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Morphological, Biochemical and Molecular Characterisations of Bacteria Isolated from Water and Submerged Painted Boat Hulls in Badagry Lagoon, Lagos State, Nigeria

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ABSTRACT: The submerged surfaces of boats act as microbial seed banks which introduce non-indigenous microbial strains in the marine habitat. This study focuses on the morphological, biochemical and molecular characterisation of bacteria isolated from water and submerged painted boat hulls in Badagry lagoon, Lagos state, Nigeria using standard techniques. The mean bacterial density obtained were 1.9 x 10^9 CFU/mL and 2.03 x 10^4 CFU/g for water samples and hull samples respectively. Morphological, biochemical and molecular characterisation confirmed the bacteria to be *Bacillus subtilis, B. flexus, B. cereus, Brevibacillus agri, Aeromonas punctata, Staphylococcus sciuri, B. licheniformis, Kurthia gibsonii* and *Leclercia adecarboxylata*. Results of the study showed that some of the isolates (*B. cereus, B. flexus, S. sciuri* and *L. adecarboxylata*) are pathogenic while others (*B. agri* and *A. punctate)* are opportunistic pathogens. The proportion of pathogenic strains isolated in this study is greater than the non-pathogenic strains.

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Microorganisms, due to their ubiquitous nature are available naturally in various environments. These microbes grow by using the nutrients in their surroundings for growth and reproduction. At other times, microbes form complex associations with different species of microbes around. This association helps to provide metabolic products that an individual microbe is unable to synthesise. An example is the creation of anaerobic microenvironment for the anaerobic members of the association. At other times, microbes come together to form biofilm by synthesising protective matrix which protect against antimicrobial agents. The aggressiveness of marine microflora increases when it adapts to changes in environmental conditions. It has been found that the corrosion rate of many materials is several times higher in polluted waters than in relatively clean waters and microorganisms make the main contribution to this corrosion process (Karpov *et al*., 2012). Biofouling or biological fouling is the accumulation of microorganisms, plants, algae, or small animals on wetted surfaces that have a mechanical function, causing structural or other functional deficiencies (Sojka *et al.*, 2023). Biofouling takes place on submerged surfaces after the formation of a biofilm that creates a surface onto which successively larger microorganisms can attach (Amara *et al*., 2018). Biofilms usually start with the adhesion of bacterial cells which modify the surface physicochemical properties, thus influencing the adhesion of successive colonizers such as algae, cyanobacteria, and protists (de Carlvaho, 2018). The marine ecosystems generally, have witnessed many environmentally unfriendly human activities which

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has caused major concern. For instance, formation of biofilm on the hulls of vessels such as ships and boats cause an increased energy (fossil fuels) consumption necessary to overcome frictional drag, heat and mass transfer limitations (Ogbonnaya and Ajayi, 2017). The use of fossil fuels to drive ships and boats (Schultz *et al*., 2011) causes emission of greenhouse gases such as carbon dioxide (CO_2) and nitrous oxides as well as pollutants such as sulphur oxides into the earth's atmosphere. Microbes dispersed by boat hulls are picked from one port and discharged into water at another port. The survival of these microbes may be reduced from one port to another due to changes in temperature and the salinity of the water (Qian *et al.*, 2022). Historically, biofouling was the key mechanism for translocating species and recent research indicates that it is still the single most important mechanism for the dispersal of non-indigenous marine species in many locations around the world (Coutts *et al*., 2010). Biofouling is influenced by several factors including salinity, pH, temperature, nutrient levels, flow rates and the intensity of solar radiation. These factors vary seasonally, spatially and with depth (Christine and Marlène, 2014).

Biofouling is a natural phenomenon that often appears in the forms of microfouling and macrofouling (visible on surfaces) (Bixler and Bhushan, 2012). Microfouling involves microorganisms; whereas, macrofouling involves larger organisms (e.g. invertebrates). Both natural and artificial surfaces are susceptible to the colonization of microorganisms forming a thin layer of microbial biofilms consisting of large quantities of microbial metabolites with bacterial cells embedded inside (Gregory and Bharat, 2012; Heidarian *et al*., 2019). The tendency of ships and boats to foul is related to the type of service in which they are employed, and, particularly, to the resulting time spent in port. At high speeds, the growth of some organisms previously attached is supressed, particularly if they have not been long established. Meanwhile, at higher speeds, the attached organisms may be washed away from the body of the vessel (Davidson *et al*., 2010).

Biofouling occur in stages. After the initial stage during which the submerged surface undergoes biochemical conditioning, pioneer bacteria will attach irreversibly to the surface (in a process implicating physical, chemical and biological parameters), and progressively form a multiple species biofilm. The complexity increases if microalgae (especially diatoms) and fungi are involved, as well as protists (Flemming and Wuertz 2019). Submerged Surfaces such as steel, iron, boat hulls, etc. are usually covered to some degree with biofilms that contain bacteria, microalgae, and protozoans. These biofilms when attached to boat hulls can be transferred from one part of the water to another as the boat moves in water. Sometimes these biofilms may contain pathogens which may have been picked from one port and then discharged in another port that originally do not harbour such strains of pathogens. Consequently, these pathogens pose serious epidemiological problems in the community. A fore knowledge of this microbial cross contamination will help to curb the wide spread of pathogens (through public sensitisation) and prevent possible contamination of sea foods and water. Hence, the objective of this paper was to evaluate the morphology, biochemical and molecular characteristics of bacteria isolated from water and submerged painted boat hulls in Badagry lagoon, Lagos state, Nigeria.

MATERIALS AND METHOD

The site of sampling for this study was the boat harbour in Badagry lagoon, Lagos, Nigeria. The hulls of fifteen (15) painted speed boats were sampled using sterile swab sticks. Samples were collected from each boat by aseptically swabbing 10 cm portion of the hull of each painted boat with a swab stick. Also, 20 mL of water samples were collected at three different points (A, B and C) which are 10km and 13km apart respectively. Five biochemical tests including catalase, oxidase, Gram reaction, indole and motility test were carried out on the bacterial isolates as described by Syahri *et al*., 2018.

Inoculation of samples and microbial Isolation: A seven-fold serial dilution was carried out aseptically for each water sample, while the swab sticks harboring hull samples were dislodged in 50 mL of sterilised peptone water and then thoroughly shaken for even distribution. 0.1 mL of each sample was aseptically inoculated on the nutrient agar medium in duplicates; using spread plate method. The petri plates were incubated aerobically at 37^0C for 24 hours, and visible colonies were counted and characterized after incubation.

Molecular Identification: The DNA of the isolates were prepared for 16S rRNA molecular identification. Polymerase chain reaction was carried out using 16S-27F:5'AGAGTTTGATCMTGGCTCAG-3' and (16S-1492R: 5'CGGTTACCTTGTTACGACTT-3' as forward and reverse primers. The PCR products were purified and gel electrophoresis was carried out. After sequencing and alignment, phylogenetic tree was plotted against several related bacteria isolates based on maximum likelihood and neighbour joining algorithms (Bhutia *et al.*, 2021).

RESULTS AND DISCUSSION

Bacteria population density: The population densities of the bacterial isolates from the duplicate cultured plates of hull samples were quantified. The graph plot of the log of the mean colony count shows a sinusoidal graph in Figure 1 while Figure 2 shows the population density of bacteria isolates in the water samples.

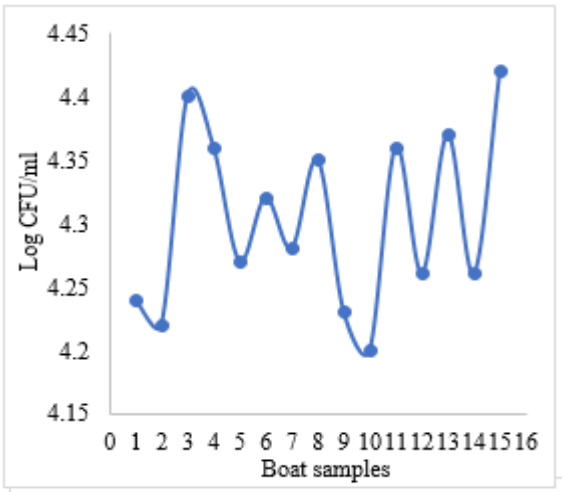

Fig. 1. Mean population Density of bacteria isolated from the boat hulls

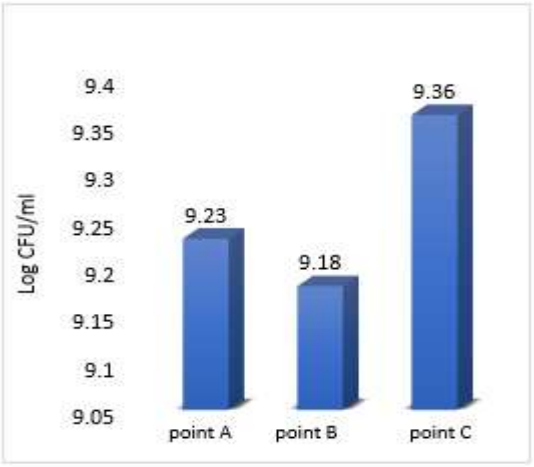

Fig. 2. Mean population density of bacteria isolated from water s

The quantitative analysis of bacteria growth from the hull samples showed that the bacteria population from each sample collected had 10^4 cells which is not very high. This value is very low when compared to the report of Basarab *et al*. (2018) who reported 10^8 cells from sample cultures at Dnieper river (Ukraine) and from wooden samples. The low value in the bacterial population density recorded in this study may be attributed to the fact that, high speed of leisure boats in the lagoon cause 'self-cleaning' on the boat hulls. Biofilms even though loosely attached to the surfaces, can maintain their presence on submerged surfaces because the surfaces are not subjected to high speed like the speed boat hulls (Christine and Marlène, 2014). However, tidal forces alone are not strong enough to wash off these biofilms due to the extracellular polymeric substances secreted by the participating organisms in the biofilm that helps the biofilm to adhere strongly in some cases to surfaces. Meanwhile, Obidi and Aina (2018), reported 10^5 bacteria cells from speed boat hulls from Ojo waterside shores, Lagos, Nigeria. This is similar although slightly higher than the value obsrved in this current study. The smooth surface of boat hulls minimizes the adhesion strength between the fouling organisms and the hulls, so that the organism can be easily removed by hydrodynamical stress during navigation at high speed. This prevents fouling of boat hulls to certain degree by reducing microbial diversity and attachment on boat hulls. Christine and Marlène, 2014, reported a situation where an initially fouled coated surface is able to self-clean when the velocity of the vessel increases.

*Identification of bacteria isolates***:** The results of biochemical tests and probable identification carried out on the bacteria isolates are presented in Table 1. Furthermore, the molecular identification of the isolates based on the analysis of the 16S rRNA of each isolate is presented in Table 2. Some of these isolates are pathogenic and could pose serious public health challenges.

Table 1: Biochemical characterisation of bacteria isolates

Isolate	Source	Catalase	Oxidase	Indole	Motility	Gram Reaction		Probable identity
BLW1	W_0		+	+	-	+	+	*Bacillus* sp.
BLB1	W_0 & H_0		+	+	-	+	+	*Bacillus* sp.
BLB3	W_0 & H_0		+	-	-	+	+	*Bacillus* sp.
BLB4	W_0 & H_0		+	+	-	+	+	*Bacillus* sp.
BLCR2	W_0 & H_0		+	+	+	+	-	*Bacillus* sp.
BLGF2	H_0		+	+	-	-	+	*Staphylococcus* sp.
BLGO2	W_0 & H_0		+	+	-	+	+	*Bacillus* sp.
BLGT1	W_0 & H_0		+	+	-	+	+	*Bacillus* sp.
BLCOD1	W_0 & H_0		+	+	+	+	-	*Bacillus* sp.

W_0, water; H_0, boat hull

Table 2: Molecular identification of bacterial isolates

Isolate	Closest relationship	Accession No.	Similarity	Request ID
BLW1	*Brevibacillus agri* Y17	KF641808.1	99%	2DJXKRNU016
BLB1	*Bacillus subtilis* H-3	KT273284.1	99%	2JDZCMD401
BLB3	*Bacillus cereus* ST06	MH475925.1	93%	2DK0Z84801R
BLB4	*Bacillus flexus* WY2	JQ936679.1	100%	2DKH12FA016
BLCR2	*Aeromonas punctata* 4LNC309	FJ940796.1	99%	2DKRF21X01R
BLGF2	*Staphylococcus sciuri* B9-58B	CP041879.1	100%	2JCR7TWD01N
BLGO2	*Bacillus licheniformis* I29	KU922363.1	95%	2DMBE08P014
BLGT1	*Kurthia gibsonii* B3	KM391941.1	99%	2JCH6F1S01N
BLCOD1	*Leclercia adecarboxylata* EGTM31	MG890203.1	99%	2DM00HYX014

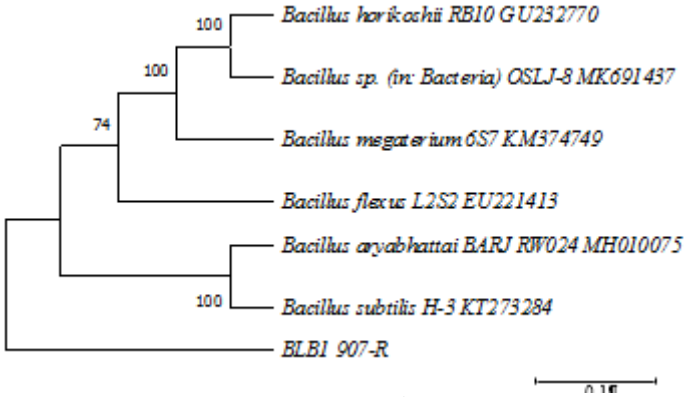

Fig. 3. Phylogenetic tree of isolate BLB1

Phylogenetic tree of bacteria isolates: The phylogenetic tree plotted against several related bacteria isolates based on maximum likelihood and neighbour joining algorithms are presented in Figures 3 -11 below.

Bacillus subtilis is one of the most studied Gram-positive bacterium. It is one of the primary colonizers of immersed surfaces and has been found to be involved in biofouling processes. However, little is known about its pathogenicity (Rummel *et al*., 2017). A study by Gu *et al*., (2019) reported virulent genes associated with toxins, adhesion, invasion, dissemination, anti-phagocytosis, and intracellular survival in *B. subtilis*. Furthermore, Celandroni *et al*., (2016) reported protease secretion in the strains of *B. subtilis* isolated from clinical samples. In addition, *nheA*, *nheB* and *nheC* genes which code for phospholipase C and sphingomyelinase which are important virulent factors were detected in the strains of *B. subtilis*. It has been reported to be able to survive in continuously changing environment due to its ability to secretes several enzymes to degrade quite a variety of substrates; hence, it is often used as an industrial cell factory for the production of vitamins, inositol, acetoin, hyaluronan and other chemicals (Olmos *et al*.,2020). *B. subtilis* can also form complex biofilms, they can be used for the production of many functional biomaterials, such as surface growth factors, antibiotics, lysozyme and antimicrobial peptides for medical materials (Duan *et al*., 2020). Although this bacterium is not a known human pathogen, Gu *et al*., (2019) studied the genome of an isolated *B. subtilis* strain and reported virulence genes which were associated with toxins, adhesion, invasion, dissemination, anti-phagocytosis, and intracellular survival. Gu *et al*., (2019) therefore, concluded that the *in vivo* pathogenicity test of *B. subtilis* provided evidence that the bacterium is capable of tissue spread and causing host death, but the underlying mechanism is unclear.

Bacillus cereus has been described to have high virulence potential. Celandroni *et al*., (2016) detected *plcA*, *sph*, and *cytK* genes which code for phosphatidylinositole-specific phospholipase C, and sphingomyelinase in *B. cereus* isolated from clinical samples. It is a foodborne pathogen that can cause emetic and the diarrheal syndrome (Messelhäußer and Ehling-Schulz, 2018). *B. cereus* infection results from consumption of food contaminated with enterotoxigenic *B. cereus* or emetic toxin. According to McDowell *et al.,* (2023), reports of respiratory infections similar to respiratory anthrax has been linked to infection of *B. cereus* harbouring *B. anthracis* gene. *B. cereus* produces several virulence factors and enters the gastrointestinal tract through ingestion, causing diarrhoea and vomiting. Yu *et al.,* (2020), explained that diarrhoea caused by *B. Cereus* is associated with four enterotoxins: haemolytic BL (encoded as HBL, *hblA*, *hblC*, and *hblD*), non-haemolytic enterotoxin (encoded as NHE, *nheA*, *nheB*,

nheC), and FM enterotoxin (*EntFM, entFM*). and cytotoxin K (*CytK*, encoded by *cytK*). Both HBL and NHE are tritoxins. *CytK* belongs to a family of β-barrel pore-forming toxins that can cause severe food poisoning, skin necrosis, haemolysis, and even death. *EntFM* refers to a cell wall peptidase (CWP) that can cause diseases such as diarrhoea. In addition to food poisoning, *B. cereus* is also associated with serious infections such as pneumonia, bacteraemia, endophthalmitis, necrotizing fasciitis, osteomyelitis and endocarditis (Ikeda *et al*., 2015).

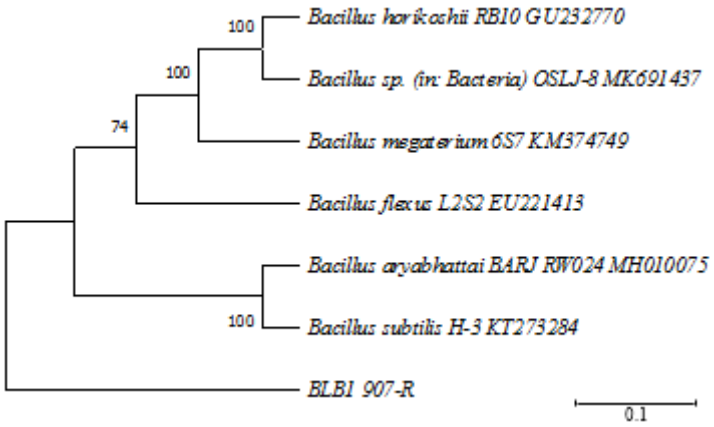

Fig. 4 Phylogenetic tree of isolate BLB3

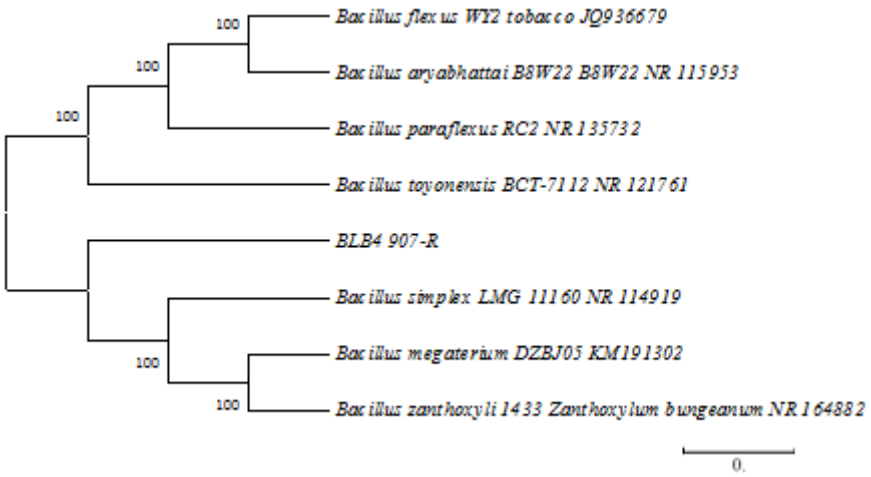

Fig. 5. Phylogenetic tree of isolate BLB4

Bacillus flexus is an aerobic, Gram-variable, rod-shaped, endospore-forming, oxidase-positive bacterium. *B. flexus* has been reported to have the ability to solubilise phosphate and hydroxyapatite; which is why this microbe is usually employed in the production of biofertilizer (Macik *et al.,* 2020). The presence of certain gene such as *nheA, nheB* or *nheC* which encode non-haemolytic enterotoxin (Cai *et al.,* 2017) in *B. flexus* suggested that this microbe is a potential pathogen. However, studies have not revealed the implication of *B. flexus* (Celandroni *et al.,* 2016). The isolation of *B. flexus* from poultry waste (Ma *et al.,* 2021) suggest this pathogen may be a potential zoonotic pathogen. The presence the gene *nheA* and *nheC* which codes for non-hemolytic enterotoxin in *B. flexus* suggests that this bacterium though not a known human pathogen carries gene that codes for virulent factors that contribute to pathogenicity (Celandroni *et al.,* 2016).

Leclercia adecarboxylata formerly identified as *Escherichia adecarboxylata* is a Gram-negative bacillus belonging to the Enterobacteriaceae family; it is an extremely rare human pathogen which affects immunocompromised individuals (Matsuura and Sugiyama, 2018). This microbe was isolated from an infected wound; and it's known to cause wound infections, abscesses, boils, peritonitis, and endocarditis in humans (Zayet *et al*., 2021). *L. adecarboxylata* has been found to cause bacteraemia in immunocompromised patients (Zamora, 2016). *L. adecarboxylata* is a ubiquitous microorganism found

in aquatic environments as well as in soil and in the commensal intestinal flora of some animals. Until now, this bacterium was considered a pathogen with low virulence and unknown pathogenicity in human infections (Alosaimi and Muhmmed, 2020). It is a rare human pathogen that occurs mostly in wounds or in contact with the aquatic environment; and has been reported to cause cellulitis (Broderick *et al.*, 2019). It is a very rare human pathogen that usually infects immunocompromised individuals, with few documented cases in adults and even fewer in paediatrics (Keyes *et al.*, 2020). Although, the clinical importance of *L. adecarboxylata* is not well established. It often causes opportunistic infections in immunocompromised patients or patients with underlying medical conditions. However, it can also be found in immunocompetent patients; although it has lower pathogenicity in immunocompetent patients (Adapa *et al.*, 2019). Although, Alosaimi and Muhmmed, (2020) reported that *L. adecarboxylata* infections are mostly non-fatal due to their excellent antibiotic susceptibility profile and low virulence factors; while a study by Li *et al.*, (2021) suggests that strains of this bacterium have shown to have multiple drug resistance; thereby raising concerns about the organism's potential as a new health threat.

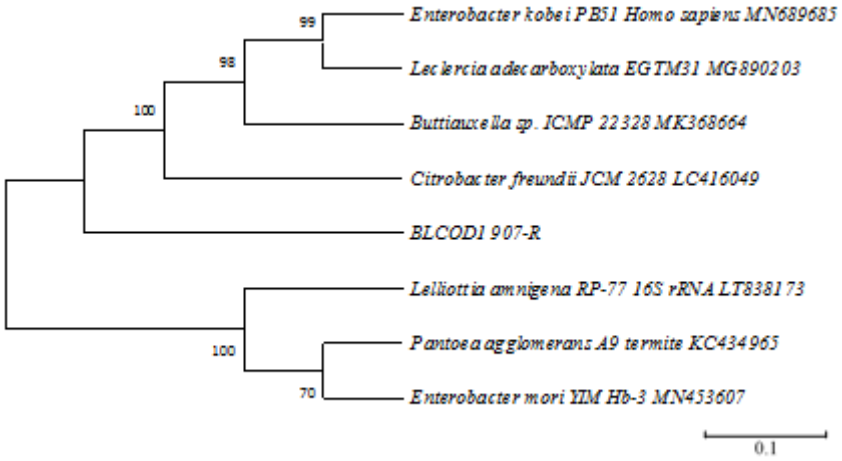

Fig. 6. Phylogenetic tree of isolate BLCOD1

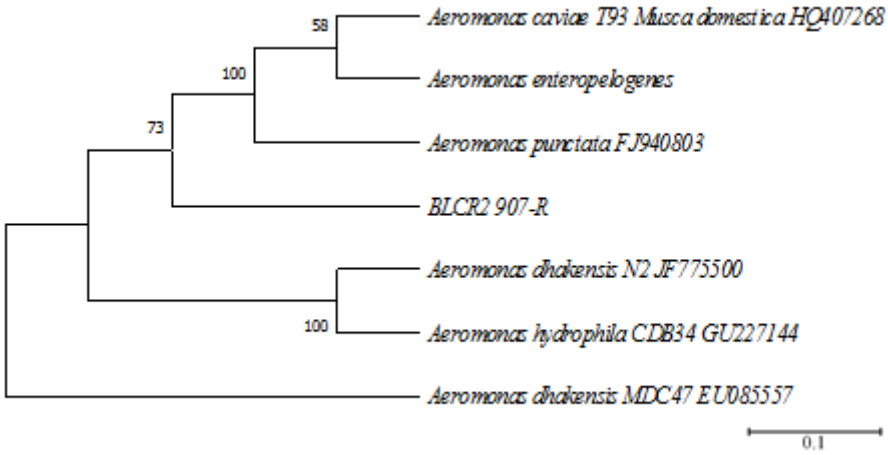

Fig. 7. Phylogenetic tree of isolate BLCR2

Aeromonas has been reported to pose serious human health challenges (Mulamattathil *et al.*, 2014). This is supported by Ahmad *et al*., (2017) and Ologbojo and Ayoola, (2015), who isolated *Aeromonas* sp. and *Pseudomonas aeruginosa* and other pathogenic bacteria strains from sea water and lagoon water samples respectively. *Aeromonas* is a family of Gram-negative, facultatively anaerobic, rod-shaped bacteria that are morphologically similar to members of the Enterobacteriaceae. Most of the described species are associated with human disease. The most reported pathogenic members of this family include: *A.*

hydrophila, *A. Caviae* and *A. veronii biovar sobria* (Qureshi and Qamar., 2020). The enterotoxin released by *Aeromonas* is divided into cytotonic and cytotoxic. They can cause infections in both healthy and immunocompromised hosts. Bacteraemia and sepsis associated with *Aeromonas* can lead to high mortality in immunocompromised patients (Martino *et al.*, 2016). According to Percival and Williams, (2014), lipopolysaccharide (LPS), S-layer, outer membrane proteins, cilia and flagella, resistance to complement-mediated lysis, and type III secretion system are important virulence factors that play crucial role in invasive diseases caused by *Aeromonas*. These virulence factors are associated with many *A. hydrophila* and *A. veronii* strain, but not in most *A. caviae* isolates. Some species of *Aeromonas* such as *A. hydrophila* and *A. Verona* produce aerolysin which cause gastroenteritis. The membrane activity of aerolysin depends on oligomerization steps that are functionally dependent on basic histidine and tryptophan residues (Liu, 2015).

Staphylococcus sciuri is an invasive and opportunistic pathogen of both human and animals, although its virulence factors are not yet clearly understood (Zeman *et al*., 2017). *S. sciuri* has been reported in cases of endocarditis, peritonitis, septic shock, urinary tract infection, and pelvic inflammatory diseases (Svec *et al*., 2016; Kengkoom and Ampawong, 2017). A study by Ahoyo *et al.*, (2013) indicated the presence of *S. sciuri* in several clinical samples collected from patients suspected to have nosocomial infections. In his study, Ahoyo noted that within his sampled population, *S. sciuri* is one the leading cause of infection among coagulase negative bacteria causing bacteraemia. This bacterium has been reported to be highly prevalent in catheter, skin diseases, wounds and areas with poor hygiene characterised by poor access to portable water (Coimbra *et al.*, 2011)

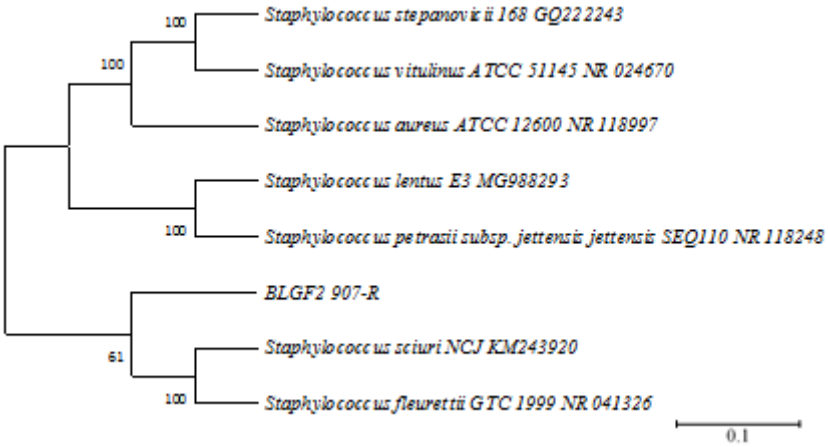

Fig. 8. Phylogenetic tree of isolate BLGF2

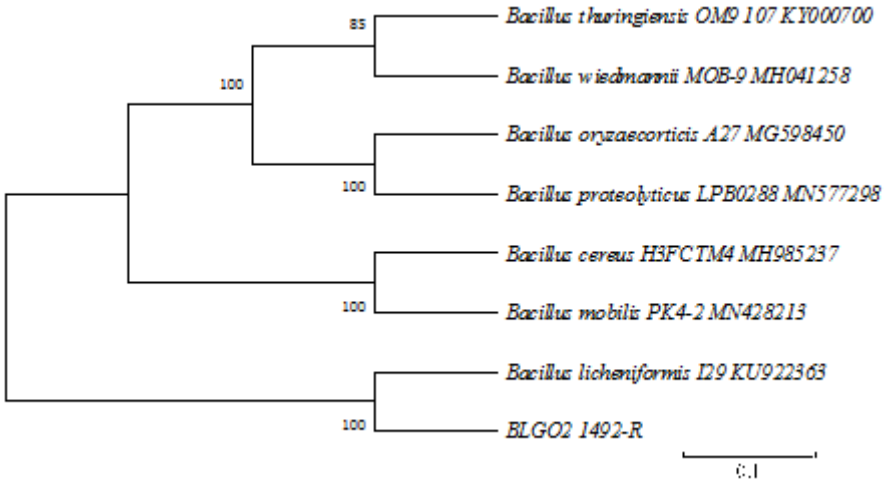

Fig. 9. Phylogenetic tree of isolate BLGO2

Bacillus licheniformis is a Gram-positive, spore-forming bacterium commonly found in soil. It has also been reported on the feathers of birds. This bacterium has been characterised as a good probiotic in animal feed as well as a potential agent for bioremediation, bioflocculation, biomineralization, anti-biofilm and biofuel production (Liu *et al.*, 2012). Although, this bacterium is considered a good probiotic agent, possible transfer of antibiotic resistant gene from this bacterium makes it unsafe as a probiotic (Muras *et al.*, 2021). *B. licheniformis* has been found to be pathogenic in immunocompromised patients causing infections such as: ventriculitis, ophthalmitis, bacteremia, peritonitis and endocarditis (Haydushka *et al.*, 2012). This microbe has been reported to be contaminant of dairy products and bread (Dhakal *et al.*, 2013). Haydushka has also reported a recurrent case of sepsis caused by *B. licheniformis* in immunocompromised patients. *B. licheniformis* can also cause foodborne gastroenteritis, an intestinal infection that can lead to a life-threatening condition called sepsis. Symptoms include abdominal pain, (acute) diarrhoea and vomiting. The onset time varies from 2 to 14 hours and does not exceed 36 hours. Toxins produced by *B. licheniformis* can damage cell membranes, deplete cellular ATP, and cause acrosomal oedema (Celandroni *et al.*, 2016).

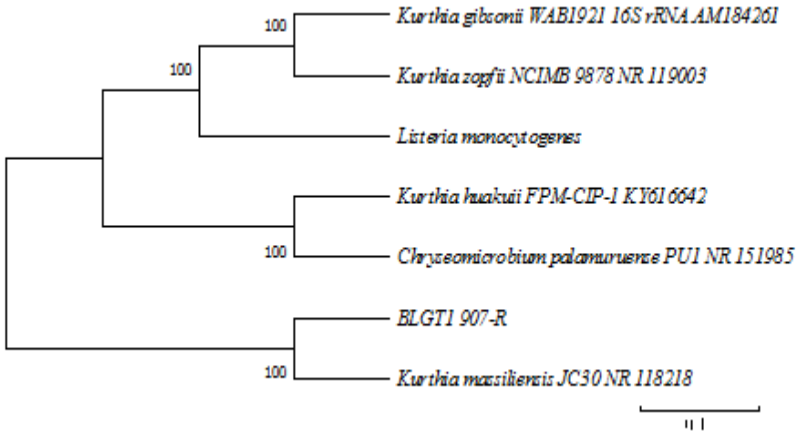

Fig. 10. Phylogenetic tree of isolate BLGT1

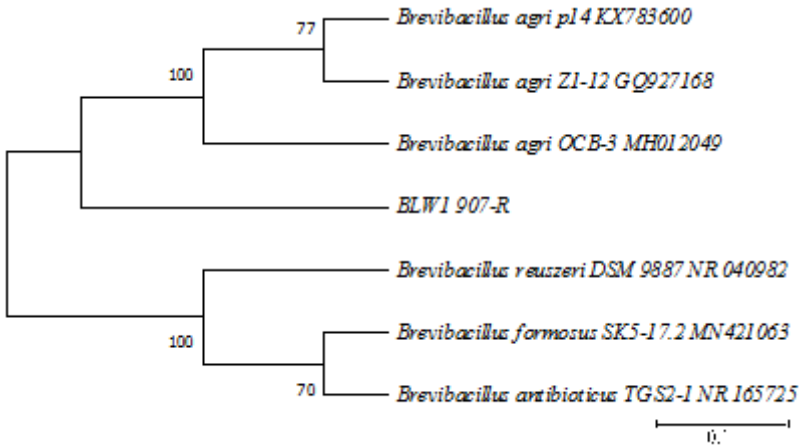

Fig. 11. Phylogenetic tree of isolate BLW1

Kurthia gibsonii has been identified as one of the players in zoonotic infections that is widely spread in sewage polluted environment (Adu *et al*., 2015). *K. gibsonii* was isolated from clinical sample of a patient diagnosed of recurrent urethritis and balanitis due to genital contact with the faecal contaminant from a female piglet (Joseph *et al*., 2016). Niveshika *et al*., (2016) reported that this microbe is greatly abundant in waters polluted with untreated waste-water, domestic water and industrial pollutant. In 2022, Lozica *et al.* reported a coinfection of *K. gibsonii* with *E. coli* in poultry birds and he concluded that *K. gibsonii* in itself is unable to cause primary infection but noted that the microbe may play a role as an

opportunistic pathogen.

Brevibacillus agri is a strong biofilm producer, non-human-pathogen and endospore-forming bacillus (Arzu and Tugba, 2020). Although it is non-human pathogen, it is pathogenic to certain pests such as *Malacosoma neustria*; hence it is used as biopesticide to improve crop yield. The report of Suneeva *et al*., (2014) who isolated *B. agri* from the urine sample of a patient diagnosed with focal pyelonephritis generated a lot of concern, and that *B. agri* may have undergone genetic mutation to cause urinary tract infection; however, as at the time of this current research, no study has indicated *B. agri* as a causal organism of pyelonephritis.

Conclusion: Indiscriminate sewage disposal into water bodies will lead to rapid biofilm attachment on the boat hulls. The movement of these boats on water surfaces will result in spread of non-indigenous microbial strains in water bodies, thereby causing point-point contamination and spread of pathogenic microbes. Consequently, this will lead to water-borne infections. The use of antibiofilm agents in boat hull paints will prevent biofilm attachment, help protect the integrity of boat hulls and ultimately reduce water-borne infections in humans.

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