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Survey of the bacterial diversity at two coastal beaches in Guyana

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Abstract. *Prashad L, Daniel R, Ram M. 2020. Survey of the bacterial diversity at two coastal beaches in Guyana. Biofarmasi J Nat Prod Biochem 18: 57-64.* The #63 Beach, Berbice and Marriott Beach, Kingston Seawall, Guyana, were surveyed for halophilic bacteria present in its waters. NaCl tolerance, temperature tolerance, and antimicrobial activity of isolates against *Staphylococcus aureus* and *Bacillus* sp. were tested. Samples of 100 mL were taken from the Shore, 5.0 m, and 10.0 m depth from both locations. The samples were plated and examined for the growth of bacteria of different pigmentation. A total of 4 halophilic isolates were found; 3 from Marriott Beach (Isolates A, B, and G) and 1 (Isolate M) from the #63 Beach. Isolates were yellow, light orange, and pink pigmentation, the 3 isolates from Marriott Beach were Gram-negative and cocci, while the one isolates from #63 Beach were Gram-positive and cocci. The optimum salinity tolerance for the Isolate G from Marriott Beach was 1.5M NaCl, Isolate M from #63 Beach 1.0M NaCl, Isolate B from Marriott Beach 1.0M NaCl, and Isolate A from Marriott Beach 0.5M NaCl. The optimum temperature for the growth of the isolates was 37°C. The isolates had no antimicrobial activity against *S. aureus* and *Bacillus* sp.

Keywords: Antimicrobial, bacterial diversity, coastal beaches

INTRODUCTION

Halophiles are organisms that require salt for their survival (DasSharma and Arora 2001). Halophilic organisms are categorized into 3 groups; slight halophiles require a salinity range of NaCl 0.2M-0.5M to survive, moderate halophiles require a range of 0.5M-2.5M, and extreme halophiles require 2.5M-5.5M. These organisms are both Eukaryotic and Prokaryotic. The Eukaryotic halophiles include algae, specifically the green algae which belong to the genus *Dunaliella*, protozoa, e.g., *Porodon utahensis* isolated from the Great Lake, and fungi. The Prokaryotes include cyanobacteria, anaerobic bacteria and archaea, and aerobic and facultative anaerobic Gram-negative bacteria, e.g., *Halomonas* and *Chromohalobacter* (DasSharma and Arora 2001). Halophiles adapt to the environment so that they can survive in this unique setting. They do so in two ways: The High-salt-in mechanism, which allows for all their intracellular proteins to be stable and active in the presence of different concentrations of salts (Ma et al. 2010). This method results in high potassium and sodium ions in their cytoplasm (Kunte et al. 2002). The other method is the Low salt organic solutes in; this results in the buildup and production of organic solutes that do not hinder the activities of normal enzymes (Ma et al. 2010). Halophiles are known for their pigmentation that varies, and sometimes this pigmentation results in the unique color seen in salt lakes due to the high growth density of these organisms. Colors include red, pink, yellow, orange, and in some rare cases, cream. Most of these organisms are usually Gram-negative, but recently, a few Gram-positive have also been discovered. Moderate halophilic bacteria are the most widespread of halophiles.

Halotolerance is the ability of organisms to survive in both environments with and without salt. They do not depend on salt being present in the environment for survival.

Halophiles are present in hypersaline environments, including salterns, salt lakes, coastal and deep-sea locations. The two most studied hypersaline lakes are the Great Salt Lake in the Western United States of America and the Dead Sea in the Middle East (DasSharma and Arora 2001). Apart from these areas, the Mediterranean Sea, The Solar Lake of Sina Egypt, and Antarctic hypersaline lakes were also surveyed (Jiang et al. 2006).

This study surveyed halophilic bacterial diversity at two of Guyana's marine beaches along the coast, the Number 63 beach and Marriott beach, to analyze the physical properties of the water in terms of pH, salinity level, and temperature from both locations of different depths and to test the antimicrobial properties of the isolates found.

MATERIALS AND METHODS

Study sites

The study was conducted at #63 beach in Region 6 and the Kingston sea wall strip directly opposite Marriott Hotel, Region 4as shown on the map.#63 Beach is a natural beach approximately 8.7 km in length and supports coconut reefs, swamps, and mangroves (CREP 2007). The strip of beach directly opposite the Marriott Hotel is a popular tourist and local area, but it has been altered by anthropogenic activities much more than #63 beaches.

Figure 1. Research sites in: #63 Beach, Berbice (A) and Marriott Beach, Kingston Seawall (B), Guyana

Collection of samples

Figure 1 shows the map highlighting the study sites. Three different depths (the shore, 5.0 m, and 10.0 m) were measured for #63 Beach and Marriott Beach. The jars were labeled accordingly and rinsed with the sample water just below the surface of the collection site three times. Water samples were collected in a jar and capped below the water's surface at both beaches. Three jars per depth were collected. Once all the samples were collected, they were placed in an ice cooler filled with ice for storage and transported back to the University of Guyana laboratory. The samples were stored in the refrigerator until further use.

Microbial analysis

Under aseptic conditions, 47.6g of Nutrient agar and 29.2g of NaCl were weighed using an electric scale and suspended in 1.7 liters of water in a conical flask to obtain 0.5M NaCl nutrient agar. The mixture was then boiled over a hot plate and was swirled constantly. After the contents within the conical flask were completely dissolved, it was placed into the autoclave for 1 hour at 121°C for sterilization. After sterilization, it was cooled to 47-50°C, and approximately 30 mL of liquefied NaCl nutrient agar was poured into sterile Petri plates and allowed to set. This step was done under strict aseptic conditions within the laminar airflow chamber. The plates were stored for use.

Culturing of microorganisms

The physical properties of the water samples from the different depths from both locations were tested using a water testing kit. The samples were filtered using Whatman filter paper#2. The filtered samples were then used for the culturing of the microorganisms. The work area was swabbed with 75% ethanol. Plating the samples was done by swabbing the filtered sample onto Petri plates. The swabbing was done in triplicates, and each Petri plate was labeled accordingly. A sterile cotton swab was placed into the filtered sample jar and was left to soak in the sample water for about 30 seconds. The soaked swab was then streaked onto the Petri plate to cover the entire surface. The plates were then sealed with scotch tape and stored at 37°C for 24 hours. The plates were inspected for colony growth. Colonies with distinct pigmentation were chosen and streaked onto 0.5M NaCl nutrient agar plates using the four-corner streaking technique and incubated at 37°C for 24 hours. Once pure colonies were obtained, the colony morphology of each isolate was recorded. The isolated colonies were stored for further testing.

Determining if the isolates are halophilic bacteria

Pure nutrient agar plates were prepared with 11.2g of Nutrient Agar and 400 mL of water. The laminar airflow was swabbed with 75% ethanol. The pure isolated colonies were each streaked onto the pure Nutrient Agar plates in a zig-zag pattern. The newly streaked plates were sealed with scotch tape and incubated for 24 hours at 37℃. The plates with the pure isolated colonies were also sealed with scotch tape and stored in the refrigerator for further use. The plates were inspected for colony growth after 24 hours.

Gram staining of isolated halophilic bacteria

A small drop of distilled water was placed in a clean glass slide center. A thin smear of the isolated bacterial colony was made on the slide in a circular direction. The smear was then fixed by swiftly heating through a Bunsen flame using a clothespin until it was dry. The slide was then flooded with crystal violet for 30 seconds and then washed with distilled water for a few seconds. After washing off crystal violet from the side, the slide was flooded with Grams of iodine for 1 minute and then decolorized by tilting the slide and drop by drop rinsing with 95% ethanol until ethanol runs clear. After decolorization, the slide was washed with distilled water for a few seconds. The slide was then stained with 5-6 drops of safranin (counter-staining) for 20 seconds, after which it was washed off with distilled water. After counterstaining, the slide was blotted and air-dried. It was then examined microscopically by using the oil-immersion objective to identify the type of bacteria colonies.

Survival of isolated halophilic bacteria under different physical conditions

Sodium chloride (NaCl) tolerance of isolates: 1.0M NaCl (8.4g nutrient agar, 17.52g NaCl, and 300 mL water) and 1.5M NaCl (8.4g nutrient agar, 26.28g NaCl and 300 mL water) nutrient agar plates were prepared and labeled accordingly. The 0.5 McFarland (1907) standard was prepared by mixing 1% barium chloride and 1% sulphuric acid of 0.09 mL and 9.95 mL, respectively. 4 test tubes with 10 mL of water were sterilized by UV radiation. The inoculum of the isolated halophilic bacteria was then prepared by mixing each bacterial with 10 mL of water until it was the same consistency as the 0.5 McFarland (1907) standard. A sterile cotton swab was used to swab the inoculum onto the 1.0M NaCl and the 1.5M NaCl nutrient agar plates, respectively. The swabbing of the plates of different molarity for each isolated halophilic bacteria was done in triplicates. The plates were labeled accordingly. The plates were sealed with scotch tape for 24 hours at 37℃; observations were made.

Temperature tolerance of isolated halophilic bacteria

The 0.5M NaCl plates were prepared. The 0.5 McFarland (1907) standard was prepared by mixing 1% barium chloride and 1% sulphuric acid of 0.09 mL and 9.95 mL, respectively. 4 test tubes with 10 mL of water were sterilized by UV radiation. The inoculum of the isolated halophilic bacteria was then prepared by mixing each bacterial with 10 mL of water until it was the same consistency as the 0.5 McFarland (1907) standard. A sterile cotton swab was used to streak the inoculum onto the 0.5M NaCl nutrient agar plates for incubation at 10℃, 37℃, and 45℃. The streaking was done in triplicates, and the plates were labeled accordingly. The plates were then sealed with scotch tape. The plates were inspected for colony growth after 48 hours.

Antimicrobial activity of isolated halophilic bacteria

Disc Diffusion Method: 6 mm discs were made. The 0.5 McFarland (1907) standard was prepared by mixing 1% barium chloride and 1% sulphuric acid of 0.09 mL and 9.95 mL, respectively. 75% ethanol was used to swab the working area. Seven test tubes with 10 mL of water were sterilized by UV radiation. The inoculum of then prepared by mixing each bacterial with the 10 mL of water until it was the same consistency as the 0.5 McFarland (1907) standard. *Staphylococcus aureus* and *Bacillus* sp. were streaked onto the entire surface of 0.5M NaCl nutrient agar plates; this step was done in triplicates and labeled accordingly. The discs were soaked in the inoculum of the isolated halophilic bacteria. 4 discs were then placed in each of the plates using a tweezer at 90° angles to the center of the Petri plates streaked with *S. aureus* and *Bacillus* sp. respectively. This was also done in triplicates. The plates were sealed with scotch tape and stored at 37℃ for 24 hours. The plates were observed for zones of inhibition, and observations were recorded.

RESULTS AND DISCUSSION

Halophiles are organisms that cannot survive without the presence of salt in the environment in which they reside; it is an evolutionary and adaptive mechanism that they developed over time to help cope with environmental stress. Tables 1-2 show the morphological characteristics of the 14 isolates found in the study sites. We found a total of 14 bacteria that were initially isolated based on their unique colony pigmentation. Of these 14 isolates, 4 were halophilic bacteria, while the remaining 10 were simply halotolerant. Due to the unique characteristic of halophilic bacteria to only grow in the presence of salt, 4 isolates, namely isolates A, B, G, and M, were unable to grow when they were streaked onto nutrient agar medium plates that were not supplemented with salt (in this case, Sodium Chloride (NaCl). Halophiles are usually of two taxonomic groupings; Archaea can tolerate high salt concentrations ranging from 20%-30% (3.5M-4.5M) NaCl and other salts, and halophilic bacteria can survive at salinity ranges from 2%-20% NaCl (Kanekar et al. 2012).

Table 1. Colony morphology of the isolated halophilic bacteria from Marriott Beach, Kingston Seawall, Guyana

Site: Marriott Beach	Colony	Morphology								
		Form	Surface	Texture	Pigmentation	Elevation	Margin	Gram Staining	Shape	Size
50 m	1 (G)	Circular	Shiny	Smooth	Light orange	Flat	Entire	Negative	Cocci	3 mm
100 m	1 (A)	Circular	Shiny	Smooth	Yellow	Flat	Entire	Negative	Cocci	2 mm
	2 (B)	Circular	Shiny	Smooth	Pink	Raised	Entire	Negative	Cocci	3 mm

Table 2. Colony morphology of the isolated halophile from # 63 Beach, Guyana.

Site: #63 Beach	Colony	Morphology								
		Form	Surface	Texture	Pigmentation	Elevation	Margin	Gram Staining	Shape	Size
100 m	1 (M)	Circular	Shiny	Smooth	Light Pink	Raised	Entire	Positive	Cocci	3 mm

Table 3. The growth density of halophilic bacteria when subjected to different temperatures. (SCALE: 0 (no growth) 1 (slight growth) 2 (medium growth) 3 (high growth) to 4 (very high growth).)

Colony	Temperature °C and growth intensity		
	10°C	37°C	45°C
A	No growth	3	1
B		4	3
G		4	1
M		4	3

Table 4. The tolerance ranges of halophilic bacteria at different NaCl concentrations. (SCALE: 0 (no growth) 1 (slight growth) 2 (medium growth) 3 (high growth) to 4 (very high growth)

Colony	NaCl range/ density of growth		Classification
	1.0 M	1.5 M	
A	0	0	Slight halophilic bacteria
B	1	0	Moderate halophilic bacteria
G	4	4	Moderate halophilic bacteria
M	2	0	Moderate halophilic bacteria

Table 5. The antimicrobial properties of halobacteria against two pathogens

Colony	Zone of Inhibition	
	Staphylococcus aureus	*Bacillus* sp.
A	No zone of inhibition	No zone of inhibition
B	No zone of inhibition	No zone of inhibition
G	No zone of inhibition	No zone of inhibition
M	No zone of inhibition	No zone of inhibition

Since the 4 isolates showed maximum activity within the NaCl concentration of 0.5M-1.5M, these isolates are identified as moderate halophilic bacteria, as shown in Table 4. The Moderate bacteria halophiles fall under the Kingdom of Eubacteria; organisms in this Kingdom are more commonly found than archaeal-type organisms and are producers of antibiotics (Eubacteria, n.d.). There was no access to 16S rRNA to identify the genus of the four isolates; these were classified as belonging to the Kingdom Eubacteria and within the Family Halomonadaceae. The Family Halomonadaceae is divided into two broadly categorized genera; the Gram-positive and Gram-negative genera (Kanekar et al. 2012).

Pigmentation

It was seen that of the 4 halophilic bacteria were found; halophilic Bacterial Isolate A was yellow-pigmented and halophilic bacterial isolate G was light orange pigmented and flat in terms of elevation, and halophilic bacterial isolate B was pink pigmented; these were isolated from Marriott Beach and halophilic bacterial isolate M, from #63 Beach, was light pink pigmented and raised in terms of elevation. The colony morphology of these halophilic isolates was circular, surfaces were shiny, the texture of the colonies was smooth, and all had entire margins, as shown in (Tables 1-2) Halophilic isolates B, G, and M were 3 mm in size while halophilic isolate A was 2 mm. The Gram stain test revealed that halophilic isolates A, B, and G, were Gram-negative and cocci shaped while halophilic isolate M was Gram-positive and cocci shaped (Figures 2-9). These findings are similar to that of Azhar et al. (2014).

Salinity tolerance

The optimum salinity range was found to be 0.5M for all the halophilic bacteria found. Halophilic bacteria A showed no growth activity at 1.0M and 1.5M NaCl (Figure 11). this is due to its intercellular enzymes being unable to survive at such concentrated salinity. Halophilic bacteria G can thrive in 0.5M, 1.0M, and 1.5M NaCl; this shows that the enzymes of this organism are able to remain stable and active at such a high concentration of salinity. Halophilic bacteria B and M had medium growth at 1.0M but no growth at 1.5M NaCl; this shows the range for their enzyme's salinity concentration tolerance.

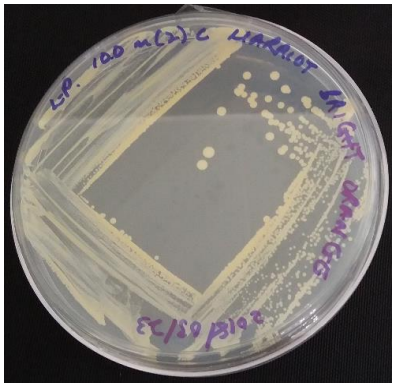

Figure 2. The halophilic bacterial isolate A**.**

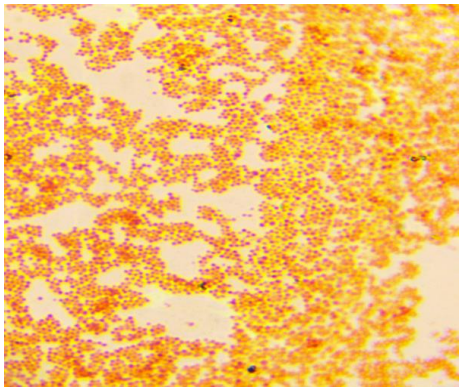

Figure 3. Gram stain of halophilic isolate A

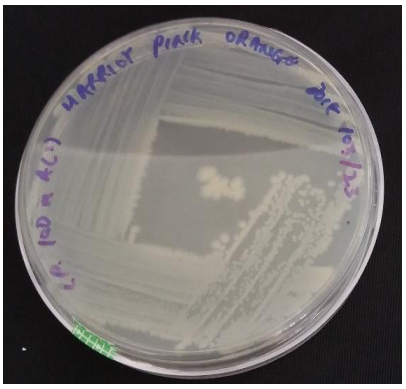

Figure 4. Halophilic bacterial isolate B

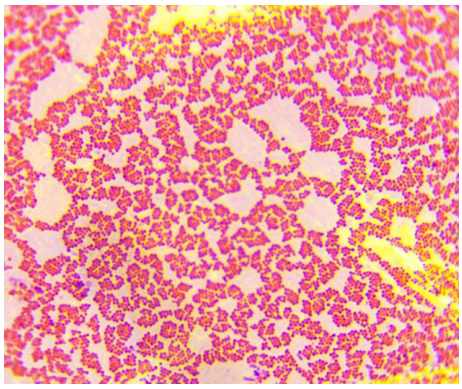

Figure 5. Gram stain of halophilic bacterial isolate B

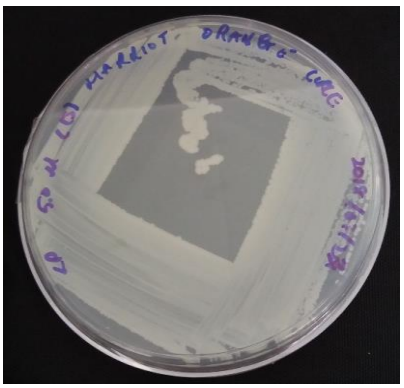

Figure 6. Halophilic bacterial isolate G.

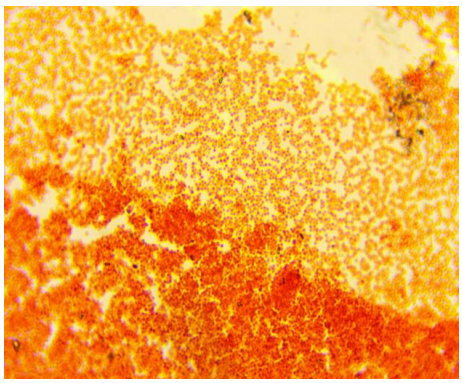

Figure 7. Gram stain of halophilic bacterial isolate G

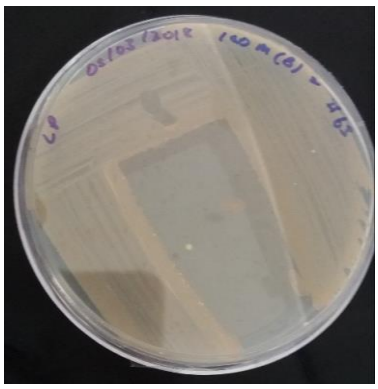

Figure 8. Halophilic bacterial isolate M.

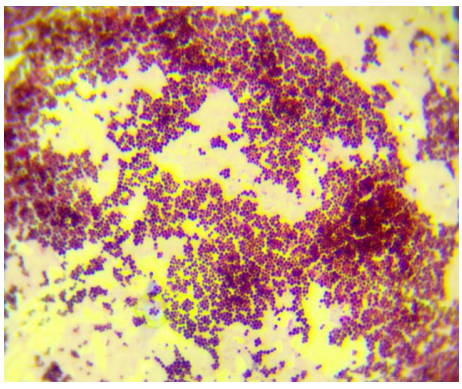

Figure 9. Gram stain of halophilic bacterial M.

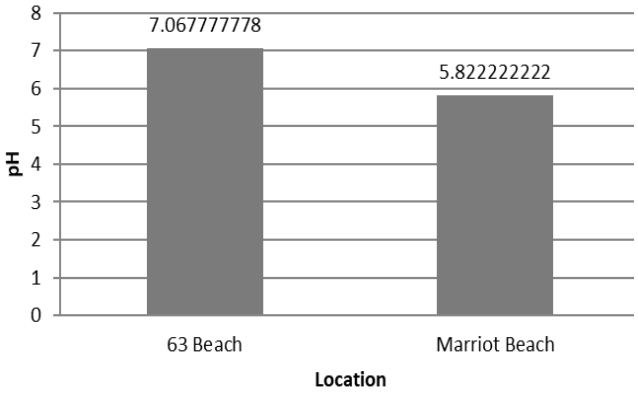

Figure 10. The average pH between the two sites. #63 Beach had a higher pH value overall when compared to Marriott Beach. Using the ANOVA testing, it was found that the p-value is 2.61E-11 which shows that there is a significant difference between the pH generally for both locations since the p-value found is lesser than the alpha value of 0.05.

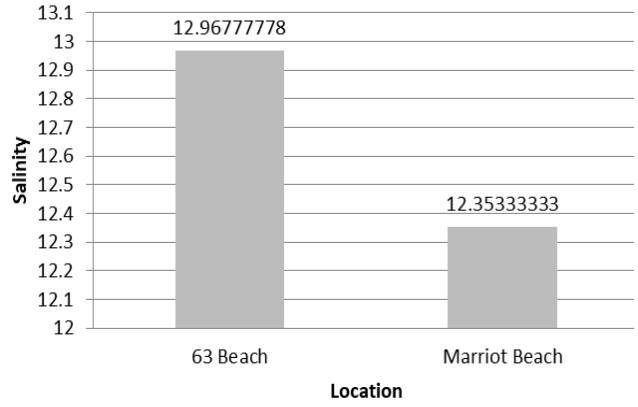

Figure 11. Average salinity between the two locations. Overall, #63 Beach had a higher salinity level than Marriott Beach. The ANOVA test shows a p-value of 0.5, greater than the alpha value of 0.05, which signifies there is no significant difference between the salinity of both locations.

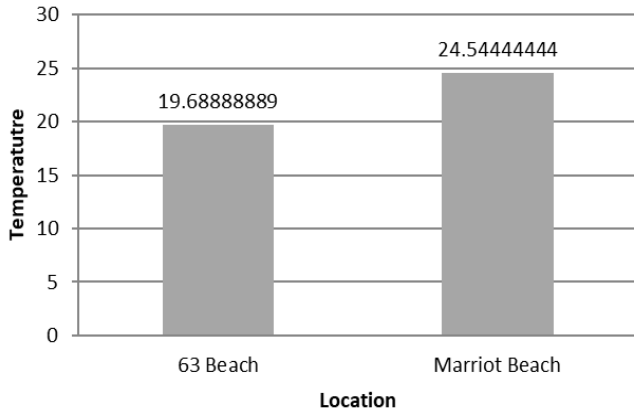

Figure 12. The average temperature of both locations. Marriott Beach has a higher average temperature for all depths than #63 Beach. From the ANOVA test, a p-value of 0.0004 was found. This shows there is great variation between the temperatures from both locations since the p-value is lower than the alpha value of 0.05.

The ability of halophiles to survive in different salinity levels compared to depends on the method they employ to adapt to their environment. Members of the Family Halomonadaceae use salt in a method that requires that the "proteins should maintain their proper conformation and activity at a near-saturating salt concentration" (Oren 2008). Therefore, the saturation level of halophilic bacteria A was 0.5M NaCl. At the same time, the saturation point for halophilic bacteria B and G was 1.0M NaCl. Halophilic Bacteria G can be further classified as a borderline extreme halophile based on the defined range by Donn Koshner. He stated that borderline halophiles thrive in the range of 1.5M to 4M salt (Oren 2008).

pH and temperature

Since 3 of the halophilic isolates found were from Marriott Beach, the null hypothesis that #63 beach will have a higher number of halophilic bacteria was rejected, and the alternative was accepted. The possible reason for this is that even though the salinity between the two locations did not have a significant difference with a p-value of 0.5, the pH varied for the depths of both locations; and was significantly different based on the ANOVA analysis; since only one halophilic bacterium was found from #63 beach of 10.0 m depth, when the pH was compared to the salinity, it was found that the pH of the 100m depth was low compared to the pH of the shore and 5.0m depth which were within the neutral range (Figure 10). Two halophilic bacteria were found in the 10.0 m depth of Marriott beach, which had low pH of 5.8 and a medium salinity level. There was a significant difference between this depth for both locations and one halophilic bacteria from the 5.0 m depth of pH 5.8 and high salinity level. A trend here is seen that most of these organisms were found where the pH was low and the salinity level high. This can be because there is a high level of carbon dioxide in the waters as it gets deeper. The presence of sodium chloride increases as the water becomes deeper because carbon dioxide reacts with water to form carbonic acid (Rose et al., 2016). Sodium chloride dissociates the carbonic acid and thereby frees the hydrogen ions, which results in a lower pH for the water (Reddi 2013). This also explains why the dissolved oxygen content for Marriott beach was lower than that of #63 Beach as the depth increased.

Generally, there was a significant difference between the temperature and the pH of Marriott beach and #63 Beach (Figure 12). The ANOVA analysis found that the temperature between the two locations had a variance p-value of 0.0004. Marriott Beach has a higher general temperature compared to #63 beach. This is due to two variables; the first one being that Marriott Beach is more exposed to pollution and carbon emissions and waste disposal as it is closer to the city compared to #63 Beach, which is more located in the countryside where carbon emissions and waste disposal is not that prevalent in the water; the second being the time which the samples were taken, the #63 Beach samples were collected earlier in January when temperatures are generally much lower while the Marriott samples were taken in March as the

temperature became higher. The temperatures showed significant variance for both locations' 5.0m and 10.0m.

The halophilic bacteria were tested for their temperature tolerance range by subjecting them to grow under 10°C, 37°C, and 45°C (Tables 3-4). The optimum temperature for growth of the 4 halophilic bacteria found was 37°C. Halophilic bacteria B and M had high growth at 45°C as well. Therefore, these halophilic bacteria are mesophilic as they survive within the range of 35°C to 45°C. Mesophiles are the most common type of bacteria found; they are able to thrive in a temperature range of 20°C to 45°C (Eddleman 1998). At 45°C, Halophilic bacteria A and G had less growth. This is due to the enzymes present in these organisms being denatured; the optimum temperature for enzymatic activities is 37.5°C; this finding corroborates with Schneegurt (2012), where it was found that the temperature range of 35°C-45°C was best for growing halophilic bacteria. For Halophilic bacteria B and M, the enzymes they produced were able to tolerate temperature at the extreme end of the mesophilic scale and were not easily denatured; the increase in temperature resulted in an increase in metabolic activities, which increased metabolic activity in the rapid growth of their cells. The halophilic bacteria showed no growth at 10°C because, at lower temperatures, the movement of molecules within the cells becomes slower and enzymatic reactions can no longer be carried out accurately. Eventually, cellular activities cease (Blamire 2000).

Antimicrobial properties

Staphylococcus aureus is a Gram-positive, cocci, pathogen found in the environment and even on the human skin. This bacterium causes a series of potentially fatal infections if it enters the human bloodstream (Taylor and Unakal 2017). *Bacillus* sp. is also Gram-positive, but it is rod-shaped. This bacterium is also a pathogen that leads to numerous infections when it enters the human body (Turnbull 1996). The halophilic bacterial isolates from Marriott and #63 Beach showed no antimicrobial activity against these pathogens (Table 5). Therefore, the alternative hypothesis is accepted and the null rejected. The finding here correlates with the findings of Irshad et al. (2013), where 5 of the halophilic isolates that were found in his study had no antimicrobial effects on pathogens; this can be due to the release of chemical toxins induced by deleterious microorganisms and mechanical stress (Velho-Pereira et al. 2012). Halophilic bacteria that thrive at very high salinity are most commonly known to inhibit the growth of pathogens. Sometimes, they are known only to release chemo toxins if it feels threatened by invading species (Ventosa et al. 1998).

In conclusion, Marriott Beach had a significant number of halophilic bacterial isolates compared to #63 Beach. The halophilic bacterial isolates belong to the Family Halomonadaceae. The 3 halophilic bacterial isolates A, B, and G, from Marriott Beach were Gram-negative, cocci, and isolate M from #63 Beach was Gram-positive, cocci. The four isolates were circular, had a shiny surface, smooth texture, and had entire margins. Marriott beach: Isolate A was yellow-pigmented and flat elevation; Isolate B was pink pigmented and raised in elevation, while Isolate G was light orange pigmented and flat in elevation. #63 beach isolate M was light pink pigmented and raised in elevation. Isolate G from Marriott Beach and Isolate M from # 63 Beach showed maximum growth at 1.0M and 1.5M NaCl. The optimum salinity for all the halophilic bacterial isolates was 0.5M NaCl. The optimum temperature for the halophilic bacterial isolates' growth was 37°C. Isolate G, from Marriott Beach, was the only halophilic bacteria that could tolerate a temperature high of 45°C. The isolates showed no antimicrobial action against *S. aureus* and *Bacillus* sp. There were significant differences between the pH and temperature between the two locations. There was not a significant difference between the salinity of both locations.

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