_{52}O_2$	-	-	-	-	↑
24	3-methylcyclopent-2-enone	C_6H_8O	-	-	-	-	↑
25	2-(2-butoxy-2-carbonylethyl)-2-hydroxysuccinic acid	$C_{18}H_{32}O_7$	-	-	-	-	↑
26	(3R,4aR,5S)-4a,5-dimethyl-3-(propan-1--2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene Octadecyl 2-ethylhexanoate	$C_{15}H_{24}$	-	-	-	-	↑

"√" means detectable, "-"means undetected, "↑" means up-regulated and "↓"means down-regulated.

identification. Multivariate statistical analysis was performed with principal component analysis (PCA) to distinguish the samples according to metabolic similarity and with the Volcano Plot model to screen the main differential metabolites related to carotenoid metabolism pathway with respect to the classification. These were combined with KEGG database to classify the metabolism pathways of the carotenoids.

RESULTS

Metabolomics data analysis of fermentation broth at varying culture durations

Fermentation liquid composition analysis

Different groups of fermentation broth samples incubated for varying durations (0, 48, 60, 72, and 96 h) were analyzed by LC-MS. The metabolite composition of fermentation broth at different culture time is shown in Table 1.

In total, 26 putative metabolites were detected, mainly sugars, amino acids, and organic acids. But alkanes and ketones were detected in the control group (0 h). In the fermentation broth of 48 h group, L-glutamic acid, aspartic acid, alanine and other amino acids and organic acids were mainly detected. Amino acids are important for cell growth and protein synthesis in microorganisms. It is speculated that this stage is the delayed period of bacterial growth which synthesizes a large number of substances related to life activities for adapting to the environment (Luo et al., 2015). Among them, glutamic acid is the synthesis precursor of purine, pyrimidine and base, and participates in many metabolic pathways as the provider of amino group in organisms. Alanine is made from glutamate by reverse transamination and can be converted back to glutamate by alanine transaminase

(Guan et al., 2015). Glutamate decreases at 96 h, which may also be one of the reasons for the decreased alanine. Midazole-4-keto-5 propionic acid is an intermediate product of histidine metabolism, which can synthesize D-ribose through the HMP pathway in the process of histidine biosynthesis. During the process of bacterial synthesis of carotenoids through this pathway acetyl-CoA is formed and then further forms IPP. Subsequently, the presence of d-ribose was also detected in the 60 h group samples (Luo et al., 2015). After 60 h of culture, other new additions to the fermentation broth are acetic acid and diisobutyladipate. Acetic acid is one of the main byproducts of lactic acid fermentation. When the fermentation time increased to 72 h, secondary metabolites such as phenols and alcohols were detected in the fermentation broth, while the content of amino acids decreased. It is speculated that the bacteria enter a stable period and the demand for amino acids is reduced for the synthesis of bacteria. When the fermentation time reached 96 h the variety of metabolites detected were significantly reduced, and complex chemical components of cyclic hydrocarbons were detected. This indicates that with the extension of the culture time, the consumption of nutrients in the fermentation medium increased, and the growth force was gradually weakened (Wilson et al., 2005).

In the early stage of bacterial fermentation, the synthesis of components related to self-growth is to adapt to the environment for primary metabolism. After entering the rapid growth stage, a large number of bacteria propagated and began to synthesize carotenoids, resulting in the rapid increase of pigment content. In the later stage, due to the depletion of nutrients in the culture medium, the accumulation of acids, alcohols and other components that are not conducive to the growth of bacteria in the fermentation broth slowed down the metabolism (Santos and Meireles, 2010). This result is consistent with the phenomenon that occurs during the fermentation process resulting in the change of fermentation liquid from light red to red until dark red. Some components may be produced in the metabolic activity and then be decomposed and utilized to participate in the whole life process of bacteria.

Principal component analysis

To establish the differences in the metabolites of the photosynthetic bacterial fermentation broth, metabolomics analysis was carried out. The data were processed with MZmine 2 software; multivariate statistical analysis was performed with PCA to discriminate the fermentation process of the strain through score plot (Santos and Meireles, 2010). It can be seen from Figure 1 that the contribution rate of principal component 1 (PC1) is 37%, and the contribution rate of principal component 2 (PC2) is 16%. This shows that the two principal components can represent 53% of metabolite variable information. The samples fermented for 48 and 60 h came together in a heap, and are in one category. The 72 and 96 h samples were grouped into a second category that is far apart from the first. It shows that the fermentation time has a great influence on the metabolites, and the changes of the metabolites have changed in stages with the change of time. The samples of the same group are tightly clustered together, indicating that the differences between the samples are small.

Metabonomics data analysis of fermentation broth at different light intensities

Fermentation liquid composition analysis

To assess the effect of light intensity on the fermentation broth, metabolomics analysis was performed. The bacteria were cultured for 4 days at light intensities of 1000 Lux and 1500 Lux respectively, which are relatively suitable for the growth of photosynthetic bacteria. LC-MS detection on the fermentation broth was done. A total of 37 metabolites were detected and analyzed, including sugars, amino acids and organic acids (Table 2). Only 10 compounds were detected in the 1000 Lux group, significantly less than 1500 Lux group in which 37 compounds were detected. This indicates that photosynthetic bacteria are more sensitive to light (Okuda et al., 2008). At low light intensities, carotenoid and other pigments capture insufficient light energy and cause bacterial metabolism to slow down and the synthesis of substances related to growth and reproduction is reduced. Compared with 1000 Lux group, amino acid components detected in the fermentation broth of 1500 Lux group were significantly increased, including proline, l-proline, l-leucine, o-acetyl-l-serine, valine, amongst others. It has been reported that amino acids play an important role not only in cell signaling, but also in regulating the phosphorylation cascade of gene expression proteins. In addition, these amino acid precursors are mostly intermediate metabolites of the TCA cycle and EMP pathway, so the increase of these intermediates may also cause the growth of carotenoids content (Sun et al., 2010). Proline may protect the bacteria by stabilizing the spatial structure of the enzyme, and also has the structure and function of stabilizing collagen, maintaining cell osmotic pressure and increasing salt and drought resistance. L-leucine is catalyzed to form α-ketoisocaproic acid by transaminase, and then is decarboxylated to form acetyl-CoA to participate in the citric acid cycle. Leucine is more efficient than other amino acids, has faster transamination rate and the ability to completely oxidize to produce adenosine triphosphate (Luo et al., 2015). In the 1500 Lux group, intermediate metabolites of some

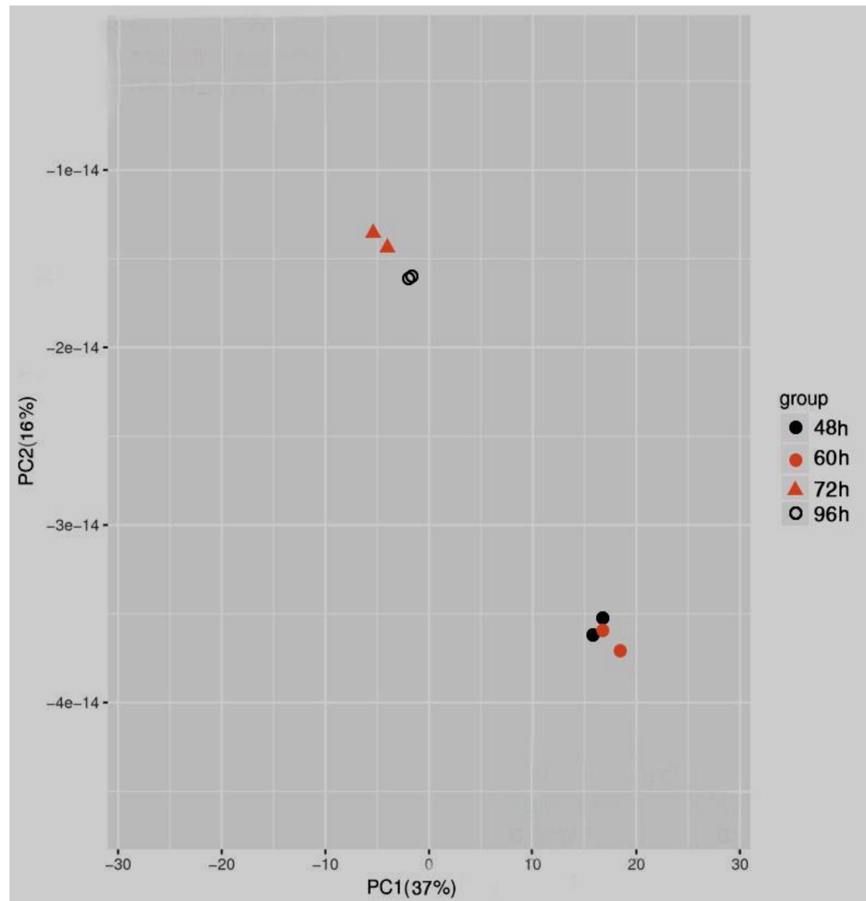

Figure 1. The principal component analysis of metabolites at different fermentation times.

purine nucleotides such as hypoxanthine, deoxyadenine nucleoside, adenine, and xanthine were detected. Some of them can participate in the tricarboxylic acid cycle as the substrate of the tricarboxylic acid cycle, and quickly provide energy through the tricarboxylic acid cycle oxidation; another part of the intermediate products can be used as allosteric effectors of related enzymes in the process of energy metabolism, which can affect other metabolic pathways by regulating enzyme activity. High content of mannitol was also detected in 1500 Lux. Mannitol is a kind of photosynthetic assimilate, which can generate mannitol-1-phosphate under the catalysis of mannitol kinase and mannitol-1-phosphate dehydrogenase, and then generate fructose-6-phosphate to enter the glycolysis cycle and participate in energy metabolism. The results showed that compared with the 1000 Lux treatment group, the metabolites in the fermentation broth cultured under the light intensity of 1500 Lux were significantly increased. The results showed that the effect of light intensity on photosynthetic bacteria was extremely large, and the increase of light intensity could promote the rapid synthesis of cell energy materials by bacteria. Many metabolites in fermentation broth participated in the synthesis of carotenoid precursor IPP (Luo et al., 2015).

Principal component analysis

The metabolites were obtained from LC-MS detection of fermentation broth with different light intensity by PCA analysis. The results of the principal component score diagram are shown in Figure 1. In the score plot, three parallels for each sample clustered together, indicating that metabolomics was appropriate for monitoring the fermentation processes and interpreting the distinctive metabolic characteristics of the samples. The samples collected at different light intensity during fermentation can be clearly distinguished. This shows that the metabolites screened by score plot can be used as potential biomarkers (Steinmann and Ganzera, 2011). It can be seen from Figure 2 that the metabolites detected in the fermentation broth under two different light intensity treatments are extremely different. The samples of different light intensity groups are completely separated in the figure; instead, they are grouped together, indicating

Table 2. Metabolite composition of fermentation broth at different light intensities.

Number	Compound name	Molecular formula	Relative value	
			1000 Lux	1500 Lux
1	3-Hydroxybutyrate	$C_7H_{11}NO_3$	-	↑
2	Hypoxanthine	$C_5H_4N_4O$	-	↑
3	Deoxyadenosine	$C_{17}H_{19}N_5O_5$	√	↑
4	Deoxycytidine	$C_{10}H_{15}N_3O_4$	-	↑
5	L-Methionine	$C_5H_{11}NO_2S$	-	↑
6	5-Hydroxy-L-lysine	$C_{12}H_{25}N_3O_6$	-	↑
7	Proline	$C_{10}H_{17}NO_5$	-	↑
8	L-proline	$C_5H_9NO_2$	-	↑
9	N-methylhistamine	$C_4H_6N_2$	-	↑
10	Triethanolamine	$C_6H_{15}NO_3$	-	↑
11	Lecithin	$C_{42}H_{80}NO_8P$	-	↑
12	D-ribose	$C_5H_{10}O_5$	√	↑
13	Xylitol	$C_5H_{12}O_5$	-	↑
14	Tetradecanoic	$C_{14}Hf_{27}O_2$	-	↑
15	hexadecanoic acid	$C_{16}H_{32}O_2$	-	↑
16	Glycerol-3P	$C_3H_9O_6P$	-	↑
17	Hexadecanoate	$C_{16}H_{38}O_2$	-	↑
18	Glutamine	$C_{32}H_{31}N_3O_8S$	-	↑
19	Adenine	$C_5H_5N_5$	-	↓
20	Xanthine	$C_5H_4N_4O_2$	√	↓
21	L-glutamine	$C_5H_{10}N_2O_3$	-	↑
22	Pseudouridine	$C_9H_{12}N_2O_6$	-	↓
23	Dihydrouracil	$C_8H_7FN_2O$	-	↑
24	Malonate	$C_{11}H_{20}O_4$	√	↓
25	Cytidine	$C_9H_{13}N_3O_5$	-	↑
26	Thymidine	$C_{10}H_{14}N_2O_5$	-	↑
27	N-acetyl-l-aspartate	$C_6H_9NO_5$	√	↑
28	L-leucine	$C_6H_{13}NO_2$	-	↑
29	O-acetyl-l-serine	$C_5H_9NO_4$	√	↑
30	D-Mannitol	$C_6H_{14}O_6$	-	↑
31	Valine	$C_5H_{11}NO_2$	-	↑
32	Glycerol	$C_3H_8O_3$	-	↑
33	Sn-glycero-3-phosphocholine	$C_{36}H_{72}O_8NP_7$	√	↑
34	Butanoic zcid	C_4H_7N	√	↓
35	UDP-N-acetyl-alpha-Dglucosamine	$C_{17}H_{27}O_{17}N_3P_2$	√	↓
36	Choline	$C_5H_{14}NO$	-	↓
37	2-Deoxy-D-ribose	$C_5H_{10}O_4$	√	↓

"√" means detectable, "-"means undetected, "↑" means up-regulated and "↓"means down-regulated.

that the metabolic differences between the treatment groups are significant.

Differential metabolite screening, functional annotation and enrichment analysis

Differential metabolites were screened for each comparison group by combining the fold change and variable importance in project (VIP) values of the Volcano Plot model. The criteria for screening included the fold change value of ≥ 2 and the VIP value of ≥ 1. The screening results are shown in Figure 3. Concisely, there were 409 significant metabolites between 1000 Lux and 1500 Lux (273 down-regulated, 136 up-regulated).

The differential metabolites were annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Figure 4). The KEGG classification results

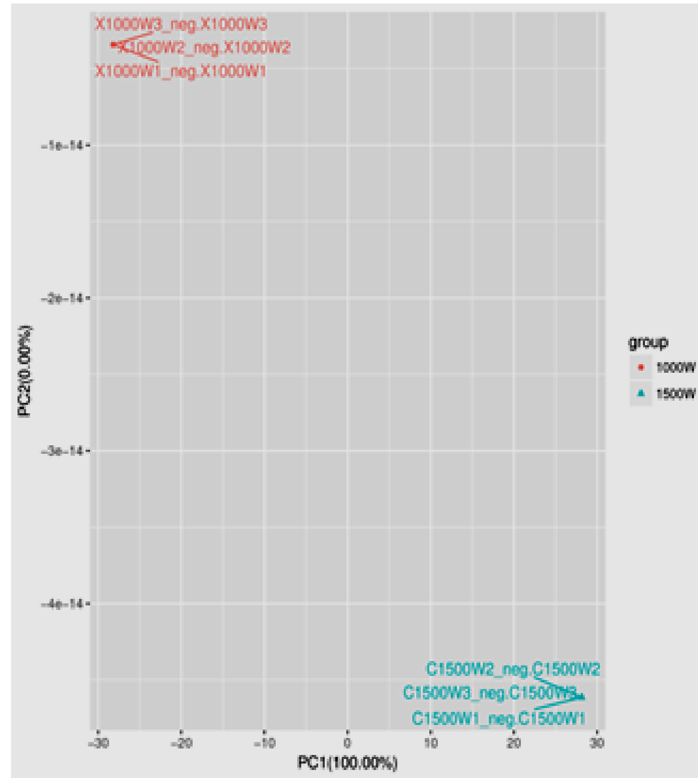

Figure 2. The principal component analysis of metabolites at different light intensities.

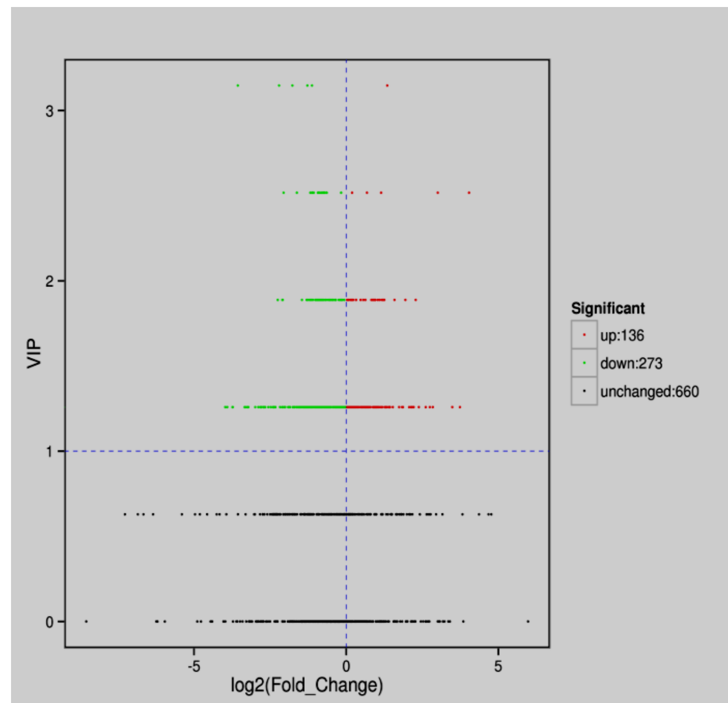

Figure 3. Volcano Plot of differential metabolites. Each spot was represented by a metabolite. The unchanged, up-regulated and down-regulated metabolites were expressed with different shade colors of black, red and blue, respectively.

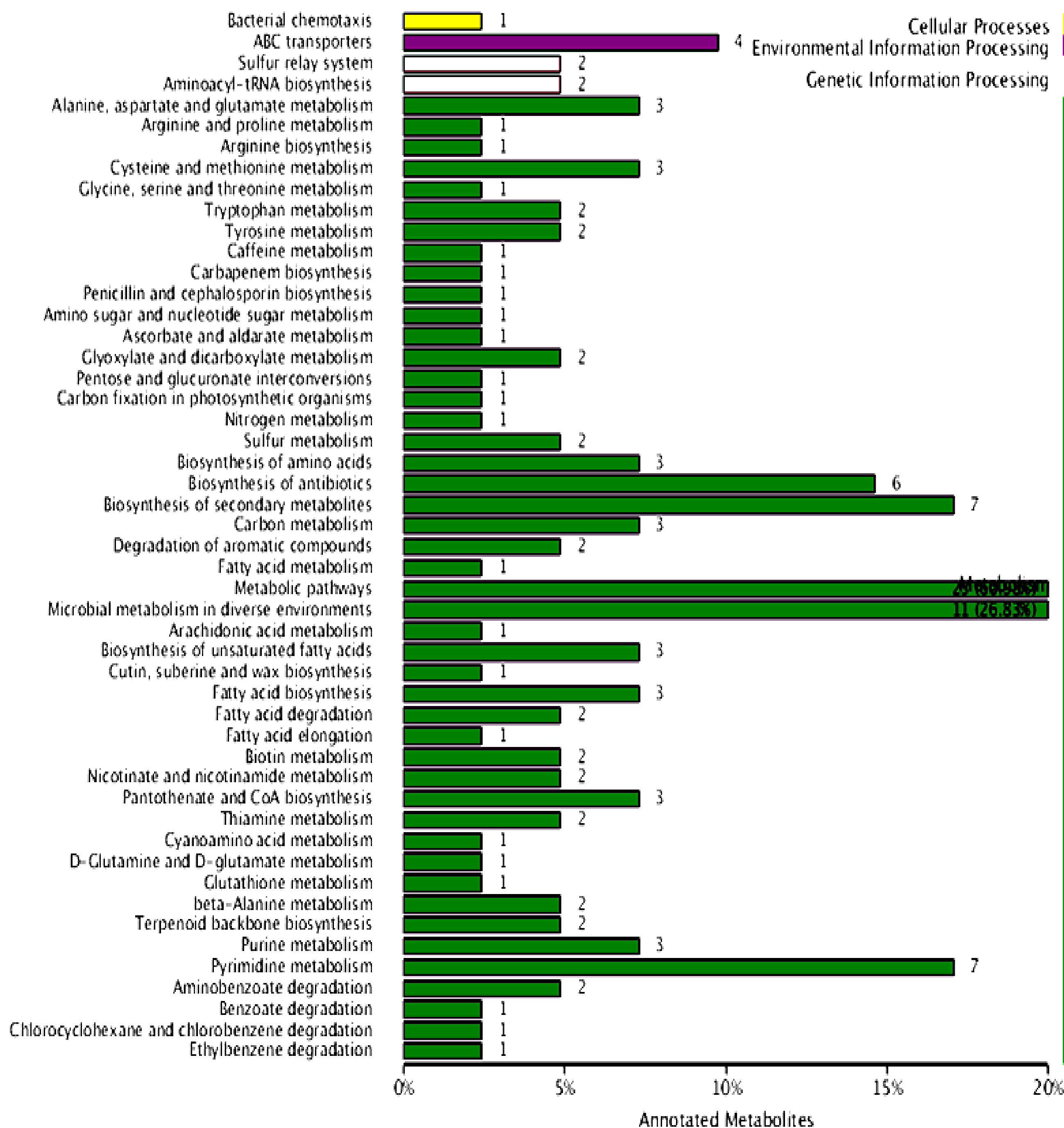

Figure 4. The differential metabolites KEGG classification of the comparison group 1000 Lux vs 1500 Lux.

indicated that the differential metabolites of comparison groups were involved in microbial metabolism in diverse environments, biosynthesis of secondary metabolites, pyrimidine metabolism, terpenoid backbone biosynthesis, carbon metabolism, biosynthesis of amino acids, pantothenate and CoA biosynthesis and other metabolic pathways. From among them, 7 differential metabolites related to the carotenoid synthesis enrichment pathway were screened (Table 3).

Pathway analysis

In order to unravel the relationships of these metabolites and their effects on the metabolic network, a possible synthetic pathway was identified for the selected differential metabolites in combination with the KEGG pathway database (Steinmann and Ganzera, 2011). The pathway model of carotenoids biosynthesis is depicted in Figure 5.

Table 3. The results of differential metabolite and metabolic enrichment pathway.

S/N	Name	Metabolic pathway
1	3-Hydroxybutyrate	Synthesis of ketone bodies
2	5-Hydroxy-L-lysine	Lysine degration
3	Glycerol-3P	Glycerophospholipid metabolism
4	hexadecanoate	Fatty acid biosynthesis
5	Tetradecanoic acid	Fatty acid biosynthesis
6	Sn-glycero-3-phosphocholine	Glycerophospholipid metabolism
7	D-Ribose	Pentose phosphate pathway

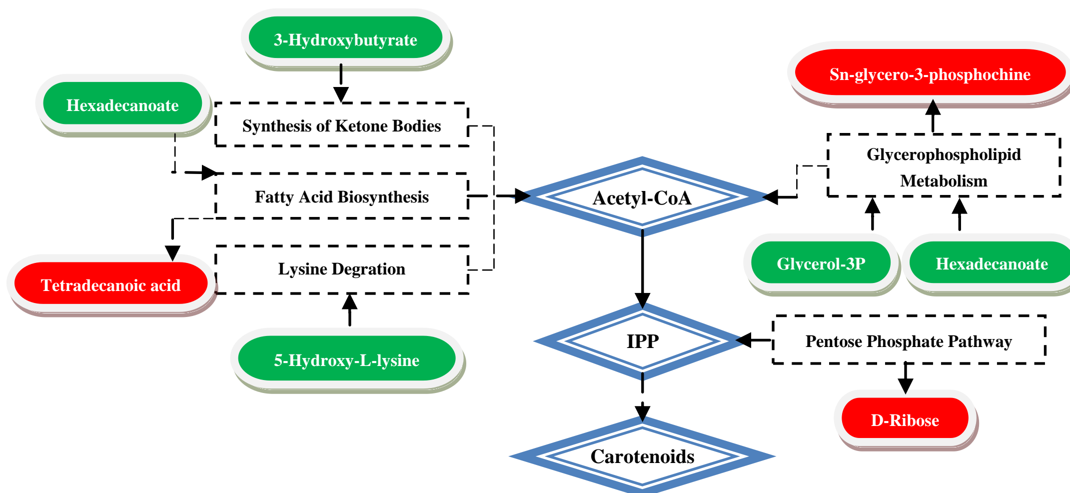

Figure 5. The pathway model of carotenoids biosynthesis. Green substances are up-regulated metabolites and red ones are down-regulated metabolites.

In Figure 5, 3-hydroxybutyrate is an up-regulated metabolite and an intermediate product in the synthesis of acetoacetate. It can be used as an intermediate product in the synthesis of acetoacetic acid and participate in the synthesis of IPP. 5-hydroxy-L-lysine can be degraded by lysine to form acetyl coenzyme A. D-ribose is related to the synthesis of the pentose phosphate pathway, bacteria produce glyceraldehyde 3-phosphate and phosphate ribose to convert to IPP through this pathway (Luo et al., 2015). It also produces a variety of important carbon-containing compounds (four-carbon, five-carbon) and multiple invertases. Sn-glycer-3-phosphocholine is a precursor substance that forms glycerol 3-phosphate and Glycerin-3-phosphate is the premise of glycerin formation. They are related to the pathway of glycerophosphatidic acid metabolism, which forms acetyl coenzyme A involved in pigment biosynthesis. Glycerol can regulate intracellular osmotic pressure as an activator of high osmotic pressure glycerol pathway. At the same time, glycerin-3-phosphate also comes from the EMP pathway, and the enhancement of the EMP pathway will also affect the levels of these two metabolites (Steinmann and Ganzera, 2011). In addition, 8 pyruvate molecules, 8 glyceraldehyde 3-phosphate molecules, 8 CTP molecules, 8 ATP molecules, and 16 NADPH molecules can synthesize a single molecule of lycopene. Tetradecanoic acid and hexadecanoate, these fatty acids undergo significant changes in 1500 Lux. This indicates that the biosynthesis of fatty acids is susceptible to environmental influences. In bacteria, the fatty acids in the peroxisome undergo b-oxidation; the resulting acetyl-CoA is converted into acetylcarnitine, and then enter the mitochondria for tricarboxylic acid (TCA) metabolism. The up-regulation of the intermediate products of the TCA cycle increased the metabolic flow of carotenoid synthesis; It is speculated that the intermediate metabolites of TCA are the carbon skeleton necessary for carotenoid synthesis. As carotenoids are intracellular metabolites, the accumulation of carotenoids increases with the bacteria growth.

DISCUSSION

In the present study, the key metabolic regulation nodes

of carotenoid synthesis in PSB-W were identified through comparative metabolomics analysis of fermentation broth subjected to different fermentation times and light intensities. As an omics technology with the widest scope of application, metabolomics is unbiased to different samples and provides a systematic depiction of microbes at the integrated level. Several techniques have been developed for metabolomics analysis, such as capillary electrophoresis–mass spectrometry (CE–MS), nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry (GC–MS), and LC–MS. LC–MS is a powerful alternative detection tool for metabolomics with the advantages of high separation efficiency, a simple pretreatment process, and high resolution (Steinmann and Ganzera, 2011). The results showed that the technique was mature and the metabonomics method was a powerful way of potential biomarker discovery (Helen et al., 2008). In this paper, we focused on the changes of PSB metabolites under different culture time and light intensity. Through the statistical analysis of samples, it was found that the synthesis of metabolites was staged, and the change of growth conditions would affect the normal synthesis and decomposition of metabolites. Based on the results of metabolic pathway enrichment and previous research, it is speculated that the synthesis of photosynthetic bacterial carotenoids uses acetyl-CoA as a precursor substance, which is catalyzed by HMG-CoA reductase to synthesize IPP and participate in the synthesis of carotenoids (Helen et al., 2008). The data acquired in this study will facilitate future functional studies of genes/pathways associated with metabolic reactions during the photosynthetic bacterial carotenoid synthesis pathway. The metabolism of microorganisms is a complex process, and deeper and more detailed research is needed in the future on the bacterial carotenoid synthesis pathway.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antibiotic susceptibility patterns of bacteria isolates from post-operative wound infections among patients attending Mama Lucy Kibaki Hospital, Kenya

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Surgical site infections account for high mortality, morbidity, and elevated costs of treatment for surgical patients. The study sought to determine the prevalence and antibiotic susceptibility patterns of bacterial isolates from postoperative wound infections among patients attending Mama Lucy Kibaki Hospital. A cross-sectional descriptive study was carried out between October 2018 and March 2019. It included patients of all age groups with surgical site infections following general, obstetrics, and gynecological surgeries. Pus swabs were obtained aseptically from 58 consented patients with clinical evidence of surgical site infections. Gram stain, culture, biochemical tests, and antibiotic susceptibility tests were done for each pus swab. The preponderant isolate was *Staphylococcus aureus* (28.2%) followed by *Escherichia coli* (15.4%). Whereas *Methicillin-resistant S. aureus* accounted for 65.4% (n=17) of the total *Staphylococcus* species. Chloramphenicol was the most sensitive drug to all the bacteria isolates. Ampicillin and amoxycillin recorded resistance rates >90% against gram-positive and gram-negative bacteria. The majority of the gram-negative rods were highly resistant. Hence, this calls for continuous monitoring of the susceptibility patterns to determine the profile of surgical site infections bacteria isolates found in the hospitals.

Key words: Surgical site infection, antibiotic susceptibility, bacteria prevalence, Mama Lucy Kibaki Hospital.

INTRODUCTION

Surgical site infections (SSI) are a worldwide problem in surgery accounting for increased deaths, morbidity, and elevated healthcare costs in surgical patients (Badia et al., 2017; Gelhorn et al., 2018). The rates of these infections worldwide vary, with most studies observing incidence rates of between 2.6 and 58% (Rosenthal et al., 2013; Apanga et al., 2014; Kaur et al., 2017). A previous report has placed the SSI incidence rate in Kenya at 37.7% with *Staphylococcus aureus* as the main isolate (Opanga et al., 2017; Okello et al., 2018). The

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pathogens however vary between settings while exhibiting variant antibiotic susceptibility pattern. For instance *S. aureus, Pseudomonas aeruginosa* (39.13%), *Escherichia coli* and *Klebsiella pneumoniae* (25.5%) are seen as the leading cause of surgical site infection in different regions of the world (Banashankari et al., 2014; Patel et al., 2019; Nwankwo et al., 2014; Billoro et al., 2019).

An increasing proportion of these infections are due to multi-drug resistant bacteria and this has complicated the treatment of SSI (Manyahi et al., 2014). This resistance is attributed to the irrational use of antimicrobial agents which has exerted selective pressure on bacteria leading to the emergence of resistant bacterial strains (Oz et al., 2014). Moreover, the decline in antibiotic development pipeline has further complicated the treatment of SSI (Nicholson, 2019). The successful treatment of these infections depends on the correct identification of etiological agents and their antibiogram profiles. However, there is a paucity of clinical microbiological data especially in developing countries that have hampered the management of these infections (Allegranzi et al., 2011).

In Kenya and the current setting, the situation is the same concerning the paucity of this essential data. Hence, this study was done to determine the prevalence and antibiotic susceptibility profiles of bacteria isolates in post-operative wound infections among patients attending Mama Lucy Kibaki Hospital. The results are intended to help clinicians when formulating a comprehensive treatment protocol for SSIs.

MATERIALS AND METHODS

Study design and setting

This was a cross-sectional descriptive study carried out at Mama Lucy Kibaki Hospital for a period of 6 months, between October 2018 and March 2019. Mama Lucy Kibaki Hospital is a level 5 public health facility located in the eastern part of Nairobi city, Kenya.

Study population

These were surgical patients with surgical site infections. These patients were drawn from the surgical wards, maternity wards and pediatric ward.

Inclusion and exclusion criteria

The study included patients of all age groups with surgical site infection following general, obstetrics and gynecological surgeries. Those patients who did not consent were excluded.

Sampling

Patients with surgical site infection were identified by surgeons during routine ward rounds and the clinical information documented in the patient file. The same information was relayed to the principal investigator through the clinicians. The identified patients were taken through the research process and informed consents obtained. A serialized structured questionnaire was used to collect additional patient clinical data.

Sample collection

The pus swabs were aseptically obtained from 58 patients with clinical evidence of surgical site infection (drainage from an incision) within the research period. After the wound immediate surface exudates and contaminants were cleansed off with sterile moistened gauze and normal saline solution. The pus was collected using a moistened sterile cotton swab from the deep viable tissues of the wound by the Levine method (Cooper, 2010). The collected swab was then placed in a tube with Stuart transport medium and the replaced cap tightened. Two pus swabs were collected from each patient, one for gram stain and the other for culture. All the swabs were carefully labeled and transported immediately to Kenyatta university laboratory in appropriate leak-proof specimen transport bags.

Bacterial culture and identification

Upon reaching the laboratory, all the pus swabs were carefully checked and their details entered into the investigator’s booklet. Smears of the pus swabs were prepared, gram stained and examined microscopically. The other swabs from each patient were inoculated on MacConkey agar and blood agar and the inoculated plates incubated at 35 to 37°C for 24 h. After incubation, the plates were examined for growth. Colony characteristics such as swarming growth and hemolysis on blood agar, changes in physical appearance in differential media, and enzymatic activities of the organisms were used for preliminary bacterial identification. Biochemical tests such as catalase, coagulase, urease, indole, methyl red, Voges Proskauer and citrate (IMViC) tests were performed for the identification of the various bacteria.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed on Mueller Hinton agar using various antibiotics by Kirby Bauer disc diffusion method as per standards prescribed in bacteriology (Hudzicki, 2016). Using a sterile swab, Mueller Hinton agar surface was uniformly coated with the suspension of the test organism matching 0.5 McFarland turbidity standard. Following incubation of test plates and controls at 35°C for 24 h, the plates were examined for confluent growth. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains. The zones of inhibitions were determined using breakpoints provided by the Clinical and Laboratory Standards Institute and British society for antimicrobial chemotherapy (CLSI, 2016; BSAC, 2013). The following antibiotics were tested for resistance; Chloramphenicol (50 ug), Vancomycin (30 ug), Amoxycillin (30 ug), Doxycycline (30 ug), Ciprofloxacin (30 ug), Cefepime (30 ug), Ceftriaxone (30 ug), Amikacin (30 ug), Gentamicin (30 ug), Oxacillin (1 ug), Cotrimoxazole (25 ug), Azithromycin (15 ug) and Ampicillin (10 ug). These antibiotics were selected based on the availability and prescription frequency of these drugs in the study area.

Data analysis

Data were entered in excel, cleaned, and the information exported to IBM statistical package for the social sciences version 20.

Table 1. Proportions of bacteria isolated from different wound sites at Mama Lucy Kibaki Hospital.

Wound site	Types and proportions of bacteria isolates		
	Gram-positive bacteria N (%)	Gram-negative bacteria N N (%)	Total isolates N (%)
Abdomen	24(51.1)	23(48.9)	47(60.3)
Lower limbs	7(30.4)	16(69.6)	23(29.5)
Arms	1(33.3)	2(66.7)	3(3.8)
Other body regions	1(20.0)	4(80.0)	5(6.4)

Descriptive statistics; mean and percentages were determined. Pearson chi-square test was used to determine the association of social demographic data and the different bacteria isolates. P-value of < 0.05 was considered to indicate statistically significant differences. Finally, the results were presented using tables.

Ethical consideration

Ethical approval was given by Kenyatta University Ethics Review Committee (KUERC) reference no PKU/700/1772. Informed consent was obtained by the signing of informed consent forms by adults. For participants below 18 years, parents or legal guardians would sign the consent forms on their behalf. All patients' records were kept anonymous.

RESULTS

Socio-demographic data of patients

A total of 58 cases of surgical site infection were observed at the facility between October 2018 and March 2019. Of these, 19 (32.8%) were men while 39 (67.2%) were women with pediatric patients accounting for 3 (5.2%) of the total cases. The patients' mean age was 31.12 years, with the youngest and eldest patient being 7 and 61 years, respectively.

Proportion of bacteria isolated from surgical wounds

A total of 78 bacteria were isolated from the culture positive swabs, with monomicrobial and polymicrobial growth occurring in 60.3% (35/58) and 34.5% (20/58) of the swabs, respectively. Whereas only 5.2% (3/58) of the swabs were culture negative. S. *aureus* 28.2% (n=22) was the prevalent isolate followed by *E. coli* 15.4% (n=12), *Acinetobacter* species (spp.) 14.1% (n=11), *Pseudomonas aeruginosa* 9.0% (n=7), *Enterobacter* spp. 9.0% (n=7), Bacillus spp. 9.0% (n=7), *Coagulase negative staphylococci* 5.1% (n=4), Proteus spp. 5.1% (n=4), *Klebsiella pneumoniae* 2.6% (n=2), *Morganella morganii* 1.3% (n=1) and *Citrobacter freundii 1.3*% (n=1).

Most of the bacteria were recovered from the abdomen 60.3% and the lower limbs 29.5%, with the arms and other body sites accounting for 3.8 and 6.4% of the total bacteria (Table 1). The majority of these isolates were gram-negative rods 57.7% (45) with gram-positive bacteria accounting for 42.3% (33) of the total bacteria. This number was high in female patients [73.1% (57)] than male patients [26.9% (21)] but the difference was not statistically significant (p=0.136).

Antibiotic susceptibility pattern of SSI bacteria isolates

In the present study, Chloramphenicol 90.9% (n=30/33), Vancomycin 87.9% (n=29/33) and Doxycycline 75.8% (n=25/33) had the highest sensitivity rates for gram-positive bacteria. However, Azithromycin 36.4% (n=12/33) and Ampicillin (3.0%) (n=1/33) recorded the lowest sensitivity rates for gram-positive bacteria (Table 2). Cefepime, Gentamicin and Amikacin showed 100% resistance to Acinetobacter spp. and *K. pneumoniae,* respectively. Chloramphenicol 53.3% (n=24/45) was the only drug that had the highest sensitivity for gram-negative rods. Multi-drug resistance (resistance to ≥ 4 antibiotics) was observed with *K. pneumoniae* and *E.coli* (Table 3). Among gram-positive bacteria, *Methicillin resistant S. aureus (MRSA)* accounted for 65.4% (n=17/26) of the total Staphylococcus species (Table 2).

DISCUSSION

The present work observed monomicrobial growth in 60.3% (35/58) of the swabs that were cultured, with polymicrobial and culture negative growth occurring in 34.5% (20/58) and 5.2% (3/58) of the swabs, respectively. These results were comparable to findings from a study carried out in Nigeria which observed single growth in 48 (75%) samples, with polymicrobial growth occurring in 16 (25%) samples (Adegoke et al., 2010). Both of these studies observed a high isolation rate of bacteria, except that in the present work three swabs failed to yield any growth which could be attributed to antibiotic use prior to sample collection.

The research revealed a total of 78 bacteria with *S. aureus* as the preponderant bacteria. These results concurred with those of a similar study conducted at Moi

Table 2. Antibiotic susceptibility pattern of gram-positive bacteria at Mama Lucy Kibaki Hospital.

Types of bacteria isolates		Tested antibiotics						
		VA, 30 ug (%)	**CIP, 30 ug (%)**	**C, 50 ug (%)**	**DO, 30 ug (%)**	**AX, 10 ug (%)**	**AZM, 15 ug (%)**	**OX, 1 ug (%)**
Staphylococcus aureus	S	90.9	77.3	95.5	86.4	4.5	50.0	27.3
	I	0.0	9.1	0.0	9.1	0.0	4.5	13.6
	R	9.1	13.6	4.5	4.5	95.5	45.5	59.1
Coagulase negative staphylococci	S	75.0	100.0	100.0	50.0	0.0	25.0	0.0
	I	25.0	0.0	0.0	25.0	0.0	0.0	0.0
	R	0.0	0.0	0.0	25.0	100.0	75.0	100.0
Bacillus species	S	85.7	28.6	71.4	57.1	0.0	0.0	-
	I	0.0	42.9	14.3	14.3	0.0	14.3	-
	R	14.3	28.6	14.3	28.6	100.0	85.7	-

VA=vancomycin, CIP=ciprofloxacin, C=chloramphenicol, DO=doxycycline, AX=ampicillin, AZM=azithromycin, OX=oxacillin, S=sensitive, I=intermediately sensitive, R=resistant.

Table 3. Antibiotic susceptibility pattern of gram-negative bacteria at Mama Lucy Kibaki Hospital.

Types of bacteria isolates		Tested antibiotics								
		C 50 ug (%)	**CIP, 30 ug (%)**	**DO 30 ug (%)**	**GEN 10 ug (%)**	**COT 25 ug (%)**	**AMX 30 ug (%)**	**CPM 30 ug (%)**	**AK 30 ug (%)**	**CTR 30 ug (%)**
Morganella morganii	S	100.0	0.0	0.0	100.0	0.0	0.0	-	-	-
	I	0.0	100.0	0.0	0.0	0.0	0.0	-	-	-
	R	0.0	0.0	100.0	0.0	100.0	100.0	-	-	-
Proteus species	S	50.0	50.0	0.0	100.0	50.0	0.0	-	-	-
	I	50.0	50.0	0.0	0.0	0.0	0.0	-	-	-
	R	0.0	0.0	100.0	0.0	50.0	100.0	-	-	-
Klebsiella pneumoniae	S	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-
	I	0.0	50.0	50.0	0.0	0.0	0.0	0.0	0.0	-
	R	50.0	50.0	50.0	100.0	100.0	100.0	100.0	100.0	-
E. coli	S	41.7	25.0	33.3	41.7	0.0	0.0	16.7	-	16.7
	I	0.0	16.7	0.0	8.3	0.0	8.3	0.0	-	0.0
	R	58.3	58.3	66.7	50.0	100.0	91.7	83.3	-	83.3
Pseudomonas aeruginosa	S	57.1	71.4	28.6	57.1	-	-	28.6	71.4	-
	I	14.3	0.0	0.0	0.0	-	-	0.0	0.0	-
	R	28.6	28.6	71.4	42.9	-	-	71.4	28.6	-
Enterobacter species	S	85.7	71.4	42.9	42.9	42.9	0.0	-	-	-
	I	0.0	14.3	0.0	14.3	0.0	0.0	-	-	-
	R	14.3	14.3	57.1	42.9	57.1	100.0	-	-	-
Acinetobacter species	S	36.4	45.5	27.3	36.4	-	-	0.0	45.5	-
	I	45.5	27.3	9.1	0.0	-	-	0.0	0.0	-
	R	18.2	27.3	63.6	63.6	-	-	100.0	54.5	-
Citrobacter freundii	S	100.0	0.0	100.0	100.0	0.0	0.0	-	-	-
	R	0.0	100.0	0.0	0.0	100.0	100.0	-	-	-

CPM=Cefepime, AK=Amikacin, CTR=Ceftriaxone, CIP=Ciprofloxacin, COT=Cotrimoxazole C=Chloramphenicol, DO=Doxycycline, GEN=Gentamicin, AMX=Amoxycillin, S=Sensitive, I=Intermediately Sensitive, R=Resistant.

Teaching and Referral Hospital-Eldoret (Okello et al., 2018). The predominance of *S. aureus* observed at the current setting maybe because this bacterium is a skin flora; therefore, its presence in surgical wounds could be as a result of endogenous contamination of the surgical site during patient skin incision. Exogenous sources such as contaminated hospital surfaces or equipment and the hands of healthcare providers could also account for *S. aureus* preponderance (Gelaw et al., 2014).

However, findings by this work were inconsistent with other studies which identified *E. coli (32.8%), P. aeruginosa and Klebsiella* species as the leading causes of surgical site infection (Namıduru et al., 2013; Kokate et al., 2017; Patel Disha et al., 2011). The difference could be attributed to the invasive nature of the procedures performed by hospitals. This is because, patients undergoing procedures involving the gastro-intestinal tract may be at risk of developing SSI as a result of endogenous contamination of the surgical site with enteric rods during surgery.

The project also saw the predominance of gram-negative bacteria over gram-positive bacteria which was analogous with findings from a study by Tuon et al. (2019). But these results were incongruous with discoveries by a separate research worker who observed the preponderance of gram-positive bacteria over gram-negative bacteria (Khyati et al., 2014). The differences in the types of bacteria isolates can be attributed to the different microbiota present in different hospital environments. Besides, the high prevalence of gram-negative rods may be due to the increase in resistance rates of gram-negative rods at the current setting.

The high resistance rates of Amoxycillin and Ampicillin recorded by the present work were in harmony with observations by other workers (Kahsay et al., 2014). On the other hand, findings on the resistance rates of gram-negative rods were comparable to the results obtained from a study in Ethiopia (Dessie et al., 2016). Additionally, an MRSA rate of 65.4% observed in the present setting was in harmony with results from a study conducted in Uganda that reported an MRSA rate of 65.9% (George et al., 2018). These current occurrences were attributed to lack of effective surveillance programs for SSI etiologies and their antibiograms.

Limitations

The design of this study could not allow for follow-up of patients which meant that some of the cases were never captured.

Conclusion

The present work noted that a majority of the drugs were resistant to gram-negative bacteria. Therefore, there is need for continuous monitoring to determine the susceptibility patterns of the most common bacteria isolates which are found in the hospitals. These findings will assist in treatment and monitoring of bacteria resistance trends across institutions.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Phytochemical screening and antimicrobial activity of *Momordica charantia* L. and *Morinda lucida* Benth extracts from Benin

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Momordica charantia and _Morinda lucida_ are Benin's pharmacopeia plants that are used traditionally for the treatment of infectious diseases. This study aims to investigate phytochemical profile and antimicrobial activity of both plants. The dried leaf powder is used for extraction with different solvents by ultrasonication (35 Hz) at room temperature for 2 h. TLC and the method based on coloring and precipitation differential reactions were used for preliminary screening. HPTLC analysis was performed on silica gel 60 F_{254}, 20.0 X 10.0 cm HPTLC plates, with Toluen: Ethyl acetate: Formic Acid: Methanol (3:4:0.8:0.7 v/v) as a mobile phase. The antibacterial and antifungal activities were assessed _in vitro_ by the method of macrodillution and solid medium agar diffusion. TLC analysis showed many spots which suggest that both of the plants extracts contain various secondary metabolites. HPTLC revealed the presence of Quercetine, cafeic acid and vanilic acid in the plants' extract. _M. charantia_ extracts have shown the largest inhibition diameters (25.00±0.00 mm) and inhibit more strains than _M. lucida_ extracts. From all the tested strains, only _P. aeruginosa_ was the most sensitive to _M. charantia_ extracts with 50% bactericidal effect.

Key words: Phytochemical screening, antimicrobial activity, *Momordica charantia* and *Morinda lucida*, Benin.

INTRODUCTION

Plants have been for centuries the source of molecules and food for humans and wildlife. One of the surveys conducted by the World Health Organization (WHO) reports that more than 80% of the world's population is still depending on the traditional medicines for various diseases (Atef et al., 2019). In the same line, most of the West African population lives in the rural areas and depends on natural resources for their own subsistence

and for their cash income (Achigan-Dako et al., 2011). Mbuni et al. (2020) also reported that rural dwellers prefer traditional medicines because of their closiness to the traditional healers and the fact that the healers understand their culture and environment as well as their patients. Indeed, these plants are used to treat all kinds of chronic diseases (Petrovska, 2012) among which are infectious diseases.

Today, there are more than 250 types of infections and food poisoning caused by bacteria and fungi (Hernández-Cortez et al., 2017). Of these infections and intoxications, the most frequently isolated pathogenic bacteria are *Staphylococci*, *Pseudomonas*, *Streptococcus* and *Escherichia coli* (Elisha et al., 2017; Hernández-Cortez et al., 2017). Otherwise, Pallavali et al. (2017) and Bassetti et al. (2018) reported that Gram-positive cocci such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus* spp. and Gram-negative bacilli such as *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Proteus* species are the most common pathogenic bacteria isolated from wound infections and are also an important cause of wound infections in diabetic individuals and infected wound following surgeries. These bacteria are also mostly responsible for toxins production. Apart from bacterial toxins, mycotoxins are fungal secondary metabolites that can cause the serious infection (Benedict et al., 2016). Indeed, fungal diseases are severe and have very high morbidity as well as up to 60% mortality for patients diagnosed with invasive fungal infection (Staniszewska, 2020). It is the case of *Candida albicans* with many virulence factors implicated in the invasive diseases, that have become common of human infections worldwide (Nouraei et al., 2020; Köhler et al., 2020).

Indeed, the treatment of infections due to bacteria and phytopathogens requires the use of several methods; the best known are the treatments with synthetic products, which is not without consequences on the environment and human health. Nowadays, there is a phenomenon of resistance of bacteria and fungi to most conventional antibiotics. Antibiotic resistance among bacterial or fungal strains strains is a serious situation. It may be so rapid that the effectiveness of common antibiotics may be lost within a span of 5 years due to genetic changes (Chandra et al., 2017). It therefore seems important to explore other alternative for fighting infectious diseases. An alternative is the use of medicinal plants. Many studies show that medicinal plants contain many biologically active secondary metabolites such as tannins (Chokki et al., 2020), terpenoid (Frezza et al., 2019), alkaloids (Vanderplanck and Glauser, 2018), glycosides (Pertuit et al., 2018), flavonoids, phenols (Frezza et al., 2019; Chokki et al., 2020) and other compounds which display various pharmacological activities: antioxidant, anti-inflammatory, anti-allergic, anti-cancer, analgesic, anti-diabetic, antibacterial, antifungal, antiviral activities (Ksouri et al., 2007; Forni et al., 2019; Senhaji et al., 2020). In the socio-economic and health context of developing countries, including Benin, the study of plants can lead to obtaining adequate and low-cost therapeutic responses, with proven scientific efficacy and optimal cultural acceptability.

Momordica charantia and *Morinda lucida* are frequently used in Benin traditional pharmacopiea against various diseases. *Morinda lucida* Benth., belonging to the family Rubiaceae is a tropical rainforest tree. It is known as xwesué (in Benin) and is one of the most used plants in the preparation of traditional medicines against fever (Lawal et al., 2012). The leaves are used as "oral teas", which are usually taken orally for the traditional treatment of malaria, and as a general febrifuge, analgesic, laxative and anti-infections (Adeyemi et al., 2014). *M. charantia* (bitter melon) is a tropical and subtropical vine of the family cucurbitaceous widely grown in India, South Asia, China, Africa (Kubola and Siriamornpun, 2008) and particularly in Benin. Leaf aqueous macerate is used without combination with other plants in the treatment of microbial and viral infections (measles virus). Considering the vast potentiality of plants as sources for antimicrobial drugs, the present research aims to carry out preliminary phytochemical screening and evaluates antimicrobial activity of *M. charantia* and *M. lucida* leaf extracts.

MATERIALS AND METHODS

Plant material

M. lucida leaf samples were collected from Agata (06°30'28''N, 002°38'44''E), which is located in the Department of Oueme, Benin, while those of *M. charantia* were collected from Dangbo (06°35'19''N, 002°33'15''E) located in the same department. A voucher specimens No. AAC8100/HNB and No. AAC8101/HNB respectively for *M. lucida* and *M. charantia* were deposited at the Benin national herbarium, University of Abomey-Calavi, Cotonou, Benin. All samples were collected in the morning at 7 am. They were air-dried (23°±2°C) for 15 days before powdered using grinder Retsch type SM 2000/1430/Upm/Smf, Haan Germany.

Preliminary screening

The preliminary phytochemical profiling of leaf powders of *M. charantia* and *M. lucida* to determine the major constituents (nitrogenous, polyphenolic, terpenic compound and glycosides) was done using a qualitative analysis based on coloring reactions and/or precipitation described by Senhaji et al. (2020).

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Preparation of plant extracts

The extracts were prepared with 9 polar solvents [water, water-ethanol 30:70 (v / v) methanol, methanol-HCl 1%, ethanol, acetone, ethyl acetate, dichloromethane] and 2 non-polar solvents (chloroform and petroleum ether). For the polar solvents, 1 g of powder in 100 mL of solvent was subjected to ultrasonication (35 Hz) at room temperature for 2 h. The same operation was carried out with non-polar solvents under reflux system. A total of 24 extracts were thus obtained, 12 per plant. In addition, the residues obtained after the ethyl acetate and petroleum ether extractions were extracted again using methanol and methanol/1% HCl. These extracts are coded Methanol-EA and Methanol/HCl-PE, respectively. Each mixture was filtered through Whatman N° 1 paper (125 mm ø, Cat No. 1001 125) and concentrated under reduced pressure using a rotary evaporator before being oven dried at 40°C. The aqueous extract was lyophilized to dryness. The extraction yields were determined by the ratio between the mass of powder and extract obtained.

Thin layer chromatography (TLC) analysis

TLC of the two plants extracts was carried out using pre-coated silica gel and alumina plate (TLC-grade ; Merck 20 20 cm, 0.2 mm thikness). Each extract was dissolved in the extraction solvent at a concentration of 1 mg/ml and about 2µl of this solution was applied 1 cm from the base of the TLC with cappillar tube. Development of the chromatograms was done in a closed tank in which the atmosphere was saturated with the eluent vapor to separate various constituents of the extract by lining the tank with filter paper wetted with the eluent and dried at the end. Solvent systems used as eluent were (1) toluen-ethyl acetate 9:1, (2) ethyl acetate-formic acid-water 8:1:1, (3) toluen-acetic acid-formic acid 5:4:1, (4) toluen-acetic acid 4:6, (5) toluen-ethyl acetate-formic acid-methanol 3:4:0.8:0.7. TLC spot was visualized under UV light fluorescent at 254 and 366 nm. The best solvent system was used for HPTLC analysis.

High performance thin-layer chromatography (HPTLC)

Chromatography was performed on silica gel 60 F 254, 20.0 X 10.0 cm HPTLC plates manufacturer Merck, with Toluen-Ethyl acetate-Formic Acid-Methanol (3:4:0.8:0.7 v/v) as a mobile phase. The standard (rutin, quercetin, galic acid, tanic acid, cafeic acid, vanilic acid and clorogenic acid) solutions (2.0 µL of 1 mg/mL) were applied to the plates as 7.0 mm bands; samples were applied with CAMAG-Linomat V automated spray on band applicator equipped with a 100 µL syringe and operated with the following settings : band length of 3.0 mm, application rate of 10 s/ µL, migration distance of 80 mm.

Assessment of antimicrobial activity

Organisms and growth conditions

10 reference strains used in this study included Gram$^+$ bacteria (*Staphylococcus aureus* ATCC 29213, *S. epidermidis* T22695, *M. luteus* ATCC 10240, *S. oralis*, *Enterococcus faecalis* ATCC 29212), Gram$^-$ bacteria (*E. coli* ATCC 25922, *Proteus mirabilis* A24974, *Proteus vulgaris* A25015, *Pseudomonas aeruginosa* ATCC 27853) and yeast (*Candida albicans* MHMR). Overnight (18h) cultures were prepared by inoculating 1 mL Muller Hinton broth with 1-2 young colonies of each organism obtained from 24 h-old Muller Hinton Agar cultures. Broths were incubated overnight at 37°C with shaking. Inocula were prepared by diluting overnight cultures in saline to approximately 10^8 cfu mL^{-1} for bacteria and 10^7 cfu mL^{-1} for *C. albicans*. These suspensions were further diluted with sterile saline as required.

Antibiogramme

The disc diffusion method described by Trinh et al. (2020) with slight modifications was used to evaluate the effects of the extracts on the strains. Briefly, two to three sterile paper discs (6 mm in diameter) were lodged, under aseptic conditions, on Mueller Hinton agar Petri dish previously flooded with the appropriate inoculum. The discs were aseptically impregnated with 25 µL of plant extract solution (30 mg/mL) and kept for 15 min at room temperature before incubation at 37°C for 24 h. After the incubation period, the dishes were examined for inhibitory zones. Each sample was performed in triplicate.

Determination of Minimal Inhibitory Concentrations (MIC)

The more effective plant extracts, which exhibited antibacterial activity at 30 mg.mL^{-1}, were used to determine their MIC using the macrodilution method described by Dah-Nouvlessounon et al. (2015). Different concentrations of the plant extracts (30, 15, 7.5, 3.75, 1.875, 0.937, 0.468, 0.234, 0.117 and 0.058 mg.mL^{-1}) sterilized through Millipore filter were prepared separately in screw tubes. To 1 mL of the above concentrations, 1 mL of the bacterial inoculum (10^6 CFU/mL) was added to obtain 2 mL as a final volume. Culture medium without samples and others without microorganisms were used in the tests as control. Tubes were incubated at 37°C for 18–24 h and growth was evaluated using turbidity measurements. The MIC is the lowest concentration of the compound at which the tested microorganism does not demonstrate visible growth (turbidity).

Dertermination of Minimal Bactericidal/Fungicidal Concentrations (MBC/MFC)

Streaks were taken from the MIC to the highest concentration of plant extracts exhibiting invisible growth, subcultured onto a fresh MH agar medium and incubated at 37°C for 18-24 h. The concentration that yielded no bacterial growth on solid medium was considered the MBC or MFC (Farshori et al*.,* 2013).

Statistical anlysis

Antibiogramme experiment was done in double and data obtained were reported as a mean ± standard deviation (SD). The data were analyzed using Graph Pad Prism 7 software. Differences of p <0.05 were considered significant.

RESULTS

Preliminary phytechemical screening

The preliminary phytochemical analysis performed on the two plants revealed the presence of several secondary metabolites (Table 1). It was noted an uneven distribution of these metabolites from one plant to another. Indeed, 78.57% of the studied secondary metabolites were present in *M. charantia* leaf powder against 71.42% in *M. lucida* leaf powder.

Table 1. Phytochemical constituents of *M. lucida* and *M. charantia* leave powdered samples.

Group of compounds	Class	*M. lucida*	*M. charantia*
Nitrogenous compound	Alkaloids	+	+
Poly-phenolics compound	Tanins	+	+
	Flavonoids	+	+
	Anthocyans	+	-
	Coumarin	+	-
	Quinonics derivate	-	+
Terpeniques compound	Triterpenoids	+	+
	Steroids	+	+
	Cardenolids	+	+
Heterosides	Saponosids (IM)	+ (152)	+ (213)
	Reducing compounds	+	+
	Free anthracénics	-	-
	O-heterosides	-	+
	Mucilags	-	+

+, Presence of secondary metabolite; -, absence of secondary metabolite; IM, Index mouss.

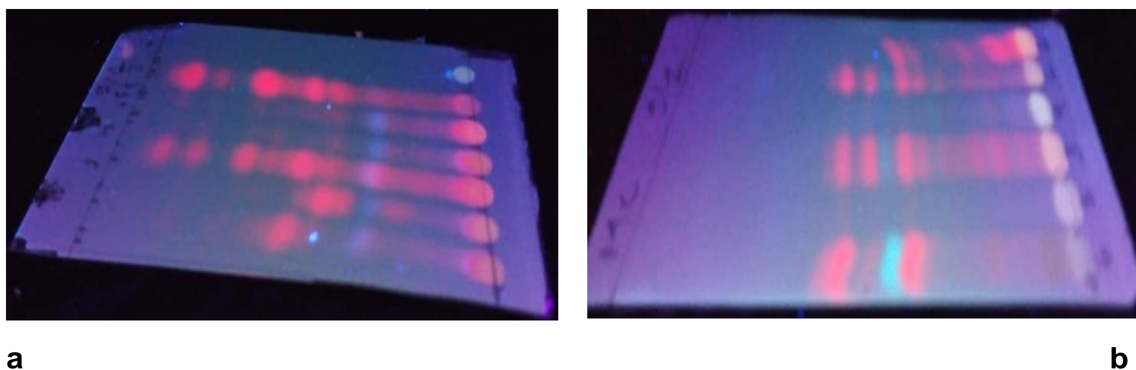


Figure 1 . Thin-layer chromatographic separations of *M. lucida* (a) and *M. charantia* (b) extracts.

Thin layer chromatography (TLC) analysis

TLC profiling showed the good separation of the metabolites with system solvent : toluen-ethyl acetate-Formic Acid-Methanol (3:4:0.8:0.7 v/v) used as eluent. As shown in Figure 1, the TLC profiling showed the presence of various components in both plant extracts. After the observations made on the plants powder, the TLC shows the efficiency of the extraction method used to extract the maximum compounds contained in the plants.

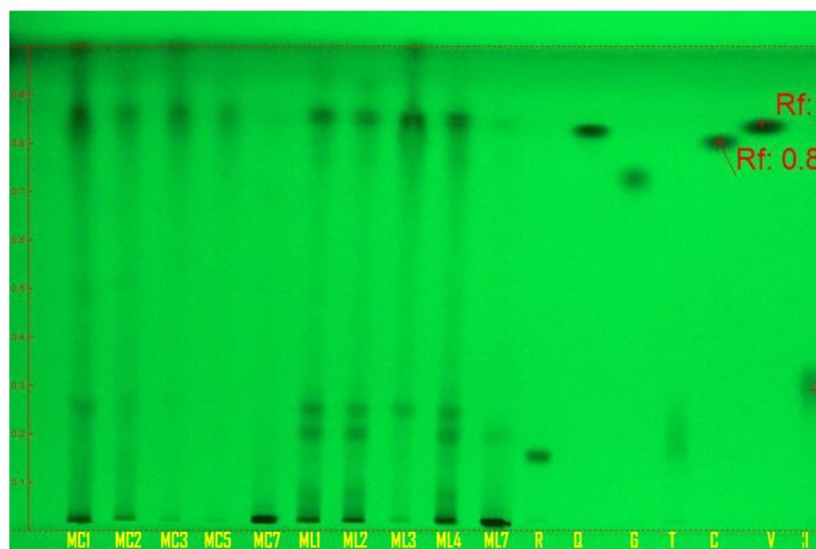
HPTLC analysis

Figure 2 shows the HPTLC profil of phenolics compunds standards and tests extracts. The florescence bands of most of the phenolics compunds are not visible at 366 nm wavelength but they are visible at 254 nm. Netherveless, the chromatograms showed that the compound available are satandard; Quercetine Rf = 0.79 was present in all of the test plant extracts. In addition, cafeic acid (Rf = 0.80) and vanilic acid were found in most of the test extracts.

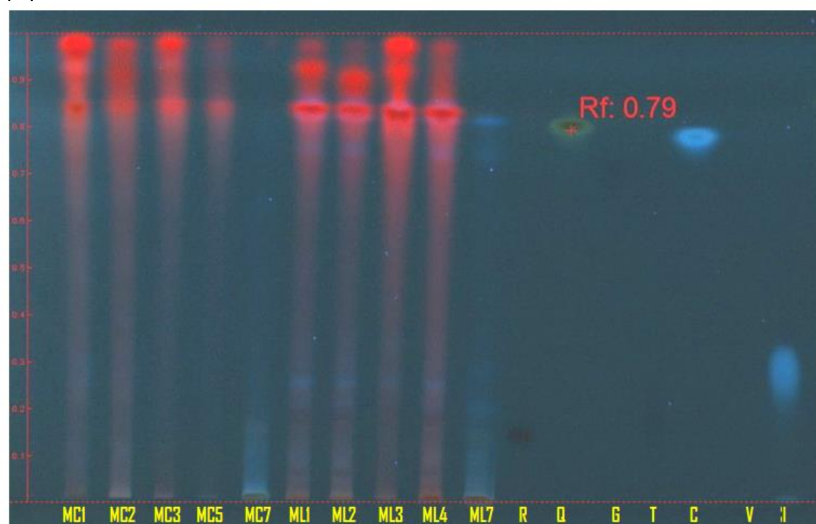
Antimicrobial activity

Sensibility test

The results of the antibiogram are presented in Table 2. The analyses have determined that 50% of *M. charantia* extracts



(a)



(b)

Figure 2. Chromatograms of extracts sample and standard in HPTLC analysis (a) under UV 254 nm, (b) under 366 nm. *M. charantia* (MC1, ethanol; MC2, methanol; MC3, ethyl acetate; MC6, Chloroforme; MC7, water); *M. lucida* (ML1, ethanol; ML2, methanol; ML3, ethyl acetate; ML4, acetone; ML7, water); Standards (R, Rutin; Q, Quercetin; G, acid Galic; T, acid Tanic; C, acid cafeic; V, acid Vanilic; Cl, acid Clorogenic).

inhibit the proliferation of at least one of the tested microorganisms against 66.66% of *M. lucida* extracts. In addition, among the strains sensitive to extracts from both plants, *M. luteus* showed more resistance to all active extracts followed by *P. vulgaris*. Inhibition diameters vary depending on strains and extract types. *M. charantia* extracts have shown the largest inhibition diameters and inhibit more strains than *M. lucida* extracts (Table 2).

For *M. charantia*, the smallest diameter (8.87±0.25 mm) was obtained with chloroform extract on *S. oralis*, while the largest diameter of inhibition (25.00 ± 0.00 mm) was obtained with methanol-EA extract on *E. coli* strain. The analysis of variance ANOVA shows a highly significant variation ($p < 0.0001$) by considering the response of the strains in terms of inhibition diameter and also a significant variation ($p < 0.05$) compared to the power of the extracts in terms of the number of inhibited strains. Regarding *M. lucida*, the smallest inhibition diameter (8.25 ± 0.50 mm) was obtained with the dichloromethane extract on *S. oralis* strain, while the largest diameter (19.28 ± 0.25 mm) was

Table 2. Inhibitory diameter (mm) of the active extracts.

Plant	Extracts	Inhibitory diameter (mm)									
		S. aur	***P. aer***	***P. mir***	***M. lut***	***S. epi***	***P. vul***	***S. ora***	***E. foe***	***E. coli***	***C. alb***
M. charantia	Dichloromethane	16.37±0.48	24.00±0.41	16.00±1.15	ni	15.87±1.18	ni	12.12±0.25	11.25±0.50	14.75±0.96	15±0.00
	Ethyl acetate	ni	17.25±0.47	ni	ni	ni	ni	ni	ni	ni	ni
	Chloroform	23.00±0.00	15.75±0.28	11±0.00	17±0.00	ni	14±0.00	8.87±0.25	10±0.00	ni	11.5±0.00
	Methanol-EA	22.38±0.25	21.25±0.50	12.13±0.25	ni	14.63±0.47	ni	17.75±0.95	24.38±0.47	25.00±0.00	16.50±0.57
	Methanol/HCl	16.88±0.25	20.50±0.57	7.50±0.40	ni	17.00±0.00	ni	21.25±0.57	24.50±0.57	20.00±0.00	16.25±0.28
	Methanol/HCl-PE	16.00±0.00	21.38±0.47	ni	ni	17.63±0.25	ni	20.00±0.00	20.88±0.25	20.50±0.57	15.00±0.00
M. lucida	Methanol/HCl	ni	Ni	ni	ni	10.25±0.50	ni	ni	ni	ni	ni
	Ethyl acetate	ni	13.12±0.25	ni	ni	ni	ni	ni	ni	ni	ni
	Dichloromethane	ni	Ni	9.62±0.25	ni	8.37±0.48	ni	8.25±0.50	14.25±0.50	ni	ni
	Methanol	ni	Ni	13.37±0.25	ni	ni	ni	ni	11±0.00	ni	12.12±0.25
	Acetone	11.00±0.00	Ni	14.13±0.25	ni	15.00±0.00	11.50±0.57	ni	17.13±0.25	ni	11.00±0.00
	Ethanol	ni	Ni	ni	ni	12±0.00	ni	ni	ni	ni	ni
	Methanol/HCl-PE	ni	Ni	ni	ni	ni	ni	ni	ni	19.28±0.25	ni
	Ethanol/water	ni	Ni	ni	ni	08.5±0.57	ni	ni	ni	ni	ni

S. aur, *Staphylococcus aureus*; P. Aer, *Pseudomanas aeruginosa*; P. Mir, *Proteus mirabilis;* M. Lut, *Miccrococcus luteus*; S. Epi, *Staphylococcus epidermidis*; P. Vul, *Proteus vulgaris*; S. Ora, *Streptococcus oralis*; E. foe, *Enterococcus foecalis*; E. Coli, *Escherichia coli*; C. alb, *Candida albicans*; ni, no inhibition.

obtained with the Methanol/HCl-PE extract on *E. coli* strain.

Minimal inhibitory concentration (MIC)

The MIC of the active extracts of the two plants are determined and presented in Table 3. These concentrations vary according to the extract types.

With *M. charantia* extracts, the lowest inhibitory concentration (0.117 mg.mL^{-1}) was obtained with the chloroform extract on the *S. aureus* strain while the highest concentration (7.5 mg/mL^{-1}) was obtained with the dichloromethane extract with *P. mirabilis* and *S. oralis*. For each extract type, a significant difference ($p < 0.05$) was observed between the tested strains. For each strain, however, there is a variation in the threshold of significance. Indeed, for the strains *S. epidermidis* and *E. coli*, no difference was observed ($p > 0.05$).

With *M. lucida*, the lowest concentration (0.234 mg.mL^{-1}) was obtained with the methanol, acetone and methanol/HCl-PE extracts respectively against *P. mirabilis*, *E. foecalis* and *E. coli* strains. On the other hand, the lowest inhibition (7.5 mg.mL^{-1}) was obtained with the dichloromethane and ethanol/water extracts on *S. epidermidis*.

Minimal bactericidal concentration (MBC)

Minimal bactericidal concentrations (MBC) of the active extracts of both plants are presented in Table 4. Like MICs, MBCs vary depending on the types of extract. With *M. charantia*, the lowest concentration (0.234 mg.mL^{-1}) was obtained with Methanol-EA extract against *E. coli* while the highest concentration obtained is greater than 30 mg.mL^{-1}. This concentration was obtained with the dichloromethane extract on the *P. mirabilis* strain. The lowest inhibitory concentration (0.468 mg.mL^{-1}) of *M. lucida* extracts was obtained with the methanol extract. The ratio of the two MIC and MBC parameters showed bactericidal and bacteriostatic effects (Table 4).

Indeed, for *M. charantia*, the dichloromethane extract has a bactericidal effect on 71.42% of the bacterial strains that are sensitive to it, followed

Table 3. Minimal inhibitory concentrations (MIC) of the active extracts of *M. charantia* and *M. lucida*.

Plants	MIC ($mg.mL^{-1}$)										
	Extracts	***S. aur***	***P. aer***	***P.mir***	***M.lut***	***S. epi***	***P. vul***	***S. ora***	***E. foe***	***E. coli***	***C. alb***
M. charantia	Dichloromethane	3.75	1.875	7.5	nd	0.937	nd	7.5	3.75	0.234	0.468
	Ethyl acetate	Nd	0.937	nd	nd	nd	nd	nd	nd	nd	nd
	Chloroform	0.117	0.468	3.75	0.937	nd	0.937	1.875	1.875	nd	1.875
	Methanol-EA	0.234	0.468	3.75	nd	1.875	nd	0.937	0.117	0.234	0.468
	Methanol/HCl	1.875	0.468	3.75	nd	0.937	nd	0.937	0.117	0.468	0.937
	Methanol/HCl-PE	3.75	0.234	nd	nd	0.937	nd	0.468	0.234	0.468	0.937
M. lucida	Methanol/HCl	Nd	nd	nd	nd	1.875	nd	nd	nd	nd	nd
	Ethyl acetate	Nd	1.875	nd	nd	nd	nd	nd	nd	nd	nd
	Dichloromethane	Nd	nd	3.75	nd	7.5	nd	3.75	0.468	nd	nd
	Methanol	Nd	nd	0.234	nd	nd	nd	nd	0.937	nd	0.468
	Acetone	3.75	nd	1.875	nd	0.937	1.875	nd	0.234	nd	3.75
	Ethanol	Nd	nd	nd	nd	1.875	nd	nd	nd	nd	nd
	Methanol/HCl-PE	Nd	nd	nd	nd	nd	nd	nd	nd	0.234	nd
	Ethanol/water	Nd	nd	nd	nd	7.5	nd	nd	nd	nd	nd

S. aur, *Staphylococcus aureus*; P. Aer, *Pseudomanas aeruginosa*; P. Mir, *Proteus mirabilis;* M. Lut, *Miccrococcus luteus*; S. Epi, *Staphylococcus epidermidis*; P. Vul, *Proteus vulgaris*; S. Ora, *Streptococcus oralis*; E. foe, *Enterococcus foecalis*; E. Coli, *Escherichia coli*; C. alb, *Candida albicans*; nd, not determined.

Table 4. Minimal bactericidal concentrations (MBC) of the active extracts of *M. charantia* and *M. lucida.*

Plants	MBC and MFC ($mg.mL^{-1}$)										
	Extracts	***S. aur***	***P. aer***	***P. mir***	***M. lut***	***S. epi***	***P.vul***	***S. ora***	***E. foe***	***E. coli***	***C. alb***
M. charantia	Dichloromethane	7.5*	1.875*	>30	nd	0.937*	nd	7.5*	7.5*	0.937	0.468
	Ethyl acetate	Nd	3.75	nd	nd	nd	nd	nd	nd	nd	nd
	Chloroform	0.937	0.468*	15	1.875*	nd	3.75	15	1.875*	nd	7.5
	Methanol-EA	1.875	1.875	30	nd	15	nd	7.5	0.937	0.234*	3.75
	Methanol/HCl	15	3.75	30	nd	3.75	nd	1.875*	0.468	7.5	15
	Methanol/HCl-PE	15	0.468*	nd	nd	3.75	nd	1.875	1.875	0.937*	7.5
M. lucida	Methanol/HCl	Nd	nd	nd	nd	7.5	nd	nd	nd	nd	nd
	Ethyl acetate	Nd	7.5	nd	nd	nd	nd	nd	nd	nd	nd
	Dichloromethane	Nd	nd	3.75*	nd	30	nd	15	1.875	nd	nd
	Methanol	Nd	nd	0.468*	nd	nd	nd	nd	3.75	nd	3.75
	Acetone	30	nd	15	nd	7.5	7.5	nd	1.875	nd	15
	Ethanol	Nd	nd	nd	nd	3.75*	nd	nd	nd	nd	nd
	Methanol/HCl-PE	Nd	nd	nd	nd	nd	nd	nd	nd	3.75	nd
	Ethanol/water	Nd	nd	nd	nd	>30	nd	nd	nd	nd	nd

With* = bactericidal effects; without* = bacteriostatic effects. S. aur, *Staphylococcus aureus*; P. Aer, *Pseudomanas aeruginosa*; P. Mir, *Proteus mirabilis;* M. Lut, *Miccrococcus luteus*; S. Epi, *Staphylococcus epidermidis*; P. Vul, *Proteus vulgaris*; S. Ora, *Streptococcus oralis*; E. foe, *Enterococcus foecalis*; E. Coli, *Escherichia coli*; C. alb, *Candida albicans*; nd, not determined.

by the chloroform extract which has a bactericidal effect on 42.85% of the sensitive bacteria. In contrast to the ethyl acetate extract which has a bacteriostatic effect, all the other extracts of *M. charantia* showed a bactericidal effect on at least one sensitive bacterial strain. Of all the tested strains, only *P. aeruginosa* was the most sensitive to *M. charantia* extracts with 50% bactericidal effect. On the other hand, *P. mirabilis* strain was the most resistant to all extracts of *M. charantia*.

Contrary to the observations made with *M. charantia*,

P. mirabilis is the strain which presented more sensitivity to *M. lucida* extracts with two bactericidal effects obtained with dichloromethane and methanol extracts. In addition to the ethanolic extract that had a bactericidal effect on the *S. epidermidis* strain, all other *M. lucida* extracts had bacteriostatic effects on all susceptible strains. The tested strains showed more resistance to *M. lucida* extracts than those of *M. charantia*.

DISCUSSION

The extracts used in this study were prepared using ultrasonication. The choice of this method is based on the fact that the mechanical effects of ultrasound induce a disruption of the cell walls. This leads to greater intraparticle penetration of the solvent into the cells, thus facilitating the rapid release of their contents and the acceleration of the kinetics extraction (Landoulsi, 2016). The efficiency of cell disruption and mass transfer are the main factors responsible for the good performance of ultrasound extraction (Romdhane, 1993). Ultrasound has the advantage of considerably reducing the extraction time and increasing the extraction yield (Bourgou et al*.,* 2016). The yields obtained during the extraction varied from one plant to another and according to the solvents. Since for the same solvent yields vary according to the plant, while the same amount of plant powder was extracted with the same amount of solvent under the same conditions, the explanation of the difference would be related to the chemical composition of the plants that would not be the same. Phytochemical screening showed that both plants are source of secondary metabolites. The preliminary screening reveals, in nitrogen compunds group, the presence of alkaloïds. This observation was similar to those made by Adomi and Umukoro (2010) in Nigeria. Tanin and flavonoid found in both plants were reported to have antibacterial, anti fungal, antiviral and antioxydant activity (Leelaprakash et al*.,* 2011; Manandhar et al*.,* 2019). Ndam et al. (2014) in Cameroon and Kazeem et al. (2013) in Nigeria have the same observations. Dandawate et al. (2016), De Oliveira et al. (2018) and Khatun et al. (2020) reported that phytochemical screening of *M. charantia* revealed the presence of secondary metabolite such as alkaloids, flavonoids, , tannins, saponins and terpenoids.

In drug analysis, analytical methods used are diversified and are still being improved to find better solutions in pharmaceutical analysis. In this study, we use also HPTLC technique. HPTLC is an analytical technique based on TLC, but with enhancements intended to increase the resolution of the compounds to be separated and to allow quantitative analysis of the compounds. The usage of HPTLC is well appreciated and accepted all over the world. HPTLC is an ideal screening tool, that not only confirms but also establishes its identity (Wang et al., 2010). A simple and reproducible method using HPTLC was successfully performed for the qualitative and quantitative analysis of medicinal plant (Yadav et al*.,* 2011; Puranik et al*.,* 2010). Therefore, HPTLC analysis showed in our extracts, the presence of some phenolics compunds such as: Quercetine Rf = 0.79 present in all of test plant extracts, cafeic acid (Rf = 0.80) and vanilic acid were found in most of the test extracts. For *M. charantia*, the same observation was made by Shodehinde et al. (2016) for quercetin and acid cafeic. In the same way, Thiruvengadam et al. (2014) found vanilic acid in *M. charantia* leaf extracts. Phytochemical studies by several authors have shown the presence of several secondary metabolites in the organs of *M. lucida* (Owolabi et al*.,* 2014; Adebayo et al*.,* 2020). The diversity of these secondary metabolites at the level of each plant gives it a wide range of biological activities. Indeed, some authors have shown the traditional use of these plants in the treatment of several diseases such as microbial and viral infections, diabetes, malaria, cancer (Ezuruike and Prieto, 2014; Kumar et al*.,* 2010). Therefore, antimicrobial activity of the extracts of these two plants was evaluated *in vitro* in our study.

The antimicrobial activity has shown that the susceptibility of the microorganisms to tested extracts varies according to the plants and the types of extracts. The variation observed at the plant level is due to the chemical composition of each plant which can be influenced by several factors such as: the soil and pedological conditions that highlight the plant's nutrition (Durand, 2007; Stewart et al*.,* 2001) on which the formation and expression of secondary metabolites depends (Fritz et al*.,* 2006). Moreover, these observations can be explained on the one hand by the conditions and harvesting period of organs. Indeed, some authors (Slimestad and Verheul, 2005; Toor et al*.,* 2006) showed the unequal distribution of secondary metabolites in plant organs between time intervals. These variations are due, among other things, to light and temperature conditions (Riga et al*.,* 2008). Moreover, regarding the extracts, the affinity that the extraction solvents exhibit according to their polarity with the phytomolecule (Bourgou et al*.,* 2016) would be the basis of the intrinsic difference (for the same plant) observed. The extracts inhibited the proliferation of microorganisms tested with inhibition diameters ranging from 7.50±0.40 to 25.00±0.00 mm. The ratio of Minimal Bactericidal Concentrations (MBC) and Inhibition (MIC) according to a previous study (Berche et al*.,* 1991) showed that some extracts have bactericidal effects which shows a good antibacterial activity. The antimicrobial activity observed with these plants is attributed to their chemical composition. In addition, other authors like Naqvi et al. (2020) showed promising antibacterial activities, >18.5 ± 0.21 mm zone-of-inhibition against *S. aureus*, and 18.4 ± 0.17 mm zone-of-inhibition against *Escherichia coli* with methanol extract of *M. charantia*. Ale (2020) reported that the aqueous extracts of different parts of *M. lucida*

were found to be effective against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa*. In addition, Olawuwo et al. (2020) investigate the *in vitro* antifungal activity of acetone and aqueous extracts of *M. lucida* (Rubiaceae) against ATCC strains of *Aspergillus fumigatus* , *A. flavus, C. albicans* and *Cryptococcus neoformans* as well as clinical isolates of *A. fumigatus* and *C. albicans* and showed that the minimum inhibitory concentration (MIC) of both extracts against tested organisms ranged from 0.11 to 2.50 mg/ml and 0.03 to 2.50 mg/ml after 48 and 72 h respectively. In the present study, MIC obtained with *M. lucida* extracts range from 0.468 to 3.75 mg/ml respectively for methanol and acetone extracts. This similitude between the results shows that *M. lucida* extract has antifungal activity.

Conclusion

From the results obtained, TLC analysis showed many spots that suggest that both plants' extracts contain many secondary metabolites. HPTLC revealed the presence of Quercetine, cafeic acid and vanilic acid in the plant extract. The presence of those compounds confers on these plants the antimicrobial activity. *M. charantia* extracts inhibited the proliferation of the test strains than those of *M. lucida* extracts. The Dichloromethane extract of *M. charantia* displays more bactericidal effect of references strains than those of *M. lucida*. The purified extracts of *M. charantia* and *M. lucida* can be useful both in food conservation and in human medicine.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effect of using treated wastewater on the bacteriological quality of raw cow's milk: A case of a farm in Northeastern Algeria

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This study aims to assess the impact of the use of treated wastewater (without chlorination) in farming and dairy cattle breeding. Milk samples were collected from a farm in northeastern Algeria. The treated wastewater from the treatment plant is used on this farm for different activities. The results obtained show that the average contamination of milks with total flora is $3.7.10^5$ CFU /ml. Fecal coliforms are present at an average value of $1.5.10^3$ CFU/ml. All of the samples (100%) were positive for the count of fecal enterococci with an average value of 2.5.10 CFU/ml. Fungal flora was present with an average value of $1.36,10^3$ CFU/ml. *Escherichia coli* was isolated in 100% of the samples with high resistance rates for beta-lactam antibiotics. The results obtained for the search for pathogens belonging to the genus *Staphylococcus* show that 64% of the isolates were coagulase-negative *Staphylococcus* and 36% of the isolates were coagulase-positive. The study of *Staphylococcus* susceptibility/resistance to antibiotics revealed high frequencies of resistance, especially to beta-lactam antibiotics and macrolides. The bacteria tested show a majority resistance for Penicillin and Oxacillin (100%). These results reflect the microbiological risk that the consumption and marketing of this milk represents for the health of consumers and the need to implement preventive measures.

Key words: Irrigation, fecal coliforms, *Escherichia coli*, *Staphylococcus* sp, antibiotic resistance, microbiological risk.

INTRODUCTION

The emergence and spread of antibiotic resistance genes among pathogenic and non-pathogenic bacteria has been a growing threat in recent decades and there is a rapid lack of therapeutic options (Li and Webster, 2018;

Figure 1. Device for the use of treated wastewater at the outlet of the treatment plant.

Barancheshme and Munir, 2018). The emergence of this resistance in bacteria in animals and their products has attracted considerable interest due to the potential of transferring this resistance to the human population (Vásquez et al*.,* 2017; McDermott et al*.,* 2018). Suspected sites of resistance transmission include wastewater treatment plants where wastewater from various sources, including municipalities, sanitary wastewater, hospital effluents, storm water runoff and industries, is mixed and treated using a multi-stage purification process (Mohammadali and Davies, 2017; Hultman et al*.,* 2018).

The use of wastewater for irrigation is observed as a way to address the imbalance between demand and supply of water. However, the literature shows that irrigation with treated wastewater is not without implications, some of which are negative (Gatto D'Andrea et al*.,* 2015; Becerra-Castro et al*.,* 2015).

Inadequately treated water from sewage systems represents both a risk to human and animal health if it is used to pasture or fodder crops grazed by livestock or otherwise consumed (Cass and Lowe, 2014). As a result, the water may contain bacteria, viruses, protozoa and helminth eggs that would be a risk to the livestock, or to humans who have contact with or consume livestock products (meat, milk, eggs, etc.) (Drechsel et al*.,* 2010). The wastewater treatment plant on the wilaya of Khenchela (Northeastern Algeria) is a low load activated sludge with a capacity of 23,000 m^3/day for 192,000 equivalent/inhabitant. A mixture of urban, industrial, agricultural, storm water runoff and hospital wastewater from the city of Khenchela is discharged to this treatment plant, only to be finally discharged without tertiary treatment and disinfection into Baghai wadi.

The owner of traditional farm uses treated water at the outlet of a wastewater treatment plant to irrigate his pasture field and breed his cows (Figure 1). On the farm, this water is used for three main activities: pasture cultivation, dairy barn farming and cleaning, and for dairy cow consumption.

The main objective of this study is to assess the risks associated with the reuse of treated wastewater in agriculture. The dairy industry is particularly concerned about the potential effect on dairy cattle and milk quality following pasture irrigation with waste water. Therefore, monitoring bacterial pathogens, their survival and transfer is of the utmost importance to ensure that milk quality is not compromised.

MATERIALS AND METHODS

Raw milk samples were obtained after a manual milking of the four healthy cows, from the lactating udder, just before the first morning milking. Milk sample (100 ml) was collected in a sterile bottle after washing and disinfecting the teats and removing the first draft. The milk samples were immediately placed at a temperature of +4°C and then quickly sent to the laboratories for an analysis of its biochemical and microbiological composition.

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FT-IR instrumental analysis

The biochemical composition of raw milk samples was performed by Fourier Transform Infrared Spectroscopy (FTIR). It is a rapid biochemical fingerprinting technique (Nicolaou et al., 2010). It can potentially be applied to produce results with the same accuracy and sensitivity as reference methods in a short period of time (Nicolaou and Goodacre, 2008). Measurements were made using a Fourier Transform Infrared Spectrometer (FTIR) (DYNASCAN Border Spectrum, Perkin-Elmer Ltd, England), equipped with a deuterated triglycerin sulfate (DTGS) detector stabilized at an optimized temperature in the far and mid infrared. Infrared spectra were recorded at 64 scans in the range of 8 300 to 50 cm^{-1} with a resolution of 4 cm^{-1}. KBr separator was used to record milk spectra. The milk sample was ground with KBr powder to be pressed into a tablet. Then, the IR spectrum was collected.

Microbiological analyses

The microbiological analyses were carried out at the Microbiology Laboratory of the University of Khenchela (Algeria). From milk previously homogenized, serial decimal dilutions were prepared in peptone saline diluent using standard methods. (ISO 6887 - 5: 2010). All raw milk samples were analyzed for the presence of total aerobic mesophilic bacteria (TAMB), fecal coliforms (CF), fecal enterococci (FE), yeasts and viable molds and for the detection of *Escherichia coli* and positive and negative coagulase *Staphylococcus* using the standard methods described below.

Total aerobic mesophilic bacteria enumeration

Total aerobic mesophilic bacteria (TAMB) were measured according to the standard method (ISO 4833-1: 2013). The Petri dishes were inoculated separately with 1 ml of each dilution to which Plate Count Agar (PCA) was added (Pasteur Institute, Algeria). After 72 h of incubation, all colonies were counted and the results were expressed in units forming colony per ml of milk (CFU/ml).

Fecal coliforms and *E. coli* counts

Fecal coliforms (FC) and *E. coli* were measured using the standard method (ISO 4831:2006 and ISO 7251:2005). Fecal coliforms were counted using the most probable number (MPN) technique in brilliant green bile (2%) broth (Pasteur Institute of Algeria). After the incubation period of 24 to 48 h at 44°C, the pattern of positive results was compared with a table of most probable numbers. The counts were expressed in units forming colony per ml of milk (CFU/ml). For the isolation and identification of *E. coli*, positive tubes showing turbidity and gas production were cultured on selective medium Hektoen agar (Pasteur Institute, Algeria) and incubated at 37°C for 24 h. Large yellow salmon colonies on Hektoen agar were suspected as *E. coli* strains and further confirmation was made by following standard microbiological techniques which include colony morphology studies, Gram staining. Biochemical analysis of *E. coli* isolates was performed using API 20E strips (BioMérieux).

Fecal *Enterococci* enumeration

Intestinal enterococci were counted using the most probable number method in Rothe broth (Pasteur Institute, Algeria). After incubation from 48 h at 37°C, the contents of the positive tubes, showing turbidity, were inoculated on BEA (*Bile Esculine Azide*) medium at 37°C for 24 and 48 h, for confirmation. Enterococcal colonies were small, translucent and surrounded by a black halo (positive esculin) (Maury, 1987).

***Staphylococcus* detection**

Staphylococcus detection was performed according to the standard method (ISO 6888-1:2003) on Baird Parker agar supplemented by egg yolk and potassium tellurite (Pasteur Institute, Algeria) by a spread plate technique; after enrichment on Giolitti Cantoni Base broth (Pasteur Institute, Algeria) (ISO, 2003). The agar plate was aerobically incubated for 24 - 48 h at 37°C. The positive result of the test is the appearance of colonies surrounded by a light halo with a black or grey center. Suspected colonies were sub-cultivated on the same selective medium plates and incubated at 37 °C for 24 h to obtain a pure culture. Pure cultures were further examined for morphological staining and cultural characteristics as well as biochemical characteristics (fermentation of mannitol, catalase and coagulase). For the identification of *Staphylococcus* species, API 20 Staph strips (BioMérieux) were used (Zangerl and Asperger, 2003).

Viable yeasts and molds enumeration

ISO 21527-1:2008 specifies a horizontal method for the enumeration of viable yeasts and molds in products intended for human consumption or animal feeding with a water activity greater than 0.95 (eggs, meat, dairy products (except milk powder), fruit, vegetables, fresh pasta, etc.), using the colony counting technique at 22 -25°C (ISO, 2008).

The spread-plate technique is strongly preferred to the pour-plate technique for enumeration of yeasts and molds in foods using dilution plating. Spread plating avoids any risk of thermal inactivation of fungal propagules which may be associated with the pour-plate technique and facilitates maximum exposure of cells to atmospheric oxygen (Beuchat, 2003). A sample of 0.1 ml of appropriately diluted sample is deposited in duplicate on the surface of the oxytetracycline glucose yeast extract agar (OGYE) (Pasteur Institute, Algeria). Then it was uniformly spread on the surface using a curved sterile glass rod. The rods must not exceed 2 mm in diameter in order to minimize the adhesion of the sample at the end of the spreading procedure. The agar plates were aerobically incubated for 5 days at 22°C (Beuchat, 2003). All colonies were counted and the results were expressed in units forming colony per ml of milk (CFU/ml).

Antimicrobial susceptibility/resistance test

An antimicrobial susceptibility/resistance test by disc diffusion on Mueller-Hinton agar (Pasteur Institute, Algeria) (Bauer et al., 1966) was performed for all *E. coli* and *Staphylococcus* isolates according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2017). The antimicrobial agents and disc charges used in this study on *E. coli* isolates were ampicillin (AMP 10 µg), amoxicillin + clavulanic acid (AMC 30 µg), ceftazidime (CAZ 30 µg), Imipenem (IMP 10 µg), Ofloxacin (OFX 05 µg), Nitrofurantoin (F 300 µg), Gentamicin (CN 10 µg), Amikacin (AK 30 µg), Colistine (CT 50 µg) and Fosfomycin (FF 200 µg) (Thermo Scientific oxoid, France).

The antimicrobial agents and disc charges used in this study on *Staphylococcus* isolates were Penicillin (P 10 µg), Oxacillin (OX 1 µg), Amikacin (AK 30 µg), Gentamicin (CN 10 µg) and Kanamycin (K 30 µg), Erytromycin (E 15 µg), Clindamycin (DA 2 µg), Pristinamycin (PT 15 µg), Ofloxacin (OFX 5 µg), Levofloxacin (LEV 5µg), Vancomycin (VD 5µg), Rifampicin (RD 5µg) and Cotrimoxazol

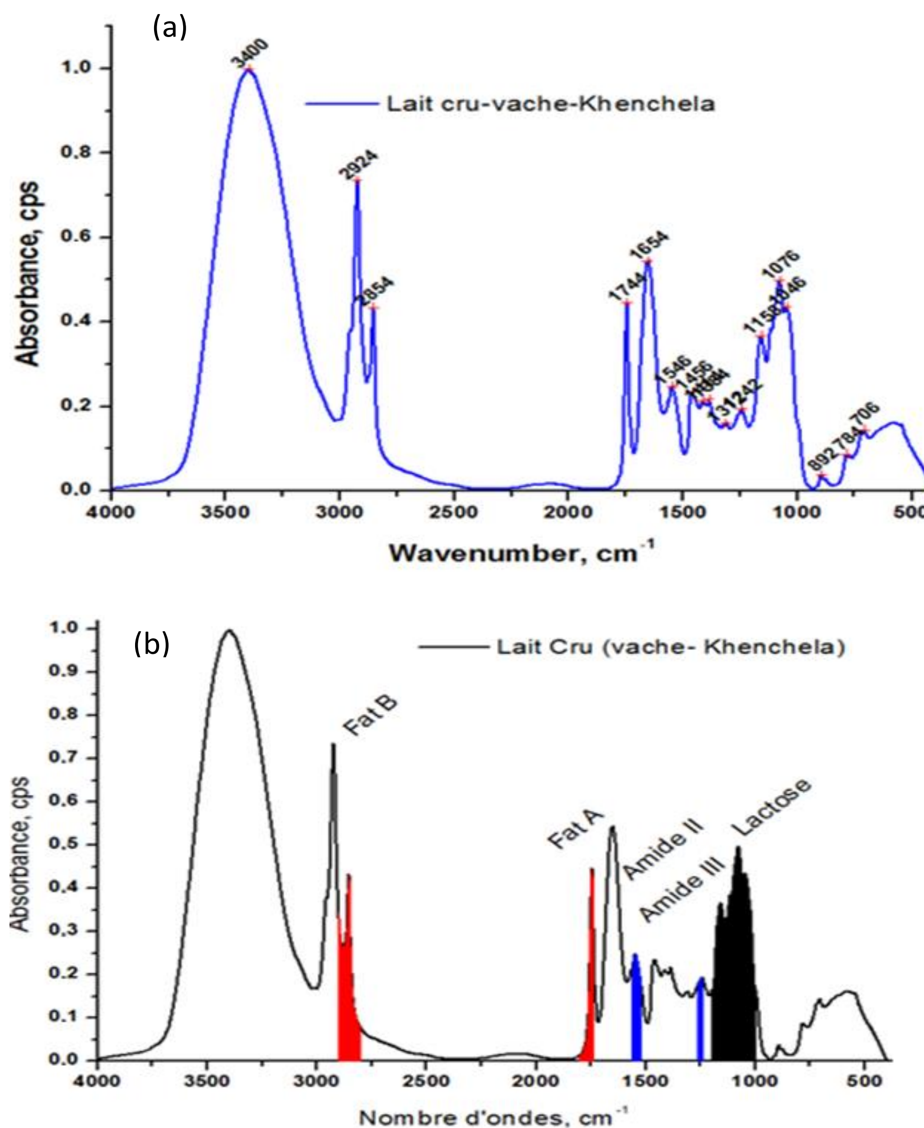

Figure 2. (a) Typical spectra raw cow milk samples obtained by Fourier transform infrared spectroscopy in selected spectral range 4 000-400 cm^{-1}. (b) Principal component analysis showing score plot of Fourier transform infrared measurements.

(SXT 1.25 µg) (Thermo Scientific oxoid, France). The area diameter for each antimicrobial agent was then transformed into sensitive, intermediate and resistant categories according to the performance standards interpretation table for antimicrobial susceptibility testing (MHPHR, 2014). For quality control, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 were used as reference strains.

RESULTS

Fourier transforms infrared (FTIR) spectroscopy

Figure 2a and b. show the Fourier transform infrared absorption spectra of raw milk samples in the spectral range of 400 - 4 000 cm^{-1}. There are numerous peaks that correspond to the different molecular bonds of milk components interacting with infrared radiation. Three main components, fat, protein and lactose, all have strong and characteristic peaks.

Determination of the fat content of milk samples from Fourier Transform Infrared (FTIR) spectra is mainly based on 2 specific regions. The spectral region between 1 725 and 1 850 cm^{-1} showed bands of low absorption due to the carbonyl group (C=O) of milk lipids (commonly called fat A). Another spectral region of medium intensity between 2 800 and 2 924 cm^{-1} absorbed infrared light due to the alkyl chain of fatty acids (commonly called fat B) (Lefier et al., 1996; Eskildsen et al., 2016). The

Table 1. Microbiological criteria for raw milk (JORA, 2017).

Raw milk	n	m	M
Total aerobic mesophilic flora	1	10^5 µo ml^{-1}	3.10^6 µo ml^{-1}
Fecal coliforms	1	10^3 µo ml^{-1}	3.10^4 µo ml $^{-1}$
Fecal enterococci	1	Absence / 0.1 ml	-
Staphylococcus aureus	1	Absence/ 1 ml	-
Clostridium sulfite-reducing agents at 46 °C	1	5× 10^1 µo ml^{-1}	$1{,}5.10^2$ µo ml $^{-1}$
Antibiotics	1	Absence	-

m: Threshold below which the product is considered to be of satisfactory quality. M: Acceptability threshold beyond which the results are no longer considered satisfactory. M = 10 m, when counting in solid media. M = 30 m, when counting in liquid medium. n: Number of units in the sample (equal to 1 for raw milk); µo ml^{-1}: microorganism per milliliter.

infrared peaks at 2 854 and 1 744 cm^{-1} observed in Fig. 2.a. could be related to the milk fat contents B and A respectively.

Milk protein is expected to have absorption bands around 1 650, 1 550 and 1 250 cm^{-1} due to amide I, amide II and amide III groups, respectively (Dagnachew et al*.,* 2013). It also has an absorbance peak in the region between 1 060 and 1 100 cm^{-1}, associated with a phosphate group bound to casein protein (Etzion et al*.,* 2004). Figure 2b shows two spectral bands of medium intensity, the peak of 1 242 cm^{-1} in the wavelength range of 1 225-1 280 cm^{-1} corresponds to the N-H bending and C-N stretching vibrations of amide III (Lei et al*.,* 2010). The other peak of 1 546 cm^{-1} in the spectral range of 1 525-1 580 cm^{-1} was obtained by bending the n-plane N-H with the C-N stretching vibrations of amide II (Moros et al*.,* 2006).

Lactose is expected to have an absorption peak in the infrared region between 1 030 and 1 150 cm^{-1} due to the presence of various C-O stretching vibrations in carbohydrates (Grappin et al*.,* 2006; Zhou et al*.,* 2006). Figure 2a shows a high intensity peak of 1 076 cm^{-1} in the spectral range between 1 000 and 1 150 cm^{-1} and this peak could be related to the lactose content of the milk. A typical water transmittance spectrum between 3 650 and 3 000 cm^{-1} was represented in the hydroxyl group (O-H) (Coitinho et al*.,* 2017) (Figure 2a).

Microbiological analyses

The average contamination of milk samples with total flora is $3.7.10^5$ CFU/ml. fecal coliforms were present at an average value of $1{,}5{,}10^3$ CFU/ml. All of the samples (100%) were positive for the count of fecal enterococci with an average value of 2.5.10 CFU / ml. For fungal flora, it was present with an average value of $1.36{,}10^3$ CFU/ml.

According to the microbiological criteria of the inter-ministerial decree of 04-10-2016 of the OJ No.: 39/17 of the Algerian Republic (Table 1) (JORA, 2017), the overall quality of the study sample is compromised. Fecal enterococci were the first causes of non-compliance: A hundred percent of the samples was unsatisfactory, the total aerobic mesophilic flora and fecal coliforms do not exceed the acceptability limit according to the Algerian standard.

E. coli isolation and identification

For *E. coli* testing, 25 isolates were isolated, purified and identified. Antibiotic resistance was tested for each of the bacteria identified against 10 different antibiotics. The values of inhibition diameters were compared with the values in the reading table (MHPHR*,* 2014). Resistance rates for each antibiotic were calculated; the results obtained are grouped in Table 2.

Seventy two percent of *E. coli* isolates were resistant to Ampicillin (AMP). Forty per cent (40%) of isolates were to Amoxicillin/Clavulanic acid (AMC). The resistance frequencies for Ceftazidime (CAZ) (third generation cephalosporin) and Imipenem (IMP) were 28 and 00% respectively. The resistance rate obtained for antibiotics belonging to the aminoglycosides class is variable, with a resistance rate of 08% for Gentamicin and 32% for Amikacin. This variability would be due to the low consumption of Gentamicin giving the existence of less toxic and more effective molecules. Eight percent of *E. coli* isolates was resistant to Colistin, 12% to fosfomycin, ofloxacin and Nitrofurantoin.

Staphylococcus isolation and identification

The second cause of non-compliance of the milk sample is the presence of *Staphylococcus*. All samples were positive for *Staphylococcus*; colonies that developed on Baird Parker agar after incubation were stained with Gram stain and staphylocoagulase tested to distinguish strains with pathogenic potential (*S. aureus)* from non-pathogenic strains. Twenty five strains were isolated and

Table 2. Antimicrobial resistance profiles of *E. coli* isolates.

Classes	Antimicrobial agents and disc charges	Resistance rate, % (n)
β - lactam	Ampicillin (AMP) (10 µg),	72 (18)
	Amoxicillin + Clavulanic Acid (AMC) (30 µg)	40 (10)
	Ceftazidime (CAZ) (30 µg)	28 (7)
	Imipenèm (IMP) (10 µg)	00 (-)
Fluoroquinolones	Ofloxacin (OFX) (05 µg)	12 3
Nitrofurans	Nitrofurantoïn (F) (300 µg)	12 3
Aminoglycosides	Gentamicin (CN) (10 µg)	08 (2)
	Amikacin (AK) (30 µg)	32 (8)
Polymyxins	Colistin (CT) (50 µg)	08 (2)
Phosphonic Acids	Fosfomycin (FF) (200 µg)	12 (3)

purified; their identification by the API 20 Staph system revealed the predominance of coagulase-negative *Staphylococcus* (64%) compared to the coagulase-positive S*. aureus* species (36%). The main species of coagulase negative *Staphylococcus* isolated and their respective frequencies were: *S. hominis* (36%), *S. xylosus* (08%), *S. warneri* (08%), *S. epidermidis* (04%), *S. chromogenes* (04%) and *S. lugdunensis* (04%).

Antibiotic resistance was tested for each of the bacteria identified against 13 antibiotics. Resistance rates for each antibiotic were calculated and the results obtained are presented in Table 3. It can be observed that resistance rates vary significantly from one antibiotic to another. The *Staphylococcus* species studied showed a 100% resistance rate to Penicillin, and Oxacillin. The resistance rate obtained for antibiotics belonging to the aminoglycosides class varies, a zero resistance rate for Gentamicin, 04% for Amikacin and 08% for Kanamycin. For macrolides; the *Staphylococcus* species studied showed 100% resistance to Erytromycin, 48% to Clindamycin and 64% to Pristinamycin. Eighty per cent (80%) of the isolates were resistant to Rifampicin and 12% to Cotrimoxazole. A resistance rate of 2% for Ofloxacin and a zero resistance rate for Levofloxacin and vancomycin. Multiple antibiotic resistance phenotypes were generated from 25 *S. aureus* isolates showing resistance to three or more antibiotics. Data indicating the predominant multiple antibiotic resistance phenotypes are shown in Table 4.

DISCUSSION

Fourier transforms infrared (FTIR) spectroscopy

The possibility of FTIR analysis for milk and dairy products has been mentioned by Lanher (1991), Van de Voort (1992) and Lefier et al. (1996). Milk FTIR spectra could possibly give more useful information on how the quality of milk is influenced by environmental factors. This could be used to define new traits and also used as a herd management monitoring tool to detect aberrations due to feeding and other environmental changes (Dagnachew et al*.,* 2013). The results obtained showed that the weight composition of water, carbohydrates, lipids and proteins in the milk samples were always balanced in descending order: The vast majority of water, carbohydrates mainly represented by lactose, lipids and finally proteins. The use of treated wastewater in the study site obviously did not influence the biochemical and nutritional composition of the milk samples.

Bacteriological qualities of raw milk

The water used on the study farm is the wastewater treated and discharged by the treatment plant; this is mixture of urban, industrial, agricultural and hospital wastewater from the city of Khenchela. On the farm, this water is used for three main activities: pasture cultivation, dairy barn farming and cleaning, and for dairy cow consumption. The main objective of this study is to assess the risks associated with the reuse of treated wastewater in agriculture. The dairy industry is particularly concerned about the potential effect on dairy cattle and milk quality following pasture irrigation with waste water. Therefore, monitoring bacterial pathogens, their survival and transfer is of the utmost importance to ensure that milk quality is not compromised.

Contamination of raw cow's milk with microorganisms is influenced by the health status and hygiene of dairy cows (Chambers, 2002; Cempírková, 2007). Due to the high nutritional value, water content and almost neutral pH of milk, many pathogenic and spoilage microorganisms can develop (Ray, 2004). The value of the total mesophilic aerobic flora of raw milk indicates a very poor quality of

Table 3. Antimicrobial resistance profile of *Staphylococcus* isolates.

Classes	Antimicrobial agents and disc charges	Resistance rate, % (n)
β - lactam	Penicillin (P) (10 µg)	100 (25)
	Oxacillin (OX) (1 µg)	100 (25)
Aminoglycosides	Amikacin (AK) (30 µg)	08 (2)
	Gentamicin (CN) (10 µg)	0
	Kanamycin (K) (30 µg)	04 (1)
Macrolides	Erytromycin (E) (15 µg)	100 (25)
	Clindamycin (DA) (2 µg)	48 (12)
	Pristinamycin (PT) (15 µg)	64 (16)
Fluoroquinolones	Ofloxacine (OFX) (5 µg)	4 (1)
	Levofloxacin (LEV) (5 µg)	0
Glycopeptides	Vancomycin (VD) (5 µg)	0
Rifamycin	Rifampicin (RD) (5 µg)	80 (20)
Sulfonamides	Cotrimoxazole (SXT) (1.25 µg)	12 (3)

Table 4. Multiple antibiotic resistant phenotypes for *Staphylococcus* isolates.

Phenotypes	Number of isolates	(%) Observed
P - OX - E	25	100
P - OX - E - RD	20	80
P - OX - E - RD - PT	16	64
P - OX - E - RD - PT - DA	12	48
P - OX - E - DA - PT - RD - SXT	3	12
P - OX - E - DA - PT - RD – SXT - AK	2	8
P - OX - E - DA- PT - RD - SXT – AK - K	1	4

raw milk compared to the required standards of 10^5 CFU/ml (JORA, 2017). In addition, the overall bacterial load was very high; 90% of the samples had a value greater than 10^5 CFU/ml of flora. This total flora load and the large number of samples exceeding the recommended limits can be attributed mainly to infected udders, unsanitary milking equipment or procedures and/or poor microbiological quality of water used for cleaning utensils and animals, as well as milk storage conditions (Chye et al., 2004; Ghazi and Niar, 2011; Singh and Gupta, 2015; Wanjala et al., 2018). The result of the fecal coliforms showed significant contamination and indicated very poor quality of raw milk compared to the required standards of 10^3 CFU/ml (JORA, 2017). In general, coliforms indicate fecal contamination and their number is proportional to the degree of pollution produced by the stool (Aggad et al., 2009). However, the presence of coliforms indicates poor hygienic and sanitary conditions during milking and subsequent handling or water supply (Yucel and Ulusoy, 2006).

Some studies have shown that cattle excreta is not a significant source of coliform contamination of raw milk, but that water used for sanitation and milking environments is considered as one of the critical sources (Kagkli et al., 2007; Martin et al., 2016). Therefore, the use of poor quality and unsanitary water during sanitation procedures can indirectly contaminate milk (Robinson, 2005).

E. coli was isolated in 100% of the samples; the presence of this bacterium in milk indicates possible contamination by contaminated manure, soil and water (Chye et al., 2004). The development of antibiotic resistance in bacteria such as *E. coli* is a serious public health problem. The results show that only one antibiotic, Imipenem, showed 100% efficacy against *E. coli* strains; of the 25 isolates tested, 72% showed resistance to at least one of the 11 antibiotics.

The highest resistance of *E. coli* isolates in this study was observed in antibiotics β-lactam. β-lactam antibiotics have low toxicity, a factor that has led to overuse of these

drugs in medical therapy (Moyane et al*.,* 2013). Few studies have noted resistance of enterobacteriaceae to the antibiotic β-lactam in milk samples (Ntuli et al*.,* 2016); a study by Geser et al*.* (2012) reported resistance to antibiotics CTX-M β-lactam in *E. coli* from milk samples.

The development of bacterial resistance to antimicrobial agents is a serious threat to human health (Zastempowska et al*.,* 2016). Although antibiotic-resistant bacteria and genes encoding antibiotic resistance have been commonly detected in wastewater and treatment system by-products, the role of wastewater treatment processes in the dissemination of antimicrobial resistance is not clear (Mohammadali and Davies, 2017). In recent years, a number of studies have focused on variables that influence the profiles of antibiotic-resistant bacteria and antibiotic resistance genes during treatment (Xia et al*.,* 2012; Yuan et al., 2014).

Hospital wastewater is likely to contribute significantly to the spread of multidrug-resistant pathogenic bacteria in wastewater treatment plants (Lien et al*.,* 2016). Due to the presence of constant sub-inhibitor levels of broad spectrum antimicrobials, hospital wastewater creates an ideal situation for the exchange of antibiotic resistance genes and their combinations between clinical pathogens and environmental bacteria (Basode et al*.,* 2018; Amador et al*.,* 2015). *Staphylococcus* was detected in all samples. The high number of isolated coagulase-negative *Staphylococcus* is believed to be due to poor milking hygiene conditions and poor quality washing water (Kouamé et al*.,* 2010; Hamiroune et al*.,* 2016).

S. aureus of environmental origin can easily colonize cow udders (Piessens et al*.,* 2011). In addition, unhygienic cow milking methods, particularly manual milking and the use of contaminated utensils, could lead to contamination of milk with *S. aureus* from foreign sources (Hamiroune et al*.,* 2016). The presence of *S. aur*eus tends to reduce the quality of milk and milk products traditionally prepared by their metabolic activities and could precipitate food poisoning due to the development of toxins that could cause disease when consumed by humans (Omoshaba et al*.,* 2018). The study of the susceptibility/resistance of *S. aureus* to antibiotics revealed high resistance frequencies, particularly for penicillin and oxacillin. The mechanism of penicillin resistance is based on the bacterium's synthesis of an enzyme called β-lactamase or penicillinase (Guérin-Faublée and Brun, 1999). This inducible plasmid enzyme hydrolyzes the β-lactam cycle of penicillins A and G and renders them inactive (Kotra and Mobashery, 1998).

Staphylococcus in hospitals, and more recently in communities (present outside the hospital environment) have developed cross-resistance between penicillins M (methicillin, oxacillin) and other β-lactamins through the production of a protein, PLP2a, which binds penicillin (PLP) and has a low affinity for these compounds (Chambers, 2001). The gene encoding PLP2a, *mec*A, is carried by a chromosomal element that also contains other genes for resistance to heavy metals and other antibiotics, which explains the multi-resistance profile of MRSA (methicillin-resistant *S. aureus*) (Dumitrescu et al*.,* 2010).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important opportunistic pathogen in humans and cattle (Omoshaba et al*.,* 2018). In this study, methicillin-resistant *Staphylococcus aureus* could be transferred from livestock to humans through milk and dairy products. Multiple antibiotic-resistant strains of *Staphylococcus*, defined as isolates resistant to three or more antibiotics, were obtained in a large proportion of the milk samples analyzed. The development of multiple antibiotic resistances in most of these isolates can be attributed to the acquisition of plasmid-mediated resistance (factor R) (Yamamoto et al*.,* 2013; Akindolire et al*.,* 2015). Usually, *S. aureus* is known to contain a number of multiple antibiotic-resistant plasmids that may explain the observed phenotypes (Yamamoto et al*.,* 2013).

These results reveal that multiple antibiotic-resistant *Staphylococcus* isolates were isolated from milk samples. It is therefore suggested that these multiple antibiotic resistant isolates can have serious health implications for people who consume such dairy products. The high number of yeasts and molds in this study may be due to poor equipment hygiene during milk handling and processing, and indicating unsanitary conditions of handling and environmental contamination (Bonfoh et al., 2003; Prejit and Latha*,* 2007). Many foodborne molds, and possibly even yeasts, can also be dangerous to human or animal health because of their ability to produce toxic metabolites called mycotoxins. Human exposure to mycotoxins can result either from the consumption of contaminated food of plant origin or from the ingestion of mycotoxins transported from animal feed into animal tissues, meat, eggs or milk (Zastempowska et al*.,* 2016). Some foodborne molds and yeasts can also cause allergic reactions or infections.

Conclusion

This study aims to assess the impact of the use of chlorine-free treated wastewater in farming and dairy cattle breeding. Milk samples were collected from a farm in Northeastern Algeria. The treated wastewater from the treatment plant is used on this farm for different activities. The results of this study indicate that the overall microbiological quality of milk samples is well below current Algerian standards; they are heavily contaminated with fecal contamination germs and pathogenic bacteria with worrying antibiotic multi-resistance profiles. The source of contamination in milk samples can be water used for three main activities: pasture farming, dairy barn operations and cleaning, and for consumption by dairy cows. The presence of

multidrug-resistant bacteria in milk can pose a serious threat to public health and has a negative effect on the treatment of infections in humans. Newborns and children appear to be more exposed to milk contaminants than adults because they consume larger amounts of milk and are more sensitive. Urgent and effective measures must be taken to ensure proper wastewater management by the services concerned and farmers. Therefore, it is recommended that training and advice be given to farm owners and workers responsible for milking, emphasizing the need for hygiene practices on farms.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Combination of genetic tools to discern *Bacillus* species isolated from hot springs in South Africa

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Using phylogenetic analysis of the 16S rRNA gene 43 Gram-positive, spore-forming bacteria of the phylum *Firmicutes* were isolated, cultured and identified from five hot water springs in South Africa. Thirty-nine isolates belonged to the family Bacillaceae, genus *Bacillus* (n = 31) and genus *Anoxybacillus* (n = 8), while four isolates belonged to the family Paenibacillaceae, genus *Brevibacillus*. The majority of isolates fell into the *Bacillus* Bergey's Group A together with *Bacillus subtilis* and *Bacillus licheniformis.* One isolate matched *Bacillus panaciterrae* which has not previously been described as a hot-spring isolate. Three unknown isolates from this study (BLAST <95% match) and three "uncultured *Bacillus*" clones of isolates from hot springs in India, China and Indonesia listed in NCBI Genbank, were included in the analysis. When bioinformatic tools: Basic Local Alignment Search Tool (BLAST), *in silico* amplified rDNA restriction analysis (ARDRA), guanine-cytosine (GC) percentage and phylogenetic analysis are used in combination, but not independently, differentiation between the complex *Bacillus* and closely related species was possible. Identification that relies solely on BLAST of the 16S rRNA sequence can be misleading.

Key words: *Bacillus*, phylogeny, ARDRA, 16S rDNA, South Africa.

INTRODUCTION

Microbes from extreme environments are interesting because they often have unique properties including extremozymes and new drug discovery potential relevant in biotechnology (Gerday, 2002; Jardine et al., 2018). López-López et al. (2013) suggested that the diversity of hot spring environments is not fully appreciated with an estimate of <1% of bacteria in hot springs that are isolated and identified using traditional culture-based methods.

Most commonly used for bacterial identification, is a comparison of the 16S rRNA (ribosomal RNA) gene sequences with known public databases, which offers no information on the physiology and biochemistry. This gene is selected because it has not changed over time and is highly conserved in different bacteria. It is however large enough (1500 bp) to allow for extraction of bioinformatic information (Janda and Abbott, 2007). Although metagenomics reveal the variety of genetic

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diversity within a microbial population, it does not take into account viability and circumstantial contamination. Therefore, any novel bacteria isolated from these unique environmental sites are essential contributions to the current database and general understanding of microbial communities. Furthermore, cultured viable bacteria are critical in understanding the biochemical potential and production of bioactive molecules related to gene expression (Handelsman, 2004).

There are additional tools to differentiate bacterial genera and species. The guanine-cytosine percentage (GC%) of the DNA of bacterial genomes varies with different genera and is useful in bacterial systematics. Also, the GC% has been correlated with the thermostability of a genome and is higher in thermophiles (Wang et al., 2006). Amplified rDNA restriction analysis (ARDRA) allows for a more accurate, rapid and efficient identification compared with the more traditional microbiological and biochemical methods (Rajendhran and Gunasekaran, 2011). A computer-simulated restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR) -amplified 16S rDNA which is the same as ARDRA, is a valid means of identifying unknown organisms (Moyer et al., 1996). The phylogenetic analysis of the 16S rRNA gene allows for maximum discrimination between closely related individual isolates taking into account each base of the entire gene, while ARDRA represents only variations in the restriction enzyme sites.

Worldwide, studies on thermophiles from hot springs have been carried out through both the use of metagenomics (López-López et al., 2013) as well as through isolation and culture-based techniques (Cihan, 2013; Khiyami et al., 2012). Metagenomic analysis has described a predominance of *Proteobacteria* and cyanobacteria in Malaysia (Goh et al., 2011), India (Sharma et al., 2014) and South Africa (Tekere et al., 2011, 2012). However, by isolation and culture, the predominant bacteria are Gram-positive spore-forming *Bacillus* and *Bacillus*-related microorganisms reported in India (Panda et al., 2016), Saudi Arabia (Khiyami et al., 2012), Armenia (Panosyan and Birkeland, 2014) and Jordan (Obeidat et al., 2012).

The classification of the genus *Bacillus* was transformed by major changes where several new genera were proposed (Ludwig et al., 2009). In addition, this is a highly diverse and expanding group, with 25 new genera being described in the past two years (Mandic-Mulec et al., 2015). The relatively new genus *Anoxybacillus,* was established in 2000 and is growing rapidly with six new species being described since 2011 (Mandic-Mulec et al., 2015). Of the 115 endospore-forming *Bacillus* isolates from geothermal regions in Turkey, *Anoxybacillus* was the most abundant, being represented by 53 isolates (Cihan, 2013) suggesting that geothermal environments could be a niche for the discovery of new *Anoxybacillus* species. Because the genera *Bacillus* and *Anoxybacillus* have been reclassified and novel species are being described at a rapid rate, there may be some incongruence and confusion when comparing the nomenclature of this group from studies prior to the reclassification, and between different studies where the new nomenclature is not taken into account.

In South Africa, more than a third of the 80 hot springs are located in the Limpopo Province. Metagenomic studies of four hot springs revealed only a very low abundance of the phylum *Firmicutes* which includes *Bacillus* and *Bacillus*-related species (Tekere et al., 2011, 2012). Various other phyla were reported but in very small percentages of the total rRNA sequences (<0.2%). The discovery that these hot springs hold a great diversity of bacteria suggests that it may be a resource for potential thermophiles that could have novel biotechnological applications. The aims of this study were to use conventional culture techniques for the isolation of bacteria and to use the 16S rRNA gene sequence analysis for genotypic identification of the isolates. Besides, the sequences were analysed for GC%, ARDRA, and phylogenetic analysis. The use of a combination of tools for identification was investigated.

MATERIALS AND METHODS

Sampling and sampling sites

Water and sediment samples from five hot springs (Tshipise, Siloam, Mphephu, Lekkerrus, and Libertas) in the Limpopo Province, South Africa were sampled. Their geographical location with GPS coordinates, average water temperature and pH conditions and local site description have been previously described (Olivier et al., 2011; Jardine et al., 2017).

Isolation of bacteria and determination of optimal growth conditions

Aliquots of 100 mL of water were passed through a 0.22 µm membrane filter and the membrane filters were then placed on the surface of different agar media (Himedia, India): nutrient agar, Actinomycete isolation agar, minimal Luria broth media, potato dextrose agar and cyanobacterial agar for 48 h at 37 and 53°C exactly as described by Jardine et al. (2017). Bacterial isolates from sediment samples were obtained using the streak plate method. Once pure cultures of the isolates were obtained, they were studied for optimal conditions of growth relating to temperature, pH and salinity in order to maintain them in the laboratory.

The optimum pH, temperature and salinity for growth of the bacteria initially isolated at 53°C were determined, by growth in nutrient broth from pH 6 to 10 in intervals of one unit, temperatures between 45 and 70°C in intervals of 5°C, and sodium chloride (NaCl) at concentrations ranging from 0 to 15% w/v, respectively. A bacterial suspension at an optical density (OD) at 600 nm (OD_{600}) of approximately 0.3 was made, and 1 mL volumes of nutrient broth were inoculated with 10 µL of the bacterial suspension, incubated under various conditions. The OD at 600 nm was measured using a spectrophotometer (Phillips PU8620 UV/VIS/NIR) to determine whether growth had occurred.

DNA extraction protocol, 16S rRNA gene sequencing and phylogeny

DNA extraction and 16S rRNA gene sequencing have been previously described in Jardine et al. (2017) without any modifications. DNA was extracted by the method described by Dashti et al. (2009), and the 16S rRNA gene was subjected to PCR with universal primers 8F, 27F and 1472R (Galkiewicz and Kellogg, 2008) with the cycling conditions as described by Jardine et al. (2017). The amplicon was Sanger sequenced with Big Dye Terminator 3.1 cycle sequencing kit (ABI) according to the manufacturer's instructions, at the African Centre for DNA Barcoding (ACDB), University of Johannesburg. The resulting consensus sequence of approximately 1400 bp was compared with those in the NCBI database (Genbank) using the Basic Local Alignment Search Tool (BLAST) (McGinnis and Madden, 2004), and EzTaxon-e (Kim et al., 2012). Isolates with a >99% match to the published sequences were identified to the species level, and those with a >97% match were identified to the genus level (Yarza et al., 2014). Alignments were made by CLUSTAL OMEGA (www.ebi.ac.uk), and manually refined using SeaView (Gouy et al., 2010). Statistical confidence in branching points was determined by 1000 bootstrap replicates. Complete and partial sequences from this study were submitted to Genbank. The Genbank accession numbers of the type strains used in the phylogenetic trees are listed in Appendix A.

Computer-simulated PCR-RFLP or amplified rDNA restriction analysis (ARDRA)

Computer-simulated PCR-RFLP patterns were generated from the approximately 1400 bp sequence of the 16S rRNA gene (using the computer program RestrictionMapper version 3 "www.Restrictionmapper.org") and restriction enzymes, *Alu1* (15 sites), *Taq1* (18 sites) (Wu et al., 2006), *HaeIII* (24 sites), *Hinf1* (21 sites), *Rsa1* (18 sites) (Wahyudi et al., 2010), *Hph1* (23 sites), *MboII* (15 sites) and *Fok1* (14 sites). The presence and absence of the simulated band was used to create a binary data file and the results were present together as a composite. Several bacterial strains from published data were included in the study, to determine the phylogenetic groups into which the isolates fell. The SeaView program was used to analyze the binary data, and a distance neighbour-joining tree was created for detection of clusters.

Guanine-cytosine (GC) content (in percentage)

The GC% for the *Firmicutes* group was calculated with the 1400bp 16S rRNA gene fragment (Yamane et al., 2011) using the ENDMEMO GC calculating tool (www.endmemo.com/bio/gcratio) for all the isolates.

Bacterial strains in this study

The 16S rRNA gene sequences of hot spring isolates from South Africa were allocated accession numbers and deposited in Genbank as indicated in Table 1. Strains of *Anoxybacillus* spp. have accession numbers: MF037806, MF037807, MF037808, MF037809, MF037810, MF037811, MF037812 and MF037813. Strains of *B. licheniformis* were assigned numbers: MF037814, MF037815, MF037816, MF037817, MF037818, MF037819, MF037820, MF037821 and MF037822. Accession numbers: MF037827, MF037828, MF037829, MF037830, MF037831, MF037832, MF037833, MF037834, MF037835, MF037836, MF037837, and MF037838 were given to *B. subtilis* strains. *Bacillus* spp. has Genbank accession numbers: MF038049, MF038050, MF038051, and MF039084. Single isolates were assigned the following accession numbers: *Bacillus pumilus* MF038052, *Bacillus panaciterrae* MF038053, *Bacillus methylotrophicus* MF038054, *Solibacillus* species MF039085, and *Aneurinibacillus* species MF040218. MF038055, MF038056, MF038057, and MF038058 were numbers allocated to four *Brevibacillus* species. All reference strains used for analyses are listed in Appendix A together with their associated Genbank accession numbers.

RESULTS

Optimal growth conditions for bacterial isolation and growth

The optimal growth conditions for the isolates were determined as the bacteria needed to be grown as inoculum for further experiments and investigations. The average optimal pH was 7, the average optimal temperature was 55°C, and the average optimal salinity was 5%. However, 19% were also able to grow in 10% salinity. These results are available in Jardine (2017).

16S rRNA gene sequencing

The contiguous sequences were compared to two databases, namely Genbank and EzTaxon-e and the highest percentage similarities and accession numbers are listed in Table 1. Values >97% suggest a match to the genus level, while a value of >99% suggests a match to species level (Yarza et al., 2014). Where no PCR product was obtained, the sequencing was not determined (nd), and in some cases, sequencing was incomplete which did not allow for a full consensus sequence to be constructed. Sequences from this study were submitted to Genbank with their relevant accession numbers as listed earlier.

Percentage guanine-cytosine (GC) content

The GC% and accession numbers for 31 *Bacillus* spp., eight *Anoxybacillus* spp., five *Brevibacillus* spp., one *Aneurinibacillus* spp., and reference strains (from Genbank) are listed in Appendix B for the approximately 1400 bp 16S rRNA gene fragment. Based on the GC% of the 16S rRNA sequences for the isolates in this study, they were grouped into four genera (*Anoxybacillus, Bacillus, Aneurinibacillus*, and *Brevibacillus*). The average and standard deviations for the GC% for these isolates together with reference strains were calculated (Appendix B) and plotted illustrated in Figure 1. The isolates that fell out of one standard deviation range of the average GC% were earmarked as potentially different from the group, that is, isolates 1T, 11T, 14S and 33Li (Appendix B as indicated by*). In all other respects, there was a

Table 1. Identification of hot spring isolates in South African using 16S rDNA sequences.

Isolate No.*	Isolation Temp °C **	GenBank BLAST	GB access No.	EzTaxon-e	EzTaxon-e Access No.	Submit Access No.	GC content***	ARDRA****	Phylogeny
3T	53	*Anoxybacillus rupiensis* 94%	KJ842629.1	*Anoxybacillus rupiensis 99%*	AJ879076	MF037806	√ (56.24)	A	*Anoxybacillus*
4T	53	*Anoxybacillus rupiensis* 99%	AM988775.1	*Anoxybacillus rupiensis 99%*	AJ879076	MF037807	√ (56.15)	A	*Anoxybacillus*
7T	53	*Anoxybacillus* sp. ATCC 99%	KJ722458.1	*Anoxybacillus rupiensis 99%*	AJ879076	MF037808	√ (56.72)	A	*Anoxybacillus*
11T	53	*Anoxybacillus* sp. 94%	KF254912.1	*Anoxybacillus rupiensis 98%*	AJ879076	MF037809	X (55.28)	C	*Anoxybacillus*
13S	53	*Anoxybacillus* sp. 99%	FN432807.1	*Anoxybacillus rupiensis 98%*	AJ879076	MF037810	√ (56.51)	A	*Anoxybacillus*
17S	53	*Anoxybacillus flavithermus* 99%	KF039883.1	*Anoxybacillus mongoliensis 98%*	EF654664	MF037811	√ (56.22)	C	*Anoxybacillus*
19S	53	*Anoxybacillus* sp. 99%	KP221933.1	*Anoxybacillus rupiensis 99%*	AJ879076	MF037812	√ (56.33)	A	*Anoxybacillus*
43T	53 S	*Anoxybacillus flavithermus* 99%	KC503890.1	*Anoxybacillus flavithermus 99%*	AVGH01000041	MF037813	√ (56.26)	C	*Anoxybacillus*
2T	53	*Bacillus licheniformis* 99%	HM631844.1	*Bacillus licheniformis ATCC 98%*	AE017333	MF037814	√ (55.4)	B	*Bacillus* group A
6T	53	*Bacillus licheniformis* 99%	KJ729823.1	*Bacillus licheniformis ATCC 99.8%*	AE017333	MF037815	√ (55.43)	C	*Bacillus* group A
8T	53	*Bacillus* sp. 98%	GU132507.1	*Bacillus licheniformis ATCC 97%*	AE017333	MF037816	√ (55.05)	B	*Bacillus* group A
10T	53	*Bacillus licheniformis* 97%	HM631844.1	*Bacillus licheniformis ATCC 98%*	AE017333	MF037817	√ (55.0)	B	*Bacillus* group A
20S	53	*Bacillus subtilis* 99%	KC634086.1	*Bacillus licheniformis ATCC 99.8%*	AE017333	MF037818	√ (55.6)	C	*Bacillus* group A
28M	53	*Bacillus licheniformis* 99%	GQ340513.1	*Bacillus licheniformis ATCC 99.8%*	AE017333	MF037819	√ (54.84)	C	*Bacillus* group A
30M	53	*Bacillus licheniformis* 99%	KJ526854.1	*Bacillus licheniformis ATCC 99%*	AE017333	MF037820	√ (55.15)	B	*Bacillus* group A
39T	37-53	*Bacillus licheniformis* 99%	KF879197.1	*Bacillus licheniformis ATCC 99.7%*	AE017333	MF037821	√ (54.52)	C	*Bacillus* group A
74T	37	*Bacillus licheniformis* 99%	JN366749.1	*Bacillus sonorensis 99.6%*	AYTN01000016	MF037822	√ (54.97)	B	*Bacillus* group A
12S	53	*Bacillus subtilis* 99%	KC634086.1	*Bacillus subtilis 99%*	AMXN01000021	MF037827	√ (54.48)	C	*Bacillus* group A
14S	53	*Bacillus* sp. 98%	CP11051,1	*Brevibacterium halotolerans 97%*	AM747812	MF037828	X (54.23)	B	*Bacillus* group A
21M	53	*Bacillus subtilis* 99%	JN585712.1	*Bacillus subtilis 99.7%*	AMXN01000021	MF037829	√ (55.04)	B	*Bacillus* group A
22M	53	*Bacillus methylotrophicus* 99%	JQ765577.1	*Bacillus tequilensis 99%*	AYTO01000043	MF037830	√ (54.64)	nd	*Bacillus* group A
33Li	53	*Bacillus subtilis* 98%	KC182058.1	*Bacillus subtilis 99.7%*	AMXN01000021	MF037831	X (54.21)	C	*Bacillus* group A
40Le	37-53	*Bacillus subtilis* 99%	KP249695.1	*Bacillus tequilensis 99.6%*	AYTO01000043	MF037832	√ (54.57)	B	*Bacillus* group A
41Li	37-53	*B licheniformis* 97%	KC429774.1	*Bacillus subtilis 96%*	AMXN01000021	MF037833	√ (54.69)	B	*Bacillus* group A
47Li	53 S	*Bacillus subtilis* 99%	KP249695.1	*Bacillus subtilis 99.9%*	AMXN01000021	MF037834	√ (54.53)	C	*Bacillus* group A
48Li	53 S	*Bacillus subtilis* 99%	NR_118486.1	*Bacillus subtilis 99.9%*	AMXN01000021	MF037835	√ (54.86)	B	*Bacillus* group A
54T	37	*Bacillus subtilis* 99%	HM753614.1	*Bacillus subtilis 98.9%*	CP002905	MF037836	√ (54.69)	B	*Bacillus* group A
78S	37	*Bacillus* sp. 99%	KF984420.1	*Bacillus subtilis 99%*	ABQL01000001	MF037837	√ (54.45)	C	*Bacillus* group A
83Li	37	*Bacillus subtilis* 97%	KF533727.1	*Bacillus subtilis 96%*	ABQL01000001	MF037838	√ (55.44)	B	*Bacillus* group A
1T	53	*Bacillus subtilis* 96%	HM367735.1	*Bacillus subtilis 96%*	AMXN01000021	MF038049	X (52.86)	C	*Bacillus* group A
15S	53	*Bacillus licheniformis* 94%	HM055609.1	*Bacillus licheniformis 94%*	AE017333	MF038050	√ (55.11)	B	*B. licheniformis*
18S	53	*Bacillus* sp. 96%	GU132507.1	*Bacillus licheniformis 95%*	AE017333	MF038051	√ (54.71)	B	*B. licheniformis*
52M	53 S	*Brevibacillus agric* 94%	JN812211.1	*Brevibacillus agri 94%*	D78454	MF039084	√ (55.09)	C	*Bacillus* group A
24M	53	*Bacillus pumilus* 99%	KJ526891.1	*Bacillus aerophilus 99.8%*	AJ831844	MF038052	√ (54.8)	A	*Bacillus* group A

Table 1. Contd.

32Le	53	*Bacillus panaciterrae* 99%	NR_041379.1	*Bacillus panaciterrae 99%*	AB245380	MF038053	√ (54.36)	C	*Bacillus* group K
77S	37	*Bacillus methylotrophicus* 99%	KP342210.1	*Bacillus methylotrophicus 99.9%*	JTKJ01000077	MF038054	√ (54.4)	C	*Bacillus* group A
73T	37	*Solibacillus sylvestris* 95%	KF441704.1	*Solibacillus sylvestris 95%*	AJ006086	MF039085	X (53.91)	C	*Solibacillus*
86Li	37	*Aneurinibacillus migulanus* 96%	NR_113764.1	*Aneurinibacillus migulanus 96%*	X94195	MF040218	√ (56.82)	C	*Brevibacillus*
16S	53	*Brevibacillus* sp. 99%	LN681596.1	*Brevibacillus formosus 97%*	LDCN01000015	MF038055	√ (54.72)	C	*Brevibacillus*
36Li	53	*Brevibacillus* sp. 99%	KM403208.1	*Brevibacillus agri 99%*	D78454	MF038056	√ (55.54)	C	*Brevibacillus*
70T	37	*Brevibacillus* sp. 99%	GQ497292.1	*Brevibacillus fluminis 99%*	EU375457	MF038057	√ (55.3)	C	*Brevibacillus*
85Li	37	*Brevibacillus formosus* 97%	KP165013.1	*Brevibacillus brevis 98%*	AB271756	MF038058	√ (54.25)	C	*Brevibacillus*
53M	53 S	*Brevibacillus* sp. 92%	GQ497292.1	*Brevibacillus fluminis 89%*	EU375457	nd	√ (56.73)	C	nd
75S	37	Uncultured bacterium 94%	KJ013386.1	*Kocuria sediminis 92%*	JF896464	nd	X (57.43)	C	nd

*Locations of hot springs: S (Siloam), T (Tshipise), M (Mphephu), Li (Libertas) and Le (Lekkerrus); **(S) Isolation from sediment. Default: isolation from water; ***(X) If outlier within the group or (√) if not using GC percentage grouping. ***ARDRA Amplified rDNA restriction analysis.

general match with the GC% and groupings into the four genera.

Figure 1 shows GC% of the family Bacillaceae including genera *Anoxybacillus* and *Bacillus*, the family Paenibacillaceae including the genera *Aneurinibacillus* and *Brevibacillus*, and unclassified *Bacillales* genus *Solibacillus* where the standard deviations of the two genera within the families did not overlap and were therefore different. Therefore, *Aneurinibacillus* could be distinguished from *Brevibacillus*, and similarly, *Anoxybacillus* could be distinguished from *Bacillus* based on GC%.

Computer-simulated amplified (16S rRNA) ribosomal RNA restriction analysis or ARDRA

In this investigation, ARDRA analysis of the eight restriction enzyme patterns was compiled resulting in an accumulative 148 sites which were aligned and analyzed using SeaView (Gouy et al., 2010) and presented as a distance neighbour-joining dendogram (Figure 2). The three main groups (A, B, C) are listed in Table 1. Group A included the *Bacillus* reference type strains determined by Bergey's classification (Ludwig et al., 2009) as well as the closely knit group of *Anoxybacillus* reference strain with *Anoxybacillus* spp. from this study. Three uncultured unknown *Bacillus* spp. previously reported from other hot spring studies (uncultured *Bacillus* clones TPB_GMAT_AC4, DGG30 and KSB12) fell into this group A together with isolate 24M. *Bacillus* spp. from this study fell into both groups B and C, however the *Brevibacillus* spp. and the single *Aneurinibacillus* spp. all fell into group C.

Phylogenetic analysis

Phylogenetic analysis of the 16S rRNA gene is commonly used for bacterial identification with greater accuracy than only a BLAST search as it defines the relationship between individual bacteria at every base. It is also more accurate than ARDRA which only provides information at the site of the restriction enzyme activity. A comparison of the three molecular tools (16S rRNA BLAST search, GC% and ARDRA) and phylogenetic tree analysis shows that there was, in general, good correlation with the grouping of the phylum *Firmicutes* into family Bacillaceae with genera *Bacillus* (n = 31) and *Anoxybacillus* (n = 8), and family Paenibacillaceae genera *Brevibacillus* (n = 3) and *Aneurinibacillus* (n = 1). A neighbour-joining phylogenetic tree of a 914 bp fragment of the 16S rRNA gene sequences between isolates from this study and representative members of type strains of *Anoxybacillus*, *Bacillus*, *Brevibacillus*, and *Aneurinibacillus* is presented in Appendix C supporting the information presented in Table 1.

In order to further discern whether the *Bacillus* spp. in the study fell into specific Bergey's groupings (Ludwig et al., 2009), a maximum likelihood phylogenetic tree (% bootstrap values based on 1000 replicates) was drawn with additional reference strains as shown in Figure 3. Isolates 18S and 15S grouped with the reference strains (none Bergey's *Bacillus* A group) and this is consistent with their low match by BLAST of 96

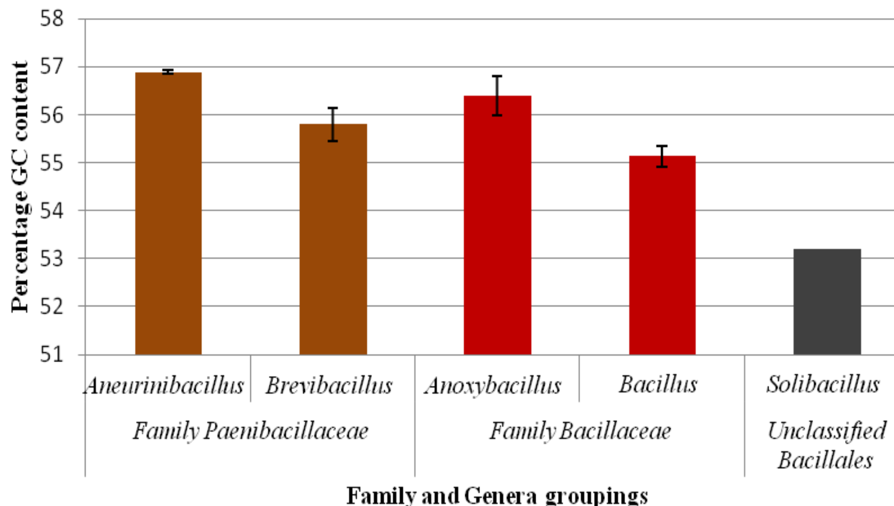

Figure 1. Average GC% of 16S rDNA sequence of isolates from this study and reference type strains of genera *Aneurinibacillus* and *Brevibacillus* (family Paenibacillaceae) and genera *Anoxybacillus* and *Bacillus* (family *Bacillaceae*) and unclassified genus *Solibacillus* showing that GC% can be used to distinguish between genera.

and 94%, respectively. Isolate 32M was confirmed as closely related to *B. panaciterrae*. Two large groups of isolates contained *B. licheniformis* reference strain, and *B. subtilis* reference strains. Type strains of *Bacillus aerophilous/B. pumilus* and *B. methylotrophicus* type strains and the corresponding new isolates from this study (24M and 77S) fell into Bergey's Group A with *B. subtilis.*

Family Paenibacillaceae genus *Brevibacillus*

Paenibacillaceae refers to "nearly *Bacillus*", and phylogenetically, the genus *Brevibacillus* is distinct from the genus *Bacillus* (Xu and Cote, 2003). Four isolates, namely 16S, 36Li, 70T, and 85Li were identified as *Brevibacillus* spp. by BLAST search and confirmed by ARDRA (Figure 2), phylogenetic analysis (Appendix C) and supported by GC% values (Table 1 and Figure 1).

Analysis of unknown isolates

The GC% of four isolates with a <97% BLAST match to published 16S rRNA sequences was significantly different from within their group, that is, isolate 11T within the *Anoxybacillus* group, and isolates 1T, 14S, and 33Li within the *Bacillus* group (Table 1). By ARDRA analysis, the difference was confirmed with 11T that was similar to *Anoxybacillus* spp. by BLAST but fell in ARDRA group C with *Bacillus/Brevibacillus* spp. but no further information could be attained with the other three isolates. Therefore, isolates that were not definitively identified by BLAST (<97%) could be further differentiated by ARDRA. Three isolates (15S, 52M and 73T) were even more poorly matched (<95%) by BLAST.

DISCUSSION

Optimal growth conditions for bacterial isolation and growth

In order to maintain the stock cultures, it was necessary to determine the optimal temperature, pH and salinity conditions for growth. The results showed that most of the bacteria preferred a neutral pH of 7, an incubation temperature ranging between 50 and 55°C, and salinity of 5% NaCl (w/v). The range of temperature was selected because the average temperature of the five hot springs was 52°C. Obeidat et al. (2012) tested eight *Geobacillus* species. from hot springs in Jordan with temperatures ranging from 48 to 62°C, and pH between 6 and 7, and found the optimal temperatures to be between 60 and 65°C, and pH 6 to 8. Zhang et al. (2011) reported on two isolates of *Anoxybacillus* with an optimal growth temperature of 55°C and pH of 8. It therefore appears that these spore-forming *Bacillus* and *Bacillus*-related organisms are robust with a tolerance for a wide range of environmental conditions. Extremophiles isolated in this study include the alkaliphilic thermophile *Anoxybacillus flavithermus* with an optimal pH of 10 and a temperature of 50°C (isolate 17S), thermophilic *Anoxybacillus rupiensis* with an optimal temperature of 60°C (isolate 13S), and halotolerant thermophiles of *B. licheniformis* that could grow in 10% (w/v) NaCl (isolates 2T, 6T and 8T)

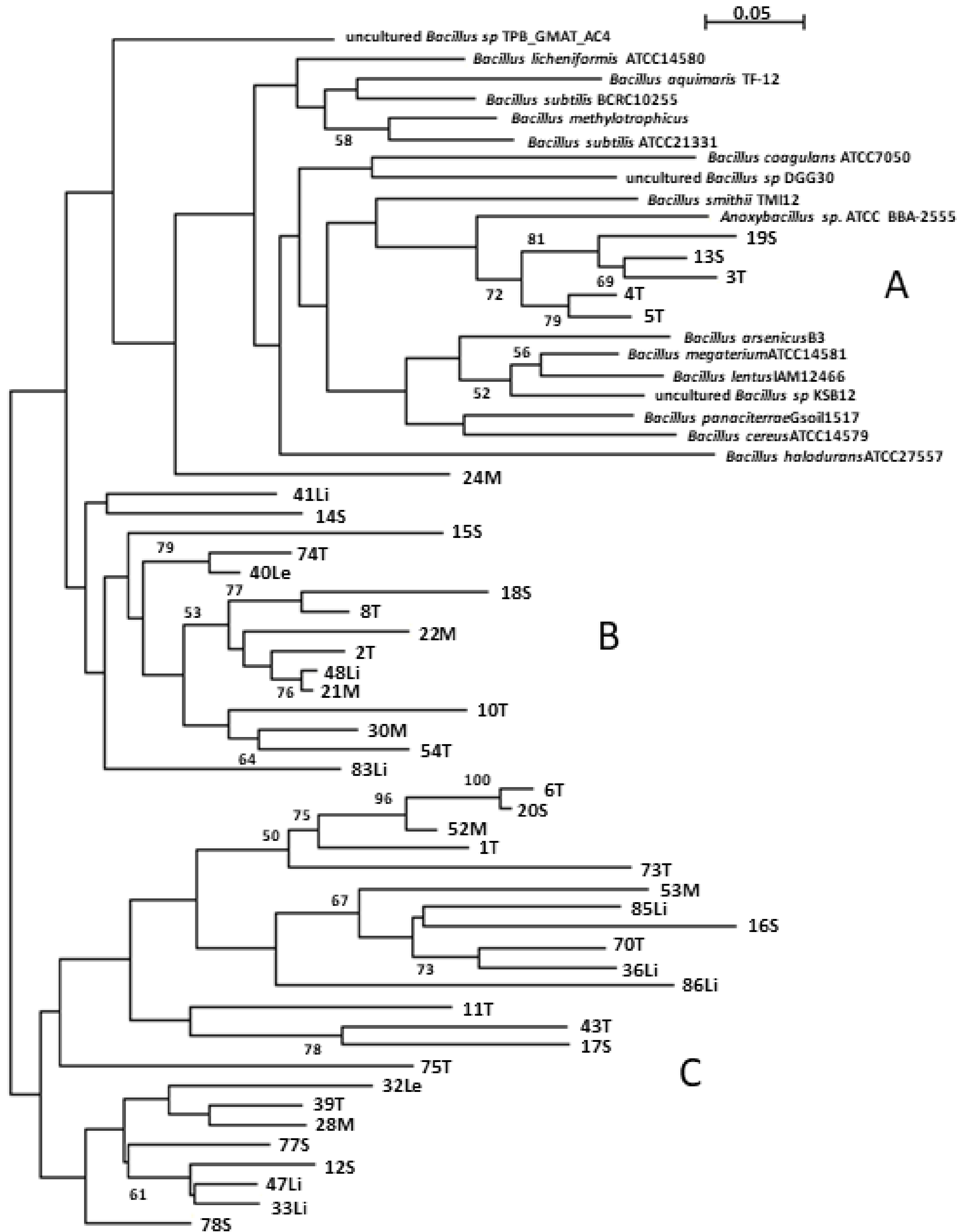

Figure 2. The combined binary data from eight different restriction enzyme digests of the 16S rDNA sequence was used to draw a neighbour-joining distance dendogram that showed >50% bootstrap of 1000 replicates. Three main clusters (A-C) are indicated in the diagram.

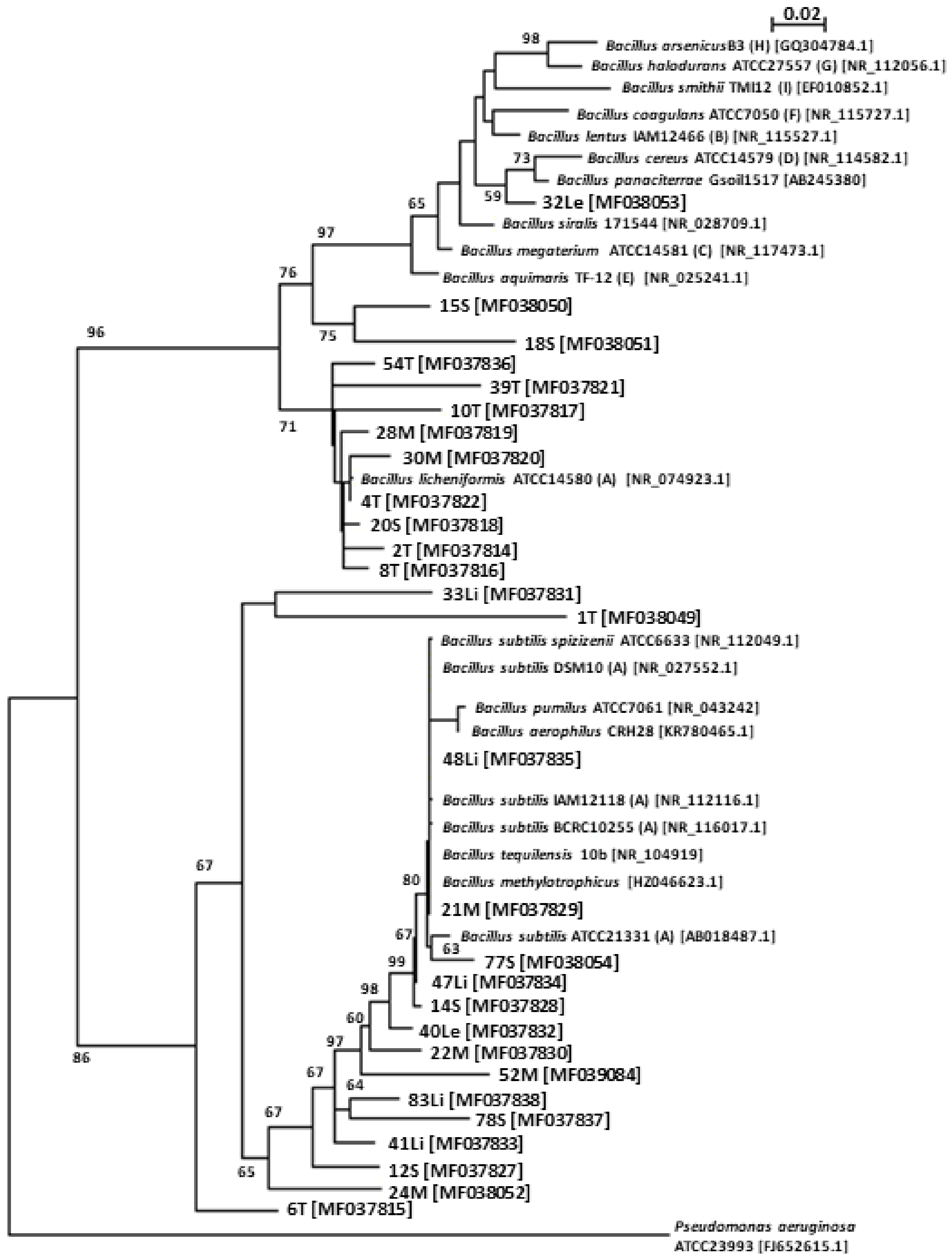

Figure 3. A maximum-likelihood phylogenetic tree of a 914 bp fragment of the 16S rRNA gene sequences of *Bacillus* isolates from this study and representative members of type strains of differently Bergey's groups A-K confirming that most isolates in this study fell into group A (*B. subtilis/B. licheniformis*). Bootstrap values (%) are based on 1000 replicates and shown for branches with more than 50% bootstrap support. Bar indicates 0.02 substitutions per 100 nucleotide positions.

(Jardine, 2017).

16S rRNA gene sequencing

Molecular techniques based on genetic sequencing have far surpassed the traditional culture methods to predict biochemical and phenotypic information of a single bacteria or a population. Handelsman (2004) elaborated that culture methods are dependent on environmental and external factors, can be time-consuming, laborious and subject to error. Phenotypic characteristics related to colony morphology, biochemical reactions, serology, pathogenicity and antibiotic resistance can vary considerably, unlike DNA that remains relatively unchanged. The 16S rRNA gene sequence has been used as the gold standard for identification of microorganisms, because it is relatively conserved in all microorganisms, with similarities to allow for PCR with universal primers but enough variability to permit differentiation between species (Rajendhran and Gunasekaran, 2011; Yıldırım et al., 2011). Conventionally, to identify unknown bacterial isolates, the 16S rRNA gene sequences are compared with those in the Genbank database (BLAST), and the closest similarities are listed in Table 1. However, the disadvantage of this tool is that public contributions create the database, and therefore it is possible that unknown sequences may be compared to misidentified or incorrectly named strains. Therefore, to confirm the BLAST results, the sequences were also compared with a more specific 16S rRNA prokaryote gene sequence database using EzTaxon-e (ChunLab USA Inc), a Web-based tool for the identification of prokaryotes (including uncultured prokaryotes). This database is manually curated and quality controlled, and thus less susceptible to be contaminated by false species identifications made by the public and hence, it would be more accurate. For example, in the case of *Bacillus* spp., the results of the BLAST search will not take into account the new reclassification of genera in 2009, and current changes within the group's nomenclature, therefore the results may be erroneous or out of date. Whichever database is used, the cut-off value for the percentage similarity is also critical. Yarza et al. (2014) and López-López et al. (2013) described with statistical proof that >97-98% allows for determination at a species level.

Other investigators have used >97% as a cut-off value (Belduz et al., 2003; Drancourt and Raoult, 2005). When the value is lower than 95%, the result cannot be accurate at the genus or species level.

Percentage guanine-cytosine (GC) content

The GC% of a fragment of DNA or the whole genome refers to the proportion of DNA that is either G-C, but not A-T, with all the bases present. The G-C bond is stronger than an A-T bond in DNA resulting in a more stable DNA molecule. The GC% of a bacterial genome and the GC% of the stem of the 16S rDNA have been correlated with optimal growth temperatures (Galtier and Lobry, 1997; Wang et al., 2006). Furthermore, the GC% varies among different genera (Muto and Osawa, 1987) which has led to its inclusion as supportive information in the taxonomic classification of bacteria. The GC% of the 16S rRNA gene was included in this study (Figure 1) to establish whether this ratio could be useful in showing which strains were similar and whether it was useful in discriminating between different genera to determine if supportive data could be generated for the discrimination of different bacteria. The results of this study showed that different genera could be distinguished from each other, *Aneurinibacillus* from *Brevibacillus*, and similarly *Anoxybacillus* from *Bacillus* based on GC% providing supportive information but cannot be used in isolation for identification at a genus level.

Computer-simulated amplified ribosomal DNA restriction analysis (ARDRA)

ARDRA is based on the number and size of fragments that are generated when a PCR product of the 16S rRNA gene is digested with a restriction enzyme, and the fragments are separated according to their lengths by agarose gel electrophoresis. The generated pattern can discriminate between species depending on the enzyme used (Rajendhran and Gunasekaran, 2011). The use of computer-simulated fragments is a valid assessment of genotyping (Moyer et al., 1996; Wei et al., 2007; Sklarz et al., 2009), and is even faster and more cost-effective than digesting with the enzyme in the laboratory. In this study, ARDRA was not performed in the laboratory but *in silico*, due to limited resources. Restriction enzymes were selected based on previous investigations of ARDRA on *Bacillus* spp. namely *Rsa1, Hinf1* and *HaeIII* (Wahuydi et al., 2010), *Alu1, Taq1* and *Rsa1* (Wu et al., 2006), *HaeIII* and *Alu1* (Rai et al., 2015) and *Hph1*, *MboII* and *Fok1* which resulted in several different fragments when processed by the online tool, Restrictionmapper. In this study, the eight restriction enzymes (*Alu1*, *Taq1, HaeIII, Hinf1*, *Rsa1, Hph1*, *MboII* and *Fok1)* used independently did not produce informative clustering since each enzyme only revealed information from 15 to 25 sites, and therefore a total of 148 sites from all the restriction enzyme patterns were analysed together. This produced a dendogram with three main groups (A, B, C) (Figure 2). There was some overlap with the maximum likelihood phylogeny tree (Figure 3) although with a much lower expected resolution. *Anoxybacillus* spp. clustered separately from the *Bacillus* spp. with a convincing

bootstrap value of 72%. *In silico* ARDRA with eight restriction enzymes could not discern the Bergey's groupings of *Bacillus* (A-K) but were useful in demonstration similarities between isolates in this study. This will be discussed further in the identification of isolates that were poorly defined by <95% match with BLAST.

Although the usefulness of ARDRA to cluster related bacteria has been described (Rahmani et al., 2006), including *Bacillus* spp. (Rai et al., 2015) from hot springs (Pagaling et al., 2012), soil environments (Wu et al., 2006; Wahyudi et al., 2010), clinical, dairy and industrial settings (Logan et al., 2002), Sklarz et al. (2009) warned that its prediction power to identify clones should be cautioned. In the latter study of computer generated ARDRA of 48 759 sequences from a ribosomal database, they reported that clones could be separated into different genera, but the clusters did not overlap with phylogenetic analysis of sequence data. This was supported and confirmed by this study. In combination with GC%, BLAST and phylogeny, *in silico* ARDRA is a useful tool for identification of bacteria using 16S rDNA, with phylogenetic analysis providing the most discriminating and accurate information. Because *in silico* findings completely reflected experimental results reported in *Lactobacillus* species (*Firmicutes*) (Oztuk and Meterelliyoz, 2015), and the shortage of publications describing computer generated ARDRA in *Bacillus* spp., the following discussions include comparisons with investigations where ARDRA was not simulated.

Phylogenetic analysis

The family Bacillaceae are distinguished by their ability to form heat tolerant endospores, and as a result, they are abundant, robust and well distributed in many environmental niches, including hot springs. The prototype *B. subtilis* was first described in 1872, and prior to the 1990s, the genus *Bacillus* mainly constituted the family Bacillaceae. However, since then, many significant taxonomic changes have occurred, which has resulted in new genera being described and several species formerly '*Bacillus"* now reclassified into other genera. Consequently, a comparison with older published literature revealed that previously named "*Bacillus"* would appear as other genera in later publications. Mandic-Mulec et al. (2015) reviewed this group in great detail. Also, this family is expanding extremely rapidly with 25 new genera described in 2013 and 2014, and a total of 62 genera listed in 2015. The genus *Bacillus* within the family Bacillaceae is the largest group with 226 species described in 2015, and it is expanding rapidly with 38 new species having been described between August 2013 and March 2015.

Some of these species are represented by only one isolate making verification challenging and increasing the complexity of *Bacillus* phylogeny. This confusion regarding the phylogeny of the genus *Bacillus* was reported by Maughan and Van der Auwera (2011) who observed that phenotypic groupings are not congruent with 16S rRNA groupings because this group is phenotypically so variable. A comparison of publications on *Bacillus* phylogeny in 1991 (Rössler et al., 1991), in 2003 (Xu and Cote, 2003) and 2009 (Ludwig et al., 2009) confirm the exploding evolutionary changes in this group's nomenclature. As a result, the nomenclature and classification of this group are challenging, and difficult to keep up to date.

Therefore, a literature review of *Bacillus* spp. isolated from hot springs will result in different nomenclature used depending on the date of publication. What may have been previously called *Bacillus* could be named "*Geobacillus*" or "*Paenibacillus*" (meaning "almost *Bacillus*") in later publications introducing incongruence between different studies. A significant proportion of publications on the identification of *Bacillus* spp. from hot springs rely on only one tool of identification, a comparison of 16S rRNA gene sequence to a public database, that is, BLAST (Ghalib et al., 2014; Obeidat et al., 2012) which has it shortcomings as previously mentioned. However, this study will show that other means of genotyping, such as phylogenetic analysis, can disprove conclusions that are based only on the BLAST tool. The consensus of an identification using the16S rRNA gene sequence on Genbank BLAST, is >97% match (Yarza et al., 2014), and if studies are not stringent in applying this cut-off value, and merely report bacterial identification based on any genetic similarity, this leads to more "misidentification" within this group.

Family Bacillaceae genus *Anoxybacillus*

As compared to other groups, the *Anoxybacillus* group is relatively new, having been established in 2000. Cihan et al. (2012) suggested that *Anoxybacillus* is the most dominant genus in hot springs. Twelve of the 15 new species of *Anoxybacillus* listed in Appendix A were isolated from hot springs. Thirty-five of the 53 isolates of *Anoxybacillus* from hot springs in Turkey showed uniquely different patterns with ARDRA compared with 12 type species (Cihan, 2013) providing further evidence that new species of *Anoxybacillus* can be found in hot springs and that differentiation from reference strains is discernible by 16S rRNA phylogeny and ARDRA. A BLAST search confirmed that eight isolates from this study were *Anoxybacillus* spp. including *A. flavithermus* and *A. rupiensis*. The neighbour-joining phylogenetic tree grouped the eight isolates, with convincing bootstrap values (Appendix C).

However, the GC% of isolate 11T differed by more

than one standard deviation from published *Anoxybacillus* spp. data (Appendix B), and the other *Anoxybacillus* isolates from this study. Results from ARDRA analysis confirmed that isolate 11T did indeed group separately from the *Anoxybacillus* cluster A (Figure 2), and requires further investigation.

Family Bacillaceae genus *Bacillus*

The majority of the isolates in this study fell within the genus *Bacillus*, more specifically into Bergey's Group A which includes *B. subtilis* and *B. licheniformis*, two very closely related species (Ludwig et al., 2009). These two species are commonly described as isolates from hot springs in many investigations. In order to ensure that the isolates did not fall into other Bergey's groups not represented in the phylogenetic tree of Appendix C, another phylogenetic tree was drawn with only the *Bacillus* isolates from this study and reference type strains from Bergey's Group B (*Bacillus lentus*), Group C (*Bacillus megaterium*), Group D (*Bacillus cereus*), Group E (*Bacillus aquimaris*), Group F (*Bacillus coagulans*), Group G (*Bacillus halodurans*), Group H (*Bacillus arsenicus*), Group I (*Bacillus smithii*) and Group J (*Bacillus panaciterrae*) (Figure 3). The sequences were obtained from published databases as listed in Appendix A. It confirmed that all the isolates in this study clustered with Group A: *B. subtilis*/*B. licheniformis* by phylogeny and that the single isolate 32Le was found not to be Bergey's Group A *Bacillus* spp. but clustered with *B. panaciterrae* as confirmed by the Genbank BLAST result. Isolate 32Le was not differentiated from the rest of the *Bacillus* reference strains with respect to GC% (Appendix B) and ARDRA clustering did not correlate with the phylogeny tree. *B. panaciterrae* is represented by only one type strain (Gsoil1517) isolated from a ginseng field (Ten et al., 2006), and has not been previously reported as an isolate from a hot spring environment. However, only a tentative conclusion can be made that this is the first report of *B. panaciterrae* being isolated from hot springs because there is only one type strain represented in this group and therefore statistically inconclusive. However, its novelty and difference from the other *Bacillus* isolates need to further investigation.

Another example of the complexity of *Bacillus* identification relates to isolate 24M which, with a BLAST search, convincingly matched (99.85%) with *Bacillus aerophilus* and *Bacillus stratosphericus.* However, these reference strains were isolated (Shivaji et al., 2006) from samples of high altitude atmospheric cryotubes. Recently, Branquinho et al. (2015) suggested that the nomenclature of these be dropped from bacterial systematics as *B. aerophilus* and *B. stratosphericus* were not represented in any type-culture collection and that they should be absorbed into the group of *B. pumilus*. However, Liu et al. (2015) reported that *B. aerophilus* was actually *Bacillus altitudinis*, and *B. stratosphericus* was a *Proteus* spp. This finding is a prime example where a Genbank BLAST result matches up to a nomenclature that is already dubious and questionable. By maximum likelihood phylogeny the placement of 24M is inconclusive and did not cluster with *B. pumilus*. *B. aerophilus* has not been reported as an isolate of hot springs although *B. pumilus* has (Aanniz et al., 2015). One needs to be aware of the fact that a bacterial isolate that has a 99.85% match to 16S rDNA sequences in databases within the public domain can be a different species.

Family Paenibacillaceae genus *Brevibacillus*

Brevibacillus is generally thought to be mesophilic although in this study isolates 16S and 36Li were isolated at 53°C. Isolation of mesophilic *Brevibacillus* from higher temperatures is typical due to the presence of heat-tolerant spores, and *Brevibacillus* spp. has been reported previously from hot springs (Derekova et al*.,* 2007; Cihan et al., 2012).

Even though there are challenges in precise identification of isolates, the aerobic Gram-positive spore-forming bacteria isolated in this study were similar to those reported in other investigations. Narayan et al. (2008) reported that of 104 isolates from hot springs in Fiji, 58% were *A. flavithermus* and 19% were *B. licheniformis/Geobacillus stearothermophilus*. *Anoxybacillus, Brevibacillus, Geobacillus*, and *Bacillus* made up the 76 isolates cultured from hot springs in Turkey (Derekova et al., 2008). Of 115 isolates, Cihan et al. (2012) listed seven genera in hot springs in Turkey which included *Anoxybacillus, Brevibacillus, Geobacillus*, and *Paenibacillus.* From hot springs in Morocco, Aanniz et al. (2015) found that 97.5% of 240 isolates were *Bacillus* spp. including *B. licheniformis* (n = 119)*, B. subtilis* (n = 6) and *B. pumilus* (n = 3).

Analysis of unknown isolates

Unknown isolates in this investigation (15S, 52M and 73T) and three "unknown *Bacillus*" sequences obtained from Genbank that remain as yet unidentified: clone TPB_GMAT_AC4; Genbank HG327138.1 from hot springs in India); clone KSB12; Genbank JX047075.1 from Indonesia and clone DGG30; Genbank AY082370.1 from China were included in the analysis.

Isolate 15S matched with *B. licheniformis* using BLAST and GC%, but not with phylogenetic analysis where it clustered with 18S supported by a 75% bootstrap value suggesting that it was possibly not a "group A type *Bacillus*". Similarly, isolate 52M also

matched to *Brevibacillus* spp. using BLAST, but did not phylogenetically or by GC% group with the *Brevibacillus* reference strain (Appendix C). ARDRA results suggested it was associated with Bergey's group A *Bacillus* and not *Brevibacillus*. Had the identification of both isolates 15S and 52M exclusively relied on BLAST results, the outcomes could be mis-identification. *Solibacillus,* an undefined member of the family Bacillaceae was included in this study because isolate 73T was similar using BLAST. Its "different" status was confirmed by a lower GC% of 53.91%, but ARDRA did not add any further discerning information. Neighbour-joining phylogenetic analysis placed isolate 73T with *Aneurinibacillus* spp. (Appendix C) and therefore this isolate could not be assigned to any genus with any degree of certainty.

Clone DGG30 from China, had a GC% of 53.2% similar to *Solibacillus* and a standard deviation different to that of *Bacillus, Anoxybacillus* and *Brevibacillus.* It clustered with the ARDRA group A suggesting it was not related to Bergey's group A *Bacillus* spp. Clone KSB12 from India was confirmed and grouped with *Bacillus* spp. by GC%; and by ARDRA, clustered with *B. megaterium* and *B. lentus* with a 56% bootstrap value suggesting that it could be related to Bergey's *Bacillus* group B or C. Indonesian isolate Clone TPB_GMAT_AC4 was confirmed to be *Bacillus* spp. by GC% but no further resolution could be obtained about its identification. Thus, published data from 16S rDNA sequences obtained from "uncultured bacteria" can be analyzed retrospectively using a combination of tools.

In conclusion, 43 isolates from Limpopo hot springs were cultured, and by comparison with 16S rDNA sequences in public databases and phylogenetic analysis, grouped into four genera: *Anoxybacillus, Bacillus, Brevibacillus*, and *Aneurinibacillus*. More specifically, the following species were identified: *A. flavithermus, A. rupiensis, B. subtilis* and *B. licheniformis.* Singular *Bacillus* spp. that are phylogenetically related to *B. panaciterrae, B. pumilus* and *B. methylotrophicus* were also identified; however, these three isolates require further characterization. All, except *B. panaciterrae* have been previously isolated from hot-spring environments. However, when the 16S rRNA gene sequences were analyzed by simulated computer-generated ARDRA using a collection of eight different restriction enzymes, additional discernment of individuals was possible. Therefore, this study shows that the use of a single molecular tool may result in a misrepresentation of *Bacillus* and *Bacillus*-related identification and that, when possible, a combination of tools should be used.

The complexity and problems regarding the *Bacillus* phylogeny were discussed. Only a small portion of the microbial diversity present in hot springs can be cultured, compared with the more comprehensive assessment of microbial diversity obtained using the metagenomic approach. Improved isolation rates could include the use of different media and different incubation conditions. Three different types of extremophiles with different properties (alkaliphilic, thermophilic and halophilic) as well as three unknowns were isolated, suggests that hot-spring water is a resource for potentially important bacteria useful in biotechnology and as a supply of novel bacteria. Hot springs sites need to be protected, conserved and maintained as valuable indigenous and pristine natural resources.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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APPENDIXES

Appendix A. List of GenBank accession numbers for 16S rRNA sequence data of reference strains and unknown isolates used in this study.

Strain or isolate	Size (bp)	GenBank or NCBI accession number
Aneurinibacillus sp. U33	1451	GenBank: KJ725179.1
Aneurinibacillus danicus NBRB102444	1501	GenBank: AB681786.1
Aneurinibacillus migulanus strain ATCC 9999	1422	NR_115593
Aneurinibacillus tyrosinisolvens	1458	GenBank: AB899818.1
Anoxybacillus sp. ATCC BBA-2555	1548	GenBank: KJ722458.1
Anoxybacillus bogrovensis strain NBIMCC 8427=DSM 17956	1341	NCBI Reference Sequence: NR_115021.1
Anoxybacillus eryuanensis strain E-112	1519	NCBI Reference Sequence: NR_117229.1
Anoxybacillus flavithermus strain WK1	1524	NCBI Reference Sequence: NR_074667.1
Anoxybacillus flavithermus subsp*. yunnanensis* strain E13	1449	NCBI Reference Sequence: NR_117774.1
Anoxybacillus gonensis strain G2	1382	NCBI Reference Sequence: NR_025667.1
Anoxybacillus kaynarcensis strain D1021	1430	NCBI Reference Sequence: NR_108265.1
Anoxybacillus kestanbolinensis strain K4	1376	GenBank: AY248711.1
Anoxybacillus pushchinoensis strain k-1	1338	NCBI Reference Sequence: NR_037100.1
Anoxybacillus rupiensis strain TSSC-4	1503	GenBank: KC759325.1
Anoxybacillus rupiensis strain TS_04	1463	GenBank: KJ842629.1
Anoxybacillus rupiensis strain TS_01	1463	GenBank: KJ842627.1
Anoxybacillus rupiensis strain A3	1428	GenBank: KC310454.1
Anoxybacillus rupiensis strain JF82	1463	GenBank: KF254911.1
Anoxybacillus rupiensis strain JF83	1510	GenBank: KC849452.1
Anoxybacillus rupiensis strain FZWP-10	1319	GenBank: JX914493.1
Anoxybacillus salavatliensis strain A343	1397	NCBI Reference Sequence: NR_104492.1
Anoxybacillus tengchongensis strain T-11	1519	GenBank: FJ438370.1
Anoxybacillus tepidamans strain R-35643	1507	NCBI Reference Sequence: NR_116985.1
Anoxybacillus thermarum strain DSM 17141	1358	NCBI Reference Sequence: NR_118117.1
Anoxybacillus vitaminiphilus strain 3nP4	1511	NCBI Reference Sequence: NR_108379.1
Anoxybacillus voinovskiensis strain TH13	1506	NCBI Reference Sequence: NR_024818.1
Bacillus aerophilus strain CRh28	1345	GenBank: KR780465.1
Bacillus aquimaris strain TF-12 (E)	1507	NCBI Reference Sequence: NR_025241.1
Bacillus arsenicus strain B3 (H)	1515	GenBank: GQ304784.1
Bacillus cereus strain ATCC 14579 (D)	1482	NCBI Reference Sequence: NR_114582.1
Bacillus coagulans strain ATCC 7050 (F)	1549	NCBI Reference Sequence: NR_115727.1
Bacillus halodurans strain ATCC 27557 (G)	1508	NCBI Reference Sequence: NR_112056.1
Bacillus lentus strain IAM 12466 (B)	1486	NCBI Reference Sequence: NR_115527.1
Bacillus licheniformis strain ATCC 14580	1545	NCBI Reference Sequence: NR_074923.1
Bacillus megaterium strain ATCC 14581 (C)	1495	NCBI Reference Sequence: NR_117473.1
Bacillus methylotrophicus	1510	GenBank: HZ046623.1
Bacillus panaciterrae Gsoil1517	1476	AC AB245380;
Bacillus pumilus strain ATCC 7061	1434	ACCESSION NR_043242
Bacillus siamensis strain IHB B 16121	1516	GenBank: KM817270.1
Bacillus siralis strain 171544	1430	NCBI Reference Sequence: NR_028709.1
Bacillus smithii strain TBMI12 (I)	1453	GenBank: EF010852.1
Bacillus subtilis ATCC 21331	1504	GenBank: AB018487.1
Bacillus subtilis subsp*. spizizenii* strain ATCC 6633	1507	NCBI Reference Sequence: NR_112049.1
Bacillus subtilis strain BCRC 10255	1468	NCBI Reference Sequence: NR_116017.1
Bacillus subtilis strain IAM 12118	1553	NCBI Reference Sequence: NR_112116.1
Bacillus subtilis strain JCM 1465	1472	NCBI Reference Sequence: NR_113265.1
Bacillus subtilis strain DSM 10	1517	NCBI Reference Sequence: NR_027552.1
Bacillus tequilensis strain 10b	1456	ACCESSION NR_104919

Appendix A. Contd.

Brevibacillus agri strain DSM 6348	1487	NCBI Reference Sequence: NR_040983.1
Brevibacillus fluminis strain CJ71	1413	GenBank: EU375457.1
Brevibacillus panacihumi strain DCY35	1473	GenBank: EU383033.1
Pseudomonas aeruginosa strain ATCC 23993	1458	GenBank: FJ652615.1
Solibacillus sylvestris HR3-23	1528	PUBMED; 10319505.
Uncultured *Bacillus* sp. clone KSB12	1504	GenBank: JX047075.1
Uncultured *Bacillus* sp. clone TPB_GMAT_AC4	1278	GenBank: HG327138.1
Uncultured *Bacillus* sp. clone DGG30	1569	GenBank: AY082370.1

The size of the 16S rRNA sequence in base pairs (bp) is also indicated.

Appendix B. Comparison of GC content (%) for the 16S rDNA sequences for reference type strains obtained from GenBank, and isolates in this study.

Isolate	Fragment size	GC content (%)	Genus	Average	SD
Anoxybacillus sp. ATCC BBA-2555	1548	56.65	-	-	-
Anoxybacillus bogrovensis strain NBIMCC 8427=DSM 17956	1341	55.78	-	-	-
Anoxybacillus eryuanensis strain E-112	1519	56.09	-	-	-
Anoxybacillus gonensis strain G2	1382	56.58	-	-	-
Anoxybacillus kaynarcensis strain D1021	1430	55.94	-	-	-
Anoxybacillus kestanbolinensis strain K4	1376	55.6	-	-	-
Anoxybacillus pushchinoensis strain k-1	1338	55.9	-	-	-
Anoxybacillus tengchongensis strain T-11	1519	56.48	-	-	-
Anoxybacillus thermarum strain DSM 17141	1358	56.55	-	-	-
Anoxybacillus vitaminiphilus strain 3nP4	1511	56.78	-	-	-
Anoxybacillus voinovskiensis strain TH13	1506	55.71	-	-	-
Anoxybacillus tepidamans strain R-35643	1507	56.67	-	-	-
Anoxybacillus salavatliensis strain A343	1397	56.4	-	-	-
Anoxybacillus flavithermus WK1 strain WK1	1524	56.56	-	-	-
Anoxybacillus flavithermus subsp. *yunnanensis* strain E13	1449	56.73	-	-	-
Anoxybacillus rupiensis strain TSSC-4	1503	56.95	-	-	-
Anoxybacillus rupiensis strain TS_04	1463	56.93	-	-	-
Anoxybacillus rupiensis strain TS_01	1463	56.73	-	-	-
Anoxybacillus rupiensis strain A3	1428	56.09	-	-	-
Anoxybacillus rupiensis strain JF82	1463	56.57	-	-	-
Anoxybacillus rupiensis strain JF83	1510	56.76	-	-	-
Anoxybacillus rupiensis strain FZWP-10	1319	56.33	-	56.40	0.41
11T *	1060	55.28	*Anoxybacillus*	-	-
4T	1407	56.15	*Anoxybacillus*	-	-
17S	1407	56.22	*Anoxybacillus*	-	-
3T	1394	56.24	*Anoxybacillus*	-	-
43T	1390	56.26	*Anoxybacillus*	-	-
19S	1406	56.33	*Anoxybacillus*	-	-
13S	1398	56.51	*Anoxybacillus*	-	-
7T	1421	56.72	*Anoxybacillus*	56.21	0.50
75S	1393	57.43	Unknown	-	-
Bacillus subtilis ATCC 21331	1504	55.12	-	-	-
Bacillus licheniformis strain ATCC 14580	1545	55.53	-	-	-
Bacillus tequilensis strain 10b	1456	55.22	-	-	-
Bacillus subtilis subsp. *spizizenii* strain ATCC 6633	1507	55.14	-	-	-
Bacillus subtilis strain BCRC 10255	1468	55.12	-	-	-

Appendix b. Contd.

Bacillus subtilis strain IAM 12118	1553	55.05	-	-	-
Bacillus subtilis strain JCM 1465	1472	55.23	-	-	-
Bacillus subtilis strain DSM 10	1517	55.31	-	-	-
Bacillus pumilus strain ATCC 7061	1434	55.16	-	-	-
Bacillus panaciterrae Gsoil1517	1476	54.74	-	-	-
Bacillus methylotrophicus	1510	55.36	-	-	-
Bacillus siamensis strain IHB B 16121	1516	55.01	-	-	-
Bacillus siralis strain 171544	1430	54.72	-	55.13	0.22
1T *	1417	52.86	*Bacillus*	-	-
33Li *	1079	54.21	*Bacillus*	-	-
14S*	1370	54.23	*Bacillus*	-	-
78S	1001	54.45	*Bacillus*	-	-
12S	1083	54.48	*Bacillus*	-	-
39T	1060	54.52	*Bacillus*	-	-
47Li	1069	54.53	*Bacillus*	-	-
40Le	1369	54.57	*Bacillus*	-	-
41Li	1386	54.69	*Bacillus*	-	-
54T	1379	54.69	*Bacillus*	-	-
18S	1411	54.71	*Bacillus*	-	-
28M	1065	54.84	*Bacillus*	-	-
48Li	1409	54.86	*Bacillus*	-	-
74T	1388	54.97	*Bacillus*	-	-
10T	1402	55	*Bacillus*	-	-
21M	1408	55.04	*Bacillus*	-	-
8T	1406	55.05	*Bacillus*	-	-
15S	1379	55.11	*Bacillus*	-	-
30M	1358	55.15	*Bacillus*	-	-
2T	1413	55.4	*Bacillus*	-	-
6T	1380	55.43	*Bacillus*	-	-
83Li	1396	55.44	*Bacillus*	54.75	0.54
24M	1303	54.8	*Bacillus*	-	-
32Le	918	54.36	*Bacillus*	-	-
77S	1057	54.4	*Bacillus*	-	-
Uncultured *Bacillus* sp. clone KSB12	1504	55.053	Unknown	-	-
Uncultured *Bacillus* sp. clone TPB_GMAT_AC4	1278	54.695	Unknown	-	-
Brevibacillus agri strain DSM 6348	1487	55.68	-	-	-
Brevibacillus fluminis strain CJ71	1413	55.56	-		
Brevibacillus panacihumi strain DCY35	1473	56.2	-	55.8	0.34
16S	1398	54.72	-	-	-
36Li	1399	55.54	-	-	-
52M	1396	55.09	Unknown	-	-
70T	1396	55.3	-	-	-
85Li	1375	54.25	-	54.95	0.58
Aneurinibacillus tyrosinisolvens	1458	56.86	-	-	-
Aneurinibacillus sp. U33	1451	56.92	-	-	-
Aneurinibacillus danicus NBRB102444	1501	56.89	-	56.89	0.03
86Li	1392	56.82	-	56.82	-
53M	1389	56.73	Unknown	-	-
Solibacillus sylvestris HR3-23	1528	53.2	-	53.2	
Uncultured *Bacillus* sp. clone DGG30	1569	53.218	Unknown	-	-
73T	1408	53.91	Unknown	-	-

(*) denotes isolates that grouped into a different ARDRA cluster, not *Anoxybacillus* and *Bacillus*.

APPENDIX C

Figure. A neighbour-joining phylogenetic tree of a 914 bp fragment of the 16s rRNA gene sequences between isolates from this study and representative members of type strains of *Anoxybacillus*, *Bacillus*, *Brevibacillus*, and *Aneurinibacillus*. Bootstrap values (%) are based on 100 replicates and shown for branches with more than 50% bootstrap support. Bar indicates 0.02 substitutions per 100 nucleotide positions.

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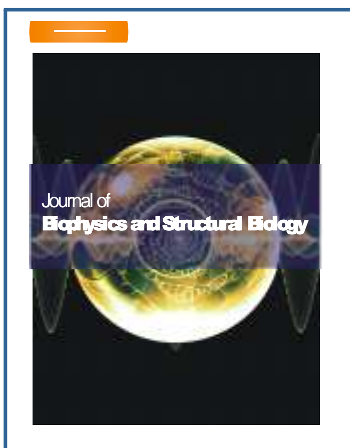

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