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Assessment of Potential Health Hazards Associated with Bacterial Diversity in Municipal Solid Wastes of Okitipupa LGA in Ondo State, Nigeria

Adeyemo I. A.* and Demehin A. A.
Biological Sciences Department, Olusegun Agagu University of Science and Technology, Okitipupa, Ondo State, Nigeria.
* Corresponding author: ia.adeyemo@oaustech.edu.ng

Abstract: This study was designed to isolate and characterize the microflora associated with selected solid waste dump sites in Okitipupa Local Government Area (LGA) of Ondo State, Nigeria. Solid waste samples were collected in triplicates from ten (10) dump sites in Okitipupa LGA at two-week interval for three months using a random sampling procedure while culture method is used to isolate the bacteria. The standard method of ASTM-D5231-92 was employed to determine the quantity and composition of wastes. The most prevalent waste was food or green wastes, which ranged from 39.40% to 19.50%, while polythene materials were the least with a range of 5.20% to 0.90%. The mean bacterial counts ranged from 9.2 x 10^6 to 4.6 x 10^6 cfu. Colonial morphology and biochemical identifications of the bacteria from the samples revealed *Serratia* sp. *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Bacillus* sp, *E. coli* and Actinomycetes. Respiratory reactions may be induced in individuals. It is then concluded that, to lower the health risks linked to microflora in solid waste dumpsites, Municipal Solid Waste (MSW) facilities should have regular inspections, clean and disinfect surfaces and equipment, make sure workers wear protective gear, and follow proper waste management and disposal practices.
Key word: Dumpsites, Environment, Microflora, Municipal solid waste, Pathogens

INTRODUCTION

Waste is defined as any substance that is discarded by an individual, home, or entity. Waste is a complex amalgamation of several substances, with only a small number of them being intrinsically detrimental to health (Abdel-Shafy and Mansour, 2018). The broad production, ever-increasing quantities of, and sustainable dispersal of solid wastes are the subject of significant worldwide concern. The vast differences in composition and the complexity that accompany them necessitate a significant amount of effort to collect, process, and dispose of them in an environmentally favourable manner (Masebinu *et al.,* 2017; Liu *et. al.,* 2019; Gelan 2021).

Worldwide, there are numerous factors contributing to the increasing volume of solid refuse generated. As national incomes increase, countries generate more wastes, rising levels of prosperity, rapid urbanization, and population growth have all contributed to an increase in the amount of garbage produced per person. Additionally, waste management is frequently managed by local administrations that possess inadequate financial, operational, and managerial resources in the majority of municipalities (Bruna, 2023). At least 33 percent of the 2.01 billion tonnes of municipal solid garbage produced annually worldwide is not managed in an environmentally favourable manner (World Bank, 2024). The average amount of waste produced per individual per day worldwide is 0.74 kilograms, with a range of 0.11 to 4.54 kilograms (World Bank, 2024). Presently, Nigeria generates 40,959 tonnes of municipal solid waste per day, which totals 14.95 MT annually (Africa Check, 2019). It is anticipated that Nigeria will generate 54.8 MT of solid waste in 2030 and 107 MT in 2050 (Africa Check, 2019). Global waste generation rates may rise to 20 billion tonnes annually by 2050 unless immediate action is taken on numerous waste management fronts. This number is still increasing in most countries of the world (Kaza *et al*., 2018).

Open dumping of municipal solid waste is prevalent in African countries, including Nigeria. Open dumping is the most affordable choice for low-income nations with solid waste collection below 50%, and approximately 95% of the collected waste is disposed of haphazardly at different dumping sites (Ohwoghere-Asuma and Aweto, 2013; Janet and Kelechi, 2016; Gelan, 2021). Multiple studies have shown

that in many towns, municipal solid wastes are indiscriminately disposed of in open areas, including major residential areas, roadsides, drainage areas, rivers, riversides, and forests. This practice has resulted in the introduction of dangerous substances, such as heavy metals, into the water and soil ecosystems (introducing various harmful substances, such as heavy metals, into the soil and water compartments (Ogwueleka, 2009; Sankoh and Yah, 2013; Hailemariam and Ajeme, 2014; Kebede *et al*., 2016). This method allows waste to be easily accessed by scavengers and animals, and the production of pollutants is not monitored. Municipal Solid Waste (MSW) is regarded as an important source of microorganisms and a significant "microbial pool", the organic content in MSW supplies the necessary nutrients for the microbiological proliferation of microorganisms, which are primarily responsible for the breakdown of waste to render it safe and stable (Janet and Kelechi, 2016; Wang *et al*., 2017) Household garbage can transfer contagious pathogenic microorganisms to the environment by direct contact, inhalation, ingestion, or indirect contact via the food chain (Fernández-García *et al.,* 2016; Marquez *et al*., 2016; Ghazaei 2022)

Solid wastes are sometimes burnt so as to decrease the amount of garbage and its ability to spread disease. However, unregulated burning can release harmful substances such as Polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) along with other pollutants (Hossain *et al*., 2011). In 2016, approximately 1.6 billion tons of carbon dioxide (CO_2) equivalent greenhouse gas emissions were produced from the treatment and disposal of solid waste. This accounted for 5 percent of global emissions and was determined based on factors such as the volume of garbage generated, its composition, and the methods used for waste management. This is mostly caused by the practice of depositing waste in open dumps and landfills without implementing landfill gas collection systems. Approximately half of all emissions are attributed to food waste. As a matter of fact, and without any changes in the industry, it is projected that solid waste-related emissions will rise to 2.38 billion tons of CO_2-equivalent per year by 2050 (GBAC, 2023)

Municipalities frequently encounter numerous challenges stemming from limited financial resources, improper coordination, and the intricate nature of municipal solid waste that surpasses the capacity of the local government to manage (Sujauddin *et al.,* 2008). The composition of municipal solid trash varies considerably between municipalities and countries. The extent of diversity mostly relies on factors such as lifestyle, economic conditions, waste management policies, and industry composition. The volume and composition of municipal solid waste are crucial factors in determining the proper treatment and management of these wastes. The provision of such information is crucial and valuable for the establishment of a municipal facility that converts solid waste into electricity (World Bank, 2024).

Securing funding for solid waste management systems poses a substantial difficulty, particularly when it comes to covering continuing operational expenses rather than initial capital investments. It is crucial to consider operating costs from the outset. The operating expenses for integrated waste management, which includes collection, transport, treatment, and disposal, typically surpass $100 per tonne in high-income countries. Low-income countries allocate a smaller amount of money to waste operations, often around $35 per tonne or more. (Lisa and Frank, 2018; World Bank, 2024). This study was therefore designed to isolate and characterize the microflora associated with selected solid waste dump sites in Okitipupa Local Government Area (LGA) of Ondo State, Nigeria Additionally, it aimed to evaluate the potential health effects of this microflora on both the environment and humans.

MATERIALS AND METHODS

Study Area: Okitipupa Local Government Area was created in 1974 in Ondo state, Nigeria. Okitipupa Local Government headquarters is located in Okitipupa township with a university, Olusegun Agagu University of Science and Technology (OAUSTECH). The Local Government Area lies between longitude 4° 31'and 4° 55'E of the Greenwich Meridian and between latitudes 6° 48 and 6° 28N of the Equator as shown in Figure 1.

Sample Collection: A random sampling method was used, with field visits for sample collection followed by laboratory-based methods for processing. Okitipupa LGA was chosen as the study area. Solid waste samples were collected from a variety of open solid waste disposal sites. One (1) kilogram of solid wastes was collected with sterile shovel from each open waste dumpsite in sterilized polythene bags throughout the ten locations in triplicates every two (2) weeks for eighteen (18) weeks from various locations in the LGA. The locations are; Okitipupa, Igodan, Okunmo, OAUSTECH, Ayeka, Sabo, Ikoya, Erinje, Idepe, and Igbodigo as shown in Figure 1 above. All samples were properly labelled and transferred to the laboratory for analysis within three (3) hours after collection.

Characterization of solid waste: Quantification/composition of waste was determined by a standard method of ASTM-D5231-92 (ASTM, 2008). American Society for Testing Materials (ASTM) has termed this technique as ASTM-D5231-92 standard technique for the analysis of the MSW composition (Worrell and Vesilind, 2011). This step involves determining the quantity and composition of solid wastes generated over a given period. It helps in understanding waste generation patterns, estimating waste management requirements, and planning appropriate waste management strategies.

Isolation of Bacteria: Bacteria isolation from the solid wastes was done on nutrient agar at 37°C for 24 h. Pure colonies were then obtained and stored. Gram staining and biochemical tests were performed for the confirmation and identification of bacteria species using the methods described by Manandhar and Sharma (2018).

RESULTS AND DISCUSSION

The composition of MSW in this research is as shown in Table 1. The observed composition of MSW varies considerably across municipalities and countries. The extent of difference mostly relies on factors such as lifestyle, economic conditions, waste management rules, and industry composition (Africa Check, 2019).

Table 1: Quantification of MSW in location sites across Okitipupa LGA

Parameters (%)	Okitipupa	Igodan	Okumo	OAUSTECH	Ayeka	Sabo	Ikoya	Erinje	Idepe	Igbodigo
Paper	12.50	21.40	18.30	20.50	17.50	33.20	16.80	16.30	38.30	37.40
Plastic	10.20	9.70	8.10	7.50	6.40	7.20	8.30	6.70	9.60	11.30
Metal	15.40	4.10	4.70	15.10	5.30	5.70	15.20	27.40	6.30	4.70
Food Wastes	25.20	39.40	36.50	31.50	29.30	38.40	27.50	28.20	24.70	19.50
Glass	12.40	4.50	7.40	5.70	18.40	3.30	18.50	7.70	9.40	10.20
Polythene Bags	2.70	1.40	3.50	5.20	1.70	1.60	2.00	1.80	3.40	0.90
Wood	7.40	3.70	7.80	4.10	3.00	2.30	1.90	2.00	1.80	2.80
Textile	6.70	7.50	7.50	1.70	11.90	2.10	1.50	1.90	4.70	10.20
Others	7.50	8.30	6.20	8.70	6.50	6.20	8.30	8.00	1.80	3.00
Total	100	100	100	100	100	100	100	100	100	100

Key MSW=, LGA=, OAUSTECH=

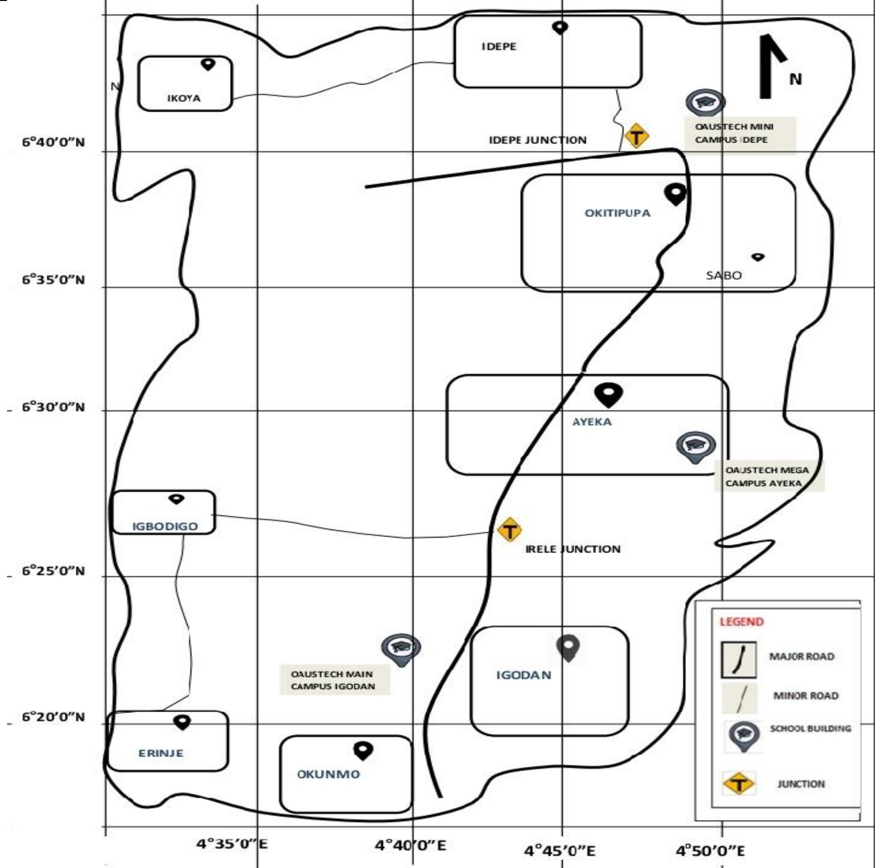

Figure 1: Map of Okitipupa LGA showing sample collection sites

Waste disposal in Okitipupa is still largely random and uncontrolled, and large quantities of waste go uncollected. It was found that, although MSW collection services owned by private organizations is available for the residents in the areas surveyed, majority of the residents are not taking the advantage of the services due to unwillingness to pay for these services. Oral discussion with inhabitants on the management of garbage collected by MSW collection agencies indicated that in most of these places, there is a lack of adequate treatment or dumping procedures for the collected wastes. On the contrary, the most prevalent method was the incineration of rubbish in open dumpsites.

This is why most residents have also opted for open and indiscriminate dumping of wastes at every available space. This same trend has also been reported by other researchers in subsharan Africa and across the globe (Agbefe *et al*., 2019; Ayeleru *et al.,* 2020; Domingo and Manejar, 2021; Muheirwe *et al*., 2023). Waste characterization revealed that food and green wastes account for the highest waste generated across the various locations ranging from 39.40% in Igodan to 19.50% at Igbodigo. This research finding aligns with that of World bank that stated that food and green waste accounted for 44% of the global waste composition in her research and that food and green waste is the largest waste produced across the countries of the world (World Bank, 2024). It has been reported that the content of waste varies depending on economic levels, which is indicative of different consumption patterns(World Bank, 2024) . In high-income countries, the proportion of food and green waste is relatively low, making up just 32 percent of the total garbage. On the other hand, these countries produce a larger amount of dry waste that has the potential to be recycled, such as plastic, paper, cardboard, metal, and glass, which accounts for 51 percent of the waste. Fifty three percent of food waste and

fifty seven percent of green waste are produced by middle- and low-income countries. As economic development levels decline, the proportion of organic waste increases as also revealed by the result of this finding where larger proportion of the wastes across all the sampled locations are green or food wastes (UNEP, 2024).

Biochemical characterization of bacterial isolates from MSW

A total of six (6) bacterial isolates were recorded in this research work. Bacterial isolates identified via cultural and biochemical characteristics included Actinomycetes (33.3%) which occurred most frequently among the isolates, followed by others – *Serratia* sp. (25%), *Pseudomonas aeruginosa* (16.7%), *Proteus mirabilis* (8.3%), *Bacillus* (8.3%) and *E. coli* (8.3%).

The mean bacterial counts isolated from solid waste dump sites across the LGA are as shown on Table 2, while the results of biochemical tests for the characterization of the bacteria are as shown on Table 3.

Table 2: Total heterotrophic counts (THC) of bacterial isolates from solid waste samples

Locations	Mean bacterial counts (Cfu/ml)
Okitipupa	5.2×10^6
Igodan,	9.2×10^6
Okunmo	6.8×10^6
OAUSTECH	6.6×10^6
Ayeka	5.6×10^6
Sabo	8.6×10^6
Ikoya	5.4×10^6
Erinje	5.8×10^6
Idepe	4.6×10^6
Igbodigo	5.6×10^6

Key: OAUSTECH = Olusegun Agagu University of Science and Technology.

Table 3: Biochemical Characteristics of the Bacterial isolates from the MSW

Isolate code	Gram stain	Shape	Spore	Capsule	Catalase	Oxidase	Glucose	Lactose	Mannitol	Xylose	Coagulase	Probable organism
SD 1	+	R	+	-	-	-	-	-	+	-	-	*Actinomycetes*
SD 2	-	R	-	-	+	-	+	-	-	+	-	*Serratia sp.*
SD 3	-	R	-	-	+	-	+	+	+	-	-	*Pseudomonas aeruginosa*
SD 4	-	R	-	-	+	-	+	-	-	+	-	*Proteus mirabilis*
SD 5	+	R	-	-	+	-	+	-	+	-	-	*Bacillus subtilis*
SD 6	-	R	-	-	+	-	+	+	+	+	-	*E. coli*

Key: - (negative), + (positive), R (Rod)

Extensive literature coverage exists regarding the health ramifications associated with specific bacteria isolated from municipal solid wastes, including *Serratia* sp., *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Bacillus* sp., *E. coli*, and actinomycetes. According to reports, while the majority of *Pseudomonas* species are saprophytic, low-virulent organisms, *P. aeruginosa* and others are recognized globally as significant human pathogens capable of causing severe nosocomial, healthcare-associated, and (less often) community-acquired infections that significantly contribute to morbidity and mortality (Hilmar and Harald, 2010; Fair and Tor, 2014). Penicillin-resistant strains of *P. aeruginosa* are notorious for providing protection against disinfectants and antibiotics via the biofilms produced (Odumosu *et al*., 2013). Reportedly, the bacterial species are capable of acquiring resistance genes that confer resistance to a variety of antibiotics that are frequently employed in the treatment of infections (Kityamuwesi *et al*., 2015. Mishandling of

pharmaceuticals and antibiotics in MSW facilities poses a potential hazard by facilitating the growth and dissemination of antibiotic-resistant strains of *Pseudomonas aeruginosa* (Nikokar *et al.*, 2013; Khosravi *et al.,* 2017; Zheng *et al.,* 2019). Other potential health complications encompass respiratory ailments, dermatological irritations, ocular infections, gastrointestinal disruptions, and systemic infections, as previously documented by Sadikot *et al.* (2005) and Barbier *et al.* (2013). In close proximity communities, the existence of *Pseudomonas aeruginosa* within MSW facilities may also present a hazard. Community members may potentially be exposed to the bacteria and develop infections or other health complications if the bacteria contaminate the surrounding environment, including air and water sources (Al-Khatib *et. al.,* 2015)

Escherichia coli isolated from municipal solid wastes in this research is in line with the reports of multiple researchers who have also isolated *Escherichia coli* from municipal solid waste (Costa *et al*., 2006; Sáenz *et al.* 2004; Paulshus *et al.* 2019). Some species of the bacterium *E. coli*, which is frequently encountered in the intestines of both humans and animals, are capable of causing illness if ingested. Foodborne illnesses, including diarrhea, abdominal cramps, and vomiting, have been associated with *E. coli* (Huang *et al.,* 2006; Majowicz *et al*., 2014). Additionally, occupational hazards resulting from water and airborne infections have been reported by health workers involved in solid waste management. (Kretchy *et al.* 2015; Kretchy *et al*. 2020; Tack *et al.* 2020). Vector infestations may result from improper solid waste management, including the attraction of flies, rodents, and cockroaches. These vectors may carry *E. coli* bacteria on their bodies, which they subsequently disseminate to other regions. This reduces the likelihood of *E. coli* transmission to humans and animals via direct contact or through the contamination of food and water sources.

In their investigation, Oshoma *et al*. (2017) isolated *Serratia* sp., *Bacillus*, *Pseudomonas*, and *E. coli* from landfill sites; which is consistent with the findings in this study. However, certain *Serratia* species are innocuous and comprise a normal component of the human microbiota, others are capable of inducing infections and presenting health hazards. Infections caused by *Serratia* can give rise to various clinical manifestations, including but not limited to pain, fever, chills, redness, edema, and sepsis, mortality, and respiratory distress (Casolari *et al*., 2005; Yoon *et al*., 2005; StewartGreco *et al*., 2012). *Bacillus subtilis* is responsible for bacteremia, endocarditis, pneumonia, and septicemia, among other infections. However, immunosuppression of the host and high-dose inoculation are required prior to *B. subtilis* infection, as the pathogen typically exploits opportunities to invade hosts (Muzumdar *et al*., 2011; Kityamuwesi *et al*., 2015; Tsonis *et al.,* 2018). The isolation of actinomycetes from solid residues in India and China, respectively, by Namrata *et al*. (2012) and Yi *et al*. (2017), is consistent with the results obtained in this study. Actinomycetes are indispensable for the decomposition of organic matter in municipal solid wastes. Although, actinomycetes generally aid in the decomposition of waste materials and the promotion of nutrient recycling, their presence in solid waste may have adverse health effects, including the production of secondary mycotoxins that are potentially toxic to humans (Lapalikar *et al.,* 2012; Cserháti *et al.,* 2013). Prior studies have documented that these toxins have the potential to induce carcinogenic effects and cause liver damage, kidney dysfunction, and neurotoxicity (Tuan *et al.,* 2003; Adeyemo *et al*., 2018; Greeff-Laubscher *et al*., 2018).

Shin-Hee *et al.* (2005) isolated *Proteus mirabilis* from retail meat waste sites in Oklahoma, while Zhongjia *et al.* (2021) also isolated *Proteus mirabilis* from Belgian broiler carcasses of retail and human stool. These findings aligns with the results of this research. *Proteus* sp. are commonly found in the intestines of humans and other animals,

and are also widely distributed in the environment. Nevertheless, the species *P. mirabilis* is frequently found to be a pathogen in urinary tract infections in humans, particularly in cases of catheter-associated bacteriuria in patients with long-term catheterization. Additionally, it is more frequently detected in the stools of patients with diarrheal disease compared to healthy patients (Sabbuba *et al.,* 2003). In addition, *P. mirabilis* has been implicated in nosocomial infections in immunocompromised persons, such as bloodstream infection, cystitis, pyelonephritis, prostatitis, neonatal meningoencephalitis, empyema, and diarrheal illness (Sabbuba *et al*., 2003; Jacobsen *et al*., 2008).

CONCLUSION

The bacteria associated with solid waste dump site in Okitipupa metropolis had been presented in this study. As a result of no functional sanitary landfill in Okitipupa metropolis, indiscriminate dumping of untreated solid waste can pose significant risks to both human health and the environment. Although, solid waste has a beneficial effect on the soil microflora and enzymatic activities, it is crucial to clean, sort and perhaps treat the wastes before disposing them off in order to minimize the health risks connected with waste dumping. This study has provided valuable insights into the bacterial communities associated with solid wastes in the dumpsites.

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Extended Spectrum Beta-lactamase Genes in Clinically Important Bacteria Isolated from Wastewater of Two Selected Tertiary Hospitals in Enugu, Nigeria

Agbo O. A.* and Momoh M. A.
Department of Pharmaceutics, University of Nigeria, Nsukka, Enugu State, Nigeria.
* Corresponding author: agbo.oluchi2024@gmail.com

Abstract: Hospital activities have resulted in increased discharge of untreated effluent into the environment, posing substantial risks to public health and the environment due to the presence of diverse harmful components, including extended spectrum β–lactamase (ESBL) producing bacteria harboring resistance genes, which are adding to the global crisis of antimicrobial resistance (AMR). This study aimed to assess the prevalence of ESBL genes in bacterial isolates from wastewater of two selected tertiary hospitals in Enugu State. A total of 20 samples were aseptically collected, transported and processed for bacteriological identification and susceptibility testing following standard procedures. Phenotypic and genotypic detection of extended spectrum beta lactamases (ESBL) were conducted following standard procedures. Screening for ESBL production was done by double disk synergy test and data obtained were analyzed using SPSS version 23. A total of 65 bacterial isolates, 41(63.1%) Gram-negative and 24(36.9%) Gram-positive, were detected from the samples. Out of these isolates, ESBL production was observed in five 5(81%) isolates of *Escherichia coli,* thirteen 13(81%) isolates of *Klebsiella* spp and one 1(20%) isolate of *Pseudomonas aeruginosa.* The ESBL encoding genes**-** *bla SHV, blaCTX-M, bla OXA, bla TEM* were found in varying levels among the *E. coli* and *Kleb.* spp isolates, while the *Pseudomonas aeruginosa* isolates were found to be harboring *bla-CTXM, bla OXA, bla TEM* genes, but *bla SHV* genes were absent. All the ESBL producers were multi-drug resistant, therefore proper treatment of hospital wastewater before discharge into the environment is highly recommended.

Key word: Antibiotics, bacteria, Enugu, extended spectrum beta lactamase (ESBL) genes**,** hospital wastewater

INTRODUCTION

The rise and dissemination of extended spectrum β -lactamases (ESBL) bacteria, once perceived as relatively harmless, have evolved into a significant resistance challenge afflicting healthcare system worldwide. This has given rise to a situation which threatens the efficacy of existing antibiotics used to combat bacterial infections, posing a serious threat to public health (Ovia *et al.,* 2023). Ja'afaru *et al.* (2023) revealed that the production of extended -spectrum β -lactamases is one of the ways bacteria have developed resistance to antimicrobial agents.

Extended spectrum beta lactamases (ESBLs) are a group of enzymes that possess the ability to deactivate the beta-lactam rings of penicillins, first, second and third generation cephalosporins, and aztreonam (Ejikeugwu *et al.,* 2016), but are inhibited by clavulanic acid, a beta – lactamase inhibitor (Bonnet, 2004; Rawat and Nair, 2010). Fernandes *et al.* (2014) identified several molecular variants of ESBLs designated as TEM-1, TEM-2, SHV, OXA, CTX-M and PER amongst others with the most prevalent types being TEM and SHV enzymes. The ESBLs are chromosomally or plasmid mediated and can easily be transferred from one bacterium to another via horizontal gene transfer (Ugah and Udeani, 2020). Therefore, organisms which produce ESBLs usually manifest resistance to multiple antibiotic classes (Giwa *et al.,* 2018), thereby posing very serious therapeutic challenges from limited treatment options, with severe, and in some cases, fatal clinical outcomes (Rawat and Nair, 2010).

Extended spectrum beta lactamases (ESBLs) are produced by Gram-negative bacteria and most strains producing them belong to the family Enterobacteriaceae, and often contain resistance determinants for other classes of antibiotics like the aminoglycosides, sulfonamides, fluoroquinolones tetracyclines, chloramphenicol, trimethoprim and sulphonamides which are readily transmissible from one strain of organism to another and between different species of Gram-negative bacteria (Munday *et al.,* 2004; Jacoby and Munoz-Price, 2005; Peirano and Pitout, 2010). Although these

transfers between bacteria can occur within the community, it is most often observed in healthcare facilities, and is a major challenge in nosocomial infections (Bello *et al.*, 2021). Oli *et al.* (2016) noted some factors that create suitable conditions for their spread within the hospital setting such as; poor hygienic practices in hospitals especially those in developing countries, indiscriminate antibiotics use, empirical antibiotic prescription and therapy not supported by the laboratory, absence of antimicrobial resistance surveillance programs and inadequate infection control practices.

Buelow *et al*. (2017) recognized hospital wastewater as a reservoir for ESBL genes, which can be disseminated into the environment, posing a risk to human health. Several cases of ESBL producing bacteria have been reported across the world in both clinical and nonclinical samples in Nigeria, Japan, South Korea, and Egypt (Ejikeugwu *et al*., 2013; Zorgani *et al.,* 2017). The ESBL-producing bacteria are responsible for the cause of community onset of urinary tract infections (UTIs) (Zorgani *et al.,* 2017). To date there is paucity of information regarding the actual disease burden and frequency of ESBL producing bacteria in Nigeria, hence this study was carried out to evaluate the presence and prevalence of extended spectrum β -lactamases genes in some clinically significant bacteria isolated from wastewater of two Tertiary Hospitals in Enugu, Nigeria.

MATERIALS AND METHODS

Sample site and collection: The wastewater samples were collected from four (4) different wastewater outlets of University of Nigeria Teaching Hospital (UNTH) and National Orthopedic Hospital Enugu (NOHE) Nigeria, with four sampling points designated: NOHE Female Medical Ward (NFMW), NOHE Amenity Ward (N-AW), UNTH Post Natal Ward (U-PNW) and UNTH Male Medical Ward (U-MMW). Samples were collected into sterile, clean dry universal containers that were tightly capped immediately after sampling and then transported to the laboratory for microbial analysis within 1–2 h from the collection time.

Isolation and characterization of test bacteria: The test samples were inoculated on chocolate agar (CHA), mannitol salt agar (MSA), cetrimide agar (CTA), Eosin - methylene blue agar (EMB), blood agar (BA), and MacConkey agar plates (MA) according to the method described by Cheesbrough (2000). These plates were incubated at 37^0C for 24 hours, and bacterial colonies on the plates were observed for shape and colour after the first 24 hours, then isolated, Gram-stained, and studied microscopically. Isolates that fermented lactose and had a greenish metallic sheen were suspected to be *Escherichia coli* and were subsequently confirmed by testing for indole production, methyl red, voges proskae, and citrate consumption (IMVIC) with other Enterobacteriaecae, using the method described by Fawole and Oso (2004). Catalase production and coagulase positive tests were performed on colonies with yellow zones on Mannitol salt agar culture. Catalase, coagulase, nitrate, oxidase, indole production, methylred test, Voges-Prauskaur, citrate utilization test, use of Kligler Iron Agar (KIA) (for double sugar fermentation and H_2S formation), pigment synthesis, and motility tests were performed on all other isolates (Cheesbrough, 2000).

Extended spectrum beta lactamase (ESBL) screening and confirmatory test: The ESBL production was phenotypically confirmed in all the bacteria clinical isolates by the double disk synergy test (DDST) method (Ejikeugwu *et al.,* 2013; Ejikeugwu *et al.,* 2016). Double disk synergy test was performed as a standard disk diffusion assay on Mueller-Hinton (MH) agar plates (Oxoid, UK) as suggested by Clinical Laboratory Standard Institute (CLSI, 2011). Antibiotic disks of amoxicillin-clavulanic acid (20/10 μg) was placed at the center of the MH agar plate, and antibiotic disks containing two third generation cephalosporins (3GC) cefotaxime (30 μg) and ceftriaxone (30 μg) was each placed at a distance of 15 mm from

the central disc (amoxicillin/clavulanic acid). The plates were incubated at 37^{0}C for 18-24 hours. Extended spectrum beta lactamase production was inferred phenotypically when the zones of inhibition of the cephalosporins (Cefotaxime 30 µg or ceftazidime 30 µg) was expanded by the amoxicillin/clavulanic acid disk (20/10 µg). However, a ≥ 5 mm increase in the diameter zone of inhibition for either of the cephalosporins tested in combination with amoxicillin/clavulanic acid versus its zone when tested alone confirmed ESBL production phenotypically (Bradford, 2001; Ejikeugwu *et al.,* 2016).

Molecular characterization of ESBL genes: The ESBL-producing isolates were subcultured in 5 ml of nutrient broth and incubated overnight at 37^{0}C for molecular characterization of ESBL genes. The whole chromosomal DNA was extracted by boiling following the procedure described by Queipo-Ortuno *et al*. (2008). The PCR amplification with specific primers targeting blaCTX-M, blaTEM, blaOXA and blaSHV genes were done using a thermocycler. Polymerase chain reaction (PCR) products were sequenced, and sequence analysis was performed using bioinformatics tools. All gene sequences were compared with data of the GenBank (NCBI) database to identify the exact b-lactamase genotype and presented in Table 1.

RESULTS

Table 2 shows the different isolates. Among the bacterial genera isolated namely *Klebsiella* spp and *E. coli* were the most prevalent Gram-negative bacteria (GNB) accounting for 16 (24.6 %), and 6 (9.2%) isolates respectively. Phenotypically, ESBL production was observed in five isolates of *E. coli*, thirteen strains of *Klebsiella* spp and one isolate of *P. aeruginosa* (Table 3). However, no presence of ESBL enzymes in *Enterobacter* spp*, Salmonella* spp*, Proteus mirabilis and Serratia marcescens* was detected. The ESBL positive isolates were found to be resistant to antibiotics in the class of aminoglycosides, cephalosporins, macrolides, penicillins and fluoroquinolones (Tables 4).

Table 1: Extended spectrum beta lactamase gene sequence of forward and reverse primers for multiplex PCR technique

Gene target(s)	Primer sequence (5' to 3', as synthesized)	Expected amplicon size (bp)
blaTEMF	5'AAACGCTGGTGAAAGTA3'	500
blaTEMR	5'AGCGATCTGTCTAT3'	
blaOXA-F blaOXA-R	5'ACACAATACATATCAACTTCGC3'	650
	5'AGTGTGTTTAGAATGGTGATC3'	
blaCTX-M F blaCTX-M R	5'CGCTTTGCGATGTGCAG3'	750
	5'ACCGCGATATCGTTGGT3'	
blaSHV F blasSHV R	5'ATGCGTTATATTCGCCTGTG3'	850
	5'TGCTTTGTTATTCGGGCCAA3'	

Note: All primers were synthesized by Inqaba Biotec™, Pretoria, South Africa

Table 2: Different bacterial isolates from the Hospital wastewaters

Bacterial isolates	Number isolated (%)	Sources
Klebsiella spp	16 (24.6)	N-AW, U-PNW, N-FMW and U-MMW
Escherichia coli	6 (9.2)	N-AW, U-PNW, N-FMW and U-MMW
Enterobacter spp	6 (9.2)	N-AW, U-PNW, N-FMW
Pseudomonas aeruginosa	5 (7.7)	N-AW, U-PNW
Salmonella spp	3(4.6)	N-AW, U-PNW
Proteus spp	2(3.1)	N-FMW
Serratia marcescens	3(4.6)	N-FMW and U-MMW
Staphylococcus aureus	8 (12.3)	N-AW, U-PNW, N-FMW and U-MMW
Coagulase-ve staphylococci	6(9.2)	N-AW, U-PNW, N-FMW and U-MMW
Streptococcus pneumoniae	4 (6.2)	U-MMW, N-FMW
Enterococcus faecalis	6 (9.2)	N-AW, U-PNW, N-FMW and U-MMW

Key: N-AW, NOHE Amenity Ward; U-PNW, UNTH Post Natal Ward; N-FMW, NOHE Female Medical Ward; U-MMW, UNTH Male Medical Ward

Table 3: Phenotypic detection of ESBL in test isolates

Organism	Number of isolates tested	Number of ESBL positive isolates (n%)	Number of ESBL negative isolates (n%)
E. coli	6	5(83)	1(17)
Klebsiella spp	16	13(81)	3(19)
P. aeruginosa	5	1(20)	4(80)
Enterobacter spp	6	0(0)	6(100)
Salmonella spp	3	0(0)	3(100)
Proteus mirabilis	2	0(0)	2(100)
Serratia marcescens	3	0(0)	3(100)

Table 4: Prevalence of ESBL genes in the test isolates

Bacteria	Number of ESBL positive isolates	Number of Isolates (%) habouring				Antibiotics resistant to
		bla SHV	*bla-CTX-M*	*bla OXA*	*bla TEM*	
E. coli	5	4(80)	5(100)	5(100)	5(100)	Cephalosporins
Klebsiella spp	13	12(92)	13(100)	13(100)	10(77)	Fluoroquinolones, Penicillins
P. aeruginosa	1	0(0)	1(100)	1(100)	1(100)	Macrolides, aminoglycosides

DISCUSSION

Hospitals play an important role in the preservation and promotion of a citizen's health. However, Azuma *et al.* (2020) reported that hospital operations cause the formation of diverse mixes of inorganic, organic, and microbiological components, which are typically discharged into the environment as wastewater effluents without previous treatment. This study evaluated the presence of ESBL-producing bacteria in hospital wastewaters, and the prevalence of ESBL encoding genes in sample isolates. Understanding the distribution of ESBL-producing bacteria is crucial for guiding empirical antibiotic therapy and implementing infection control measures in healthcare settings. In the present study, a total of 65 bacterial strains were isolated from the four different wards. Among the bacterial genera isolated were *Klebsiella* spp and *E. coli*. The two isolates appeared to be the most prevalent Gram-negative bacteria (GNB) accounting for 16(24.6%), and 6(9.2%) of the isolates respectively. This is consistent with the findings of Mirkalantari *et al*. (2020), that reported *E. coli* to be the most dominant when urine samples were investigated. However, studies have reported conflicting results regarding the predominant bacteria isolated from various clinical samples, with Ogefere *et al.* (2015); Andrew *et al.* (2017) identifying *K. pneumoniae* as the most common species in their works, Jain *et al*. (2003) reported *Enterobacter* as the most prevalent genus, and Pandey *et al*. (2020), recorded *Bacillus* spp, *Staphylococcus* spp, and *Streptococcus* spp as the most common bacteria in hospital wastewater. These variations in results might be due to differences in sampling sites, prescription patterns of healthcare practitioners in the areas, isolation procedures, amongst other factors. Among the tested species in this study, *E. coli* and *Klebsiella* spp exhibited the highest rates of ESBL positivity, with 83% and 81% of isolates being ESBL-positive, respectively. This is consistent with the study of Zhang *et al*. (2009) who recorded a significant incidence of ESBL-producing *E. coli* bacteria in hospital wastewaters. The high prevalence of ESBL production in these organisms highlight the urgent need for vigilant surveillance and antimicrobial control efforts to mitigate the spread of resistant strains and optimize therapeutic strategies. Contrastingly, *Pseudomonas aeruginosa* an opportunistic pathogen associated with hospital-acquired infections, particularly in immunocompromised individuals and those with cystic fibrosis

exhibited a lower rate of ESBL positivity, with only 20% of isolates being positive. On the other hand, the ESBL-producing *P. aeruginosa* strains are less common compared to other Gram-negative bacteria. Livermore (2009) revealed that their emergence poses a significant threat to immunocompromised patients and those with indwelling medical devices. *Enterobacter* species and the other Enterobacteriaceae tested (*Salmonella* species, *Proteus mirabilis,* and *Serratia marcescens*) displayed a striking absence of Extended spectrum beta lactamase production in this study. This may be interpreted as a favourable finding from an infection control perspective. Woodford *et al*. (2014) documented that the absence of ESBL production in these species does not negate their clinical significance or their potential to acquire resistance mechanisms through horizontal gene transfer or mutational events (Ugah and Udeani, 2020). The data presented in this study revealed that there was prevalence of ESBL genes among various bacterial isolates, along with their associated resistance profiles. Notably, all ESBLpositive *E. coli* isolates harboured genes encoding for bla-SHV, bla-CTX-M, bla-OXA, and bla-TEM, conferring resistance to cephalosporins. This corroborates the findings of Olutayo and Abimbola (2016), thus indicates a diverse repertoire of ESBL enzymes within this species. This observation also aligns with the report of Obasi *et al.* (2017), and other previous studies documenting the widespread dissemination of bla-CTX-M, bla-OXA, and bla-TEM genes among clinical *E. coli* isolates, contributing to the global burden of multidrug-resistant infections (Cantón and Coque, 2006; Adeyankinnu *et al.,* 2014; Raji *et al.,* 2015; Bello *et al.,* 2021). Similarly, *Klebsiella* species exhibited a high prevalence of ESBL genes with majority of the ESBL-positive strains harbouring bla-SHV, bla-CTX-M, bla-TEM and bla-OXA genes, conferring resistance to fluoroquinolones, and penicillin. This conforms with the reports of Clarivet *et al*. (2016), and Azuma *et al*. (2022) who also recorded the bla-SHV enzymes predominated in *Klebsiella* spp. These findings underscore the importance of vigilant surveillance and infection control practices, particularly in healthcare settings where *Klebsiella* spp. are prominent contributors to nosocomial infections (Bush and Fisher, 2011). The association of ESBL genes with resistance to macrolides and aminoglycosides underscores the complex interplay between different classes of antibiotics and the potential for cross-resistance to develop (Livermore, 2002). All of these findings point to the existence of a large number of MDR bacteria with ESBL genes in hospital settings. However, unlike other ESBLs, the bla-CTX-M family is made up of a diverse and complicated set of enzymes which are now significantly more common in Enterobacteriaceae than other forms of ESBLs in Europe and other areas of the world (Azuma *et al*., 2022).

CONCLUSION

This study found a substantial population of both Gram negative and Gram positive multiantibiotic resistant bacteria in wastewater effluents from the two tertiary hospitals used. Most ESBLs, were found in higher proportions in *Klebsiella* spp, and *E. coli*. Some or all of the GNB recovered from the four hospital wards have ESBL producing genes. A major public health worry is that multi-drug resistant isolates from hospital wastewaters serve as a reservoir of resistance genes that could be transferred to other vulnerable bacteria. In view of these findings, proper treatment of hospital wastewater is recommended before discharge into the environment.

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Antibiotic Susceptibility Pattern of Bacterial Species Isolated from Selected Underground Water Bodies in Ohaukwu Metropolis, Ebonyi State, Nigeria

Awoke O. O.[1] Edeh P. A.[2*] Ekuma U. O.[3] Ede J. U.[4] Ituma M. A.[1] Awoke C. U.[5] Anene C. C.[6] and Onwa N. C[1]

1. Department of Applied Microbiology, Ebonyi State University, Abakaliki Nigeria
2. Department of Microbiology, Kingsley Ozumba Mbadiwe University Ideato Imo State Nigeria
3. Department of Microbiology, Federal University of Technology Owerri, Nigeria
4. Department of Biology/Biotechnology, David Umahi Federal University of Sciences, Uburu Ebonyi State Nigeria
5. Department of Industrial Chemistry, Ebonyi State University, Abakaliki Nigeria
6. Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka Nigeria

* Corresponding author: anyigoredeh@gmail.com

Abstract: Infections caused by using contaminated water are common in developing nations. Indiscriminate use of antibiotics has led to increased spread of antibiotic-resistance bacteria, even in underground water. This study investigated the susceptibility pattern of bacterial isolates from borehole water and hand-dug wells in Ohaukwu, Ebonyi State, Nigeria. Twenty (20) water samples were collected at random from hand-dug wells and underground boreholes located throughout Ohaukwu Local Government Area, and were examined for their physicochemical parameter, presence of bacteria as well as the antibiogram of the bacteria isolates using standard techniques. Our test results showed most of the physicochemical parameters were within permissible limits. The bacteriological analysis however exceeded the WHO limit of 0Cfu/100ml for drinking water with the presence of *Escherichia coli* 22 (42.5%), *Pseudomonas* spp. 3 (8.9%), *Klebsiella* spp.15 (36.0%), Salmonella spp. 15 (36.0%), and *Staphylococcus aureus* 6 (12.6%) make up the percentage distribution of the bacteria isolates. The antibiogram analysis revealed that most isolates were resistant to Mupirocin (97%), Bacitracin (91%), Gentamycin (84%) and Clindamycin (76.3%), but were sensitive to Ofloxacin (99.4%), Ceftazidime (86%) and Amikacin (72.3%). The study revealed that while *Salmonella* species was susceptible to Amikacin (72%), Ceftazidime (82.1%), and Ofloxacin (86.9%), it was resistant to the other three tested drugs. The resistance profile and low bacteriological quality of the drinking water sources evident in this study, is of great importance for the public health to the people resident in Ohaukwu Local government Area of Ebonyi State. It emphasizes the need for public education campaigns against the risks of building substandard wells and boreholes, and maintaining stringent standards for sanitation and personal hygiene.

Key word: Groundwater, antibiotics, resistance, Ebonyi

INTRODUCTION

A new era in the practice of contemporary medicine began with the discovery of antibiotics (Zainab *et al.,* 2020; Ejaz *et al.,* 2021). The development of antibiotic resistance by pathogenic organisms has presented a counterattack to this achievement, though. This therapeutic achievement is currently under jeopardy due to the notable rise in antibiotic resistance among common bacterial infections, which puts critically sick patients' chances of recovery at risk and creates a serious public health emergency (Pazda *et al.,* 2020). The global community recognizes the emergence of resistance among the most significant bacterial diseases as a serious danger to public health. Recent researches have shown that the environment has a significant role in the establishment of resistant infections and the spread of resistant bacteria, contributing to the rising healthcare issue caused by antibiotic resistance (Larsson and Flach, 2022).

It is estimated that seventy percent (70%) of the Earth's surface is made up of water (Atobatele and Owoseni, 2023). This underground water may be found almost anywhere on Earth if one digs deep enough, but most accessible groundwater is generally found within 1 km of Earth's surface (Atobatele and Owoseni, 2023). In many parts of the world, it is regarded as the most

important source of public water supply (Zainab *et al.,* 2020). Underground water is made up of water bodies that are not directly exposed to the atmosphere, but are submerged within the earth's crust. Examples include wells, springs, and so on. It is generally believed that groundwater is the purest form of naturally occurring water since the passage of water through the soil sediments tends to retain contaminating agents, (Orogu *et al.,* 2017). The fact that ground water is not always free of microorganisms however, defeats this assumption as recent studies have shown that the quality of groundwater in most urban areas in Nigeria is deteriorating fast due to contamination with pathogenic microorganisms (Ekhosuehi *et al.,* 2018). It has been demonstrated that pathogenic organisms, including bacteria and fungi, are prevalent in the soil and eventually the ground water. However, because several contaminants have the ability to enter the aquifers, anthropogenic activities, particularly those related to agriculture and industry, pose a threat to groundwater quality (Zainab *et al.,* 2020). Some recently identified types of environmental pollutants, such as pharmaceuticals and personal care products (PPCPs), have drawn attention from the scientific community and the general public because of their possible bioactive qualities and unclear consequences on the aquatic environment, (Pompei *et al.,* 2019). Groundwater quality pollution has been linked to well construction and placement, well proximity to residential waste disposal sites, abattoirs, and sanitary systems such septic tanks and pit latrines (Ekhosuehi *et al.,* 2018; Obayiuwana and Ibekwe, 2020).

Access to clean water is a global concern as water is responsible for a variety of human illnesses, including urinary tract infections, wound infections, gastroenteritis, meningitis, septicemia, and pneumonia. A lot of enterobacterial infections are distributed by faecal-oral transmission and are commonly associated with poor hygienic conditions (Olufeyikemi and Abimbola, 2020; Uzairue *et al.,* 2023). Countries with poor water decontamination have more illness and death from enterobacterial infection. Infections gotten through the consumption and use of contaminated water are common in poor and developing nations (Atobatele and Owoseni, 2023). A significant issue for humanity is the availability of sufficient and high-quality water, particularly in developing and impoverished countries. It is now extremely difficult to meet all of the water requirements in terms of quantity, quality, and consistency due to the population growth in most towns and cities and the ensuing rise in demand for social amenities (Atobatele and Owoseni, 2023). In the past, only bacteria linked to illnesses like cholera and typhoid fever were a major issue when it came to water contamination. However, more recently, it has been reported that newly identified microbial pollutants, including microbial strains carrying virulence genes and antibiotic resistance determinants designated as severe public health risk, represent more serious problems to water safety (Ateba *et al.,* 2020), especially when the organisms develop resistance to antibiotics.

In Nigeria, particularly in the state of Ebonyi, the government is unable to offer purified water via pipes, people resort to groundwater for their water needs. The most readily available sources of potable water for residents of Ohaukwu Local Government Area in Ebonyi State, similar to many other African and Nigerian communities, are boreholes, wells, and streams. These water sources may be home to pathogens that cause diseases like cholera, diarrhea, typhoid fever, river blindness, and schistosomiasis, among others (Onifade *et al.,* 2019). Most people agree that one of the purest sources of water is groundwater. Pipe borne and groundwaters have been shown to contain pathogenic bacteria, including *Escherichia coli*, *Aerobacter aerogenes*, *Klebsiella* sp., *Pseudomonas* sp., *Proteus* sp., *Staphylococcus* sp., and *Acaligenes* spp. (Ogu *et al.,* 2017; Ekhosuehi *et al.,* 2018). Groundwater and pipe-borne *Acaligenes* sp.

have been isolated (Ogu *et al.,* 2017; Babatunde *et al.,* 2022). Particularly in individuals with impaired immune systems, these isolates, *Pseudomonas aeruginosa* and *Klebsiella* sp., have the potential to cause infections. Public health is at risk due to the spread of new pollutants like bacteria and genes that resist antibiotics. One unattractive side effect of this is the rise in the prevalence of bacterial illnesses in the general population. The fact that viruses that persist longer and are distributed throughout the environment pose a higher risk to public health than those that are contagious is the reason for their significance (Tangwa *et al.,* 2019). Since antibiotic-resistant genes and bacteria settle in aquatic environments, the spread of these resistant bacteria in the environment poses a serious risk to public health. Any direct or indirect contact with contaminated water for drinking or recreational purposes increases the risk of antibiotic-resistant pathogens harming and infecting the human population (Tangwa *et al.,* 2019).

The existence of antibiotic-resistant genes and bacteria in treated and untreated drinking water has drawn more attention in recent years (Sanganyado and Gwenzi, 2019). This study is necessary because there are no known comprehensive studies on the antibiogram pattern of bacterial species isolated from specific underground water bodies in residential environments in Ohaukwu Local Government Area of Ebonyi State, Nigeria. While many studies of antimicrobial resistance around the state have focused more on environments and samples considered to be antibiotic resistance hotspots, such as sewage, Abattoir effluents, municipal wastewater, medical environments, and effluents, this one will address these issues. This study was designed to ascertain the bacteriological quality and antibiotic susceptibility pattern of bacterial species isolated from selected underground water bodies in Ohaukwu metropolis, Ebonyi State, Nigeria.

MATERIALS AND METHODS

Study area: This study was carried out in Ohaukwu Local Government Area of Ebonyi State, Nigeria. Ohaukwu Area Council is one of the 13 Area Councils in Ebonyi State, South-East, Nigeria and it has three main communities namely Izhia, Ngbo and Effium with an estimated human population of over 196,000 (NPC, 2006). The area lies within latitudes 6^0 3' N to 6^0 50' N and longitudes 7^0 80' E to 8^0 00' E with climatic conditions such as rainy season (March-October) and dry season (October-February). Two distinct vegetative regions exist in the study area: The Savannah in the Northern part of the study area, and tropical rainforests in the southern parts. More than 70% of the inhabitants of Ohaukwu metropolis engage in economic activities such as petty trading, subsistent agriculture, hunting and fishing.

Underground water sample collection: A total of twenty (20) underground water samples were randomly collected from different locations at Ohaukwu metropolis (Ngbo, Izhia) aseptically. Ten (10) water samples from boreholes and ten (10) samples from hand dug wells used by households and public outlets/markets as a source of drinking, cooking and bathing purposes. Samples from the wells were aseptically collected by lowering a clean plastic container tied to a synthetic rope down the well. Samples from borehole water were also aseptically collected by first pushing the handle of the borehole so that water will flow for about 2 minutes, before putting the sterile beaker to collect the water samples. All the water samples (100 ml each) collected in beaker were labeled and transported to Microbiology laboratory unit of Ebonyi State University Abakaliki, Nigeria for bacteriological analysis. Samples were analyzed within 4 h of collection.

Physicochemical analysis of the underground water samples: A portion of the water samples collected for the physicochemical analysis such as total dissolved solids, turbidity, total alkalinity and temperature were determined according

to American Public Health Association (APHA) methods.

Culture, isolation and identification of bacteria: The water samples were shaken thoroughly and one milliliter from each sample location was added to 9 ml sterile peptone water, and serially diluted up to 10^{-4}. Thereafter, 0.1 ml was aseptically collected from all the dilutions and inoculated on sterile nutrient agar (Oxoid, UK). This was done by using a sterilized, flamed wire loop to get a colony of the organism, then streak on the new petri dish containing nutrient agar (Oxoid, UK), medium, it was incubated at 37^0C for 24 hours. Pure culture was prepared by sub-culturing the organisms in new petri dishes containing new sterile MacConkey agar, Eosin methylene blue agar (Flumedia, UK) and Mannitol salt agar. At the end of incubation, the number of distinct colonies were counted and used to calculate the bacterial load of each organism. The total viable counts of the colonies were counted and recorded with all the colony counts expressed as Cfu/m of water sample. Plates that showed significant growth were separated for biochemical and Gram staining. Identification of isolated bacteria to species level was carried out using Gram stain, microscopic examination and biochemical tests such as catalase test, coagulase test, citrate test, indole test, VP test, motility test, methyl red, oxidase test, and nitrate reduction test as described by (Cheesebrough, 2010).

Antibiogram: Bacterial species isolated were tested for their sensitivity against a total of seven antibiotics by means of M2-A6 disc diffusion method recommended by the National Committee for Clinical Laboratory Standards, NCCLS (NCCLS, 2004) using Mueller-Hinton agar. Aliquots of the test isolates were exposed to gentamicin (10 µg), mupirocin (5 µg), amikacin (30 µg), bacitracin (10 µg), ofloxacin (5 µg), ceftazidime (30 µg), and clindamycin (10 µg) (Oxoid UK) and incubated at 37°C for 24 h. The diameter zone of inhibition (DZI) around each disc was measured in millimeter using a transparent plastic rule, and interpreted accordingly. Test isolates were classified as resistant, intermediate or susceptible based on the zone of inhibition following the standard interpretive chart (NCCLS, 2004).

Statistical analysis: The mean and standard error of the mean were calculated. Relationships between variables of physicochemical parameters were determined by Pearson correlation. Analysis was carried out with the aid of Statistical Package for Social Science (Version 21) and antimicrobial susceptibility profile of isolated bacteria against commonly used antibiotics were analyzed using one-way analysis of variance (ANOVA) with the level of significance set at $p<0.05$.

RESULTS

The findings in Table 1 below shows the bacterial loads of borehole water samples from different locations in Ohaukwu L.G.A of Ebonyi State, Nigeria. From the samples code, BWS2 had the highest counts of 7.2×10^4 cfu/ml followed by BWS10, BWS8, BWS1 and BWS4, while BWS5 and BWS3 had the least count of 8.5×10 cfu/ml and 8.8×10^2 CFU/ml respectively. The bacterial loads of well water samples from different locations in Ohaukwu L.G.A of Ebonyi State. From the samples code, WWS6 and WWS1 had the highest count of 4.0×10^5 cfu/ml and 2.3×10^5cfu/ml respectively followed by WWS3, WWS5, WWS10 and WWS2 while WWS8, WWS9 and WWS7 had the least count of 5.2×10 cfu/ml, 3.0×10^2 cfu/ml and 3.1×10^2cfu/ml respectively. Table 2 below shows the morphological, microscopic and biochemical characteristics of bacteria isolated from 10 borehole water samples examined. The probable isolates were *Escherichia coli, Staphylococcus aureus, Staphylococcus* sp, *Pseudomonas* sp, and *Klebsiella* sp. The findings of morphological, microscopic and biochemical characteristics of bacteria isolated from 10 borehole water samples examined revealed that the probable isolates were *Escherichia coli, Staphylococcus aureus, Staphylococcus*

sp*, Pseudomonas* sp*, Salmonella* sp *and Klebsiella* sp as shown in Table 3.

Table 3 illustrates the distribution of bacterial isolates from borehole water samples from different locations in Ohaukwu metropolis of Ebonyi State. The findings shows that the borehole water habours various degrees of microbes such as *Escherichia coli, Pseudomonas* spp*, Staphylococcus aureus* and *Klebsiella* spp. Samples tagged BWS8 and BWS1 has greater percentage bacteria distribution, while BWS9 had the least percentage distribution. It also revealed the distribution of bacterial isolates from hand dug well water samples from different locations in Ohaukwu metropolis of Ebonyi State. The findings showed the presence of *Escherichia coli, Salmonella spp, Pseudomonas* sp*, Staphylococcus aureus* and *Klebsiella spp.* In the hand dug well water at varying degrees. From the table below, samples WWS1 and WWS4 has a greater percentage of bacteria distribution while WWS5 has the least percentage distribution. Table 4 below depicts the findings of the physicochemical parameters of underground borehole and well water samples in Ohaukwu. The minimum, maximum, standard deviation and mean value of the temperature, pH, total dissolved solid (TDS), turbidity and alkalinity were measured and the mean temperature of the borehole water samples was 28.45°C and the mean pH value of the borehole was at 7.42, while the turbidity value of the borehole was 5.85 NTU. The mean value of total dissolved solids for the borehole source was 250.14 mg/l and the mean value of total alkalinity of the borehole of 304.30 mg/l was recorded for borehole water samples. Similar to the borehole water samples, the minimum, maximum, standard deviation and mean value of the Temperature, pH, Total dissolved solid (TDS), Turbidity and Alkalinity were measured and the mean temperature of the well water samples was 28.90°C and the mean pH value of the well was at 6.85 while the turbidity value of the borehole was 5.20 NTU. The mean value of total dissolved solids for the well water sample was 230.20 mg/l and the mean value of total alkalinity of the well sample of 256.90Mg/l was recorded for well water samples. The findings were compared with WHO standard value and the findings obtained shows that the physicochemical parameter of these underground waters meets the WHO standard value for safe drinking water as shown in Table 4 below.

Table 1: Bacterial loads of the borehole water sample (BWS) and well water samples (WWS)

	Samples Code	Bacterial load (cfu/mL)
	BWS1	9.4×10^3
	BWS2	7.2×10^4
	BWS3	8.8×10^2
Borehole water samples	BWS4	6.3×10^4
	BWS5	8.5×10^1
	BWS6	1.12×10^2
	BWS7	1.09×10^2
	BWS8	9.6×10^3
	BWS9	1.02×10^2
	BWS10	7.1×10^4
Well water samples	WWS1	2.3×10^5
	WWS2	2.7×10^3
	WWS3	2.8×10^4
	WWS4	4.2×10^4
	WWS5	3.4×10^3
	WWS6	4.0×10^5
	WWS7	3.1×10^2
	WWS8	5.2×10^1
	WWS9	3.0×10^2
	WWS10	3.8×10^3

Key: BWS= Borehole water sample; WWS= well water samples

Table 2: Morphological and biochemical characteristics of bacterial species isolated from the borehole water sample and well water samples

Morphological Characteristics				Microscopic Characteristics	Biochemical Test									Probable Organism
Source	Cell Shape	Cell arrange-ment	Colour	Gram reaction	Motility Test	Catalase Test	Citrate Test	Coagulase Test	Indole Test	Oxidase Test	Methyl red	Nitrate reduction test	VP Test	
Bore hole water samples	Rod	Single	Pink	-	+	+	-	-	+	-	+	+	-	*Escherichia coli*
	Cocci	Cluster	Golden-yellow	+	-	+	+	+	-	-	+	+	+	*Staphylococcus aureus*
	Cocci	Cluster	Yellow	+	-	+	+	-	-	-	+	+	+	*Staphylococcus* sp
	Rod	Pairs	Greenish	-	+	+	+	-	-	+	-	+	-	*Pseudomonas* sp
	Rod	Single	Shiny and dark pink	-	-	-	+	-	+	-	-	+	+	*Klebsiella* sp
Well water samples	Rod	Pair	Pink	-	+	+	-	-	+	-	+	+	-	*Escherichia coli*
	Cocci	Cluster	Golde-yellow	+	-	+	+	+	-	-	+	+	+	*Staphylococcus aureus*
	Cocci	Cluster	Golden-yellow	+	-	+	+	-	-	-	+	+	+	*Staphylococcus* sp
	Cocci	pairs	Greenish	-	+	+	+	-	-	+	-	+	-	*Pseudomonas* sp
	Rod	chains	Grayish white	-	+	+	-	-	-	-	+	+	-	*Salmonella* sp
	Rod	Single	Shiny pink	-	-	-	+	-	+	-	-	+	+	*Klebsiella* sp

Key: + = Positive Reaction; - = Negative Reaction

Table 3: Percentage distribution of bacterial species from the borehole water samples (Bws) and well water samples (Wws)

Source	Isolates	BWS1 (%)	BWS2 (%)	BWS3 (%)	BWS4 (%)	BWS5 (%)	BWS6 (%)	BWS7 (%)	BWS8 (%)	BWS9 (%)	BWS10 (%)
Borehole water samples	*E. coli*	2 (10)	1 (5)	0 (0.0)	3 (15)	2 (10)	3 (15)	0 (0.0)	5 (25)	0 (0.0)	7 (35)
	S. aureus	3 (15)	2 (10)	5 (25)	0 (0.0)	3(15)	0 (0.0)	4 (20)	2 (10)	2 (10)	0 (0.0)
	P. sp	7 (35)	3 (15)	1 (5)	2 (10)	4 (20)	0 (0.0)	2 (10)	6 (30)	3 (15)	5 (25)
	K. sp	3 (15)	2 (10)	6 (30)	4 (20)	1 (5)	6 (30)	0 (0.0)	4(20)	0 (0.0)	1 (5)
	Total	15(75)	8 (40)	12(60)	9 (45)	10(50)	9 (45)	6 (30)	17(85)	5 (25)	13 (65)
Well water samples	Isolates	WWS1 (%)	WWS2 (%)	WWS 3 (%)	WWS 4 (%)	WWS 5 (%)	WWS 6 (%)	WWS 7 (%)	WWS 8 (%)	WWS 9 (%)	WWS10 (%)
	E. coli	5 (25)	3 (15)	2(10)	3 (15)	0 (0.0)	1 (5)	0 (0.0)	3 (15)	0 (0.0)	4 (20)
	S. aureus	7 (35)	2 (10)	8 (40)	2 (10)	3(15)	2 (10)	4 (20)	0 (0.0)	2 (10)	5(25)
	S. sp.	3 (15)	4 (20)	0 (0.0)	3(15)	3(15)	4 (20)	0 (0.0)	1 (5)	2 (10)	6 (30)
	P. sp.	4 (20)	3 (15)	4 (20)	5 (25)	0 (0.0)	1 (5)	6 (30)	2 (10)	5(25)	2(10)
	K. sp.	1 (5)	3 (15)	4 (20)	6 (30)	1 (5)	5 (25)	0 (0.0)	5(25)	3 (15)	0 (0.0)
	Total	20(100)	15 (75)	18 (90)	19 (95)	7 (35)	13 (65)	10 (50)	11 (55)	12(60)	17 (85)

Table 4: Physicochemical parameters of the underground borehole water and well water samples analyzed

	Borehole water samples					Well water samples					
Sample Code	Temp	pH	TDS	Turbidity	Total alkalinity	Sample code	Temp	pH	TDS	Turbidity	Total alkalinity
BWS1	28.40	6.80	350.0	2.30	330	WWS1	32.30	6.60	320.0	3.20	295
BWS2	28.10	6.60	250.0	5.50	343	WWS2	29.20	6.40	210.0	4.50	324
BWS3	29.00	6.50	310.0	6.50	345	WWS3	29.00	6.30	280.0	5.50	320
BWS4	28.40	7.80	320.0	7.40	334	WWS4	30.40	7.10	310.0	6.40	240
BWS5	28.50	6.40	215.0	5.50	270	WWS5	32.50	5.40	210.0	5.30	250
BWS6	28.20	7.80	240.0	5.70	370	WWS6	29.20	6.80	220.0	5.60	360
BWS7	29.00	7.40	230.0	6.50	170	WWS7	29.70	7.40	260.0	6.30	210
BWS8	28.70	6.80	240.0	5.40	336	WWS8	31.60	6.70	240.0	5.40	305
BWS9	28.30	8.40	250.00	5.40	265	WWS9	29.30	7.20	230.00	5.10	235
BWS10	28.60	8.50	220.0	5.30	280	WWS10	30.00	8.00	220.0	5.40	270
Mean	28.45	7.42	250.14	5.85	304.30	Mean	28.90	6.85	230.20	5.20	256.90
STD	0.45	0.82	46.33	1.18	22.16	STD	0.72	0.64	41.24	1.12	16.10
MIN	28.10	6.40	215.0	2.30	170	Min	29.00	6.30	210.00	3.20	210
MAX	29.00	8.50	350.0	7.40	370	Max	32.50	8.00	320.0	6.40	360
WHO (2006) (≤)	26.00-32.00	6.50 – 9.20	500	2.20 – 7.50	500	WHO (2006) (≤)	26.00-32.00	6.40 - 8.90	500	2.20 -8.30	500

Key: Temp = Temperature; pH = Acidity/Alkalinity; TDS= Total Dissolved Solid; BWS= Borehole water samples; WWS = well water samples; STD = Standard deviation; MIN= minimum; Max=maximum

Table 5: Antibiogram pattern of different bacteria isolated from the underground water samples

Antibiotics	Conc. (µg)	*Escherichia coli*				*Pseudomonas spp*				*Staphylococcus aureus*				*Klebsiella spp*				*Salmonella spp*			
		Borehole water sample n = 8		Well water sample n = 12		Borehole sample n = 7		Well water sample n = 9		Borehole sample n = 6		Well water sample n = 8		Borehole sample n = 3		Well water sample n = 5		Borehole sample n = 5		Well water sample n = 14	
		R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)	S (%)
DA	2	8(100)	0(0.0)	9(75)	3(25)	5(71.4)	2(28.6)	8(88.9)	1(11.1)	4(66.7)	2(33.3)	7(87.5)	1(12.5)	1(33.3)	2(66.7)	5(100)	0(0.0)	3(60)	2 (40)	8(57.1)	6(42.9)
B	10	6(75)	2(25)	8(66.7)	4(33.3)	7(100)	0(0.0)	9(100)	0(0.0)	5(83.3)	1(16.7)	8(100)	0(0.0)	3(100)	0(0.0)	4(80)	1(20)	4(80)	1(20)	10(71.4)	4(28.6)
OFX	5	4(50)	4(50)	1(8.3)	11(91.7)	3(42.9)	4(57.1)	0(0.0)	9(100)	2(33.3)	4(66.7)	3(37.5)	5(62.5)	1(33.3)	2(66.7)	2(40)	3(60)	1(20)	4(80)	3(21.4)	11(78.6)
CAZ	30	0(0.0)	8(100)	5(41.7)	7(58.3)	1(14.3)	6(85.7)	7(77.8)	2(22.2)	1(16.7)	5(83.3)	2(25)	6(75)	0(0.0)	3(100)	0(0.0)	5(100)	0(0.0)	5(100)	6(42.9)	8(57.1)
MUP	5	5(62.5)	3(37.5)	7(58.3)	5(41.7)	4(57.1)	3(42.9)	6(66.7)	3(33.3)	2(33.3)	4(66.7)	5(62.5)	3(37.5)	2(66.7)	1(33.3)	4(80)	1(20)	5(100)	0(0.0)	10(71.4)	4(28.6)
CN	10	7(87.5)	1(12.5)	9(75)	3(25)	6(85.7)	1(14.3)	5(55.6)	4(44.4)	4(66.7)	2(33.3)	8(100)	0(0.0)	3(100)	0(0.0)	4(80)	1(20)	3(60)	2(40)	7(50)	7(50)
AK	30	2(25)	6(75)	2(16.7)	10(83.3)	2(28.6)	5(71.4)	2(22.2)	7(77.8)	0(0.0)	6(100)	2(25)	6(75)	1(33.3)	2(66.7)	2(40)	3(60)	1(20)	4(80)	5(35.7)	9(64.3)

Where**:** DA= Clindamycin, B = Bacitracin, OFX = Ofloxacin, CAZ = Ceftazidime, MUP = Mupirocin, CN = Gentamicin, AK = Amikacin

Table 6: Percentage antibiotics susceptibility/resistance profile of the bacteria species isolated from the underground water samples

Antibiotics	Conc. (µg)	Borehole water samples		Well water samples	
		Total No of species Susceptible (%) n = 29	Total No of species Resistant (%) n = 29	Total No of species Susceptible (%) n = 48	Total No of species Resistant (%) n = 48
Amikacin	2	23 (79.31)	6 (20.69)	35 (72.92)	13 (27.08)
Bacitracin	10	4 (13.79)	25 (86.21)	9 (18.75)	39 (81.25)
Ceftazidime	5	27 (93.10)	2 (6.90)	28 (58.33)	20 (41.67)
Clindamycin	30	8 (25.59)	21 (72.41)	11 (22.92)	37 (77.08)
Gentamicin	5	6 (20.69)	23 (79.31)	15 (31.25)	33 (68.75)
Mupirocin	10	11 (37.93)	18 (62.07)	16 (33.33)	32 (66.67)
Ofloxacin	30	18 (62.07)	11 (37.93)	39 (81.25)	9 (18.75)

DISCUSSION

The underground water samples from hand-dug wells and borehole water in Ohaukwu Local Government Area were assessed using physicochemical and microbiological methods. The underground water was found to contain some bacteria of public health importance. The total aerobic plate count of bacteria from boreholes and well water samples with total aerobic plate count ranged from 4.3×10^2 cfu/ml to 7.5×10^5 cfu/ml in the borehole samples while well samples ranged from 9.4×10^2 cfu/ml to 1.12×10^5 cfu/ml). This finding is in line with the findings of Ekhosuehi *et al.* (2018) on the microbial quality of borehole and well water in Ijebu-Ode and Ago-Iwoye communities in South Western Nigeria, but disagrees with the result reported by Onuoha *et al*. (2017) from bacterial species in Surface Waters in Afikpo, Ebonyi South Eastern Nigeria, which exceeded the WHO limit of cfu/ml for drinking water (WHO, 2011).

The findings support earlier research on the microbiological quality of potable water sources in the Ekosodin Community, Benin City, Nigeria, by demonstrating that the underground water samples were polluted with many mesophilic bacteria of public health significance (Ekhosuehi *et al.,* 2018). Similar to the reports of Orogu *et al.* (2017), Atobatele, and Owoseni (2023); Babatunde *et al.* (2022); Ogu *et al.* (2017), and Gao and Sui (2021), our findings showed that most bacteria present in these underground water samples examined where from the Enterobacteriaceae group.

In this study, the findings revealed a percentage distribution of *Escherichia coli* 22 (42.5%), *Pseudomonas* spp. 3 (8.9%), *Salmonella* spp. 15 (36.0%), and *Staphylococcus aureus*. 6 (12.6%) Which suggests a potential faecal contamination of the underground waters in Ohaukwu Local Government Area of Ebonyi State. Similar investigations also demonstrated that surface water bacteria are involved, as previously reported from water samples by Atobatele, and Owoseni (2023); as well as Ajoke, and Adetokunboh (2018). Since these are intestinal bacteria, their existence suggests that residents of Ohaukwu Local Government Area of Ebonyi State do not practice good hygiene which may be the cause of their underground water contamination. The high total aerobic bacterial counts in the water samples under investigation may result from the study area's poor hygiene standards, as some boreholes and wells were drilled within 15 meters of sanitary facilities like pit latrines, soak-away pits, and septic tanks, where the underground water sources were located. In other cases, the study location's basic observation revealed that the majority of the

indigenous people lack proper sewage systems and toilet facilities, hence resort to the use of any nearby bush or open space to dispose of their waste and defecate.

The presence of *Pseudomonas*, *Salmonella*, and *Staphylococcus* species have been linked to a variety of human illnesses, their presence is especially noteworthy. The *E. coli* isolation may have resulted from faecal contamination of underground water and storage environments. There have been reports linking *Escherichia coli* to several water-borne illnesses, including diarrheal disorders. In this investigation, *Pseudomonas* species and *Staphylococcus aureus* were also identified. *Pseudomonas* species have been linked to nosocomial infections, such as urinary tract infections after catheterization, eye and ear infections that can be dangerous in hospitalized patients, diarrhea patients who drink untreated water, and localized and/or generalized infections after surgery or burns (Sanganyado and Gwenzi, 2019).

Since *Staphylococcus aureus* may cause a variety of diseases, including foodborne poisoning, its identification is also crucial for public health. Other investigations have documented the isolation of *Staphylococcus aureus* from water samples. *Staphylococcus aureus* produces poisonous chemicals called enterotoxins, which is commonly associated with gastroenteritis. The presence of this bacterium in underground water is indicative of inadequate sanitation practices by the residents.

For drinking water, a pH between 6.5 and 7.5 is suitable. The pH levels of the well and borehole water were found to be, 7.42 and 6.85 respectively, the lowest and highest values. According to our findings, the average pH values of the well and borehole are 6.85 ± 0.64 and 7.42 ± 0.82, respectively, and there is a significant difference between them at $p<0.05$. The world Health Organization's (WHO) range (6.5 to 7.5) is not met by the well water quality. The pH levels of the well and borehole water samples under investigation varied from 6.10 to 6.90. During the study period, no notable differences were observed. Every drinking water source examined fell between 5.0 and 9.5 on the stated criterion, with an average value of 6.62±0.25. The weakly acidic nature of drinking water may be traceable to some dissolved matter in the water. This finding shows that the pH of these waters has an acidic tendency (pH below 7). The water sources (well) with pH below 6.5 may be attributed to the discharge of acidic products into this source by the agricultural and domestic activities. Studies have shown that 85% of all underground water worldwide is related to the geological nature of the aquifer formations and the lands traversed (Gothwal and Shashidhar, 2014; Karimi *et al.,* 2023).

Water temperature is a physical and ecological characteristic that significantly impacts both living and non-living elements of the environment, hence influencing organisms and the efficiency of an ecosystem. The well's average temperature of 28.90 ± 0.72 degrees Celsius is much higher than the borehole water's (28.45 ± 2.55). However, our finding shows that there is no significant difference (28.90 ± 0.72), even at $p<0.05$, between the well and borehole water. The highest and minimum values (28.10 and 32.50) were found in the well and borehole water, respectively. They all, however, exceed the WHO's recommended drinking water temperature of 25°C.

This study revealed that the turbidity values of the water Samples measured were somewhat greater than the WHO advised standard. The measurement of total dissolved solids (TDS), indicates whether or not the sources of drinking water included all suspended materials. The TDS was between 150.00 and 260.00. From the foregoing, it is evidently clear that there were no appreciable variations when compared to World Health Organization (WHO) standard value for drinking water. average TDS levels were 211.47±26.02 mg/l, within the 1000 mg/L drinking water standard. With the exception of the well sample designated W8, which has a high

degree of turbidity and TDS, all drinking water sources from all boreholes and wells studied had TDS levels within the advised ranges and might not have any detrimental effects. For appropriate monitoring and treatment, however, regions with significant fluctuation in physicochemical parameters and microbial burden can be identified in the research.

An antibiotic susceptibility test of the isolated species of bacteria against commonly used antibiotics showed different levels of bacterial susceptibility pattern. The widely used antibiotics that were evaluated showed a general order of antibacterial ineffectiveness: Mupirocin (97%) > bacitracin (91%) > gentamycin (84%) > clindamycin (76.3%). The majority of the isolates tested positive for more than three different antibiotics, according to the findings of the antibiotic susceptibility testing. This result is consistent with the findings of recent studies conducted in Ife East local government area (LGA), Ile-Ife, Osun State, Nigeria, by Babatunde *et al.* (2022) and Atobatele, and Owoseni (2023) on the distribution of various antibiotic-resistant Gram-negative bacteria in potable water from hand-dug wells in Iwo, Nigeria.

Majority of the bacterial species isolated were sensitive to Amikacin (72.3%), Ceftazidime (86%), and Ofloxacin (99.4%). It also showed variations in the susceptibility and patterns of resistance to popular antibiotics amongst the bacterial isolates. Ninety-five percent of the *Escherichia coli* isolation from the Ogiri Playground borehole was sensitive to amikacin and ceftazidime. The findings of this study are in line with the reports of Babatunde *et al.* (2022) and Odonkor *et al.* (2022) who had previously reported a 91.75% susceptibility profile *Escherichia coli* isolates from various water sources. Furthermore, it was discovered that the vast majority of the isolated bacteria were resistant to Bacitracin, Clindamycin, Gentamycin, and Mupirocin, and very susceptible to Ofloxacin, Ceftazidime, and Amikacin. It implies that these medications may not be useful in treating illnesses brought on by these bacteria.

The finding that *Bacillus* and *Staphylococcus* isolated from well waters in Iworoko-Ekiti, Nigeria, were completely resistant to ampicillin and Bacitracin is consistent with the result of Ogunleye *et al.* (2017). This result, however, conflicts with the reports of Soge (2009); as well as Samie *et al.* (2011) who reported that Ampicillin resistance rates in South Africa and Uganda were 92% and 50%, respectively. The *Pseudomonas* species isolated from the Okwo borehole in this study, exhibited varying degrees of sensitivity to antibiotics, Ceftazidime, and Amikacin, ranging from 95% to 86%. The majority of the antibiotics examined in this study were shown to be resistant in *Escherichia coli* isolates, although were found to be 99% sensitive to Ofloxacin, 87.4% susceptible to Ceftazidime, and 78.6% susceptible to Amikacin.

Salmonella species found in the water sources examined, with significant prevalence rates of 25% and 45% found in the Ekwashi playground well and the Ogiri playground borehole in Ohaukwu city, respectively. The *Salmonella* species isolated in this study, were found to be 86.9% Sensitive to Ofloxacin, 82.1% susceptible to Ceftazidime, and 72% susceptible to Amikacin. Despite this, three of the tested antibiotics were ineffective against the *Salmonella* isolates. Since *Salmonella* species are not very stable in aquatic settings, their presence in drinking water suggests recent contamination from human.

Therefore, the majority of the bacteria isolates from water sources are becoming resistant to routinely used antibiotics, as previously reported by Morshed *et al.* (2018); Sanganyado and Gwenzi (2019); Ateba *et al.*, 2020; Gao and Sui (2021); and Babatunde *et al.,* 2022) which this study validates. Any previous research on the bacterial diversity of some underground water in Ohaukwu, the capital of Ebonyi State, is improved by the findings of this

study. The findings from this study, outside the various physicochemical parameters that showed some variations from the world health organization (WHO) standards, suggests that the underground waters harbour microorganisms of public health importance. More disturbing, it revealed that the isolates exhibited drug resistance to commonly used antibiotics tested. Generally, the findings from this study suggests a contamination of the underground water sources and the occurrence of antibiotics resistant bacteria which greatly impacts the public health of the residents.

CONCLUSION

The results of these study have shown that the underground water samples analyzed had physicochemical properties that were under the acceptable drinking water standard limit. The bacteriological analysis showed that several bacteria of significance to public health were present in underground water samples in Ohaukwu Local Government Area, Ebonyi State. These bacterial species, which included *Salmonella* spp., *Pseudomonas* spp., *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus aureus*, may contribute to the spread of potentially harmful organisms to consumers, particularly in light of the antibiotic resistance that some of these organisms have shown. According to our study, the isolates were resistant to Bacitracin, Clindamycin, Gentamycin and Mupirocin, but were mostly sensitive to Ofloxacin, Ceftazidime, and Amikacin.

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Physicochemical Qualities of Ogbese River, Ovia North East LGA, Edo State, Nigeria

Idemudia I. B.* Ovenseri A. N. and Ekhaise F. O.
Applied Environmental Bioscience and Public Health Research Group, Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.
* Corresponding author: iyore.idemudia@uniben.edu

Abstract: Ogbese river is the primary source of drinking water for the community's inhabitants. It is located in Ogbese town, Ovia North East Local Government Area of Edo State, Nigeria. This study aimed to evaluate the physicochemical parameters which are of public health significance from Ogbese river in Ovia North East Local Government Area of Edo State, Nigeria. Three sampling points, upstream, midstream, and downstream, were chosen with intervals of 100 meters apart, and water samples were collected against the water current. Samples for physicochemical analyses were collected in sterile clean containers, appropriately labelled, and analyzed using standard analytical methods. The results showed that temperature ranged from 26.00 ±1.00^{0}C - 27.00 ±1.00^{0}C, pH ranged from 6.63 ±0.12 - 8.20 ±0.10, total dissolved solids ranged from 24.83 ±1.53 mg/ml - 156.77 ±16.47 mg/ml and conductivity ranged from 48.00 ±3.00 µS/cm - 224.07 ±166.53 µS/cm respectively. The results of the physicochemical analysis showed that the parameters sampled apart from Temperature and pH values were all below the permissible limit. Concerted efforts should be put in place, by the relevant authorities, to checkmate the public health menance associated, with the consumption of water from Ogbese river, Nigeria.
Key word: Dissolved solids, heavy metals, Ogbese river, public health

INTRODUCTION

Water is one of the most important natural resources which is essential in the life of all living organisms from the simplest plants and microorganisms to the most complex living system known as the human body (WHO, 1991; 2011). Water resources are vital in various aspect of life; human consumption, cultivation of plants, environmental management and economic development (Akhtar *et al.,* 2021). The management of water pollution and water resources, play a vital role at both local, national and international level. This is because the various uses of potable water, affects the quality and quantity of its availability (Ogedengbe and Akinbile, 2004).

Pollution-causing activities have caused severe changes in aquatic environments over the last few decades and have affected the safe use of river water for drinking and other purposes in recent times. Several contaminants pollute the river water hence it is of great concerns in our communities because rivers play a vital role in shaping up the natural, cultural, and economic aspects of any country (Sharma *et al.,* 2020). Contamination of water arises from various human activities (agricultural, industrialization and urbanization) from industrial and residential areas which eventually goes back to the water bodies and results in the water quality degradation as well as outbreak of diseases (Akhtar *et al.,* 2021; Bashir *et al*., 2020).

Ogbese River is about 150 meters from the community. Some of the anthropogenic activities in and around Ogbese river include washing of cars, motor bikes, farm produce, clothes, bathing, open defecation and urination, which are capable of causing deleterious deviation from the standard values of both the physicochemical quality of the river (Olayinka *et al*., 2021). Anthropogenic activities bring almost contamination and subsequent pollution to our varied ecosystems (Bashir *et al*., 2020).

It is noteworthy that, there have been limited reports of public health cases amongst the consumers of water from Ogbese river. Several reasons could be adduced for this, ranging from poor diseases data management system, insignificant number of casualties, acquired immunity over time, natural ability of the river to self – purify amongst other factors, which are not within the scope of this study. However, findings from this study could provide prophylactic measures, whenever there is an incidence of an epidemic associated with the

consumption of water from Ogbese River, Nigeria.

MATERIALS AND METHODS

Study area: River Ogbese lies between longitude 5°26' and 6°34' and latitude 6°43'E and 7°17' E. The River runs through Ogbese town, a town which is about five kilometers from Akure, in Akure North Local Government Area of Ondo State, Nigeria. River Ogbese is one of the major perennial rivers in South Western Nigeria. It took its source from Awo Ekiti in Ekiti State. It flows for approximately 22 km from its source to meet River Ose which is 265 km long and discharges into the Atlantic Ocean through an intricate series of creeks and lagoons (Ajakaye *et al*., 2017).

Sample collection: The samples were collected from three sites; upstream midstream and downstream approximately 100 m apart using three sterile 4 l plastic containers labelled A, B and C respectively. The samples in containers were transported to the laboratory within 2 hours with an iced pack.

Physicochemical analysis: The physicochemical properties of the river water samples were determined using standard methods for analysis of water. The parameters determined were: temperature, which was determined *in-situ* using an infra-red thermometer. A portion of the water sample was used for determination of pH and electrical conductivity of the water. pH metre (model 300408.1, Denver Instrument Company, Bohemia, New York, USA) was calibrated using millesimal buffers of pH 4.0, and 7.0 before taking the measurements. The electrical conductivity of the samples was determined on site using a multi-parameter analyzer (Hach model C0150). Total Dissolved Solids and total suspended solid were determined using multiparameter analyzer (Hach model C0150). Turbidity was determined by pouring twenty-five milliliters (25ml) of water sample into the cuvette and read at zero in the spectrophotometer at 450 nm. Dissolved Oxygen, Biological Oxygen Demand and Chemical Oxygen Demand were determined using the method described by APHA (1999). Determination of sulphate concentration was carried out using the turbidometric method. Concentration of phosphate in the water samples was determined by the molybdenum blue method. Ammonium nitrate was determined by indophenol method involving oxidation with sodium hypochlorite and phenol solution. Colour was determined using atomic absorption spectrophotometer (AAS) (Perkin Elmer Analyst 200). FME (1991); WHO (1991); AOAC (2000).

RESULTS

Physicochemical parameters of water samples

Table 1 shows the physicochemical parameters of Ogbese river water at different months of sampling. The findings revealed that temperature had a range of 26.00 ± 1.00^{0}C – 27.00 ± 1.00^{0}C, pH ranged from 6.63 ± 0.12 – 8.20 ± 0.10, total dissolved solids had a range of 24.83 ± 1.53 – 156.77 ± 16.47 mg/ml. The conductivity ranged from 48.00 ± 3.00 - 224.07 ± 166.53 µS/cm, while alkalinity had a range of 11.33 ± 1.53 – 17.33 ± 1.15. The dissolved oxygen and biochemical oxygen demand ranged from 0.73 ± 0.06 – 0.83 ± 0.32 mg/l and 1.37 ± 0.21 – 1.73 ± 0.15 mg/l respectively, while the chemical oxygen demand values ranged from 31.33 ± 3.06 – 45.00 ± 3.00 mg/l. The temperature and pH were within the Nigerian Standard of Drinking Water Quality (NSDWQ) permissible limits of 25^{0}C – 30^{0}C and 6.5 – 8.5 respectively. The total dissolved solids, conductivity and alkalinity were significantly below the NSDWQ permissible limits of 500 mg/ml, 1000 µS/cm and 100 respectively.

Physicochemical parameters in terms of dissolved nutrients of Ogbese River

Table 2 shows the physicochemical parameters in terms of dissolved nutrients of Ogbese River over the months of sampling. The findings revealed that nitrate content had a range of values from 1.18 ± 0.10 – 1.40 ± 0.10 mg/ml, phosphate ranged from

0.42 ± 0.04 - 0.64 ± 0.04 mg/ml. The sulphate content ranged from 3.67 ± 1.15– 4.00 ± 0.00 mg/ml. These parameters were significantly below the NSDWQ permissible limits of 50 mg/ml for nitrate and phosphate and 100 mg/ml for sulphate.

Heavy metal content of Ogbese River

Table 3 shows the physicochemical parameters in terms of the heavy metal content of Ogbese River over the months of sampling. The result revealed that lead and cadmium were below detectable limits. However, zinc had a range of values from 0.33 ± 0.05 (mg/ml) – 0.37 ± 0.10 (mg/ml). The chromium content ranged from 0, 03 ± 0.00 (mg/ml) – 0.07 ± 0.02 (mg/ml). The zinc and chromium were significantly below the NSDWQ permissible limits of 3 mg/ml.

Table 1: Physicochemical properties of Ogbese river water sampled from January to March, 2019

Parameters	January	February	March	NSDWQ	WHO
Temperature (°C)	26.00±1.00[a]	27.00±1.00[a]	26.00±1.00 [a]	25-30	-
pH	6.63±0.12[a]	8.20±0.10[b]	6.70±0.00[a]	6.5-8.5	6.8-7.5
Suspended solid (mg/l)	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	3	0.02
Turbidity	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	5	5
Total dissolved solid(mg/l)	24.83±1.53[a]	156.77±116.47[a]	25.17±0.58[a]	500	5
Total solid (mg/l)	24.83±1.53[a]	156.77±116.47[a]	25.17±0.58[a]	-	5
Conductivity (µS/cm)	48.00±3.00[a]	224.07±166.53[a]	59.33±1.15[a]	1000	100
Alkalinity	15.00±3.00[ab]	11.33±1.53[a]	17.33±1.15[b]	100	8.2
DO (mg/l)	0.77±0.15[a]	0.83±0.32[a]	0.73±0.06[a]	-	10
BOD (mg/l)	1.37±0.21[a]	1.40±0.36[a]	1.73±0.15[a]	-	10
COD (mg/l)	31.33±3.06[a]	34.67±10.69[a]	45.00±3.00[a]	-	10

Same alphabets across rows indicate no significant difference ($p>0.05$)

Keys: DO = dissolved oxygen BOD = biological oxygen demand, COD = Chemical oxygen demand, pH = potential of hydrogen, ^{0}C = degree Celsius, NSDWQ = Nigeria Standard for Drinking Water Quality, WHO = World Health Organization.

Table 2: Physicochemical parameters of dissolved nutrients sampled from January to March, 2019

Parameters	January	February	March	NSDWQ	WHO
Nitrate	1.18±0.10a	1.36±0.54a	1.40±0.10a	50	0.02
Phosphate	0.48±0.06a	0.42±0.04a	0.64±0.04b	-	0.05
Sulphate	4.00±0.00a	3.67±1.15a	3.67±1.15a	100	0.05

Same alphabets across rows indicate no significant difference ($p>0.05$)

Keys: NSDWQ = Nigeria Standard for Drinking Water Quality, WHO = World Health Organization.

Table 3: Physicochemical parameters of heavy metals sampled from January to March, 2019

Parameters	January	February	March	NSDWQ	WHO
Lead	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.01	0.001
Cadmium	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.003	0.001
Zinc	0.33±0.05a	0.37±0.10a	0.35±0.03a	3	0.005
Chromium	0.06±0.01a	0.03±0.00a	0.07±0.02a	-	0.001

Same alphabets across rows indicate no significant difference ($p>0.05$)

Keys: NSDWQ = Nigeria Standard for Drinking Water Quality, WHO = World Health Organization.

DISCUSSION

It is the collective responsibility of every stakeholder, to ensure that the quality of the source of potable water, is deliberately maintained in order to sustain its continuous availability. The situation becomes even more critical, as human population and activities grow around the surface water,

especially if it is the major source of water to the entire community (Ogedengbe and Akinbile, 2004).

The temperature of Ogbese River obtained in this study ranged between 26^0–27^0C. A similar range of temperature was reported by Pang *et al.* (2017). The variation in the temperature of surface waters, is capable of converting carbon (iv) oxide to oxygen and availability of more oxygen, can consequently enhance the growth rate of aerobic (Pang *et al.,* 2017).

The findings of the Total Dissolved Solids recorded ranged between 24.83 – 156.77 mg/l and it was significantly at variant with the findings of Zhang *et al.* (2017), who recorded a much higher TDS range of values. This difference in TDS value, may have been due to two mutually inclusive factors, which are seasonal variation and volume of water. During the rainy season, the volume of the river water increases, which may cause a decrease in the TDS value. Conversely, during the dry season or early rainy season, the volume of the river water decreases and there is a corresponding increase in TDS values. An increase in the total dissolved solids of surface water, has been reported to proportionately cause an increase in the overall salinity of the environment. Consequently, the high salinity of the environment may ultimately result to cell death or plasmolysis (Zhang *et al.,* 2017). Low TDS values were recorded in this study, the conductivity value of this study ranged from 48.00 ± 3.00 µS/cm– 224.07 ± 166.53 µS/cm. These values were at variant with the study of Gadhia *et al.* (2012) as well as Akinbile and Omoruyi (2018), who separately reported a relatively, lower and higher electrical conductivity mean ± SD values of 32.27 ± 4.84 µS/cm and 1102.75 ± 414.53 µS/cm respectively, during the dry season. Generally, the electrical conductivity of a water body depends on the volume of water, which is influenced by amount of rainfall and the temperature. The higher the temperature and lower the volume of water, the lesser the electrical conductivity. This is because the level of ionization is relatively higher, when the surface temperature of the river is high and the volume of water is low Gadhia *et al.* (2012). Therefore, the variations in one or both of these factors, may have significantly accounted for the differences observed, in the values of electrical conductivity. The findings of the turbidity of Ogbese River, recorded in this study was quite high.

Throughout the period of sampling and analysis, the values obtained were 0.00± 0.00 NTU. Firstly, it was significantly below the NSDWQ permissible limit of 5 NTU. Secondly and comparatively, while Gadhia *et al.* (2012) recorded a relatively high mean value of 152 ± 48.28 NTU; Borthakur and Singh (2020), reported a mean turbidity value of 29.35 ± 2.01 NTU. Turbidity is the degree of transparency or light penetration and scattering of a water body. Therefore, several factors may influence the turbidity of a water body including the concentration and presence of suspended organic and inorganic materials such as planktons and other microscopic organisms, silt, clay and so on. In addition, tidal waves, decayed organic material and algal bloom may also negatively impact the turbidity of a water body (Davies-Colley and Smith, 2007; Bilotta and Brazier, 2008).

The alkalinity values recorded in this study ranged between 11.33 ± 3.00 – 17.33 ± 1.15. This was relatively higher than that reported by Idowu *et al.* (2020), who recorded a mean ± SD alkalinity value of 10.3 ± 1.7. This variation in values recorded may be due to the volume of water and the concentration of bicarbonates and other salts dissolved in the water.

The dissolved oxygen of Ogbese River in this study, had a range of values from 0.73 ± 0.06 mg/l – 0.83 ± 0.32 mg/l. Dissolved oxygen was not detected (ND), in the findings of Okoya *et al.* (2016). The values obtained in this study were significantly lower, than that obtained from the findings of Akinbile and Omoruyi (2018), who reported dissolved oxygen values ranging from 4.70 – 16.00 mg/l and a mean ± SD value of 10.23 ± 3.70 mg/l. Meanwhile,

Idowu *et al*. (2020), recorded yet a higher dissolved oxygen values ranging from 22.5 – 25.2 mg/l from four (4) sampling sites and a mean value of 23.5 mg/l. The variation in the amount of dissolved oxygen from the various findings and this study are due to several factors. For instance, the higher the photosynthetic activities in the river, the lower the temperature, the higher the amount of dissolved oxygen. The higher the temperature, the lower the amount of dissolved oxygen. In addition, low salinity cumulative effect of tidal waves/wind velocity, may also increase the amount of dissolved oxygen Gadhia *et al*. (2012). The biochemical dissolved oxygen (BOD) recorded in this study ranged from 1.37 ± 0.21 mg/l – 1.73 ± 0.15 mg/l. In their separate findings, Okoye *et al.* (2016) reported a much higher mean ± SD BOD value of 23.50 ± 0.71, while Borthakur and Singh (2020), reported values ranging from 2.00 – 28.00 mg/l and a mean ± SD value of 13.29 ± 5.74 mg/l. Again, the variations in these BOD values, may be due to several factors including the temperature, pH, types and presence of aerobic organism, organic and inorganic materials (Gadhia *et al*., 2012).

Generally, there is a direct correlation between DO and BOD levels of a water body. The chemical oxygen demand (COD) values recorded in this study ranged from 31.33 ± 3.06 mg/l – 45.00 ± 3.00 mg/l. Borthakur and Singh (2020), reported similar range of COD values 18.00 – 49.00 mg/l and a mean ± SD value of 35.70 ± 7.63 mg/l in their findings, during the dry season of their study. Gadhia *et al*. (2012) reported a much higher COD value of 118.00 ± 31.72 mg/l, during the pre – monsoon (dry) season of their study. In their assertion, COD is in indication of the level of organic matter contamination and decomposition. Such pollutants could either be of domestic or industrial source.

For the dissolved nutrients, the amount of nitrate concentration recorded in this study ranged between 1.18 ± 0.10 mg/l – 1.40 ± 0.10 mg/l. These values were significantly lower than the findings of Okoye *et al.* (2016), who recorded a mean ± SD value of 17.00 ± 0.01 mg/l, during the dry season of their sampling. Conversely, Borthakur and Singh (2020), reported a range of nitrate values of 0.01 – 0.03 mg/l and a mean ± SD value of 0.51 ± 0.063 mg/l, which were significantly lower than that obtained from this study. The variation in the concentration of dissolved nitrates in the river, is determined by the amount of nitrogenous salts either from decayed organic matter, increase in phytoplankton excretory products, and reduction of nitrates and oxidation of ammonia as well as recycling of inorganic fertilizers, run off from neighboring farms (Gadhia *et al*., 2012). The dissolved phosphate levels recorded in this study had a range of 0.42 ± 0.04 mg/l – 0.64 ± 0.04 mg/l. These values were at variance with the findings of Borthakur and Singh (2020), who reported a low dissolved phosphate range of 0.02 – 0.09 and a mean ± SD value of 0.34 ± 0, 47 mg/l. High phosphate levels of surface waters, have been implicated as a major cause of algal bloom and excessive growth of aquatic plants, which can influence the turbidity. Considering the low phosphate levels of Ogbese river, from different findings, it tends to establish a correlation between the low phosphate levels and the low turbidity in NTU reported so far. The dissolved sulphate in Ogbese river recorded in this study ranged between 3.67 ± 1.15 mg/l – 4.00 ± 0.00 mg/l. These values were within the range of the findings of Okoye *et al.* (2016) who reported a dissolved sulphate values ranging from 0.01 – 26.00 mg/l, at 9 different sampling sites.

In the case of heavy metals, lead and cadmium were not detected in this study. This finding was in agreement with the study done by Elsherief *et al.* (2014) who also reported that lead was not detected in their study, especially during the dry season. However, while the amount of cadmium was not mentioned by Enabulele *et al.* (2022), it was detected in trace amounts at all the points (from upper to lower stream)

throughout the month of February and only at the lower stream in the month of March (Olayinka *et al*., 2021). The values for the amount of zinc obtained in this study, ranged from 0.33 ± 0.05 mg/l – 0.37 ± 0.10 mg/l. Although Fronsolet *et al*. (2008), detected zinc from their findings, the values obtained were not only in trace amounts, but significantly lower than that reported in this study. The values of chromium obtained in this study, were in trace amounts and they ranged from 0.03 ± 0.00 – 0.07 mg/l. It was however, not detected by Elsherief *et al.* (2014) throughout the period of their study, except once with a value of 0.010 ± 0.000 mg/l, at the lower stream in the month of February.

The presence of heavy metals in water exerts a great impact on both the water and the sediments as well. As heavy metal persists in the environment, it results to bioaccumulation and a higher degree of toxicity to all forms of life. Comparatively, the level of their concentration in sediments, over time is much more than in the water. A more worrisome characteristic feature of heavy metals is, their non – biodegradability, which makes them relatively very lethal both within and outside a living system, from microscopic to the most complex, which is the human body (Olayinka-Olagunju *et al.,* 2022). Bioaccumulation and biomagnification of heavy metals, within edible aquatic life form like fishes, crabs, and prawns and so on, have been reported to be highly carcinogenic (Yadev *et al.,* 2008). Untreated chemical effluents from industries, waste water from washing of car and motor bike exhausts inside the river as well as mixing of crude oil contaminated water with fresh water are the commonly implicated sources of heavy metal contamination of surface water like Ogbese River Olawusi *et al*. (2014).

CONCLUSION

Ogbese river is a major source of water for both domestic, agricultural and other purposes. Seasonal variation and changes in the volume of water, coupled with other mechanisms, by which the river self- purify itself are ineffectual to maintain the portability of the water. This is against the backdrop that, findings from this research, has reiterated the negative impact of natural and anthropogenic processes on the contamination of Ogbese river. The water must be properly treated to meet NSDWQ or WHO physicochemical permissible limits before consumption.

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Pathogens Effects of Herbicides (Atrazine, Gramaxone and Glyphosate) on Soil Physicochemical and Microbiological Properties

Jafaru M. I.[1*] Doughari J. H.[1] and Mohammed A.[2]

1. Department of Microbiology, School of Life Sciences, Modibbo Adama University, Yola, Adamawa State, Nigeria
2. Department of Pharmaceutical Science, Federal Polytechnic Mubi, Adamawa State, Nigeria

* Corresponding author: aliyumohammed1234@gmail.com

Abstract: The studies on the effects of the herbicide (atrazine, glyphosate and gramaxone) on soil physico-chemical properties was carried out between the months of August 2022 to January2023. Two sites forest and a garden were selected in Federal Polytechnic Mubi, Adamawa State, Nigeria using randomized complete block design (RCBD). The herbicides had significantly increased the amounts of soil organic carbon, organic matter, pH, sodium, potassium, bulk density, particle density, percentage moisture, available phosphorus and percentage nitrogen at $p< 0.001$ level of confidence, at same time lowering the values of available phosphorus (6.27 ppm), magnesium (2.19 ppm), sodium (0.36 ppm), potassium (2.10 ppm), iron (0.25 ppm) and manganese (7.21 cmol k^{-1}). Bacterial species isolated at the study sites showed *Pseudomonas aeruginosa, Pseudomonas putida, Bacillus subtilis, Bacillus sphaericus, Staphylococcus aureus, Klebsiella, Escherichia coli, Azotobacter nigricans, Flavobacterium aquaticum, Micrococcus loteus, Salmonella typhimurium*, and *Proteus vulgaris.* Forest soil was found having the highest percentage of (52.75 %), while, the garden (47.25 %) of soil bacterial isolates. The herbicides caused a decrease in the soil bacterial counts and that gramaxone at higher dosage caused the highest decrease while glyphosate the least. Garden soil was found as having higher bacterial counts compared with the forest. Month of November/120th day ($157.5x10^{-3}$ ±13 cfu/g) had the highest counts, followed by August, while December and January had the lowest which may be attributed due to seasonal variation from rainy to dry season.

Key word: Herbicide, soil, biodegradation, absorption, bacteria

INTRODUCTION

In the history of agriculture, more time, energy and money have been devoted to weed control than any other agricultural activity. The focus was on controlling persistent perennial weeds such as thistles, and quack grass with inorganic compounds such as sodium chlorate, sodium arsenate, and sodium metaborate tetra hydrate (Souzaa *et. al*., 2024). High cost, high rates and re cropping issues limited their use to small patches (Holm and Johnson, 2009). Prior to the invention of herbicides, manual weeding by hand was the most widely used weed management technique in Nigeria. However, it takes a lot of time and effort, physically taxing, and often expensive (Imoloame, 2014). According to estimates, manual weeding consumed between 40 and 60 percent of the cost of production, and in addition to being expensive, labour availability was unpredictable, making timeless weeding challenging to achieve and increasing yield loss. In areas like Sub-Saharan Africa (SSA), which is made up of Nigeria, Ghana, Benin, and Senegal, where modern input uptake has historically been limited and crop yields low, farmers may benefit from using modern agricultural inputs like pesticides and fertilizers to increase productivity (Sheahan *et al.* 2017). Herbicide application is a vital strategy of weed control. The effects of these chemicals on the non-target soil microorganisms are very intense; have adverse impact on physicochemical parameters of the soil, which in turn affect soil fertility and plant growth (Tudararo-Aherobo and Ataikiru, 2020). The term "pesticide" specifically refers to chemical compounds that change the biological functions of living things considered to be pests, whether these are weeds, harmful plants, insects, or fungi (Damalas, 2009). Herbicides have many different brand names, which farmers are familiar with; grouping them together is the simplest method to identify them. phenoxys, atrazines, substituted urease, triazoles, arbamates, and bipyridiliums are the most significant and prevalent categories (Souza

et. al., 2024). Pesticides can also be divided into categories based on their intended use, mode of action, duration of effect, or chemical function. Examples include insecticides (used to kill insects), herbicides (used to kill weeds), fungicides (used to kill fungus), and nematocides (used to kill nematodes) (Van Bruggen *et al.,* 2021). An ideal pesticide must not permeate into ground water, be recyclable, and harmful exclusively to the creature it is intended to kill. This is regrettably rarely the case, and the widespread usage of pesticides is concerning (Krishnasamy *et al.*, 2019). All agricultural toxins are dumped in the soil, which also acts as the primary habitat for the majority of microbial species, including bacteria, actinomycetes, and fungus, whose activities affect soil fertility (Tudararo and Ataikiru, 2020). The presence of organic carbon generally affects how most herbicides behave (Torrents and Jayasundra, 1997). The volatility of an herbicide is the rate at which it transforms from a liquid or solid into a gas which is determined by its vapor pressure. These herbicides are less likely to persist than herbicides with high vapor pressures because volatility rises with temperature (Wyskowska and Kitcharski, 2003). According to studies, the persistence of the chemical in the environment, the amount, the frequency of application, and the herbicide's toxicity all affect how contaminated the soil is with these chemicals (Tudararo and Ataikiru, 2020). Solubility, adsorption, volatility, and degradation are some pesticide qualities that have an impact on how quickly these chemicals reach groundwater. Atrazine, ($C_5H_{14}CIN_5$), is a chlorinated systemic selective herbicide that is used extensively throughout the world to eradicate weeds in crops including sugar cane, maize, cornifers, pineapples, and others (Febrina, *et al.,* 2017). It has a half-life of 13 to 261 days in soil, 100 days in rivers, and 10 days in sea water, making it extremely persistent in these environments (Jiangwei, 2019). The average half-life of atrazine in microbially active soil is 2.4 times shorter than in sterile soil. N-(phosphonomethyl) glycine, also known as glyphosate or Roundup, is a post-emergence, non-selective herbicide that is used to kill weeds (Van Bruggen *et al.,* 2021). The non-selective herbicide is frequently used (Chantana *et al.*, 2016). The features of paraquat, a non-selective contact herbicide, were found in 1955 (Febrina *et al.,* 2017). Soil texture, soil permissibility, organic matter content, soil site conditions, depth to underground water, geologic and climatic conditions are among the soil characteristics that influence pesticide transport (Sebiomo *et al.*, 2020).

MATERIALS AND METHODS

Description of the Study Area: The soil samples were collected in two distinct places that is a biological garden and forest area of Federal Polytechnic Mubi, Mubi North Local Government Area in Adamawa State, Nigeria. The Department of Biological Science's Biological Garden and Forest (which have never been treated with pesticides).

Sample Collection: Soil samples were collected following the composite method as described by Jaiswal, 2003. About 10 g of soil was collected after removal of debris at the depth of 0-30 cm using soil auger and placed in polythene bags. The samples were placed in the refrigerator. Petri dishes, conical flasks distilled water, plate count agar were used for bacterial culture and enumeration using colony counter. Herbicides were obtained ADADP stores, Yola. Soil analysis was carried out using pH meter, Flame Photometer, Kjedahl apparatus, Atomic Absorption Spectrometer

Experimental Design and Soil Sample Treatment: The soil plots from the two study locations (the Forest and the Biological Garden), were subjected to a randomized complete block design (RCBD) in the experimental layout. The test land was divided into equal sections that were each one meter square (1 $m^{2)}$ in size. For suitable spaces (alleys) between plots, one meter (1 m) of free ground was left on either side of each plot (Figure 2). Each of the farmlands

under study was divided into one-meter-square (1 m^2) subplots, with a one-meter gap of open space between each subplot to produce gaps. Atrazine was measured and diluted in 5.6 milliliters (ml) of distilled water to a concentration that was twice the manufacturer's concentration (6 kg/ha) and sprayed on subplot one. Subplots 2 and 3 were treated with the same concentrations by mixing 0.3 milliliters of gramaxone or glyphosate with 5.6 milliliters of distilled water. Subplot 4 was left untreated and served as the control. Atrazine, gramaxone, or glyphosate were each diluted with 0.6 ml each of the three herbicides with 5.6mls of distilled water before being sprayed on subplots 5, 6, and 7. In each of the subplots, soil samples were taken before and after the treatment for a total of six months, and were then taken to the microbiology laboratory Adamawa State University, Mubi for analysis.

Determination of Physicochemical Properties of Soil samples: The soil particle size distributions was determined using the hydrometer method of soil mechanical analysis to separate the soil sample into sand, silt and clay particles (Jaiswal, 2003). Soil pH, presence of organic matter, organic carbon, soil nitrogen, bulk density, particle density, porosity, moisture content were determined also as described by Jaiswal (2003). Soil exchangeable bases such as calcium, magnesium were determined using Atomic Absorption Spectrophotometer(AAS), model; sp-AA3604F produced by Drawell Scientific while sodium and potassium using Flame Photometer model; PFP7 by Janway with their appropriate lamps as described by Jaiswal (2003).

Heterotrophic Bacterial Counts and Biochemical Characterization of Bacterial Isolates: Using the pour plate procedure as outlined by Cheesbrough (2006), 0.1 ml of the 10^{-4} soil dilution was pipetted into triplicate plate count agar plates. The inoculated plates were then kept in an incubator for 24 to 48 hours at 37ºC. Discrete colonies that appeared on the plates were then counted using a colony counter (√Henry and Paul (1971) and Cheesbrough (2006) and the results were multiplied by the reciprocal of the dilution factor. The colonies were expressed as colony forming units (cfu/g) per gram of soil. Biochemical tests such as Grams staining reactions, catalase, Indole, motility, Voges Proskuer, methyl-red, urease, and citrate tests were carried out to identify the bacterial isolates as described by Cheesbrough (2006), and the results compared with a standard table based on Bergeys Manual of Determinative Bacteriology by Bergey and John (1994).

RESULTS

Effects of Herbicides on Soil Physicochemical Properties

The findings obtained on the effect of the three herbicides (atrazine, gramaxone and glyphosate) on soil physicochemical properties (Table 1) showed no significant effect of increase or reduction at $p< 0.001$ level of confidence on soil particle size distribution (sand, silt and clay), magnesium, porosity and manganese on all the three herbicides treated soils. Gramaxone treated soil showed slight increase of silt (20.20 %), while atrazine treated soils had the highest amounts of clay (7.84%) and sand (72.68%). The herbicides had caused a significant increased at $p< 0.001$ level of confidence on soil organic carbon, organic matter, pH, sodium, potassium, bulk density, particle density, percentage moisture, available phosphorus and percentage nitrogen. Percentage organic matter (1.90 %) was the highest in glyphosate and lowest at atrazine treated soil. Organic carbon (1.17%) were recorded highest at atrazine treated soils while gramaxone treated soil had the least (1.17%). The present findings is in agreement with those of Blu *et al.* (2019) and Sebiomo and Banjo, 2020 observed that the values of potassium, magnesium, calcium, percentage organic matter, percentage organic carbon and percentage nitrogen were higher in herbicide treated soil than the control. The analysis of variance (ANOVA) interaction on the effects of the

herbicides, its concentration, site of application and the control on soil physicochemical properties (Table 2) were found to have no significant difference on soil particle size distribution (sand, silt and clay), bulk density, particle density, percentage moisture content ,soil porosity, magnesium and potassium. The herbicides, its concentration, site of application and the control were found to have increased the amounts of soil organic carbon, soil pH, calcium and zinc, soil organic matter, percentage nitrogen, percentage moisture, available phosphorus, sodium, iron, cupper and manganese at $p < 0.01$ compared with the control and is in conformity with results obtained by Sebiomo and Banjo, 2020. The soil textural class for both forest and garden were sandy loam which shows appreciable amount of sand. Blu *et al*. (2019) attributed to the predominance of sand as a result of the humid tropical nature of Nigerian soils. Control soils were found having the highest percentages of organic carbon and organic matter in forest (1.61 %, organic matter 2.35 %) and (garden organic carbon 1.57 %, organic matter (2.72 %), while gramaxone treated with 6 kg/ha. had the lowest (O C. 0.46 % and O M 1.54%). According to Landon (1991) organic carbon above 20 % is rated as very high, 10-20 % as high, 2-4 % low and less than 2 % as very low. Thus, percentage organic carbon in both the herbicide treated and control soils are very low. This could be attributed to poor root growth of weeds due to their suppression by herbicides. Available phosphorus, sodium, potassium, iron, zinc, copper and manganese in control soils of both forest and garden soil were found significantly lower at $p > 0.01$ confidence level than herbicide treated sites. Forest control had high values of available phosphorus (6.27 ppm), magnesium (2.19 ppm), sodium (0.36 ppm), potassium (2.10 ppm), iron (0.25 ppm) and manganese (7.21 cmol k^{-1}) compared to garden and herbicide treated soils (Table 2). This observation is in agreement with the findings made by Sebiomo and Banjo (2020), who reported significant increase in iron, zinc, potassium, available phosphorus, sodium and manganese concentrations in soils treated with atrazine, primextra, glyphosate and gramaxone as compared with the control. Effect of period of treatment monitored for six months from August to January (30th to 180th day) on the soil physicochemical properties showed that at 99% confidence level of significant effect on all the other soil properties except on soil particle size distribution (Table 3). Highest values of organic carbon and organic matter were recorded in the month of December (150th day after herbicide treatment) (O C 1.91 %) and (O M 2.88 %) and the lowest in September (60th day) which agrees with the findings made by Rhoda *et al*. (2009) in which the authors asserted that long term use of herbicides may result to depletion of these micro and macro elements from the soil. Singh (2002) rated percentage nitrogen (< 0.15% as low, 0.2% as medium and > 0.2 % as high). Available phosphorus was also found in low quantities at both sites with average mean of 8.95 ppm as the highest in gramaxone treated soil which can be considered as low based on Singh (2002) rating (when phosphorus is < 8 ppm low, 8 – 20 ppm medium, > 20 high).

Isolation and characterization of bacterial isolates from herbicide treated and control soils

The bacterial species isolated at the study sites (Table 4) showed *Pseudomonas aeruginosa, Pseudomonas putida, Bacillus subtilis, Bacillus sphaericus, Staphylococcus aureus, Klebsiella, Escherichia coli, Azotobacter nigricans, Flavobacterium aquaticum, Micrococcus loteus, Salmonella typhimurium*, and *Proteus vulgaris.* Sebiomo and Banjo (2020) isolated *P. aeruginosa, P. putida, B. subtilis P. vulgaris, P. florescence* bacteria and some fungi in herbicide treated soils in Ijebu Ode, Nigeria, and also Muhiuddin *et al*. (2017) obtained similar organisms as observed in the present study. All the bacterial isolates were found widely distributed at the forest as well as garden soils and also in atrazine, gramaxone and glyphosate treated soils with exception of

Flavobacterium aquaticum (found only at the garden) and *Proteus vulgaris* (absent at the forest treated with atrazine). Forest soil was found having highest percentage of (52.75 %), while, the garden (47.25 %) of soil bacterial isolates. Ijah *et al*. (2021) found that the bacteria are not evenly distributed at the treated sites and the control which is in agreement with the present findings and also that of Nwadike, and Jibola-Shittu (2020). Percentage abundance of each bacterial isolate from each of the two study sites (Table 4) revealed *Pseudomonas aeruginosa, Pseudomonas putida,* and *Bacillus subtilis* as the most common bacterial species. Findings obtained and analyzed using ANOVA showed no significant differences in the frequency distribution of *Pseudomonas aeruginosa, Pseudomonas putida, Flavobacterium aquaticum, Klebsiella, Bacillus sphaericus, Bacillus subtilis and Salmonella typhimurium* in both atrazine, glyphosate and gramaxone treated soils. Significant differences at 95% confidence level were found in the frequency of distribution in the herbicide treated soils on Escherichia coli, *Micrococcus loteus, Azotobacter nigricans, Staphylococcus aureus* and *Proteus vulgaris* (Table 5). Highest frequency of distribution of *B. subtilis, E. coli, P. aeruginosa* (17.5) and *Klebsiella* (17.0) were found in atrazine treated soils. The lower values were recoded for *P. vulgaris, M. loteus and A. nigrican* in gramaxone treated soils with each having 2.5 frequency of distribution. P. vulgaris was completely absent in forest atrazine treated soils but present in all garden soils which can be attributed to the inhibition of the organisms by atrazine and its ability to develop resistance to the herbicides when continually in use on the site. Nwadike and Jibola-Shittu (2020) had similar results with the present findings in which they observed species of *Proteus vulgaris* and *Acinetobacter* were isolated only on in soils with lower dosages of herbicides and not found in higher dosages. The authors suggested that higher herbicide dosages may lead to disappearance of some species

Effects of Herbicides on Total Heterotrophic Bacterial Counts (THBC)

Results of one way Analysis of Variance (ANOVA) (Figure 3) plotted on the effects of herbicides on total heterotrophic bacterial counts showed that the control soil had highest bacterial counts ($273.03 \times 10^3 \pm 32$ cfu/g), followed by glyphosate at 3 kg/ha (300 ppm) ($161.0 \times 10^3 \pm 57$ cfu/g) and gramaxone at 6 kg/ha (600 ppm) ($44.39 \times 10^3 \pm 22$ cfu/g) having the least. This is line with findings made by Mazhari and Ferguson (2018), Sebiomo *et al.* (2020) and also Nwadike and Jibola-Shittu (2020) in which the authors reported higher bacterial counts in glyphosate than in gramaxone. Period of soil treatment with the herbicide (Months/ Days) showed the month of November/120th day ($157.5 \times 10^3 \pm 13$ cfu/g) having the highest HBC counts, followed by August/30th day ($133.0 \times 10^3 \pm 72$ cfu/g), while December and January/150th and 180th days had the lowest ($97.376 \times 10^3 \pm 57$ cfu/g) and ($103.776 \times 10^3 \pm 645$ cfu/g) respectively (Figure 4). Garden soil was found to have a higher THBC ($130.6.5 \times 10^3 \pm 19$ cfu/g), while forest had ($115.78 \times 10^3 \pm 66$ cfu/g). Garden soil was found as having higher bacterial counts compared with the forest (Figure 5). Mazahri and Ferguson (2018) had similar results with the present findings, and observed that the effect of the herbicides gramaxone and glyphosate led to reduction in bacterial population and colony counts were dependent on concentration of the herbicide as also was observed by Nwadike and Jibola-Shittu (2020). Chantana *et al.* (2016) reported that gramaxone is toxic to soil microorganisms and was implicated in reduction of bacterial population which also agrees with the present findings. Adomako and Ayeampong (2016) also reported reduction in bacterial population when treated with gramaxone. Mazhari and Ferguson (2018) reported that glyphosate treatment was found to cause an increase in bacterial population.

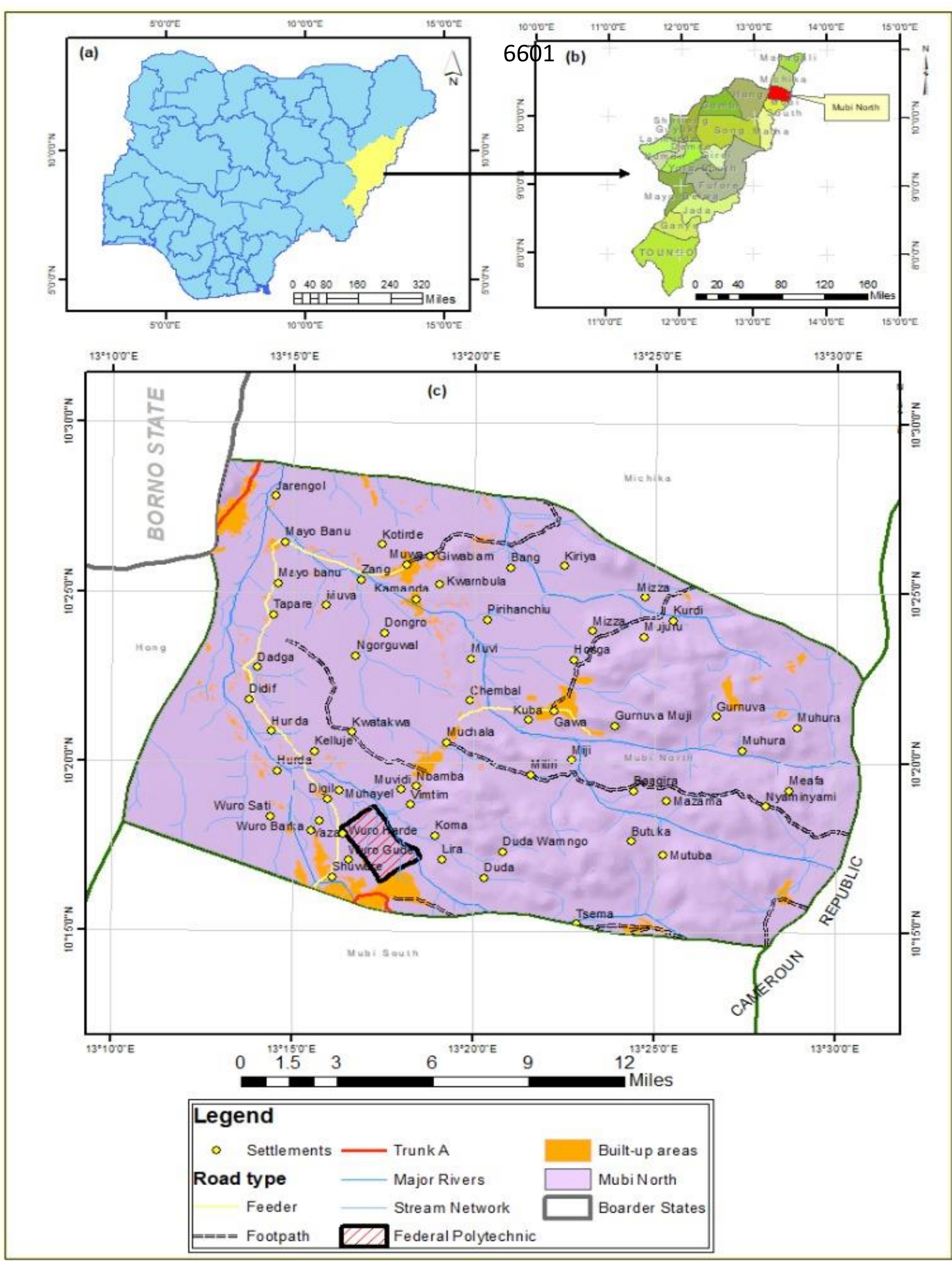

Figure 1: Map of Mubi and Study Area

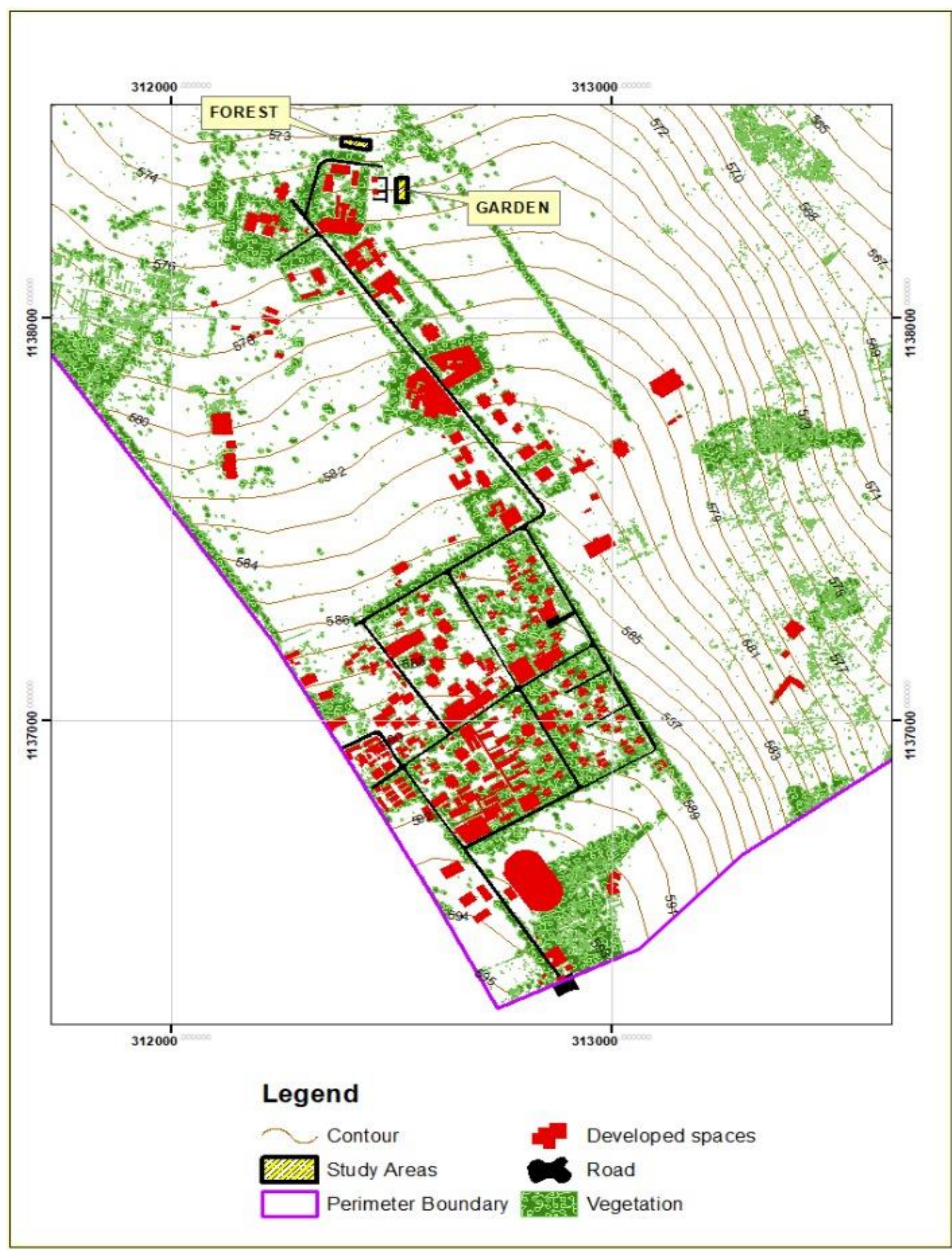

Figure 2: Study sites (Adebayo and Tukur, 1999)

Table 1: Effect of Herbicides on Physicochemical Properties of Soil samples

Soil Properties	Atrazine	Herbicides Glyphosate	Gramaxone	±SEM	LOS
Silt (%)	1 9.67	19.96	20.30	0.42	NS
Clay (%)	7.84	7.73	7.25	0.43	NS
Sand (%)	72.68	70.28	71.97	1.57	NS
Organic Carbon (%)	1.17^{a}	1.16^{b}	1.15^{b}	0.14	***
Organic Matter (%)	2.15^{a}	1.90^{b}	1.73^{c}	0.02	***
Nitrogen (%)	0.45^{a}	0.37^{c}	0.38^{b}	4.11	***
Available Phosphorus(ppm)	8.92^{a}	8.57^{b}	8.95a	0.06	***
Bulk Density (gcm^{-3})	0.31^{a}	0.31^{a}	0.25^{b}	2.10	***
Particle Density (gcm^{-3})	1.89^{b}	1.97^{a}	1.79^{c}	0.03	***
Moisture (%)	2.52^{a}	2.42^{a}	2.32^{c}	0.03	***
Porosity (%)	0.16^{a}	0.15^{b}	0.16^{a}	1.57	NS
pH	6.18^{b}	6.22^{b}	6.31^{a}	0.03	***
Magnesium (ppm)	3.04	3.05	3.05	0.04	NS
Sodium (ppm)	1.02	1.49	1.26	0.26	***
Calcium (ppm)	3.42a	3.40a	3.07b	0.02	***
Potassium (ppm)	0.33b	0.45a	0.33b	0.12	***
Iron (ppm)	8.00c	8.20a	8.07b	0.02	***
Zinc (ppm)	4.19b	4.34a	4.13b	0.05	***
Cupper (ppm)	0.17c	0.18b	0.24a	3.62	***
Manganese (ppm)	8.87	8.99	8.69	0.03	NS

Key: $^{a, b, c}$ Means in the same row bearing different superscript (s) is significantly (P < 0.05) different, SEM = Standard error of the mean, LOS = Level of significant, NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001 (SPSE, 2012)

Table 2: Interaction effects of herbicides, its concentration and site on soil physicochemical properties

Soil Properties	Forest C*C*F	A*3*F	A*6*F	L*3 F	L*6*F	G*3*F	G*6*F	C*C*G	A*3*G	Garden A*6*G	L*3*G	L*6*G	G*3*G	G*6*G	±SEM	LOS
Silt (%)	20.21	19.20	18.35	19.20	19.05	19.05	19.10	20.20	19.75	19.05	19.75	19.00	17.45	18.15	1.08	NS
Clay (%)+	4.15	3.70	4.85	4.05	4.20	3.90	4.85	4.16	7.80	3.40	7.58	4.20	4.05	6.10	0.07	NS
Sand (%)	75.65	77.05	75.40	76.95	76.95	77.05	76.05	75.65	72.80	77.55	72.45	76.85	77.00	75.75	1.96	NS
Organic Carbon (%)	1.61^{a}	0.6^{cd}	1.30^{ab}	0.60^{def}	0.54^{def}	0.60^{def}	0.46^{ef}	1.57^{a}	0.78^{cde}	0.71^{cde}	0.78^{cd}e	0.49ef	0.53def	0.63def	0.16	*
Organic Matter (%)	2.35^{b}	1.89^{c}	2.25b	0.97f	0.93fg	1.87c	1.54g	2.72a	1.33de	1.27e	1.34de	0.83fg	0.75d	0.89fg	0.10	***
Nitrogen (%)	0.52^{cd}	0.39^{cde}	0.30b	0.39^{cde}	0.38^{cde}	0.39^{cde}	0.42^{cd}	0.41^{c}d	0.32ef	0.42^{cd}	0.32^{ef}	0.40cd	0.42cd	0.44c	0.04	***
Available Phosphorus(ppm)	6.27	11.20	13.64	11.21	10.66	11.11	12.56	6.16	11.23	10.24	11.23	11.68	11.17	11.48	0.84	NS
Bulk Density (gcm^{-3})	0.88^{ab}	0.87b	0.87b	0.87b	0.87b	0.88ab	0.87b	0.88ab	0.88ab	0.87b	0.88ab	0.87b	0.87b	0.88ab	9.13	NS
Particle Density (gcm^{-3})	2.19	2.17	2.17	2.46	2.18	2.13	1.57	1.70	2.23	2.18	2.19	2.19	2.13	2.18	0.22	NS
Moisture (%)	5.15	4.85	5.05	4.75	4.90	4.75	5.15	5.15	4.90	5.00	4.85	4.95	4.75	4.15	0.20	NS
Porosity (%)	0.42	0.44	0.41	0.38	0.41	0.41	0.44	0.42	0.45	0.41	0.42	0.41	0.41	0.41	0.02	NS
pH	7.15	6.3^{ab}	6.15^{b}	6.35^{ab}	6.2^{b}	6.35^{ab}	5.35^{ab}	6.95	6.16^{b}	6.35^{ab}	6.15^{b}	6.30^{ab}	6.35^{ab}	6.45^{a}	0.11	*
Magnesium (ppm)	2.19	2.65	2.65	2.75	2.75	2.65	2.40	2.20	2.45	2.40	2.45	2.55	2.72	2.90	0.13	NS
Sodium (ppm)	0.36	0.55^{cd}	0.25^{f}	1.30^{a}	1.35^{a}	0.25^{f}	0.75^{b}	0.32	0.35^{bf}	0.45^{de}	0.75^{bf}	0.40ef	0.35ef	0.65bc	0.07	***
Calcium (ppm^{1})	2.10^{de}	2.25de	3.35a	3.15a	2.35	2.45e	2.30de	2.13de	2.65^{b}c	2.45^{be}	2.70b	2.40cde	2.35de	2.45bcd	0.14	*
Potassium (ppm)	0.25	0.35	0.25	0.45	0.30	0.25	0.35	0.20	0.35	0.35	0.35	0.30	0.55	0.50	0.10	NS
Iron (ppm^{1})	3.69^{b}	3.71b	5.65^{a}	3.72b	3.70b	3.78b	3.70b	3.56b	3.92b	3.73b	3.93b	3.66b	3.57b	3.73b	0.65	***
Zinc (ppm^{-1})	7.71^{bc}	6.79f	7.71^{bc}	6.85f	7.70bc	7.84b	7.27e	7.37e	7.37e	7.84b	7.37e	8.24a	7.58c	7.56cd	0.07	*
Cupper (ppm^{1})	0.12^{ef}	0.15^{de}	0.15^{de}	0.14ef	0.16^{cd}	0.14ef	0.15^{4ef}	0.11b	0.16^{ef}	0.17^{c}	0.14ef	0.17c	0.16cd	0.13ef	8.17	***
Manganese ($cmolk^{-1}$)	7.21^{bcd}	8.32^{cd}	8.27^{bcd}	7.84^{d}	9.04^{a}	8.52^{ab}c	7.24e	7.15^{e}	8.28bc	8.95^{a}	8.28bc	8.21e	8.54abc	8.72ab	0.26	***

Key: $^{a, b, -- g}$ Means in the same row bearing different superscript (s) is significantly (P< 0.05)different, SEM= Standard error of the mean, LOS = Level of significant, NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001 , A= Atrazine, L= Glyphosate, G = Gramaxone, F= Forest, G = Garden, Control = (0kg), 3= 3kg, 6= 6kg(SPSE, 2012).

Table 3: Effect of Herbicides Period of Treatment (Months) on Physicochemical Properties of Soil

Physicochemical Properties	Months							
	August	September	October	November	December	January	± SEM	LOS
Silt (%)	19.52	17.95	20.03	22.78	20.03	21.21	0.59	NS
Clay (%)	4.36	5.66	7.12	9.29	10.62	8.68	0.610.1	NS
Sand (%)	76.13	71.23	72.89	68.49	71.12	69.96	2.23	NS
Organic Carbon (%)	1.07b	1.12	1.07b	1.27b	1.91	1.18b	0.19	***
Organic Matter (%)	1.92b	1.32	1.76a	2.03a	2.88b	1.80c	0.03	***
Nitrogen (%)	0.33	0.46a	0.45a	0.39c	0.35d	0.33	5.82	***
Available Phosphorus(ppm)	9.12b	8.83c	9.70a	8.45d	8.47d	8.30d	0.09	***
Bulk Density (gcm^{-3})	0.82	0.57	0.45	0.19	0.21	0.10	2.98	***
Particle Density (gcm^{-3})	2.20a	2.22a	1.90b	1.72c	1.82b	1.42d	0.04	***
Moisture (%)	4.98a	4.47b	2.56c	1.57d	0.72e	0.22	0.04	***
Porosity (%)	0.41a	0.25b	0.25b	0.01c	0.14c	0.21c	2.22	***
pH	6.24b	6.25b	6.12c	6.11c	6.31ab	6.39a	0.04	***
Magnesium (ppm)	2.44e	2.52e	2.84d	3.08c	3.50b	3.91a	0.05	***
Sodium (ppm)	0.50	1.32	1.03	1.18	1.82	1.69	0.37	NS
Calcium (ppm)	2.62f	3.18e	3.34d	3.45c	3.54b	3.63a	0.03	***
Potassium (ppm)	0.29d	0.34cd	0.36bc	0.39ab	0.42a	0.42a	0.02	***
Iron (ppm)	7.38d	7.73c	8.16b	8.19b	8.53a	8.57a	0.03	***
Zinc (ppm)	3.65d	4.04d	4.23b	4.31b	4.52a	4.59a	0.07	***
Cupper (ppm)	0.17cd	0.15e	0.16de	0.18bc	0.19b	0.34a	5.12	***
Manganese (ppm)	7.64f	8.49e	8.99d	9.16c	9.59a	9.26b	0.04	***

Key: $^{a, b, c}$ Means in the same row bearing different superscript(s) is significantly (P< 0.05)different, SEM= Standard error of the mean, LOS = Level of significant, NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001(SPSE, 2012).

Table 4: Effects of herbicides on soil bacterial percentage abundance

Bacteria	% abundance in Forest			% abundance in Garden		
	Atrazine	Gramaxone	Glyphosate	Atrazine	Gramaxone	Glyphosate
p. aeruginosa	18(14.28)	18(14.63)	18(16.07)	18(13.43)	18(12.08)	18(13.63)
P. putida	17(12.49)	17(13.82)	16 (14.28)	13(9.70)	14(9.39)	15(11.36)
B. subtilis	18(14.28)	18(14.63)	18(16.07)	18(13.43)	18(12.08)	17(12.87)
B. sphaericus	14(11.11)	13(12.19)	12(10.71)	12(8.95)	10(6.71)	15(11.36)
F. aquaticum	0(((0.00)	0((0.00)	0(0.00)	8(5.97)	12(8.05)	11(8.33)
S.typhimurium	3 (2.38)	2(1.62)	3(2.67)	5(3.73)	8(5.37)	5(3.78)
E. coli	18(14.28)	16(13.01)	11(9.82)	18(13.43)	18(12.08)	13(9.84)
Klebsiella sp	17(12.49)	18(14.63)	17(15.18)	18(13.43)	17(11.41)	18(13.63)
M.loteus	5(3.96)	6(4.87)	4(3.57)	6(4.47)	5(3.35)	2(1.51)
P. vulgaris	0(0.00)	2(1.62)	2(1.78)	10(7.46)	7(4.70)	6(4.54)
S. aureus	9(7.14)	7(5.69)	7(6.25)	11(8.21)	13(8.79)	6(4.54)
A.nigrican	7(5.55)	6(4.87)	4(3.57)	7(5.22)	9(6.04)	6(4.54)
Total	126	123	112	134	149	132
Freest Total/Percentage=361(47.25 %)				Garden =403 (52.75 %)		

Table 5: Effect of Herbicides on Frequency of Bacterial Isolates

Bacterial Isolates	Herbicides			±SEM	LOS
	Atrazine	Glyphosate	Gramaxone		
Pseudomonas aeruginosa	17.50	17.50	17.50	0.50	NS
Flavobacterium aquaticum	5.75^{b}	5. 25^{a}	6.00^{a}	0.41	NS
Escherichia coli	17.50^{a}	16.50^{a}	11.50^{b}	0.50	***
Klebsiella sp	17.00^{a}	17.00	17.00	0.50	NS
Micrococcus loteus	5.00^{a}	5.00^{a}	2.50^{b}	0.50	**
Azotobacter nigricans	6.50^{a}	6.00^{a}	2.50^{b}	0.50	**
Pseudomonas putida	15.00	14.50	14.50	0.76	NS
Bacillus sphaericus	12.50	12.00	13.00	0.50	NS
Staphylococcus aureus	9.500^{a}	9.500^{a}	6.00^{b}	0.50	***
Bacillus subtilis	17.50	17.50	17.00	0.50	NS
Salmonella typhimurium	3.50	4.50	3.50	0.50	NS
Proteus vulgaris	4.70^{a}	4.00^{a}	2.50^{b}	0.46	***

Key: $^{a, b, c}$ Means in the same row bearing different superscript (s) is significantly (P< 0.05)different, SEM= Standard error of the mean, LOS = Level of significant, NS = Not significant, * = P<0.05, ** = P<0.01, *= P<0.001 (SP

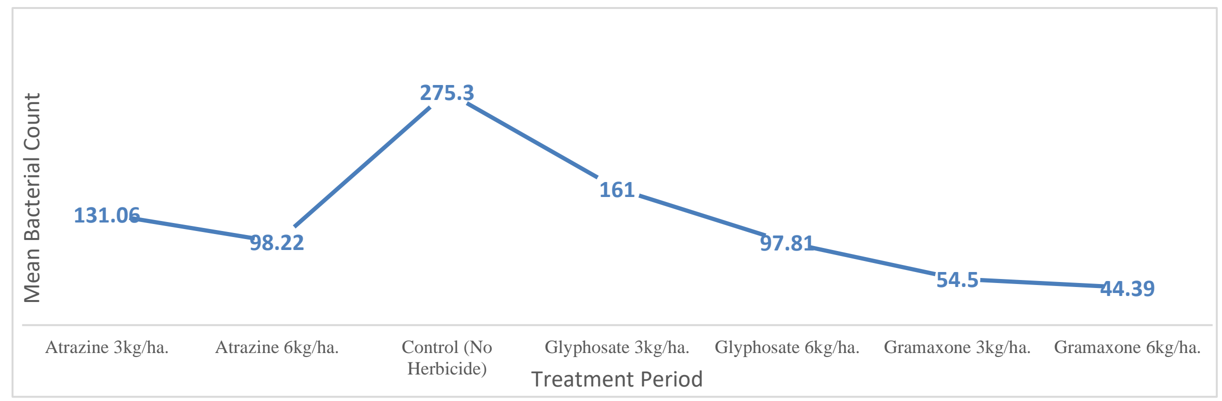

Figure 3: Effects of Herbicide Dosage on Soil Heterotrophic Bacterial Count

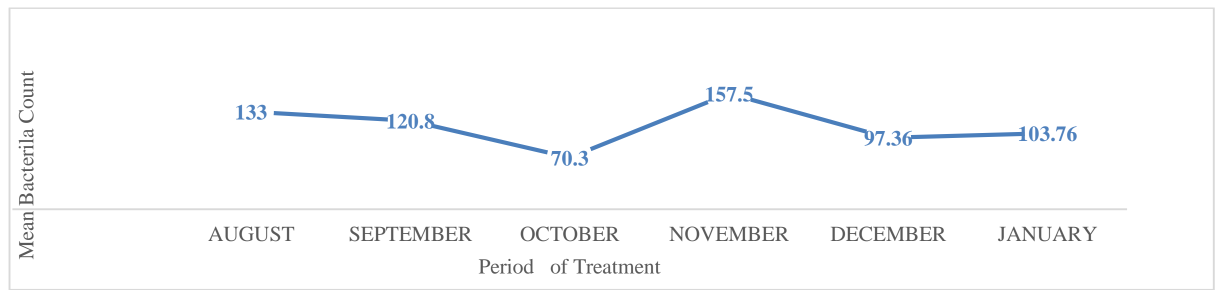

Figure 4: Effects of Herbicide Period of Treatment on Soil Heterotrophic Bacterial Count

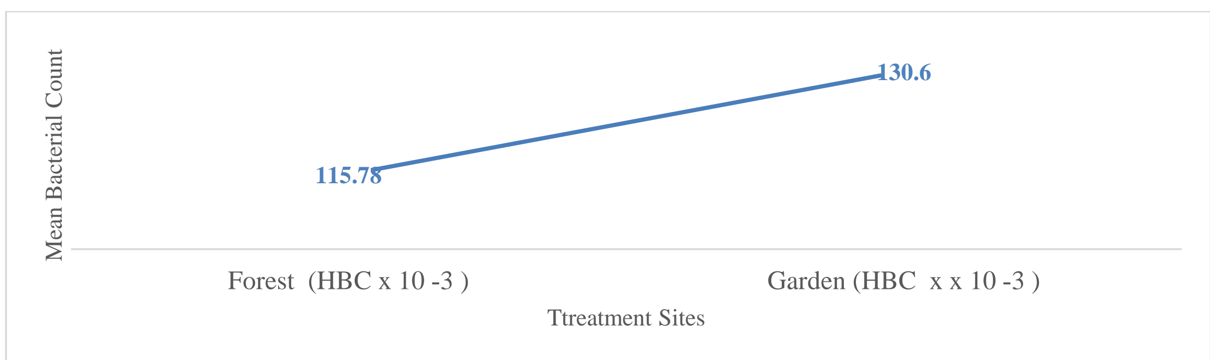

Figure 5: Effects of Herbicide Site of Treatment on Soil Heterotrophic Bacterial Count

CONCLUSION

The herbicides had significantly increased the amounts of soil organic carbon, organic matter, pH, sodium, potassium, bulk density, particle density, percentage moisture, available phosphorus and percentage nitrogen at $p< 0.001$ level of confidence, at same time lowering the values of available phosphorus (6.27 ppm), magnesium (2.19 ppm), sodium (0.36 ppm), potassium (2.10 ppm), iron (0.25 ppm) and manganese (7.21 cmol k^{-1}). The herbicides caused a decrease in the soil bacterial counts and that gramaxone at higher dosage caused the highest decrease while glyphosate the least. Garden soil was found as having higher bacterial counts compared with the forest. Period of soil treatment showed the month of November/120th day ($157.5\text{x}10^{-3}$ ±13 cfu/g) having the highest counts, followed by August, while December and January had the lowest which may be attributed due to seasonal variation from rainy to dry season and also the ability of bacteria to become adapted with the herbicides presence.

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Screening and Optimization of Biosurfactant Production by *Bacillus subtilis* from Restaurant Wastewater Contaminated Soil

John W. C.[1*] Joshua V. I.[1] Ihum T. A.[2] Sikiru G. K.[1] Dinchi P. T.[1] and Ishaya M.[3]
1. Federal College of Forestry, Jos, Nigeria.
2. Nigeria Store Products Research Institute (NSPRI) Ilorin, Kwara State, Nigeria.
3. Federal College of Forestry Resources Management, Maiduguri, Nigeria.
* Corresponding author: jowaltee@gmail.com

Abstract: Biosurfactant production from *Bacillus subtilis* RT9(4)B isolated from restaurant wastewater contaminated soil was investigated. The isolated *Bacillus subtilis* RT9(4)B was screened for biosurfactant production using the emulsification index, oil displacement, zone of haemolysis, surface tension and a positive drop collapse method. Effects of carbon and nitrogen sources, pH, temperature and incubation periods on biosurfactant production were determined by optimization. The biosurfactant characterization was by Fourer Transform Infra-Red and Gas Chromatography Mass Spectrometry analyses. Preliminary assessment showed that the emulsification index was 56.41 ± 1.30 %, oil displacement, 6.02 ± 1.24 mm, zone of haemolysis, 5.12 ± 1.06 mm, surface tension, 29.46 ± 0.62 mN/m and a positive drop collapse test. At optimal conditions of temperature (40 ℃), pH (7), carbon and nitrogen sources (lactose, 20 g/l and urea, 1.5 g/l respectively), the surfactant reduced surface tension up to 11.10 ± 0.78 mN/m and the emulsification index rose to 95.51 ± 2.66%. The highest biosurfactant produced was 3.73 ± 0.19 g/L at 72 h. *Bacillus subtilis* RT9(4)B produced lipopeptide type biosurfactant containing hexadecanoic, octadecanoic acid, peptides, aliphatic, alkyl and esters.

Key word: *Bacillus subtilis*, biosurfactants, emulsification index, restaurant wastewater, surface tension

INTRODUCTION

Biosurfactant is one of the very important bio-based microbial products. It is a naturally produced surface active compound capable of reducing tensions between different phases (Danyelle *et al*., 2016). It is produced by a wide range of microorganisms including bacteria, fungi and algae. *Bacilli* form the dominant bacterial biosurfactant producers. *Bacillus* species have long history of use in biotechnology. Virtually, all species of *Bacilli* have been implicated in one bio-based production or the other irrespective of whether they are pathogenic or not. *Bacillus subtilis* is non-pathogenic and generally regarded as safe (GRAS) (Josh *et al*., 2013) and their use in biosurfactant production could offer some special advantages.

Production of surface-active agents by biological means has attracted attentions recently. Environmental consciousness and concern have driven minds back to bio-based products. There are many reasons for the preference of biosurfactant to chemically-synthesized surfactants. On the one hand, their production is environmentally friendly. Besides, their products are biodegradable and less toxic. Furthermore, biosurfactants are stable over a wide variety of environmental factor (Paraszkiewicz *et al*., 2019). However, production of biosurfactants is faced with bottlenecks relating to lack of industrial availability (Fenibo *et al*., 2019). Chemically synthesized surfactants are non-biodegradable and toxic to the environment (Mulligan *et al*., 2014). Production of biosurfactant is not cost-effective.

Lipopeptide as a type of biosurfactant is a powerful cyclic lipopeptide with surface activities (Plaza *et al*., 2015). It is made up of fatty acids and seven amino acids (Pecci *et al*., 2010). Lipopeptide share the other general biosurfactant properties of environmental friendliness, biodegradable, low toxic and non-hazardous (Jacques 2011). In addition, lipopolypeptide specifically have better foaming properties and higher selectivity than their synthetic counterparts (Jacques 2011) and are active at extreme environmental conditions (Pacwa-Płociniczak *et al*., 2011).

The study investigated the production, characterization, and optimisation of biosurfactant produced by *Bacillus subtilis* RT9(4)B isolated from soil contaminated with restaurant wastewater with a view to

getting biosurfactant that can conveniently replace chemically derived surfactants.

MATERIALS AND METHODS

Isolation of Bacillus species: Ten gram of restaurant wastewater contaminated soil sample was suspended in 90 ml of sterile distilled water contain in 100 ml Erlenmeyer flask and amended with 1 ml engine oil. The medium was incubated at 25˚C for 48 hours on a rotary shaker (Model S150, Bartoworld, scientific, 1995) at 150 rmp. After incubation, the medium was serially diluted by transferring 1 ml of the stock solution into 9 ml of sterile distilled water in a test tube using a sterile pipette. One ml from the dilutions (10^{-1} to 10^{-6}) was transferred aseptically to sterile Petri-dishes. The sterilized nutrient agar (Oxoid CM0929, Hanis, UK) was allowed to cool to 45-50ºC before pouring into the Petri-dishes. The inoculated plates were incubated at 30 °C for 48 hour. Pure cultures were obtained by sub-culturing and stored on Nutrient agar (Oxoid CM002, Hampshire, England) slants at 4 °C refrigeration.

Identification of bacterial isolates: The selected isolate *Bacillus subtilis* RT9(4)B was identified through 16S rRNA sequencing. The protocol was followed according to the manufacturer's (QIAquick® Gel Extraction kit, Qiagen, Hilden, Germany) manual. Standard primers 8F:5-AGAGTTTGATCCTGGCTCAG-3 and 1492R:5 GGCTACCTTGTTACGACTT-3 and 27F:AGAGTTTGATCMTGGC and 1492R:5-GGCTACCTTGTTACGACTT-3 were used (Sreethar *et al*., 2014).

Screening of the Bacillus isolates for biosurfactant production: Screening of *Bacillus* isolates for surfactin production were carried out using nutrient broth (30 mL) in 100 mL flask inoculated with 3 mL McFarland 0.5 standardized pure culture grown on Nutrient broth for 24 hours. The inoculated culture media were incubated at 30 °C on a rotary shaker at150 rpm for 72 hours. The cultures were centrifuge (Model 80-213, 2000) at 3000 rpm for 30 minutes to obtain cell free supernatant. The supernatants were collected and cells discarded. The various supernatants were used for emulsification stability, drop collapse and oil spread ability.

Screening of Bacillus species for biosurfactant production

Determination of blood haemolytic ability: Sterilized Blood agar base was allowed to cool for 45 ºC and 20 ml aseptically collected goat blood was added, mixed gently and poured on Petri dishes. A 24 h freshly grown cultures were point inoculated using wire loop at the centre of the blood agar plates. The plates were incubated at 30 ºC for 24 h. The area of clear zone around the colonies were measured using meter rule as reported by El-Shahawy (2014).

Emulsification stability test (EI24): Kerosene (2 ml) was added to the same amount of cell free supernatant obtained through centrifugation, vortexed for 2 minutes using an electronic vortex machine (Model XH-B, 2012), and allowed to stand for 24 hours. The E_{24} index was given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) x 100. Emulsification stability test of culture samples was measured after 24 h (Balogun and Fagade, 2010) and values obtained recorded.

Drop collapse assay: The method of Seema and Nakuleshwar (2012) was adopted for the drop collapse assay. Ten microliters of cell free broth was dropped in the centre of a vegetable oil (Grand Cereal, Jos, Nigeria) drop on a clean glass slide. After one minute the drops was examined visually. The destabilizations of cell free broth dropped indicated positive result while non-destabilized drop indicated negative result. Activity of collected supernatant was compared with water as control (Seema and Nakuleshwar, 2012).

Determination of oil spreading ability: A 20 ml distilled water were dispensed in Petri plates. One millilitre of crude oil was dropped in the centre of the plates containing the distilled water. This was followed by dropping 20 µl of the supernatant of the culture of *Bacillus* isolate

at the centre of the crude oil. Ring formation due to displacement of crude oil was measured using a meter rule and a 20 μl distilled water was used as control as documented by Hasham *et al.* (2012).

Surface tension measurement: Surface tension was measured using a KSV Sigma 702 tensiomenter. All measurements were made on the cell-free broth obtained by centrifuging the culture at 10000 rpm for 15 minutes. Ten millilitre of each cell free broth was transferred into a clean 20 mL beaker and placed onto the tensiometer platform. A platinum wire ring was submerged into the solution and then slowly pulled through the liquid-air interface, to measure the surface tension (mN/m). Between each measurement, the platinum wire ring was rinsed with water and flamed with Bunsen burner (Mulligan *et al*., 2014).

Determination of cell dry weight: The initial weight of sterile Petri dish was taken using electronic weighing balance. Residue from centrifuged culture broth was poured on the sterile Petri dish and dried in a hot air oven for 1h at 180°C. After drying the final weight of the plate was measured and the weight of the cell dry weight calculated using the following formula:

Cell dry weights = Final weight of the plate after drying - initial weight of the empty plate (Mulligan *et al*., 2014).

Preparation of mineral salt medium (MSM) for optimization study: Mineral salts medium (MSM) was prepared as described by Atlas (2010). The trace element solution was prepared first by adding components (0.232 g H_3BO_3, 0.174 g $ZnSO_4.7H_2O$, 0.116 g $FeSO_4(NH_4)_2SO_4.6H_2O$, 0.096 g $CoSO_4.7H_2O$, 0.022 g $(NH_4)6Mo7O_{24}.4H_2O$, 8.0 mg $CuSO_4.5H_2O$, 8.0 mg $MnSO_4.4H_2O$) to 1.0l of distilled water. The solution was then mixed thoroughly. The trace element solution (5.0 ml), was mixed with 12.5 g K_2HPO_4, 3.8 g KH_2PO_4, 1.0 g $(NH4)_2SO_4$, 0.1 g $MgSO_4.7H_2O$ and 1000ml of distilled water. The solution was mixed thoroughly, gently heated to boil. The pH of the medium was adjusted to 7.0 using 1 M HCl and 1 % NaOH. The mixture was then autoclaved at 121 ºC for 15 min and cooled to 45 - 50 °C.

Determination of the effect of pH on biosurfactant production: This was done using fifty millilitres of sterile 40 g/l glucose mineral salt medium (Atlas, 2010) with varying initial medium pH from 6.0 ~ 10.0. The medium pH was adjusted using 1 M HCl and 1 % NaOH and then followed by inoculation with 3 mL of overnight nutrient broth culture (McFarland 0.5 standardized pure culture grown on Nutrient broth for 24 hours to obtain 1 x 10^8 CFU/ml). The media were incubated in an incubator shaker (Series F 200, England) for 72 h at 150 rpm.

Influence of incubation temperature on biosurfactant production: This was carried out using fifty millilitres of sterile 40 g/l glucose MSM (pH adjusted to 7.0). The fermentation medium was inoculated and incubated as described previously in screening for bacterial biosurfactant production. The rotary shaker (Bartoworld, Scientific, Model S150, 1995) temperature was regulated at different (25 - 45 ºC) interval for 72 h. After which the biosurfactant production was determined.

Determination of the effect of incubation time on biosurfactant production: In 250 ml Erlenmeyer flasks, 50 mL sterile 40 g/l glucose mineral salt medium were dispensed. The media were inoculated with 3 ml overnight culture of the strains under study. The fermentation media were previously adjusted to pH 7 before inoculation. The flasks were incubated at 30 ºC under shaking condition at 150 rpm for 120 h and biosurfactant production taking at 24 h interval.

Effect of different carbon sources on biosurfactant production: Effects of glucose, lactose, dextrose, and soluble starch at concentrations 10, 20, 30, 40, and 50 g/l were examined on biosurfactant production by *Bacillus subtilis*. To 1000 ml mineral salt medium, the carbon sources (glucose, lactose, dextrose and soluble starch) were added individually, the fermentation medium pH was adjusted to 7.0. This was then followed by inoculation with 3 ml of

overnight nutrient broth culture (McFarland 0.5 standardized pure culture grown on nutrient broth for 24 hours to obtain 1 x 10^8 CFU/ml) and incubated under shaking (Orbital Shaker, Series F200, England) condition at 150 rpm at 30 ℃ for 72 h. After which biosurfactant production was determined.

Effect of different nitrogen sources on biosurfactant production: Effect of different concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 g/l) of yeast extract, urea and proteose peptone on biosurfactant production were determined. To a 1000 ml mineral salt medium, varying concentrations of these nitrogen sources were added individually. The fermentation medium pH was adjusted to 7. The medium was inoculated with 3 ml overnight nutrient broth culture and was incubated under shaking condition at 150 rpm, 30 ℃ for 72 h. After which biosurfactant production was determined.

Extraction of biosurfactant: To extract the crude biosurfactant, the culture supernatants was centrifuged at 10000 rpm for 20 minutes at 4 ℃. Then, the pH of the supernatant was reduced to 2.0 using 0.5M HCl after the supernatant was collected. The collected supernatant was stand for 24 hours at 4ºC to precipitate. Equal volume of chloroform: methanol (2:1) was added, mixture was shaken vigorously and left to stand overnight. The resulting white coloured sediments were collected the following day (Anitha *et al*., 2015) for characterization.

Preliminary separation by column chromatography: The Column was loaded with 50 grams of slurry of silica gel. Aliquot of biosurfactant was fractionated using varying ratios (13:20 v/ v, 20:15 v/v, 15:25 v/v, 20:30 v/v and 10:15 v/v) of established solvent system (Chloroform and methanol). About 2 ml of eluent were collected at 10 minutes' interval. A total of 32 different fractions were collected for further purification.

Thin layer chromatography of partially purified fractions: Aluminium TLC sheets covered with silica gel prepared commercially were used. The plates were cut to fit 5 × 5 cm size. Drop of eluents were placed at distance of 0.5 cm from the bottom of the TLC plate. The plate was then placed in a chromatographic tank containing mixture of 15:25 ratio of Chloroform and methanol earlier determined as the best resolution. The plates were spread using sulfuric acid. Fractions with the same retention factor were pooled together (Mulligan *et al*., 2014).

Structural characterization and identification of biosurfactant: Structural classification of the biosurfactant was carried out using fourer transform infra-red (FTIR) and gas chromatography mass spectroscopy (GC-MS). The FTIR instrument (Buck scientific M530 USA) was equipped with a detector of deuterated triglycine sulphate and beam splitter of potassium bromide. The software of the Gram A1 was used to obtain the spectra. One milligram of the biosurfactant sample was mixed thoroughly with 100 mg of homogenized porcelain-milled Potassium bromide (KBr). During measurement, FTIR spectra was obtained at frequency regions of 4,000 – 600 cm^{-1} and co-added at 32 scans and at 4 cm^{-1} resolution. The FTIR spectra were displayed as transmitter values (Jain *et al*., 2012).

Ten milligrams (10 mg) of biosurfactant was mixed with 5 % HCl-methanol reagent. The reaction was stopped with addition 1 mL of sterile H_2O. The samples were recovered with methanol and 1 mL of samples were injected into a gas chromatograph (30 m × 0.25 mm ID × 0.25 mm). The carrier gas was Helium at a flow rate of 1.5 $mLmin^{-1}$ and the working temperature of the GC injector was 260 ˚C. The gradient temperature was set as range from 60 to 260 ℃ at a speed of 5 ℃ min^{-1}, through an isothermal phase of 10 min at the end of the analysis. The electron impact ion source was sustained at 200 ˚C. Mass spectra were recorded at 70 keV. The mass spectra were obtained with a m/z range: 40–700 ultra-high-resolution mode with an acquisition speed of 6 spectra/second. The identification of components was done in scan mode by

using NIST11 and Wiley8 library and the target mass spectra obtained from sample were compared with mass spectra obtained from the library as recently reported by Parthipan *et al*. (2017).

Statistical analysis of data obtained: The data obtained were presented in graphs, tables and charts. Data from biosurfactant production screening were statistically interpreted using Chi-square and Analysis of Variance (ANOVA). Optimization studies data were also analysed using analysis of variance (ANOVA) at 99 % confidence level. Means were separated using Duncan test. The means were compared using one-way ANOVA to indicate any significant difference among parameters and the variables.

RESULTS AND DISCUSSION

Preliminary screening of *Bacillus subtilis* for biosurfactant production

Bacillus subtilis was subjected to four preliminary screening assays in order to determine their biosurfactant production potential. These include haemolytic activity, oil collapse, emulsification index and oil spreading assays. This preliminary characterization showed on Table 1 indicated that *Bacillus subtilis* RT9(4)B among other *Bacillus* species had the highest β- haemolysis with 5.12 ± 1.06 mm zone of lyses. The initial emulsification index (E_{24}) was 56.41 ± 1.30 %. *Bacillus subtilis* displaced crude oil by 6.02 ± 1.24 mm with a positive drop collapse test. In previous reports, one or more screening assay is used as index for biosurfactant production (Kiran *et al*., 2010). Hence, Antoniou *et al.* (2015) used drop collapse test for preliminary determination of biosurfactant production from marine hydrocarbon-degrading bacteria using crude oil as carbon substrate. The tests in the present study have been applied by Anaukwu *et al.* (2015) and Sidkey *et al.* (2016).

β-haemolysis has been used as an attribute for biosurfactant producing potential by microorganisms. Consequently, El-Shahawy (2014) reported that the ability to haemolyse blood is a characteristic used in identifying biosurfactant production by microbes. In addition, Carrillo *et al*. (1996) have linked blood haemolysis with biosurfactant production and Eduardo *et al*. (2015) had also reported β-haemolysis for *Bacillus subtilis*. In line with the above, Roy *et al*. (2014) quantified the zone of clearing and noted a direct relationship between the zone of clearing and medium biosurfactant content. The β-haemolytic zone of clearing obtained in the present study of 5.12 ± 1.06 mm was lower than 8.7 mm previously obtained (Akintokum *et al*., 2017) for a related bacterium.

Table 1: Blood haemolysis of bacterial isolates

Isolate codes	Haemolysis	Zone of Haemolysis (mm)
RT9(4)B	Beta	5.12 ± 1.06^a
RT6(4)B	Beta	1.70 ± 2.57^c
RT1(3)A	Gamma	0.00 ± 0.00^d
RT8(3)C	Beta	2.82 ± 3.10^b
RT10(5)C	Apha	0.00 ± 0.00^d
RT7(4)B	Beta	2.55 ± 2.80^b
RT3(3)A	Gamma	0.00 ± 0.00^d
RT9(4)A	Beta	5.57 ± 0.55^a
RT4(4)C	Gamma	0.00 ± 0.00^d

Means with different superscripted alphabets along the columns are significantly different. Values are expressed as means±SE (Standard error of means). **Significant difference level at 0.01.

Table 2: Biosurfactant production parameters of the bacterial isolates

Isolate codes	Drop collapse	Oil spreading (mm)	Emulsification index (%)
RT9(4)B	+	6.02 ± 1.24[a]	56.41 ± 1.30[a]
RT6(4)B	-	0.00 ± 0.00[d]	0.00 ± 0.00[f]
RT1(3)A	-	0.00 ± 0.00[d]	0.00 ± 0.00[f]
RT8(3)C	+	2.62 ± 2.88[cb]	33.22 ± 3.40[d]
RT10(5)C	-	0.00 ± 0.00[d]	0.00 ± 0.00[f]
RT7(4)B	+	4.62 ± 0.4[ab]	48.95 ± 1.22[c]
RT3(3)A	-	0.00 ± 0.00[d]	0.00 ± 0.00[f]
RT9(4)A	+	5.87 ± 0.61[a]	51.25 ± 1.62[b]
RT4(4)C	-	0.00 ± 0.00[d]	0.00 ± 0.00[de]

Means with different superscripted alphabets along the columns are significantly different. Values are expressed as means±SE (Standard error of means). **Significant difference level at 0.01.

Table 3: Effect of different initial medium pH, temperature and incubation time on growth and biosurfactant production by *Bacillus subtilis* RT9(4)B.

Treatment	Variable	Emulsification index (%)	Surface tension (mN/m)	Cell dry weight (g/L)
pH	6	32.39 ± 1.97[d]	65.53 ± 1.74[a]	0.32 ± 0.08[b]
	7	49.13 ± 0.54[a]	55.16 ± 1.02[c]	0.53 ± 0.04[a]
	8	42.00 ± 0.36[b]	59.20 ± 1.61[b]	0.36 ± 0.06[b]
	9	34.83 ± 1.39[c]	63.81 ± 3.02[a]	0.30 ± 0.07[b]
	10	29.81 ± 1.00[e]	66.68 ± 1.51[a]	0.09 ± 0.10[c]
Temperature (°C)	25	37.41 ± 0.66[d]	58.61 ± 3.00[a]	0.47 ± 0.13[cd]
	30	43.82 ± 1.11[c]	55.30 ± 0.97[ab]	0.83 ± 0.04[bc]
	35	60.47 ± 1.08[b]	39.97 ± 4.81[c]	1.16 ± 0.10[b]
	40	63.34 ± 0.73[a]	31.84 ± 1.68[d]	1.54 ± 0.09[a]
	45	56.13 ± 2.31[c]	42.80 ± 2.86[b]	1.15 ± 0.23[d]
Incubation Period (h)	24	35.73 ± 0.86[a]	48.12 ± 0.86[a]	0.13 ± 0.03[c]
	48	54.21 ± 1.09[b]	35.59 ± 2.18[b]	0.61 ± 0.31[b]
	72	84.44 ± 0.21[a]	31.94 ± 1.20[c]	1.03 ± 0.19[a]
	96	85.51 ± 2.66[a]	30.10 ± 0.78[cd]	0.70 ± 0.02[b]
	120	84.19 ± 1.03[a]	29.36 ± 0.82[d]	0.80 ± 0.02[ab]

Means with different superscripted alphabets along the same column for each test parameter are significantly different. Values are expressed as means ± SE (Standard error of means). **Significant difference level at 0.01.

In furtherance to the determination of the biosurfactant production ability of the *Bacillus* species, culture broth of *Bacillus subtilis* RT9(4)B tested positive for drop collapse (Table 2). Theoretically, in the presence of surfactant, a drop of culture supernatant spreads over oil surface as the interfacial tension between the droplet and oil surface is reduced (Batiata *et al*., 2006). The result of oil spreading technique indicated that the organism had oil spreading up to 6.02 mm (Table 2). Chandran and Das (2010) reported oil displacement to be a function of surfactant presence in the broth and that the diameter of the oil displacement is directly proportional to the activity of the surfactant. The oil spreading result of the present study is higher than that reported Nur and Mohammed (2015) for a related *Bacilli*, probably signifying more biosurfactant activity.

Emulsification index (EI24) activity of the organism was also determined as a preliminary test for the detection of biosurfactant producing potential (Table 2). Although not all bio-emulsifiers are biosurfactant producer, percentage emulsification has a direct correlation with biosurfactant production as high percent EI24 translates to high biosurfactant activity. The preliminary EI24 (56.41 ± 1.30 %) in this study is lower than that presented by

Ainon (2013) (EI24, 68 %) but higher than 50% obtained by Al-Wahabi *et al*. (2014) with related species of bacteria. The ability of the present organism to emulsify kerosene implies that it can be applied in various emulsion industries. The *Bacillus subtilis* RT9(4)B showed excellent biosurfactant production potential among other *Bacillus* species during the preliminary screenings. This necessitated the used of the isolate for further study.

Biosurfactant production by *Bacillus subtilis* RT9(4)B under varying pH, temperature and incubation periods

Effects of growth parameters on biosurfactant production by *Bacillus subtilis* isolated from soil contaminated with restaurant effluent was determined with a view to finding the optimum conditions for growth and biosurfactant production. Table 3 showed the effects of pH, temperature and incubation periods on biosurfactant production by *Bacillus subtilis*. The optimum pH for the *Bacillus subtilis* was 7.0.

Subsequent increase in pH led to a significant reduction in biosurfactant production. To grow and/or to produce metabolite, whether primary or secondary, a definite pH requirement is necessary. As the initial medium pH increased, both bacterial growth and biosurfactant production decreased. At this optimum pH (pH 7.0) the cell dry weight of 0.53 ± 0.04 g/l were obtained in the present study. Few related studies also obtained maximal biosurfactant at pH 7.0 (Husam and Ahmed, 2013).

Also, the effect of temperature on biosurfactant production is presented in Table 3. Maximum biosurfactant production was observed at 40 ºC. Similarly, the effect of different incubation periods on biosurfactant production potential of *Bacillus subtilis* is shown in Table 3. There is a strong dependence of microorganisms on some of the physicochemical parameters for secondary metabolite biosynthesis. In this study, biosurfactant production at 40 ºC indicated the highest EI24 activity of 63.34 ± 0.73 %, cell dry weight of 1.54 ± 0.09 g/l and reduction of inter surface of 31.84 ± 1.68 mN/m as shown in Table 3. Therefore, 40 ºC was recorded as the optimum temperature for biosurfactant production. This observation implies that the organism is moderately thermophilic and thus required high temperature for biosurfactant production. In a similar assessment, Dhail (2012) obtained optimum temperature range of 30 to 40 ºC while, Antoniou *et al*. (2015) obtained a non-temperature dependent biosurfactant.

The influence of incubation period on the bacterial growth and biosurfactant production was determined through a time course-study. The findings showed that increased incubation period increased biosurfactant production by *Bacillus subtilis*. Highest biosurfactant production was obtained at 96 h with a slight decline at 120 h (Table 3). This result could imply that using *B. subtilis* and optimizing substrates is required for optimal biosurfactant harvest. Previous study by Husam and Ahmed (2013) revealed that four days were needed for maximal biosurfactant production while an elevated increase up to nine days were obtained by Khopade *et al*. (2012). These biosurfactant production assessments were indirect as emulsification indices were measured rather than biosurfactant itself.

Biosurfactant production by *Bacillus subtilis* RT9(4)B under different concentrations of carbon and nitrogen sources

The effects of glucose, lactose, dextrose, and soluble starch at concentrations 10, 20, 30, 40, and 50 g/l were examined on biosurfactant production by *Bacillus subtilis*. Among the carbon sources, 20 g lactose was the most suitable for *Bacillus subtilis* biosurfactant production, followed by 50 g glucose (Table 4). The bacterium gave the highest biosurfactant production based on result of emulsification index, surface tension and cell dry weight of 85.65 ± 0.98 %, 27.76 ± 1.23 mN/m and 1.53 ± 0.00 g/l respectively at 20 g/l lactose (Table 4).

Consequently, 20 g/l lactose was recorded as the obtained optimal carbon source in this

study. While Antoniou *et al.* (2015), obtained biosurfactant independent of culture biomass quantity and carbon substrate using marine hydrocarbon-degrading bacteria. This study revealed that carbon source determines biosurfactant production. The study supported the reports of Amalesh *et al*. (2012) on lactose carbon substrate and Raza *et al*. (2007) on the dependence of biosurfactant production on carbon source.

The effect of the nitrogen source concentrations examined on biosurfactant production potential of *Bacillus subtilis* showed that 1.5 g urea gave the highest values followed by yeast extract (Table 5). Emulsification index, reduction of medium surface tension and cell dry weight value were 86.35 ± 1.02 %, 28.46 ± 0.62 mN/m, 2.05 ± 0.25 g/l respectively at 1.5 g/l urea. These findings were similar to report of Agarry *et al*. (2015), in which urea at 1.5 g/l concentration for biosurfactant production by *Bacillus subtilis*. This present work showed that an increase in urea concentration above 1.5 g/l reduced biosurfactant production.

Growth of *Bacillus subtilis* RT9(4)B under optimal medium condition

In Figure 1, 20 g of lactose and 1.5 g of urea were used as the optimum concentrations of carbon and nitrogen sources for biosurfactant production by *Bacillus subtilis* RT9(4)B. Following application of optimized conditions including pH 7, temperature 40 ^oC and carbon and incubation period at 96 h, maximum production was obtained as indicated by emulsification index (95.51 ± 2.66%) and surface tension (11.10±0.78 mN/m) reduction values. Overall, biosurfactant produced by *Bacillus subtilis* was 3.73 ± 0.19 g/l at 72 h. The optimisation led to approximately 13.2% reduction in surface tension (30.70 ± 1.42 to 27.10 ± 0.78 mN/m) and a 69.3% rise in the emulsification index (56.41±1.30 to 95.51 ± 2.66%) from the preliminary test values. The present optimisation result is related to the work of Nitschke and Pastore (2004); Sharma *et al.* (2015) who reported biosurfactant maximum yield of 3.2 g/l at 72 hours. This is in agreement with the study of Danashekar and Natarajan (2011) who found a highest EI24 values of, 75, 66.6 and 70% with Petrol, Kerosene and Diesel respectively in the culture samples. A basic advantage observed in this study is that culture conditions could be optimised leading to maximum biosurfactant yield. Consequently, *Bacillus subtilis* of the present study is a good candidate for biosurfactant production.

FT-IR profile of *Bacillus subtilis* RT9(4)B biosurfactant

The FTIR profile of *Bacillus subtilis* surfactant is presented in Table 6. Peaks, transmission and functional groups of the associated compounds. The notable functional groups were alkyl (CH_2- and –CH_3 chains), aliphatic, carbonyl, esters and peptides. The FT-IR findings of the present investigation is similar to that obtained by Faria *et al*. (2011) and Ibrahim *et al*. (2013) based on their lipopeptide reports.

GC - MS profile of biosurfactant produced by *Bacillus subtilis* RT9(4)B

The GC-MS profile showed that the compound produced by *Bacillus subtilis* was a lipopeptide derivative. The findings revealed the presence of 25 major peak (Table 7). The major compounds identified included Oleic acid (30.95%), Octadecanoic acid (25.90%), Cyclododecanol, 1-aminomethyl- (18.28%), Trimyristin (9.00%), Methyl stearate (7.79%), Stearic acid hydrazide (3.52%) and n-Hexadecanoic acid (1.09%).

Other compounds were present in relatively lower amounts. Combining the FT-IR and GC-MS findings, the functional groups including peptide presence and the numerous fatty acids preclude a lipopeptide-type biosurfactant. The characterisation profile of the present study is similar to the finding obtained by Donio *et al*. (2013) and Ibrahim *et al.* (2013) which was a confirmation of the presence of the identified compounds.

Table 4: Effect of different Carbon sources concentrations on growth and biosurfactant production by *Bacillus subtilis* RT9(4)B.

Treatments	Concentrations (g/l)	Emulsification index (%)	Surface tension (mN/m)	Biomass (g/l)
Soluble starch	10	28.04 ± 0.74^{e}	67.59 ± 1.92^{a}	0.07 ± 2.69
	20	32.83 ± 1.91^{d}	55.33 ± 0.59^{b}	0.07 ± 0.04
	30	42.77 ± 1.19^{c}	44.85 ± 1.04^{c}	0.07 ± 0.09
	40	46.59 ± 1.39^{b}	46.51 ± 2.76^{c}	0.08 ± 0.04
	50	54.07 ± 1.71^{a}	40.74 ± 3.30^{d}	0.08 ± 0.46
Glucose	10	55.87 ± 2.12^{c}	45.62 ± 0.87^{c}	0.11 ± 0.04^{bc}
	20	45.47 ± 1.52^{d}	57.04 ± 0.96^{a}	0.08 ± 0.10^{cd}
	30	44.16 ± 2.19^{d}	50.85 ± 0.42^{b}	0.07 ± 0.17^{d}
	40	63.38 ± 1.60^{b}	31.20 ± 3.52^{d}	0.29 ± 0.11^{b}
	50	77.79 ± 0.73^{a}	28.38 ± 2.06^{e}	0.59 ± 0.22^{a}
Lactose	10	76.52 ± 0.56^{b}	30.70 ± 1.42^{d}	0.96 ± 0.12^{b}
	20	85.65 ± 0.98^{a}	30.76 ± 1.72^{d}	1.53 ± 0.00^{a}
	30	76.23 ± 0.97^{b}	34.40 ± 1.20^{c}	0.52 ± 0.17^{c}
	40	65.97 ± 0.74^{c}	40.38 ± 0.59^{b}	1.52 ± 0.08^{a}
	50	51.00 ± 1.29^{d}	45.62 ± 1.58^{a}	1.44 ± 0.21^{a}
Mannose	10	66.70 ± 0.64^{a}	34.39 ± 0.82^{d}	0.12 ± 0.03^{c}
	20	51.76 ± 1.35^{b}	41.06 ± 0.60^{c}	0.48 ± 0.03^{a}
	30	50.47 ± 1.09^{b}	41.36 ± 1.15^{c}	0.17 ± 0.05^{c}
	40	42.24 ± 1.44^{c}	56.75 ± 0.59^{b}	0.30 ± 0.10^{b}
	50	26.71 ± 1.06^{d}	61.37 ± 0.82^{a}	0.57 ± 0.06^{a}

Means with different superscripted alphabets along the same column for each test parameter are significantly different. Values are expressed as means ± SE (Standard error of means). **Significant difference level at 0.01.

Table 5: Effect of different nitrogen source concentrations on growth and biosurfactant production by *Bacillus subtilis* RT9(4)B

Treatments	Concentration (g/l)	Emulsification index (%)	Surface tension (mN/m)	Cell dry weight (g/l)
Yeast extract	0.5	39.61 ± 1.13^{e}	62.61 ± 1.31^{a}	0.77 ± 0.06^{d}
	1.0	78.51 ± 0.99^{a}	27.25 ± 0.95^{d}	1.65 ± 0.09^{a}
	1.5	60.10 ± 0.09^{c}	31.58 ± 1.48^{c}	1.14 ± 0.08^{c}
	2.0	63.89 ± 0.51^{b}	30.80 ± 1.20^{c}	1.38 ± 0.08^{b}
	2.5	42.00 ± 0.77^{d}	51.51 ± 1.23^{b}	1.14 ± 0.03^{c}
Urea	0.5	58.91 ± 1.92^{e}	42.62 ± 1.92^{a}	0.66 ± 0.07^{c}
	1.0	75.31 ± 0.90^{c}	33.40 ± 1.15^{b}	1.27 ± 0.11^{b}
	1.5	86.35 ± 1.02^{a}	28.46 ± 0.62^{d}	2.05 ± 0.25^{a}
	2.0	81.05 ± 1.49^{b}	30.67 ± 1.51^{c}	1.43 ± 0.09^{b}
	2.5	69.72 ± 0.44^{d}	35.12 ± 1.86^{b}	0.80 ± 0.02^{c}
Peptone	0.5	55.73 ± 0.86^{b}	35.95 ± 2.18^{c}	0.13 ± 0.03^{a}
	1.0	64.21 ± 1.09^{a}	28.12 ± 0.86^{d}	0.08 ± 0.03^{b}
	1.5	44.44 ± 0.21^{c}	56.94 ± 1.20^{a}	0.08 ± 0.03^{c}
	2.0	43.84 ± 1.14^{c}	55.10 ± 0.78^{ab}	0.06 ± 0.02^{e}
	2.5	44.19 ± 1.03^{c}	54.36 ± 0.82^{b}	0.07 ± 0.02^{d}

Means with different superscripted alphabets along the same column for each test parameter are significantly different. Values are expressed as means ± SE (Standard error of means). **Significant difference level at 0.01.

Table 6: The FTIR profile of *Bacillus subtilis* RT9(4)B biosurfactant

S/No.	Peaks	Transmission (%)	Functional group
1	3490.006	35	Peptides
2	3001.030	65	Aliphatic
3	2065.125	80	Aliphatic
4	1642.420	45	Carbonyl
5	1432.404	65	Alkyl
6	1252.720	75	Alkyl
7	1112.254	15	Esters
8	913.529	20	CH_2

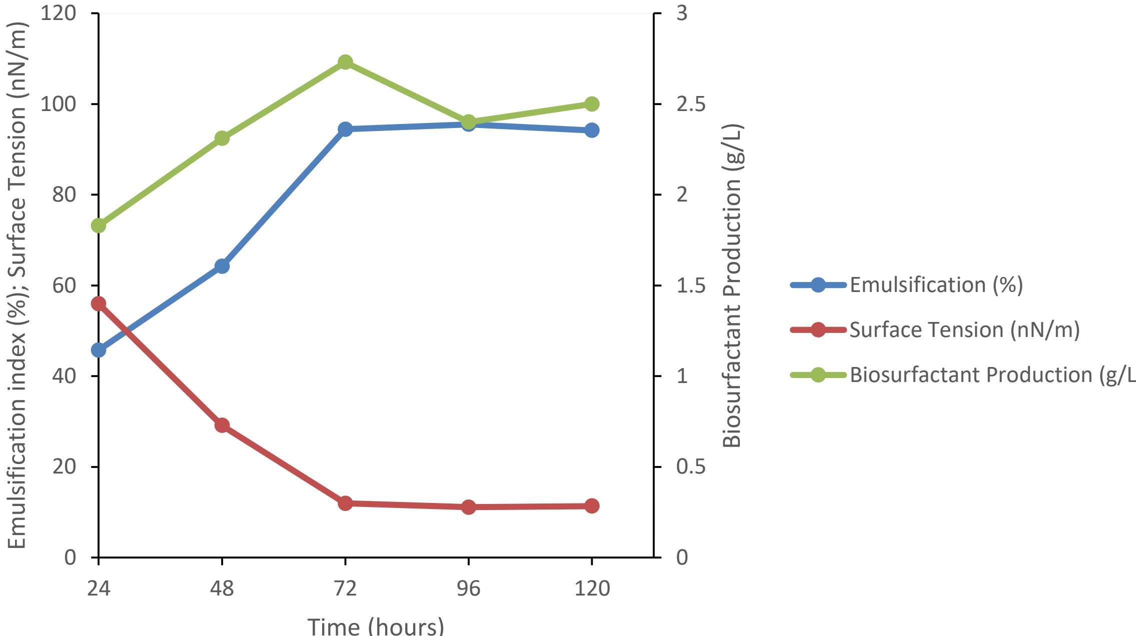

Figure 1: The emulsification index, surface tension and biosurfactant production profiles of *Bacillus subtilis* RT9(4)B grown in optimised medium.

Table 7: GC-MS Profile of Surfactin Produced by *Bacillus subtilis* RT9(4)B

No.	Retention time	Peak area (%)	Name of compounds
1	5.359	0.70	Tert-Butyl isopropyl disulfide, perfluoro
2	9.585	0.73	Cyclotetrasiloxane, octamethyl-
3	15.594	0.07	Plumbane, diethyldimethyl-
4	16.524	0.09	Cyclopentasiloxane, decamethyl-
5	18.540	0.04	Cyclotrisiloxane, hexamethyl-
6	33.544	0.08	Undecanoic acid, 10-methyl-, methyl ester
7	37.421	0.33	Dodecanoic acid
8	37.963	0.03	Nonanedioic acid, dimethyl ester
9	41.530	0.08	Tridecanoic acid, 12-methyl-, methyl ester
10	44.515	0.25	Tetradecanoic acid
11	48.818	0.25	Pentadecanoic acid, 14-methyl-, methyl ester
12	51.804	1.09	n-Hexadecanoic acid
13	54.362	0.18	9-Octadecenoic acid, methyl ester, (E)-
14	54.983	7.79	Methyl stearate
15	56.960	30.95	Oleic Acid
16	57.464	35.90	Octadecanoic acid
17	58.084	0.03	2,7-Octadien-1-ol
18	59.092	0.12	Pentanoic acid, propyl ester
19	61.883	0.03	9-Decenoic acid
20	62.310	0.06	Trimyristin
21	64.481	3.52	Stearic acid hydrazide
22	64.830	0.13	Undecane, 5,6-dimethyl-
23	65.140	0.25	Tridecane
24	65.838	9.00	Trimyristin67.040 18.28
25	67.040	18.28	Cyclododecanol, 1-aminomethyl-

CONCLUSION

This study revealed that soil contaminated with restaurant wastewater is a good source of biosurfactant producing *Bacillus* species. At optimal conditions of temperature (40 ºC), pH (7), carbon and nitrogen sources (lactose, 20 g/l and urea, 1.5 g/l respectively), there was an approximately 23.2% reduction in surface tension (14.46 ± 0.62 to 11.10 ± 0.78 mN/m) and a 69.3% rise in the emulsification index (56.41±1.30 to 95.51 ± 2.66%). The highest biosurfactant produced was 3.73 ± 0.19 g/l at 72 h. Furthermore, the *Bacillus subtilis* RT9(4)B produced lipopeptide type biosurfactant and therefore a potential xxcandidate for biosurfactant production of industrial application.

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Water Quality Assessment and Antibiotic Susceptibility Testing of Bacteria Isolated from Borehole Water Supplies within a Residential Environment in Elele, Rivers State, Nigeria

Mbah E. I.* **and Amuchie N. E.**
Department of Microbiology, Madonna University, Elele, Rivers State, Nigeria.
* Corresponding author: mbahedith@yahoo.com

Abstract: Borehole water supplies within residential settings in southern Nigeria are a major source of drinking water for low- and middle-class families. This study was designed to assess the water quality, phenotypic characterization and antibiogram profiling of bacteria isolated from some borehole water supplies within residential environments in Elele, Rivers State, Nigeria, that low and some middle-class families rely on as their source of drinking water. A total of 30 borehole water samples were collected at various geo-referenced points and subjected to physicochemical analysis and bacteriological before antibiotic susceptibility tests using standard methods. Results of the physicochemical analysis revealed that most water quality parameters fell within the safe limits of the World Health Organization (WHO) except for chromium (2·0mg/L) and fluoride (25-100mg/L) which were above their permissible limits of 0·05 and 1·5 mg/L respectively. Predominant pathogenic bacteria; *Escherichia coli* (7, 50·0%), *Klebsiella pneumoniae* ssp *pneumoniae* (2, 14·3 %), and *Enterobacter* spp (5, 35·7 %) experimentally annotated and confirmed by the VITEK® 2 Compact system, were all multidrug-resistant, MDR (100 %), phenotypes. However, this study unveiled the high rate of sensitivity (100%) for *Klebsiella pneumoniae* ssp *pneumoniae* and *Enterobacter* spp to ofloxacin, streptomycin and gentamycin 100 % resistance to meropenem and chloramphenicol. *E. coli* showed varying sensitivity and resistance levels to the test antibiotics. This study therefore, offers insight to the diversity of the predominant MDR bacterial phenotypes in borehole water within the study area and the use of ofloxacin, streptomycin and gentamycin in the treatment of waterborne infections caused by MDR *Klebsiella pneumoniae* ssp *pneumoniae* and *Enterobacter* spp within the study area. Also, regular evaluation of chromium and fluorine levels in these water supplies is needed to encourage the early development of an intervention strategy once detected above the WHO permissible limit.
Key word: Borehole water, WHONET software, multidrug-resistant bacteria.

INTRODUCTION

Borehole water supplies within different residential settings in Southern Nigeria are a major source of drinking water for the low and some middle-class families. These boreholes are mostly privately owned and there exist no adequate monitoring to validate the efficacy of water treatment (example' Sediment filtration, water softening, disinfection, cleaning, iron removal and so on.), frequency of treatment cycles and also, the maintenance of water piping systems that would ensure that water quality parameters attain the drinking water permissible limits of World Health Organization (WHO). Yusuf *et al*. (2014) documented that unsafe drinking water supplies in Nigeria are due to uncoordinated efforts of various federal, state and local agencies. Waterborne diseases like cholera, dysentery and typhoid fever which can spread rapidly within a population are associated with the contamination of drinking water (Mbah *et al*., 2016; Kumar *et al*., 2022). World Health Organization, WHO, and United Nations International Children's Emergency Fund, UNICEF, (2021) reported that 70% of drinking water in Nigeria at the point of consumption is contaminated. Waterborne diseases arising from the consumption of contaminated water are often caused by pathogens like; viruses, protozoa, bacteria and intestinal parasites. Most of these pathogens shed into the groundwater through fecal contamination to initiate diseases like cholera, typhoid fever and diarrhea (Obuekwe *et al*., 2021). It is however a known fact that the burden of waterborne disease is one of the leading causes of mortality worldwide. Approximately 6.3 percent of mortality worldwide occurs as a result of unsafe water consumption, inadequate sanitation and poor hygiene (WHO, 2015; Kumar *et al*., 2022) and out of which 4 percent of this global

disease burden could be preventable through the provision of safe water supply, good sanitation and hygiene. In Nigeria, only about 90 million people have access to improved water (Yusuf *et al.,* 2014) and poor access to improved water and sanitation contributes majorly to high mortality and morbidity rates among children under five (UNICEF, 2022). Also, UNICEF (2022) further documented that the consumption of contaminated water and the use of poor sanitary conditions in Nigeria increases the vulnerability of the populace to waterborne diseases which leads to an annual mortality of more than 70,000 children under the age of five. Microbial contamination of borehole water supplies in Southern Nigeria mostly occurs through seepages of environmental waters into the groundwater aquifer, especially with a sharp rise in the groundwater table during the peak wet seasons (Mbah and Okafor, 2021; Unamba *et al*., 2016) resulting to the inundation of the environment and subsequent destruction of some borehole wells. Antimicrobial resistance (AMR) is the leading cause of mortality worldwide and with the highest burden documented in the low-resource settings (Murray et al., 2022). The emergence and distribution of multidrug-resistant (MDR) bacteria within the clinical and environmental compartments is a major global concern to public health. These MDR bacteria are predominantly members of the Enterobacteriaceae (Teklu *et al*., 2019). The increasing rise of antibiotic resistance in the environmental compartments have been linked to high anthropogenic activities (Finley *et al*., 2013; Mbah *et al*., 2016; Gekenidis *et al*., 2018). Drinking water systems are a major source through which MDR bacteria and other waterborne pathogens are disseminated to the populace (Teklu *et al*., 2019; Falodun *et al*., 2017), through a fecal-oral route. MDR bacteria such as *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Enterococcus* spp etc. isolated from borehole water supplies have been well documented (Odonkor *et al*., 2022; Obuekwe *et al.,* 2021; Mbah and Okafor, 2021). Globally, *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Streptococcus pneumonia, Acinetobacter baumanii and Pseudomonas aeruginosa* are the six leading MDR bacteria responsible for 929,000 deaths which were attributable to antimicrobial resistance (AMR) and 3.57 million deaths associated with AMR in 2019 (Murray *et al*., 2022). Thus, many researchers are now screening different classes of antibiotics and other good-quality antimicrobials on emerging clinical and environmental isolates of MDR bacteria so as to queue into their national action plan in overcoming this challenge. Following the World Health Organization (WHO) recommendations on the use of AWaRe (Access, Watch, Reserve) classification tool (WHO, 2019) in tackling antimicrobial resistance in achieving antimicrobial stewardship, there is an urgent need to continue evaluating the efficacy of all Watch group antibiotics on all emerging MDR bacteria showing resistance to the Access group antibiotics before further screening with other essential medicines in the Reserved group. In Rivers State, studies on residential borehole water supplies that low- and middle-class families rely on as their sole source of drinking water are limited. It has been ascertained that borehole water supplies at Elele, Rivers State, are emerging hotspots for MDR bacteria (Mbah and Okafor, 2021). However, little is known about the effectiveness of antibiotics on these superbugs within the study area. Thus, this study aimed at investigating the drinking water quality and the sensitivity of isolated MDR bacteria from residential borehole water supplies at Madonna University, Elele against commonly used antibiotics.

MATERIALS AND METHODS

Study sites: The sampling sites selected for this study are located in Madonna University, Elele, and its environs (Figure 1) which is situated in Rivers State. The ten (10) sampling sites used in this study were geo-referenced as follows:

SM (5°08'23.1"N, 6°49'46.2"E);

EH (5°08'13.9"N, 6°49'43.9"E);
FI (5°08'11.0"N, 6°49'39.2"E);
MI (5°08'04.5"N, 6°49'29.9"E);
SC (5°07'57.8"N, 6°49'37.7"E);
EQ (5°08'02.7"N, 6°49'46.5"E);
RC (5°08'09.6"N, 6°49'40.0"E);
BF (5°08'08.0"N, 6°49'41.3"E);
JS (5°08'01.6"N, 6°49'42.0"E);
FL (5°08'27.0"N, 6°49'36.7"E).

This residential environment harbor a good mix of the low, middle and high-income families working within the University community and its environs.

Sample collection: A volume of 1000 mL of borehole water samples from the ten different sites were collected aseptically with sterile screw capped plastic sample containers of 1000 mL capacity. Before each water collection, cotton wool soaked with 70% (v/v) ethanol was used to sterilize the nozzles of the borehole taps to ensure no contamination entered the water (Azuonwu, *et al.,* 2020). Once the taps were turned on, water was allowed to flow for about 2-3 minutes before collection into the well labeled sterile containers and then, transported to the laboratory in an ice packed container. All water samples were processed within six (6) hours of collection in the Department of Microbiology, Madonna University Elele Nigeria.

Physicochemical analyses: On-site physicochemical analyses of the water samples were conducted using both probes (Hanner instrument, USA) and paper-based sensors. The physicochemical analysis that was carried out included; pH, temperature, electron conductivity and total dissolved solids while total alkalinity, hardness, lead, copper, iron, mercury, chromium, bromine, residual chlorine, fluoride, sulfite, nitrate, and nitrite were determined using paper-based biosensors. (Dhanaji *et al*., 2016; Busa *et al*., 2016).

Bacteriological analysis of water: The total cultivable heterotrophic bacterial counts were evaluated by the use of standard methods of pour plating using nutrient agar medium (Enaigbe *et al.,* 2019). The plates were incubated at 37°C for 24 h and distinct colonies were used to calculate the cultivable bacterial load (colony forming units per ml, cfu/mL). Pathogenic bacteria were also isolated from all drinking water samples collected by employing the membrane filtration technique. Approximately 100 ml of each water samples were filtered through 0.45 µm pore size, 47 mm diameter sterile membrane filter (Millipore, Billerica, MA, USA). The membrane filters were then inoculated on solidified Eosin Methylene Blue (EMB) agar media and incubated at two temperatures (37°C and 44.5°C) for 18-24 h. The temperature at 44.5°C allowed for the selection of all *E. coli* present in the water samples. All presumptive colonies were picked, sub-cultured on EMB and characterized by experimental annotation (gram staining, morphological and biochemical characteristics) and VITEK® 2 Compact system, (version 9.02). The use of VITEK® 2 Compact system for the confirmation of all experimentally annotated phenotypes were done at the Medical Laboratory Unit, Calabar Teaching Hospital, Calabar, Cross River, Nigeria.

Antibiotic susceptibility tests of bacterial isolates: Antibiotic susceptibility test of bacterial isolates was carried out using the Kirby–Bauer disc diffusion technique as described by Wakil and Mbah (2012) and Mbah and Okafor (2021). Nitrofurantion (100 µg), Ciprofloxacin (10 µg), Chloramphenicol (10 µg), Gentamicin (10 µg), Ofloxacin (10 µg), Meropenem (10 µg), Pefloxacin (10 µg), Ceftriaxone (30 µg), Amoxicillin (30 µg), and Streptomycin (10 µg); (Oxoid, UK) were used for this test. This was done by introducing about 3-5 well isolated pure young colonies of 18-24 h into normal physiological saline and homogenized to create a bacterial suspension. The turbidity of the various bacterial suspensions was adjusted to the turbidity of 0.5 McFarland standard against a contrasting black and white background and the bacterial load (1-2$\times10^8$ cfu/mL) was confirmed using a spectrophotometer of 1 cm light path at 625 nm to obtain an

absorbance reading of 0.08-0.13. The bacterial suspensions were then inoculated uniformly unto the surface of Mueller Hinton agar plates using sterile swab sticks immersed in the suspension and pressed against the side of the test tubes to remove excess fluid. The antibiotic discs were carefully placed on the dry surface of inoculated plates with the aid of sterile forceps and incubated at 37˚C for 18-24 h. Zones of inhibition were measured in millimeters and mean zones of inhibition recorded were interpreted as susceptible (s), intermediate (I) or resistant (R) following the guidelines of the Clinical and Laboratory Standards Institute (2021). Isolates that were resistant to three or more antimicrobial classes were regarded as MDR.

Statistical analysis: The data for physicochemical parameters of the borehole water supplies within the residential environment were summarized in means ±standard deviation. Distribution of pathogenic bacteria supplies were analyzed in percentages. WHONET data analysis and interpretation were conducted on datasets generated from the antimicrobial susceptibility tests.

RESULTS

Physicochemical and bacteriological analysis of borehole water supplies

The findings obtained from the water quality analysis conducted revealed that all physicochemical parameters including the total cultivable heterotrophic bacteria (TCHB) fell within the permissible limit of World Health Organization (Table 1) except for chromium (2.0 mg/L) and fluoride (25-100 mg/L) across all study sites. A total of 14 pathogenic bacteria were isolated and identified by both experimental annotations and VITEK® 2 Compact systems. From the results, *Escherichia coli* (7, 50.0%), *Klebsiella pneumoniae* ssp *pneumoniae* (2, 14.3%), and *Enterobacter* spp (5, 35.7%) were the prevalent pathogenic bacteria found and with *Escherichia coli* as the most dominant bacteria. However, *Enterobacter cloacae* ssp. *cloacae, Enterobacter cloacae* ssp. *dissolvens* and *Enterobacter aerogenes* were detected as the diverse species of Enterobacter in the water supplies (Figure 2).

Antibiotic susceptibility tests of the bacterial isolates

The WHONET-19 analysis of the antimicrobial resistance profiles of bacterial pathogens isolated from the borehole water supplies meant for human consumption within the residential area categorized all tested bacterial pathogens presented in this study as medium priority pathogens. However, due to their high level of resistance to carbapenem, WHONET-15 classified them as high priority pathogens. The antibiotic susceptibility profiles of members of the Enterobacteriaceae involved in this study presented the percentage of isolates that are resistant (including a 95 % confidence interval), intermediate, and susceptible in Fig. 4 (a-d). Out of the ten antibiotics (AMX, MEM, STR, PEF, CIP, CRO, CHL, GEN, OFX and NIT) used for the study; all tested bacterial pathogens were found to be resistant to more than three antibiotics. All isolates that were resistant to at least 3 of the 10 antibiotics categories tested were grouped as multidrug resistant (MDR) phenotypes. In this study, all isolates that their sensitivity to antibiotics was in the intermediate category were also regarded as resistant phenotypes. However, the sensitivity of these MDR bacteria to some antibiotics was also observed. ofloxacin and streptomycin had the highest susceptibility rate for *Klebsiella pneumoniae* ssp *pneumoniae* (100 %), while it was ofloxacin, streptomycin and gentamycin for *Enterobacter aerogenes* (100 %), ofloxacin for *Enterobacter clocae* (100 %) and gentamycin (85.7 %), streptomycin (71.4 %) and ofloxacin (71.4 %) for *E. coli* respectively. *Klebsiella pneumoniae* ssp *pneumoniae* demonstrated 100% resistance to meropenem and chloramphenicol while *Enterobacter aerogenes* showed 100% resistance to meropenem. In addition, *Enterobacter clocae* showed 100% resistance to chloramphenicol while *E. coli*

isolates were 85.7% resistant to meropenem and nitrofurantoin respectively.

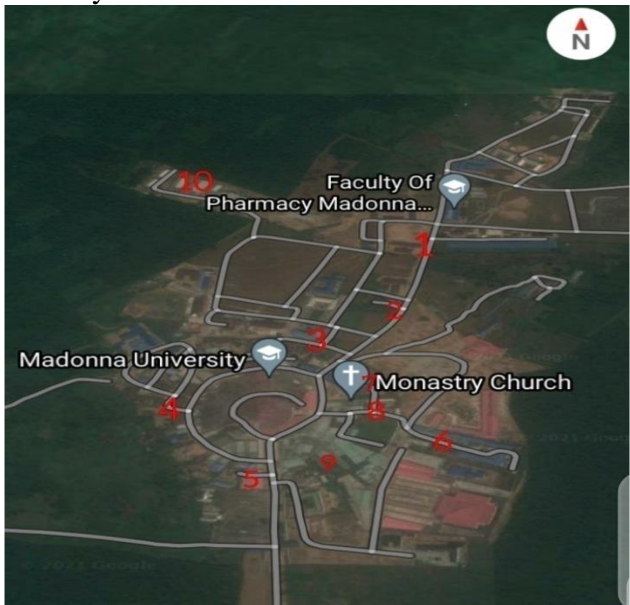

Figure. 1. A Map of the geo-referenced points within the study location. NB: 1. St. Mathias Lodge, SM, (5°08'23.1''N, 6°49'46.2''E); 2. Engine house, EH, (5°08'13.9''N, 6°49'43.9''E); 3.Female Internship Lodge, FI, (5°08'11.0''N, 6°49'39.2''E); 4. Male Internship Lodge, MI, (5°08'04.5''N, 6°49'29.9''E); 5. Special Canteen Lodge, SC, (5°07'57.8''N, 6°49'37.7''E); 6. European Quarters, EQ, (5°08'02.7''N, 6°49'46.5''E); 7. Reconciliation Lodge, RC, (5°08'09.6''N, 6°49'40.0''E); 8. Benefactor Lodge, BF, (5°08'08.0''N, 6°49'41.3''E); 9. Jesus the Savior Lodge, JS, (5°08'01.6''N, 6°49'42.0''E); 10. Factory Lodge, FL, (5°08'27.0''N, 6°49'36.7''E)

Table 1: Physicochemical and bacteriological analysis of borehole water supplies

Parameters	Unit	SM	FI	JS	SC	EH	EQ	BF	MI	RC	FL	WHO/NSDWQ Limit (2017)
Temp.	^{o} C	26±02	24±02	25±2.5	25±2.5	23±01	25±2.5	25±2.5	24±02	24±02	28.±02	Ambient
P^{H}		7.3±07	7.0±06	7.6±0.7	7.0±06	7.0±06	7.2±0.7	7.3±07	7.1±06	7.2± 0.7	7.4±0.	6.5-8.5
EC	(S/cm)	26±02	28±2.8	8.0±.0.8	16±1.6	26±2.6	27±2.7	26±2.6	18±1.7	28±2.8	36±3.6	1000
TDS	mg/L	13±1.3	12±1.2	4.0±0.4	9.0±0.9	12±1.2	14±1.4	13±1.3	08±0.8	14±1.4	20±02	500
T. alkalinity	mg/L	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	-
Hardness	mg/L	50±5.0	50±5.050•0	125±12	50±5•0	125±12	0.0	50±5.0	50±5.0	50±5.0	50±5.0	150
Iron	mg/L	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0.1
Mercury	mg/L	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0.006
Chromium	mg/L	02±0.2	02±0.2	02±0.2	02±0.2	02±0.2	02±0.2	02±0.2	02±0.2	02±0.2	02±0.2	0·05
Bromine	mg/L	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0.05
Nitrate	mg/L	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	50
Nitrite	mg/L	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	3.0
Cl	mg/L	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	5.0
Fluoride	mg/L	100±10	25±2.5	100±10	50±5.0 50•0	100±10	50±5.0 50•0	100±10	50±5.0 50•0	50±5.0	25±2.5	1.5
Sulfate	mg/L	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	0·0	50
TCHB	Cfu/mL	18 X10^{-1}	4·0X 10	2·2X 10^{1}	1·2X10^{1}	2·5X10^{0}	1·2X10^{0}	1·1.X10^{0}	6·0X10^{-1}	1·5X10^{0}	2·3X10^{0}	400

Data are presented in mean ±Standard deviation. SM: St. Mathias Lodge; FI: Female internship Lodge; JT: Jesus the Saviors Lodge; SC: Special canteen Lodge; EH: Engine house; EQ: European Quarters; BF: Benefactors Lodge; MI: Male internship Lodge: RC: Reconciliation Centre Lodge; FL: Factory Lodge; TCHB: Total Cultivable Heterotrophic Bacteria.

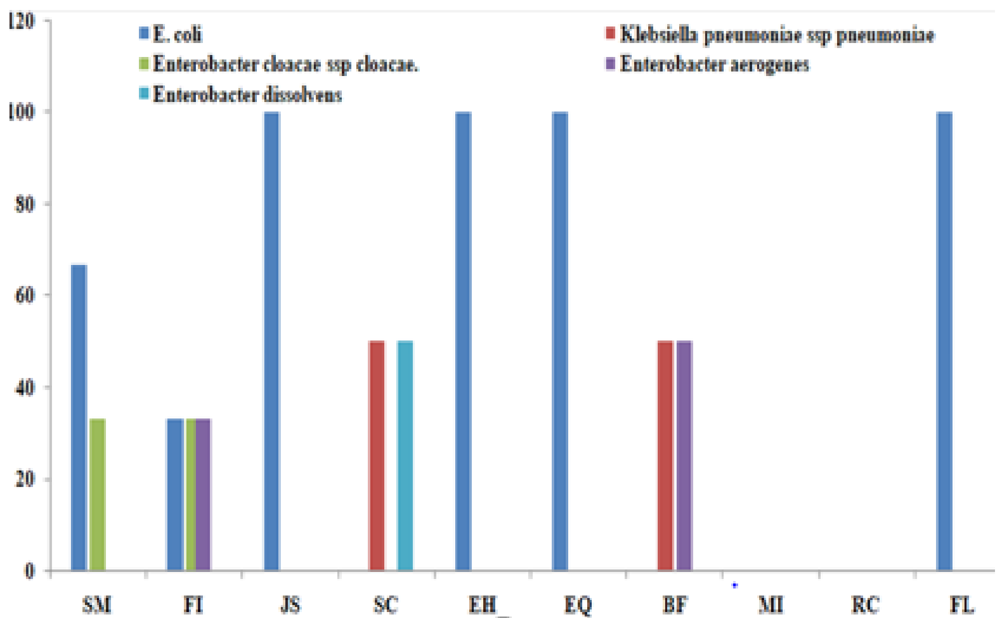

Figure 2. Distribution and relative abundances of pathogenic bacteria in borehole water supplies within the residential area. Key: SM: St. Mathias Lodge; FI: Female internship Lodge; JT: Jesus the Saviors Lodge; SC: Special canteen Lodge; EH: Engine house Lodge; EQ: European Quarters; BF: Benefactors Lodge; MI: Male internship Lodge: RC: Reconciliation Centre Lodge; FL: Factory Lodge

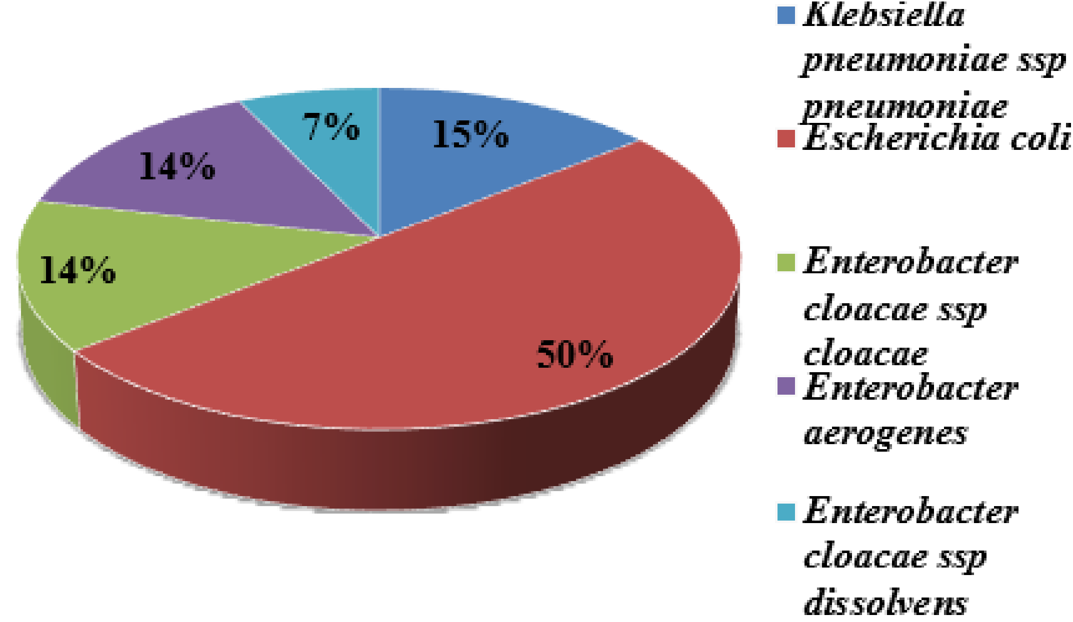

Figure 3: Percentage abundances of pathogenic bacteria recovered from all the borehole water supplies.

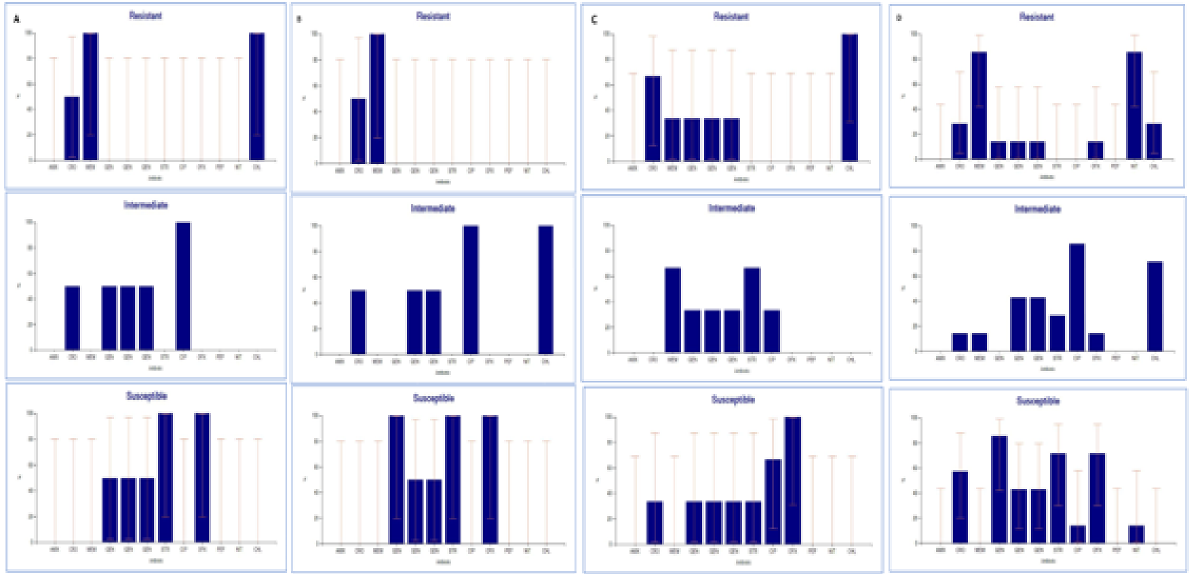

Figure 4. Antibiotic resistant patterns of *Klebsiella pneumoniae* ssp *pneumoniae* (A), *Enterobacter aerogenes* (B), *Enterobacter clocae* (C), and *E. coli* (D) from residential borehole water supplies. Key: Amoxicillin (AMX), Meropenem (MEM), Streptomycin (STR), Pefloxacin (PEF), Ciprofloxacin (CIP),Ceftriaxone (CRO), Chloramphenicol (CHL), Gentamycin (GEN), Ofloxacin (OFX) and Nitrofurantoin

DISCUSSION

This study focused on the water quality assessment and antibiogram profiling of bacteria isolated from some borehole water supplies within residential environments in Elele, Rivers State, Nigeria, that low and some middle-class families rely on as their source of drinking water. This study observed the presence of MDR bacteria and high levels of chromium (2.0 mg/L) and fluoride (25-100 mg/L) in the borehole water supplies that serve as a major source of drinking water for low-and middle- class families.

In Nigeria, microbial quality of drinking water is often guided by the WHO (World Health Organization) and NSDWQ (Nigeria Standard for Drinking Water Quality) water quality guidelines. Following these guidelines for drinking water quality (NSDWQ, 2017; WHO, 2017), the results obtained from this study showed that all the borehole drinking water samples obtained within the residential area of Madonna University, Elele and its environs, had the mean concentration values of the tested parameters fall within the safe limits of WHO and NSDWQ except for chromium (2.0 mg/L) and fluoride (25-100 mg/L) which were above their permissible limits of 0.05 and 1.5 mg/L for chromium and fluorides respectively. Similar results on high floride concentration above the WHO permissible limit have been described by other authors (Ram *et al*. 2017, Dongzagla *et al*. 2019; Egor and Birungi, 2019 & Giwa *et al.* 2021) on studies done in borehole drinking water situated in different residential environments. Drinking water is a major source of fluoride intake to the human body (Dongzagla *et al.* 2019). Fluoride is an element that is very important to the human health; however, its high accumulation in the body may lead to Fluorosis (Waziri *et al.* 2012). The risk of population exposure to high fluoride is largely due to the consumption of large volumes of water during the hot weather (Dongzagla *et al.* 2019). The occurrence of severe dental and skeletal fluorosis has been documented by Giwa *et al.* (2012) in four communities within the North-eastern states of Nigeria.

For the high level of chromium concentrations (2.0 mg/L) above the WHO limit observed from all the tested borehole water samples used in this study, a similar observation of high chromium concentrations in borehole and well water samples in Mgbuoshimiri community in Rivers State have been previously documented by Odu *et al*. (2020).

This study characterized 14 pathogenic bacteria isolated from 30 borehole water supplies within residential environments in Elele, Rivers State of Nigeria by the use of phenotypic methods. *Escherichia coli* (7, 50.0%), *Klebsiella pneumoniae* ssp *pneumoniae* (2, 14.3 %), and *Enterobacter* spp (5, 35.7 %) were the diverse Gram-negative bacteria found and with *Escherichia coli* as the most dominant phenotypes. The occurrence of these Gram-negative bacteria in the borehole water supplies suggests the degradation and fecal pollution of the drinking water supplies within this residential area in Elele community. According to Mbah *et al*. (2016), exposure to drinking water contaminants and its resultant health effect is a major concern to public health. Previous reports have shown that the ingestion of fecal contaminated water may trigger waterborne diseases like, gastroenteritis (Folorunso *et al.,* 2014; Mbah *et al*., 2016)

This study found out that all members of the Enterobacteriaceae isolated were of the multidrug resistant phenotypes. Resistance to different antibiotics can contribute to the existence of newly emerging multidrug-resistant bacteria which may get transmitted to different individuals, causing illnesses that may be difficult to treat (Ayandiran *et al.,* 2014). However, despite the level of antibiotics resistance demonstrated by most bacterial isolates, some bacterial phenotypes were found to show susceptibility to some antibiotics used in this study. Ofloxacin and streptomycin had the highest susceptibility rate for *Klebsiella pneumoniae* ssp *pneumoniae* (100%), while it was ofloxacin, streptomycin and gentamycin for *Enterobacter aerogenes* (100%), ofloxacin for *Enterobacter cloacae* (100%) and Gentamycin (85.7%), ofloxacin and streptomycin (71.4%) for *E. coli*. The bactericidal activity of ofloxacin, streptomycin and gentamycin on Gram negative bacteria contaminating drinking water resources in Nigeria is of great interest to public health. Similarly, to the effectiveness of ofloxacin to most of our bacterial phenotypes, Eddeh-Adjugah *et al*. (2022) documented that amongst all pathogenic *E. coli* isolated from several borehole water taps in Port-Harcourt, Rivers State, ofloxacin showed the highest susceptibility rate of 100 %. Olorunleke *et al.* (2022) also revealed that ofloxacin had the highest susceptibility rate for all *E. coli* isolates recovered from livestock and in-contact human in Southeastern Nigeria.

CONCLUSION

Consumption of borehole water supplies by low- and middle-class families within the residential settings in Elele community in Rivers State, Nigeria should be discouraged due to the occurrence of MDR bacteria and high levels of chromium (2.0 mg/L) and fluoride (25-100 mg/L) above the WHO limits for safe water quality that have been found in this study. Awareness campaigns on high levels of chromium and fluoride above the WHO permissible limits for safe water quality in Elele community is highly recommended. This would encourage families to screen their borehole water supplies for the presence of high levels of chromium and fluorides and then seek for intervention strategies to help reduce their levels.

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Microbiological Quality of Sediment and Water Samples from Selected Surface Waters in Anambra State, Nigeria

Okaa A. I. Ogu C. T.* and Chukwujekwu A. G.
Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria
* Corresponding author: ct.ogu@unizik.edu.ng

Abstract: Water occupies about 70% of the earth's surface and is one of the most demanded of all urban and rural amenities, thus indispensable for human activities. Microbiological properties of water and sediment samples from four surface waters in Anambra state Nigeria were investigated for the evaluation of their pollution level. A total number of twenty (24) samples, with three (3) samples per sampling station were collected and examined in this study. Standard plate counts method was used to estimate the total aerobic, heterotrophic and coliform counts. The total aerobic heterotrophic bacterial counts obtained varied in each station from 3.6 x 10^4 cfu/ml – 2.23 x 10^5cfu/ml in the rainy season and from 3.2 x 10^4cfu/ml – 2.1 x 10^5cfu/ml in the dry season. The microorganisms found included *Escherichia coli, Micrococcus* sp., *Pediococcus* sp., *Citrobacter* sp., *Planococcus* sp., *Flavobacterium* sp., *Mucor* sp., *Allescheria* sp. *and Saccharomyces* sp. The average high iron content ranging from 6.4 – 52.55 mg/l was greater than the World Health Organization (0.3 mg/l) permissible range for drinking water. There was a negative correlation between the alkalinity value with total coliform of water, but positive correlation between the alkalinity values and the total coliform counts of the sediment and water. There was a significant difference at $P < 0.05$ between the mean seasonal alkalinity values of the water and sediments. The findings revealed that the selected surface waters are highly contaminated with pathogenic microorganisms with poor physicochemical characteristics and remains unsafe for human consumption.This necessitates the need for primary prevention measures to be identified.
Key word: Water, sediment, surface waters, analysis

INTRODUCTION

Surface water is a natural water source which collects from water running across the surface of the ground. As this water runs across the ground surface, it picks up microorganisms, organic matter and minerals. The type and number of microorganisms is a reflection of the condition of the water. Some of the microorganisms found in water may be regarded as indigenous, while others may be considered as contaminants (Okaka and Ene, 2005). Microorganisms are widely distributed in nature and are found in most natural waters. Their abundance and diversity may be used as a guide to the suitability of water for fish, animals, recreational and amenity purposes. Sediment is matter (Sand, dirt, gravel) that settles to the bottom of a water body after euthrophication. Euthrophication is any increase in the concentration of available nutrients: it may be man-made as in sewage discharge into stream or natural as with rain water washings (Chukwura, 2001). Generally, excessive amount of organic matter produced in eutrophic bays, high amounts of primary production results in appreciable amounts of organic matter input into the sediment where active involvement of biological, microbiological and chemical processes decompose the organic matter (Smetacek *et al*., 1991). Microbiologically mediated decomposition process play a vital role in the mineralization of organic matter and is expected in sediments receiving high load of organic matter because the oxygen transport within the sediment is limited. Hence, these sediments are always under reduced conditions because of stratification and high organic matter input (Blackburn, 1991).

Water pollution is one of the most important environmental problems faced by the third world countries (Barry, 2000). The use of bacteria as water quality indicators can be viewed in two ways: first the presence of such bacteria can be taken as an indication of faecal contamination of the water and thus as a signal to determine why such contamination is present, how serious it is, and what steps can be taken to eliminate it:

second, their presence can be taken as an indication of the potential danger of health risks that faecal contamination poses. The higher the level of indicator bacteria, the higher the level of faecal contamination and the greater the risk of contracting the disease (Villa,2000). Increasing industrialization and urbanization has led to a wide scale contamination of surface waters from industrial effluents, domestic sewage discharge and excessive usage of fertilizers and pesticides (Haruvy, 1997). Domestic wastes vary in composition and often contain millions of bacteria per milliliters. The continuous discharge of untreated sewage around homes into surface water source is a serious health hazard because the causative agents of bacillary dysentery, cholera, enteric fever and other diarrheal diseases are carried in sewages (Chukwura, 2001).

MATERIALS AND METHODS

Sample sources and collection: Eight samples on average (4 of water and 4 of sediment) were collected from the following locations; Obizi sediment, Obizi water, Nkisi sediment, Nkisi water, Ebenebe river sediment, Ebenebe river water, Agulu lake sediment and Agulu lake water. Each sample for physiochemical analysis was collected using a clean 2-litre plastic container with screw cap and at the point of collection, the container was rinsed with the sample.

Microbiological Analysis –

Isolation: Isolation was carried out by spread plate method according to the method of Ogbulie *et al*. (1998). Bacteria were isolated from sediment and water after serial dilution using nutrient agar plates. The plates were incubated at room temperature for 24 hours after which the colonies developed on the plates were sub-cultured on fresh nutrient agar. The purified isolates were maintained on nutrient agar slant for further tests. Moulds were isolated from the sediments and water after serial dilution using Sabouraud dextrose agar (SDA) plates. The plates were incubated at room temperature for 48 hours after which the developed colonies were sub cultured. Yeasts were isolated from the sediment and water using yeast extract agar (YEA). The plates were incubated at room temperature for 48 hours, developed colonies were sub-cultured on fresh yeast extract agar plates for purification and purified isolates maintained on slants.

Estimation of Escherichia coli counts using the membrane filteration technique: Approximately 2 ml of membrane enriched Lauryl sulphate was added to the absorbent pad contained in a Petri dish. The Petri dish was covered until the sample was filtered through the membrane. The millipore filter was placed on a filtration unit and damped in position, and the samples (10 ml diluted with 90 ml of sterilized distilled water) were drawn through the millipore filter with the aid of vacuum pump. The filter disk handled with sterile forceps was placed on absorbent pad previously saturated with membrane enriched lauryl sulphate. Incubation was carried at $44^{0}C$ for 24-48 hours after which yellow colonies were counted (Chesbrough, 2006).

Identification of isolates: Identification of bacterial isolates was based on cultural, morphological and biochemical tests (Chesbrough, 2006). Moulds were identified based on colonial morphological features-lactophenol blue mount preparation and slide culture technique. Yeast isolates were identified based on colonial morphological features and sugar fermentation characteristics (Ogbulie *et al*., 1998).

RESULTS AND DISCUSSION

The result of the morphological and biochemical characterization of the bacterial isolates in rainy season were recorded as *Micrococcus* spp., *Escherichia coli, Pediococcus* spp., *Citrobacter* spp., *Planococcus* spp., and *Flavobacterium* spp. respectively (Table 1). *Escherichia coli* was found in all the sampling stations during this period. The dry season showed a decrease in the number of bacterial genera obtained and *E.coli* was found in most of the sampling stations during this period (Table 2). The

fungal genera isolated were *Mucor* spp. and *Allescheria* spp. (moulds) and *Saccharomyces* sp. (Yeast) (Table 3 and 4). The presence of enteric pathogens in water constitutes health hazards and the presence of *E. coli* in both seasons indicated that there was recent contamination of faecal origin as at the time of sampling. The results of the total aerobic heterotrophic, coliform and *E.coli* counts of the sediment and water (Tables 5 and 6) showed that total higher aerobic and coliform counts were recorded in sediment than in that of water for both seasons. This could be attributed to the fact that a high percentage of microorganisms are attached to solid particles which provide protection against environmental factors and these microrganisms could settle down towards the segments faster than the planktonic ones (Fang *et al*., 2018). Environmental conditions in sediment are quite different than in the water column due to reduced sunlight, lower temperature fluctuation, protection against predators, increased nutrient organic carbon availability and more colonizable surfaces enhancing microorganisms' persistence and survival (Fang *et al*., 2018).The higher total aerobic, heterotrophic and coliform counts recorded in the rainy season indicated that run- offs from land increased the bacterial load of the surface water sources during this period (Okpokwasili and Akujobi, 1996). Floods and run-offs represent greater pollution sources than that of humans since the frequencies of human trips to the streams and rivers for the purpose of fetching water for domestic purposes decrease in rainy season (Okpokwasili and Akujobi, 1996). Individuals without pipe-borne water in their homes resort to rain water harvesting from rooftops during the season. The fact that water sampled were obtained at the areas of the streams from which users fetched their water meant that these were the points where humans made direct contact with the water sources. This falls in line with the reports of Blum *et al.* (1987) and Okpokwasili and Akujobi (1996). The selected surface waters are highly contaminated with pathogenic microorganisms with poor physicochemical characteristics and remains unsafe for human consumption. This necessitates the need for primary prevention measures to be identified in order to reduce health risks associated with exposures to pathogenic microorganisms in surface waters and sediments.

Table 1: Morphological and biochemical characteristics of bacterial isolates (Rainy season)

Isolates	Morphological Characteristics	Gram stain	Catalase	Voges proskauer	Methyl Red	Urease	Indole	Oxidase	Motility	Spore stain	Citrate	Glucose	Fructose	Manitol	Galactose	Identity
A	Smooth Sphercal Cells in pairs Clusters	+	+	+	+	+	+	+	+	-	+	**A**	**A**	**AG**	**AG**	***Micrococcus* spp.**
B	Moist small rods in Singles	-	+	-	+	+	+	-	-	-	+	**A**	**A**	**AG**	**AG**	***E. coli***
C	Small, Smooth Spherical cells in	+	-	-	+		+	+	-	-	+	**A**	**A**	-	-	***Pediococcus* spp.**
D	Smooth, Shiny Surface entire edge	-	+	-	+	+	+	-	+	+	+	**AG**	**AG**	**AG**	**AG**	***Citrobacter* spp.**
E	Smooth, Yellow, spherical Cells in pairs	+	+	-	-	+	-	-	+	-	+	**A**	**A**	-	**A**	***Planococcus* spp.**
F	Smooth, small Colonies, short Slender rods	-	+	-	-	-	+	-	-	-	+	**A**	-	**A**	**A**	***Flavobacterium* spp.**

Key: Source of Isolates, + Positive result A – 1b, 2a, 2b,3a,3b,4b, -Negative result B – 1a – 4b, A Acid Production C – 1b, 2b, 3b, 4b, G Gas production D – 1a, 2a, 2b, 3b, 4b, E – 1a, 1b, 2b, 3b, 4b, F – 1b, 2b, 3b, 4b

Table 2: Morphological and biochemical characteristics of bacterial isolates (Dry season)

Isolates	Morphological Characteristics	Gram stain	Catalase	Voges proskauer	Methyl Red	Urease	Indole	Oxidase	Motility	Spore stain	Citrate	Glucose	Fructose	Manitol	Galactose	Identity
G	**Smooth, Moist Shiny surface Entire edge, small Short rods in Single pairs**	**-**	**+**	**-**	**+**	**+**	**+**	**-**	**+**	**-**	**-**	**A**	**AG**	**AG**	**AG**	***E. coli***
H	**Transluscent Smooth Entire, small Colonies, short Slender rods**	**-**	**+**	**-**	**-**	**-**	**+**	**+**	**-**	**-**	**+**	**A**	**A**	**A**	**A**	***Flavobacterium* sp.**
I	**Yellow, Smooth Spherical cells in Singles and pairs**	**+**	**+**	**-**	**-**	**+**	**-**	**-**	**+**	**-**	**+**	**A**	**-**	**A**	**-**	***Planococcus* sp.**
J	**Small, Smooth Cells in pairs of Cocci**	**+**	**-**	**-**	**+**	**-**	**+**	**-**	**-**	**-**	**+**	**A**	**-**	**-**	**-**	***Pediococcus* sp.**

Key: Source of Isolates, + Positive result G – 1a, 1b, 2b,,3b, 4b, -Negative result H – 1a, 1b, 2a, 2b, 3b,4b, A Acid Production I – 3b, 4b, G Gas production J – 1a, 2a, 2b,3a, 3b, 4b.

Table 3: Morphological and sugar fermentation characteristics of yeast isolates (Rainy and Dry season)

Isolates	Colour	Elevation	Shape	Glucose	Fructose	Maltose	Mannitol	Lactose	Sucrose	Galactose	Identity
X	Milkish	Raised	Ovoid	A	A	A	A	A	A	A	*Saccharomyces* sp.
Y	Milkish	Raised	Ovoid	AG	AG	-	A	A	A	AG	*Saccharomyces* sp.

Key: Source of Isolates, -Negative result X – 1b, 2b, 4b, A Acid Production Y – 2b, 3b, G Gas production

Table 4: Mould isolates (Rainy and Dry season)

Isolates	Macroscopic Characteristics	Microscopic Characteristics	Probable Identity
1	Rapid growth spreads and Fills the plate, whitish gray And fluffy mycelium	Spores are borne within their fruiting bodies enclosed in sac-like structure sporangia	*Mucor* sp.
2	Soft, mouse-grey, flur like Colony	coenocytic hypha, spores borne singly on branched conidiophores with each conidiophores ending in a single conidium	*Allescheria* sp.

Key to source of Isolates: Isolate 1- Both seasons, Isolate 2- Majorly in rainy season except one sample site

Table 5: Total aerobic heterotrophic coliform and *E. coli* counts of the sediment and water (Rainy season)

Sampling Stations	Total aerobic bacterial Count ($\times 10^3$ cfu/ml)	Total coliform count ($\times 10^3$ cfu/ml)	*E. coli* count (cfu/100ml)
1a	72	5	27
1b	36	2	20
2a	85	25	33
2b	54	6	15
3a	223	31	21
3b	105	17	10
4a	133	45	24
4b	69	19	13

Table 6: Total aerobic heterotrophic coliform and *E. coli* counts of the sediment and water (Dry season)

Sampling Stations	Total aerobic bacterial Count ($\times 10^3$ cfu/ml)	Total coliform count ($\times 10^3$ cfu/ml)	*E. coli* count (cfu/100ml)
1a	46	10	15
1b	32	4	8
2a	57	15	-
2b	31	10	6
3a	174	20	-
3b	55	12	4
4a	210	32	-
4b	62	14	10

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Heavy Metals Sludge from Septic Tank Sewage: Implication for Use as Fertilizer

Oyem I. M.[1*] and Oyem H. H.[2]
1. Department of Biological Sciences University of Delta, PMB 2090, Agbor Delta State, Nigeria.
2. Department of Chemical Sciences, University of Delta, PMB 2090, Agbor Delta State, Nigeria.
* Corresponding author: mirian.oyem@unidel.edu.ng

Abstract: Sludge contains nutrients, heavy metals, and metalloids some of which are essential macronutrients, such as N, P, K, Ca, Mn and other essential trace elements, such as Cu, Fe and Zn. This paper examines the heavy metals within the septic tank system and their eventual accumulation as components of sludge with implication for agricultural use. Sludge samples from the inlet and outlet chambers of the septic tank were randomly collected from three study locations in the south-south region of Nigeria. The composite samples were made for each of these study locations from which nine trace metals (Fe, Zn, Mn, Pb, Cu, Cr, Cd, Ni and Va) were analysed. The concentrations of the metals were determined using standard methods for water and wastewater examination. Results showed that sludge samples from the anaerobic bioreactors contained a high concentration of various trace metals. Iron had the highest mean concentration level of 556 ± 0.2 mg/kg – 3391 ± 1.13 in the raw sewage sludge sample, while vanadium was the least in abundance with mean concentrations of 0.86, 0.77, and 1.52 mg/kg in locations A, B and C respectively in the inlet chamber sludge samples. Although, sludge is variously used for agricultural purpose, there is need for treatment to reduce the concentration levels of heavy metals prior to its use as fertilizer as a matter of public health importance.
Key word: Fertilizer, food chain, heavy metals, septic tank, sewage sludge

INTRODUCTION

Heavy metals are elements whose densities are high typically above 5 g/cm^3 (Hawkes, 1997; Chu, 2018; Ali *et al.*, 2019; Briffa *et al.*, 2020; Sayo *et al.*, 2020). Examples of heavy metal elements include lead, zinc, chromium, cadmium, arsenic, mercury, vanadium, manganese, and iron. These metals are found in the environment from geogenic and anthropogenic sources (Tchounwou *et al.*, 2012; Ibrahim and Ibrahim, 2016). The metals exist in the air, surface and groundwater, and soil. Thus, can be transported through the soil to plants and up the food chain to man (Nkwunonwo *et al.*, 2020), as well as through air and water pollution. Man is the major receiver of these metals in the environment from the uptake of these heavy metals through plants, surface and groundwater, and airborne pollutants (He *et al.*, 2005; Usman *et al.*, 2020). This makes man a veritable bio-accumulator of these heavy metals which eventually arrive at the septic tank.

Septic tanks are digestion chambers where anaerobic bacteria breakdown organic matter from human domestic activities is treated in a two-step process effected by acid- and methane-forming microorganisms (Abatenh *et al.*, 2017). The process of microbial remediation of heavy metals in the sewage of septic tank systems is dependent on the pH of the system (Ademoroti, 1996; Abatenh *et al.*, 2017). This therefore, determines the threshold and balance of equilibrium between acid-loving bacteria (acidophiles) and bacteria that thrive in alkaline environment (methanogens). Bio-precipitation of trace metals onto cell surfaces by microbes is favoured by acidic conditions (Ademoroti, 1996; Canovas *et al.*, 2003; Jin *et al.*, 2004; Yuncu *et al.*, 2006; Hassan *et al.*, 2009; Igiri *et al.*, 2018; Osman *et al.*, 2019; Rose *et al.*, 2019; Delangiz *et al.*, 2020;). By convention, septic tank systems are designed to have two chambers; that is, the raw (inlet) chamber and the semi-treated (outlet) chamber. Hence, the components of the septic tank be classified into suspended solids, effluent and sludge.

For septic tank to work optimally, it should be evacuated every 3 to 5 years, however, inspection should be conducted biannually to confirm that the system is working optimally, and that there are no leaks. It is

also necessary to check that the levels of sludge and scum in the tank has not risen drastically (USEPA, 1997) that would necessitate evacuation should it exceed 25 percent of the working liquid capacity of the tank or the level of the layer of scum is within three inches of the bottom of the outlet baffle (USEPA, 1994). These situations make the sludge readily available for utilization for agricultural purpose as organic fertilizers.

Sludge is the visco-solid component of sewage which is about 57.0 per cent water (Ademoroti, 1996). The precipitation of both the solvated and insoluble substances leads to the development of sludge (Diep *et al.*, 2018; Zhang *et al.*, 2021). Sludge is composed of proteins, carbohydrates, pathogens and heavy metals (Zhang *et al.*, 2021). Even though sewage sludge contain a deluge of plant nutrients, the heavy metals content can be a problem (Oudeh, 2002). Therefore, there is a need to treat accumulated sludge in a wastewater before safe disposal especially into water bodies, which is usually the practice. The essence of treating sludge is to reduce the amount of organic matter, potentially toxic heavy metals and the population of disease-causing microorganisms. The most common treatment options include anaerobic digestion. Others, include aerobic digestion and composting. Treated sludge can be disposed off or utilized as fertilizers for agricultural land, providing cheap manure for farmers (El sokkarry, 1993). Waste water sludge is a useful source of nitrogen, phosphorus and organic matter. However, there are limits to what can be applied. For example application of waste water sludge should not exceed 250 kg nitrogen per hectare per annum, particular within nitrate vulnerable zones (EEC, 1986; USEPA, 1997).

Fertilizers are substances which are formulated to aid plant growth, where the normal soil may lack sufficient nutrient to support plants growth and development. Application of inorganic fertilizer to crops has become difficult owing to its expensive nature and sometimes unavailability. Sludge therefore, provides cheap and readily available means of supplying essential nutrients to plants – Green Science. However, as beneficial as the use of sludge as fertilizer is, the process is frost with some misgivings as waste water sludge contains large concentration of heavy metal than that present in most soils (Gomez-Canela *et al.,* 2012). Once applied, the heavy metals will accumulate in the top soil until it is picked up by plants.

However, the case is being made by green environmentalists for organic fertilizers to make up the heavier source of fertilizers use by farmers. Reasons given for this include, the advantage organic fertilizers have in terms of their composition apart from it having a natural source. Manure as organic fertilizers as often called, possess minerals, organic matter, moisture content, and microbes, some of which could be beneficial to the soil and plant eventually, unlike the inorganic fertilizers whose components are hewn out of source rocks and lack the same compositions as organic fertilizers (Zhou *et al*., 2022). In spite of the fact that during the formation of manures by microbes, methane gas (a greenhouse gas) is formed which adds to the burden of global greenhouse atmospheric load, manure from septic tanks – a pH controlled anaerobic digester can mitigate this problem (Oyem *et al*., 2022), while providing plant with the requisite nutrients via sludge. This paper focused on ascertaining the levels of heavy metals present in septic tank sludge and the implications of using sludge as fertilizers.

MATERIALS AND METHODS

Sample area: Sludge samples for analysis were collected from different septic tanks in three locations in Agbor in Delta State, Nigeria. Agbor has a population of 162,594 (National Bureau of Statistics, 1977), with an area covering 436 sq. kilometres.

Sample collection: Sludge samples from septic tank sewage were collected from three locations in Agbor in 1 L plastic bottles. Samples were immediately taken to the

laboratory in cool ice packs. Samples were then made into a composite sample for analyses using the APHA (1995) method of wastewater analysis.

Heavy metal analyses: Heavy metals ions present in the sample were analysed using Atomic Absorption Spectrometry (AAS) instrument, model EDX 800, manufactured in Japan. The source of radiation used was a Hallow lamp which emitted the wavelength characteristics of the metal, using a different lamp source for each metal and directed through a flame into a monochromator that selected the preferred wavelength. A photomultiplier tube was the detector and converted the incident signal into an electric signal. The metals were identified at varying wavelengths and current. In all, nine (9) heavy metal ions were analysed and values obtained were recorded.

RESULTS AND DISCUSSION

Iron recorded the highest concentration of 1410 mg/kg and 615 mg/kg in both the inlet and outlet chambers of the septic tanks sampled in the immediate locality. This would be expected for a region known for its high Fe content from its geology (Avwunudiogba, 2000). The soil is characteristically reddish brown in colour and clearly depicts high iron content. One would therefore expect a high Fe content in this environment, especially in the plants cultivated on this soil and its groundwater. It is therefore not surprising that the difference between the Fe content and other heavy metal ions is significantly high.

Zinc had the second highest heavy metal content of the nine analysed in the sludge component of the study area after iron. Zinc is a trace metal needed in the body's biochemistry, it is necessary for certain important functions of the body (Roohani et al., 2013). Its presence in septic tank to this extent may not be associated entirely to diet and supplements but, also through groundwater (Oyem *et al*., 2015) from ancient corrugated zinc roofing sheets as well as zinc oxide in most ointments, creams, lotions, shampoos and antiseptics (NCBI, 2017). Decreased levels of Zn in the semi-treated sludge samples compared to the raw sewage samples was observed. These findings are in tandem with reports by Duan *et al*. (2017); Steinhardt and Egler (2018) and Agoro *et al*. (2020), who noted that sludge was a rich deposit of toxic metals and though were within normal range yet in agricultural soils may be a potential risk for human safety.

Manganese concentration in sludge sample analysed was 45.8 mg/kg in the inlet chamber and 17.3 mg/kg in the outlet chamber. These values were the third highest in the study's findings, translating to a significant proportion of the heavy metal load in the environment, a similar pattern was observed as in Zinc.

The sludge sample in the inlet chambers had Cu levels of 13.2 mg/kg and 5.38 mg/kg in the outlet chamber. The Cu is a component of some enzymes in the body besides being ubiquitous as component of many electrical appliances (Mydy *et al*., 2021). Although, naturally a trace metal, its presence in sludge to this concentration is telling and significant.

Chromium in this study represented as total chromium had a slightly significant value of 1.50 mg/kg and 1.20 mg/kg in both chambers of the septic tanks respectively. Chromium in the environment is treated with concern because of it deleterious effect on health (Oyem *et al*., 2014; Georgaki and Charalambous, 2022). Although, the values recorded in this study is benign, it is nonetheless considerable.

The concentration of cadmium in the study for the inlet and outlet chambers were 0.02 mg/kg and 0.01 mg/kg. Cadmium presence is often associated with man's industrial activities, and so in areas were Cd concentration is significant, anthropological sources are readily adduced (Singh and Liu, 2024). In this study, Cd concentration is not significant, and this signifies non-industrial activities in the area.

In the inlet chambers' sludge samples ranged from 0.35 to 0.90 mg/kg in the septic tank sewage in the area. Again, these values are

low and do not depict pollution of the area arising from industrial activities (Jakubus and Graczyk, 2020).

In sludge sample Pb concentrations were slightly significant but, not suggestive of serious pollution. The Pb toxicity is treated with serious concern because of its effect on humans. The Pb causes a damage to the central nervous system (WHO, 2023). Therefore, Pb values of 0.90 and 0.50 mg/kg in the inlet and outlet chambers is of slight concern.

Vanadium was the least abundant heavy metal analysed in this study. Vanadium is present in the inlet sludge samples in concentrations of 0.50 mg/kg and 0.30 mg/kg in the study. Vanadium in this area can be traced to the activities of auto mechanics which are present in the area (Wnuk, 2023).

The main risk related to agricultural use of sewage sludge are the potential presence of heavy metals, pathogens and pollutants enrichment in soil, plants and animal pastures and the subsequent entry into the food chain (Ternes *et al*., 2004 and Gomez-Canela *et al.,* 2012).

Heavy metals, pathogens and organic pollutants can also affect soil functioning and biodiversity (Harrison *et al*., 2006 and Roig *et al*., 2012). According to literature, low application doses of sludge did not cause a significant increase in the heavy metal concentration in soil (Singh and Agrawal, 2008). On the contrary, low metal sludge has beneficial effect on microbial biomass, organic carbon and on soil microbial activity (Usman *et al*., 2012). Excessive application of sewage sludge to soil has been found to increase the bioavailability of heavy metals that have a negative effect on soil (Singh and Agrawal, 2008 and Usman *et al*., 2012). However, the option of heavy metal removal using genetically engineered microorganisms has been explored in a recent study (Diep *et al*., 2018).

Table 1: Heavy metals concentrations (mg/kg) in both chambers of the septic tanks in the study locations

S/N	Metal ion (Analyte)	Inlet chamber (mg/kg)	Outlet chamber (mg/kg)
1.	Fe	1410 ±0.20	615 ±2.00
2.	Zn	110 ±0.20	66.2 ±0.20
3.	Mn	45.8 ±0.20	17.3±0.10
4.	Cu	13.2 ±0.20	5.38 ±0.20
5.	Cr	1.50 ±0.02	1.20 ±0.02
6.	Cd	0.02 ±0.01	0.01±0.05
7.	Ni	0.90 ±0.20	0.35 ±0.01
8.	Pb	0.70 ±0.01	0.50 ±0.02
9.	V	0.50 ±0.02	0.30 ±0.02

CONCLUSION

Heavy metals distribution in the sludge components of septic tank systems was studied to understand their composition within the system. Results showed that sludge samples from the anaerobic bioreactor contained a high concentration of various trace metals. This has high implications for the use of sludge as compost material in Agriculture. Although sewage sludge is a good source of nutrients for plant growth, the presence of heavy metals in sludge can reduce its use. However, the heavy metals found in this study are not those with great health implications. That notwithstanding, the need to ensure proper treatment of sludge prior to its use as fertilizer is a matter of public health importance.

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Evaluation of Probiotic Properties of Lactic Acid Bacteria Isolated from Some Fermented Foods

Abdulsalam R.[1*] Bala J. D.[1] Abalaka M. E.[1] and Ocheme O. B.[2]
1. Department of Microbiology, Federal University of Technology, Minna, Niger State, Nigeria
2. Department of Food Science Technology, Federal University of Technology, Minna, Niger State, Nigeria
* Corresponding author: a.ramatu@futminna.edu.ng

Abstract: Probiotics are live microorganisms which when consumed in adequate quantity; promote the health of the consumer. This study was carried out to evaluate probiotic properties of lactic acid bacteria (LAB) isolated from fermented foods (wara, ogi, orange juice, pickles, sauerkraut and grape juice). A total of eighty four isolates were identified as LAB species by morphological, Gram staining and short biochemical tests. All isolates were characterized for probiotic properties including NaCl tolerance, bile tolerance, sugar fermentation, proteolytic activity, acid tolerance, antibiotic susceptability assay and adherance to stainless plates. All isolates survived in 2%, 4%, 6.5% and 8% NaCl concentrations. Four isolates were tolerance to pH 1.5, 2.0, and 3.0 for 24 and 48 hours. The four acid tolerant isolates were found to tolerant 0.3% bile salt for 24 hours with 85 to 99% rate of survival. Results of fermentation test showed that most isolates fermented all sugars. All strains digested casein by producing protease enzyme in skim milk agar plate. All the four isolates were found inhibiting some pathogenic bacteria to varying degrees and also displayed varying susceptibility to different antibiotics. The *in vitro* adherence to stainless steel plates of the 4 screened isolates were ranged from 32.83 to 37.70% adhesion rate. The phylogenetic analysis and the 16S rDNA sequencing assigned all the four efficient LAB isolates with probiotic properties to genus *Lactobacillus, Leuconostoc* and *Weissella* and were identified as *Lactobacillus plantarum*, *Lactobacillus plantarum, Leuconostoc mesenteriodes* and *Weissella paramesenteroides.* The four LAB strains were found to be potentially useful to produce probiotic products. Further study is needed to find specific probiotics with specific benefit from fermented foods.
Key word: Probiotic, Pickles, Wara, Acid tolerance*, Lactobacillus plantarum.*

INTRODUCTION

Probiotics are live microorganisms that confer health benefit to human when administered in adequate quantity, generally by improving or restoring the gut microflora (Maftei *et al.,* 2024). Probiotics seem to function by influencing both intestinal epithelial and immune cells of the gut, but the details of these effects are still being unraveled (Sudan *et al.,* 2022). Therefore, probiotics, through their effects on the host immune system, might ameliorate diseases triggered by disordered immune responses. However, the beneficial effects of probiotics can vary between strains; therefore the selection of most suitable ones is crucial for their use in the prevention or treatment of specific diseases (Han, *et al.,* 2024). In order to exert their functional properties, probiotics need to be delivered to the desired sites in an active and viable form (Rabetafika *et al.,* 2023).
Probiotics effects can be strain specific, but may have more than one health benefits depending on their delivery method, host response or interaction with other microbes (Zommiti *et al.,* 2020). This study aims to evaluate the *in vitro* probiotic properties of lactic acid bacteria (LAB) isolated from fermented foods products wara, ogi, orange wine, pickles, sauerkraut and grape wine with respect to their potential probiotic properties against some pathogenic bacteria.

MATERIALS AND METHODS

Production of the fermented food Wara (Cheese): The sodom apple (*Calotropis procera*) stem were weighed and carefully washed with distilled water and sliced with a sterile knife. The sliced stem was manually mixed with disilled water in order for the extract to be collected. The mixture was further sieved with a sterile sieve cup to collect the extract. Fifty millilitres (50 ml) of the extract was added to the 1 L raw milk and mixed manually. The mixture was sieved to remove the sheaves and was transfer in a metallic pot place on hot plates,

heating was carried at a regulated temperature of 68 ^oC for 20 min. After the heating clotting occurs, the resulting mixture was cooled to renneting temperature of 31 ^oC. The curd was separated from the whey (noncoagulated part of the mixture) using a decontaminated sieve cup (Ogunlade, 2019).

Sauerkraut: The spotted and defective cabbage heads were trimed off. Cabbage was prepared by removing the outer leaves and core. The cabbage was shredded with a sterile knife into 2 mm x 10 mm. The shredded cabbages were weighed (1 kg) followed by the addition of 1.5 % non-iodized sodium chloride. The shredded cabbage salt was placed in alternating layers in a sterile glass jar. Pressure was applied to gently squeeze out the brine and 100 ml of water was added. The glass jar was covered with sterile lids and incubated at 27^oC for 7 days for the natural fermentation of the cabbage. During fermentation process the lactic acid bacteria were isolated; temperature and pH of the fermentation medium were recorded for confirmation of ideal process at 12 hrs interval for 7 days (Bhosale and Sapre, 2021).

Orange juice: The fruits were washed, manually peeled, cut into halves with sterile knife using hand gloves and their seeds removed. The cut oranges (mesocarp) were pressed with a hand juicer squeezer to extract the juice. The juice and pulp obtained were homogenized (blended) in a sterile hand Monilex blender. The homogenate was clarified manually using a sterile muslin cloth to obtain a clear juice During fermentation process the lactic acid bacteria were isolated, temperature and pH of the fermentation medium were recorded for confirmation of ideal process at 12 hrs interval for 7 days (Abdulla *et al.,* 2021).

Pickles: Cucumber was washed under running water.The cucumber was shredded with a sterile knife into 5 mm x 10 mm. The shredded cucumber were weighed (1 kg) followed by the addition of 1.5 % non-iodized sodium chloride. The shredded cucumber salt was placed in alternating layers in a sterile glass jar. Pressure was applied to gently squeeze out the brine and water was added (Zeng *et al.,* 2020). The glass jar was covered with sterile lids and incubated at room temperature for 7 days for the natural fermentation of the cucumber. During fermentation process the lactic acid bacteria were isolated; temperature and pH of the fermentation medium were recorded for confirmation of ideal process at 12 hrs interval for 7 days.

Ogi: Ogi was produced from sorghum by adapting the method described by Eke-Ejiofor, (2018) and Banwo**,** *et al.* (2022). The sorghum grain was cleaned by hand to remove dirt like stones and chaff. Five hundred gram (500g) of cereal was steeped in 2 liters of distilled water in a sterile plastic buckets and allowed to ferment for 2 days at room temperature. The fermented grain was separated from the steeping water by decanting. Afterwards, the grain were wet milled using a blender and the resulting slurry passed through a sterile muslin cloth, and washed with excess water. The by-products in the muslin cloth were discarded, and the starch in the bucket were covered with muslin cloth and allowed to settle at 27^oC. The sieved starch was allowed to sediment and ferment naturally. During fermentation process the lactic acid bacteria were isolated; temperature and pH of the fermentation medium were recorded for confirmation of ideal process at 12 hrs interval for 7 days.

Biochemical characterization of LAB: The isolated bacteria were characterized biochemically with Gram staining, catalase, indole, methyl- red, motility, citrate utilization test, following the procedures as explained by Erdoğmuş *et al.* (2021) and Vasiee *et al.* (2022).

Growth at different NaCl concentrations: The LAB isolates were tested for their tolerance different NaCl concentrations. For this purpose, 2%, 4%, 6.5% and 8% NaCl concentrations were used for testing. Similarly, test tubes with 5 ml of modified MRS broth containing bromecresol purple indicator were prepared according to the appropriate concentrations and were

inoculated separately with 50 µl of 1% of each overnight culture of LAB and incubated at 37°C for 48 h. The change of the color from purple to yellow was considered as proof of cell growth (Mulaw *et al.,* 2019).

Low pH tolerance test of LAB: The LAB were grown separately overnight in 5 ml MRS broth at 37°C under anaerobic conditions. A volume of 1 ml of 10^7 cfu/ml of each overnight-grown culture was inoculated into 10 ml of MRS broth to give an initial inoculum level of 10^6 cfu/ml. The culture was then centrifuged at 5000 rpm for 10 min at +4°C. The pellets were washed twice in phosphate buffer (pH 7.2). The pellets were resuspended in 5 ml MRS broth which was adjusted to pH values of 1.5, 2.0 and 3.0 using 1 N·HCl to simulate the gastric environment. The test tubes were incubated at 37°C for 24 and 48 hours. After an appropriate incubation period, 1 ml of the culture was diluted in sterile 9 ml phosphate buffer prepared according to the manufacturer's instruction (0.1 M, pH 6.2) in order to neutralize the medium acidity. Briefly, a 100 µl aliquot of the culture and its 10-fold serial dilutions were plated on the MRS agar medium. The inoculated plates were incubated at 37°C for 24 to 48 h under anaerobic condition using an anaerobic jar and the grown LAB colonies were expressed as colony-forming units per milliliter (cfu/ml). A positive control consisting of regular MRS broth inoculated with the culture was used (Gheziel *et al.* 2019). The survival rate was calculated as the percentage of LAB colonies grown on MRS agar compared to the initial bacterial concentration:

$$\text{Survival rate (\%)} = \frac{\text{Log CFN}_1}{\text{Log CFN}_0} \text{ X } 100$$

Where N_1 is the viable count of isolates after incubation and N_0 is the initial viable count

Tolerance to bile salts: To estimate bile tolerance of acid-tolerant LAB, the isolates were separately grown overnight in MRS broth at 37°C under anaerobic conditions. Each culture with the initial concentration of 10^6cfu/ml was then centrifuged at 5000 rpm for 10 min at 4°C. The pellets were washed twice in the phosphate-saline buffer (PBS at pH 7.2). Cell pellets were resuspended in MRS broth supplemented with 0.3% (w/v) bile salt. Samples were taken at 24 h from the onset of incubation to determine the survivability of cells as (Mulaw *et al.,* 2019), positive control consisting of plain MRS broth without bile salts inoculated with each separate culture was simultaneously set up. After appropriate incubation, 1 ml of each separate culture was diluted separately in sterile 9 ml phosphate buffer (0.1 M, pH 6.2) in order to neutralize the medium. Concisely, a 100 µl aliquot of the culture and its 10-fold serial dilutions were plated on MRS agar medium. Plates were incubated at 37°C for 24 to 48 h under anaerobic condition using an anaerobic jar. The survival rate was calculated as the percentage of LAB colonies grown on MRS agar compared to the initial bacterial concentration:

$$\text{Survival rate (\%)} = \frac{\text{Log CFN}_1}{\text{Log CFN}_0} \text{ X } 100$$

Where N_1 is the viable count of isolates after incubation and N_0 is the initial viable count

Sugar fermentation test: The ability of the isolates to ferment various sugars was determined using 1g sugar in 100ml MRS broth. (Glucose, Sucrose, Fructose, Lactose, Mannitol, xylose and sorbitol were used). Two drops of phenol red was used as indicator. Ten millilitre (10ml) of media was dispensed into a testtube and Durham's tube was inverted into each testtube. Isolates were inoculated and incubated at 37°C for 24 h and 1 testtube of each fermentation broth was kept uninoculated as a negative control and setup was observed for colour change and gas formation (Vasiee *et al.,* 2022).

Casein digestion test: The protease activity was performed using MRS agar plate containing 1% skim milk solution. Bacterial cultures were inoculated and incubated for 48h at 37°C. Clear zones around the cultures indicated protease activity (Gao *et al*, 2022). *Pseudomonas* spp. and *Klebsiella* spp. were

used as positive and negative control, respectively.

Antimicrobial activity against bacteria pathogens: Antibacterial activity of the acid-bile-tolerant LAB strains against some pathogens was determined using the agar-well diffusion method with some modifications of the protocol indicated by Zapa´snik *et al*. (2022). The selected acid-tolerant LAB isolates were inoculated from slants to fresh MRS broth containing 1% glucose and incubated overnight at 37°C. The overnight active culture broth of each isolate was centrifuged separately at 5000 rpm for 10 min at 4° C. The cell-free supernatant from each separate culture was collected as a crude extract for the antagonistic study against some food-borne pathogens. The pure cultures of pathogenic bacteria were inoculated from slants to brain heart infusion broth. After 24-hour incubation at 37°C, a volume of 100 µl of inoculum of each test bacteria was swabbed evenly over the surface of nutrient agar plates with a sterile cotton swab. The plates were allowed to dry, and a sterile cork borer (diameter 5 mm) was used to cut uniform wells in the agar. Each well was filled with 100 µl culture-free filtrate obtained from each of the acid-bile-tolerant LAB isolates. After incubation at 37°C for 24 to 48 hours, the plates were observed for a zone of inhibition (ZOI) around the well. The diameter of the zone of inhibition was measured by calipers in millimeters, and a clear zone of 1 mm or more was considered positive inhibition (Benkova *et al.,* 2020).

Bacterial adhesion to stainless steel plates: The adherence assay of the lactic acid bacterial isolates was determined on stainless steel plates with some modifications by Mulaw et al. (2019). Lactic acid bacteria were cultured in sterile MRS broth. Thereafter, the overnight bacterial culture (500 µl) was deposited in a test tube, which was then filled with 450 µl of MRS broth, wherein the sterile stainless steel plate was deposited, and the test tubes were then incubated for 24 h at 37°C. The stainless steel plate was removed under aseptic conditions, washed with 10 ml of sterile 1% peptone water, and left for 5 min in a sterile 1% peptone water tube. The plate was then washed again in the same conditions and vortexed for 3 min in a sterile 1% peptone water tube (6 ml) consecutively to detach the bacterial cells adhering to the steel plate surface. The cell number was determined by counting on MRS agar after 24 h of incubation at 37°C. Simultaneously, the total initial cell numbers were estimated to calculate the percentage of adhered bacterial cells for each LAB.

Antibiotic susceptibility assay: Each of the acid -tolerant and antagonistic lactic acid bacteria isolates was assessed for its antibiotic resistance by the disc diffusion method against some antibiotics (ciprofloxacin, norfloxacin, chloramphenicol, erythromycin, gentamycin, ampiclox, amoxil, streptomycin, rifampicin, levofloxacin). In this case, a volume of 100 µl of actively growing cultures of each acid-tolerant and antagonistic lactic acid bacteria was swabbed evenly over the surface of nutrient agar plates with a sterile cotton swab. After drying, the antibiotic discs were placed on the solidified agar surface, and the plates were left aside for 30 min at 4° C for the diffusion of antibiotics and then anaerobically incubated at 37°C for 24 to 48 h. Resistance was defined according to the disc diffusion method by using the above antibiotic discs, and the diameters of zones of inhibition were measured using calipers (Khan *et al.,* 2019), the zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S (≥21 mm); intermediate, I (16– 20 mm), and resistance, R (≤15 mm) (Gheziel *et al.* 2019).

Extraction of genomic DNA of the LAB: Single colonies grown on medium were transferred to 1.5 ml of liquid medium grown on a shaker for 48 h at 28 ºC and centrifuged at 4600g for 5 min. The resulting pellets were resuspended in 520 µl of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then

added (Adebowale *et al.,* 2021). The mixture was incubated for 1 hour at 37 ºC, then 100 µl of 5 M NaCl and 80 µL of a 10% CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 h. The DNA was collected by centrifugation at 13000g for 10 min, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours till ethanol drops disappeared completely. The dried sediment was dissolved in 30 µl TE buffer.

Polymerase chain reaction (PCR) of 16S rDNA: Polymerase chain reaction amplification of the extracted DNA was carried out with the 16S primer in a total volume of 25 µl containing 100mg of genomic DNA. Two point five microlitre (2.5 µl) of 10 x PCR buffer, 1 µl of 50mM $MgCl_2$, 2 µl of 2.5mM dNTPs (Thermo Scientific), 0.1 µl Taq polymerase (Thermo Scientific). One microlitre (1 µl) of DMSO, 1 µl each of forward and reverse primers and 11.3 µl H_2O. Touch-down PCR was used for amplification as follows: Initial denaturation step of 5 minutes at 94 ºC, follow by 9 cycles each consisting of a denaturation step of 20 seconds at 94 ºC, annealing step of 30 seconds at 65 ºC, and an extention step of 72 ºC for 45 seconds. This was followed by another 30 cycles each consisting of a denaturation step of 20 seconds at 94 ºC, annealing step of 30 seconds at 55ºC, and an extention step of 72ºC for 45 seconds. All amplification reactions were performed in a GeneAmp@ PCR System 9700, Applied Biosystems. Polymerase chain reaction amplicons were loaded on 1.5% agarose gel and run at 100volts for 2 hours (Lee, *et al.,* 2023).

DNA Sequencing: The amplicons were selected from the amplified products and purified using manufacturer's protocol, sequencing was performed using a big dye terminator cycle sequencing kit (Applied ioSystems), Unincorporated dye terminators were then purified and precipitated using ethanol EDTA solution. The pellets were then resolved in HiDi formamide buffer. Sequencing was performed using 3130 x 1 Genetic Analyser. The resulting pattern was then compared with the 16s rRNA nucleotide sequences present in BLAST tool of Genbank at NCBI (Sharma *et al.,* 2020).

RESULTS AND DISCUSSION

All the selected four potential probiotic bacteria were identified as LAB on the basis of their morphological, biochemical, and physiological characteristics, catalase negative and Gram positive rod. All tested isolates grows at 15°C, 37°C, 45°C and tolerate 2%, 4%, 6.5% and 8% NaCl concentration (Table 1). These findings were consistent with a previous study of Goa *et al.* (2022), testing the abilities of isolates growing at 15° C and 45°C.

Sugar fermentation and protease activity tests of the isolates

The isolates exhibied both heterofermentative and homofermentative types (Table 2) this finding is in accordance with Mulaw *et al.* (2019) who isolated Lactobacillus species from Ergo and found all isolates were grouped as homofermentative and heterofermentative types. The four selected isolates fermented and produce gas from glucose. Isolates CA4 fermented fructose, sucrose, manitol, sobitol and xylose except for lactose. Isolates NO5, W6 and W7 fermented all the sugars except for xylose. Isolates NO5, W6 and W7 would be useful for lactose intolerant people who cannot metabolize lactose due to lack of β-galactosidase enzyme. Lactose and dextrose utilization by LAB was confirmed by lactose and glucose utilization test. Lactic acid bacteia digests casein in order to grow in milk and subsequently utilize the degradation products (Aimee, 2022). In this present study, all the isolated strains were found to digest casein indicating LAB produced protease enzyme. Raveschot *et al.*

(2020) reported in their study that, Lactobacilli species utilized casein by proteolytic activity.

pH tolerance and bile salt tolerance tests of the isolated LAB

Out of 84 isolates, 6 isolates (7.14%), 12 isolates (14.28%), and 14 isolates (16.67%) tolerated pH values of 1.5, 2 and 3 for 24h, respectively. Upon further extension of the incubation period to 48 h, 5 and 8 isolates (tested at pH 1.5 and 2) survived, while 11 survived from 14 isolates with the extension of incubation period to 48 h at pH 3.0 (Table 3). Therefore, out of the total 84 LAB isolates, 4 (4.76%) isolates survived pH 1.5 upon exposure for 24 and 48 hours, and the mean value of the treatments was significantly different at $p < 0.05$ (Table 4). Among the fou selected isolates, 1 (25%) was isolated each from cabbage and ogi sample and 2 (50%) were isolated from wara. The survival rate of the isolates was ranged from 41.46 to 90.53% at different pH values for 24 and 48h incubation periods (Table 4). Isolates, like W6 and W7 were found highly tolerant and persisted above 50% for both 24 and 48h at pH 1.5. Isolate Ca4 and O5 upon exposure to pH 1.5 could not grow above 50%. However, incubation at low pH resulted in a significant decrease in the survival rate of all LAB isolates as reported in another study by Das *et al.* (2020) the authors observed that the viable counts of all lactic acid bacteria were significantly affected by low acidity, especially at pH 2.0.

The four acid tolerant LAB isolates showed high tolerance to bile salt conditions (85% to 95%) (Table 4). Similar to the present findings, the results in other studies have revealed that all the isolated strains displayed high tolerance to bile salt conditions and the survival rates of Lactobacillus strains ranged from 88% to 92% (Haghshenas *et al.,* 2023). In a related study, Akalu, *et al.* (2017) have also shown that out of the 30 tested LAB isolates, 17 Lactobacillus isolates obtained from Ethiopian traditionally fermented Shamita and Kocho showed remarkably high tolerance to an environment containing 0.3% bile salt. Inaddition, Biswal, *et al.* (2021) has reported that all of the LAB isolates demonstrated a high level of tolerance to bile salts by displaying surviving percentage above 50% on exposed to 0.3% bile salts after 24 h at 37°C.

Antimicrobial activities of the isolated LAB

The selected four potential probiotic lactic acid bacterial (W7, CA4, W6 and O5) exhibited varying degree of antagonism against *Staphylococcus aureus, Escherichia coli, Salmonella typimurium,* and *P. aeruginosa* (Table 5). All the selected potential probiotic LAB strains exhibited strong antimicrobial activity against the pathogens. Isolate O5 displayed the highest antagonistic activity against *E. coli*, *S. typhimurium* and *P. aeruginosa* with the zone of inhibition ranged from 20.08 to 20.85 mm in diameters, also isolates W7, CA4, W6 and O5 displayed highest antagonistic activity against *Staphylococcus aureus*, *E. coli, Salmonella typhi* and *P. aeruginosa* with the zone of inhibition ranged of 21.1mm, 21.00mm, 21.00 mm and 21.40 mm diameters respectively. In agreement to this study, Dejene *et al.* (2021) have verified that all the LAB isolates originated from Borde and finfish belonging to the genera *Lactobacillus,* found to inhibit the growth of the test strains such as *S. aureus, Salmonella spp.,* and *E. coli* with zone of inhibition that ranged from 15 to 17 mm in diameters. In addition, Gheziel *et al.* (2019) had demonstrated that 6 *Lactobacillus plantarum* strains isolated from faecal samples exposed high antibacterial activity against potential foodborne pathogens *E. coli* and *Staph. aureus*. Most LAB strains showed inhibitory activity against the growth pathogenic microorganisms either by competing with pathogenic bacteria for food or by producing antimicrobial compounds such as hydrogen peroxide, diacetyl, bacteriocins, naturally protective organic acids, and specific substances, such as antiviral peptides or low-molecular-weight peptides (Souza, 2021).

Among the main important characteristics of probiotic bacteria, adhesion to the intestinal mucosa is required. The screened probiotic LAB isolates possesses *in vitro* adherence property to stainless steel plates with the adhesion rate ranged from 32.83 to 37.70%. Isolate CA4 showed the highest (37.70%) adherence rates. However, isolate O5 showed the least 32.83% adherence rate (Table 6). In agreement with this study, Mulaw *et al.* (2019) have reported that the adhesion rate of lactic acid bacteria to stainless steel plates ranged from 32.75 to 36.30%, El-Jeni *et al.* (2020) have also revealed that the adhesion rate of lactic acid bacteria to stainless steel plates ranged from 32 to 35%.Generally, this suggests that the LAB isolates have a potential capacity to colonize the gastrointestinal (GI) tract mucosa.

Antibiotic susceptibility profile of the isolated LAB

All of the tested four Lactobacillus strains were found to be resistant to all the antibiotcs. However, CA4 and O5 are susceptible and intemediate to streptomycin and levofloxcin respectively but displayed resistance to other antibiotics (Table 6). These findings were in agreement with the report obtained by Yao *et al.* (2022), that isolates of LAB from curd were found to be resistant to erythromycin, streptomycin, gentamycin, ciprofloxacin and Norfloxacin. Similarly, Ferdouse *et al.* (2023) have reported that out of 120 isolates of LAB from four different Indonesian traditional fermented foods, 16 isolates were resistant to erythromycin. In line with this, Peng *et al.* (2023) observed that among the 12 Lactobacillus species obtained from Chinese fermented foods, 5 isolates were sensitive to kanamycin, 7 resistant to erythromycin, 9 resistant to ampicillin, and 8 isolates resistant to tetracyclin. On the contrary Tigu *et al.* (2016) reported that all of the LAB isolates obtained from traditional fermented condiments such as Datta and Awaze were susceptible to ampicillin, erythromycin, and tetracycline.

Identification of Probiotic LAB Isolates by 16S rRNA Gene Sequencing of the isolated LAB

The 16S rRNA gene sequences of the 4 LAB isolates with the best potential probiotic properties showed the highest homology to the known species of bacteria in the database (Figure 1). Accordingly, CA4 showed 99.80% similarity with *Leuconostoc mesenteriodes* NO5 showed 99.79% match with *Lactobacillus plantarum*, W6 showed 99.90% match with *Weissella paramesenteroides* and W7 showed 98.61% homology with *Lactobacillus plantarum*. Li, *et al.* (2023) have identified *Lactobacillus plantarum* with potential probiotic with anti-obesity and antioxidant anti-obesity and antioxidant properties using 16S rRNA genes analysis. Similarly, Zhao *et al.* (2022) have revealed the strain level identification of lactic acid bacteria with potent probiotic properties isolated from some Sichuan pickle using phylogenetic estimation of 16S rDNA genes.

Table 1: Morphological, biochemical and physiological characterization of the isolates

Isolates	Gram staining	Shape	Catalase	Citrate	Methyl red	Indole	Spore staining	Motility	Temperature (°C)			NaCl concentration (%)			
									15	37	45	2	4	6.5	8
CA4	+	Rod	-	-	-	-	-	-	+	+	+	+	+	+	+
NO5	+	Rod	-	-	-	-	-	-	+	+	+	+	+	+	+
W6	+	Rod	-	-	-	-	-	-	+	+	+	+	+	+	+
W7	+	Rod	-	-	-	-	-	-	+	+	+	+	+	+	+

Key:- = negative, + = positive

Table 2: Sugar fermentation and protease activity tests of the isolates

Isolate	Sugar							Protease activity
	Glucose	Fructose	Sucrose	Lactose	Manitol	Sorbitol	Xylose	
CA4	$A^{+}G^{+}$	$A^{+}G^{+}$	$A^{+}G^{-}$	$A^{-}G^{-}$	$A^{+}G^{-}$	$A^{+}G^{-}$	$A^{+}G^{-}$	+
NO5	$A^{+}G^{+}$	$A^{+}G^{+}$	$A^{+}G^{+}$	$A^{+}G^{-}$	$A^{+}G^{+}$	$A^{+}G^{+}$	$A^{-}G^{-}$	+
W6	$A^{+}G^{+}$	$A^{+}G^{+}$	$A^{+}G^{+}$	$A^{+}G^{-}$	$A^{+}G^{+}$	$A^{+}G^{+}$	$A^{-}G^{-}$	+
W7	$A^{+}G^{+}$	$A^{-}G^{-}$	$A^{+}G^{+}$	$A^{+}G^{+}$	$A^{+}G^{+}$	$A^{+}G^{-}$	$A^{-}G^{-}$	+

Key: - = negative, + = positive, A = assimilation, G = gas

Table 3: pH tolerance patterns of the isolates at different pH values after 24 and 48 hours exposure

Source	No. Isolate	No. of survived isolates (%) 24 hours			48 hours		
		1.5	2.0	3.0	1.5	2.0	3.0
Cabbage	14	1(7.14%)	2(14.28%)	2(14.28%)	1(7.14%)	1(7.14%)	2(14.28%)
Cucumber	14	-	1(7.14%)	2(14.28%)	-	1(7.14%)	2(14.28%)
Grape wine	14	-	-	1(7.14%)	-	-	1(7.14%)
Ogi	14	1(7.14%)	3(21.43%)	2(14.28%)	1(14.28%)	2(14.28%)	2(14.28%)
Orange wine	14	-	-	2(14.28%)	-	-	1(7.14%)
Wara	14	4(28.57%)	6(42.86%)	5(35.71%)	2(14.28%)	4(28.57%)	3(21.43%)
Total	84	6(7.14%)	12(14.28%)	14(16.67%)	4(4.76%)	8(9.52%)	11(13.09%)

Table 4: Percentage survival of probiotic LAB at different pH levels and 0.3% bile salt

Isolates	No. of survived isolates 24 hours			48 hours			Bile tolerance 24 hours
	1.5	2.0	3.0	1.5	2.0	3.0	0.3%
Ca4	47.35 ± 0.00^{b}	70.51 ±0.28^{c}	83.52 ± 0.70^{c}	43.68 ± 1.41^{d}	65.39± 0.01^{b}	78.64± 0.05^{ab}	95.00±1.41^{a}
O5	43.38 ±0.07^{c}	76.89 ± 0.70^{b}	74.00 ± 0.00^{d}	48.50 ± 0.14^{c}	66.50± 0.00^{b}	73.42 ± 0.0^{4b}	85.5±0.70^{c}
W6	63.79 ± 0.70^{a}	87.19 ± 0.08^{a}	87.13 ± 0.02^{b}	55.34 ± 0.00^{a}	73.02± 0.07^{a}	86.60 ± 0.04^{a}	94.5±2.12^{ab}
W7	61.55 ± 1.41^{a}	73.47± 0.58^{b}	90.53 ± 0.21^{a}	51.46 ± 0.07^{b}	70.11± 0.13^{a}	83.85 ± 0.07^{a}	91.5±0.70^{b}

Key: Values are presented as mean ± standard deviation of three replicates. Values with different superscripts along column are significantly different at $p < 0.05$.

Table 5: Antimicrobial activities of the isolates against some pathogenic bacteria and adhesion of LAB to stainless steel plate

Diameter of inhibition zone (mm)					
Isolstes	*S. aureus*	*E. coli*	*S. typhimurium*	*P. aeruginosa*	Adherence (%)
Ca4	17.00±0.00^{b}	19.15±0.03^{e}	18.65±0.01^{a}	20.00±0.00^{b}	37.70
O5	17.05±0.03^{b}	20.08±0.04^{d}	20.85±0.09^{b}	20.40±0.06^{c}	32.83
W6	14.00±0.00^{a}	16.06±0.03^{a}	19.05±0.03^{ab}	16.35±0.20^{a}	35.84
W7	9.50±0.06^{c}	17.00±0.00^{b}	20.65±0.03^{b}	20.05±0.03^{bc}	37.33

Key: Values are presented as mean ± standard error of mean (SEM) of three replicates. Values with different superscripts along column are significantly different at $p < 0.05$.

Table 6: Antibiotic susceptibility profile of the isolates

Isolstes	CH	CPX	S	NB	APX	LEV	E	CN	AMX
Ca4	R	R	S	R	R	R	R	R	R
O5	R	R	R	R	R	I	R	R	R
W6	R	R	R	R	R	R	R	R	R
W7	R	R	R	R	R	R	R	R	R

CPX- Ciprofloxacin, NB- Norfloxacin, CH- Chloramphenicol, E- Erythromycin, CN- Gentamycin, APX- Ampiclox, AMX-Amoxil, S- Streptomycin, RD- Rifampicin, LEV- Levofloxacin. Zone of inhibition (diameter in mm) for each antibiotic was measured and expressed as susceptible, S (≥21mm); intermediate, I (16–20mm); and resistance, R (≤15mm).

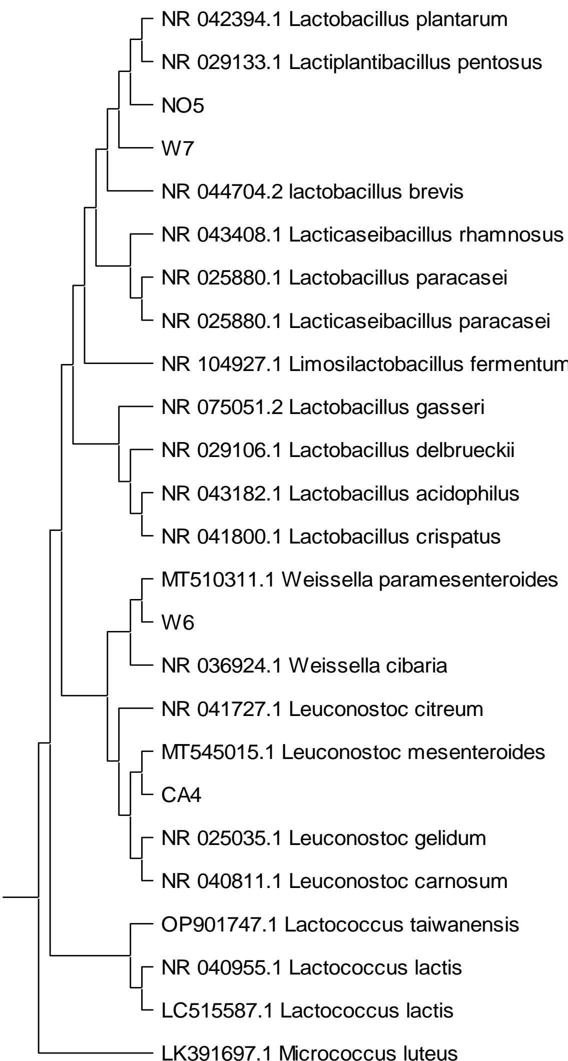

Figure 1: Neighbour-joining phylogenetic tree for LAB based on the 16S rRNA gene sequences. The relationship between isolated strains and related species were shown

CONCLUSION

The four LAB isolates from Wara, Cabbage and Ogi were the more desirable health-promoting bacteria with better acid and bile resistance, adherence property antimicrobial activity and antibiotics susceptibility test. It is suggested that these strains can be good candidates for food industries as the preservation of acidic foods at an industrial scale and can also serve as biopreservation of chilled food, which is mostly contaminated by spoilage pathogen. Since the increasing use of antibiotics leads to collateral damage to the host by disturbing the normal intestinal microbiota; therefore, consumption of fermented foods such as fermented milk and other enhances the proliferation of healthy GIT microbiota on the one hand and prevents the growth of undesirable microorganisms.This study reveals the functional properties of probiotics in fermented foods. Therefore, this fermented food product could be considered a valuable resource for probiotic strain screening and starter culture application.

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Detoxification of Poultry Feed Using *Candida tropicalis* Isolated from Palm Wine

Elesin M. A.* **Akinyele B. J. and Oluwole O. R.**
Department of Microbiology, Federal University of Technology, Akure, Ondo State, Nigeria.
* Corresponding author: motunrayoelesin@gmail.com

Abstract: Aflatoxin contamination in poultry feed poses a significant threat to both poultry health and food safety. This study aimed to explore the potential of using *Candida tropicalis*, isolated from palm wine, to detoxify aflatoxins in poultry feed ingredients. In this study, *Candida tropicalis* was isolated from palm wine samples collected within Akure metropolis, Ondo State, Nigeria. The isolated yeast strain was identified morphologically, through sugar fermentation tests and molecularly. Concurrently, toxigenic *Aspergillus flavus* strains was isolated from contaminated poultry feed ingredients and was confirmed for aflatoxin production through quantification using thin layer chromatography (TLC). The ability of the isolated *Candida tropicalis* to degrade aflatoxins was assessed by inoculating toxigenic *A. flavus* into formulated poultry feed, followed by treatment with a suspension of *C. tropicalis*. The detoxification efficacy was evaluated by comparing the aflatoxin levels in treated and untreated feed samples. The *in vivo* effects of detoxified feed on broiler chickens, dividing them into five dietary groups and their haematological and liver enzyme parameters were monitored using standard techniques. Broilers fed with *Candida tropicalis*-treated feed showed improved hematological profiles, including higher white blood cell counts, compared to those fed with aflatoxin-contaminated feed. Additionally, liver enzyme activity was closer to normal in broilers consuming detoxified feed, demonstrating the protective effects of *Candida tropicalis.* Findings of the research demonstrates the potential of *Candida tropicalis*, as an effective biological agent for mitigating the effects of aflatoxins in poultry feeds.

Key word: Aflatoxins, *Candida tropicalis***,** detoxification, haematological parameters

INTRODUCTION

Poultry meat is becoming more and more popular as the world's population grows at a rapid pace. This is mostly because it is readily available and has a magnificent reputation as a protein source (Bilal *et al*., 2023). The growing number of people consuming chicken products means that concerns about microbial contamination must be addressed (Danbappa *et al*., 2018). Poultry products are at high risk of contamination from their feed, which can affect both animal health and consumers (Suleman *et al.,* 2022). Despite being dry, poultry feed provides a favourable environment for microbial growth, especially fungi and bacteria, due to its nutrient content, environmental factors like moisture and temperature further promote contamination (Gicheha *et al.,* 2021).

Mycotoxins which are harmful compounds produced by moulds, are a common contaminant in feeds. These toxins, including aflatoxins from *Aspergillus*, deoxynivalenol and fumonisin from *Fusarium*, and ochratoxin from *Penicillium*, are dangerous to both poultry and humans (Ochieng *et al.,* 2021). Among these, aflatoxins are particularly concerning due to their lower tolerance levels in poultry feed, causing symptoms like reduced growth, lower egg production, and increased mortality rates (Okasha *et al.,* 2024). Aflatoxins refer to a collective term for a group of chemically similar compounds with similar toxicity levels, including aflatoxin B1, B2, G1, and G2. Among these, AFB1 is usually the most commonly found in feedstuffs. In terms of toxicity, the order is typically Aflatoxin B1 > aflatoxin B2 > aflatoxin G1 > aflatoxin G2 (Nazhand *et al.,* 2020). Many studies have been conducted to determine the teratogenic, carcinogenic, mutagenic, and growth-inhibiting effects of aflatoxins in poultry in order to assess its toxicity. The detrimental consequences of aflatoxins on the body's hematological and histological systems have also been well documented (Oguz, 2011). Preventing the growth of moulds and contamination by aflatoxins in feed and feedstuffs is paramount.

However, in cases where contamination occurs despite preventive measures, it is crucial to decontaminate aflatoxins before

using these materials. To combat aflatoxins, biological detoxification using yeasts has emerged as an effective method. Yeasts can break down aflatoxins and also preserve feed quality. It also promote growth in broilers and enhances immunity by raising macrophages and antibodies. Yeasts cell wall derivatives are known to bind aflatoxins, reducing their harmful effects. Additionally, yeast produces enzymes that degrade aflatoxins by breaking down their chemical structure (Bilal *et al.,* 2023).

Palm wine, a fermented product of various palm trees, serves as an excellent and accessible source of yeast (Sarma *et al.,* 2022). Common yeast species from palm wine include *Saccharomyces* and *Candida*, which have shown promise in detoxifying poultry feeds (Djeni *et al.,* 2020). This study aims to assess the effectiveness of yeast isolated from palm wine in detoxifying poultry feed, while also evaluating the impact of detoxified feed on the hematological and histological health parameters of broilers.

MATERIALS AND METHODS

Isolation and identification of yeast from palm wine samples: Freshly tapped palm wine samples were obtained in sterile containers from various locations within Akure metropolis, Ondo State, Nigeria, including Ipinsa, Ibule, Ilara, and Oba-ile and transported to the laboratory for microbial analysis. Yeast isolation was carried out by serially diluting 1 ml of each sample in 9 ml of sterile distilled water. From the diluted suspensions, 0.1 ml was aseptically transferred onto sterile yeast extract agar (YEA) plates. The plates were incubated at 28±2°C for 48 hours, after which yeast colonies were enumerated. Pure cultures of the yeast isolates were obtained through repeated streaking on YEA. The yeast isolates were identified based on morphological characteristics, sugar fermentation profiles and molecular techniques (Olaniyi *et al*., 2019).

Isolation and identification of toxigenic fungus from the poultry feed samples: The suspected contaminated feed ingredient sample was homogenized to increase the surface area for fungal growth. The homogenized sample was plated on potato dextrose agar (PDA). The plate was incubated at room temperature (usually around 30-37°C) for five (5) days to allow fungal colonies to grow. To get pure fungal cultures, individual colonies were sub-cultured onto sterile PDA plates and observed for growth (Olaniyi *et al.,* 2019).

Quantification of aflatoxin in toxigenic fungi using thin layer chromatography: The method described by Leszczynska *et al*. (2001) was used in which, 50 g of the ground sample was weighed, filtered, and concentrated. Silica gel G was spread on a 20x20 cm plate and spotted with different volumes of the sample extract and standard aflatoxins. The plate was developed in a solvent system and the disintegrated sample was extracted with a methanol-water mixture to separate aflatoxin. The sample was homogenized, centrifuged, and diluted with phosphate buffer at pH=7.2. The absorbance of the solution was measured at 450 nm using a UV/Visible spectrophotometer, and the aflatoxin content was calculated using a prepared standard curve

Identification of yeast and toxigenic fungi isolate using molecular method: Genomic DNA of yeast and *Aspergillus* was extracted from the fungal samples with the Zymo Research Quick-DNA fungal/bacterial kit. The Internal Transcribed Spacer (ITS) region was amplified using OneTaq Quick-Load 2X Master Mix and specific primers. The amplified PCR products were enzymatically cleaned using the EXOSAP method. Sequencing was carried out in both directions with the Brilliant Dye Terminator Cycle Sequencing Kit, and the DNA fragments were purified using the ZR-96 DNA Sequencing Clean-up Kit. The purified sequences were analyzed on an ABI 3500xl Genetic Analyzer, and the resulting .ab1 files were processed using DNASTAR software. Sequence identification was conducted via BLAST search in the

GenBank database of the National Centre for Biotechnology Information (NCBI) (Stephen *et al.,* 1997).

Screening of yeast isolate for aflatoxin degrading enzymes: Laccase production was assessed by culturing each fungal strain on potato dextrose agar (PDA) plates supplemented with 0.02% guaiacol and 0.5% tannic acid, which were sterilized separately and incorporated into the medium before solidification. Additionally, 0.01% Remazol Brilliant Blue R (RBBR) was used as an indicator. The plates were incubated at 30°C for 7 days (Senthivelan *et al*., 2019). Chitinase activity was evaluated using the dinitrosalicylic acid (DNS) method, with colloidal chitin serving as the substrate. A mixture of 1.0 ml enzyme solution, 1.0 ml of 0.5% colloidal chitin, and 1.0 ml of 0.1 m citrate buffer (pH 7.0) was incubated at 37°C in a Wincom shaker water bath (Model-WBS-C2) at 280rpm for 30 minutes. The reaction was terminated by adding 2 ml of DNS reagent, followed by heating the mixture in a boiling water bath for 10 minutes. After cooling, the mixture was centrifuged at 10,000 rpm for 10 minutes at room temperature, and the absorbance of the supernatant was measured at 540 nm (Gonfa *et al*., 2023).

Inoculation of toxigenic Aspergillus flavus into the formulated poultry feed and detoxification with Candida tropicalis: Eighty kilograms (80 kg) of formulated poultry feeds were divided into four portions. The first portion contained feed inoculated with 500 ml of spore suspension of toxigenic *A. flavus*, while the second portion contained feed inoculated with 500 ml of spores suspension of toxigenic *A. flavus* plus 500 ml cell wall suspension of *Candida tropicalis*. The third portion contained feed inoculated with 500 ml cells wall suspension of *Candida tropicalis*, while the fourth portion contained feed that was not inoculated with either fungal spores or *Candida tropicalis* (control) (Ibitoye *et al.,* 2021). These detoxified feed samples were used for *in vitro* assessment and then administered to broiler chickens in accordance with International Standards of Animal Welfare. Fifteen four-week-old broilers were sourced from a commercial hatchery and divided into five groups, each containing three broilers. The groups were assigned as follows: Group 1 was fed with a basal diet (uninoculated feed), Group 2 was fed with *A. flavus*-contaminated feed, Group 3 was fed with *A. flavus*-contaminated feed treated with *Candida tropicalis*, Group 4 was fed with *Candida tropicalis*-inoculated feed, and Group 5 was fed with commercially purchased feed.

Quantitative analysis of aflatoxins in the formulated poultry feed samples: Disintegrated sample was extracted with 10 ml of methanol–water mixture (7:3) to separate aflatoxin. To that end, the rest was homogenized for 10 min at room temperature and the resultant deposit was centrifuged. An aliquot (100 µl) of the supernatant was diluted with 600 µl of phosphate buffer at pH = 7.2. The samples were incubated for 30 min at room temperature in the darkness. Then, 50 µl of tetramethylbenzidine and 50 µl of urea peroxide were added and incubated again for 30 min in darkness. The reaction was terminated by adding 100 µl of the stop reagent. The absorbance of solution was measured at a wavelength of 450 nm, using Longmed UV/Visible spectrophotometer (Model-SHZ82). The content of aflatoxins was calculated using prepared standard curve.

Haematology of the blood and serum biochemistry: At the end of the study, blood samples were collected directly from the axillary veins of the wing, obtaining 3 cc of blood into a sterilized glass tube containing Ethylene diamine tetra acetic acid (EDTA) and another glass tube without anti-coagulant for haematological and serum biochemistry (liver enzymes) respectively. Blood samples were separated, centrifuged, and frozen at 100℃ for the serum analysis (Hidayat *et al*., 2020).

Statistical analysis of data obtained: All generated data were subjected to one way analysis of variance (ANOVA) using

Statistical package for social sciences (SPSS) version 23.0. Treatment means were compared using Duncan's new multiple range test and differences were considered significant at P>0.05.

RESULTS

Total yeast counts and characteristics of yeast isolates

Figure 1 displays the total yeast counts from palm wine samples collected from various locations. The sample from Ibule exhibited the highest yeast count of 2.46×10^{2} cfu/ml, while the sample from Oba-Ile had the lowest count of 1.50×10^{2} cfu/ml. Table 1 presents the cultural and morphological characteristics of yeast isolated from different locations. Table 2 details the sugar fermentation profiles of the yeast isolates using carbon substrates such as galactose, glucose, sucrose, maltose, and raffinose. The colour change from red to yellow after 48 hours of fermentation suggests acid production.

Molecular identification of the yeast and fungi isolates

The yeast isolate was confirmed to be *Candida tropicalis* and the fungus as *Aspergillus flavus* using molecular methods. The sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI) as shown in Table 3.

Quantity of degrading enzymes present in *Candida tropicalis* isolates

Table 4 depicts the quantities of the degrading enzymes namely laccase and chitinase present in *Candida tropicalis.* The relatively high quantity of laccase (4.851 mg/ml/min) and the presence of chitinase at 1.211 mg/ml/min in *Candida tropicalis* could lead to both direct degradation of aflatoxins and inhibition of aflatoxin-producing fungus, contributing to safer feed and improved poultry health.

Concentration of aflatoxin in the toxigenic *Aspergillus flavus* isolated from the feed ingredients

The aflatoxin B1 (AFB1) content of the fungal biomass was quantified and determined to be 1.563 μg/mg using thin layer chromatography while AFB2 was not detected.

Quantitative values of aflatoxins in the formulated feed samples

The quantitative values of aflatoxins in Table 5 indicates high aflatoxin B1 production in formulated feed with *A. flavus*, low production in yeast and *A. flavus*, and no aflatoxin B1 production in the control and yeast-only samples.

Haematological parameters and liver enzyme test of broilers fed with different diets

Table 6 and 7 shows the haematological parameters and liver enzyme test of broilers from the different diet groups. The hematological parameters suggest that broilers fed with feed containing toxigenic *A. flavus* (A.F) exhibit signs of immunosuppression and potential anemia. The introduction of *Candida tropicalis* (C.T) as a treatment appears to ameliorate some of these adverse effects, as evidenced by improved red and white blood cell counts and platelet parameters. The findings statistically had no significant differences ($p < 0.05$).

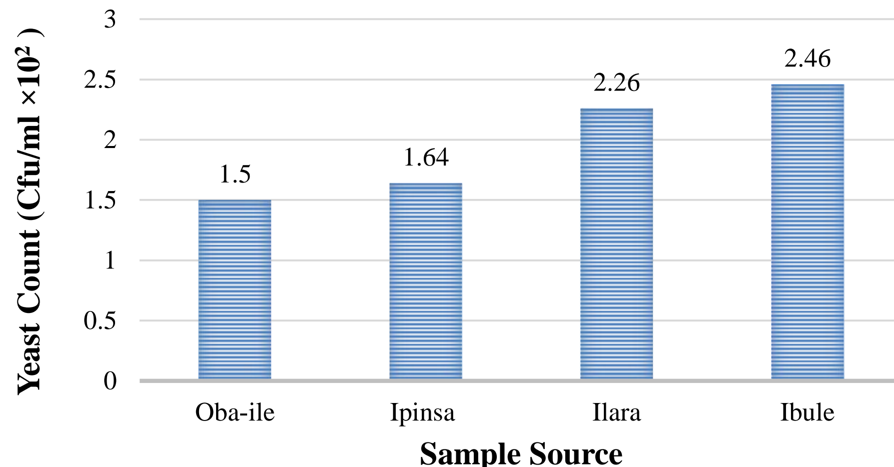

Figure 1: Total yeast counts from palm wine obtained from different locations

Table 1: Morphological and microscopic characteristics of yeast isolates

Isolate code	Microscopic structure	Shape	Color	Elevation	Margin	Opacity	Surface
IP	Long chain	Oval	Cream	Slightly raised	Regular	Opaque	Smooth, glossy
IB	Short chain	Oval	Cream	Slightly raised	Regular	Opaque	Smooth, glossy
IL	Short chain	Oval	Cream	Slightly raised	Regular	Opaque	Smooth, glossy
OB	Short chain	Oval	Cream	Slightly raised	Regular	Opaque	Smooth, glossy

Key: Ip = Ipinsa ; Ib = Ibule ; Il = Ilara and Ob = Oba-Ile

Table 2: Sugar fermentation activity of the yeast isolates

Isolate code	Lactose	Glucose	Arabinose	Xylose	Sucrose	Raffinose	Galactose	Maltose
IP	-	+	-	-	+	+	+	+
IB	-	+	-	-	+	+	+	+
IL	-	+	-	-	+	+	+	+
OB	-	+	-	-	+	+	+	+

Key: Ip = Ipinsa ; Ib = Ibule ; Il = Ilara ; Ob = Oba-Ile ; + = fermentable and - = non-fermentable

Table 3: Molecular identification of the isolated fungi and yeast (Blast prediction)

S/N	Sample ID	Organism	Sequence length (bp)	% Identity	Accession no of BLAST hit	E-value	Alignment score	Highest query coverage (%)
1	F	*Aspergillus flavus*	595	99.83%	MT645322.1	0.0	≥200	100%
2	Yeast	*Candida tropicalis*	523	99.24%	MZ363119.1	0.0	≥200	99%

Key: F- *Aspergillus flavus*

Table 4: Quantity of aflatoxin degrading enzymes in *Candida tropicalis*

Enzyme in *Candida tropicalis*	mg/ml/min
Laccase	4.851
Chitinase	1.211

Table 5: Quantitative values of aflatoxins in the formulated feed sample

Sample	Aflatoxin content µg/mg
A. flavus	1.50 ± 0.00[b]
Candida tropicalis only	0.00 ± 0.00[a]
Candida tropicalis and A.flavus	0.12 ± 0.00[a]
Control (formulated)	0.00 ± 0.00[a]

Data are represented as mean ± standard error where n=2. Mean of groups with the same superscript across the column are not statistically significant (P> 0.05)

Table 6: Haematological parameters of broilers fed with different formulated diets

LAB	WBC (10^9/L)	RBC	PLT	P-LCR (%)	MPV (fL)	NEU (%)	EOS (%)	BAS (%)	HCB (g/L)
C1	3.08±0.03[b]	2.48±0.55[a]	164.5±99.5[a]	26.85±3.25 a	8.3±0.00[a] b	51±1.00[a]	1.00±0.00[a]	0.50±0.50[a]	7.25± 0.15[a]
C2	3.42±0.02[b]	2.33±0.01[a]	188.5±100.5[a]	26.85±3.25 a	8.5±0.70[a] b	50.5±2.50[a]	1.00±0.00[a]	1.00±0.00[a]	8.80±0.10[ab]
A.F	2.46±0.24[a]	2.53±0.08[a]	72.5±0.50[a]	14.6±5.90[a]	7.7±0.10[b]	56.50±1.50 a	0.50±0.50[a]	0.00±0.00[a]	9.35±0.05[b]
A.F-C.T	2.45±0.25[a]	2.53±0.08[a]	72.5±0.50[a]	19.9±4.60[a]	7.0±0.10[b]	56.50±1.50 a	0.50±0.50[a]	0.00±0.00[a]	9.35±0.05[b]
C.T	2.90±0.30[a]	2.50±0.57[a]	185±102.0[a]	24.6±0.10[a]	8.5±0.40[a]	50.50±2.50 a	0.50±0.50[a]	0.50±0.50[a]	10.25±1.05[b]

Data are represented as mean ± standard error where n=2. Mean of groups with the same superscript across columns are not statistically significant (P> 0.05). Keys: Basophil-Bas(%); Eosinophil-Eos(%); Hemoglobin Concentration-HCG(G/L); Mean Platelet Volume-MPV(Fl); Platelet-PLT(10^9/L); Plateletcrit-Large Cell Ratio- P-Lcr(%); Red Blood Cell Or Erythrocyte-RBC(10^{12} /L); White Blood Cell-WBC(10^9 /L); Neutrophil-NEU(%); Broilers Fed With *C.Tropicalis*- C.T; Broilers Fed With Commercially Purchased Feed- C2; Broilers Fed With The Basal Diet (Bd) (Uninoculated Sample)-C1; Broilers Fed With Toxigenic *A. Flavus*-A.F; Broilers Fed With Toxigenic *A. Flavus* & Treated With *C.Tropicalis*- A.F&Ct

Table 7: Liver enzyme markers

LAB	ALP (U/L)	AST (U/L)	ALT (U/L)	LDH (U/L)
C1	58.20±0.00[ab]	142±33.01[a]	390.19±7.48[a]	36.05±0.53[a]
C2	58.35±40.94[ab]	154.6±53.503[a]	396.78±23.52[a]	29.98±8.05[a]
A.F	101.85±20.58[ab]	184.6±11.08[a]	406.35±6.64[a]	48.57±5.72[a]
A.F & C.T	58.20±0.00[b]	177.90±17.22[a]	401.11±1.07[a]	42.50±14.31[a]
C.T	29.15±0.07[a]	145.88±43.357[a]	399.75±4.20[a]	40.54±5.63[a]

Data are represented as mean ± standard error where n=3. Mean of groups with the same superscript across the columns are not statistically significant (P> 0.05). Keys: Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Lactate dehydrogenase (LDH), Broilers fed with toxigenic *A. flavus* and treated with *C.tropicali*s (A.F & C.T), Broilers fed with toxigenic *A. flavus* (A.F), Broilers fed with *C.tropicalis* (C.T), Broilers fed with the basal diet (BD) (uninoculated sample) (C1) and Broilers fed with commercially purchased feed (C2)

DISCUSSION

In commercial poultry farming, aflatoxins present serious financial difficulties. There is proof that aflatoxin contamination in animal diets has a detrimental effect on animal productivity (Mgbeahuruike *et al*., 2016). Given that between 65 and 75% of the costs associated with producing chickens are related to feed, changes in feed quality would inevitably impact the productivity of poultry (Hassan *et a*l., 2021). The significant presence of fungi can be linked to the low water activity in animal feed and the characteristics of the contaminating fungal

genera. These fungal species might have originated from carry-over of fungi that were already present in the field. Additionally, handling and other post-harvest processes may also be key contributors to the contamination (Danbappa *et al.,* 2018).

Aflatoxins, particularly aflatoxin B1 (AFB1), have been shown to adversely affect poultry health. Studies indicate that AFB1 can lead to hepatotoxicity, resulting in liver damage and impaired growth rates in broilers (Bilal *et al.,* 2023). Reducing the detrimental effects of aflatoxins in animal nutrition may be accomplished through the use of yeast to detoxify feeds contaminated with the toxin (Oluwole *et al.,* 2023).

Palm wine is a great medium for microorganisms like yeasts and bacteria to develop and proliferate with its high nutrient content. The isolation of diverse yeast populations from palm wine were previously published by Boboye *et al.* (2008) and Oluwole *et al.* (2023). *Candida tropicalis* is a common microorganism in various fermented foods. Its ability to bind aflatoxin indicates its potential use in removing toxins from cereals during fermentation. Magnoli *et al*. (2016) identified *Candida tropicalis* as one of the most effective non-Saccharomyces yeasts for aflatoxin binding, along with other strains like *Clavispora lusitaniae* and *Pichia anomala. Candida tropicalis* possesses enzymes that play a crucial role in the degradation of aflatoxins, particularly through its metabolic pathways. In this study, laccase and chitinase enzymes were found present and quantified in the isolated *Candida tropicalis*. Numerous studies have focused on the measurement of these enzymes which are involved in the decomposition of aflatoxin. These studies have also examined the enzymes' effectiveness in combating aflatoxin B1 (AFB1). When attempting to target aflatoxin breakdown, Dellafiora *et al*. (2017) stressed how crucial it is to comprehend the distinct actions of laccase isoforms. The authors showed that *Trametes versicolor* laccase (enzymes) had the ability to break down toxic substances like aflatoxins, which is similar with findings in this study. During which a decrease in the aflatoxin B1 level in the *Candida tropicalis* + *A.flavus* feed sample was observed, compared to the aflatoxin B1 level in the *A. flavus* feed sample. The formulated feed sample treated solely with *C. tropicalis* also had undetectable levels of aflatoxin B1. In another study conducted by Xiong *et al*. (2022), a new laccase (enzyme) produced from *Bacillus amyloliquefaciens* B10 was also shown to be capable of digesting aflatoxin. The authors identified key active site residues and successfully cloned and expressed the laccase gene in *E. coli*. This provided a strong foundation for further development and optimization of the enzyme for practical applications. Notably, the laccase exhibited high degradation activity, suggesting its effective utilization under a range of environmental conditions commonly found in food and feed storage.

The physiological state of an animal is reflected in its haematological constituents in relation to both its internal and external surroundings (Mulatu *et al.,* 2019). Reactive oxygen species (ROS), which are created by AFB1, have the potential to harm cells, including those found in the bone marrow where red blood cells are synthesized. The generation of white blood cells (WBC) and red blood cells (RBC) may be hampered by this injury. Findings in this study indicates that aflatoxin contamination leads to a significant reduction in WBC counts, highlighting its immunosuppressive effects. The treatment with *Candida tropicalis* shows some improvement in WBC counts, suggesting a partial protective effect, though not enough to fully counteract the suppression caused by aflatoxins. This is consistent with the findings of Riahi *et al*. (2021) where the mycotoxin binder was shown to stabilize certain haematological parameters but, did not completely negate the effects of mycotoxins. Another study conducted by Basmacioglu *et al*. (2005) found that broilers' hemoglobin levels and lymphocyte counts decreased when fed with an aflatoxin-contaminated diet. In this recent

study, exposure to toxigenic *Aspergillus flavus* resulted in a higher RBC count but, significantly lower hemoglobin levels, indicating anemia and stress from aflatoxin toxicity. Treatment with *Candida tropicalis* only had a lower RBC count showing partial recovery towards normal hemoglobin levels, although not completely restoring RBC health. Yeast supplementation has been shown to positively influence hematological parameters in poultry affected by aflatoxicosis. Specifically, the inclusion of yeast in the diet can lead to improvements in red blood cell counts, hemoglobin levels, and overall blood health, which are often negatively impacted by aflatoxin exposure (Oguz, 2011).

Yeast supplementation has been linked to lower levels of liver enzymes such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are indicators of liver health. A reduction in these enzymes suggests less hepatic stress and better overall liver function (Hassan, 2021). Broilers exposed to toxigenic *Aspergillus flavus* (A.F) in this recent study showed higher levels of liver enzymes, suggesting liver damage or stress due to aflatoxins. This is similar to a study done by Bhatti *et al.* (2016), which showed that broilers fed dietary contamination with 0.1, 0.2, and 0.6 mg/kg aflatoxin B1 had an elevated blood concentration of ALT compared to broilers given a non-contaminated food. In another study by Rashidi *et al.* (2020), a substantial increase in the blood levels of ALT was observed when feeding broilers an aflatoxin B1-contaminated food at a rate of 0.5 mg/kg in comparison to the control group. The group treated with both *A.flavus* and *Candida tropicalis* showed similar ALP levels but, still elevated AST, ALT, and LDH levels. *Candida tropicalis* treatment helped normalize ALP levels but, did not completely mitigate liver damage caused by aflatoxins. The findings of this study align with that of Riahi *et al.* (2021), where the presence of mycotoxins (OTA and T-2) also resulted in altered liver enzyme activities, suggesting liver stress. The authors further reported the inclusion of the Multicomponent mycotoxin detoxifying agent (MMDA) improved some liver enzyme activities but did not fully reverse the negative effects of the mycotoxins on liver function. The group treated solely with *Candida tropicalis* had the lowest liver enzyme levels, suggesting *Candida tropicalis* treatment alone did not stress the liver and may have a protective effect. This study is in contrast to a study carried out by Hashmi *et al.* (2006) where yeast sludge did significantly improve the levels of serum total protein, serum albumin, and alanine transaminase (ALT) in the broilers. Notably, while *Candida tropicalis* may not completely eliminate the effects of aflatoxins in the diet, it plays a significant role in reducing their impact.

CONCLUSION

This study underscores the significant threat posed by aflatoxins in poultry feed, which can severely impair broiler health, growth performance, and liver function thus affecting poultry business. The findings align with previous research, highlighting the widespread occurrence of aflatoxin B1 in contaminated feed and its adverse effects on broiler chickens. The study confirms that dietary contamination with toxigenic *Aspergillus flavus* thereby altered hematological parameters and elevated liver enzyme activities. Importantly, the research demonstrates the potential of *Candida tropicalis*, a yeast strain isolated from palm wine, as an effective biological agent for mitigating the effects of aflatoxins in poultry feed. The yeast treatment significantly reduced aflatoxin B1 levels in formulated feed, reduced some of the adverse hematological and liver enzyme changes induced by aflatoxins. Although, *Candida tropicalis* did not completely reverse the effects of aflatoxin exposure, it provided a protective effect, particularly in reducing the severity of liver damage.

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Biopreservative Effects of Jack Fruit Seed and Bark on Fruit Juices

Itaman V. O.* Osaro-Matthew R. C. and Okorie E. E.
Department of Microbiology, College of Natural Science, Michael Okpara University of Agriculture,Umudike, Abia State, Nigeria
* Corresponding author: itamanvivien@yahoo.com

Abstract: Fruit juices are drinks consumed worldwide as it contain vitamins, minerals, amino acids, dietary fibers, sugars and bioactive compounds which are important for the wellbeing and health of humans. Fresh fruits are minimally processed to obtain juices which render it perishable products and hence the need to preserve these juices. This study was aimed at investigating the biopreservative potential of jackfruit seed and bark on freshly prepared juices (watermelon, pineapple and pawpaw). The colony count was observed during the 96 hours storage period at 0 hour, 48 hours and 96 hours by standard spread plate count method. The samples were screened for total Heterotrophic Bacterial Count (THBC), Total Coliform Count (TCC), Total Lactic Acid Bacteria Count (LAB) and Total Fungal Count (TFC) according to the standard methods for the enumeration of bacteria and fungi. The microbial counts of the juices preserved with jack fruit seeds ranged from 1.0^{f} x 104 to 1.18^{b} x 10^{7} cfu/ml while those preserved with jackfruit bark ranged from 1.3^{e} x 10^{4} to 8.8^{e} x 106 cfu/ml. Jack fruit seeds exerted more biopreservative effects on the pineapple juice, while jackfruit bark had more biopreservative effects on the watermelon juice. The isolated microorganisms included *Escherichia coli, Bacillus* species, *Salmonella* species, *Staphylococcus aureus, Citrobacter* species, *Micrococcus* species, *Lactobacillus* species, *Rhizopus* species, *Aspergillus* species and *Penicillium* species. *Bacillus* sp were the most occurring bacteria in all fruit juices, followed by *Staphylococcus aureus* and *Lactobacillus* sp. while the most occurring fungi was *Penicillium* sp. Some of the microorganisms detected in this study can cause spoilage, food borne illness and pose great risks to human health, so there is need for mitigating the potential risks to consumer health and improving the quality of fruit juices by the addition of natural antimicrobials of plant such as jackfruit to fruit juices which are with little or no negative health consequences. In conclusion, jack fruit seed and bark has been found quite effective as a biopreservative and therefore, could serve as a good candidate for use in extending the shelf life of fruit juices.
Key word: Fruit juices, biopreservative, jackfruit, microbial count, human health

INTRODUCTION

The growing awareness on the need for healthy living and the consumption of healthy, safe and nutritive fruits and vegetables enhanced with natural sources as biopreservative rather than with synthetic preservatives has raised a need for wholesome and nutritive fruits (Qadri *et al*., 2015).

Fruits serve as excellent sources of vitamins, minerals, amino acids, dietary fibers, sugars and bioactive compounds which are important for the wellbeing and health of humans (Allaqaband *et al.,* 2022; Munekata *et al.,* 2023). Also, these components of fruits provide an excellent environment that supports the growth of a range of microorganisms. Examples of fruits include apple, banana, watermelon, pineapple, strawberry, grape, orange, mango, guava and pawpaw. The minimal processing of fresh fruits to make juices render them perishable products that will deteriorate rapidly compared with intact fruits (Mao *et al*., 2006).

Watermelon (*Citrullus lanatus* (Thunb.)) fruit which has about 93% water content is consumed worldwide. It is a rich source of vitamin C, A and B, amino acid, antioxidants and carotenoid lycopene which serve as important nutrients required for healthy living (Rani *et al*., 2019; Naz *et al*., 2014; Asante *et al*., 2020). Pineapple (*Ananas comosus* L.) fruit is a tropical fruit that is consumed worldwide. It contains calcium, potassium, vitamin A, C and carotene, organic acids, bromelain, phosphorus, carbohydrates, sugars, crude fiber, water and other essential compounds (Debnath, 2012; Lagnika *et al*., 2017) which are of immense benefits to human health. The fruits also possess antioxidant, therapeutic and medicinal properties. Pineapple fruits are either consumed fresh or as fresh pineapple juice. The fruits are also used in the production of different food items such as jam, syrup, jelly and pickles (Hossain *et al.,*

2015). Pawpaw (*Carica papaya* L.) fruit is commonly found in the tropical regions of the world. It is a good source of components such as minerals, iron, calcium, vitamins A, B and C which are beneficial to human health. Pawpaw fruits also have medicinal properties which aid in the treatment of various ailments (Dutta *et al*., 2010; Olusegun *et al*., 2016)

Jackfruit (*Artocarpus heterophyllus* Lam) belong to Moraceae family and grow mostly in countries such as India, Bangladesh, Sri Lanka, Burma, Malaysia and Brazil and some parts of Nigeria (Jagadeesh *et al*., 2007). The jackfruit is unusually large in size with each fruit weighing up to 30-35 kg. The ripe fruit consist of three parts which include: the skin (fibrous portion), the pulp (bulbs) and the seeds whose seed and pulp are edible (Albi and Jayamuthunagai, 2014). The pulp when ripe appears soft and yellow to brownish colour with distinct flavour which is sweetish and liken to banana-like flavour and taste (Elevitch and Manner, 2006). Jackfruit is a rich source of several high-value compounds that has potential beneficial physiological activities (Jagtap *et al*., 2010). It contains high level of proteins, starch, calcium, dietary fibre, vitamins and minerals (Ramli *et al*., 2021). Jackfruit has antibacterial, antifungal, antidiabetic, anti-cancer, anti-osteoporotic, anti-inflammatory, and antioxidant activities (Shanmugapriya *et al*., 2011; Khan *et al*., 2021; Palamthodi *et al*., 2021). Different parts of jackfruit tree are been used in ethno-medicine for the treatment of ailments such as diabetes, diarrhoea, dermatitis, malarial fever, asthma, tapeworm infection and anaemia (Araújo and Lima, 2010).

The presence of bioactive compounds in fruits enhances their beneficial effect (Galaverna *et al*., 2008; Vinuda *et al*., 2010). The functional significance of jackfruit (pulp) is associated with the phytochemicals it contains which include: phenolic compounds, carotenoids, flavonoids, volatile acids, sterols, and tannins (Ranasinghe *et al*. 2019; Chandrika *et al*. 2004; Amadi *et al*. 2018). In order to preserve and boost or improve the nutritional quality of fresh products, the use of "green" technologies is preferable (Davachi *et al*., 2021). Preserving food help to sustain it's freshness, quality and colour. Biopreservation involve the extension of shelf life and enhancement of food's safety by the use of natural or controlled microbiota and/or antimicrobial compounds. Hence, this study determines the preservative potential of jack fruits seeds and bark on some fruit juices.

MATERIALS AND METHODS

Sample collection: Fresh matured pineapple, watermelon and pawpaw fruits for juice extraction were purchased from a local market (isi-gate Market) in Umuahia while the jackfruit and its stem bark were obtained from a local farm located at Olokoro in Umuahia, both in Abia state, Nigeria. The Samples were authenticated in the Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Abia state and further transported in ice bags to the Laboratory for analysis.

Sample preparation: The Jackfruit was sorted, washed and sliced open and the seeds separated from the pulp manually using a sterile knife. The de-hulled seeds were washed with sterile water and dried while the Jackfruit stem bark was separated from the plant using sterile knife, washed with sterile water, and dried. The seeds and the bark were oven dried at 60° C for 8 hours, according to the methods of Amadi *et al*. (2018). The dried seed and stem bark samples were ground into powdered form with the aid of an industrial blender (Philips HL1646 model) (Amadi *et al*., 2018).

Preparation of plant part extract: Fifty grams (50 g) each of the powdered samples was soaked in 500 ml distilled water in a conical flask, kept on a shaker for 24 hours. The extracts were filtered through Whatman filter paper (No.1) and concentrated to dryness with the aid of a rotary evaporator (Laborota 4000-efficient, Hedolph city, Germany). The extracts from each sample were kept in the refrigerator at 4°C for analysis or prior to use (Silva *et al*., 2014 and Adeogun *et al*., 2016).

Production of fruit juices: Healthy watermelon, pineapple and pawpaw fruits with unbroken skin were thoroughly washed under a running tap water to remove dirt and then, rinsed with distilled water. The fruits were aseptically cut open with a sterile knife after surface disinfecting them with 70% v/v ethanol solution. The seeds were removed and the flesh (pulp) cut into chunks and scooped into a sterile blender, and blended with distilled water to make 1:1 (w/v) mixture. The juice was obtained by sieving the homogenized blend through a two-fold muslin cloth to get a clear juice (Boone *et al*., 2005; Gwana *et al*., 2014).

Experimental/ treatment set-up

Set-up A: 100% watermelon juice (Control), 95% of watermelon juice + 5% of jack fruit bark/seed, 90% of watermelon juice + 10% jack fruit bark/seed, 85% of watermelon juice + 15% of jack fruit bark/seed, 80% of watermelon juice + 20% of jack fruit bark/seed.

Set-up B: 100% Pineapple juice (Control), 95% of Pineapple juice + 5% of jack fruit bark/seed, 90% of Pineapple juice + 10% jack fruit bark/seed, 85% of pineapple + 15% of jack fruit bark/seed, 80% of pineapple + 20% of jack fruit bark /seed.

Set-up C: 100% Pawpaw juice (Control), 95% of Pawpaw juice + 5% of jack fruit bark/seed, 90% of Pawpaw juice + 10% jack fruit bark/seed, 85% of pawpaw + 15% of jack fruit bark/seed, 80% of pawpaw + 20% of jack fruit bark/seed.

Microbial study/analysis during storage: The colony counts were observed during the 96 hours storage period at 0 hour, 48 hours and 96 hours by standard spread plate counts method. The total Heterotrophic Bacterial Counts (THBCs), Total Coliform Counts (TCCs), Total Lactic Acid Bacteria Counts (LABs) and Total Fungal Counts (TFCs) were done according to the standard methods for the enumeration of bacteria and fungi (Michael and Joseph, 2004). The isolates were identified using standard microbiological (morphologically and biochemically) methods (Cheesbrough, 2006).

Statistical analysis: All data obtained were analyzed using one-way analysis of variance (ANOVA). Descriptive statistics in form of means and standard deviation were also used to assess the data. The significance differences between the variables at $p < 0.05$ were determined using Duncan's range test.

RESULTS

Enumeration of total microbial load (cfu/ml) in fruit juices during storage or preserved with jack fruit seed and bark

The bacteria and fungi population enumerated during the storage period is presented in Table 1 and Table 2. The total Heterotrophic Bacterial Counts (THBCs), Total Coliform Counts (TCCs), Total Lactic Acid Bacteria Counts (LABs) and Total Fungal Counts (TFCs) of fruit juices prepared from watermelon, pineapple and pawpaw and treated/preserved with different ratio of jack fruit seed and bark blend shows a decrease in the number of bacteria, coliforms and fungi present during the 96 hours storage period. The control (fruit juices without the jack fruit blend) which was run along with these to detect the efficacy of the bio-preservative effect of the jack fruit seeds showed an increase in the number of organisms present in the juices as the storage period advanced (Table 1and 2).

Characterization and identification of isolates

The morphological and biochemical studies on the bacteria and fungi isolates present during the storage period revealed isolates to be *Escherichia coli, Bacillus* species, *Salmonella* species*, Staphylococcus aureus, Citrobacter* species, *Micrococcus* species, and *Lactobacillus* species while the fungi isolates characterized were *Rhizopus* species*, Aspergillus* species and *Penicillium* species.

Microbial distribution during preservation or storage of fruit juices

Table 3 and 4 depicts the distribution of the isolates during the preservation period of 96 hours. *Bacillus* sp were the most occurring bacteria in all fruit juices analyzed, followed by *Staphylococcus aureus* and *Lactobacillus* sp. while the most occurring fungi was

Penicillium sp. During the preservation of the fruit juices with the seed blend, *Bacillus* sp occurred most with a distribution rate of 27.3% in both watermelon and pawpaw fruit juices preserved with 5% jack fruit seed while for the preservation with the bark, *Bacillus* sp also had the highest distribution of 25% in watermelon juice preserved with 5% jack fruit bark.

Table 1: Total microbial load (cfu/ml) of fruit juice preserved with jack fruit seed

Key: JFS- Jack Fruit Seed; WMJ- Watermelon juice; PPJ-Pineapple juice; PWJ-Pawpaw juice; NG- No growth, THBC-The total Heterotrophic Bacterial Count, TCC- Total Coliform Count, TFC- Total Fungal Count, LAB-Total Lactic Acid Bacteria Count, Values with different superscript down a column are significantly different from each other

Sample code	24hrs				48hrs				96hrs			
	THBC	TCC	TFC	LAB Count	THBC	TCC	TFC	LAB Count	THBC	TCC	TFC	LAB Count
A Control (100% WMJ)	$1.33^{a} \times 10^{7}$	$4.8^{b} \times 10^{4}$	$4.2^{a} \times 10^{4}$	NG	$1.58^{a} \times 10^{7}$	$7.8^{a} \times 10^{4}$	$5.1^{a} \times 10^{4}$	$2.8^{f} \times 10^{4}$	$2.13^{a} \times 10^{7}$	$8.4^{a} \times 10^{4}$	$5.8^{a} \times 10^{4}$	$3.4^{c} \times 10^{4}$
B (95%WMJ: 5% JFS)	$1.18^{b} \times 10^{7}$	$4.2^{c} \times 10^{7}$	$3.6^{b} \times 10^{4}$	NG	$1.02^{d} \times 10^{7}$	$3.9^{d} \times 10^{4}$	$3.1^{c} \times 10^{4}$	$1.7^{g} \times 10^{4}$	$8.4^{e} \times 10^{6}$	$3.6^{d} \times 10^{4}$	$2.3^{d} \times 10^{4}$	$1.0^{f} \times 10^{4}$
C (90%WMJ: 10% JFS)	$1.03^{d} \times 10^{7}$	$3.3^{d} \times 10^{4}$	$3.1^{c} \times 10^{4}$	NG	$8.8^{e} \times 10^{6}$	$2.8^{ef} \times 10^{4}$	$2.7^{d} \times 10^{4}$	NG	$7.1^{f} \times 10^{6}$	$2.5^{f} \times 10^{4}$	$1.8^{e} \times 10^{4}$	NG
D (85%WMJ: 15% JFS)	$8.3^{e} \times 10^{6}$	$2.6^{e} \times 10^{4}$	$2.2^{e} \times 10^{4}$	NG	$7.4^{f} \times 10^{6}$	$2.4^{g} \times 10^{4}$	$1.6^{f} \times 10^{7}$	NG	$6.9^{f} \times 10^{6}$	$1.8^{g} \times 10^{4}$	$1.1^{f} \times 10^{4}$	NG
E (80% WMJ: 20% JFS)	$7.7^{f} \times 10^{6}$	NG	NG	NG	$7.1^{f} \times 10^{6}$	NG	NG	NG	$6.2^{g} \times 10^{6}$	NG	NG	NG
A Control (100% PPJ)	$8.1^{e} \times 10^{6}$	$3.6^{d} \times 10^{4}$	NG	NG	$1.14^{c} \times 10^{7}$	$5.6^{c} \times 10^{4}$	$2.1^{e} \times 10^{4}$	$3.5^{d} \times 10^{4}$	$1.28^{c} \times 10^{7}$	$7.1^{c} \times 10^{4}$	$2.9^{c} \times 10^{4}$	$3.9^{b} \times 10^{4}$
B (95% PPJ: 5% JFS)	$7.3^{g} \times 10^{6}$	$2.4^{e} \times 10^{4}$	NG	NG	$6.9^{g} \times 10^{6}$	$2.3^{g} \times 10^{4}$	$1.3^{f} \times 10^{4}$	$2.4^{f} \times 10^{4}$	$6.4^{g} \times 10^{6}$	$2.2^{f} \times 10^{4}$	$1.1^{f} \times 10^{4}$	$2.1^{e} \times 10^{4}$
C (90% PPJ: 10% JFS)	$7.1^{g} \times 10^{6}$	NG	NG	NG	$6.7^{g} \times 10^{6}$	NG	NG	NG	$5.8^{h} \times 10^{6}$	NG	NG	NG
D (85% PPJ: 15% JFS)	$5.4^{h} \times 10^{6}$	NG	NG	NG	$5.1^{i} \times 10^{6}$	NG	NG	NG	$5.1^{i} \times 10^{6}$	NG	NG	NG
E (80% PPJ: 20% JFS)	$3.6^{i} \times 10^{6}$	NG	NG	NG	$3.2^{j} \times 10^{6}$	NG	NG	NG	$2.9^{j} \times 10^{6}$	NG	NG	NG
A Control (100% PWJ)	$1.16^{b} \times 10^{7}$	$5.2^{a} \times 10^{4}$	$2.6^{d} \times 10^{4}$	$6.8^{a} \times 10^{4}$	$1.42^{b} \times 10^{7}$	$6.4^{b} \times 10^{4}$	$3.8^{b} \times 10^{4}$	$7.3^{a} \times 10^{4}$	$1.68^{b} \times 10^{7}$	$7.8^{b} \times 10^{4}$	$4.3^{b} \times 10^{4}$	$8.4^{a} \times 10^{4}$
B (95% PWJ: 5% JFS)	$1.08^{c} \times 10^{7}$	$5.0^{a} \times 10^{4}$	$2.4^{d} \times 10^{4}$	$6.1^{b} \times 10^{4}$	$1.01^{d} \times 10^{5}$	$3.8^{d} \times 10^{4}$	$2.2^{e} \times 10^{4}$	$4.8^{b} \times 10^{4}$	$1.02^{d} \times 10^{7}$	$3.2^{e} \times 10^{4}$	$1.9^{e} \times 10^{4}$	$3.7^{b} \times 10^{4}$
C (90% PWJ:10% JFS)	$3.6^{f} \times 10^{6}$	$4.9^{ab} \times 10^{4}$	$2.4^{d} \times 10^{4}$	$4.2^{c} \times 10^{4}$	$7.4^{g} \times 10^{4}$	$3.3^{e} \times 10^{4}$	$2.0^{e} \times 10^{4}$	$3.9^{c} \times 10^{4}$	$2.7^{i} \times 10^{6}$	$3.0^{e} \times 10^{4}$	$1.8^{e} \times 10^{4}$	$3.4^{c} \times 10^{4}$
D (85% PWJ: 15% JFS)	$7.9^{g} \times 10^{5}$	$4.3^{c} \times 10^{4}$	NG	NG	$6.9^{h} \times 10^{4}$	$3.1^{e} \times 10^{4}$	NG	$3.1^{e} \times 10^{4}$	$6.2^{g} \times 10^{4}$	$3.0^{e} \times 10^{4}$	NG	$2.9^{d} \times 10^{4}$
E (80% PWJ: 20% JFS)	$7.4^{i} \times 10^{5}$	$4.0^{c} \times 10^{4}$	NG	NG	$6.1^{j} \times 10^{4}$	$3.0^{e} \times 10^{4}$	NG	$3.0^{e} \times 10^{4}$	$5.3^{i} \times 10^{4}$	$2.2^{f} \times 10^{4}$	NG	NG

Key: JFS- Jack Fruit Seed; WMJ- Watermelon juice; PPJ-Pineapple juice; PWJ-Pawpaw juice; NG- No growth, THBC-The total Heterotrophic Bacterial Count, TCC- Total Coliform Count, TFC- Total Fungal Count, LAB-Total Lactic Acid Bacteria Count, Values with different superscript down a column are significantly different from each other.

Table 2: Total microbial load (cfu/ml) of fruit juice preserved with jack fruit bark

Sample code	24hrs THBC	TCC	TFC	LAB Count	48hrs THBC	TCC	TFC	LAB Count	96hrs THBC	TCC	TFC	LAB Count
A Control (100% WMJ)	$2.60^{a} \times 10^{7}$	$8.1^{a} \times 10^{4}$	1.6^{c} x 104	NG	$2.80^{a} \times 10^{7}$	$8.4^{a} \times 10^{4}$	$2.6^{b} \times 10^{4}$	$2.2^{d} \times 10^{4}$	$3.04^{a} \times 10^{7}$	$8.8^{a} \times 10^{4}$	$4.2^{b} \times 10^{4}$	$3.7^{c} \times 10^{4}$
B (95%WMJ: 5% JFB)	$1.51^{b} \times 10^{7}$	$7.8^{b} \times 10^{4}$	NG	NG	$1.26^{c} \times 10^{7}$	$6.9^{b} \times 10^{4}$	$1.9^{d} \times 10^{4}$	$1.7^{f} \times 10^{4}$	$1.13^{d} \times 10^{7}$	$5.02^{b} \times 10^{4}$	$2.1^{d} \times 10^{4}$	$2.8^{d} \times 10^{4}$
C (90%WMJ: 10% JFB)	$1.36^{d} \times 10^{7}$	$7.0^{c} \times 10^{4}$	NG	NG	$1.18^{e} \times 10^{7}$	$6.4^{d} \times 10^{4}$	NG	NG	$1.03^{e} \times 10^{7}$	$3.9^{h} \times 10^{4}$	NG	NG
D (85%WMJ: 15% JFB)	$1.08^{g} \times 10^{7}$	$6.8^{c} \times 10^{4}$	NG	NG	$8.3^{i} \times 10^{6}$	$5.1^{e} \times 10^{4}$	NG	NG	$8.0^{g} \times 10^{6}$	$4.8^{f} \times 10^{4}$	NG	NG
E (80% WMJ: 20% JFB)	$9.2^{h} \times 10^{6}$	$5.3^{e} \times 10^{4}$	NG	NG	$8.1^{i} \times 10^{6}$	$4.7^{f} \times 10^{4}$	NG	NG	$7.3^{h} \times 10^{6}$	$4.2^{g} \times 10^{4}$	NG	NG
A Control (100% PPJ)	$1.53^{b} \times 10^{7}$	$6.8^{c} \times 10^{4}$	$1.8^{c} \times 10^{4}$	NG	$1.64^{b} \times 10^{7}$	$7.2^{c} \times 10^{4}$	$3.1^{a} \times 10^{4}$	$3.3^{b} \times 10^{4}$	$1.78^{c} \times 10^{7}$	$7.7^{c} \times 10^{4}$	$3.8^{c} \times 10^{4}$	$4.2^{b} \times 10^{4}$
B (95% PPJ: 5% JFB)	$1.43^{c} \times 10^{7}$	$6.4^{d} \times 10^{4}$	$1.8^{c} \times 10^{4}$	NG	$1.16^{e} \times 10^{7}$	$6.0^{d} \times 10^{4}$	$1.7^{d} \times 10^{4}$	$1.9^{e} \times 10^{4}$	$1.03^{e} \times 10^{7}$	$5.1^{e} \times 10^{4}$	$1.3^{f} \times 10^{4}$	$2.2^{e} \times 10^{4}$
C (90% PPJ: 10% JFB)	$1.28^{e} \times 10^{7}$	$5.3^{e} \times 10^{4}$	$7.0^{e} \times 10^{3}$	NG	$9.6^{g} \times 10^{6}$	$4.7^{f} \times 10^{4}$	$1.8^{d} \times 10^{4}$	NG	$8.8^{f} \times 10^{6}$	$4.3^{g} \times 10^{4}$	$2.1^{d} \times 10^{4}$	NG
D (85% PPJ: 15% JFB)	$8.6^{i} \times 10^{6}$	$4.9^{f} \times 10^{4}$	NG	NG	$8.1^{i} \times 10^{6}$	$3.9^{g} \times 10^{4}$	NG	NG	$7.2^{h} \times 10^{6}$	$3.3^{i} \times 10^{4}$	NG	NG
E (80% PPJ: 20% JFB)	$8.2^{j} \times 10^{6}$	$4.3^{g} \times 10^{4}$	NG	NG	$7.8^{j} \times 10^{6}$	$3.1^{h} \times 10^{4}$	NG	NG	$6.8^{i} \times 10^{6}$	$2.6^{j} \times 10^{4}$	NG	NG
A Control (100% PWJ)	$1.49^{c} \times 10^{7}$	$4.7^{f} \times 10^{4}$	$2.6^{a} \times 10^{4}$	$3.1^{a} \times 10^{4}$	$1.61^{b} \times 10^{7}$	$5.2^{e} \times 10^{4}$	$3.4^{a} \times 10^{4}$	$4.2^{a} \times 10^{4}$	$2.12^{b} \times 10^{7}$	$6.3^{d} \times 10^{4}$	$4.8^{a} \times 10^{4}$	$5.7^{a} \times 10^{4}$
B (95% PWJ: 5% JFB)	$1.26^{e} \times 10^{7}$	$4.0^{g} \times 10^{4}$	$2.2^{b} \times 10^{4}$	$2.8^{b} \times 10^{4}$	$1.22^{d} \times 10^{7}$	$3.8^{g} \times 10^{4}$	$2.1^{bc} \times 10^{4}$	$2.4^{d} \times 10^{4}$	$1.17^{d} \times 10^{7}$	$3.1^{i} \times 10^{4}$	$1.8^{de} \times 10^{4}$	$2.4^{e} \times 10^{4}$
C (90% PWJ:10% JFB)	$1.18^{f} \times 10^{7}$	$3.7^{h} \times 10^{4}$	$1.8^{c} \times 10^{4}$	$2.8^{b} \times 10^{4}$	$1.06^{f} \times 10^{7}$	$3.3^{h} \times 10^{4}$	$1.7^{d} \times 10^{4}$	$2.8^{c} \times 10^{4}$	$1.02^{e} \times 10^{6}$	$2.4^{j} \times 10^{4}$	$1.6^{e} \times 10^{4}$	$2.5^{de} \times 10^{4}$
D (85% PWJ: 15% JFB)	$9.6^{h} \times 10^{6}$	$3.1^{i} \times 10^{4}$	$1.8^{c} \times 10^{4}$	$1.6^{c} \times 10^{4}$	$8.8^{h} \times 10^{6}$	$2.9^{i} \times 10^{4}$	$2.3^{b} \times 10^{4}$	$2.2^{d} \times 10^{4}$	$8.0^{g} \times 10^{6}$	$2.0^{k} \times 10^{4}$	$2.0^{d} \times 10^{4}$	$1.8^{f} \times 10^{4}$
E (80% PWJ: 20% JFB)	$9.4^{h} \times 10^{6}$	$2.6^{j} \times 10^{4}$	$1.3^{d} \times 10^{4}$	$1.3^{c} \times 10^{4}$	$7.9^{j} \times 10^{6}$	$1.8^{j} \times 10^{4}$	$1.3^{e} \times 10^{4}$	$1.9^{e} \times 10^{4}$	$7.0^{i} \times 10^{6}$	$1.8^{k} \times 10^{4}$	$1.0^{f} \times 10^{4}$	$1.5^{f} \times 10^{4}$

Key: JFB- Jack Fruit Bark; WMJ- Watermelon juice; PPJ-Pineapple juice; PWJ-Pawpaw juice; NG- No growth
THBC-The total Heterotrophic Bacterial Count, TCC- Total Coliform Count, TFC- Total Fungal Count, LAB-Total Lactic Acid Bacteria Count, Values with different superscript down a column are significantly different from each other.

Table 3: Distribution of microorganisms in 96 hours jackfruit seeds preserved juices

Samples	*E.coli*	*Bacillius* sp	*Salmonella* sp	*Staphylococcus aureus*	*Citrobacter* sp	*Micrococcus* sp	*Lactobacillus* sp	*Rhizopus* sp	*Aspergillus* sp	*Penicillium* sp	Total
WMJ 100:0 (Control)	1(4.5)	5 (21)	1(4.5)	3 (13)	3 (13)	2 (9)	3 (13)	0 (00)	3 (13)	2 (9)	23 (100)
WMJ 95%: JFS 5%	0 (00)	3(27.3)	1(9.1)	2(18.2)	1(9.1)	1 (9.1)	2(18.2)	0 (00)	1(9.1)	0 (00)	11 (100)
WMJ 90%: JFS 10%	0 (00)	2 (50)	0 (00)	1 (25)	0 (00)	0 (00)	1 (25)	0 (00)	0 (00)	0 (00)	4 (100)
WMJ 85%: JFS 15%	0 (00)	1(100)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	1 (100)
WMJ 80%: JFS 20%	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)
PPJ 100:0 (Control)	1(4.8)	4 (19)	3(14.3)	4 (19)	3(14.3)	2 (9.5)	0(00)	1 (4.8)	0(00)	3(14.3)	21(100)
PPJ 95%: JFS 5%	0 (00)	2 (25)	1(12.5)	2 (25)	1(12.5)	1(12.5)	0(00)	0(00)	0(00)	1(12.5)	8(100)
PPJ 90%: JFS 10%	0 (00)	2(66.7)	0(00)	1(33.3)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	3(100)
PPJ 85%: JFS 15%	0 (00)	1(100)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	1(100)
PPJ 80%: JFS 20%	0 (00)	0 (00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)
PWJ 100:0 (Control)	1 (4)	6 (24)	2 (8)	3 (12)	2 (8)	1 (4)	3 (12)	2 (8)	2 (8)	3 (12)	25 (100)
PWJ 95%: JFS 5%	1(9.1)	3(27.3)	1 (9.1)	1 (9.1)	0(00)	0(00)	2(18.2)	1(9.1)	0(00)	2(18.2)	11(100)
PWJ 90%: JFS 10%	0 (00)	2 (50)	0(00)	0(00)	0(00)	0(00)	1 (25)	0(00)	0(00)	1 (25)	4(100)
PWJ 85%: JFS 15%	0 (00)	1(100)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	1(100)
PWJ 80%: JFS 20%	0 (00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)

Key: WMJ = watermelon Juice; PPJ = Pineapple Juice; PWJ = Pawpaw juice; JFS = Jack Fruit Seed

Table 4: Distribution of microorganisms in 96 hours jackfruit bark preserved juices

Samples	*E.coli*	*Bacillus* sp	*Salmonella* sp	*Staphylococcus aureus*	*Citrobacter* sp	*Micrococcus* sp	*Lactobacillus* sp	*Rhizopus* sp	*Aspergillus* sp	*Penicillium* sp	Total
WMJ 100:0 (Control)	1(4)	4 (16)	2(8)	4 (16)	4 (16)	3 (12)	2 (8)	1 (4)	1 (4)	3 (12)	25 (100)
WMJ 95%: JFB 5%	0 (00)	3(25)	1(8.3)	3(25)	2(16.7)	1 (8.3)	1(8.3)	0 (00)	0 (00)	1 (8.3)	12 (100)
WMJ 90%:JFB 10%	0 (00)	1(33.3)	0(00)	1(33.3)	0 (00)	0 (00)	1(33.3)	0 (00)	0 (00)	0 (00)	3 (100)
WMJ 85%:JFB 15%	0 (00)	1(100)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	1 (100)
WMJ 80%:JFB 20%	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)	0 (00)
PPJ 100:0 (Control)	1(5.5)	3(16.7)	3(16.7)	2(11.1)	2(11.1)	3(16.7)	1(5.5)	0 (00)	0(00)	3(16.7)	18(100)
PPJ 95%: JFB 5%	0 (00)	2(25)	1(12.5)	1(12.5)	1(12.5)	1(12.5)	0(00)	0(00)	0(00)	2(25)	8(100)
PPJ 90%: JFB 10%	0 (00)	1(33.3)	0(00)	1(33.3)	0(00)	0(00)	0(00)	0(00)	0(00)	1(33.3)	3(100)
PPJ 85%: JFB 15%	0 (00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)
PPJ 80%: JFB 20%	0 (00)	0 (00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)
PWJ 100:0 (Control)	1 (4.3)	4(17.4)	1 (4.3)	2 (8.7)	1 (4.3)	2 (8.7)	3(13.1)	2 (8.7)	3(13.1)	4(17.4)	23 (100)
PWJ 95%: JFB 5%	0 (00)	2(22.2)	0 (0.0)	1(11.1)	0(00)	1(11.1)	1(11.1)	1(11.1	1(11.1)	2(22.2)	9(100)
PWJ 90%: JFB 10%	0 (00)	1 (50)	0(00)	0(00)	0(00)	0(00)	0 (00)	1(00)	0(00)	1 (50)	2(100)
PWJ 85%: JFB 15%	0 (00)	1(100)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	1(100)
PWJ 80%: JFB 20%	0 (00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)	0(00)

Key: WMJ = watermelon Juice; PPJ = Pineapple Juice; PWJ = Pawpaw juice; JFS = Jack Fruit Bark

DISCUSSION

Fruits such as pineapple and watermelon contain high concentrations of vitamins, minerals, amino acids, and sugars, thereby providing an excellent environmental condition for the development of a wide range of microorganisms that can lead to the spoilage of these fruits (Rahman *et al*., 2011; Khanam *et al*., 2018). Hence, to minimize or prevent the spoilage of these fruits, the use of natural preservatives is imperative.

In this study, the bacterial and fungal population enumerated showed a decrease in the microbial counts of fruit juices treated with jackfruit seeds and bark during the 96 hours storage period.

As the days of storage progressed, the rate of deterioration of the fruit juices declined**.** Microbial populations were lower in the preserved juices compared to unpreserved/ untreated fruit juices (controlled samples). This may be as a result of the contents in jackfruit which can inhibit and cause decline in the growth of the microorganisms in the juices (Burci *et al*. 2018). This is in accordance with the findings of Fauzi, *et al*. (2013), which states that jackfruit wood has antimicrobial substances which can affect microorganism activities and act as bacteriostatic in lower concentration and as bactericidal in high concentration. It may also be due to the synergistic effects of the bioactive phytochemical constituents in the extracts. This agrees with the findings of Adeogun *et al*. (2016) who attributed the activity of natural antimicrobials present in *Thaumatococcus daniellii* to be due to the synergy among the avalanche of phytoconstituents present in the plant extracts which was also observed in this study.

The various concentrations of the treatment ratios is directly proportional to the microbial loads obtained as the higher the treatment concentration, the lower the microbial load. This suggests that the higher the concentration of treatment addition, the more effective the preservation of the stored juice would be. The decrease in the bacterial load could also be as a result of the decrease in the pH of the juice as the storage period progresses as most bacteria such as those present in this study do not grow at low pH, while the reduction in fungi load could be due to deposited antimicrobials (Ike *et al*., 2020).

From this study, *Escherichia coli, Bacillus* species, *Salmonella* species*, Staphylococcus aureus*, *Citrobacter* species, *Micrococcus* species, *Lactobacillus* species, *Rhizopus* species*, Aspergillus* species and *Penicillin* species were isolated. These contaminating

microorganisms comprises of both spoilage and potential pathogenic organisms. The presence of microorganisms in the fruit juices may be as a result of the quality of the raw materials used, the equipment used for processing, handling practices employed, unsanitary storage conditions used and the environment (Afroz *et al*., 2013). In this study, the presence of *E. coli* and *Salmonella* was identified only in very few numbers and in a smaller part of the samples. The presence of *E. coli* and *Salmonella* in fruit juices are of great concern as these pathogens are of feacal origin and have been associated with a lot of outbreaks related to fruit juices (Raybaudi-Massilia *et al*., 2009). *Bacillus* species were detected across all the fruit juice samples investigated and were the most occurring microorganism in all fruit juices preserved and analyzed. This could be due to the presence of their endospores which are able to tolerate environmental stresses (Willey *et al*., 2008; Ndip and Njom, 2019). *Staphylococcus* sp are common contaminants that are often introduced into foods from handlers of the foods and the environment. The presence of *Staphylococcus* spp in the juice could be attributed to post process contamination. Lactic acid bacteria (LAB) regularly occur in juices that are unpasteurized (Oliveira *et al*., 2006). *Lactobacillus* and *Leuconostoc* have been reported as important spoilage microorganisms in products that are acidic (Keller and Miller, 2006). Lactic acid bacteria (LAB) produces lactic acids in fruit juices along with small amounts of acetic and gluconic acids, ethanol and carbon dioxide, thereby resulting in the alteration of the flavour of the juice (Jay and Anderson, 2001) and reduction in the pH of the juices. Moulds which are also spore formers and common food contaminants from the environment are able to cause spoilage of fruit juices by causing loss of juice cloud (Lawlor *et al*., 2009). Amidst these, some moulds such as *Aspergillus* and *Penicillium* species have the potential of producing mycotoxins which pose great risks to the health of humans who consume the juices. *Rhizopus* species are also known to cause the spoilage of fresh fruits, juices and vegetables (Moss, 2008). Hence, in order to prevent spoilage and food borne illness, there is need to control the existence/ presence of these organisms in fruit juices even in low numbers (Mudgil *et al*., 2004; Oranusi *et al.,* 2007).

In general, the distribution of microbial population during storage was quite low and some totally disappeared at the end of the 96 hours of storage. Less numbers of microorganisms were isolated compared to unpreserved fruit juices (controlled samples). The low microbial counts could be associated to the antimicrobial effect of jackfruit seed and bark. The added ratios of the jackfruit seeds and bark might have prevented or halted the growth of microbes. Sousa *et al*. (2021) have reported the bactericidal (antimicrobial) effect of jackfruit dry leaf extracts against *E. coli,* methicillin resistant *S. aureus* and *S. enterica.* Also, Ruiz-Monta´nez *et al.* (2015) and Burci *et al*. (2018) have reported the effective antimicrobial activity of jackfruit against microorganisms such as *Streptococcus mutans*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis, Pseudomonas aeruginosa, Penicillium italicum* and *Candida albicans* which are similar to the microorganisms isolated from this study.

CONCLUSION

The demand for fresh wholesome fruit juices has been on the increase because of their health benefits. The major challenge with fresh fruit juices is the presence of microorganisms which are potentially hazardous to public health and their limited shelf life resulting in continuing usage of synthetic chemicals as preservatives. Hence, by addressing these concerns, the potential risks to consumer health can be mitigated and the safe consumption of fruit juices ensured by the addition of natural antimicrobials of plant such as jackfruit to fruit juices which are with little or no negative health consequences. Jack fruit seed and bark extracts has been found quite effective as a

bio-preservative, therefore could serve as a good candidate for use in extending the shelf life of fruit juices. This is based on the ability of the extracts to effectively prevent or inhibit the growth of spoilage and pathogenic microorganisms isolated in this study and making the consumption of wholesome juices realistic. This will make maintaining the quality of fruit juices and avoiding food borne disease outbreaks an easy task.

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Profiling Phytochemical Constituents and Antibacterial Efficacy of Ethanol Extract *of Anacardium occidentale* Linn (Cashew) Slender Branches

Jesse I. A.[1*] Istifanus T.[1] Mafe A. N.[1] Oloninefa S. D.[2] Attah F.[3] and Targema B. T.[4]

1. Department of Biological Sciences, Taraba State University, Jalingo, Taraba State, Nigeria
2. Department of Biological Sciences, Kogi State University Kabba, Kogi State, Nigeria
3. Department of Microbiology, Confluence University of Sceince and Technology, Osara, Kogi State, Nigeria
4. Department of Biological Sciences, Applied Parasitology Unit, Taraba State University, Jalingo, Taraba State, Nigeria

* Corresponding author: jesseinnocent75@gmail.com

Abstract: Antibiotic resistance in most bacterial infections remains a threat to humanity. This necessitates the search for natural sources of remedy from *Anacardium occidentale* Linn (cashew) slender branches. The plant slender branches were air dried for four weeks at 25°C and process for extraction. Cold maceration method was used to obtain the extract using ethanol as menstruum. Antibacterial susceptibility test (AST) of the extract was carried out against *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* obtained from the department of Biological Science Laboratory Ciprofloxacin and dimethyl sulfoxide (DMSO) were used as positive and negative controls respectively. Ethanol extract at 200 mg/ml, shows 17.5±0.5 mm zone of inhibition against *P. aeruginosa*, 16±1.0 mm against *E. coli*, 14±0.0 mm against *P. mirabilis* and 11±1.0 mm against *S. aureus*. *Pseudomonas aeruginosa* was the most susceptible, while *S. aureus*, the most resistant to the extract. The findings of MIC and MBC revealed that the extract was bactericidal at 250 mg/ml. Profiling the phytochemical constituents revealed the presence of alkaloids, flavonoids, quinone, phenols, saponins, and carotenoids in the extracts. High Performance Liquid Chromatography shows the presence of quercetin a flavonoid, chlorogenic acids a phenol and testosterone which could be responsible for its antibacterial activity. This study reveals that *A. occidentale* Linn slender branch has antibacterial activity and could be use as precursor for drugs development.

Key word: Bacteria, extract, resistant, phytochemical

INTRODUCTION

The *Anacardium occidentale* Linn. (cashew), belong to the family anacardiaceae (Kannan *et al.,* 2009). It is a tree that grows up to 1.5 m in height with thick tortuous trunk and woody branches native to northeast Brazil with great economic and medicinal value (Rajesh *et al*., 2009), but is now cultivated extensively in all tropical areas, notably in India and East Africa. The main producing countries of cashews are India, Cote d'Ivoire and Vietnam (Salas-Salvadó and Pascual-Compte*,* 2023). *Anacardium occidentale* is commonly called Cashew in English, "Yazawa" in Hausa, "Okpokpo" in Igbo and Kaju in Yoruba (Arekemase *et al*., 2011). *Anacardium occidentale* (L) leaves is consumed fresh in some regions of Asian, American and African countries. The slender branches are used in some regions of Nigeria as tooth brush (Oviasogie *et al.,* 2016). It has been used in folk medicine to treat gastrointestinal disorders (acute gastritis, diarrhoea), mouth ulcers as well as throat problems according to Kudi *et al*. (1999). Sadiq *et al*. (2009) reported that *Anacardium occidentale* leaves, stems and bark extracts are used extensively for the treatment of diarrhoea, dysentery and colonic pain. Natural antimicrobials can be found in the ethanolic extracts of cashew leaf such as flavonoids, tannins, saponins, anthocyanins, and alkaloids. In *in vitro* experiments, tannins in cashew leaf has been reported to have antimicrobial and fungicidal substances (Arekemase *et al.,* 2011; Anand *et al*., 2015). Compounds such as flavonoids and quercetin in cashew leaves are also known as natural antimicrobials that can protect the body from pathogen attack (Ajileye *et al*., 2015). Hassan *et al*. (2019) concluded in their study that leaf extracts of *Anacardium occidentale* dissolved in distilled water and

ethanol had good potential for the development of antibacterial drugs for urinary tract pathogens like *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* after an *in vitro* cup-plate method of agar diffusion technique experiment.

Infectious diseases are responsible for 45% of deaths in low- income countries and 50% of premature death worldwide (Gangoue, 2007). In addition, among the death caused by microorganisms, bacterial infections account for 70% of cases (Antimicrobial Resistant Collaborators, 2022). To control these pathogens, antibiotics are frequently used but, unfortunately, the emergence of antibiotic-resistant bacteria has put an end to this wave of optimism (Adejuwon *et al*., 2011). About 64% of people infected with multidrug resistant *Staphylococcus aureus* (MRSA) infections are more likely to die than people infected with drug-sensitive species (WHO, 2022). On the contrary, mortality because of drug-resistant strains of *Pseudomonas aeruginosa* infections is ever-increasing, accounting for about 11% of hospital-acquired bacterial infections (Nwobodo *et al*., 2020). According to the WHO facts sheet on antimicrobial resistance, resistance to ciprofloxacin, an antibiotic often used to treat urinary tract infections, ranged from 8.4% to 92.9% for *Escherichia coli* (Yenehun *et al*., 2021). To face this increasing inefficiency observed with available antibacterials, it is essential to seek a novel broad spectrum action antibacterial substance with more effective action. Hence, this study seeks to investigate the phytochemical profile and antibacterial efficacy of *A. occidentale* Linn slender branches extract against *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa***.**

MATERIALS AND METHODS

Study area: This study was carried out in the Department of Biological Science Laboratory, Taraba State University, Jalingo, Taraba State, Nigeria. Jalingo is located on latitude 8°54’ to 9°01’N and longitude 11°22’ to 11°30’E (Garba *et al.,* 2018).

Collection of plant materials: The plant materials were collected from Taraba State University farm, identified and authenticated by a botanist in the Department of Biological Sciences, Taraba State University, Taraba State Nigeria.

Preparation of plant extract: The authenticated slender branches of *A. occidentale* were air-dried for four weeks at 25ºC. The samples were ground using mortar and pestle into fine powder. Cold maceration extraction of the plant was made as described by Thomas *et al*. (2012) using ethanol as menstruum in a standard volumetric flask. Hundred grams of the plant sample was measured into a beaker containing ethanol. The set-up was left for 48-72 hours with frequent agitation. It was then filtered with a muslin cloth. The filtrate was evaporated to dryness in a rotary evaporator at 45ºC. The percentage yield of the extract (Y%) was calculated using the formula reported by Abbas *et al.* (2021):

$$Yield\ (\%) = \frac{Weight\ of\ solvent\ free\ extract}{Dried\ extract\ weight} \times 100$$

Sterility test of the dried extract: The slender branch extract was tested for the growth of contaminants. The extract was inoculated on nutrient ager and checked for sterility. The plate was incubated at 37ºC for 18-24 hours. The plates were observed for any sign of visible growth. No growth on plates indicates a sterile extract and successful extraction process.

Medium and test organisms: Pure isolates of *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa,* were obtained from the Biological Science Laboratory’s culture bank and authenticated using standard microbiological techniques according to the method of Efuntoye *et al*. (2010).

Antibacterial susceptibility testing: The antibacterial activity of the extract on the confirmed pure cultures of *Staphylococcus aureus, Proteus mirabilis, Escherichia coli,* and *Pseudomonas aeruginosa* was determined by the standard agar well

diffusion technique. The crude extract reconstituted using 20% DMSO to give the following concentration 200 mg/ml, 150 mg/ml, 100 mg/ml, and 50 mg/ml (Jesse *et al.,* 2021). Agar well diffusion method was carried out using nutrient agar and cork borer (8mm) to bore hole through the agar and appropriately label. The inoculum was manually adjusted to equal a 0.5 McFaland standard that was freshly prepared using barium sulphate and sulphuric acid. Incubation for 18-24 hours, the antibacterial efficiency of the slender branch-extract was determined by measuring the zone of inhibition formed around the well with a transparent metre rule (Cheesebrough, 2000; Caleb *et al.,* 2022).

Minimum inhibitory concentration and Minimum bactericidal concentration of extracts: The MIC of the extract was determined according to the micro broth dilution technique. Standardized suspensions of the test organism were inoculated into a series of sterile tubes of nutrient broth containing two-fold dilutions of leave extract and incubated at 37ºC for 24 hours. Alongside broth and extract control test tube were prepared. The MIC was read as the least concentration that inhibited the visible growth of the test organisms NCCLS (2000). The MBC was determined by selecting tubes that shows no visible growth during MIC determination and sub cultured onto nutrient agar plates using the spread plate technique and incubate for 18-24 hours at 37ºC. The least concentration, at which no growth was observed, was observed and recorded as the MBC (Ibekwe *et al*., 2001; Jesse *et al.,* 2021).

Phytochemical screening of A. occidentale slender branch extracts: The preliminary qualitative phytochemical screening was carried out using the method previously described by Trease and Evans, (1989) and Abalaka *et al*. (2010) with slight modification.

Detection of Alkaloids: Wagner's test: Wagner's reagent was added to the extraction if a brown-reddish formation is observed, and it indicates the presence of alkaloids (Kaur *et al.*, 2016).

Detection of Phenols: Add few drops of ferric chloride to 10mL of extract. A bluish-black colour indicates the presence of phenol (Shah *et al.,* 2015).

Detection of Flavonoids: Add few drops of sulphuric acid to the extracts, and the formation of orange colour indicates the presence of flavonoids (Roghini & Vijayalakshmi 2018).

Detection of Saponins: A 0.5 mg of the extract was mixed vigorously with 5 mL of distilled water. The formation of frothing indicates the presence of saponins (Mir *et al*., 2015)

Detection of Quinone: Take 1 gram of the extract and dissolve it in 5 ml of distilled water. Transfer 1 ml of this solution to a 5-milliliter test tube. Add 1 ml of concentrated sulfuric acid to the test tube. The formation of a red color indicates the presence of Quinone (Mir *et al*., 2015).

Detection of Carotenoid: Place 1 gram of the extract into a test tube. Add 1 ml of chloroform to the test tube and shake vigorously. Filter the mixture using Whatman filter paper. Add 85% sulfuric acid to the filtrate. The presence of a blue-colored precipitate at the interface indicates the presence of carotenoids (Roghini & Vijayalakshmi 2018).

Statistical analysis of data obtained: Data generated were subjected to statistical analysis. Results are expressed as mean values ± standard error (S.E.) of duplicate determinations. Data were analyzed using one-way Analysis of Variance (ANOVA) and Duncan's New Multiplier Range Test for mean separation at a 5% level of significance with Statistical Package for Social Sciences (SPSS) software version 21. Differences were considered significant at $p < 0.05$ (Jesse *et al.,* 2021).

RESULTS

Yield of extract *A. occidentale*

The percentage yield of ethanol extract obtained from *A. occidentale* Linn. slender branches extract to be 8.38%.

Antibacterial susceptibility of the isolates

The findings of the Antibacterial Susceptibility Test (AST) of *A. occidentale* Linn. slender branch ethanol extracts against *E. coli, S. aureus, Proteus mirabilis* and *Pseudomonas aeruginosa.* The concentrations of the extract used were 50, 100, 150 and 200 mg/ml. *Escherichia coli* had the following zone of inhibition 12.5±0.5, 14.0±0.5, 14.5±0.5 and 16.0±1.0 mm. *Staphylococcus aureus* was only susceptible at a concentration 200 mg/ml with 11.0±1.0 mm zone of inhibition. Zone of inhibition of 11.5±1.5, 12.5±0.5 and 14.0±0.0 mm was reported for *P. mirabilis,* while 13.0±0.0, 14.5±0.0, 15.0±0.0 and 17.5±0.5 mm zones of inhibition on *P. aeruginosa.* Ciprofloxacin exhibited 19.5±05, 20.0±0.0, 19.5±0.5 and 27.5±0.5 mm zones of inhibition against *E. coli, S. aureus, P. mirabilis* and *P. aeruginosa* respectively. No inhibition was observed for DMSO.

Minimum inhibitory concentration (MIC) and Minimum bacteriacidal concentration (MBC) of *A. occidentale* Linn. slender branches extract

The minimum inhibitory concentration on *E. coli, P. mirabilis* and *P. aeruginosa* was 125 mg/ml while for *S. aureus* it was 250 mg/ml. The four isolates had MBC at 250 mg/ml as revealed in Table 2.

Phytochemical compositions of the plant extracts Table 3, showed the results of the qualitative phytochemical screening of the plant extracts. It was observed that alkaloid, flavonoid, phenol, quinone, and saponins were the present while carotenoid and quinone were absent.

High performance liquid chromatography (HPLC) of *A. occidentale* Linn. slender branches ethanol extract

Figure 1 reveals the chromatogram of *A. occidentale* Linn. slender branches ethanol extract, quercetin, testosterone, and chlorogenic acids were confirmed present by HPLC analysis. For a gram of the slender branch extract, 6.0% of quercetin, 2.6% of testosterone, and 91.3% of chlorogenic acids were observed to be present.

Table 1: Antimicrobial susceptibility test (AST) of ethanolic extract of *A. occidentale* slender branch against some bacteria

Conc./Control (mg/ml)	Bacteria zones of inhibition (mm)			
	E. coli	*S. aureus*	*P. mirabilis*	*P. aeruginosa*
50	12.5±0.5	-	-	13.0±0.0
100	14.0±0.5	-	11.5±1.5	14.5±0.0
150	14.5±0.5	-	12.5±0.5	15.0±0.0
200	16.0±1.0	11.0±1.0	14.0±0.0	17.5±0.5
Cipro	19.5±0.5	20.0±0.0	19.5±0.5	27.5±0.5
DMSO	0±0.0	0±0.0	0±0.0	0±0.0

Key: - = No zone of inhibition, DMSO; Dimethyl sulfoxide (negative control), CIPRO; Ciprofloxacin (positive control), *zone of inhibition size mean ± standard error of mean of duplicate determination

Table 2. MIC and MBC of *Anacardium occidentale* slender branches extract

Bacteria	MIC (mg/ml)	MBC (mg/ml)
E. coli	125	250
S. aureus	250	250
P. mirabilis	125	250
P. aeruginosa	125	250

Key: MIC; minimum inhibitory concentration, MBC; minimum bactericidal concentration.

Table 3. Phytochemical profile of *Anacardium occidentale* slender branch crude extract

Phytochemical components	Availability (Present/Absent)
Alkaloid	++
Carotenoid	-
Flavonoid	+
Phenol	++
Quinone	-
Saponin	+

Key: + = Present, - = Absent

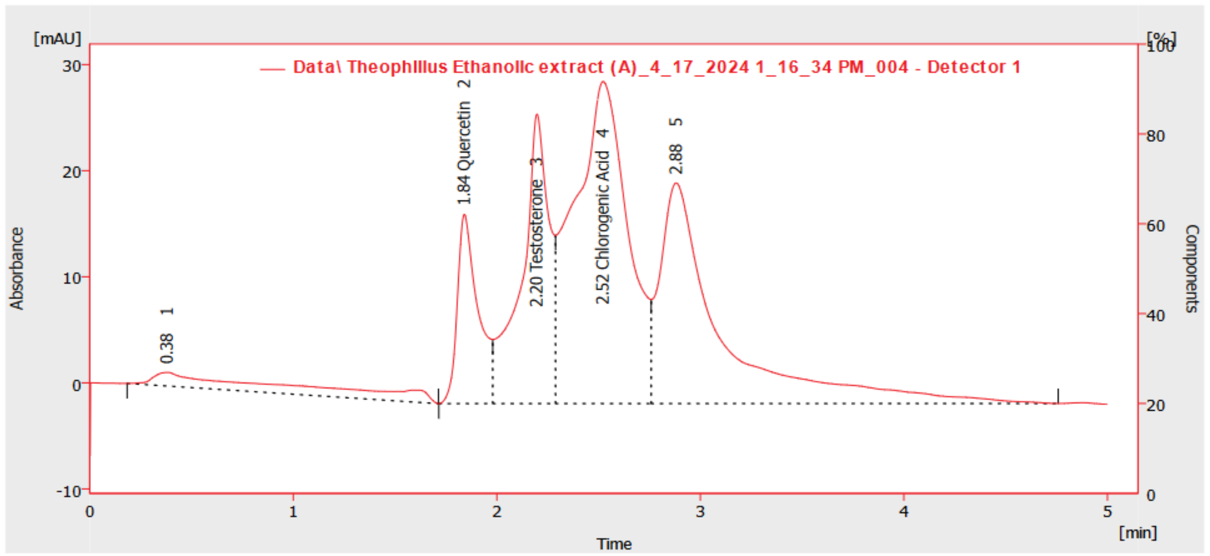

Figure 1: Chromatogram of *A. occidentale* Linn. slender branches ethanol extract

DISCUSSION

In this study, *A. occidentale* L. slender branches ethanol extract had 8.38% yield. The low percentage yield of the ethanol extract could be attributed to the high amount of husks in the plant slender branches. A contrasting findings was reported by Jesse *et al.* (2021) who observed 33.08% for petroleum ether and 30.31% yield for n-hexane extract of *A. occidentale* L. shell oil. The difference could be attributed to the type of menstruum used, plant part and method of extractions.

The findings of the antibacterial susceptibility test of the ethanol extract shows that *P. aeruginosa* (17.5±0.5 mm) was the most susceptible follow by *E. coli* (16±1.0 mm), *P. mirabilis* (14±0.0 mm) and then *S. aureus* (11±1.0 mm). This reveals that the extract possesses antibacterial substances, which agrees with the work of Sadiq *et al.* (2009) on the same plant. The extracts activity against *E. coli* justifies the traditional use of *A. occidentale* in the treatment of diarrhea. This is similar to the study of Ayepola and Ishola (2009), where the aqueous extract of the same plant have activity on the same test bacteria. Similarly, Chabi *et al.* (2014) and Anand *et al.* (2015) independently reported the ethanol leaf extract of *A. occidentale* to have antibacterial activity against the following bacteria pathogens *Enterococcus faecalis, S. aureus, Streptococcus mutans,* and *E. coli*. This could be due to certain compounds that could be responsible for their activity. Furthermore, the extract activity aligns with the work of Abalaka *et al.* (2009) and Oloninefa *et al.* (2018) on plants extracts.

Ciproflaxacin was use as positive control which give the greatest zone of inhibition as compare to the crude extract, this is no surprise because of the level of its purity. The crude extract might perform better than positive control if the extract is further refined and devoid of impurities (Parasa *et al.,* 2011). In the case of the negative control, DMSO gave the expected result because is know not to have any antimicrobial activity (Oloninefa *et al.,* 2016).

The MIC and MBC of the extract reveals that at certain concentration it is bacteriostatic and at higher concentration is bactericidal and this finding is at par with the studies of Akash *et al.* (2009). This shows that the extract can completely kill the test bacteria and effectively treat the infection cause by these pathogens (Ochei and Kolhatkat, 2010).

The phytochemical profile of the slender branches extract reveals the presence of

alkaloids, flavonoids, phenols and saponins. This was equally reported Goncalves *et al.* (2005). The presence of tannins, alkaloids, saponins, terpenes and flavonoids in *A. occidentale* leaves and the slight difference in phytoconstituents could be due to the season in which the plants materials were harvested, method of extraction and the phytochemical analysis that were carried out. Quercetin, testosterone and chlorogenic acids were confirmed in the extract by HPLC analysis. These bioactive compounds are known to have antimicrobial activities, alkaloids with anti-diarrhoeal effect, flavonoids inhibits *Vibrio cholerae*, *Streptococcus mutans*, S*higella* species, and viruses, and other phenolic compounds that are antibacterial and antifungal (Oloninafa *et al.,* 2018). Ajileye *et al*. (2015) reported that compounds such as quercetin and chlorogenic acids members of flavonoids and phenols family respectively in cashew leaves are also known as natural antimicrobials that can protect the body from pathogen attack. Thus, the antibacterial activity of the extracts on the test organisms may be due to the presence of these phytochemical components.

CONCLUSION

Anacardium occidentale Linn. slender branches extract has high (++) phytoconstituents profile. The High-Performance Liquid Chromatography (HPLC) shows that quercetin a flavonoid and chlorogenic acids such as phenol are present in large amount (++), hence its antibacterial activity. Further study needs to be done to purify and isolate the actual compound(s) that could be responsible for its activity.

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Evaluation of Fungi Infestation of Stored White and Yellow Cassava Garri in Jos North Metropolis

John W. C.[1*] Ishaya M.[2] Ihum T. A.[3] Joshua V. I.[1] Dinchi P. T.[1] and Sikiru G. K.[1]
1. Federal College of Forestry, Jos, Nigeria
2. Federal College of Forestry Resources Management, Maiduguri, Nigeria.
3. Nigerian Stored Products Research Institute (NSPRI) Ilorin, Kwara State, Nigeria.
* Corresponding author: jowaltee@gmail.com

Abstract: Food security is required national development and for human survival. However, it is important that food security should not be seen only in the perspective of availability but also on safety. This study determined the mycological quality of selected white and yellow Garri sold within Jos North metropolis. The study was conducted at the biology laboratory of Federal College of Forestry, Jos. A total of 24 samples (12 white and 12 yellow) were purchased from Katako, Terminus, Busa buji and New market. The sample were collected in sterile polyethylene nylon. The fungi were isolated using prepared and sterilized Patatose dextrose agar. Identification of fungi was carried using microscopic method. Disk Diffusion Technique on Muller Hilton Agar (MHA) was used to determine fungal sensitivity. Data were statistical analyses using ANOVA and t-test at *p*value ≥ 0.05. The means was separated using Duncan's Multiple Range Test. The result revealed white Garri from Terminus has the highest fungal load of 14.50± 1.26 Sfu/gl. Busa buji showed the lowest value of 5.50±1.53 Sfu/gl. The Fungi isolated were *Aspergillus niger, Curvilaria* sp*, Penicillium* sp*, fusarium oxysparum, Geitricum* sp and Macrophrosis. *Aspergillus* sp had the highest frequency of occurrence (28.95% and 23.68%) in both white and yellow Garri while Macrophrosis had the least frequency occurrence of 2.63%. The pH values of white and yellow Garri ranges between 5 and 6 and moisture content showed that yellow Garri had the highest moisture content of 21.76 ± 1.24. The isolate were more sensitive to keteconarzole than fluconazole and Griseofulvin. The findings revealed that consumers are exposed to the risk of fungal infestation. Therefore renewed vigilance on the efficiency of garri processing condition, handling and storage is required.
Key word: Garri, fungi, pH, moisture content, consumers

INTRODUCTION

Cultivation of cassava (*Manihot esculanta* Crantz) is majorly needed for human consumption. It is an important staple food in Tropical Africa including Nigeria (Chinwe *et al.,* 2016). Worldwide, Nigeria is among major cassava producing countries (Obi et al., 2022). Cassava production is geared towards alleviation of the food crisis in developing regions and is suitable because of its sufficient nutrient, high energy yielding, availability, tolerance and adequate farming methods in Africa (Chinwe *et al.,* 2016; Obi *et al*., 2022).

Garri is a fermented bye product of cassava (*Manihot esculenta* Crantz) tubers and is well known, it is widely consumed in West and Central Africa. Ten million tons of Cassava is estimated to be cultivated in Nigeria alone per annum (Okafor *et al.*, 2018). In Nigeria and most West African countries, Garri is one of the commonly consumed and preferred Cassava products due to it affordability, easy to cook and long shelf life (FAO, 2010; Oluwafemi and Udeh, 2016; Awoyale *et al*., 2021). Majority of the cassava harvested from farms in Nigeria are being transformed into Garri (Adebayo *et al*., 2012; Okolo and Makanjuola, 2021).

However, as nutritious and acceptable as Garri can be, the unhygienic methods experienced in processing of the cassava roots into Garri predisposes the product to a lot of microbial contaminations and, this has serious health implications. Microbial contamination of garri could arise due to processing conditions, storage methods, and storage containers (Akindele and Abimbola, 2018). The high carbohydrate content of Garri encourages fungal growth (Aguoru *et al*., 2014). Moulds such as *Penicillium, Aspergillus*, *Rhizopus*, *Fusarium*, *Mucor* and *Cladosporium* have been implicated with processed garri (Aguoru *et al*., 2014; Ezekiel *et al*., 2020; Orpin *et al*., 2020; Tersoo-Abiem *et al*., 2020; Obi *et al*., 2022). Report has suggested that moisture content create suitable condition for mould proliferation and contamination of garri during storage

(Halliday *et al*., 1967). *Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium* spp presence in garri has been reported in many studies can also produce aflatoxins (Ezekiel *et al*., 2020; Tolulope *et al.,* 2020), which have serious effects on health especially when consume in high dosage.

Garri is largely sold in open market and consume without any form of pre-treatment which may endanger consumer's health (Okafor *et al*., 2018). Development of moulds in Garri may lead to changes in the nutritive quality, organoleptic and microbiological content, finally leading to product spoilage. Based on the negative health effects of post-harvest fungal deterioration of Garri, it becomes important to supply baseline information on the types of fungal flora associated with Garri in Jos and possible management measures. This study was aimed at determining the mycological quality of selected white and yellow Garri sold within Jos North metropolis for consumer's awareness.

MATERIALS AND METHODS

Study area: The study was carried out at Biology Lab of Federal College of Forestry Jos North Local Government Area of Plateau State located at Northern Guinea Savannah at Latitude $9^0$55 and $8^0$55 Longitude. It has an average elevation of 1.250m above sea level and stands at a height of about 600m, above surround plain. The average temperature ranges between 21^0C and 25^0C. The climate of the state is cool due to its high altitude. Rainy season is usually April to September while dry season is October to March. The mean annual rainfall is 1.260mm (Wuyep and Daloeng, 2020).

Sample collection: A total of 24 samples (12 Yellow and 12 White garri 20 gram each) were collected randomly from three major markets within Jos metropolis. Samples were collected in labelled sterile polyethylene bags and transported to Biology laboratory of Federal College of Forestry Jos for analysis after three days.

Sample preparation and isolation of fungi from garri: Ten (10) grams of potato dextrose agar was dissolved in 250mls of distilled water, the dissolved medium was thoroughly mixed using a sterilized glass rod and autoclaved at 121°C for 15minutes. One (1) gram of each sample was suspended in 9 ml of sterilized distilled water in a test tube, the samples were homogenized and a ten (10) fold serial dilution technique was carried out by dispensing 1ml of the suspension into another 9 ml of distilled water up to the 10th test tube. One (1) milliliter of the diluents were collected and dropped on the already solidified Potato Dextrose Agar in a glass plates. Incubation of the inoculated plates were done at 25°C for 7 days and were examined after 3 days for possible growths.

Cultural characteristics: Macroscopically, the fungal isolates were characterized according to size, growth pattern, colour, pigmentation and texture (Tolulope *et al*., 2020).

Microscopy identification of fungi from garri: The fungal isolates were identified by picking small portion of the subculture fungi. The picked culture were teased and placed on a grease free clean glass slide containing Lactophenol cotton blue stain, and covered with a cover slip. The slide was observed under microscope with 10x and 40x objective lens. The presence of conidial heads, conidiosphores, philiades and rhizoids were recorded (John *et al*., 2016).

***Antibiogram assay of isolated fungi*:** The method described by Olabode *et al*. (2016) was adopted. This was carried out by disk diffusion technique on Muller Hilton Agar (MHA). Methylene blue was added to the surface of the agar and allowed to air dry prior to the inoculation of the fungal isolates. The fungi were inoculated by dipping a sterile swab into the standardized inoculums suspension (0.5 McFarland standards, 10^6 cells/ ml) and streaked over the agar surface. The plates were allowed to dry for 15mins at ambient temperature and the prepared antifungal drugs in 10 mg/ml was applied. The zones of inhibitions of the isolates were

taken using meter ruler after 72 hours of incubation to determine sensitivity.

Determination of moisture content of garri sample: This was done by a modification of method describe by Aguoru *et al*. (2014). The moisture content of each of the garri samples was determined immediately after collection. This was carried out by weighing 5.0g of the garri and drying in an oven maintained at 60°C for 10 h to obtain a constant weight. This was followed by placing the sample in a desiccator to cool before re-weighted. The difference in weight of the garri was taken to determine the moisture content.

pH determination of garri sample: The pH of the different samples of yellow and white garri was examined following method described by Ogiehor and Ikenebomah (2010). Ten grams of each sample was homogeneous in 10 ml of distilled water and the pH of the suspension determined using a glass electrode pH meter (Hach pH 1500).

Analysis of data collected: The various data obtained from this study was subjected to statistical analyses: ANOVA and t-test was used to compare significant difference between the treatments at *p*value ≥ 0.05. The means was separated using Duncan Multiple Range Test (DMRT).

RESULTS

Total viable fungal counts of white and yellow garri obtained from different markets in Jos North

The findings in Table 1 showed the Total Viable Fungal counts of white and yellow garri from the different markets. White garri collected from Terminus market gave the highest fungal load of 14.50 ± 1.26 sfu while Busabuji market showed the lowest value of 5.50 ± 1.53 sfu. The findings of the White garri indicated significant difference between the various markets at $p \leq$ value 0.05 while the findings of the Yellow garri indicated no significant difference between the various markets at *p*-value 0.05. The results in Table 1 also indicated that Yellow garri has the highest fungi load of 19.31 ± 0.51 compare to White garri with 9.23 ± 0.96. The result of the t-test indicated significant difference at p value ≤ 0.01.

Macroscopic and microscopic features of the fungi isolates from Yellow and White garri

Plate 1 demonstrated the fungi identification (Macroscopic and Microscopic features) from yellow and white garri collected from the various markets. The probable fungi isolate identified include *Aspergillus niger*, *Cuvurlaria lunata*, *Penicillum* sp, *Fusarium oxysporum* and *Geotricum* sp.

Frequency Distribution and Percentage of Fungal Isolates from Garri

Table 2 revealed Frequency Distribution and Percentage of Fungal Isolates from Garri. A total of 7 fungi genera were isolated from garri obtained from the various markets. Penicillum species with 28.95% gave the most frequently occurring mould compared to other species. *Microsphaerosis*sp showed the least occurring with 2.63%.

Changes in pH content of white and yellow garri at ambient temperature.

Table 3 indicated the results of Changes in pH content of white and yellow garri at ambient temperature. Results showed white garri from Katako market with the highest pH value of 6.15 ± 0.04 but statistically, the findings showed there was no significant different in pH among the various markets. Also the comparative study indicated no significant different existed in pH values between yellow and white garri.

Changes in moisture content of white and yellow garri at ambient temperature.

Table 4 indicated the changes in moisture content of white and yellow garri at ambient temperature. Fingings showed yellow garri from Terminus market with the highest moisture value of 21.76 ± 1.24 but statistically, the findings showed there was no significant different in moisture among the various markets. White garri obtained from Terminus market indicated lowest moisture value of 11.79 ± 0.87 but statistically, the findings showed there was no significant different in moisture among the various markets. The comparative study indicated significant different existed in

Moisture values between yellow and white garri from Terminus and Busabuji market.

Antifungal sensitivity (Zone of Inhibitions (mm) At Day 3 Incubation.

The findings in Table 5 indicated the Antifungal sensitivity of the various fungal isolates after 3 days of incubation. *Geotrichum* sp and *Microsphearosis* sp showed the highest sensitivity by zone of inhibition of 4.07 ± 0.09, 4.13 ± 0.03 and 4.23 ± 0.09 mm to Ketoconazole, Fluconazole and Griseofulvin respectively. *Aspergillus niger* and *Fusarium oxysporum* were resistant to Griseofulvin showing 0.00 ± 0.00 mm zone of inhibition. The lowest zone of inhibition to Ketoconazole and Fluconazole of 1.20 ± 0.06 and 0.47 ± 0.07 mm were exhibited by *Aspergillus niger* and *Penicillum* sp respectively. The findings revealed there was significant difference between the isolates at *p* value ≤ 0.01.

Antifungal sensitivity (Zone of Inhibitions (mm) At Day 4 Incubation

Table 6 revealed the Antifungal sensitivity of the various fungal isolates after 4 days of exposure. *Microsphaerosis* sp gave the highest sensitivity with zone of inhibition of 4.33 ± 0.03, 4.17 ± 0.07 and 4.10 ± 0.00 mm to Ketoconazole, Fluconazole and Griseofulvin respectively. *Asperrgillus niger* was resistant to Griseofulvin with 0.00 ± 0.00 mm zone of inhibition after 4 days of exposure. The lowest zone of inhibition to Ketoconazole and Fluconazole of 1.43 ± 0.03 and 0.40 ± 0.10 mm were exhibited by *Asperrgillusniger*. This study in revealed that there was significant difference between the isolates at *p* value ≤ 0.01.

Antifungal sensitivity (Zone of Inhibitions (mm) At Day 5 Incubation

Table 7 showed the Antifungal sensitivity of the various fungal isolates after 5 days of exposure. The findings indicated *Asperrgillus niger* was resistant to Griseofulvin showing 0.00 ± 0.00 mm zone of inhibition after 5 days of exposure. *Microsphaerosis* sp gave the highest sensitivity with zone of inhibition of 4.30 ± 0.12, 4.13 ± 0.03 and 4.17 ± 0.07 mm to Ketoconazole, Fluconazole and Griseofulvin respectively. The lowest zone of inhibition to Ketoconazole and Fluconazole of 1.47 ± 0.12 and 0.47 ± 0.12 mm were exhibited by *Asperrgillus niger*. There was significant difference between the isolates at *p* value ≤ 0.01.

Comparison of the efficacy of the fungicides on the fungal isolates

The findings in Table 8 revealed the comparison of the efficacy of the fungicides on the fungal isolates. These shows that isolates were more sensitive to Ketoconazole than Fluconazole and Grisofulvin. Griseofulvin exhibited the lowest efficacy against the fungal isolates. Increase in the days of exposure of the isolates to the antibiotic led to increased zone of inhibition. *Asperrgillus niger* recorded the highest resistant value of 1.47 ± 0.12 mm against the Ketoconazole compare to other isolates after 5 days. The results showed significant differences between the antibiotics compared at *p*value ≤ 0.05 and 0.01.

Table 1: Total viable fungal counts of white and yellow garri obtained from different markets in Jos North

Markets	White Garri (sfu g^{-1} x 5)	Yellow Garri (sfu g^{-1} x 5)	t-test	*P*-value
Terminus	14.50 ± 1.26^{a}	23.50 ± 2.18	3.576	0.023*
Katako	6.50 ± 2.25^{c}	14.93 ± 2.83	2.333	0.080
New market	10.67 ± 2.40^{b}	15.67 ± 4.91	0.915	0.412
Busa buji	5.50 ± 1.53^{c}	22.67 ± 3.18	4.866	0.008**
Average (TFU)	9.23 ± 0.96	19.31 ± 0.51	9.302	0.001**
ANOVA	4.073	1.620		
P-value	0.033*	0.244		

Key: * Significant difference exists at *P* ≤ 0.05; ** Significant difference exists at *P* ≤ 0.01, Values are mean of triplicate readings. Mean values were separated using Duncan Multiple Range Test. Mean values with different superscripts in the same column are significantly different

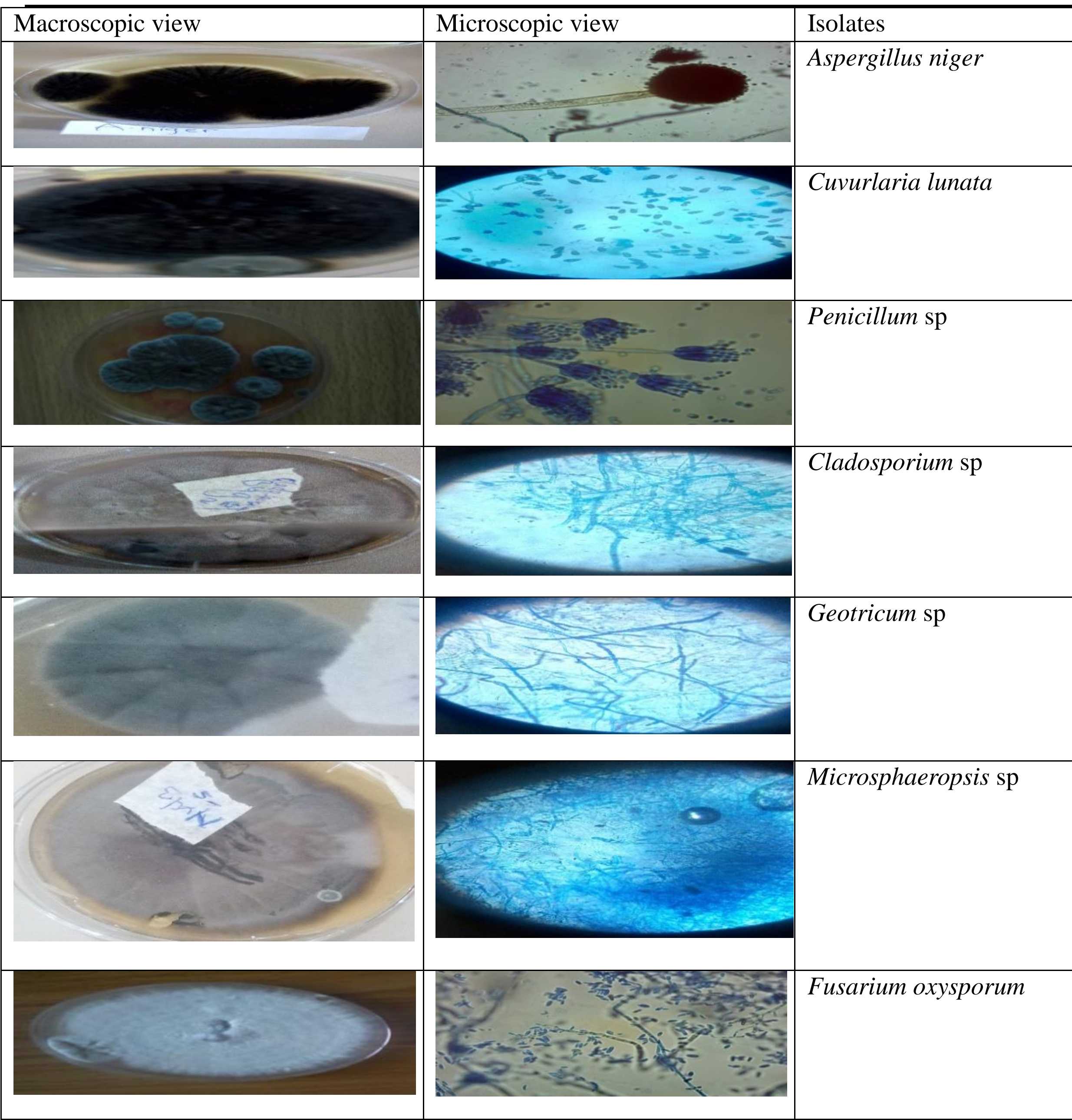

Plate 1: Macroscopic and microscopic features of the fungal isolates from Yellow and White garri

Table 2: Frequency Distribution and Percentage of Fungal Isolates from Garri

S/N	Isolates	Frequency occurrence	Percentage (%)
1	*Microsphaerosis* sp	1	2.63
2	*Cladosporium* sp	2	5.26
3	*Cuvurlaria* sp	4	10.53
4	*Geotrichum* sp	4	10.53
5	*Fusarium oxysporum*	7	18.42
6	*Asperrgillus niger*	9	23.68
7	*Penicillum* sp	11	28.95
Total		38	100

Table 3: Changes in pH content of white and yellow garri at ambient temperature

Markets	pH (Mean ± SEM)			
	White	Yellow	t-test	*P*-value
Terminus	5.47 ± 0.63	5.21 ± 0.40	0.347	0.746
Katako	6.15 ± 0.04	5.29 ± 0.37	2.316	0.082
New market	5.26 ± 0.37	5.47 ± 0.68	0.280	0.793
BusaBuji	5.67 ± 0.32	5.14 ± 0.43	0.979	0.383
ANOVA	0.900	0.086		
P-value	0.482	0.966		

Key: * Significant difference exists at $P \leq 0.05$; ** Significant difference exists at $P \leq 0.01$, Values are mean of triplicate readings. Mean values were separated using Duncan Multiple Range Test. Mean values with different superscripts in the same column are significantly different

Table 4: Changes in moisture content of white and yellow garri at ambient temperature

Markets	Moisture (Mean ± SEM)			
	White	Yellow	t-test	*P*-value
Terminus	11.79 ± 0.87	21.76 ± 1.24	6.573	0.003**
Katako	13.16 ± 0.35	16.95 ± 2.57	1.462	0.218
New market	15.18 ± 1.95	16.05 ± 2.26	0.293	0.784
Busabuji	14.45 ± 0.01	20.15 ± 0.55	10.405	< 0.001**
ANOVA	1.900	2.118		
P-value	0.208	0.176		

Key: * Significant difference exists at $P \leq 0.05$; ** Significant difference exists at $P \leq 0.01$, Values are mean of triplicate readings. Mean values were separated using Duncan Multiple Range Test. Mean values with different superscripts in the same column are significantly different

Table 5: Antifungal sensitivity (Zone of Inhibitions (mm) At Day 3 Incubation

Isolate	Day 3		
	Ketoconazole	Fluconazole	Griseofulvin
Asperrgillus niger	1.20 ± 0.06^{e}	0.40 ± 0.06^{g}	0.00 ± 0.00^{f}
Cuvurlaria sp	1.73 ± 0.09^{d}	0.90 ± 0.06^{f}	0.40 ± 0.06^{e}
Penicillum sp	2.10 ± 0.06^{c}	1.70 ± 0.06^{e}	0.50 ± 0.06^{e}
Cladosporium sp	3.67 ± 0.09^{b}	3.70 ± 0.06^{b}	3.67 ± 0.09^{c}
Geotrichum sp	4.07 ± 0.09^{a}	4.00 ± 0.06^{a}	4.00 ± 0.06^{b}
Microsphaerosis sp	4.13 ± 0.03^{a}	4.13 ± 0.03^{a}	4.23 ± 0.09^{a}
Fusariumoxysporum	3.77 ± 0.07^{b}	3.00 ± 0.06^{c}	3.07 ± 0.03^{d}
ANOVA	240.505	700.875	1232.198
P-value	< 0.001**	< 0.001**	< 0.001**

Key: * Significant difference exists at $P \leq 0.05$; ** Significant difference exists at $P \leq 0.01$, Values are mean of triplicate readings. Mean values were separated using Duncan Multiple Range Test. Mean values with different superscripts in the same column are significantly different

Table 6: Antifungal sensitivity (Zone of Inhibitions (mm) At Day 4 Incubation

Isolate	Day 4		
	Ketoconazole	Fluconazole	Griseofulvin
Asperrgillus niger	1.43 ± 0.03^{d}	0.40 ± 0.10^{e}	0.00 ± 0.00^{f}
Cuvurlaria sp	2.07 ± 0.12^{c}	1.07 ± 0.09^{d}	0.57 ± 0.03^{e}
Penicillum sp	2.20 ± 0.12^{c}	1.77 ± 0.12^{c}	0.67 ± 0.03^{d}
Cladosporium sp	3.90 ± 0.06^{b}	3.87 ± 0.09^{b}	3.57 ± 0.07^{b}
Geotrichum sp	4.10 ± 0.10^{ab}	4.03 ± 0.09^{ab}	3.80 ± 0.40^{ab}
Microsphaerosis sp	4.33 ± 0.03^{a}	4.17 ± 0.07^{a}	4.10 ± 0.00^{a}
Fusariumoxysporum	4.27 ± 0.09^{a}	3.10 ± 0.12^{c}	3.00 ± 0.06^{c}
ANOVA	229.066	291.421	169.575
P-value	< 0.001**	< 0.001**	< 0.001**

Key: * Significant difference exists at $P \leq 0.05$; ** Significant difference exists at $P \leq 0.01$, Values are mean of triplicate readings. Mean values were separated using Duncan Multiple Range Test. Mean values with different superscripts in the same column are significantly different

Table 7: Antifungal sensitivity (Zone of Inhibitions (mm) At Day 5 Incubation

Isolate	Day 5		
	Ketoconazole	Fluconazole	Griseofulvin
Asperrgillus niger	1.47 ± 0.12^{d}	0.47 ± 0.12^{e}	0.00 ± 0.00^{f}
Cuvurlaria sp	2.03 ± 0.09^{c}	1.27 ± 0.12^{d}	0.60 ± 0.06^{e}
Penicillum sp	2.67 ± 0.07^{b}	1.90 ± 0.06^{c}	0.77 ± 0.09^{d}
Cladosporium sp	4.07 ± 0.09^{a}	3.97 ± 0.07^{a}	3.60 ± 0.06^{b}
Geotrichum sp	4.10 ± 0.10^{a}	4.07 ± 0.09^{a}	4.13 ± 0.07^{a}
Microsphaerosis sp	4.30 ± 0.12^{a}	4.13 ± 0.03^{a}	4.17 ± 0.07^{a}
Fusarium oxysporum	4.13 ± 0.09^{a}	3.07 ± 0.09^{b}	3.00 ± 0.06^{c}
ANOVA	153.100	294.866	1113.210
P-value	< 0.001**	< 0.001**	< 0.001**

Key: * Significant difference exists at $P \leq 0.05$; ** Significant difference exists at $P \leq 0.01$, Values are mean of triplicate readings. Mean values were separated using Duncan Multiple Range Test. Mean values with different superscripts in the same column are significantly different

Table 8: Comparison of the efficacy of the fungicides on the fungal isolates

Day	Isolate	Ketoconazole	Fluconazole	Griseofulvin	*P*-value
3	*Asperrgillusniger*	1.20 ± 0.06^{a}	0.40 ± 0.06^{b}	0.00 ± 0.00^{c}	< 0.001**
	Cuvurlariasp	1.73 ± 0.09^{a}	0.90 ± 0.06^{b}	0.40 ± 0.06^{c}	< 0.001**
	Penicillumsp	2.10 ± 0.06^{a}	1.70 ± 0.06^{b}	0.50 ± 0.06^{c}	< 0.001**
	Cladosporiumsp	3.67 ± 0.09	3.70 ± 0.06	3.67 ± 0.09	0.943
	Geotrichumsp	4.07 ± 0.09	4.00 ± 0.06	4.00 ± 0.06	0.746
	Microsphaerosissp	4.13 ± 0.03	4.13 ± 0.03	4.23 ± 0.09	0.422
	Fusariumoxysporum	3.77 ± 0.07^{a}	3.00 ± 0.06^{b}	3.07 ± 0.03^{b}	< 0.001**
4	*Asperrgillusniger*	1.43 ± 0.03^{a}	0.40 ± 0.10^{b}	0.00 ± 0.00^{c}	< 0.001**
	Cuvurlariasp	2.07 ± 0.12^{a}	1.07 ± 0.09^{b}	0.57 ± 0.03^{c}	< 0.001**
	Penicillumsp	2.20 ± 0.12^{a}	1.77 ± 0.12^{b}	0.67 ± 0.03^{c}	< 0.001**
	Cladosporiumsp	3.90 ± 0.06^{a}	3.87 ± 0.09^{a}	3.57 ± 0.07^{b}	0.031*
	Geotrichumsp	4.10 ± 0.10	4.03 ± 0.09	3.80 ± 0.40	0.676
	Microsphaerosissp	4.33 ± 0.03^{a}	4.17 ± 0.07^{b}	4.10 ± 0.00^{b}	0.021*
	Fusariumoxysporum	4.27 ± 0.09^{a}	3.10 ± 0.12^{b}	3.00 ± 0.06^{b}	< 0.001**
5	*Asperrgillusniger*	1.47 ± 0.12^{a}	0.47 ± 0.12^{b}	0.00 ± 0.00^{c}	< 0.001**
	Cuvurlariasp	2.03 ± 0.09^{a}	1.27 ± 0.12^{b}	0.60 ± 0.06^{c}	< 0.001**
	Penicillumsp	2.67 ± 0.07^{a}	1.90 ± 0.06^{b}	0.77 ± 0.09^{c}	< 0.001**
	Cladosporiumsp	4.07 ± 0.09^{a}	3.97 ± 0.07^{a}	3.60 ± 0.06^{b}	0.009**
	Geotrichumsp	4.10 ± 0.10	4.07 ± 0.09	4.13 ± 0.07	0.864
	Microsphaerosissp	4.30 ± 0.12	4.13 ± 0.03	4.17 ± 0.07	0.355
	Fusariumoxysporum	4.13 ± 0.09^{a}	3.07 ± 0.09^{b}	3.00 ± 0.06^{c}	< 0.001**

Key: * Significant difference exists at $P \leq 0.05$; ** Significant difference exists at $P \leq 0.01$Values are mean of triplicate readings. Mean values were separated using Duncan Multiple Range Test. Mean values with different superscripts in the same row are significantly different

DISCUSSION

The moulds isolated from both the yellow and white garri sample were *Aspergillus niger*, *Penicillium sp, Fusarium oxysparum, Curvularia lurana, Geotricum sp, Microsphearopsis arundinis*. Similar moulds were reported by other studies in different parts of Nigeria (Olopade *et al.,* 2014; Okolo and Makanjuola, 2021; Obi *et al*., 2022). The findings of these study is an evident that garri sold in open market was infested with fungi. The slight difference in fungal species from other studies might be due to varying production process (Obi *et al*., 2022). The fungi recorded in this study may be due unhygienic practice involved in processing and handling garri. These processes exposes cassava and it products to fungal spores (Ogugbue *et al*., 2011).

The Yellow Garri in this study indicated higher fungal load compared to White garri, this is contrary to the work of Onyeke *et al.* (2010), which reported fewer colony in yellow garri compared to white garri. The presence of palm oil support fungal growth (Enemuor *et al*., 2012). The high fungal load

in yellow garri could attributed to high content of degradable sugar associated with palm oil used in processing yellow garri (Schmidt and Michele, 2020). The variation could also be due to difference in oxygen and carbon dioxide permeability and water vapour (Ebidor *et al*., 2015)

Penicillium spp and *Aspergillus niger* recorded the highest occurrence in the garri sample (yellow and white), supported by the study of Rabiu *et al*. (2021). The high occurrence of *Aspergillus* species in both Garri could be linked to their abundance and wide distribution in nature. The presence of these fungal species in garri studied indicates poor sanitary processes in garri production and handling and this pose a serious health challenge to consumers. *Aspergillus, Fusarium* and *Penicillum* isolated in this study are major food and environment contaminant. The ability of these fungi to produce spores and mycotoxin make them very potent (Oranusi *et al.,* 2013; Olopade *et al.,* 2014) and of medical important.

The percentage moisture content of yellow and white Garri in this study revealed higher moisture content compared with previous studies (Halliday *et al*, 1967; Ogugbue and Obi 2011; Aguoru *et al*., 2014). In this study it was observed that yellow Garri recorded higher moisture content than white Garri, contradicting the work reported by Aguoru *et al*. (2014) and Rabiu *et al*. (2020). The disparity observed in moisture content could be attributed to variation in temperature, extent of dry frying/roasting and storage condition of the finished product (Okolo and Makanjuala, 2021). Aguoru et al. (2014) reported that the major important factor that could encourage mould contamination and proliferation in Garri was the high initial moisture content. Moisture is one of the factor that could lead to fungal infestation and multiplication in Garri (Halliday *et al*., 1967).

The pH values of white and yellow garri in this study ranges from 5-6. The findings shows no significant in pH among the various markets and comparative analysis indicates no significant different between yellow and white garri. The pH values recorded in this study was within the ranges of those reported by Olapade *et al*. (2018). A study conducted by Orji *et al*. (2016) in Ebonyi State, Nigeria reported pH value of 5.47 to 6.61 which is agreement with this study. The pH obtained in this study is a reflection of the presence of high fermentative fungi (Okolo and Makanjuala, 2021). This condition could create an enabling environment for fungal growth and proliferation.

The antibiogram study with antifungal agent on the fungal isolates obtained indicated that the fungi were sensitive to the antifungal agents used. The antifungal agents used in this study are similar to the report of Bamidele *et al.* (2014) and Mohammed *et al*. (2019) who used antifungal drugs: fesovin, fluconazole, itraconazole, griseofulvin, and ennotab vab to study the antibiogram against different fungal isolates. The findings in this study contradict the report of Mohammed *et al*. (2019), who demonstrated zone of inhibition as high as 17mm. This variation could be due to difference in antifungal agent and concentrations used. Maurizio and Posteraro (2018) had earlier reported that the fungal isolates sensitive and resistance were dose dependent to antifungal agents used. The fungal isolates reacted differently to the antifungal agent as the days of exposure increased. According to Mohammed *et al*. (2019) it could be attributed to physiology and enzyme produced by some of the fungi. Variation in genetic makeup and species diversity could also cause difference in sensitivity. The high fungal load observed in this study could pose a great health challenge to consumer if not mitigated. This group of fungi have been implicated in several diseases due to mycotoxin production. The ingestion of toxin produced by some of these fungi leads to acute and chronic toxicity that may compromise human organs. Hence the need for hygienic approaches in processing and handling of garri sold in Jos and Nigeria at large.

CONCLUSION

The present study indicated high fungal load and different species of fungi in garri sold in Jos, Plateau state. Yellow garri recorded the highest fungi load in this study compare to white garri. The pH obtained in this study reflected presence of high fermentative fungi. The antibiogram of the fungal isolates were said to be sensitive while some were resistant to antifungal drugs. The presence of fungi associated with Garri could be attributed to the production processes of the products especially the yellow garri. Therefore, proper processing techniques and handling that provides adequate hygiene and very low relative humidity and moisture is needed to prevent moulds proliferation and survival in garri that causes public health concerns consumers.

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Quality Improvement of Watermelon-*Clerodendrum volubile* Extract Wine Produced via Sequential Malolactic Fermentation by *Saccharomyces cerevisiae* and *Lactobacillus delbrueckii*

Kazeem M. O.[1*] Aderoboye Y. O.[1] Oyinlola K. A.[2] and Maiangwa J.[3]
1. Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Nigeria.
2. Department of Microbiology, University of Ibadan, Oyo State, Nigeria
3. Department of Microbiology, Kaduna State University, Kaduna, Nigeria
* Corresponding author: kazeem.mo@unilorin.edu.ng

Abstract: Herbal infusions medicinal benefits in wine and the impact of malolactic fermentation on wine quality is of high significance. The study aimed at improving the quality of watermelon wine with *Clerodendrum volubile* extract using *Saccharomyces cerevisiae* and *Lactobacillus delbrueckii* subsp *bulgaricus. S. cerevisiae* and *L. delbrueckii* isolated from palm wine and yoghurt, respectively were used in this study. Fermentation must was prepared in various dilution ratios ranging from 95:5, 90:10 and 85:15 (watermelon to *C. volubile).* Static fermentation was carried out for 5 days with *S. cerevisiae* followed by malolactic fermentation with *L. delbrueckii* and then fermentation with *S. cerevisiae* for 23 days at room temperature. Physicochemical, phytochemical, mineral, and sensory properties were observed. Noticeable was pH decrease (5.21 -.3.33), increased titratable acidity (0.05-0.69 g/l), decreasing reducing sugar (0.59-0.011 mg/ml), temperature (30.5-24.2°C) and increasing total dissolved solids (19.7-48.9°B). Wine fermented with *S. cerevisiae* (D) had the highest phenolic content (481.68±0.37 mg/100ml), while vitamin C increased (20.2±0.73 - 29.28±0.70) with increase in *C. volubile* concentration. The Na^{+} was most abundant (51.71 mg/100ml), while Ca^{2+} (5.23 mg/100ml) was improved. Watermelon wine (D and H) showed the least (1.38±0.5%) alcohol content while wine C and G recorded the highest. Organoleptic properties of wine E received the highest preference rating for flavour, colour and taste. Therefore, the nutritional and sensorial properties of Watermelon-*C. volubile* wine can be improved through sequential malolactic fermentation.

Key word: *Clerodendrum volubile, Lactobacillus delbrueckii, Saccharomyces cerevisiae*, Watermelon, wine

INTRODUCTION

Wine, an alcoholic beverage, is made by fermenting fruit juices. During the wine making process, yeast turns the sugar in the fruit juices into alcohol, carbon dioxide, and organic acids, which then react to generate aldehydes, esters, and other chemical compounds that aid in the preservation of the wine (Aminu *et al.*, 2018). In recent years, several researchers have turned their attention to production processes involving fermentations with mixed yeast inocula in an attempt to improve the organoleptic characteristics of the resulting wines and to reduce the alcohol content. Hence, the use of non-*Saccharomyces* species as starters, along with various *S. cerevisiae* strains improved considerably various wine characteristics, such as: physicochemical properties, the composition and concentration flavour, aroma of the final product and others (Dias *et al.*, 2020). Malolactic fermentation (MLF) is a natural process that occurs as a result of the metabolic activity of lactic acid bacteria (LAB). Malolactic fermentation plays three roles in wine making: it reduces wine acidity due to malic acid, stabilizes wine by removing a possible carbon source, and increases aroma and flavour modification (Virdis *et al.,* 2020) *Oenococcus, Leuconostoc,* and *Pediococcus*, are the only genera associated to wine (Holzapfel and Wood, 2014; Zheng *et al.*, 2020). Due to its high tolerance for low pH, high ethanol concentrations and scarcity of nutrients, *O oeni* is the main LAB of choice in winemaking. However, with increasing temperatures during growth and harvest, and a consequent rising pH trend for many wines, other LAB have the potential to become a valid alternative to *Oenococcus*, playing an important role in the modifications of wine aroma (Krieger-Weber *et al.*, 2020; López-Seijas *et al.*, 2020).

Currently, there is little or no information on the use of *L. delbrueckii* subsp *bulgaricus* for malo -lactic fermentation (MLF). Wine production from grapes are commonly reported in the literature (Gavahian *et al.*, 2022), due to their natural chemical equilibrium, which aids in the fermentation process without the addition of sugars, acids, enzymes, or other nutrients. However, due to the climatic restrictions of grape, current research is directed towards producing similar and/or better wines from other tropical fruits sources. Fruits such as pawpaw, banana, cucumber and other fruits have been used as single or mixed fruit for wine production (Ogodo *et al.*, 2015). Watermelon (*Citrullus lanatus*) is a fruit which belongs to the family of Cucumbitacea, mostly grown in the northern part of Nigeria. It is nominally 60% flesh and about 90% of the flesh is juicy which contains 7 to 10 % (w/v) sugar. Thus, over 50 % of the watermelon is readily fermentable liquid (Ozcelik and Yavuz, 2016). Nutritional profile of watermelon is full array of nutrients, including carbohydrates, sugar, soluble and insoluble fiber, sodium, vitamins, minerals, fatty acids, amino acids According to Ozcelik and Yavuz (2016), a serving cup of watermelon contains 12.31 mg of vitamin C, 864.88 IU of vitamin A, 170.24 mg of potassium and 45.60 calories.

Clerodendrum volubile also known as white butterfly (Lamiaceae) is a shrub-like climbers' native to Africa. It is predominantly grown as ornamental plant in tropical West Africa including Ghana, Ivory Coast, Sierra Leone and Nigeria (Ajao *et al.*, 2018). *C. volubile* is usually 1–4.5 m long, stem terete or round, leaves oblong, commonly 1.5–15.5 cm long and 0.6–6 cm wide. It is commonly known as "Marugbo" or "Eweta" among the Ikale, Ilaje and Apoi people found in Southern-senatorial district of Ondo State, South West Nigeria. The leaf of is commonly consumed as vegetables sweet aroma and taste and have great nutritional value as well as herbal and medicinal value. According to Ogunwa *et al.* (2015), proximate analysis of *C. volubile* vitamins, minerals and crude protein essential for healthy living. The anti-diabetic, anti-hyperlipidemic and anti-hypertension action of *C. volubile* have been demonstrated by Erukainure *et al.*, (2016). In addition, the anti-inflammatory, anti-cancer, analgestic and anti-microbial activity of *C. volubile* extract have been previously demonstrated (Afolabi *et al.*, 2019; Okaiyeto *et al.*, 2021). Based on our previous knowledge, this will be the baseline research on the use of watermelone-*C. volubile* extract for wine production. Therefore, the current study aimed at evaluating the physichochemical, nutritional and organoleptic properties of water melon-*C. volubile* extract for wine production using sequencial fermentation with *S. cerevisiae* and *L. delbrueckii.*

MATERIALS AND METHODS

Sample collection: *Clerodendrum. volubile* was obtained at Moferere Market Ondo West Local Government of Ondo State, Nigeria, while watermelon was purchased from a native vendor at Tanke, Ilorin, Kwara state, Nigeria Fresh palm wine was obtained from Asa Dam, Ilorin, Nigeria, while REV yoghurt fermented with *L. debdrueckii subsp bulgaricus* was purchased at Palms Mall, Ilorin, Kwara State, Nigeria.

Isolation and identification of yeast cell and Lactobacillus sp.: Yeast extract peptone dextrose (YPD) and lactobacillus De man Rogosa and Sharpen Agar (LMRS) were used as medium for isolation. The fermentation yeast and *Lactobacillus* were isolated from 25 ml palm wine and yoghurt, respectively using pour plate technique. The yeast and bacteria strain were identified according to the method of Franklin *et al.* (2019) which involves morphological and cultural characterization.

Sugar fermentation test: A colony of the isolate was inoculated into 10 m phenol red fermentation broth containing sugars (lactose, maltose, mannitol, glucose, and fructose) and incubated at 27°C for 24 – 72 h. A positive sugar fermentation result was

indicated by a yellow colouration while the retention of initial red colour denotes negative fermentation.

Preparation of C. volubile extracts (infusion): The extraction process was conducted using the aqueous extraction method as described by Chawafambira, (2021). *C. volubile* leaves were sorted, washed thoroughly with tap water, oven dried at 60 °C and blended. Thereafter, 500 g of the powdered leaves were then boiled in 2 l sterile distilled water, strained using muslin cloth, and the liquid extract was stored at 4°C in a chiller until further use.

Pulp extraction and preparation of watermelon must: Ripe watermelon fruits were thoroughly washed and was disinfected using 70% ethanol to remove dirt, microorganisms and its vegetative form adhering to the surface of the watermelon. The watermelon was cut opened with a sterile knife, the pulp was cut into pieces and blended, then sieved with a muslin cloth to obtain the juice. Then 0.15 g of sodium metabisulfite ($Na_2S_2O_2$) was added to prevent fermentation of the must by autochtonous organisms as well as inhibit pathogenic microorganism (Chawafambira, 2021).

Fermentation of must to wine: Fermentation was carried out in a 2 l fermentation vessel. The wine was produced from mixtures of water melon and *C. volubile* extract at different ratios (90:5, 90:10, 85:15, 100: 0) and the sugar content was then adjusted by addition of 40 g sucrose. Aerobic fermentation was initialed by inoculation of 4 ml starter culture of *S. cerevisiae,* allowed for 5 days fermentation at 27°C, while anaerobic fermentation continued for 23 days by inoculation of 4 ml *L. delbrueckii.* After fermentation, the wine was chilled to 5°C, racked with minimum exposure to the air, and clarified. The fermented wine was then centrifuged at 10000 rpm for 10 min.

Proximate analysis of watermelon must and C. volubile extract: The moisture, ash, crude fibre, fat, crude protein and, total carbohydrate were determined Falade *et al.*, (2014). The watermelon must was air dried for 7 days while the, *C. volubile* leaves were blended to powder prior to analysis.

Determination of pH total dissolved solid (TDS) and total titratable acidity (TTA) of watermelon must and C. volubile extract wine: The pH and TDS of the wine samples were determined using a digital pH meter (Omega PHH44) and digital refractometer (ATAGO PAL-1) (AOAC, 2005). Total titratable acidity was determined in a 20:100 (wine:water) sample by titration with 0.1 M NaOH (Feldsine *et al.*, 2002).

Reducing sugar and specific gravity of watermelon must and C. volubile extract wine: The reaction of wine sample with dinitrosalicylic acid (DNS) in boiling water and further spectrophotometry was used for reducing sugar analysis (Miller, 1959). The specific gravity was determined using specific gravity bottle (Joseph *et al.*, 2019).

Mineral, phytochemical and alcohol content of watermelon must and C. volubile extract wine: The determination of Ca^{2+}; Na^{+}, Fe^{2+}, K^{+} and Mg^{2+} and total flavonoids were carried out as described by AOAC (2005). Total phenolic and vitamin C content were determined using spectrophotometric amd titrimetric method, respectively (Ndawula *et al.,* 2004: Vijay and Rajendra 2014). Alcohol content was determined using the iodoform test technique (Tsegay *et al.,* 2018).

Sensory evaluation of the produced wine: Sensory evaluation of the watermelon-*C. volubile* wines was carried out by 8 panelists . This was done in order to ascertain its acceptability. The organoleptic parameters that were evaluated include taste, aroma, colour, clarity and overall acceptability using five-point hedonic scales. From the scale 5 indicates extremely like and 1 indicates extremely dislike (Wakil and Kazeem, 2012; Animu *et al.*, 2018).

RESULTS

The study was aimed at investigating the nutritional and health benefits of watermelon-*C. volubile* wine and the impact

of *L. delbrueckii* on the organoleptic properties of the resulting product.

Morphological characterization of starter isolates

The colonial, morphological and microscopic characterization of *S. cerevisiae* and *L. delbrueckii* is illustrated in Table 1. The yeast isolate present creamy, non-motile, oval shape and budding. On the other handthe LAB strain was creamy, Gram positive, rod and non-motile. Both isolates were able to ferment glucose, sucrose, maltose, fructose and lactose.

Proximate composition of wine

The findings shows that *C. volubile* contained high crude fiber, total ash, crude protein and crude lipid content (29.34%, 10.98%, 14.89% and 10.12%). However, watermelon must has higher carbohydrate and moisture content (50.36% and10.30%) in comparison with *C. volubile* extract Table 2.

Physicochemical properties of watermelon-*C. volublie* extract wine

There was a decrease in pH with increase in fermentation period from initial day to the 28th day. (Table 3). The highest pH values were observed in samples before fermentation specifically in sample C (5.21), while the lowest pH values were obtained in wine with sequential fermentation with *S. cerevisiae* and *L. delbrueckii,* with the lowest (3.33) specifically recorded in wine sample E and F. The pH profile was recorded in order before fermentation > 5 days fermentation with *S. cerevisiae* > 28 days fermentation with *S. cerevisiae* > 28 days fermentation with *S. cerevisiae* and *L. deibrueckii.* The titratable acidity increase with increase in fermentation from initial day to 28 days with the highest (0.69) obtained in wine sample E and F, while the lowest (0.05) was recorded in wine sample C. Overall, the titratable acidity of wine samples was higher in samples with sequential fermentation with *S. cerevisiae* and *L. delbrueckii* compared with samples fermented with only *S. cerevisiae.* The reducing sugar content of the wine samples decreased with increase in fermentation time. The highest reducing sugar (0.59 mg/ml) was observed in wine sample B before fermentation which reduce to (0.167 mg/ml) after 5 days fermentation with *S. serevisiae.* Reducing sugars were detected below minimum in the 28 days fermented wine samples. There was a decrease in temperature from 30.5°C in wine sample B and C before fermentation to 24.2°C in samples F, G and H of 28 days sequential fermentation with *S. cerevisiae* and *L. delbrueckii.* The co-fermentation wine samples experienced lower temperatures than fermentation with *S. cerevisiae* alone. The specific gravity decrease with increase in fermentation days. The highest specific gravity were recorded in wine samples before fermentation, with the highest (1.03 kg/m^3) from wine sample C. while the lowest specific gravity (0.97 kg/m^3) was observed in wine samples D (28 days fermentation with *S. cerevisiae* and H (28 days) fermentation with *S. cerevisiae* and *L. delbrueckii*).

Phytochemical constituents of watermelon-*C. volublie* extract wine

The total phenol content ranged from 261.36 ± 0.46 unit in sample E to 481.68 ± 0.37 unit, with the highest obtained in wine produced from watermelon using *S. cerevisiae* (D) Table 4. It is worth to note that the phenol content increased with increase in concentration of the *C. volubile* extract concentration for the MLF (E-H). However, a decrease was observed with the A-C wine sample. The total flavonoids content ranged from 25.42 ± 0.95 unit in sample H to 48.94 ± 0.34 unit in sample C. In this case there was an increased with increase in *C. volubile* concentration for both fermentation with *S. cerevisiae* (A-C) and MLF (E-G), except for the D and H that recorded declination. Wine samples produced from watermelon alone contained the lowest flavonoid content for both types of fermentation. The vitamin C content increased with an increase in *C. volubile* with the highest (29.28 ± 0.70 units) obtained in sample (C) and the lowest (17.22) in wine from watermelon alone (H),

fermented with *S. cerevisiae* and *L. delbrueckii.*

Mineral composition of watermelon-*C. volublie* extract wine

The mineral composition (calcium, potassium, magnesium sodium and iron) of the wine samples is shown in Table 5. The K^+ composition of all the eight wine shows slight difference in concentration, ranging from 4.64± 0.014 (mg/100g) to 4.7 ± 0.00 (mg/100g), with the highest K^+ composition recorded in wine C and the lowest K^+ composition recorded wine E and F . The Ca^{2+} content raged from 3. 96 ± 0.00 (mg/100g) to 5.23± 0.012 (mg/100g) with the highest Ca^{2+} recorded in wine F and the lowest in wine C. The Mg^{2+}content ranged from 5.65 ± 0.001 (mg/100g) to 6.07 ± 0.01(mg/100g) with the highest Mg^{2+} recorded in wine G and the lowest in wine D. The Na^{2+} composition ranged from 51.71 ± 0.00 (mg/100g) to 55.21 ± 0.00 (mg/100g), with the highest Na^{2+} composition recorded in wine A and the lowest Na^{2+} composition recorded wine C. The Fe^{2+} composition shows slight difference in concentration, ranging from 0.08 ± 0.003 (mg/100g) to 0.23 ± 0.001(mg/100g) with the highest Fe^{2+} composition recorded in wine G and the lowest recorded in the control wine E and H.

Alcohol content of watermelon-*C. volublie* extract wine

The incorporation of *C. volubile* extract resulted in increased alcohol concentration as observed in wine A, B, C, E, F, G ranging from 2.74% to 2.76% with wine D and H having lower alcohol concentration of 1.38 %. Hence co-fermentation with *S. cerevisiae* and *L. delbrueckii* has no effect on alcohol concentration of the wine samples examined in the current study.

Organoleptic characterization of watermelon-*C. volublie* extract wine

The findings of the sensory properties of the wine samples is as shown in table 6. The wine sample E was rated highest (4.5) in terms of flavour while the lowest rating (1.8) was observed with wine sample C. Overall, wine with lower concentrations of *C. volubile* (5 and 10) had higher flavour rating which reduced when the *C. volubile* concentration was increased to 15. Based on the taste evaluation, the wine sample E also had the highest rating (4.0), while the lowest rating (2.0) was observed in wine sample C. The taste rating reduced with increase in *C. volubile* concentration. Similarly, the wine sample E was rated highest (4.4) in terms of colour, while sample G was rated the lowest (1.8). Overall, the wine sample E with best combination of watermelon- *C. volubile* ratio (95:05) with sequential *S. cerevisiae* and *L. delbruekii* fermentation presented the best organoleptic properties in terms of flavour, colour and taste.

Table 1: Morphological and biochemical characterization of wine starter cultures

Test	*L. delbrueckii*	*S. cerevisiae*
Colony on culture media	Creamy, slimy, raised entire circular colony	Creamy, big raised, circular colony
Motility	Non motile	Non motile
Gram staining	Gram positive rod	NA
Budding	NA	Multiple budding
Lactophenol cotton blue stain	NA	Oval shape
Turbidity in broth	Slightly turbid	Slightly turbid
Growth on LMRS media	Positive	Nil
Glucose	Positive	Positive
Sucrose	Positive	Positive
Fructose	Positive	Positive
Maltose	Positive	Positive
Mannitol	Negative	Negative

Table 2. Proximate composition of *C. volubile* extract and watermelon pulp

Sample	Crude protein (%)	Crude lipid (%)	Moisture (%)	Crude fiber (%)	Carbohydrate (%)	Total ash (%)
C. volubile	14.89±0.03	10.12±0.8	10.14±0.35	29.34±1.35	39.16±0.09	10.98±0.71
Water melon	11.57±0.01	10.12±0.8	10.3±0.167	3.48±0.22	50.36±1.27	8.63±1.72

Table 3. Physicochemical properties of watermelon-*C. volubile* extract wine

Wine	Parameters					
	pH	TTA	RS (mg/ml)	TDS (°Brix)	TEMP (°C)	SG
	Before fermentation					
A	5.06±0.00	0.09±0.0	0.56±0.00	19.7±0.00	29.5±0.00	1.02±0.0
B	5.14±0.00	0.071±0.0	0.59±0.00	20.6±0.00	30.5±0.00	1.01±0.0
C	5.21±0.00	0.05 ±0.0	0.55±0.00	23.0±0.00	30.5±0.00	1.03±0.00
D	4.99±0.00	0.12±000	0.38±0.00	23.1±0.00	28±0.00	1.02±0.00
	After 5 days fermentation with *S. cerevisiae*					
A	3.73±0.00	0.42±0.00	0.192±0.00	18.6±0.00	26.7±0.01	0.99±0.00
B	3.80±0.00	0.44±0.00	0.1670.00	22.2±0.00	25.3±0.04	0.99±0.00
C	3.88±0.00	0.43±0.00	0.175±0.00	21.3±0.00	24.7±0.06	0.99±0.00
D	3.89±0.00	0.33±0.01	0.172±0.00	22.5±0.00	24.4±0.00	0.99±0.00
	After 28 days fermentation with *S. cerevisiae*					
A	3.39±0.037	0.56±0.00	0.09±0.00	46.5±0.00	26.6±0.00	0.98±0.00
B	3.43±0.06	0.54±0.00	0.11±0.00	44.2±0.00	26.4±0.00	0.98±0.00
C	3.57±0.053	0.55±0.00	0.08±0.00	48.3±0.02	24.2±0.00	0.98±0.00
D	3.86±0.00	0.54±0.01	0.09±0.00	46.3±0.01	27.3±0.00	0.97±0.00
	After 28 days fermentation with S. cerevisiae and *L. delbrueckii*					
E	3.33±0.00	0.69±0.00	0.08±0.00	48.7±0.00	24.2±0.00	0.98±0.00
F	3.33±0.00	0.69±0.00	0.11±0.00	45.4±0.00	24.0±0.00	0.98±0.00
G	3.44±0.00	0.60±0.00	0.09±0.00	47.8±0.00	24.2±0.00	0.98±0.00
H	3.69±0.00	0.58±0.02	0.11±0.00	48.9±0.00	25.9±0.00	0.97±0.00

Key: Watermelon to *C.volubile* dilution ratio TTA= total titrable acidity, RS= reducing sugar, TDS= total dissolved solid, TEMP= temperature SG= specific gravity, Values are means of triplicate readings ±SD, Blend formulation - Watermelon: *C. volubile,* A =95:05, B =90:10, C =85:15, D =100:00 (control), E =95:05, F =90:10, G =85:15, H =100:00 (control), A to D: wine fermented with *S. cerevisiae,* E to H: wine fermented with *S. cerevisiae* and *L. delbrueckii*

Table 4. Phytochemical constituent of wine sample

Wine sample	Total phenol (%)	Total flavonoids (%)	Vit C (mg/100g)
A	372.3 ± 0.5	36.0 ± 0.14	20.2 ± 0.73
B	353.2 ± 1.83	44.33 ± 1.5	24.59 ± 1.30
C	346.69 ±0.3	48.94 ± 0.3	29.28 ± 0.70
D	481.7 ± 0.37	33.08 ± 0.3	19.98 ±0.27
E	261.5 ± 0.46	28.45 ± 0.6	17.9 ± 0.45
F	278.7± 0.37	37.13 ± 0.0	22.81 ± 0.21
G	328.6 ± 0.62	42.44 ± 1.7	24.59 ± 1.30
H	421.6 ± 0.54	25.42 ± 0.9	17.22 ± 0.04

Values are means of triplicate readings ±SD, Blend formulation - Watermelon: *C. volubile* A =95:05, B =90:10, C =85:15, D =100:00 (control), E =95:05, F =90:10, G =85:15, H =100:00 (control), A to D: wine fermented with *S. cerevisiae,* E to H: wine fermented with *S. cerevisiae* and *L. delbrueckii*

Table 5. Mineral composition of water melon-*C. volubile* extract wine (mg/100g)

Wine	Minerals				
	K^+ (mg/100g)	Ca^{2+}(mg/100g)	Mg^{2+} (mg/100g)	Na^+ (mg/100g)	Fe^{2+} (mg/100g)
A	4.65 ± 0.0	4.22 ± 0.1	5.95± 0.00	55.21 ± 0.0	0.1 ± 0.0
B	4.65 ± 0.0	4.11 ± 0.0	5.955± 0.00	54.36 ± 0.3	0.22 ± 0.0
C	4.7 ± 0.0	3.96 ± 0.0	6.05 ± 0.01	51.71 ± 0.0	0.195 ±0.1
D	4.66± 0.0	4.94 ±0.1	5.65 ±0.001	52.11± 0.01	0.13± 0.0
E	4.64±0.02	4.01 ± 0.0	5.66 ± 0.01	52.06± 0.01	0.08 ± 0.01
F	4.64± 0.0	5.23± 0.0	5.92± 0.01	52.19 ± 0.0	0.11 ± 0.1
G	4.66 ± 0.0	4.23 ± 0.1	6.07 ± 0.01	52.13 ± 0.0	0.23 ± 0.1
H	4.65 ± 0.2	5.11± 0.0	5.79 ± 0.03	52.14± 0.0	0.08 ± 0.0

Values are means of triplicate readings ±SD. Blend formulation - Watermelon: *C. volubile* A =95:05, B =90:10, C =85:15, D =100:00 (control), E =95:05, F =90:10, G =85:15, H =100:00 (control), A to D: wine fermented with *S. cerevisiae,* E to H: wine fermented with *S. cerevisiae* and *L. delbrueckii*

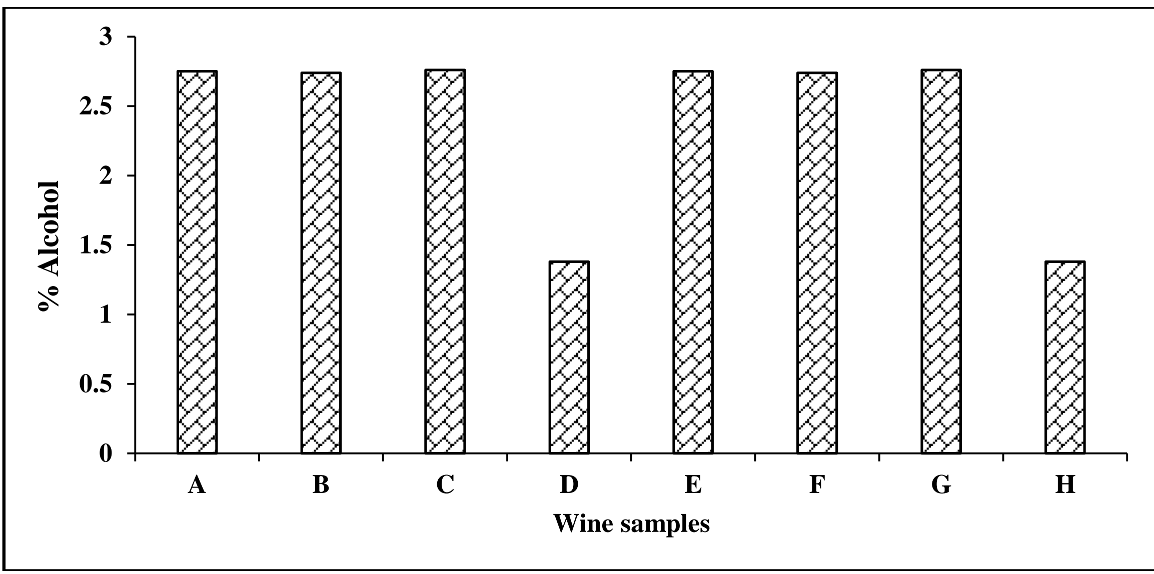

Figure: 1 Alcohol content of water melon-*C. voluble* extract wine.
Values are means of triplicate readings ±SD, Blend formulation - Watermelon: *C. volubile,* A =95:05, B =90:10, C =85:15, D =100:00 (control), E =95:05, F =90:10, G =85:15, H =100:00 (control), A to D: wine fermented with *S. cerevisiae,* E to H: wine fermented with *S. cerevisiae* and *L. delbrueckii*

Table 6. Mean scores of sensory evaluation of wine samples fermented with *S. cerevisiae* and *L. delbrueckii*

Parameters	Wine samples							
	A	B	C	D	E	F	G	H
Flavour	4.0	4.3	1.8	2.6	4.5	4.1	2.3	3.4
Taste	2.5	3.6	2.0	3.1	4.0	3.3	2.1	4.0
Colour	3.4	3.6	2.1	3.0	4.4	3.1	1.8	4.0

Acceptability rating 1–5: 1 = very bad and 5 = very good. Values are means of triplicate readings ±SD, Blend formulation - Watermelon: *C. volubile,* A =95:05, B =90:10, C =85:15, D =100:00 (control), E =95:05, F =90:10, G =85:15, H =100:00 (control), A to D: wine fermented with *S. cerevisiae,* E to H: wine fermented with *S. cerevisiae* and *L. delbrueckii*

DISCUSSION

Clerodendrum volubile is consumed by in some part of Nigeria due to its nutritional and health benefits. Several research articles have pointed out its medicinal and nutritional properties. Some species of lactic acid bacteria possess the ability to carry out malolactic fermentation, which is lacking in fermentation yeast, but which contributes greatly to the physicochemical and sensory parameters of the wine. This study clearly showed an improved watermelon wine produced with *C. volubile* via sequential fermentation with *S. cerevisiae* and *L. delbrueckii* subsp. *bulgaricus*. The isolates fermented different sugars as confirmed by (Amoroso *et al.* (1989) and Timmermans *et al.* (2022) The proximate content observed in this study, was in agreement with Ajao *et al.* (2018),who reported similar crude protein, lipid, carbohydrate and ash content, while Sadiq *et al.* (2021) reported similar proximate content with watermelon.

The pH is a desirable attribute during fruit fermentation because it creates a conducive environment favorable for the fermenting yeast while creating an undesirable environment for spoilage organism. Several studies has confirmed this claim (Yusufu *et al.*, 2018). Aminu *et al.* (2018), reported acidity of watermelon in the range of 5.14 to 4.90. Acidity plays a vital role in the overall stability and characteristics of the wine. The inverse correlation between the pH and the TTA shows that pH decrease lead to increase in acidity of watermelon juice-*C. volubile* wine. This is in agreement with the studies of Yusufu *et al.* (2018) who reported

related findings in wine produced from watermelon and ginger. Also study carried out by (Awe and Nnadoze, 2015) and (Yusufu *et al.*, 2018) on date palm and watermelon with ginger extract respectively, shows comparable results.

The declination in specific gravity observed in this study have previously been reported by and Soibam *et al.* (2016) and Chawafambira (2021) who separately reported reduction in specific gravity of wine produced from *U. kirkiana* juice and *Lippia javanica* and wine produced from watermelon and sugarcane. Prolong fermentation contributed to the reduction in reducing sugar in the wine samples. This is in agreement with Chawafambira (2021), who reported similar findings in wine produced from *U. kirkiana* juice and *Lippia javanica*. The *C. volubile* and malolactic fermentation contributed to the higher mineral composition of watermelon wine compared with the wine produced from watermelon and ginger (Yusufu *et al.*, 2018). Maarman (2014), reported increased minerals in wine fermented with co-culture of yeast and LAB. It is worth to note that the malolactic fermentation improved the calcium content of wine F compared with wine B (fermented with *S. cerevisiae* only). This could be that the dilution ratio is compatible with the natural requirement of *L. delbrueckii*.

Wine sample rich in phenolic compounds and flavonoids have impact on the overall taste and flavour of the wine. In this study, wine samples produced via sequential fermentation has lesser phenolic and flavonoids content which could be due to the activities of the *Lactobacillus* specie. However, the *C. volubile* concentrations has positive impact in improving the phytochemicals. Reports has shown that flavonoids has an antioxidizing power, which prevents browning and spoilage thus help protects wine from oxidation. Also, wine samples without *C. volubile* extract (wine D and H) has lesser vitamin C content, this indicates that *C. volubile* contributed to the vitamin C composition of the wine. The overall acceptability ratings decrease in the highest concentration of *C. volubile*. There were differences among the wine samples with respect to flavour, taste and colour, and similar findings was reported by Chawafambira (2021) in wine produced from *U. kirkiana* juice- *L. javanica* extract wine. However, wine F was more preferred in terms of the sensory properties evaluated.

CONCLUSION

The study revealed that incorporation of *C. volubile* improved the nutritional and physicochemical properties of the watermelon wine. Sequential malolactic fermentation with *S. cerevisiae* and *L. delbrueckii* subsp *bulgaricus* improved the organoleptic properties of the wine. Therefore, production of wine and by extension other wines with incorporation of of *C. volubile* coupled with fermentation with *S. serevisiae* and *L. delbrueckii* should be encouraged based on its overall acceptability.

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Purification and Characterization of *Digitaria exilis* Protease (White Acha)

Ogu C. T.[1*] Mbah N. K.[2] and Odibo F. J. C.[1]
1. Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, Nigeria.
2. Department of Microbiology and Parasitology, David Umahi Federal University of Health Sciences, Uburu, Nigeria.
* Corresponding author: ct.ogu@unizik.edu.ng

Abstract: The protease of malted *Digitaria exilis* (white acha) species was extracted, purified and characterized. The enzyme was extracted with 200 ml of 0.1M citrate phosphate buffer (pH 7) containing 0.4% (w/v) cysteine and 0.86% NaCl, purified by dialysis against 4M sucrose, Ion exchange chromatography on CM sepharose and gel filtration chromatography on Sepharose 4B gel. On ion exchange chromatography and gel filteration chromatography, the white acha protease was purified 2.21 folds with a specific activity of 362 Umg^{-1} protein. The relative molecular weight of the protease was estimated to be 88,000 daltons by Gel filtration. The white acha protease was optimally active at 50ºC and pH 7, but retained about 40% of its activity at 70ºC (30 mins) and pH 8. Appreciable stimulation ($P<0.05$) of the white acha protease was only achieved by Mn^{2+}, while the other metal ions (Zn^{2} ,Ba^{2+} , Fe^{2+} ,Cu^{2+} ,Ca^{2+} ,Sr^{2+} & Hg^{2+}) were inhibitory. Guanidine chloride, n-bromosuccinamide and EGTA were inhibitory ($P<0.05$) to the acha protease, while sodium sulphite and 2-mercaptoethanol (2-Me) were stimulatory with striking stimulation obtained with 2-ME. A significant effect ($P<0.05$) of inhibitors on acha protease was recorded. The enzyme exhibited broad specificity (70 – 100%) in the hydrolysis of various proteins (Bovine serum, albumin, casein, egg albumin and gelatin) and showed strongest affinity for casein when its km (0.188 mg/ml) and Vmax (0.208 U/mol) values were obtained, respectively. Therefore, *Digitaria exilis* protease can be useful in food industries if harnessed.

Key word: *Digitaria exilis,* enzymes, extraction, proteases, purification

INTRODUCTION

The world continues to depend and receive sustenance from grain crops (Conklin and Stilwell, 2007). Thus, research and development on acha cereal grains is experiencing renewed interest in Africa and the rest of the world, particularly for its flavour and nutritional qualities (Jideani *et al*., 2000; koreissi *et al*., 2007). Acha proteins have composition similar to that of rice (Temple and Bassa, 1991; Jideani and Akingbala, 1993), but have relatively high sulphur amino acid (Methionine and cysteine) content (De Lumen *et al*., 1993). Sulphur amino acids are crucial for proper heart function and nerve transmission and cereals are an essential source of these amino acids for people with low meat intake (Cirad, 2006). These and other attributes of acha show the uniqueness of the grains and their potential in contributing significantly to whole grain diets. In view of the recent trend in developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment, food, pharmaceutical, detergent industries, and in several bioremediation processes. Acha is potentially an important source of neutraceuticals such as antioxidant phenolics and cholesterol-lowering waxes (Jideani, 1999). The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents. Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structure-function relationship in the synthesis of peptides and in the sequencing of proteins (Chiplonkar *et al*., 1985). Therefore, this study was aimed at characterizing the white acha protease for industrial application.

MATERIALS AND METHODS

Acha grains used in this study were bought from a neighbourhood market in Onitsha, Anambra state, Nigeria and identified by Botany Department, Nnamdi Azikiwe

University, Awka, Anambra State, Nigeria.

***Enzyme extraction*:** Acha malt grists (20 g) of 4th day of germination was used. The ground samples were extracted with 200 ml of 0.1 M citrate phosphate buffer, pH 7, containing 0.4% (w/v) cysteine and 0.86% sodium chloride for 2 hours using a rotary shaker at 250 rpm.. The obtained extract was centrifuged at 4500 rpm in an Eppendorf refrigerated centrifuge for 30minutes at 4°C and the supernatant was retained for enzyme purification (Ogbonna *et al*., 2003).

Enzyme assay: Protein assay was done by the method described by Upton and Fogarty (Upton and Forgarty, 1977). Casein (1 % w/v) in 0.3 M citrate phosphate buffer was used as the substrate. One milliliter each of casein solution and enzyme in a test tube was incubated in a thermostatically controlled water bath at 40°C (30 mins). The enzyme reaction was stopped by the addition of 6 ml of 5 % (w/v) trichloroacetic acid (TCA). The mixture was allowed to stand for 10 minutes and then filtered through Whatman No 1 filter paper. To 1 ml of the filtrate were added 3 ml of 7.5 %(w/v) solution of sodium carbonate followed by 1 ml of a 1:3 dilution of Folin-Ciocalteau reagent. A blank was prepared as described in enzyme assay except that 1 ml of water was used in place of the enzyme. After standing the mixture for 30 mins at room temperature, optical density values were read at 660nm in a UV visible spectroscopy (Jenway, 6405). One unit of protease activity is that amount of enzyme releasing 1 mg of tyrosine from casein per minute under the assay condition.

Protein estimation: Total protein was estimated by the dye-binding method of Bradford (1976) using BSA as standard at wavelength of 595 nm.

Purification of enzyme: Prior to enzyme purification process, the enzyme solution was concentrated through an overnight dialysis at 4°C using 6 M sucrose solution. The enzyme concentrate was centrifuged at 4500 rpm (4°C) and the supernatant applied to the column (1.7x35 cm) containing the CM-Sepharose previously equilibrated with phosphate buffer pH 7. After a wash with the buffer, proteins were eluted with 250 ml of phosphate buffer (pH 7.0) and equal volume of 0.5 M NaCl solution in buffer. Elution was done at 10 ml per 15 mins. A total of 30 fractions were collected and protein assayed for enzyme activity and protein. The protein absorbance values were read using an Eppendorf Biophotometer at 280 nm and fractions 6-9 which showed enzyme activity were pooled. The enzyme concentrate was applied to a column (2x55 cm) packed with Sepharose 4B which was equilibrated with about 200 ml of 0.1 M citrate phosphate buffer and elution continued with 0.1 M of citrate phosphate buffer at a flow rate of 1 ml/4.5 mins. The fractions (10ml per tube) with protease activity were pooled and their enzyme activities determined.

Estimation of molecular weight: The molecular weight of the protease was determined by the method of Andrews (1964) using Sepharose 4B column (2 cm by 55 cm) equilibrated with 0.1 M citrate phosphate buffer (pH 7.0). Marker proteins (0.2 mg/ml of each protein) consisted of bovine serum albumin (mol wt. 66,500 daltons), *Aspergillus niger* amyloglucosidases (mol. wt. 97,000 daltons) and *Bacillus licheniformis* amylase (mol.wt. 55,200 daltons) (Roche diagnostic).

Temperature activity profiles and stabilities: The temperature activity profiles of white acha protease was determined by reacting 0.2 ml of the enzyme with 0.2 ml of 1% (w/v) casein in buffer at various temperatures (40°C, 50°C, 60°C, 70°C) for 30 mins. For the temperature stabilities, 0.2 ml of the enzyme contained in thin-walled test-tubes was incubated at different temperatures for 30 mins. The enzymes were promptly chilled in ice-chips and the remaining activities assayed.

pH activity profiles and stabilities: The pH activity profiles of acha protease were determined by incubating separately 0.2 ml of the enzyme with 0.2 ml of 1%(w/v) casein in various buffers (0.1M acetate buffer pH 3 – pH 5 and 0.1M citrate phosphate buffer pH 6 – pH 8) at 50°C for

30mins. The enzyme activity was assayed. For pH stabilities, 0.2 ml of the enzyme was incubated with 0.2 ml of the various buffers of different pH values for 24 hours at room temperature. Thereafter, 0.2 ml of casein solution was added and the enzyme assay carried out against a blank at 50°C.

Effect of metal ions and inhibitors: The effects of various cations ($FeSO_4$, $CaCl_2$, $BaCl_2$, $MnSO_4$, $CuSO_4$, $SrNO_4$, $ZnSO_4$, $HgSO_4$) and some inhibitors such as 2-mercaptoethanol (2-ME), ethylene glycoltetraaceticacid (EGTA). n-bromosuccinamide (NBS), guanidine hydrochloride (Guanidine) and sodium sulphite (NaS) on the activities of the proteases were examined. The reaction mixtures contained 0.2 ml of 1mM concentration of the reagents solutions, 0.2 ml of the enzymes and 0.2 ml of substrate (casein). The mixtures were incubated at 50°C for 30 mins to assay for enzyme activity. A control was set up without the salts and inhibitors.

Effect of substrate concentration on enzyme activity: The various concentrations (0 – 1 mg) of the protein substrates (BSA, egg albumin, gelatin and casein) in 0.1M phosphate buffer, pH 6 for the white acha purified enzyme were prepared. To 0.2 ml of the substrate was added 0.2 ml of the enzyme which was incubated at 50°C for 30 mins. Enzyme activity was determined as described earlier. The km values of the enzymes were determined by the Lineweaver-Burk linear transformation of the Michalis-Menten equation.

Statistical analysis: Analysis of variance (ANOVA) was used to analyze the effects of various cations and inhibitors on the properties of the purified enzymes. The significance level used was $P \leq 0.05$.

RESULTS AND DISCUSSION

Enzyme purification

The elution profile of white acha protease on CM Sepharose ion exchange chromatography showed major peaks of protease activity between fractions 6-9 (Figure 1) with 10% retention of the overall activity, a 1.6 and 1.5-fold purification and final specific activity of 311 and 340 Umg^{-1} protein, respectively (Table 1). Similarly, on gel filtration chromatography, major peaks were recorded between fractions 7-10 (Figure 2) with 6% retention of the overall e Umg^{-1} protein, respectively (Table 1)

The relative molecular weight of the acha protease was estimated to be 88,000 daltons. Thus, the acha protease could be said to be a high molecular weight protein.

A summary of purification of the proteases shows that about 6% of the original activity was recovered after gel filteration chromatography, but its specific activity increased (362 Umg $protein^{-1}$) along the purification steps. This specific activity value was lower than that obtained (402.1 Umg $protein^{-1}$ from a sorghum protease (KSV8-11 variety) on Q- Sepharose ion exchange chromatography and gel filtration chromatography on sephadex G-100 (Ogbonna *et al*, 2003).

The relative molecular weight (88,000 daltons) is comparable with that of an acid proteinase (80, 000) from germinated sorghum (Garg and Virupaksha, 1970), but higher than that of a protease (62,000 daltons) from a sorgum malt variety (Ogbonna *et al*., 2003) and protease (35,000-40,000 daltons) from two millet varieties (Taylor, 2004) .

The protease enzyme from white acha species demonstrated optimal activity and stability at 50^0C; such feature places the enzyme at a good advantage for industrial purposes**.** The white acha protease demonstrated pH activities from pH 3.0 to 7.0 with an optimum at pH 6 but was optimally stable at pH 4. Most malt enzymes are known to be metal ion dependent for their activity (Barett, 1995).

Inhibitors give the clearest evidence to the type of catalytic site which forms the basis for the classification of the enzymes (Odibo, 1987). The purified white acha protease was significantly stimulated by 2-ME which is generally known as an activator of enzymes and also contains an –SH group. Another stimulatory effect was obtained with sodium

sulphite which contains sulpur and this stimulatory effect supports the presence of sulphur amino acids (methionine and cysteine) in white acha grain. The white acha protease was inhibited by guanidine hydrochloride; an inhibitor of serine proteases and other enzymes. Ethylene glycol tetraacetic acid (EGTA); a chelating agent, inhibited the protease and must have removed the Mn^{2+} from the active site thereby inhibiting it. The inhibition of the proteases by n-bromosuccinamide indicates the presence of tryptophan group in the active site of the enzyme. The white acha protease could be classified as a metalloprotease. The white acha protease obeyed the rule modeled by Michaelis-menten by producing linear plots, thus permitting the determination of Km and Vmax showing that white acha protease enzyme had strong affinity for proteins and the strongest affinity for these enzymes was demonstrated by gelatin and casein. The relative rate of hydrolysis of proteins by the enzyme occurred at varying rates (63-100%) which indicated broad substrate specificity.

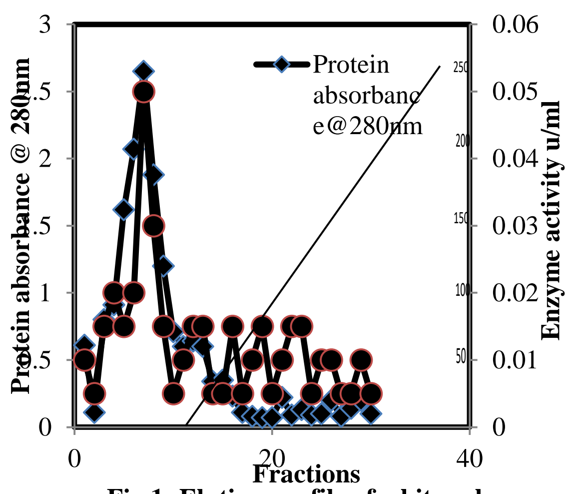

Fig.1: Elution profile of white acha malt protease on CM Sepharose Ion exchange chromatography

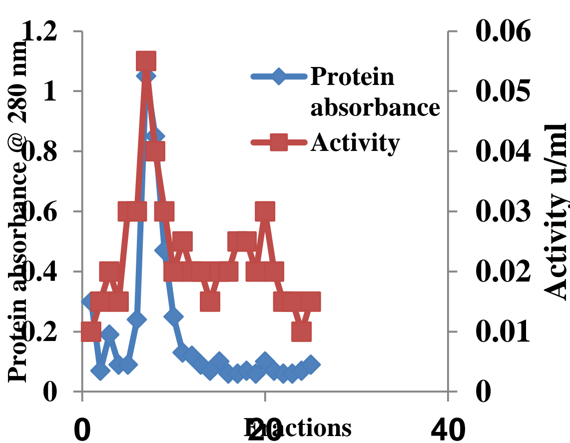

Fig.2: Elution profile of white acha malt protease on 4B Gel filtration Chromatography

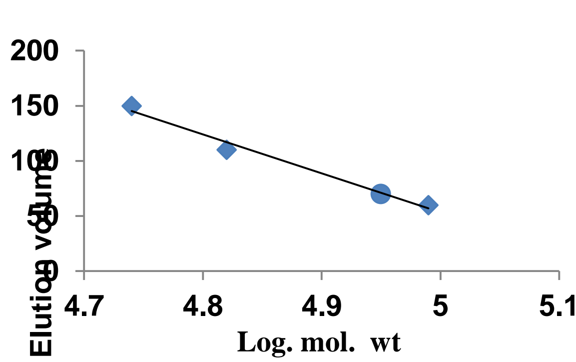

Fig.3 Estimation of relative molecular weight of protease from acha species using Sepharose 4B.

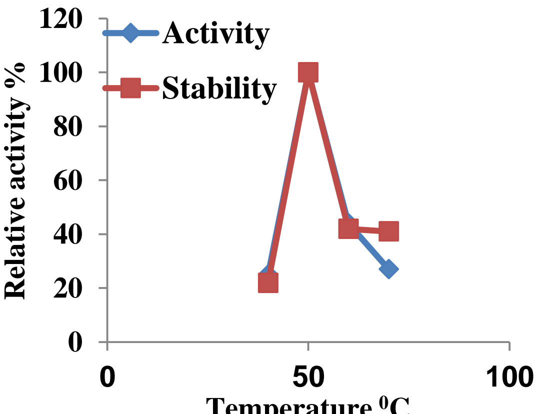

Fig.4: Temperature effect on activity and stability of white acha malt protease

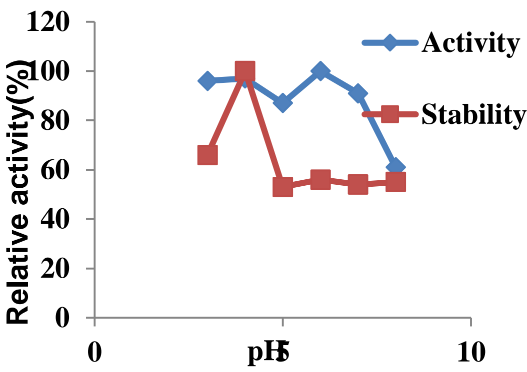

Fig. 5: pH effect on the activity and stability of white acha malt protease

Table 1: Purification summary of Protease from white Acha malt

Purification Steps	Vol (ml)	Total enzyme activity (U/ml)	Total protein (mg)	Specific activity (Umg protein^{-1})	Yield (%)	Fold
Crude enzyme	100	2520	15.4	164	100	1
Dialysis in 6M Sucrose solution	40	1172	4.56	257	46.5	1.56
Ion exchange Chromatography	10	258	0.83	311	10.23	1.89
Gel filtration Chromatography	8	152	0.42	362	6.03	2.207

Table 2: Effect of metal ions on the white and acha proteases

Metal ions (1×10^{-3}M)	White acha P < 0.05 protease % remaining activity
None	100
$SrNO_3^-$	95±1
$ZnSO_4$	56±2
$MnSO_4$	111±2
$BaCl_2$	63±1
$FeSO_4$	59±2
$CuSO_4$	65±1
$CaCl_2$	64±1
$HgSO_4$	53±2

Key: Values represent mean ± standard deviation of duplicate determinations

Table 3: Effect of some inhibitors on acha malt proteases (% relative activity)

Inhibitors (1×10^{-3})	White acha P < 0.05 protease
None	100
EGTA	85±1
NBS	97±2
NaS	125±2
Guanidine	57±1
2-ME	1137±3

Key: Values represent mean ± standard deviation of duplicate determinations, EGTA – Ethylene glycol tetraacetic acid, NBS - N-Bromosuccinamide, NaS - Sodium sulphate, Guanidine – Guanidine hydrochloride, 2-ME – 2-Mercaptoethanol

CONCLUSION

White acha malt protease (enzyme) qualities recorded in this study are such that can be useful, for example, its thermal activities and stabilities, pH broad range and specificity of the enzyme reflects its potential as a good protein source.

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Assessment of Lactic Acid Bacteria Treatments on some Biochemical Indices Associated with Ulcerative Colitis Induced in Wistar Rats

Bamigbade B. O.[1*] Oladejo B. O.[1] and Omayone T. P.[2]
1. Department of Microbiology, Federal University of Technology, Akure, Nigeria
2. Department of Physiology, Federal University of Technology, Akure, Nigeria
* Corresponding author: bamigbadebukky01@gmail.com

Abstract: Ulcerative colitis (UC) is an idiopathic inflammatory bowel disease that affects the colonic mucosa and it's clinically portrayed by diarrhea, abdominal pain and so on. Lactic acid bacteria (LAB) are one of the most significant groups of probiotic organisms, commonly used in fermented dairy products. These group of organisms enhance lactose digestion, stimulate the immune system, prevent and treat diarrhea. In the present study, the therapeutic effects of *Lactiplantibacillus plantarum* PQ104969 and *Lactiplantibacillus plantarum* PP893151 on acetic acid-induced ulcerative colitis was evaluated in Wistar albino rats. Acetic acid-induced ulcerative colitis was achieved by intrarectal administration of 5% acetic acid after acclimatization. Wistar rats were then treated orally with either 1 ml of normal saline, *L. plantarum* PQ104969 (5×10^7 Cfu/ml), *L. plantarum* PP893151 (5×10^7 Cfu/ml) or prednisolone (2 mg/kg) once a day for 7 days. Disease activity index (DAI) was recorded daily after colitis induction by assessing the symptoms. The rats were sacrificed on day 3 and 7 by cervical dislocation, and colon tissues were isolated for the biochemical analysis of oxidative stress parameters. Depletion of total glutathione (GSH) levels in the colitis group was significantly restored in the *L. plantarum* PP893151 treated groups, while *L. plantarum* PQ104969 regulated the expression of proteins, thus alleviated inflammatory response. Both lactic acid bacteria inhibited neutrophil infiltration to suppress myeloperoxidase activity in order to mitigate inflammatory reaction and oxidative stress development in acetic acid induced ulcerative colitis. Hence, *Lactiplantibacillus plantarum* associated with indigenous fermented foods could be used as an alternative treatment of Ulcerative Colitis.

Key word: Acetic acid, inflammation, ulcerative colitis, *Lactiplantibacillus plantarum*, oxidative stress

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the digestive tract, with ulcerative colitis and Crohn's disease being major forms (Rajendiran *et al*., 2018). These diseases can negatively impact tissues and organs. Some research suggests that chronic inflammation could also play a role in a range of conditions, from cancer to asthma (Oladejo *et al*., 2024). Ulcerative colitis (UC) is an idiopathic inflammatory bowel disease that affects the colonic mucosa and is clinically portrayed by diarrhea, abdominal pain and hematochezia. The predominance of IBD including ulcerative colitis, is generally higher with an estimation of 250 cases per 100,000 individuals in western countries however, it is becoming common in other parts of the world due to the adoption of western lifestyle (Baumgart and Sandborn, 2007).

Lactic acid bacteria (LAB) are non-sporing, non-respiring Gram-positive cocci or rods that produce lactic acid as the primary end product during carbohydrate fermentation. Lactic acid bacteria and their probio-active substances have numerous beneficial effects on the gastrointestinal tract, preventing the adherence, establishment, and replication of various enteric mucosal pathogens through various antimicrobial mechanisms (Naidu *et al*., 2010). Lactic acid bacteria are a well-known type of probiotic bacteria, including the genera such as *Lactobacillus*, *Lacticaseibacillus*, *Lactiplantibacillus* and so on. Lactic acid bacteria can produce high levels of lactic acid and other metabolites, which possess anti-inflammatory properties (Hao *et al*., 2023).

Steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) are used to manage inflammatory conditions, but their use is limited due to high costs and adverse effects (Jargalsaikhan *et al*., 2019). The NSAIDs include naproxen, ibuprofen, and aspirin and so on. Corticosteroids, a type of steroid hormone, decrease inflammation and suppress the immune system. But, long-term use of corticosteroids can cause vision problems, high blood pressure, and osteoporosis (Santos-Longhurst, 2018).

Thus, there is a need to come up with other substances or drugs that can treat or reduce inflammatory reactions (Mohd *et al*., 2021). The discovery of anti-inflammatory drugs obtained from natural sources is a rational and effective approach to the treatment of inflammatory conditions because they are safe, effective, biocompatible, and cost-efficient treatment options for inflammatory diseases (Oladejo *et al*., 2024). The purpose of this study was to assess the potential of *Lactiplantibacillus plantarum* strains in mitigating oxidative stress associated with acetic acid-induced ulcerative colitis in Wistar rats.

MATERIALS AND METHODS

Preparation of LAB species isolated from "Pap" (maize gruel): Strains of *Lactiplantibacillus plantarum* were isolated from "Pap" (Maize gruel; prepared from fermented maize-Yellow or white and are popularly consumed in Tropical Africa) samples in this study. The organisms were identified using morphological characterization and biochemical tests. The strains were confirmed by molecular characterization as *Lactiplantibacillus plantarum* PQ104969 and *Lactiplantibacillus plantarum* PP893151 (Bin Maslam *et al*., 2018).

Animals, chemicals, and treatments: The study involved 50 Wistar albino male rats, each weighing 160g. They were kept in cages for 2 weeks to acclimate before the experiments. The rats were fed with standard rat food and water, following the ethics committee's requirements of the Federal University of Technology Akure, Nigeria. Ethical permission was sought from FUTA Research Ethical Committee (FUTA/ ETH/23/101). The study involved rats in different groups, including Group A, which was neither induced nor treated, Group B, which was colitis-only, Group C and D, which were colitis-induced and treated with 5×10^7 Cfu/ml of *L. plantarum* PQ104969 and *L. plantarum* PP893151 respectively. Group E, which was colitis-induced and treated with prednisolone (2 mg/kg). Colitis was induced in rats by using 5% acetic acid through rectal administration. This was achieved through the use of a flexible plastic catheter (outer diameter of 2 mm) which was inserted rectally into the colon 8 cm proximal to the anus of each fasted rats. The rats were then sacrificed on days 3 and day 7 and their blood and colon were analyzed for oxidative stress (Omayone *et al*., 2018)

Biochemical assays of the colon: The colon was removed and homogenized in a Tris-KCl buffer solution of pH 7.00. The homogenate was then centrifuged at 5000 g for a duration of 10 minutes. The resulting supernatants were collected for the assessment of protein levels and various enzymatic activities (Omayone and Olaleye 2022).

Determination of protein concentration in the colon: The Biuret method, as described by Gornal *et al.* (1949), was used to determine protein concentrations in various samples, with a slight modification involving potassium iodide being added to prevent precipitation of Cu^{2+} ions as cuprous oxide as reported by Omayone and Olaleye 2022. The sample homogenates were diluted 10 times with distilled water, 1 ml was then added to 3 ml of Biuret reagent in triplicate. The mixture was incubated at room temperature for 30 minutes. Absorbance was read at 540 nm from spectrophotometer using distilled water as blank.

Determination of myeloperoxidase (mpo) activity in the colon: Myeloperoxidase activity was determined according to the method of Xia and Zweier (1997). Two thousand (2000) µl of O-dianisidine and H_2O_2 mixture was pipetted in the cuvette and 70 µl of sample (serum) was subsequently added to it. The reaction mixture was read at 0 second, 30 seconds and 60 seconds respectively at 460 nm wavelengths. One unit of MPO activity can be defined as the quantity of enzyme able to convert/degrade 1 µmol of hydrogen peroxide to water in one minute at room temperature.

Determination of glutathione (gsh) level in the colon: The method of Beutler *et al.*

(1963) was followed in estimating the level of reduced glutathione (GSH). Sample of 0.2 ml was added to 1.8 ml of distilled water and 3 ml of the precipitating agent was mixed with the sample. This was centrifuged at 3,000 g for 4 minutes. Thereafter, 0.5 ml of the supernatant was added to 4.5 ml of Ellman reagent. A blank was prepared with 0.5 ml of the diluted precipitating agent and 4 ml of phosphate buffer and 0.5 ml of Ellman's reagent. The absorbance of the reaction mixture was read from spectrophotometer within 30 minutes of colour development at 412 nm against a reagent blank.

Ethical approval: Ethical approval was obtained from the Centre for Research and Development (CERAD) of The Federal University of Technology, Akure; FUTA/ETH/23/101.

Statistical analysis of the results: All data were expressed as mean ± standard error mean (SEM). Statistical comparison was performed across the groups using Graph Pad Prism 9 and Microsoft Excel 2019. The differences across the group were accessed by means of analysis of variance (ANOVA). For all tests, the significant differences were taken as $P<0.05$.

RESULTS

Effect of Lactobacilli administration on body weights of rats

Weights of rat's post-colitis induction showed no significant changes across all groups as shown in Figure 1.

Effect of Lactobacilli administration on myeloperoxidase (mpo) activity in rat's colon

The study revealed on day 3, group A (negative control) maintained a low MPO activity, while group B (colitis only) maintained a very high level. Group D (plantarum B2 treated group) had a relatively lower MPO activity as compared to group C (plantarum B3 treated group), while the standard drug (group E) had the highest MPO activity among the treatment groups. Following 7 days treatment, there was a tremendous decrease of MPO activity in group D and group E than that of group C as shown in Figure 2.

Protein concentration in rat's colon

The concentration of protein in the colon of Group A (negative control) remained constant throughout the period of the experiment, with an observation of a highly similar effect on the protein concentration in the Group C (plantarum B3 treated group) and Group E (Prednisolone treated group). Group D (plantarum B2 treated group) and Group B (colitis only group) showed increase protein concentration. Groups of rats that were sacrificed on day 7, showed no significant difference from the control group. This is shown in Figure 3.

Effect of Lactobacilli administration on glutathione (gsh) level in rat's colons

The concentration of glutathione antioxidant molecule in Group A rats' colon remained constant, with a slight decrease in the colitis group on day 3. The *L. plantarum* PQ104969 treatment exhibited similar effects to colitis only, while oral *L. plantarum* PP893151 administration significantly increased GSH levels, with slight increases observed at day 7. This is shown in Figure 4.

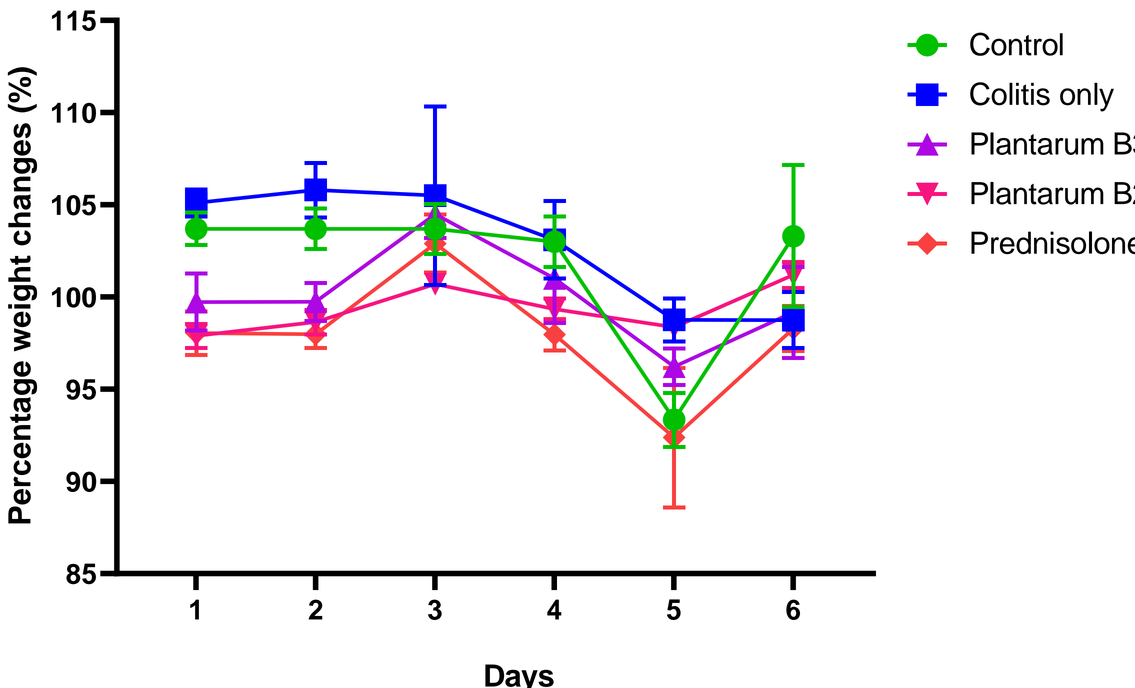
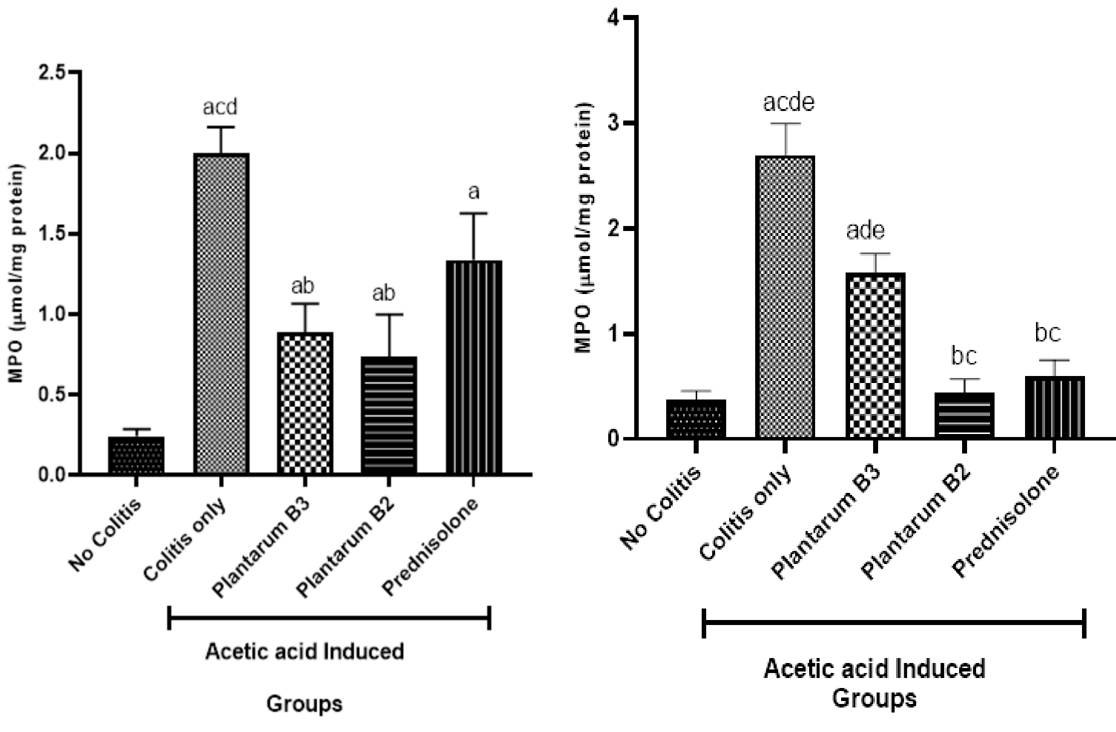
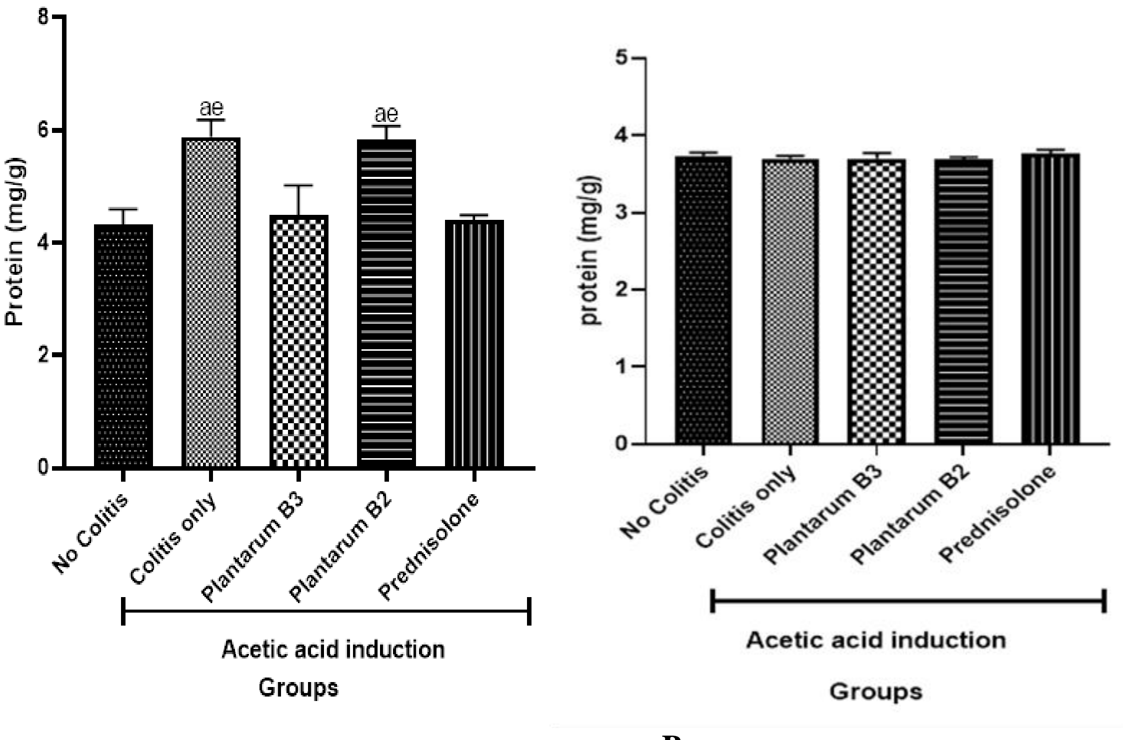


Figure 1: Change in body weights of experimental rats (g) for a period of time (Days). Mean values ± standard error for five rats per group (n = 5) are shown for each group. Group A: control; Group B: Colitis only; Group C-D: fed with *L. plantarum* PQ104969 and *L. plantarum* PP893151 respectively; Group E: treatment with prednisolone (positive control) (significant at P<0.05).



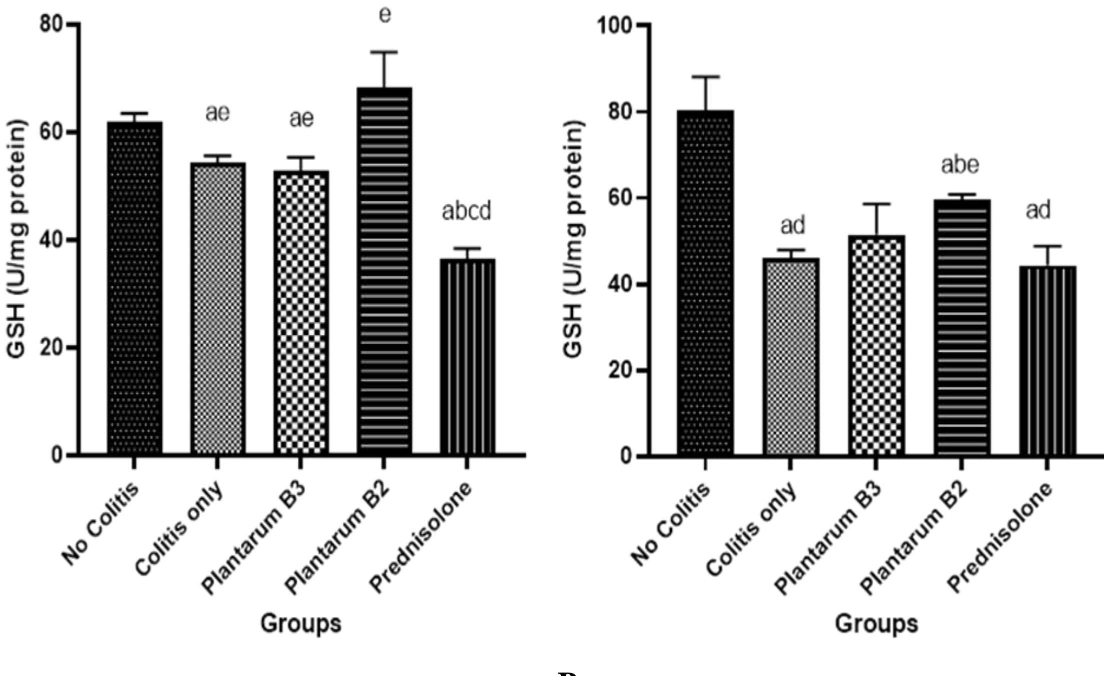
A **B**

Figure 2: Effect of *Lactobacilli* administration on myeloperoxidase (MPO) activity in the colons of rats sacrificed on day three (A) and seven (B). Mean values ± SEM of MPO values in acetic acid-induced colitis ($p < 0.05$; $n = 5$). a, b and c indicate significant difference when compared to control, colitis only, plantarum B3, plantarum B2, prednisolone respectively from One-way ANOVA and Tukey's post-tests.



A B

Figure 3: Effect of *Lactobacilli* administration on protein concentration in the colon of rats sacrificed rats sacrificed on day three (A) and seven (B). Mean values ± SEM of protein concentration values in acetic acid-induced colitis ($p < 0.05$; $n = 5$). a, b and c indicate significant difference when compared to control, colitis only, plantarum B3, plantarum B2, prednisolone respectively from One-way ANOVA and Tukey's post-tests



A B

Figure 4: Effect of lactobacilli administration on glutathione (GSH) concentration in the colon of rats sacrificed on day three (A) and seven (B). Mean values ± SEM of GSH values in acetic acid-induced ulcerative colitis ($p < 0.05$; n = 5). a, b, c, d and e indicate significant difference when compared to No colitis, colitis only, plantarum B3, plantarum B2, Prednisolone respectively from One-way ANOVA and Tukey's post-tests.

DISCUSSION

Acetic acid-induced ulcerative colitis is a reproducible model for inducing colitis in rats, similar to human ulcerative colitis, potentially useful for studying its pathophysiology and treatment (Renata *et al*., 1992). *Lactiplantibacillus plantarum* has been reported for its use in the treatment of various inflammatory disorders (Hao *et al*., 2023). The study examines the therapeutic effect of *Lactiplantibacillus plantarum* on acetic acid-induced ulcerative colitis in Wistar albino rats.

In this study, the body weights of the rats in the treatment groups showed no significant variation from those of the control group. An increased level of MPO in the blood have been linked to inflammation and oxidative damage (Davies and Hawkins, 2020). Inflammatory reactions result in raising myeloperoxidase levels, through the significant influx of neutrophils into the tissue (Oladejo *et al*., 2024). Lactic acid bacteria treatment reduced MPO levels in rats treated with LAB compared to colitis-only groups, suggesting inhibiting neutrophil activation and infiltration.

Increase in protein concentration indicates potential malabsorption or malnutrition, linked to chronic gastro-intestinal diseases like Crohn's and ulcerative colitis. This offers valuable information regarding the severity of the inflammation (Oladejo *et al*., 2024). The study found that *L. plantarum* PQ104969 treatment reduced protein concentration, suggesting it relieves inflammation by inhibiting protein increase.

The Glutathione (GSH), a non-enzymatic antioxidant, maintains cellular redox homeostasis by scavenging reactive oxygen species (ROS) (Oladejo *et al*., 2024). It supports immunological functions and eliminates electrophilic compounds and peroxidases through catalytic activities. In this study, *Lactiplantibacillus plantarum* PP893151 enhances GSH antioxidant activity, protecting the colon from lipid peroxidation caused by oxidative stress.

CONCLUSION

The study found that lactic acid bacteria treatment can mitigate inflammatory reactions and oxidative stress in acetic acid-induced ulcerative colitis. The *L. plantarum* PQ104969 regulates protein expression, while *L. plantarum* PP893151 increases GSH concentration, scavenging reactive oxygen species, suggesting it could be a potential therapeutic agent.

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Anti-Bacterial Properties of Leaf Extracts of *Moringa olifera* and *Alchornea cordifolia* Against Biofilm-Forming Strains of *Pseudomonas aeruginosa*

Enya E.* Ekundayo E. O. and Madukwe E. U.
Department of Microbiology, College of Natural Sciences, Michael Okpara University of Agriculture Umudike, Abia State, Nigeria
* Corresponding author: emmy_enya12@yahoo.com

Abstract: Medicinal plants represent potential sources of new antibacterial agents against drug resistant bacteria. The aim of this study was to investigate the antibacterial activities of aqueous and ethanol extracts of the leaves of *Moringa oleifera* and *Alchornea cordifolia* against strains of *Pseudomonas aeruginosa* expressing various virulence and biofilm-forming characteristics. Five hundred grams (500 g) of each of the powdered plant materials was soaked in 1,500 ml of ethanol/water respectively for 24 h at room temperature. The extracts were filtered using non-adsorbent muslin cloth into a clean beaker. The filtrates were dried by evaporating off the solvent at 50 ^{o}C in a hot air oven. The inhibitory activities of the extracts were tested against the strains using the agar well diffusion and microbroth dilution assays. The crude ethanolic and aqueous extracts of the leaves of *Moringa olifera* and *Alchornea cordifolia* produced inhibition zones ranging from 10.0 mm to 20.0 mm at a concentration of 200 mg/ml. The extracts of *Alchornea cordifolia* had higher inhibitory effects on all the test isolates. The antibacterial activities of the extracts varied according to the genetic determinants carried by the various strains with the zone sizes decreasing to between 7.5 mm to 13.0 mm for some strains. The wild-type strain (PA14-GFP) carrying the green fluorescent protein was susceptible to the aqueous and ethanol extracts of both plants while some of the strains carrying mutations for biofilm formation were less susceptible to the plant extracts. It is remarkable that the ethanol extract of *A. cordifolia* had appreciable inhibitory activity against all isolates including strains like PA01-L-wt-PqSR and PA01-L-wt which carry mutations for biofilm formation. The minimum inhibitory concentration against these strains ranged from 6.25 mg/ml to 25 mg/ml while the minimum bactericidal concentration was between 12.5 mg/ml for the ethanol extracts and 25 mg/ml for the aqueous extracts. Findings of the study provides justification for further research on the potentials of these medicinal plants as sources for antibacterial and antibiofilm compounds.
Key word: Antibacterial, Biofilm, Alchornea, Moringa, Antibiotics

INTRODUCTION

Plants have been the main source of pharmaceuticals and healthcare products in many countries (Jamshidi-Kia *et al.*, 2018). This is so because plants possess enormous reservoir of compounds that have numerous reported biological activities including antimicrobial properties (Ruban and Gajalaksmi, 2016). This potential and the growing public health concern resulting from development of multi-drug resistant bacterial strains suggests that investigating plants can yield valuable and innovative pharmaceutical medicines. The medicinal components in plants and plant products are usually in the form of phytochemicals, which are found in all plants (Ruddaraju *et al.,* 2020; Vaou *et al.,* 2021). These secondary metabolites are crucial therapeutic agents in the development of new drugs. Pharmaceuticals can be made by synthesizing, compounding, or transforming these phytochemicals. Plant extracts are the foundation of most pharmacotherapy systems and have been utilized to treat a variety of illnesses (Ayena *et al.,* 2021).

One of the most drug-resistant bacteria observed in healthcare settings is *Pseudomonas aeruginosa*, which is also a major contributor to nosocomial infections (Kamali *et al.,* 2020; Labovska, 2021). According to D'Abbondanza and Shahrokhi (2021), it is one of the pathogens linked to severe burn infections, post-operative surgical site infections, and urinary tract infections caused by catheter use.

Furthermore, due to its high level of antibiotic resistance, which is prominently enabled by its ability to form dense exopolymeric matrices known as biofilms and a variety of other virulence determinants, *P. aeruginosa* can cause a variety of infections in humans that are typically difficult to treat (Waldrop *et al.*, 2014; Gupta *et al.,* 2016). *Pseudomonas*

aeruginosa poses a significant clinical risk due to its resistance to multiple antibiotics (Sonmezer *et al.*, 2016). Therefore, to control these biofilm-mediated diseases, anti-infective medicines that are effective against planktonic and biofilm microbial species must be explored. Multidrug-resistant (MDR) microorganisms pose a serious risk to the welfare and health of people (Lehtinen *et al.,* 2019). Healthcare professionals face challenges when treating infections caused by multi-drug resistant species, such as *P. aeruginosa*, because treating these infections might lead to higher patient morbidity (Kebede *et al.*, 2021).

Alchornea cordifolia has been routinely utilized as a local remedy for cold (Ebenyi *et al.,* 2017). More importantly, according to Siwe *et al.* (2016), it is also utilized to treat illnesses caused by several bacterial and parasitic species. It is also used as a sedative, an antispasmodic, and an antidote to poisons. The leaves and stem bark are the portions that are most commonly used in medicine, however the leaf has greater potency (Ebenyi *et al.*, 2017). Anti-inflammatory properties have been observed in the crude aqueous/methanolic extract of *A. cordifolia* leaves (Djimeli *et al.* 2017).

Discovery of novel pharmaceutical agents is very important for the control of this organism and other pathogenic drug resistant clinical isolates. The emergence of antibiotic-resistant strains among community-acquired diseases and the unfavourable side effects of some medications (Lehtinen *et al.,* 2019) highlight the necessity of screening the numerous overlooked plant materials. *Moringa oleifera* has been used for centuries as a miracle tree and traditional remedy for many diseases. This plant is one of the most valuable multifunctional trees in the world as several component of it may be used to make food, medicine, cosmetics, or even purify water (Mursyid *et al.,* 2019). The leaf of *Moringa oleifera* exhibits antibacterial, antifungal, antihypertensive, antihyperglycemic, antitumor, anticancer, and anti-inflammatory properties from a pharmaceutical standpoint (Deyno, 2014). The search for and creation of novel antimicrobial drugs is thus an important endeavor. Hence this study was undertaken to evaluate the potentials of leaf extracts of *Moringa oleifera* and *Alchornea cordifolia* in controlling growth of biofilm forming strain of *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Source of Isolate: The *Pseudomonas aeruginosa* strains were laboratory strains obtained from Dr. Blessing Oyedemi of the Department of Biological Sciences, University of Bristol, United Kingdom.

Collection and Extraction of Plant Material: Fresh leaves of *Alchornea cordifolia* and *Moringa oleifera* were collected from Umudike Village, Abia State, Nigeria. The medicinal plants were authenticated by a Plant Taxonomist in the Department of plant science and Biotechnology, Michael Okpara University of Agriculture, Umudike Abia State, Nigeria. The plant materials were air dried for two weeks on cardboards on the floor of a well-ventilated shed used for drying plant materials. The dried parts were pulverized to fine powder using a mechanical grinder. The powdered leaf materials were sieved, weighed and stored in airtight containers at room temperature until they were extracted.

Extraction of plant materials: Five hundred grams (500 g) of each of the powdered plant materials was soaked in 1,500 ml of ethanol/water respectively for 24 h at room temperature. The extracts were filtered using non-adsorbent muslin cloth into a clean beaker. The filtrate was dried by evaporating off the solvent at 50°C in a hot air oven over a period of two days

Screening of the Extracts for Antibacterial Activity: Exactly 0.4 g of each crude extract was reconstituted in 2 ml of dimethyl sulphoxide (DMSO) to obtain extract concentration of 200 mg/ml. This was serially diluted in 2-folds to obtain the following lower extract concentrations: (100, 50, and 25) mg/ml.

Screening of Extracts and Fractions for Antibacterial Activity: The antibacterial activities of the extracts were assessed by the agar well diffusion assay as previously described (Allotey-Babington *et al*., 2014) with slight modifications. Briefly, a stock solution of 200 mg/ml of each of the plant extracts was made in dimethyl sulfoxide (DMSO). Further dilutions were made to obtain concentrations of 50 mg/ml and 25 mg/ml. The test organisms were reactivated by streaking out on a freshly prepared nutrient agar plate. An aliquot of 100 µl of suspension of each *Pseudomonas aeruginosa* isolate standardized to 0.5 MacFarland standard was aseptically inoculated unto Muller-Hinton agar plate using a cotton swab to create a lawn of the organisms. Wells were created on the agar surface using a flame sterilized cork-borer of 6 mm diameter. An aliquot of 50 µl of each of the plant extracts was loaded into each well. A strain of *Pseudomonas aeruginosa* (ATCC 27853) was used as a control.

The minimum inhibitory concentration (MIC) was determined by microbroth dilution technique. Serial dilutions of the plant extracts were made in test tubes containing sterile Mueller-Hinton broth, to obtain concentrations of 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/ml). The test tubes were inoculated with 50 µl of suspension of the test bacterium standardized to McFarland standard tube No. 0.5. The inoculated tubes were incubated aerobically at 37°C for 18-24 h. After incubation, the tubes were examined for turbidity. The tube with the lowest concentration of extracts which showed no turbidity was recorded as the MIC value for the tested extract. The minimum bactericidal concentration (MBC) was determined by streaking the contents of the two last tubes with no turbidity, separately, on freshly prepared nutrient agar plates. The MBC is the concentration in the tube from which no growth was observed after 18-24 h of incubation (Ekundayo *et al.,* 2020).

RESULTS

The growth of the microorganisms used in this study was inhibited by both the ethanolic and aqueous extracts of the leaves of *Moringa oleifera* and *Alchornea cordifolia*. The zone of inhibition ranged from 10.0 mm to 18.5 mm at the 200 mg/ml concentration for the aqueous extract of *Moringa oleifera*. *Alchornea cordifolia* had the highest inhibitory effect on all the test isolates with zone of inhibition ranging between 10.0 mm to 20.0 mm at the 200 mg/ml concentration. Also, the zone of inhibition decreased to between 7.5 mm to 15.0 mm at the 100 mg/ml concentration (Table 1).

Table 1: Diameter of zone of inhibition (mm) of ethanol extracts of Alchornea cordifolia and Moringa oleifera against the Pseudomonas aeruginosa isolates

Isolate code	Alchornea cordifolia			Moringa oleifera			Control (mm)
	200 mg/ml	100mg/ml	50 mg/ml	200 mg/ml	100 mg/ml	50 mg/ml	
PA14-GFP	18.0	13.0	9.0	0.0	0.0	0.0	33
PA01-L-wt	14.0	11.0	0.0	0.0	0.0	0.0	33
PA01-L-wt-PqSR	16.0	7.5	0.0	0.0	0.0	0.0	35
PA01-N-GFP	16.0	10.0	0.0	0.0	0.0	0.0	41
PA14-wt	20.0	15.0	0.0	12.0	0.0	0.0	45
PA01-Rhl L-Lux	15.0	0.0	0.0	0.0	0.0	0.0	35

Table 2: Diameter of zone of inhibition (mm) of aqueous extracts of *Alchornea cordifolia* and *Moringa oleifera* against the *Pseudomonas aeruginosa* isolates

Isolate code	*Alchornea cordifolia*			*Moringa oleifera*			Control
	200mg/ml	100mg/ml	50mg/ml	200mg/ml	100mg/ml	50mg/ml	(mm)
PA14-GFP	15	0.0	0.0	13.0	10.0	0.0	33
PA01-L-wt	12	0.0	0.0	16.0	10.0	0.0	33
PA01-L-wt-PqSR	15	0.0	0.0	0.0	0.0	0.0	35
PA01-N-GFP	13	0.0	0.0	18.5	12.0	8.0	41
PA14-wt	17	8.0	0	17.0	14.0	7.0	45
PA01-Rhl L-Lux	17	0.0	0.0	0.0	0.0	0.0	35

Table 3: MIC and MBC values (mg/ml) of Ethanol extract of *Alchornea cordifolia* and *Moringa oleifera* against the *Pseudomonas aeruginosa* isolates

Plants	Organisms	100	50	25	12.5	6.25	3.12	MIC (mg/ml)	MBC (mg/ml)
Alchornea cordifolia	PA14-GFP	-	-	-	-	+	+	12.5	25
	PA01-L-wt	-	-	-	+	+	+	25	25
	PA01-L-wt-PqSR	-	-	-	-	+	+	6.25	12.5
	PA14-wt	-	-	-	-	+	+	12.5	25
Moringa oleifera	PA14-GFP	-	-	-	+	+	+	25	25
	PA01-L-wt	-	-	-	+	+	+	25	25
	PA01-L-wt-PqSR	-	-	-	-	+	+	12.5	25
	PA14-wt	-	-	-	-	+	+	12.5	25

Key: +: growth of the organism indicated by turbidity in the broth medium; -= Absence of growth of the test organism shown by no form of turbidity in the medium.

Table 4: The MIC and MBC values (mg/ml) of aqueous extract of *Alchornea cordifolia* and *Moringa oleifera* against the *Pseudomonas aeruginosa* isolates

Plants	Organisms	100	50	25	12.5	6.25	3.12	MIC	MBC
Alchornea cordifolia	PA14-GFP	-	-	-	+	+	+	25	25
	PA01-L-wt	-	-	-	+	+	+	25	25
	PA01-L-wt-PqSR	-	-	-	-	+	+	12.5	25
	PA14-wt	-	-	-	-	+	+	12.5	25
Moringa oleifera	PA14-GFP	-	+	+	+	+	+	50	50
	PA01-L-wt	-	+	+	+	+	+	50	50
	PA01-L-wt-PqSR	-	-	+	+	+	+	25	50
	PA14-wt	-	+	+	+	+	+	50	50

+: growth of the organism indicated by turbidity in the broth medium; -= Absence of growth of the test organism shown by no form of turbidity in the medium.

DISCUSSION

Development of medications with the potential to inhibit biofilm formation by bacteria could serve as a major therapeutic target for the treatment of a variety of bacterial infections. Finding naturally occurring plant-based chemicals that can prevent the production of biofilms is a possible substitute (Slobodnıkova *et al.*, 2016).

The growth of the microorganisms used in this study was inhibited by both the ethanolic and aqueous extracts of the leaves of *Moringa oleifera* and *Alchornea cordifolia*. The activity of the extracts were observed to be concentration dependent. These parts of the plants have medical potential because of their capacity to prevent the growth of certain diseases through leaf extracts.

However, the ethanol extracts of *Moringa oleifera* at the respective concentrations tested had no observable activity against any of the test isolates. The findings of this study is comparable to the report of by Nugraha *et al.* (2020) and Ilanko *et al.* (2019). Results for the aqueous extract varied from those of Shoba *et al.* (2014) and Emmanuel *et al.* (2014) who reported no inhibition or a mean disk diffusion zone of 9.5 mm respectively, while Onsare *et al.* (2013) reported inhibition zones up to 24 mm.

The water extract of *A. cordifolia* had antibacterial activity on all strains of *Pseudomonas aeruginosa* only at the highest concentration. The MIC values of the water extract was 25 mg/ml for both PA14-GFP and PA01-L-wt, while PA01-L-wt-PqSR and PA14-wt had an inhibitory concentration of 12.5 mg/ml each. The findings of this study are consistent with a prior work by Ebenyi *et al.* (2017), which found that *A. cordifolia's* aqueous extract has antibacterial activity against *P. aeruginosa* at a minimum inhibitory concentration (MIC) of 25 mg/ml. The findings of this study are also in agreement with the report of Boniface *et al.* (2016) and

Djimeli *et al.* (2017). The antibacterial efficacy of *A. cordifolia's* aqueous extract against *P. aeruginosa* was also reported by Gatsing *et al.* (2010), with an MBC value of 50 mg/ml and a MIC of 25 mg/ml.

The ethanol extract of *A. cordifolia* had the most remarkable antimicrobial activity on all isolates at the highest concentration tested. The activity was observed to decrease as the concentration decreased. Its minimum inhibitory concentration was 6.25 mg/ml for PA01-L-wt-PqSR and 25 mg/ml for PA01-L-wt. This is in line with the findings of Ngoupayo *et al.* (2015), who reported the antimicrobial activity of the ethanol extract of *A. cordifolia*. The authors reported an MIC value 25 mg/ml of and an MBC value of 50 mg/ml for *P. aeruginosa*. Earlier studies had reported promising antimicrobial properties of this plant in another investigations (Abdullahi and Ali, 2019).

The aqueous extracts of *Moringa oleifera* was found to be more active than the ethanol extract. This observation in line with the submission of Saadabi and Abu (2011) who reported that the aqueous extracts of *Moringa olifera* have inhibition potential against many pathogenic bacteria, such as; *Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* in a dose dependent manner. These findings corroborate previous studies that the therapeutic agents derived from plants are used as an important alternative or complementary treatment of infectious diseases (Bakal *et al.,* 2017).

The findings of this study provide direction for the creation of novel medications that may be able to meet therapeutic needs. It could serve as a lead to finding other suitable antibiofilm medication that either promotes the dispersal of preformed biofilms or prevents the development of new biofilms *in vivo*.

The susceptibility of the isolates to the study plant extracts implies that chemical compounds in the extracts can be further developed to fight against these drug resistant microorganisms (Uttu *et al.*, 2015).

CONCLUSION

The parent wild type strain PA14-GFP was observed to be susceptible to the aqueous and ethanol extracts of both plants as against some of the mutated strains, further affirming the role of biofilms in mediating drug resistance. However, the activity observed against some of the mutated isolates by these extracts could be regarded as a boost in the search for other medicinal agents against drug resistant organisms.

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Multi-Drug Resistance, HIV/AIDS Coinfection and Risk Factors Associated with *Mycobacterium tuberculosis* Infection in Nigeria: A Systematic Review

Kashibu E.[1*] Doughari J. H.[2] and Ja'afaru M. I.[2]
1. Department of Medical Laboratory Science, Taraba State University, PMB 1167, Jalingo, Taraba State, Nigeria.
2. Department of Microbiology, Faculty of Life Sciences, Modibbo Adama University, PMB 2076, Yola, Nigeria.
* Corresponding author: Emmanuel.kashibu@yahoo.com

Abstract: Multi-drug resistant tuberculosis (MDR-TB) and human immunodeficiency virus (HIV) have emerged as major public health challenges facing tuberculosis control programme particularly in Asia and Africa. In Nigeria, the seroprevalence of HIV is 4.4%, the third highest infection burden in the world, with 21% of all tuberculosis patients dually infected with TB and HIV. The impact of MDR-TB is likely to increase if adequate measures are not taken. Despite the high prevalence of MDR-TB in Nigeria, not much effort has been made at tackling the problem. This paper reviews the burden of MDR-TB and the factors that are responsible for the problem particularly in Nigeria. Internet search of studies on MDR-TB was done and those relevant for this study were reviewed. The major risk factors of MDR-TB in Nigeria are previous TB treatment and HIV/AIDS coinfection. Efforts should be made towards proper diagnosis of HIV/AIDS and MDR-TB and adequate treatment given where causes are treatable. Patients should be adequately counseled and where facilities for diagnosis and treatment are inadequate, the government can provide and subsidize the cost for ease of management and treatment.
Key word: Tuberculosis, multi-drug resistance tuberculosis, TB/HIV confection, risk factors.

INTRODUCTION

Pulmonary tuberculosis is a global emergency, one third of the world's population has latent TB infection with about 10% of this population developing active pulmonary tuberculosis (PTB) during their life time (Omote *et al.,* 2018). In 2016 10.4 million new cases was recorded globally with estimated death figure of 1.4 million persons at approximately 4,105 mortality rate per year (Chandreskaran *et al.,* 2017*).* There are several chemotherapeutic agents (antibiotics) available and are used for the treatment of PTB, these agents were helping to record success story against PTB until the advent of drug resistant strains and HIV/AIDS pandemic (Abiodun *et al.,* 2015). Drug resistance PTB can be classified as mono (resistance to a single anti TB drug, multi (resistance to rifampicin and isoniazid) and extensive (resistance to rifampicin, isoniazid, fluoroquinolones and kanamycin) (Ullah *et al.,* 2016). Drug resistance PTB is a serious public health issue. Globally 3.7% of newly diagnosed and 20% of previously treated cases of PTB was estimated to be caused by multi-drug resistant tuberculosis (MDR-TB) strains in 2022. Higher figures were reported for central Asia and Eastern-Europe and the presence of extensive TB strain have been reported in 92 countries (Yoon *et al.,* 2013).

It is believed that immune response is associated or may be responsible for susceptibility to tuberculosis (Chandreskaran *et al.,* 2017*).* Immune responses are broadly divided into two (innate and acquired). While innate immunity includes physical barrier, complement system, macrophages, monocyte system and natural killer cells, acquired immunity comprises of humeral immunity (B-lymphocytes/T-lymphocytes dependent and production) and cell medicated/ T-lymphocytes dependent and functions in antigen presentation as well as cytoxic activities). Immunological parameters such as total white cell count, absolute neutrophil count, absolute lymphocyte counts, monocyte to lymphocytes ratio and neutrophil to lymphocyte ratio are been used as susceptibility markers to certain disease (Yoon *et al.,* 2013). There are several reports associating susceptibility of PTB with these parameters (Afzal *et al.,* 2010).

Drug resistant tuberculosis and its rising incidence and prevalence have become a

global health burden with serious threat to the world economic growth (Naranbhai *et al.,* 2014). In 2016, there was an estimated 4.1% of new cases and 19% previously treated cases of multi-drug resistant tuberculosis. This figure has increased by an annual rate of more than 20% (Lange *et al.,* 2018). According to a world Health Organization 2017 report, 240,000 people died from multi-drug resistant tuberculosis and countries that reported at least one strain of extensive drug-resistant tuberculosis rose from 92-123 (WHO, 2017). Several factors have been linked to the emergence of multi-drug resistant tuberculosis. But within the last three decades, acquisition or emergence of drug resistant tuberculosis has been linked to previous TB treatment, use of inferior regimes, poor adherence to anti-TB drugs, poor access to healthcare and largely due to HIV/AIDS pandemic (Oneydum *et al.,* 2017). In Nigeria, similar risk factors have been associated with emergence of multi-drug resistant tuberculosis. The Economic, social and psychological burden associated with drug resistant tuberculosis therapy is enormous. Patients have to cope with limited treatment options, longer duration of therapy regimes with increased toxicity and the economic burden for those accessing care from private practitioners (Thomas *et al.,* 2016).

Method of Literature Search

PubMed and Google scholar were used to search for literature on multi-drug resistance, HIV/AIDS coinfection and risk factors associated with *Mycobacterium tuberculosis* infection. A total of 1,220 articles were initially obtained. Out of these 48 articles were retrieved and included in the review.

Burden of Multi-Drug Resistant Tuberculosis in Nigeria

According to the World Health Organization (WHO) global report 2016, among 10.4 million incidence of TB cases worldwide, 3.9% are estimated to have had rifampicin or multi-drug resistant tuberculosis (MDRTB) in 2015 (WHO, 2016). Also, 21% of previously treated TB cases were estimated to have MDRTB in the same year (WHO, 2016). Nigeria is one of the countries that is included among the 30 high burden countries for multi-drug resistant tuberculosis (WHO, 2016) The estimated incidence of MDRTB in Nigeria is 4.3% among new cases and 2.5% among previously treated case (WHO, 2016). However, with the advent of newer molecular diagnosis techniques for TB and the current advocacy for a country-wide roll-out by the Nigeria TB program and other partners (FMH, 2015 WHO, 2016), Several studies have reported on the rates of DRTB in different cohorts of TB patients across various setting in Nigeria. A study in the Mid-Western Nigeria in 2018 showed a prevalence of 3.3% which is lower than the national prevalence (Kome *et al.,* 2018). In 2018, a study carried out in South-West of Nigeria recorded a prevalence rate of 23.4%. This is high compared to what was reported in studies from Southern and Northern part of the country (Kuyinu *et al.,* 2018; Vain *et al.,* 2014). However, study from another Southern State in Nigeria indicated a high prevalence than what was found in the Northern part of Nigeria (Osman *et al.,* 2012). In North Eastern Nigeria a study carried out in 2017 showed a prevalence rate of 5.9%. This is comparable to other studies in Nigeria which showed prevalence of 7. 1% and 8.6% respectively (Moss *et al.,*2012, *Rasaki et al*., 2014). This high prevalence can be attributed to improper prescription of anti-TB treatment regimes, inadequate drug supply poor quality of drug, high default and treatment failure rate (Blondal *et al.,*2017, Jindani *et al.,* 2004*,* Van der Werf *et al.,*2012). In addition, once selected, drug resistant strain of multi-tuberculosis may be transmitted in the community. Despite the high prevalence of multi-drug resistant TB recorded in Nigeria, no significant efforts have been made in tackling the problem.

Multi-Drug Resistant TB and HIV/AIDS Co-Infection

Drug resistant tuberculosis (DR-TB) is defined as case of TB excreting bacilli that are resistant to one or more anti-TB drug. Drug resistant tuberculosis can exist in

different forms including; mono drug resistant TB, poly drug resistant TB, rifampicin resistant TB, multi- drug resistant TB and extensively drug resistant TB (WHO, 2019). Studies have shown that there is a dynamic interaction between TB and HIV infection. Tuberculosis accelerates the progression of disease in people living with HIV (PLHIV) and people living with HIV have increased susceptibility to TB infection. Tuberculosis has been found to be the major cause of death in PLHIV and TB has been found to be responsible for failure of TB control programs to achieving targets particular in high burden countries. TB and HIV co-infection enhances the risk of acquiring multi-drug resistant TB strains (Wells *et al.,* 2007, Dubrovina *et al*., 2008).

Global Burden of TB and HIV Co-infection

The incidence of drug resistant TB (DR-TB) and HIV coinfection has increased over the past decades as both are strongly linked. It is estimated that PLHIV, especially with fewer 200/cm^3 CD4 counts, show 9-fold increased risk of developing active TB compared to HIV negative patient (Kolo, 1991). World Health Organisation in 2016 reported that 8.6% (7.4-10%) of 10 million incident cases with active TB had coinfection with HIV in 2018. Sub-Saharan Africa is the region with the highest burden of co-infection cases comprising 10% of global co-infected cases of the 30% countries burdened with TB and HIV co-infection. Tuberculosis infection has been detected in 70% of patients with HIV with South Africa reporting the highest number of incidence cases of coinfection (177,000) followed by India (92,000) and Mozambique (N85,000) Also, in 2018, 0.25 million (16.8% of a total 1.5 million deaths from TB shared co-infection with HIV (Kolo, 1991). Out of 862,000 new cases of TB among PLHIV, 477,461 (56%) were established and 86% were placed on antiretroviral therapy (ART) (Kome *et al.,* 2018). In Nigeria, a study in Zaria, North West of the country in 1991 showed a prevalence of 19, 13, and 29% of newly diagnosed patients that are multi-drug resistant (Abdullahi A, 2006). In addition, resistance to isoniazid, streptomycin and pyrazinamide by the isolates had increased to 29, 14, 42% by 2006 (Abdullahi, 2006). This suggests that multi-drug resistant must have been in existence in newly diagnosed patients in Nigeria for some time. But, the WHO without an actual survey put the prevalence in Nigeria at 1.9 and 9.3 for new and previously treated patients respectively. World Health Organization probably underestimated the Nigeria MDR-TB burden. Thus, this brings to fore the need for good clinical practices and cohort drug sensitivity test (DST) survey. It is likely that MDR-TB emerged in Nigeria in 1990s as reported by Idigbe *et al.,* 1999 who reported that 5% of isolated strains were not responding to anti-TB treatment and were resistant to one or more of the drugs used, with 38% being resistant to isoniazid, although 2% were resistant to rifampicin at the time and were not associated with HIV infection (Idigbe *et al.,* 1992).

Association between DR-TB Epidemiology and HIV and Risk factors

There are several epidemiological reasons that DR-TB may be associated with HIV. Among the reasons are i) rapid progression of disease due to harbouring of drug resistant strains, particularly in the immune compromised compared to the immune competent state, ii) malabsorption of anti-TB drugs such as rifampicin and ethambutol, leading to drug resistance and treatment failure, iii) early reactivation of an infection due to increased vulnerability in an immune compromised state acquired from community or institution transmission, iv) direct contact with DR-TB cases, suggesting primary or transmitted resistance, v) co-founding by common risk factors such as intravenous drug abuse imprisonment, vi) low socio-economic status, vii) alcoholism, viii) frequent hospitalization, ix) repeated exposure to DR isolates and x) poor adherence to treatment. The effect of global burden of DR-TB and HIV co-infection has not been effectively defined due largely to lack of sufficient data. The primary reasons

for the lack of data is that HIV infection and anti-TB drug susceptibility testing (DST) are not adequately accessed from joint surveillance under routine conditions. Epidemiological studies from different countries have shown discordant associations. There have been differences in setting, demographic profile, methodology and analysis of data. In the fourth WHO international union against tuberculosis and lung disease global drug surveillance report, 24 countries reported data on MDR-TB by HIV status (Busillo *et al.,* 2011*)* Of these countries only eleven (11), majority from East European and Central Asian regions, reported strong association between HIV and drug resistance. This has made existing data to be sparse as implementation of tests such as automated GeneXpert MTB/RIF assays and polymerase chain reaction-based line probe assay (LiPAS) remain sparse and sub-optimal, particularly in resource-limited settings assumed to have a high burden disease (Busillo *et al.,* 2011) . Community based surveillance at national level should be conducted for all regions worldwide to estimate the burden co-infection in near future.

Table 1: Ten-year study reports on multi-drug resistant tuberculosis in Nigeria

Authors	Study Design and topics
Omote *et al.* (2018)	Pulmonary tuberculosis among suspected cases in Delta State, south Southern Nigeria
Abiodun *et al.* (2015)	Incidence of HIV and Pulmonary tuberculosis co-infection among patients attending out-patients clinic in a Nigerian Hospital.
Oneydum *et al.* (2017)	Prevalence of drug resistant tuberculosis in Nigeria.
Kome *et al.* (2018)	Multi-drug resistant *Mycobacterium tuberculosis* in Port-Harcourt Nigeria.
Kuyinu *et al.* (2018)	Characteristics of *Mycobacterium tuberculosis* positive patients screened of drug resistant tuberculosis.
Osman *et al*. (2012)	Resistance of *Mycobacterium tuberculosis* for first- and second-line Anti Tuberculosis drugs.
Rasaki *et al.* (2014)	Rifampicin Resistant Tuberculosis in a second Health Institution in Nigeria.
Kolo *et al*. (1991)	Bacteriological and drug sensitivity studies on *Mycobacterium* isolates from patient and their close contacts in ABU Teaching Hospital, Zaria.
Idigbe *et al.* (1992)	Resistance to anti-tuberculosis drugs in treated patients in Lagos Nigeria.
Akinyele *et al.* (2020)	Risk factors associated with MDR-TB among Tuberculosis patients in Ibadan

Risk Factors Associated with Multi-Drug Resistant TB

Drug resistant tuberculosis is a serious global health issue with alarming morbidity and mortality figures. There are established risk factors associated with MDR-TB and this include; i) previous TB infection, ii) poor TB drug regime, iii) poor adherence to TB treatment, iv) contact with MDR-TB patient and v) HIV pandemic. Despite the publicity by health workers on the importance of taking precautional measures regarding this risk factors, DR-TB is still with us. This may not be unconnected with the socio-economic condition globally in diagnosed cases of MDR-TB and lack of awareness and inaccessibility of health services. For example, in Ethiopia in 2012, WHO estimated that the number of patients in Ethiopia tested for MDR-TB was <1% of new and <4% of retreatment case (WHO, 2015).

Previous Tuberculosis treatment

Most cases in Nigeria showed a strong association between the occurrence of MDR-TB and previously treated TB with anti-TB drugs (Akinyele *et al*., 2020). This occurs when there is a history of incomplete or inappropriate TB treatment regimens lasting at least 1 month (Gomes *et al.,* 2018). This may be because prior inadequate anti-TB treatment only suppresses the growth of susceptible bacilli and does not affect other resistant strains, leading to suitable conditions for the dominant multiplication of

pre-existing drug resistant mutants (Mc Konen *et al.,* 2015).

Alcohol

Alcohol has been found to be a risk factor occurrence of MDR-TB. Several reports indicated that the use of alcohol increases the risk of developing MDR-TB due to poor adherence to treatment impairment of immune responses and increased risk of adverse drug effects (WHO, 2015, Shin *et al.,* 2010).

Smoking

Smoking has been found to contribute significantly to development of MDR-TB (Gomes *et al.,* 2018). This is because smoking and alcohol have been found to go together. And this increases the risk of developing MDR- TB by poor adherence to treatment and impairment of the immune response and also increasing the risk of adverse drug effect (WHO, 2015, Shin *et al.,* 2010).

HIV/AIDS

The HIV/AIDS has been found in most studies to be significant in the development of MDR-TB *d*espite the availability and access to antiretroviral therapy and prophylaxis to infectious diseases (Marahatta *et al.,* 2015, Andrew *et al.,* 2021). But, findings from a study in South West Nigeria showed that positive HIV status contributes relatively to development of drug resistant tuberculosis (Akinyele *et al.,* 2020). This is an agreement with report of (Mishal *et al.,* 2017) who revealed there is high rate of DR-TB among HIV infected persons. This is as a result of the fact that HIV weakens the immune system and give rise to the rapid progression of latent TB (Mishal *et al,* 2017, Andrew *et al.,* 2021).

Gender

Some studies have shown significant statistical associations between gender and MDR-TB (Fasutini *et al.,* 2006) others show no significant association (Akinyele *et al.,* 2020). A study carried out in Ibadan, Western Nigeria specifically showed that there is no significant statistical association between gender and MDR-TB, though more than two thirds of the MDR-TB cases were male. This was so because the male gender due to the nature of their work are exposed more to external environment than the female and as such are more prone to risk to MDR-TB. On the contrary, a in South Africa reported that more female (63.6%) than males (36.4%) were infected with MDR-TB. They submitted that the reason for this observation was because the male gender had quick access to health care services any time males suffer illness than female. This is because culturally, the female depends on other members of the family to have access to health care as they cannot freely express their help problems (Gomes *et al.,* 2018).

Occupation

The occupation of patients has also been found to have a significant statistical relationship to acquiring MDR-TB. A study in Nigeria showed a significant association with the occurrence of MDR-TB and the occupation of the patients (Akinyele *et al.,* 2020). This is contrary to a study carried out in the United States of America in which no significant difference was found in participant's occupations and occurrence of MDR-TB (Mesfin *et al.,* 2014). The type of job is connected with the income and is an indicator of the socio-economic status of an individual. Several findings indicated high burden of MDR-TB among individuals of low socio-economic status (F.M.H, 2015, Marta *et al.,* 2014). This could be explained because if a family has low income, they might have limited access to medical treatment and health care services. Also, the overcrowded and poor living conditions may facilitate the spread of infectious diseases.

CONCLUSION

Multi-drug resistant tuberculosis in Nigeria has an incidence rate of 4.3% among new cases and 25% among previously treated cases. Nigeria also ranks among the 30 high burden countries for multi-drug resistant tuberculosis in the world . Efforts should be made towards the diagnosis and treatment of the infection. Given that health care facilities are limited in Nigeria, but with the advent of newer molecular diagnostic techniques for

TB diagnosis and the current advocacy for a country wide roll out by the Nigeria TB programme and other partners, this problem could be surmounted. Also, improper prescription of anti-TB treatment regimens, inadequate drug supply, poor quality of drugs, high default and treatment failure rates could be taken care of by increasing direct observation treatment (DOT) centers and provision of treatment clinics whose patients are isolated and treated for the required period of time so as to take care of the associated problems. The government should make anti- retroviral therapy (ART) available for HIV/AIDS patients, since there is significant statistical association between HIV/AIDS and MDR-TB. More HIV diagnostic and counseling units should be provided in most health facilities. Other risk factors can be controlled through public health enlightenment programs in both the urban and rural areas in Nigeria.

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Molecular Detection of Buruli Ulcer among Patients with Various Degrees of Skin Ulcerative Lesions Revealed Zero Prevalence in Parts of Jigawa State, Northern Nigeria

Kashim Y. A.[1] Dogara M. M.[2] Mienda B. S.[1] Nwafor T. E.[3] Akinwale O. P.[3] Adeniyi K. A.[2] and Abubakar H. H.[4]

1. Department of Microbiology and Biotechnology, Federal University Dutse, Ibrahim Aliyu By-Pass, 720223, Dutse, Jigawa State, Nigeria.
2. Department of Biological Sciences, Federal University Dutse, Ibrahim Aliyu By-Pass, 720223, Dutse, Jigawa State, Nigeria.
3. Department of Public Health and Epidemiology, Nigerian Institute of Medical Research, P.M.B. 2013, Yaba, Lagos, Nigeria.
4. Department of Medical Laboratory (Microbiology Unit), General Hospital, Dutse, Jigawa State, Nigeria.

* Corresponding author: kassimadeiza@gmail.com

Abstract: The indolent, necrotizing Buruli ulcer disease is a chronic condition caused by *Mycobacterium ulcerans*, an environmental bacteria that can damage the skin, tissues, and bones. It is now recognized as one of the 20 Neglected Tropical Diseases targeted for elimination by the year 2030. Public health continues to face difficulties due to its mechanism of transmission that is yet to be completely unravel. From December 2019 to September 2023, a hospital-based study was conducted to ascertain the prevalence of Buruli ulcer in parts of Jigawa State, Nigeria. A purposive sampling technique was adopted in selecting and diagnosing 382 samples from patients with various degrees of skin ulcerative lesions across all age groups and gender. The diagnosis of all the samples initially involved the use of Ziehel Nelseen staining procedure. Thirty-five (35) samples with symptoms suspected to be Buruli ulcer were diagnosed using real-time quantitative polymerase chain reaction (qPCR) method. The results were negative for the 382 samples diagnosed using Ziehel Nelseen staining procedure as well as the 35 suspected Buruli ulcer using real-time PCR. This study concludes that despite testing samples with obvious ulcerative lesions suspected to be Buruli ulcer from patients, both Ziehel Nelseen staining procedure and real-time PCR revealed negative results. It is recommended that more samples suspected to have symptoms of Buruli ulcers should be diagnosed using real-time PCR and also make use of both IS2404 and IS2606.

Key word: Public health, Buruli ulcer, *Mycobacterium ulcerans,* Neglected Tropical Disease.

INTRODUCTION

The bacteria *Mycobacterium ulcerans* is the aetiologic agent that causes Buruli ulcer (BU), a chronic, indolent, necrotizing cutaneous illness that is categorized by the World Health Organization (WHO) as one of the skin-related Neglected Tropical Diseases (NTDS) (Mitja *et al.,* 2017). After leprosy and tuberculosis, it is the most prevalent mycobacterial illness (Keragala *et al.,* 2020). Large ulcers, typically on the limbs, are the result of the infection's devastation of soft tissue and skin (Peetermans *et al.,* 2020). Clinically speaking, a BU begins as a papule, nodule, plaque, or oedematous lesion that gradually spreads to a large area of skin ulceration (Yotsu *et al.,* 2018). Surprisingly, considering the degree of tissue loss, the lesion typically causes little to no pain. Buruli ulcer is distinct from other mycobacterial infections in that the pathophysiology of the illness can be attributed to mycolactone, a lipid-like and diffusible exotoxin, rather than the bacterium (Yotsu *et al.,* 2018). In the endemic areas, Buruli ulcer is highly feared and stigmatized due to the unsightly abnormalities it leaves behind. It is also often linked to witchcraft and curses (Osei and Duker, 2015).

Currently, PCR of IS2404, IS2606, and ER is used to detect M. ulcerans DNA for BU diagnosis confirmation. BU can also be confirmed by histology, culturing, and microscopic identification of acid-fast bacilli in lesions. The goal of the World Health Organization is to have PCR confirmation for more than 70% of cases that are reported. (Yotsu *et al.,* 2018). The difficulty in

confirming cases persists because many endemic areas lack convenient access to facilities needed for these testing. New diagnostic tools are now being developed for case management as well as early case detection, with a focus on those that might be used in the field. The loop-mediated isothermal amplification (LAMP) test is one of them. (Ablordey *et al.,* 2012; Beissner *et al.,* 2015), applications of thin-layer chromatography in mycolactone detection (Wadagni *et al.,* 2015), and tests for antigen detection (Dreyer *et al.,* 2015).

At least 27 nations in Africa, Asia, South America, and the western Pacific region have patchy foci of Buruli ulcer (Joshi *et al.,* 2021). Most of the cases that have been recorded are from West and Central Africa (Singh, 2019). Earlier research had shown that Buruli ulcer is endemic in Nigeria, particularly among those who live close to bodies of water. However, most investigations were all conducted in Nigeria's southern regions. There is no documented study on Buruli ulcer in Jigawa State, Northern Nigeria, particularly among the people that live near water features. It is against this background that this study was conducted to determine the prevalence of Buruli ulcer in parts of Jigawa State, Nigeria.

MATERIALS AND METHODS

Study area: Jigawa State was created on 28th August, 1991 from the old Kano Stae, Nigeria. The state is situated between latitudes 11.00ºN and longitudes 8.00ºE to 10.15ºE, and ies in the northwest part of Nigeria. It is bordered on the west by Katsina and Kano States, on the east by Yobe and Bauchi States, and on the north by the international boundary with the Niger Republic. As shown in Figure, 1 the state is divided into three senatorial Districts namely: Northwest, Central and Northeast. The study was conducted in secondary health facilities in Northwest and Central Senatorial districts. The state has two distinct seasons: the dry season, which runs from October to May, and the rainy season, which runs from June to September. The hot temperature during the rainy season is approximately 42°C, while the low temperature is 10°C (Okereke *et al.,* 2015). The state ranks eighth in terms of population according to the 2006 census.

The state has three types of health facilities: the primary health care which is under the purview of the Local Government, secondary health care is overseen by the State Government (the state has sixteen general hospitals and one specialty hospital), and tertiary health care is under the purview of the Federal Government and there is only one Federal Medical Center (Dogara and Ocheje, 2016; Makinde *et al.,* 2018). State-level health policy development and execution, as well as translation of Federal health policies for implementation, fall under the purview of the Ministry of Health and the Agency for Primary Health Care. A primary health center is intended to be available in every ward of the state, as the state currently functions under a single roof system (Makinde *et al.,* 2018). Patients identified with various degrees of ulcerative lesions are admitted into General Hospitals for care.

Six secondary healthcare facilities were selected, they include General Hospital Dutse, General Hospital Birnin Kudu, General Hospital Jahun, General Hospital Ringim, General Hospital Gumel and General Hospital Kazaure, as shown in Figure 1.

Study design: A cross-sectional hospital-based study was conducted. Two out of the three senatorial districts were selected for the study. The samples were collected from selected secondary healthcare facilities within the two senatorial districts of Northwest and Central of the state.

Sample size determination: The study adopted a purposive sampling technique in the selection of the patients whose samples were collected from the study population. A total of three hundred and eighty-two (382) skin related ulcer patients were screened. Specifically, thirty-five (35) samples were collected from patients who presented

symptoms mimicking Buruli ulcer for the molecular analyses (Portaels and World Health Organization, 2014). Samples were collected from the patients between December 2019 and September 2023.

Screening of samples for the detection of *Mycobacterium ulcerans*

Microscopic examination: Microscopic diagnoses by direct smear examination with Ziehl-Neelsen staining to detect the presence of acid-fast bacilli (AFB) was done using the quantification of smears by the method locally used for the diagnosis of tuberculosis (Portaels, 2001). The positive control sample was collected from the tuberculosis (Tb) positive stock at the Tb Unit of Dutse General Hospital, Jigawa State, Nigeria. Self-sputum was collected and smeared, stained, and subsequently used as the negative control. Using a sterile swab stick and fine needle aspirate (FNA), ulcerative lesions, papules and nodules were collected respectively from suspected patients and then smeared on a clean grease free slide using sterile technique. The smear was air dried, and heat fixed by passing through an open blue f lame. The smear was covered with the carbol fuchsin stain. The stain was heated until vapour rose for 5 minutes. The smear was washed with clean water and 3% v/v acid alcohol was applied to the smear for 5 minutes to decolorize the smear to pale pink colour. The smear was rinsed with clean water. Malachite green stain was applied to the smear and allowed to stain for 2 minutes. The stain was rinsed with clean water. Back of the slide was wiped to clean and then placed in the rack for the smear to air-dry. The smear was examined microscopically, using the ×100 oil immersion objective. Confirmed positive and Negative tuberculosis samples were used as controls. Negative Microscopy confirms the absence of acid-fast bacilli.

Molecular analyses: The analyses were performed at the Molecular Parasitology Research Laboratory of the Nigerian Institute of Medical Research (NIMR), Yaba Lagos State, Nigeria, a reference laboratory for Buruli ulcer diagnosis and research. Deoxyribonucleic Acid (DNA) extraction was performed by bacterial lysis with the use of Genolysis kit (reference; 51610. Hain LifeScience).

***Sample collection and processing*:** Thirty-three (33) swab and two (2) fine needle aspiration (FNA) samples were aseptically collected into collection tubes. Each tube was labelled for ease of identification. Subsequently each swab sample collected from lesion on the patients' limbs (upper or lower) was transferred into 15 ml conical tube, was rehydrated with 2 ml of sterile water and vortexed. This was left to stand for 5 minutes and re-vortexed to increase the concentration of the sample. The swabs were gently discarded from the 15 ml tube leaving the content. Thereafter, 400 µl content obtained from 2 ml swab content was measured and transferred into a 1.5 ml tube with screw cap for DNA extraction and purification. The FNA samples, were vortexed and a 400 µl each was taken and transferred into a 1.5 ml tube with screw cap for DNA extraction (Nigerian Institute of Medical Research, 2023).

Deoxyribonucleic acid (DNA) extraction and purification procedure: The DNA extraction was performed using the Genolysis kit (Hains Lifescience, Nehren, Germany). A 400 µl of specimen suspension was placed into 1.5 ml tubes with screw cap for each sample, Thereafter, tubes were centrifuged at 12000 g for 15 minutes at room temperature. The supernatants were carefully discarded using a P1000 filter tip leaving the pellets at the bottom of the conical tubes. The pellets were re-suspended in 400 µl of distilled water and centrifuged at 12000 g for 15 minutes at room temperature. The supernatant was carefully discarded, and the pellets were re-suspended in 50 µl of A-LYS buffer and incubated for 10 minutes at 95°C. The tubes were centrifuged for 10 seconds to pellet the suspension. Then, 50 µl of buffer A-NB was added and the tubes were kept at 4°C prior to PCR/amplification procedure (Nigerian Institute of Medical Research, 2023).

Real-time quantitative PCR (RT qPCR): The RT qPCR was performed as described by Fyfe and Lavender (2022). The method is recommended by the WHO for *M. ulcerans* detection (Bretzel and Beissner, 2018), based on primers IS2404TF and IS2404TR. The primers set employed for quantification is depicted in Table 1.

Quantitative PCR (qPCR)/amplification condition/procedure: The qPCR/amplification reaction was performed in a total volume of 20 µl reaction in Bio-Rad thermal cycler machine (Bio-Rad Laboratories, Marnes- la- Coquette, France). Five micro liter (5 µl) of 5x Hot fire Pol probe qPCR mix (Master mix) was added to 11.5 µl water (PPI), followed by 1.5 µl of forward primer and 1.5 µl of reverse primer including 0.5 µl template DNA. The qPCR amplification procedure is presented as follows: The sample was heated at initial denaturation temperature of 95°C for 10 minutes at 1 cycle, followed by final heating at 95°C for 15 seconds at 60°C for 1 minutes at 40 cycles. The detection was made through Taqman probe. The constitution of total reaction volume for the qPCR reaction is shown in Table 2.

Statistical analysis of data: Results of the analysis were converted to percentages and presented on tables.

Ethical approval: The study was conducted under human right ethical approval sought from Jigawa State Ministry of Health with reference number: MOH/SEC.3/S/819/1, prior to commencement. Study protocol was submitted for approval by the ministry. Permission was sought from the Medical Directors of all the six (6) hospitals where this research was conducted. Sample collection from the participants was conducted following verbal and documented informed consent by each of the participant.

RESULTS

Prevalence of Buruli ulcer in parts of Jigawa State, Nigeria

The observations from both microscopic examination (Ziehel Nelseen staining procedure) and molecular analyses (Real-Time PCR) which were all negative are shown in the Tables 3 and Table 4.

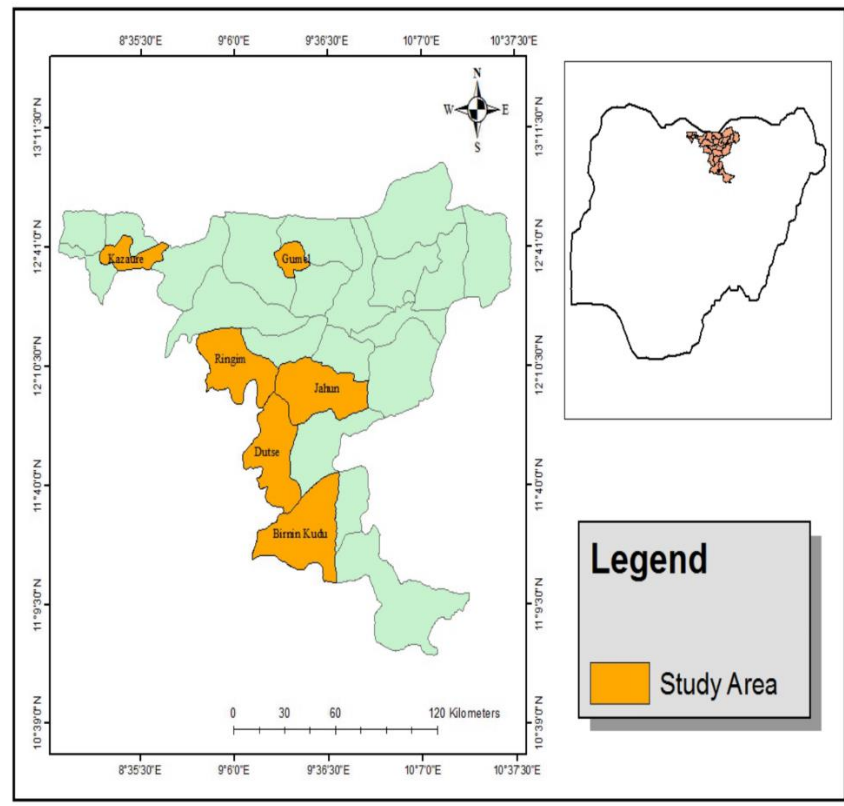

Figure 1: Map of the study area. Source: Geographic Information System (GIS) Unit, Department of Environmental Management and Toxicology, Federal University Dutse, Dutse, Nigeria

Table 1: Primer Set Information for IS2404 Gene

Primer	Primer Sequence (5′–3′)	Primer Length	Fragment Size (bp)
IS2404TF (5′)	AAAGCACCACGCAGCATCT	19	276
IS2404TR (3′)	AGCGACCCCAGTGGATTG	18	330

Fyfe and Lavender (2022)

Table 2: Constitution of total reaction volume for the qPCR reaction

Component	Volume (µl)
Master mix (5x)	5
F Primer	1.5
Reverse Primer	1.5
DNA template	0.5
Water	11
Total volume	20

Table 3: Prevalence of Buruli ulcer in relation to age group, gender and health facility

Variable	Number Tested	Number of Positive	Percentage Positive (%)
Age group (Years)			
0 – 5	13	0	0
6 – 11	48	0	0
12 – 17	127	0	0
18 and above	194	0	0
Gender			
Male	265	0	0
Female	117	0	0
Health Facility			
Birnin Kudu GH	67	0	0
Dutse GH	60	0	0
Gumel GH	60	0	0
Jahun GH	65	0	0
Kazaure GH	65	0	0
Ringim GH	65	0	0

Key**:** GH = General Hospital

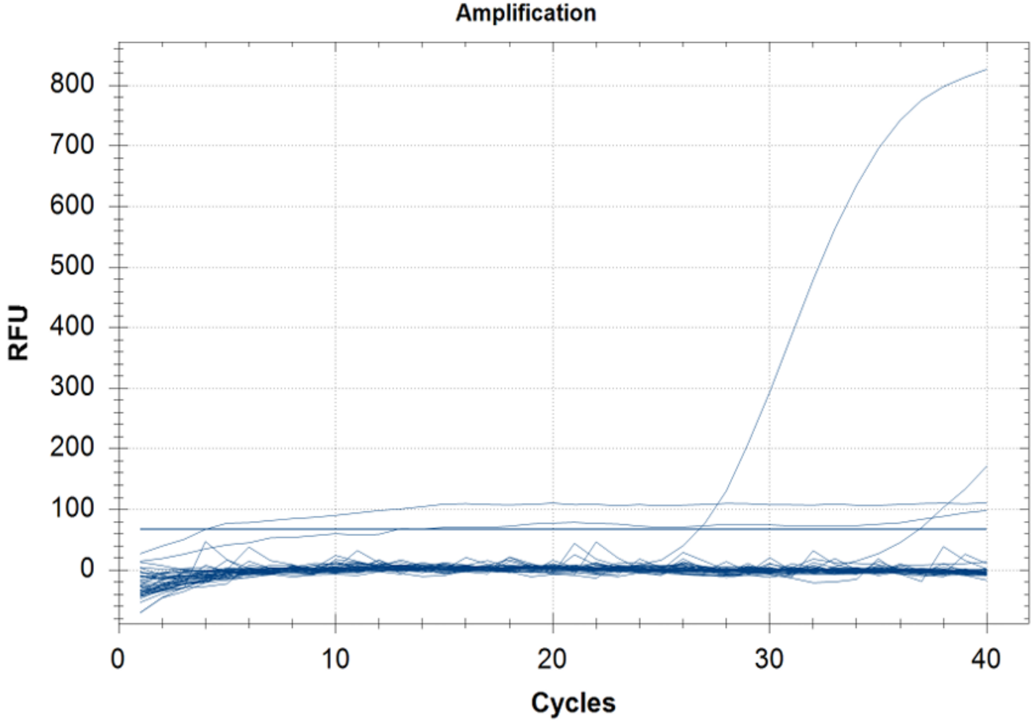

Figure 2: The qPCR amplification plot

Table 4: The qPCR Quantification Parameters

S/N	Sample ID	Type of Specimen	Target Gene	C_q	C_q Mean	PCR Status
1	3449	Swab	IS2404	N/A	0.00	Negative
2	3450	Swab	IS2404	N/A	0.00	Negative
3	3451	Swab	IS2404	N/A	0.00	Negative
4	3452	Swab	IS2404	N/A	0.00	Negative
5	3453	Swab	IS2404	N/A	0.00	Negative
6	3454	Swab	IS2404	N/A	0.00	Negative
7	3455	Swab	IS2404	N/A	0.00	Negative
8	3456	Swab	IS2404	N/A	0.00	Negative
9	3457	Swab	IS2404	N/A	0.00	Negative
10	3458	Swab	IS2404	N/A	0.00	Negative
11	3459	Swab	IS2404	N/A	0.00	Negative
12	3460	Swab	IS2404	N/A	0.00	Negative
13	3461	Swab	IS2404	N/A	0.00	Negative
14	3462	Swab	IS2404	N/A	0.00	Negative
15	3463	Swab	IS2404	N/A	0.00	Negative
16	3464	Swab	IS2404	N/A	0.00	Negative
17	3465	Swab	IS2404	N/A	0.00	Negative
18	3466	FNA	IS2404	N/A	0.00	Negative
19	3467	Swab	IS2404	N/A	0.00	Negative
20	3468	FNA	IS2404	N/A	0.00	Negative
21	3469	Swab	IS2404	N/A	0.00	Negative
22	3470	Swab	IS2404	N/A	0.00	Negative
23	3471	Swab	IS2404	N/A	0.00	Negative
24	3472	Swab	IS2404	N/A	0.00	Negative
25	3473	Swab	IS2404	N/A	0.00	Negative
26	3474	Swab	IS2404	N/A	0.00	Negative
27	3475	Swab	IS2404	N/A	0.00	Negative
28	3476	Swab	IS2404	N/A	0.00	Negative
29	3477	Swab	IS2404	N/A	0.00	Negative
30	3478	Swab	IS2404	N/A	0.00	Negative
31	3479	Swab	IS2404	N/A	0.00	Negative
32	3480	Swab	IS2404	N/A	0.00	Negative
33	3481	Swab	IS2404	N/A	0.00	Negative
34	3482	Swab	IS2404	N/A	0.00	Negative
35	3483	Swab	IS2404	N/A	0.00	Negative
36	Water	N/A	IS2404	N/A	0.00	Negative
37	Negative Control	N/A	IS2404	N/A	0.00	Negative
38	Negative Control	N/A	IS2404	N/A	0.00	Negative
39	Positive Control	N/A	IS2404	26.75	26.75	Positive

Key: N/A = Not Applicable; FNA = Fine Needle Aspirates; C_q = Cycle of quantification; ID = Identity; PCR = Polymerase Chain Reaction

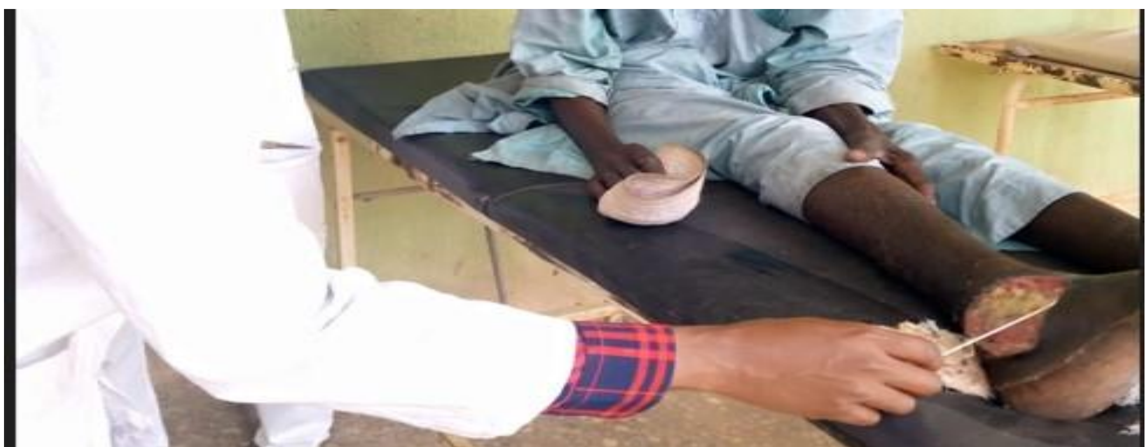

Plate 1: Sample of Patient from General Hospital Dutse

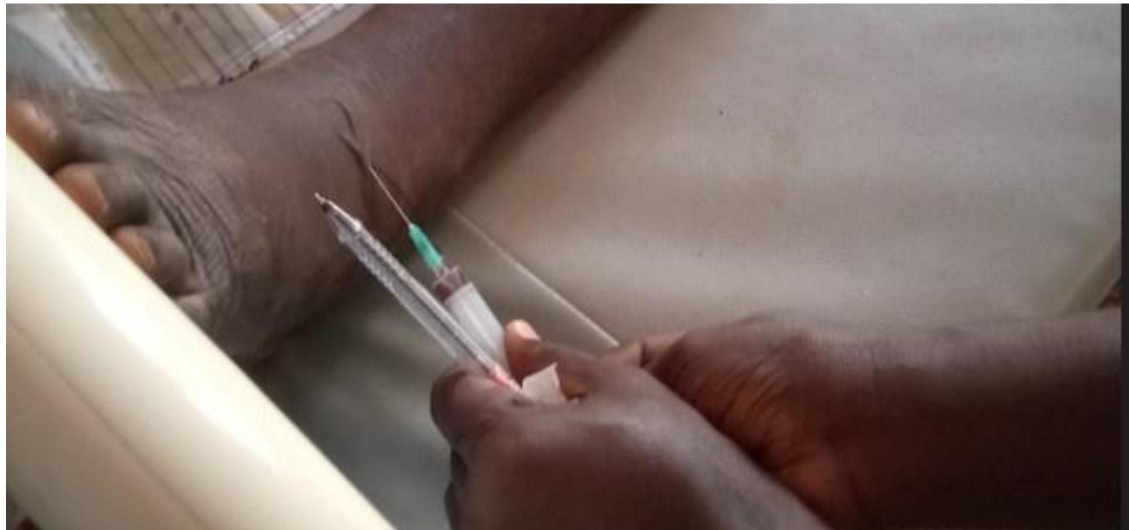

Plate 2: Sample of Patient from General Hospital Ringim

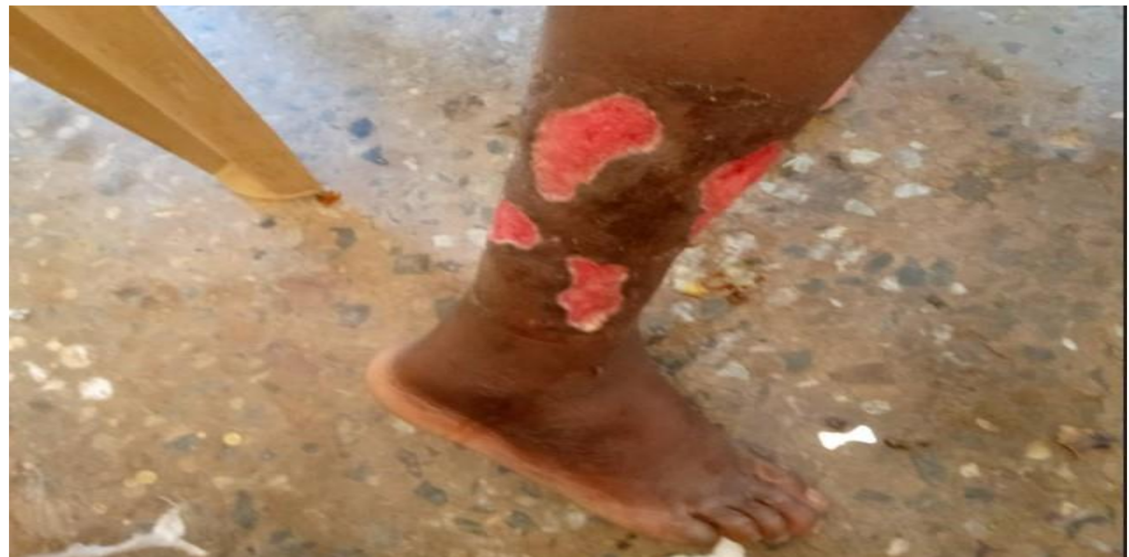

Plate 3: Sample of Patient from General Hospital Kazaure

Plate 4: Sample of Patient from General Hospital Gumel

Plate 5: Sample of Patient from General Hospital Birnin Kudu

Plate 6: Sample of Patient from General Hospital Jahun

DISCUSSION

Finding from this study is different from that of Ukwaja *et al.* (2016) who reported the crude prevalence of 18.7 per 100,000 in their study communities and much higher rate 41.4 per 100,000 in Ogoja. However, this study agrees with one of the commonly held notions by heath policymakers in Nigeria that Buruli ulcer disease is not endemic in the country anymore (Ukwaja *et al.,* 2016).

The findings from this study also differs from the number of cases from the following regions, Nkpo Hamida village, Igbo-Eze North Local Government Area of Enugu State (1 case); Iburu village, Ohaozora Local Government Area of Ebonyi State (1 case), Akoju village, Ikwo Local Government Area of Ebonyi State (1 case); Amazunze village, Nkanu East Local Government Area of Enugu State (1 case); Okro Mbokho village, Eastern obolo, Akwa Ibom State (1 case); Oron village, oron Local Government Area of Akwa Ibom State. (1 case); and (1 case) in Ugwu Tank, Awka South Local Government Area of Anambra State (Okechukwu *et al.,* 2007).

Orujyan *et al*. (2022), reported the protective effect of neonatal Bacille Calmette-Guérin (BCG) vaccination against severe forms of BU disease, although there are conflicting reports on its effectiveness. The zero prevalence of BU in parts of Jigawa state may be attributed to the administration of BCG vaccine as a component of routine immunization schedule. This findings also revealed that Buruli ulcer is not endemic in Jigawa state, Northern Nigeria, despite the availability of

various degrees of skin ulcerative lesions in the study facilities.

Although reports of the community's attitude towards Buruli ulcer illness as not being a medical condition exist, the zero prevalence may possibly be a result of the study being conducted entirely in hospitals (Kashim *et al.,* 2024). Such a bad impression might have discouraged many BU patients from visiting medical facilities, which would have limited the number of opportunities to interact with BU patients. Another common belief regarding buruli ulcers is that they are spiritual or paranormal illnesses brought on by witchcraft. (Osei and Duker, 2015). These beliefs may have also caused BU patients to seek care from native or herbalist doctors, so obstructing their access to healthcare institutions and potentially making it more difficult to include them in this study.

CONCLUSION

This study diagnosed Buruli ulcer using molecular and microscopic approaches, but nonetheless, the findings show that there is no Buruli ulcer in the study area. Sampling in this study was hospital-based, future researcher should focus on community sampling. More samples suspected to have symptoms of Buruli ulcers should be diagnosed using real-time PCR and make use of both IS2404 and IS2606.

Acknowledgement

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Bacterial Infections of the Upper Respiratory Tract of Different Breeds of Dog in Abeokuta, Ogun State, Nigeria

Lifowora S. O.[1] Adeyosoye O. I.[2*] and Akintokun A. K.[3]

1. Biotechnology Centre, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.
2. Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.
3. Department of Microbiology, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.

* Corresponding author: soji.adeyosoye@gmail.com

Abstract: Dogs are domestic animals as well as human pets with potential zoonotic respiratory infections. Nasopharyngeal samples were collected from a total of fifty-five (55) dogs at the Veterinary Teaching Hospital (VTH), Federal University of Agriculture, Abeokuta (FUNAAB), Nigeria. Ten (10) breeds were examined for probable bacteria responsible for upper respiratory tract infection (URTI) and antibiotic resistance among the isolates. The isolates per breed with age as a factor, were microbiological screened, while antimicrobial susceptibility test (AST) was performed by Kirby-Bauer's disc diffusion method with their minimum inhibitory concentration (MIC). The URTI was highest (49.0%) for dogs younger than 12 months, while the least percentage of 5.5% was recorded for dogs between ages 6 and 10 years. The decreasing order of URTI rate based on breed was; Alsatian (43.6%)>Boerboel (20.0%)>Italian mastiff>Terrier cross (1.8%). The frequency of occurrence of ten identified bacterial species were *Escherichia coli* (83.1%), *Citrobacter freundii* (73.4%), *Staphylococcus aureus* (67.5%), *Klebsiella oxytoca* (65.2%), *Bacillus subtilis* (57.6%), *Staphylococcus saprophyticus* (40%), *Pseudomonas aeruginosa* (38.2%), *Streptococcus* spp. (18.2%), *Proteus mirabilis* (14.5%) and *Haemophilus* spp. (5.8%). All the isolates expressed significant differences ($P<0.05$) across all the parameters tested and were also 100% resistant to at least one of the antibiotics tested. Percentage susceptibility rate (%) to nitrofurantoin (100), ciprofloxacin and amoxycillin (90.0), ceftriaxone (10.0), while augmentin was completely resisted by all the isolates (0%). The study revealed that most pet-dogs in the sampled area were potential carriers of antibiotic-resistant bacterial strains. More public awareness aimed at curtailing the spread of these pathogenic agents is highly recommended.

Key word: Bacterial infection, breeds of dog, human pets, upper respiratory tract

INTRODUCTION

Genetic evidence shows an evolutionary split between the modern dog's lineage and the modern wolf's lineage around 100,000 years ago, but the oldest fossil specimens genetically linked to the modern dog's lineage dated to approximately 33,000-36,000 years ago (Laura *et al*., 2017; Mietje *et.al*., 2009). Dogs are domestic animals having close contact with humans (Dewey and Bhagat, 2002; Natalie *et al*., 2019) and are becoming more economically important day after day as dogs are being used as pets, security, game hunting and amazingly, as meat in some parts of the country (Druzhkova *et. al*., 2013). Through genetic selective breeding, dogs have been characterized into hundreds of various breeds, and shows more behavioral and morphological variation than any other land mammal (Spady and Ostrander, 2008; Erin *et al*., 2019). The common breeds include; Akita, Boerboel, Bloodhound, Boxer, Bouvier Des Flanders, Bulldog, Bull Mastiff, Chihuahua, Chowchow, Dobermann, German Shepherd, Labrador Retriever, Neapolitan Mastiff, Pit Bull Terrier, Rottweiler, West Highland White Terrier and so on (AKC, 2022).

In Nigeria currently, for security reasons and due to lack of adequate knowledge of implications of zoonosis, there is an increasing population of dog owners, with dogs living freely among people, especially children. This close relationship of pet animals to their owners may constitute potential public health hazard (Ajuwape *et al*., 2006; Paul *et al*., 2020). The upper respiratory tract of dogs includes the nasal cavity, larynx, pharynx and bronchi. These regions harbour many microorganisms due to constants inhalation of potentially contaminated air (Ajuwape *et al*., 2006; Tress *et al*., 2017). Coughing and dyspnea are commonly associated with primary

problems of the respiratory tract and may also occur secondary to disorders of other organs or systems (Buonavoglia and Martella, 2007; Shair *et al*., 2022).

Both young and aged animals are at risk of developing respiratory diseases. At birth, the respiratory systems are incompletely developed; thus, facilitates the introduction and spread of pathogens within the lungs and the alveolar flooding may occur. In aged animals, chronic degenerative changes that disrupt normal mucociliary clearance and immunologic allergy may render the lungs more vulnerable to airborne pathogens and toxic particles (Windsor and Johnson, 2006; Brasier *et al*., 2024).

Commensally known bacteria such as; *Pasteurella multocida*, *Bordetella bronchiseptica*, Streptococci, Staphylococci, Pseudomonads and coliform spp. are autochthonous to the canine nasal passages, nasopharynx and upper trachea, and at least intermittently in the lungs, without causing clinical signs (Windsor *et al.*, 2004; Qekwana *et al*., 2020). Opportunistic infections by these bacteria may occur when defense mechanisms are compromised by infections with primary pathogens (distemper, par influenza virus, or canine type 2 adenovirus in dogs), or diseases such as congestive heart failure and pulmonary neoplasia (Knotek *et al.*, 2001; Yondo *et al*., 2023). Secondary bacterial infections complicate the management of viral respiratory infections of dogs (Maboni *et al*., 2019).

The pathogens may continue to reside in the respiratory tract of convalescent animals. When stressed, these animals may relapse; and can also act as a source of infection for others. Poor management practices (overcrowding) are often associated with poor hygienic and environmental conditions, and the resultant stress increases both the incidence and severity of infections. Conditions that favour the spread of infections often occur in catteries, kennels, pet shops, boarding facilities, and human shelters (Buonavoglia and Martella, 2007).

Poor handling of the dog nasal mucus and sputum have been found to be a major route of spread of bacteria from domestic dog to its handlers (Guardabassi *et al*., 2004). Although, more than 10% of dogs may carry *Staphilococcus aureus* and other pathogenic bacteria which may infect humans (Guardabassi *et al*., 2004). Therefore, there is a need to further prevent increasing resistant bacteria pathogen from infecting human beings. This study aimed at profiling the antimicrobial susceptibility pattern of bacteria associated with the upper respiratory tracts in domesticated dogs in Abeokuta, Ogun State.

MATERIALS AND METHODS

Study area: The study was conducted at the Veterinary Teaching Hospital (VTH), Federal University of Agriculture, Abeokuta (FUNAAB), Ogun State, Southwest Nigeria, lying within Latitude 70114’07’N and Longitude 3026’15’E (Adenubi *et al*., 2022).

Sample collection and transportation: A total of fifty-five (55) nasopharyngeal samples were aseptically collected from dogs brought to FUNAABVTH for treatment, usually, between the hours of 9:00 am – 11:00 am and immediately transported using sterile swabs from the upper respiratory tracts. The samples were transported in sterile maximum recovery diluent at a maintained at temperature (25°C) in Thermos flask (Master chef vacuum flask MC-F808) to the laboratory for microbiological assessment immediately.

Culturing, characterization, isolation and identification bacterial species: Aliquots of serially diluted nasopharyngeal suspension were inoculated onto sterile nutrient agar plates, blood-enriched agar and MacConkey agar for isolation of aerobic bacteria. The plates were invertedly incubated at 35°C for 48 h. The isolates were identified based on their colonial, morphological and biochemical characteristics according to standard methods (Barrow and Feltham, 1993; Cheesbrough, 2006), while the identification was done according to the

description of Bergey's manual of determinative bacteriology (Bergy, 2000).

Antimicrobial susceptibility screening of the bacterial isolates: Different classes of antibiotics were tested against the isolates by the Kirby-Bauer disc diffusion method on Mueller-Hinton agar according to the method of Hudzicki (2009), using; augmentin (20 µg), ceftriaxone (30 µg), nitrofurantoin (30 µg), gentamicin (10 µg), cotrimoxazole (30 µg), ofloxacin (10 µg), ciprofloxacin (10 µg), amoxicillin (10 µg), pefloxacin (10 µg), and tetracycline (30 µg) respectively. One hundred microliter of 0.5 MacFarland standardized broth culture (average of 1.6 x 10^7) of each isolate was spread on Mueller-Hinton agar and allowed to air-dry aseptically. Each antibiotic disc was carefully placed at about 20 mm distance from each other on the inoculated agar and incubated invertedly at 35°C for 24 h. The zones of inhibition were measured and interpreted as sensitive (S), intermediate (I) and resistant (R), according to the interpretation chart of CLSI (2018).

Minimum inhibitory concentration (MIC) of bacterial isolates: Standard broth micro-dilution method was used to determine the MIC of the isolates. All the isolates were tested against the following antibiotics dilution ranges in micro tubes; cotrimoxazole (0.5-64µg/ml), augmentin (0.5-32µg/ml), gentamycin (0.5-64 µg/ml), ciprofloxacin (0.5-64 µg/ml), ceftriaxone (1-64 µg/ml), pefloxacin (0.25-128 µg/ml), ofloxacin (0.25-128 µg/ml), tetracycline (0.25-64 µg/ml), nitrofurantoin (0.25-64 µg/ml) and amoxycillin (0.25-64 µg/ml). Each antibiotic was serially diluted in 1% peptone of 100 µl according to their respective ranges and equal volume of 100 µl of overnight 0.5 MacFarland standardized broth culture was added to all the dilution ranges from well 1 to well 10 and incubated at 35°C for 24 h. Turbid wells were indicated to have growth, while clear wells were identified to have no growth after incubation. The MIC of each antibiotic to the resistant organism was noted as the highest dilution showing no growth. The respective MIC of each isolate was determined and interpreted according to CLSI (2018) recommended guidelines.

Statistical analysis of the obtained data sets: Descriptive statistics was used to present the rate of the isolation of the bacteria using percentages, average and bar chart, while Chi-square was used to determine the significant of the bacteria isolates obtained from the dogs examined at 95% confidence interval of p-value less than 0.05 using SPSS version 16 of 2003.

RESULTS AND DISCUSSION

Majority of the dogs examined were young puppies which are often carried around, played with and used for sporting activities. Percentage occurrence of URTI was highest (43.0) in Alsatian and least (2.0) in Italian mastiff, Mongrel, Caucasian, Neapolitan mastiff and Terrier cross respectively (Figure 1). These severe infections such as; pneumonia or bronchopneumonia occur when dogs come in contact with pathogenic bacterial agents (Timoney, 2004). Table 1 shows both cultural and morphological characteristics of 10 bacterial isolates obtained from the nasal swab of the dogs examined, while biochemical responses of each isolate are presented in Table 2 respectively.

Escherichia coli recorded highest (83.1%) while *Haemophilus sp.* had the least occurrence (5.8%) as shown in Figure 2. The highest percentage occurrence recorded for *E. coli* and *C. freundii* could have resulted from faecal transmission of pathogens through unhygienic handling of animal discharges. Also, unrestrained dogs occasionally scavenge food crumbs in refuse and dirty areas thereby contracting *E. coli* which could easily be transmitted. *Staphylococcus aureus* is an important cause of human nosocomial and community-acquired infections globally and it is usually found responsible for numerous respiratory infections in dogs (Gortel *et al.,* 1999). The transmission of *S. aureus* between pet dogs and their owners was reported by Manian, (2003). A lot of health risks are posed by

contracting *S. aureus* infection from dog through nasal discharge such as sputum, mucus, blood and saliva (Pesavento *et al.*, 2008).

As shown in Table 3, all the isolates were resistant to at least one of the antibiotics tested. Complete susceptibility to nitrofurantoin (100%), ciprofloxacin and amoxycillin (90.0%) were recorded, while 0.0% and 10.0% susceptibility rate were recorded for augmentin and ceftriaxone respectively. The antibiotic susceptibility pattern of the nasal bacterial isolates depicted increasing rate of antibiotic resistance among the bacteria resident in dogs. In the last few years, methicillin-resistant *Staphylococcus aureus* (MRSA) has gained world-wide attention as a human pathogen in hospitals and in communities (Bourély *et al.*, 2019). Recent reports confirmed MRSA infection and colonization in dogs and cats (Walther *et al.*, 2008), thereby, indicating that the resistant strain is becoming a pathogen of animals as well as human involving wounds and post-operative infections (Weese *et al*., 2006).

Streptococcus species which is found as commensally organism found on the tonsils, URT, skin and urinogenital tract of dogs is now considered an opportunistic pathogen (Gibson and Richardson, 2008).

Table 4 shows minimum inhibitory concentration (MIC) of antibiotic agents against the bacterial isolates. The MIC ≥16 µg/ml is interpreted as resistant according to CLSI (2018) guideline and resistance rate of 100% was recorded to augmentin, 90% resistant to ceftriaxone and 60% to gentamycin. No resistant was shown to nitrofurantoin (0.0%). This may suggest a dangerous trend for public health as many bacterial isolates from dog which infect humans may be very difficult to treat as a result of their drug resistance.

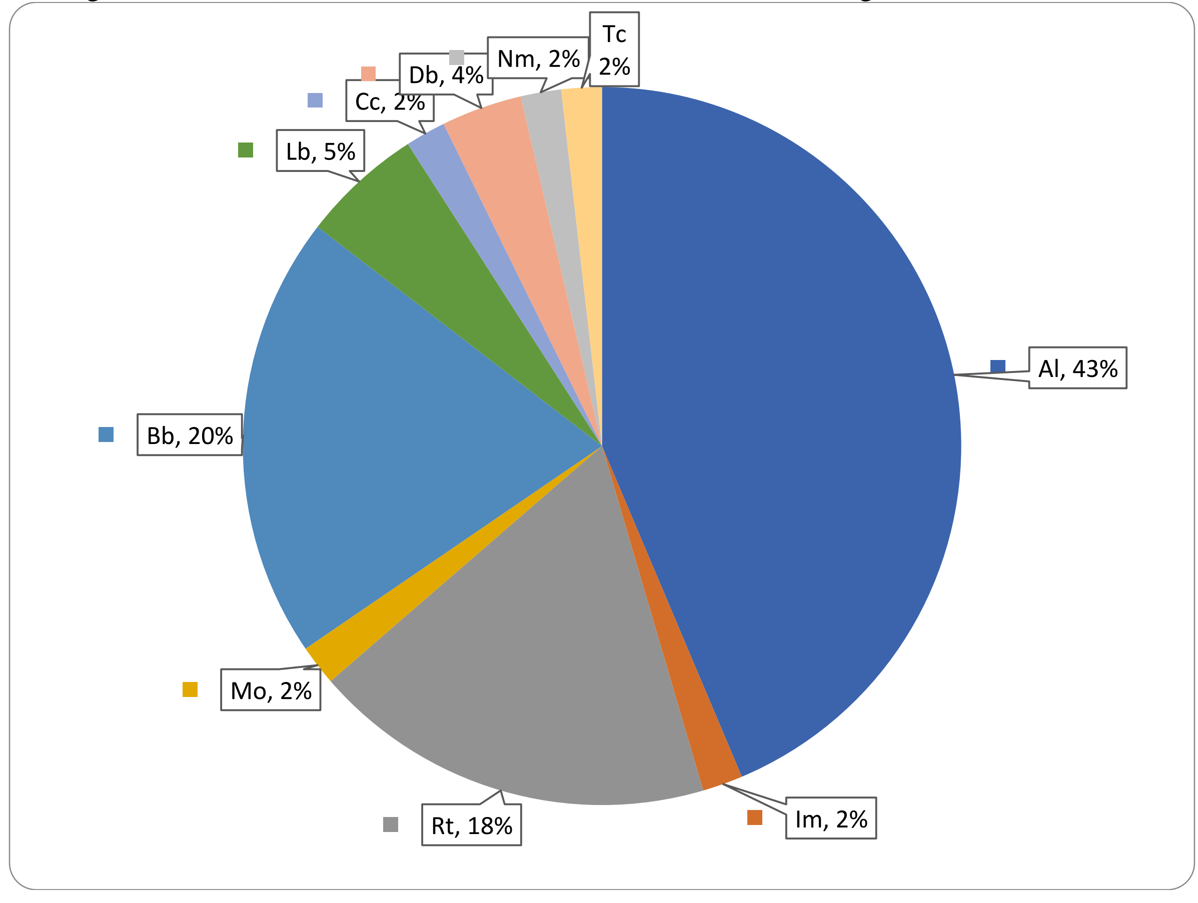

Figure 1: Percentage occurrence per breed of URTI
Al: Alsatian, Im: Italian mastiff, Rt: Rottweiler, Mo: Mongrel, Bb: Boerboel, Lb: Labrador, Cc: Caucasian, Db: Doberman, Nm: Neapolitan mastiff, Tc: Terrier cross

Table 1: Cultural and morphological characteristics of the bacterial species isolated from Upper Respiratory Tracts of Dogs

Isolate code	Cultural characteristics	Morphology
B1	Raised, wet, non-haemolytic, round	GNB
B2	Small, Non-Haemolytic, Round	GNB
B3	Small, translucent, partial haemolysis	GNC
B4	Small, non-lactose fermenter, round	GNB
B5	Yellow, round, non-haemolytic	GPC
B6	Very small, translucent, haemolytic	GPC
B7	Golden yellow, small, haemolytic	GPC
B8	Large, mucoid, wet	GNB
B9	Big, large, wet	GPB
B10	Small, rough, non-lactose fermenters	GNB

Key: GNB = Gram negative bacilli, GPC = Gram positive Cocci

Table 2: Biochemical characteristics of the bacterial isolates from Upper Respiratory Tracts of Dogs

S/N	Code	Gram	Motility	Glucose	Lactose	Mannitol	Maltose	Indole	Methyl Red	Voges Proskauer	Citrate	H_2S	Sucrose	Urea	Oxidase	Coagulase	Catalase	Probable Isolate
1	B1	-	+	+	+	+	+	+	+	-	-	-	NA*	-	-	NA*	+	*Escherichia coli*
2	B2	-	+	+	+	+	+	-	+	-	+	+	-	-	-	-	+	*Citrobacter freundii*
3	B3	-	+	+	+	+	+	+	+	-	-	-	NA*	-	-	NA*	+	*Haemophilus* species
4	B4	-	+	+	-	+	+	-	+	+	-	+	+	+	+	NA*	+	*Pseudomonas aeruginosa*
5	B5	+	-	+	+	+	+	NA*	+	-	+	-	+	+	-	-	+	*Staphylococcus saprophyticus*
6	B6	+	-	+	+	+	+	NA*	+	-	+	-	+	+	-	-	-	*Streptococcus* sp.
7	B7	+	-	+		+	+	NA*	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
8	B8	-	-	+	+	+	+	-	-	-	+	-	+	-	-	-	+	*Klebsiella oxytoca*
9	B9	+	+	+	+	+	+	NA*	-	+	NA*	NA*	+	-	-	NA*	+	*Bacillus subtilis*
10	B10	-	+	+	-	-	-	-	+	-	+	+	+	+	-	NA*	+	*Proteus mirabilis*

Key: + = positive reaction, - = negative reaction, NA = not analyzed

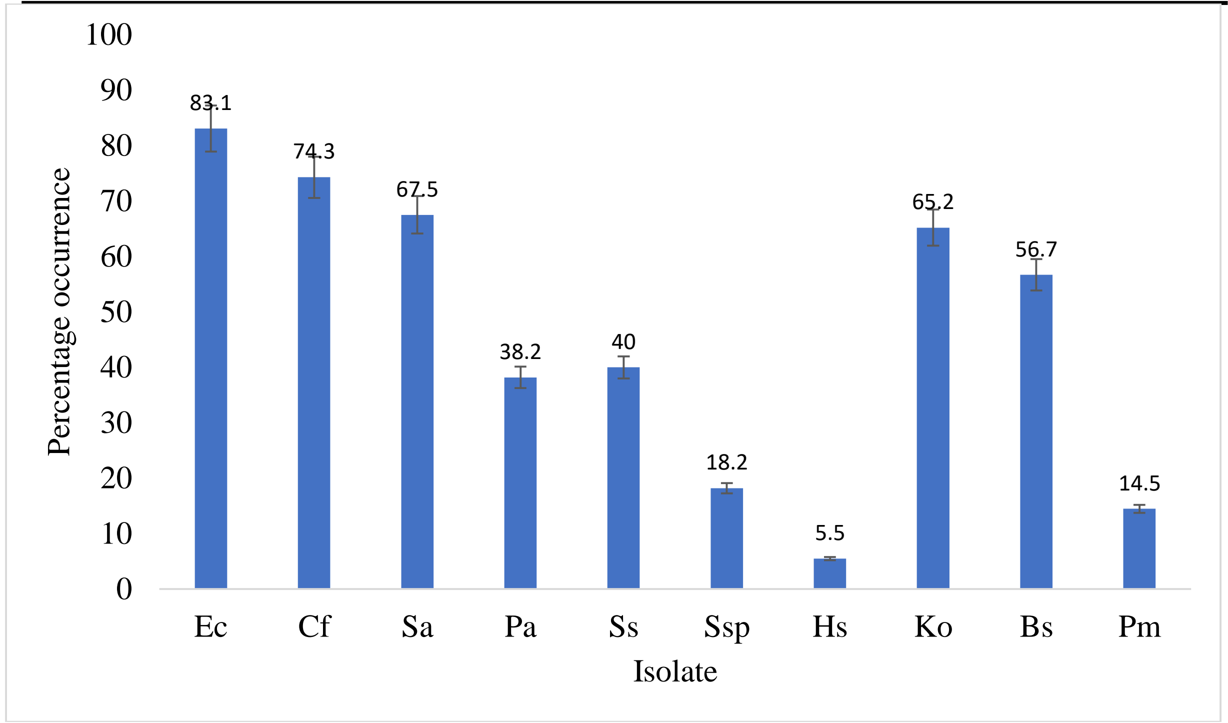

Figure 2: Percentage occurrence of bacterial species isolated from URT of selected dogs
Keys: Ec = *Escherichia coli,* Cf = *Citrobacter freundii,* Sa = *Staphylococcus aureus,* Pa = *Pseudomonas aeruginosa,* Ss = *Staphylococcus saprophyticus,* Ssp = *Streptococcus sp.,* Hs = *Haemophilus species,* Ko = *Klebsiella oxytoca,* Bs = *Bacillus subtilis,* Pm = *Proteus mirabilis.*

Table 3: Antibiotics susceptibly pattern of the bacterial isolates from Upper Respiratory Tracts of Dogs

Isolates	Augmentin	Ceftriaxone	Nitrofurantoin	Gentamycin	Cotrimoxazole	Ofloxacin	Amoxycillin	Ciprofloxacin	Tetracycline	Pefloxacin
Escherichia coli	R	R	S	R	R	R	S	S	R	R
Citrobacter freundii	R	R	S	R	R	R	R	R	S	R
Staphylococcus aureus	R	R	S	R	S	S	S	S	R	R
P.aeruginosa	R	R	S	R	S	S	S	S	S	R
Stapylococcus saprophyticus	R	R	S	R	S	S	S	S	S	S
Streptococcus spp	R	S	S	S	S	S	S	S	R	R
Haemophilus species	R	R	S	S	S	S	S	S	S	S
Klebsiella oxytoca	R	R	S	S	S	S	S	S	S	R
Bacillus subtilis	R	R	S	R	S	S	S	S	S	R
Proteus mirabilis	R	R	S	S	S	S	S	S	S	R
Total susceptibility rate (%)	0.0	10.0	100.0	40.0	80.0	80.0	90.0	90.0	70.0	80.0

Key: R =Resistant, S=Sensitive. (CLSI, 2018).

Table 4: Minimum inhibitory concentration (MIC) of antibiotics against the bacteria isolated from Upper Respiratory Tracts of Dogs

Isolates	Augmentin	Ceftriaxone	Nitrofurantoin	Gentamycin	Cotrimoxazole	Ofloxacin	Amoxicillin	Ciprofloxacin	Tetracycline	Pefloxacin
	MIC≥16 µg/ml									
Escherichia coli	32	32	16	64	64	16	4	2	32	16
Citrobacter freundii	32	32	2	16	16	32	32	32	2	32
Staphylococcus aureus	64	64	1	64	2	2	4	1	16	2
P. aeruginosa	32	32	2	32	2	1	1	2	1	16
Staphylococcus saprophyticus	32	32	2	16	2	2	2	2	2	2
Streptococcus sp.	32	2	2	1	2	1	2	2	32	32
Haemophilus species	32	32	2	2	1	1	1	2	2	4
Klebsiella oxytoca	32	32	2	2	2	1	2	2	2	32
Bacillus subtilis	32	16	1	16	1	8	8	4	4	32
Proteus mirabilis	32	32	2	8	4	4	8	8	8	16
Total susceptibility rate	0.0	10.0	100.0	40.0	80.0	80.0	90.0	90.0	70.0	80.0

CONCLUSION

Dogs and other pet animals living closely with humans could transmit fatal respiratory infections. Also, abuse of antibiotics on dogs could increase drug resistance in bacteria of medical importance associated with domestic dogs. Public awareness on health risks other than bites from pet dogs, regular vaccination and adequate treatment of infected dogs are highly recommended.

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Prevalence of *Helicobacter pylori* in Nigeria (2010-2023)

Mohammed O. A.*
Department of Microbiology, Edwin Clark University, Delta State, Nigeria.
* Corresponding author: ayus5love@yahoo.co.uk

Abstract: *Helicobacter pylori* the aetiologic agent of peptic ulcer, also associated with gastritis and gastric mucosa-associated lymphoid tissue lymphoma, has been reported to be prevalent in some developing countries. Taking into consideration its mode of transmission which is faecal oral, it is important for developing countries to be alert on the nation's own prevalence, as a high rate could facilitate increase in transmission which would be detrimental to public health. This review, analyzes on available related research published online between 2010-2023, to identify the prevalence rate of *H. pylori* in Nigeria. The research papers collected for this review represented the North, South, East and West regions of Nigeria. Thus, showing a representative overview of prevalence rates of *H. pylori* in Nigeria. The range identified was 20%-80% prevalence rate, with most over 50% and the highest percentage prevalence of *H. pylori,* reported in the Northern region of Nigeria. Risk factors observed included among others, overcrowding, low socioeconomic class, water sources and toilet facility. Therefore taking into consideration the economic status of Nigeria, the risk factors and prevalence showed that a large proportion of Nigerians are at risk and may not have the facilities required to prevent this infection. As a result, it may be futile to limit the *H. pylori* prevalence management to personal efforts of individuals, rather more governmental and non-governmental groups should be set up and financed to aid the control of the spread of *H. pylori* infection.
Key word: *H. pylori*, prevalence rate

INTRODUCTION

Nigeria, a country in West Africa with 356,700sq miles and a population count of over 216 million people (National bureau of statistics, 2022). Though a developing country in many areas, its microbial prevalence and distribution is still in need of more studies compared to developed countries. Such studies would aid the general health and wellbeing of Nigerians as well as alert health practitioners to the microbial status of their environment.

Helicobacter pylori is a Gram-negative microaerophilic bacterium which has been identified to be a cause of peptic ulcer, associated with gastritis and gastric mucosa-associated lymphoid tissue lymphoma (Forman *et al*, 1990, Morris *et al*, 1991). Other diseases have been associated to *H. pylori* infection, a 2007 study, evaluated the prevalence of *H. pylori* infection in 80 patients with diabetics mellitus and its correlation to diabetic vascular complication. It was reported that diabetic patients are at risk of being infected with *H. pylori* and its infection could associated with the development of microvascular and macrovascular complications in Diabetic mellitus cases (Hamed et al, 2007)

This pathogenic bacterium has been isolated in different areas in Nigeria. A 2017 global review of *H. pylori* prevalence, identified that though more than half the world's population is infected, Africa has the highest pooled *H. pylori* prevalence and a high prevalence of 87.7% was observed in Nigeria (Hooi *et al*, 2017). Also some features of these bacteria aiding colonization in the gastric epithelium are: the polar-sheathed flagella, mobility, chemotaxis, its shape, adherence and persistence, (Sabbagh *et al*, 2019). Diagnostic test is dependent on clinical condition, and other factors like cost and sensitivity. There are invasive and non invasive diagnostic tests, with the invasive tests including rapid urease test, endoscopy with biopsies of gastric tissues for histology. While non invasive test include fecal antigen test and urea breath test (Sabbagh *et al*, 2019) though there is an array of methodology to diagnose *H. pylori* infection, the gold standard for detecting *H. pylori* was reported to be undetermined (Sabbagh *et al*, 2019). Due to the high continental prevalence of *H. pylori* and its adverse health complications, it is important to understand its prevalence in Nigeria and factors that has been observed to be associated with its incidence. This review

itemises the previous research that has identified *H. pylori* as a pathogen amongst the Nigerian population, its prevalence per region and shows the risk factors and circumstances associated with this pathogen in Nigeria.

MATERIALS AND METHODS

The review was done using search engines such as Google, Scopus, Google Scholar and searching words such as *Helicobacter pylori* and Nigeria. Research articles found were scrutinised and findings were reflected in this article.

RESULTS AND DISCUSSION

This review, identified research from different regions in Nigeria collating findings for the prevalence of *H. pylori.*

Western region

A study on *H. pylori* was done at the University college Hospital Ibadan Nigeria, which is a hospital reported to serve as a referral centre for a substantial part of south western region of Nigeria. The study was aimed at detecting the prevalence of *H. pylori* among dyspeptic patients and its association with gastroduodenal pathologies using gastric biopsy histology and rapid urease test. It involved 86 adult patients with dyspeptic symptoms undergoing endoscopy (Jemilohun *et al.*, 2010). *Helicobacter pylori* was observed in 64% of patients and gastritis was observed in 60.5% of the sample population. The study also emphasized that serious gastroduodenal pathologies such as gastric cancer were low in prevalence (14%) irrespective of the high prevalence of *H. pylori.* It was also observed that 63.5% of endoscopic gastritis had *H. pylori* infection (Jemilohun, *et al*, 2010).

Harrison *et al.* (2017) also detected *H. pylori* in 577 samples obtained from hospitals in 3 south west states inclusive of Lagos and one northern state in Nigeria. Urease blood test (UBT) was carried out, as well as *H. pylori* isolation. The highest UBT positive patients were observed to be females, but more *H. pylori* isolates were obtained from males. With respect to age there was a continuous increase of *H. pylori* positive samples with increasing age from age 10 up until age 60 after which, a gradual decrease was observed. Age groups 0-10, 91-100 and 100 -110 were observed to be *H. pylori* negative, which could be because sample size was less than 3. In total, 35% were positive to UBT and about 48% were negative the others were on borderline or not recorded. Lagos was observed to have the highest UBT positive and *H. pylori* isolated (Harrison *et al.,* 2017). The *H. pylori* prevalence in healthy children residing in Lagos was observed to be 63.6%. The study also reported a significantly high risk of *H. pylori* infection amongst the low socio economy class, thus affecting children in such areas (Senbanjo *et al.,* 2014). No significant protective effect against *H. pylori* was observed in breast feeding in this research. But, it was observed that increase in the duration of breast feeding reaching 17 months resulted to a decrease in the prevalence of *H. pylori* infection (Senbanjo *et al.,* 2014). Thus, indicating a dose response protective effect of IgA antibodies in breast milk, but it needed to be confirmed with a larger sample size (community based). *H. pylori* infection was three and a half times more prevalent with children with recurrent abdominal pain (RAP) than those without RAP (Senbanjo *et al.*, 2014). An institutional based, cross-sectional study was conducted amongst 2 randomly selected secondary schools at Ikeja LGA, Lagos. Out of 100 participants 59% prevalence rate of *H. pylori* was observed and majority was over the age of 15 years. The study did not detect an association between drinking water sources, defecation, hand washing with *H. pylori* prevalence (Mynepalli *et al*., 2014).

South region

Furthermore, the seroprevalence of *H. pylori* infection in the south-south region of Nigeria (Delta State) was determined among asymptomatic subjects and shown to be 52.5%. No significant difference in prevalence amongst different age groups and between gender was observed. However, *H. pylori* was observed to be associated with

geographical region, people located at Delta state central were observed to be at higher risk. This link to geographical location was stated that it could reflect differences in social/hygiene factors and environmental factors (Omosor *et al.,* 2017). Mbang *et al.* (2019) determined the prevalence of *H. pylori* infection amongst dyspepsia patients in Calabar. The study involved 115 patients of which 42.6% was positive for *H. pylori* infection, which is slightly lower than the global prevalence. The study deduced that gradual urbanization with access to portable water may be linked to the relative lower prevalence of *H. pylori* when compared to some other regions of the country. This study, contrary to others also found a higher prevalence of *H. pylori* in patients with tertiary level of education, though not statistically significant. No gender predilection was observed, sibling number also did not contribute to *H. pylori* prevalence. Higher trend of *H. pylori* infection was observed in this study in 4th and 5th decade of life supporting other studies in this review (Mbang *et al.*, 2019)

Seroepidemiology of *H. pylori* infection among children seen in a tertiary hospital in Uyo, southern Nigeria was observed to be 30.9%. It also detected the early acquisition of *H. pylori* as 13% of infants less than one year old were observed to be seropositive, lowest age prevalence was observed in ages 11-15 years and the highest in ages 6.0 -10 years thus contrasting with other research that have shown increasing prevalence with increase in age. Taking into consideration that this study was majorly on children, thus prevalence in Uyo adults was not reflected. No gender difference was observed in the seroprevalence in this study. Seroprevalence was observed to be higher within the lower social class. Household with 10-12 members had almost twice the prevalence rate compared to households of 1-3 members. *Helicobacter pylori* was also strongly associated with domestic water supply, type convenience used in the home and waste disposal method. Borehole and well water as drinking water sources were more linked to *H. pylori* prevalence compared to pipe borne water. The use of pit latrine and open system of waste disposal also was associated to high *H. pylori* level compared to water system. Number of siblings had no significant effect on prevalence rates associating it with the effect of number of persons already discussed, suggests that other factors of close family contact and not necessarily sleeping together maybe responsible for the association of high prevalence of this infection with high household population (Etukudo *et al.,* 2012).

A 2022 report recorded a 55% prevalence rate of *H. pylori* in Rivers State University Teaching Hospital Port Harcourt. It was a hospital based study consisting of 242 participants who were patients with presumptive gastritis (Agi *et al.*, 2022).

The prevalence of *H. pylori* amongst 115 dyspepsia patients in Calabar was also studied and result showed that 42.6% of the participants were positive for *H. pylori* and borderline result was observed in 6.1% of patients, no disparity was observed with gender in this study (Mbang *et al.*, 2019)

Eastern region

Ezugwu and Chukwubuike (2014), reported on the epidemiology of *H. pylori* infection among dyspepsia patients in south-eastern Nigeria. The faecal samples were collected from dyspepsia patients in densely populated areas of five states within the south east zone of Nigeria. Approximately 76% were positive of the total population (643 patients) sampled for the presence of *H. pylori*. The research further detected that age groups over 40 years had the highest frequency of isolates. Gender, population density, water sources, toilet facility and educational level were also factors analysed to detect their influence on the spread of *H. pylori* (Ezugwu and Chukwubuike, 2014). The research identified that *H. pylori,* was more prevalent amongst those in an overcrowded environment, those that used stream water and those with water closet toilet as well as patients with low level of education (Ezugwu and Chukwubuike, 2014).Thus, depicting that prevalence of *H. pylori* is

facilitated by old age and poor living conditions and personal hygiene which could be reflected in lack of hand washing, lack of regular toilet cleaning, use of water without boiling. Though, this could be as a result of lack of knowledge as those with only a primary education showed higher *H. pylori* isolation rates (Ezugwu and Chukwubuike, 2014). The high prevalence of *H. pylori* observed in the study is a cause of concern; however, the study was majorly on dyspepsia patients. However, the epidemiological factors concerned could as well be the living conditions of asymptomatic individuals. As a result, educating the lower social class of proper disposal of human waste could help control the spread of *H. pylori,* as well as digging of wells far away from pit toilets and septic tanks to avoid contamination of water sources. These recommendations made by the authors, could aid reduce the spread of *H. pylori* even in asymptomatic individuals.

The seroprevalence of *H. pylori* infection was determined in Owerri amongst children. The study population was a hundred and twenty children aged 6 months to 15 years studied from March to June 2016. Questionnaire was used to document the sociodemographic data, while *H. pylori* infection was detected using a rapid test kit. Findings showed *H. pylori* prevalence at 20.0%, increase in prevalence was also observed with increasing age with highest observed between the age 10-15 years age group (P=0.001). Low socio economic class (SEC) was also observed to be significant risk factor in the prevalence of *H. pylori* infection (Emerenini *et al.,* 2021). Another study at Owerri was conducted to determine the epidemiology and risk factors for acquisition of *H. pylori* among 384 participants, symptomatic and asymptomatic for peptic ulcer. For the purpose of providing baseline data and creating awareness for effective management and prevention of infection caused by this pathogen. Both faecal and blood samples were collected to detect the presence of ulcer and *H. pylori* infection respectively, a questionnaire was used to document sociodemographic characteristics of each participants. Findings showed seroprevalence of *H. pylori* infection and ulcer prevalence over 70% age groups between 41-50 had 100% *H. pylori* prevalence. Smoking and gender were not observed to be significantly associated seroprevalence of *H. pylori* (Okoroiwu *et al*., 2022)

Peptic ulcerative individuals in Nnewi were examined for seropositivity to *H. pylori* and Prevalence rate for *H. pylori* was determined to be 51.4% (Chukuma *et al*., 2020).

North region

More research on *H. pylori* prevalence was carried out in Kano, Nigeria (Bello *et al.,* 2018). It was observed that *H. pylori* prevalence was very high (over 80%) and an increased prevalence was observed more in male subjects. People at risk of this infection were majorly those whose water source is from the wells and ponds and those from a lower social class. Cigarette smoking and multiple occupants per room (over 3) were also stated to be factors strongly associated to *H. pylori* infection (Bello *et al*., 2018).

Further research was also carried out in the Northern Nigeria, but specifically on University students. The sample population were undergraduate students from Nassarawa State University Keffi (Ishaleku and Ihiabe, 2010). The *H. pylori* seropositive samples were observed in 54% of the population. A significantly higher prevalence rate was observed more in females. Students aged between 31- 40 were reported to have higher seroprevalence of about 86%, which was reported to be a major concern as it could enhance transmission due to the high interpersonal social activities usually associated with this age group (Ishaleku and Ihiabe, 2010).

Mustapha *et al.* (2011) also determined the prevalence *H. pylori* infection among dyspeptic patients at a tertiary hospital in Northern Nigeria which was observed to be 77.1%. In addition, 125 dyspepsia patients were examined at Maiduguri teaching hospital, for *H. pylori* infection using

different methods. Histological samples showed 80% *H. pylori* prevalence while, serological test showed 93.6% prevalence (Olokoba *et al*., 2013).

CONCLUSION

This review was aimed at knowing the present spread of *H. pylori* in Nigeria between 2010 – 2023 by pulling all the research done at different geographical regions of Nigeria. Research observed that *H. pylori* prevalence has been observed in the North, South, West and East of Nigeria at a range of 20%-80%with most over 50%. The highest prevalence was observed in the northern region. This prevalence rate should be a major concern to public health management irrespective of the health status of the research participants. The rate of *H. pylori* prevalence at the reported level puts the general public at risk due to the mode of transmission of *H. pylori* which is faecal oral. Thus, lack of amenities facilitating hygiene and programmes educating the public its risk factors would result in an increase of the spread of this infection caused by bacterium. Senbanjo *et al.* (2014) reported that there is a high risk of *H. pylori* infection amongst the low economy class. Taken, into consideration that about half the Nigerian population are at the poverty line, it means that a large proportion of the Nigerian population is at risk. Thus, it is a public health problem and should be addressed at governmental levels and by non-governmental groups, not left for individuals. Nigerian population at the poverty line, despite the enlightment may not have the means required to avoid environmental factors like overcrowding, drinking good water, even maintaining good personal hygiene due to cost. As basic as using good water source is, this review has shown that a proportion of research participants use wells and ponds as their water sources which was observed to be linked to *H. pylori* prevalence. Thus, it may be futile to limit the *H. pylori* prevalence management to personal efforts of individuals, rather more governmental groups should be set up and financed to aid the control of the spread of *H. pylori* infection.

Furthermore, a virtual repository for the nation should be made available and health institutions should be mandated to submit monthly data on infections detected during diagnosis. This would aid identify potential epidemic cases and facilitate early prevention.

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In-vitro Biofilm Formation and Antimicrobial Resistance of Metallo Beta-lactamase Producing *Pseudomonas aeruginosa* of Clinical Origin

Nwankwo F. M.* Iroha I. R. Awoke O. A. Ugbo E. N. and Nwankwo L. N.
Department of Applied Microbiology, Faculty of Science, Ebonyi State University, Abakaliki, Ebonyi State, Nigeria.
* Corresponding author: mbamfidelis@gmail.com

Abstract: *Pseudomonas aeruginosa* is an important cause of morbidity and mortality in hospitalized patients and patients with underlying medical conditions. The prevalence of biofilm formation and muti-drug resistant strains of *P. aeruginosa* isolates has been on the increase. This study was aimed at *in-vitro* biofilm formation in metallo beta-lactamase producing *Pseudomonas aeruginosa* of clinical origin. A total of 590 different clinical samples were used for this study, during which the samples were collected from different units of Alex-Ekwueme Federal Teaching Hospital and Mile 4 Hospital all in Abakaliki. Standard microbiological methods were used to identify the isolates. The isolated *P. aeruginosa* were further subjected to imipenem-ethylene diamine tetractic acid combine disc test (CDT) to ascertain the metallo beta-lactamase production, biofilm assay using tube method to determine the ability of isolates to form biofilm. The isolates were also subjected to antibiotics susceptibility studies against different classes of antibiotics through disc diffusion method. Out of the 590 samples collected and screened, fifty nine (59) isolates were identified and characterized as *P. aeruginosa.* Thirty four (34) were metallo beta-lactamase (MBL) producer, and 21 were biofilm producers. The antibiogram of the biofilm producing *P. aeruginosa* revealed high resistance rate to cefoxitin (95.2%), nalixidic acid (85.7%), cefepime (80.9%), piperaciilin (80.9%), ofloxacin (76.2%), colistin (76.2%), amikacin (76.2%), tetracycline (71.4%), amoxicillin (71.4%), and ceftriaxone (66.7%). Strict implementation and adherence to antibiotics stewardship in the hospital setting is highly recommended to control and manage the rise antibiotic resistance.
Key word: *In-vitro*, biofilm, metallo beta-lactamase, isolates

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic pathogen in nosocomial infections and responsible for high mortality rates in burn centers, cystic fibrosis, pneumonia (Altoparlak, 2004; Lipovy, 2010). Infections caused by *P. aeruginosa* are difficult to treat, as the majority of isolates exhibit innate resistance to several antibiotics, due to poor outer-membrane permeability, constitutive expression of various efflux pumps and production of antibiotic inactivating enzymes (Lambert, 2002). Among these, the important roles of various β-lactamases such as AmpC, extended-spectrum β-lactamases (ESBL) and carbapenemases have been reported (Mesaros, 2007; Kumar, 2012). AmpC β-lactamase is responsible for resistance to cephalosporins and ESBLs confer resistance to all β-lactams except for the carbapenem family. Carbapenemases, particularly metallo beta-lactamases (MBL), hydrolyze all β-lactam antibiotics with the exception of monobactams. Co-existence of multiple β-lactamases in clinical isolates of *P. aeruginosa* is common, causing resistance to almost all β-lactam antibiotics (Upadhyay, 2010).

Another important factor contributing to the pathogenesis of *P. aeruginosa* in causing fatal infections is its potential to form biofilms on biotic and abiotic surfaces (Karatuna, 2010). The bacterial populations in biofilms are usually more resistant to antibiotics and host-mediated clearance strategies compared to their planktonic counterparts, giving rise to chronic infections that are notoriously difficult to eradicate (Costerton, 1995; Mah, 2003). Bacteria growing in biofilms produce one or more extracellular polymeric matrices which hold the cells of the biofilm community together. Polysaccharides are important components of the biofilm matrix, as they contribute to the overall biofilm architecture and to the resistance of biofilm-grown bacteria to certain antibacterial agents.

At least three exopolysaccharides have been shown to be involved in biofilm formation by *P. aeruginosa,* including alginate, *psl*,

and *pel* (Ghafoor, 2011). Among these, *psl* is a mannose-rich polymer with an essential role in the initial steps of biofilm formation by non-mucoid *P. aeruginosa* as well as in its maintenance. *psl* forms a helical structure around *P. aeruginosa* cells which increases the cell-to-surface and cell-to-cell interactions necessary for biofilm formation (Jackson, 2004; Ma, 2006). Synthesis of *psl* is mediated by the *psl* gene cluster (pslA-pslO) and pslA has been reported to be the first and most important gene necessary for *psl* synthesis (Matsukawa, 2004; Overhage, 2005). To the best of our knowledge this research study is the first documented epidemiological analysis of *in-vitro* biofilm formation and antibiotics resistance pattern of MBL producing clinical isolates of *P. aeruginosa* in a tertiary hospitals in Abakaliki, Ebonyi state. As such our present study, therefore, would be useful to understand the dynamics of biofilms forming MBL *P. aeruginosa* strains in our study center.

MATERIALS AND METHODS

Study area: This study was carried out Alex Ekwueme Federal University Teaching Hospital Abakaliki and Mile 4 Missionary Hospitals all in the capital city of Ebonyi State, Nigeria. Ebonyi State is located in the South Eastern part of Nigeria. The state shares boundary with Benue, Cross River, Abia and Enugu States. It is between longitude 7°30' and latitude 60°45' E and about 3,242,500 in population mainly farmers and traders.

Sample collection: The samples used were collected from Alex-Ekwueme Federal Teaching Hospital and Mile 4 Hospital as follows: wound (185) using sterile swab stick to rotate on the wound surface in a zigzag motion, urine (230) through the use of well labeled sterile asptic container to collect early morning urine, ear swab (90) by the use of sterile cotton swab deeped into the ear canal while sputum (50) the patients was asked take a deep breaths, then force out deep cough and expectorate into a sterile screw-top container and ear (35) were from Mile 4 hospital using previous method. The collected specimens were transported to the Department of Applied Microbiology Laboratory unit, Faculty of Science, Ebonyi State University, Abakaliki within two hours of collection for bacteriological analysis. Then specimens were cultured in nutrient broth and to nutrient agar for isolation and identification of *P. aeruginosa*.

Isolation, identification and Characterization of the isolates: The various clinical samples were collected from patients who visited the hospitals and inoculated in a sterile nutrient broth. This was incubated for 24 hrs at 30°C and observed for turbidity. Test tubes with turbidity was aseptically streaked on agar plate and incubated at 37°C for 24 hrs. The colonies they were subcultured to obtain pure cultures which were observed for colony morphology. Gram staining and biochemical tests such as catalase test, oxidase test, citrate utilization, indole test and sugar fermentation test were carried out on the pure cultures. Culturally morphologically and biochemically identified *P. aeruginosa* was done and further characterized by PCR using 16s rRNA specific primers (Lamont, 2003).

Antibiotic susceptibility studies of the bacterial isolates: Antibiotic sensitivity of the biofilm producing metallo beta-lactamase *P. aeruginosa* isolates was determined using Kirby – Bauer disc – diffusion method as described by CLSI (2022). Briefly, a sterile swabs stick was used to inoculate the test organism onto Mueller-Hinton agar. Sterile forceps was used to carefully distribute the following antibiotic disc, meropenem (MEM10µg), ceftriaxone (30µg), ofloxacin (5µg), tetracycline (30µg), piperacillin/TAZ (40µg), amikacin (30µg), colistin (10µg), cefoxitin (30µg), amoxicillin/clavulanic (30µg), nalidixic acid (30µg), imipenem (10µg), cefepime (10µg) (Oxoid, UK) evenly on the inoculated plates at a distance of 30 mm. The plates were placed on the bench for 30 minutes to allow pre-diffusion of the antibiotics, inverted and incubated

aerobically at 35ºC for 18-24 hours. The zones of inhibition was measured using a meter rule recorded as milliters (mm) and compared with CLSI guidelines on antimicrobial susceptibility studies (Chigbu, 2003).

Determination of matallo beta-lactamse production of the isolates: Using imipenem-ethylene diamine tetracetic acid combine disc test (CDT). A lawn culture of the test isolate was prepared and allowed to dry for five minutes. Two imipenem (10 µg) discs, one with 0.5 M EDTA and other a plain imipenem disc, was placed on the surface of agar plates approximately 30 mm apart. The plates was then incubated overnight at 37ºC for 16-18hrs and then observed for an increase in zone diameter of >7 mm around the imipenem-EDTA disc in comparison to imipenem disk alone which indicates the production of MBL (Chigbu, 2003).

Biofilm assay of the bacterial isolates: A loopful of the test organisms was inoculated in 10 ml of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 hrs. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method was done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong (Chigbu, 2003).

Ethical consideration: Ethical clearance for this study was obtained from the institutional ethical committee and research committee of Ministry of Health Abakaliki Ebonyi State, Health Committee Assigned Number: HC/032

RESULTS

Table 1: Prevalence of *Pseudomonas aeruginosa* from different clinical samples

Location	specimen	No. sample	No. Isolates (%)
AE-FETHA 1	Wound	80	9(11.2%)
AE-FETHA 1	Urine	130	6(4.6%)
AE-FETHA 1	Ear swab	70	9(12.8%)
AE-FETHA 2	Wound	105	18(17.1%)
AE-FETHA 2	Urine	100	4(4%)
AE-FETHA 2	Ear swab	20	4(20%)
Mile 4	Sputum	50	5(10%)
Mile 4	Ear swab	35	4(11.4%)
Total		590	59(10%)

Key: AE-FETHA, Alex Ekwueme Federal Teaching Hospital

Table 2: Age distribution of *Pseudomonas aeruginosa* among patients from AE-FETHA and Mile 4 hospitals

Variable	Total samples	Total isolates	AE-FETHA (%)	Mile 4 (%)
Age				
0-20	98	7	5(71.4)	2(28.6)
21–40	175	21	16(76.2)	5(23.8)
41–60	180	11	4(36.4)	7(63.6)
61+	137	20	13(65)	7(35)
Total	590	59	38(64.4)	21(35.6)

Key: AE-FETHA, Alex Ekwueme Federal Teaching Hospital

Table 3: Frequency of isolation of MBL producing *Pseudomonas aeruginosa* from different clinical samples

Location	Specimen	Isolates	MBL (%)
AE-FETHA 1	Wound	9	3(33.3%)
AE-FETHA 1	Urine	6	2(33.3%)
AE-FETHA 1	Ear swab	9	5(55.5%)
AE-FETHA 2	Wound	18	13(72.2%)
AE-FETHA 2	Urine	4	3(75%)
AE-FETHA 2	Ear swab	4	3(75%)
Mile 4	Sputum	5	2(40%)
Mile 4	Ear swab	4	3(75%)
Total		59	34(57.6)

Key: AE-FETHA, Alex Ekwueme Federal Teaching Hospital

Table 4: Antibiogram of biofilm producers from the MBL producing *Pseudomonas aeruginosa*

Antibiotics	No of isolates	Susceptible (%)	Resistance (%)
Imipenem	21	11(52.4)	10(47.6)
Meropenem	21	13(61.9)	8(38.1)
Ceftriaxone	21	7(33.3)	14(66.7)
Tetracycline	21	6(28.6)	15(71.4)
Ofloxacin	21	5(23.8)	16(76.2)
Piperacillin	21	4(19.1)	17(80.9)
Colistin	21	5(23.8)	16(76.2)
Cefoxitin	21	1(4.8)	20(95.2)
Amoxicillin	21	6(28.6)	15(71.4)
Nalidixic Acid	21	3(14.3)	18(85.7)
Cefepime	21	4(19.1)	17(80.9)
Amikacin	21	5(23.8)	16(76.2)

Table 5: Distribution of MBL biofilm forming *Pseudomonas aeruginosa* from different clinical samples

Location	Specimen	MBL	Biofilm positive (%)
AE-FETHA 1	Wound	3	1(33.3%)
AE-FETHA 1	Urine	2	0(0%)
AE-FETHA 1	Ear swab	5	3(60%)
AE-FETHA 2	Wound	13	9(69.2%)
AE-FETHA 2	Urine	3	1(33.3%)
AE-FETHA 2	Ear swab	3	2(66.6%)
Mile 4	Sputum	2	2(100%)
Mile 4	Ear swab	3	3(100%)
Total		34	21(61.8)

Key: AE-FETHA, Alex Ekwueme Federal Teaching Hospital

DISCUSSION

In this study, the total of 59(10%) samples collected from (AE-FETHA 1 and 2; and Mile 4) were colonized with *P. aeruginosa* isolates and the highest frequency distribution of 20% was observed in ear swab samples compared to the frequencies across the isolates. This is similar to the 10.5% reported in Zaria, northern Nigeria by Olayinka *et al.* (2004) while contrary to the 20.3% as reported in a study conducted by Savas *et al.* (2005) in India and 30% in

Pakistan by Nadeem *et al.* (2009) as this similarity could be attributed to the prevalence of nosocomial infection. This study revealed that *P. aeruginosa* prevalence rate to be 11.2% and 17.1% in wound samples of AE-FETHA 1 and 2 respectively, 4.6% and 4% in urine samples of AE-FETHA 1 and 2, 12.8% and 20% in ear swab of AE-FETHA 1 and 2 respectively, while 10% and 11.4% in sputum and ear swab from mile 4 respectively. This is largely different from the study of Ndip *et al.* (2005), but similar to the 17.2% wound sample as report of 17.85% compared to Ekrem *et al.* (2014). The highest rate of 76.2% and 71.4% prevalence of *P. aeruginosa* was observed among patients within the age range of 0-20 and 21-40 in AE-FETHA 1 and 2 respectively and 63.6% at the age range of 41-60 in Mile 4 hospital was recorded in this study. This is similar to the report of Okon *et al.* (2009) on resistance pattern of *P. aeruginosa* isolated from clinical specimens in a tertiary hospital in northeastern Nigeria.

This study examined the prevalence rate across the age distribution, it showed greatest percentage 76.2 %(21-40), 71.4 %(0-20) in AE-FETHA. This likely indicates that several youthful activities can contribute to the emergence and spread of bacterial pathogens like *Pseudomonas aeruginosa.*

In this study, the total of 34(57.6%) out of the whole isolates were implicated to have produced metallo beta-lactamase (MBL) enzyme. The highest rate of 75% was recorded in urine and ear swab samples collected from AE-FETHA urine and ear swab samples and Mile 4 ear swab samples. This finding is in tandem with reports of studies in and outside Nigeria by Umar *et al*. (2020) and Peshattiwar *et al.* (2011) with similar 75% prevalence rate as reported in this sudy. Biofilm-forming bacteria play a significant role in the development of chronic and recurrent infections that pose challenges for treatment. Managing and effectively treating biofilm-associated infections is of utmost importance in healthcare settings (Da Costa Lima, 2017). The distribution of biofilm forming *P. aeruginosa* from different clinical samples revealed in this study that, 0(0%) in AE-FETHA 2 (ear swab), 100% of MBL producing isolates from sputum and ear swab samples from Mile 4 hospital. This implies that those isolates harbour greater percentage of gene mediating biofilm formation in them which is in contrast to the report of Da Costa Lima *et al.* (2017).

This study also examined the antibiotic susceptibility of *P. aeruginosa* and it was found that cabapenem class of antibiotics has the highest effect on the majority of the isolates. The antibiogram findings showed low level of resistance to members of carbapenem class of antibiotics, specifically imipenem and meropenem of 47.6% and 38.1% respectively. The high susceptibility pattern of these drugs could be associated to less drug abuse by the population being that the cost of these antibiotics prevents patient's self-medication. However, recent studies by Ranjbar *et al.* (2011) and Hamze *et al.* (2012) in Lebanon revealed a high resistance of 97.5% and 33.3% of *P. aeruginosa* to imipenem respectively, thus, demonstrating the evolution of imipenem-resistant strains of *P. aeruginosa*. Nevertheless, imipenem remain a potent anti-pseudomonal antimicrobial agent in Nigeria contrary to other report outside Nigeria where high imipenem resistance is prevalent (Odumosu, 2012). However, the antibiogram of the biofilm formers revealed high resistance rate of cefoxitin 20(95.2), nalixidic acid 18(85.7), cefepime 17(80.9), piperaciilin 17(80.9), ofloxacin 16(76.2), colistin 16(76.2), amikacin 16(76.2), tetracycline 15(71.4), amoxicillin 15(71.4), ceftriaxone 14(66.7), this is in disagreement to an international multicenter study done by Micek *et al.* (2015).

In the present, it is interesting to note that MBL *P. aeruginosa* isolated from ear swab and sputum samples in Mile 4 hospital apparently are all biofilm formers. Meanwhile 0% biofilm formation was seen in urine samples from AE-FETHA 1.

CONCLUSION

This study reported the presence of biofilm formation in metallo beta-lactamase producing *Pseudomonas aeruginosa* isolated from Alex-Ekwueme Federal Teaching Hospital and Mile 4 Hospital. Metallo beta-lactamase producing *Pseudomonas aeruginosa* had percentage prevalence of 57.6 and biofilm producing *Pseudomonas aeruginosa* had a prevalence of 61. 8%. Thus, stringent measure is needed to tilting proper usage of antibiotics in the patient treatment at hospital and strict implementation and adherence to antibiotics stewardship in the hospital setting is highly recommended.

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Occurrence of Metallo-beta-lactamase in Multidrug Resistant *Escherichia coli* Isolated from Donkey Slaughter Market

Ugbo E. N.[1*] Ugbo A. I.[2] Agumah B. N.[1] Nwojiji E. C.[2] Ewah C. M.[3] Ogbonna I. P.[2] and Ekpono C. U.[2]

1. Department of Applied Microbiology, Faculty of Science, Ebonyi State University, P.M.B. 053, Abakaliki, Ebonyi State, Nigeria.
2. Department of Microbiology and Parasitology, Faculty of Allied Health Sciences, David Umahi Federal University of Health Sciences, P. M. B. 211 Uburu, Ebonyi, Nigeria.
3. Department of Biochemistry, Faculty of Science, Ebonyi State University, P.M.B. 053, Abakaliki, Ebonyi State, Nigeria.

* Corresponding author: ugbonuel2001@yahoo.com; emmanuel.ugbo@ebsu.edu.ng

Abstract: Animals used in food production have been identified as one of the major primary sources of antibiotic resistant pathogenic bacteria within the Enterobacteriaceae family. The *Escherichia coli* harboring metallo-β-lactamases (MBLs) is a serious threat to global health. This study was aimed at the occurrences of metallo-beta-lactamase in multidrug resistance *Escherichia coli* isolated from donkey slaughter market. A total of 75 swab samples were collected from equipment used in donkey slaughters and *E. coli* was identified using Eosin Methylene Blue Agar (EMBA), MacConkey Agar (MCA) media and other microbiological standard techniques. The *E. coli* isolates were tested for multidrug resistance (MDR) using disk diffusion method and multiple antimicrobial resistances index (MARI) were calculated. The presence of metallo-beta-lactamase (MBL) in multidrug resistance *E. coli* was confirmed using modified Hodge test method. Thirty (40.0%) swab samples were positive for *E. coli*, knife had 8(32.0%), table had 12(48.0%) and slab harbored 10(40.0%). The isolates were resistance to tetracycline (40.0%-80.0%), gentamicin (25.0%-30.0%), erythromycin (40.0%-50.0%), ampicillin (70.0%-75.0%) and ciprofloxacin (20.0%-37.5%). Three major antibiotics resistance pattern were revealed from the 14 (46.7%) isolate that were MDR-*E. coli* which includes; TE-CN-E-AMP-CIP, TE-E-AMP-CIP and TE-CN-E-AMP. This study revealed the occurrences of MBLs in MDR- *E. coli* to be 5(35.7%). The presence of MBLs in MDR *E. coli* isolated from donkey creates strong threat to the treatment of such infection in clinical setting and it calls for an urgent veterinary surveillance program to monitor antibiotics used as growth enhancers in animal production.

Key word: Metallo-beta-lactamase, MDR *E. coli*, donkey slaughter equipment, public health.

INTRODUCTION

The livestock industry especially donkey plays a vital role in various regions such as in Asia, Middle-east, Africa like Nigeria, where it is a significant source of meat and milk consumption (FAO, 2019). Despite its importance, concerns about food safety and potential zoonotic diseases have been raised some years ago (Eyitayo *et al*., 2018). One of the potential risks associated with donkey meat is the presence of pathogens, such as *Escherichia coli* which can lead to gastrointestinal illnesses in humans when the contaminated product is consumed (USDA-FSIS, 2021). The transmission of *E. coli* from animals to humans has been a subject of increasing public health concern, particularly when it involves the food production chain (CDC, 2021). The improper handling and processing of donkey meat can potentially introduce pathogenic *E. coli* and other bacterial contamination (Wang *et al*., 2017). The donkey scientifically known as *Equus africanus asinus* is a domesticated member of the horse family, Equidae (Orhan *et al.,* 2012). Donkeys have been used as a work animal for at least 5000 years. They are mostly found in under developed countries where they are used principally as draught or pack animals.

In developing countries donkeys are valued in particular for their ability to survive under harsh conditions (Swai and Bwanga, 2008). Interestingly, donkey faeces is sometimes used in rural communities to rub/coat the inner walls of mud buildings where human beings live, as local fertilizers and this creates a strong potential for contamination and/or infection of those persons who perform this work and people who live in

such houses, touch these surfaces and farmers who use it for crop productions (Pritchard *et al*., 2019). Furthermore, it has been established that donkeys shed *E. coli* (Jesse *et al*., 2015) which poses a great risk to the people handling these animals directly or indirectly, it could also expose these people to diseases caused by this organism. In developing countries, including Nigeria, Ethiopia, animals are commonly slaughtered and processed under unhygienic conditions and these further compromises the microbiological quality and safety of the meat obtained from the animals (Bello *et al*., 2015; Dulo *et al*., 2015).

Food-producing animals such as donkey harboring multidrug resistance genes together with genes that mediate the production of some high-profile antibiotic hydrolyzing enzymes such as metallo-beta-lactamases (MBLs),extended spectrum beta-lactamases (ESBLs) and AmpC enzymes possess health risks to the human population particularly due to their potential contribution to the spread of multidrug resistant microorganisms in the community (Ejikeugwu *et al.,* 2018). MBL-encoding genes are usually carried by mobile genetic elements that facilitate horizontal gene transfer (HGT) between bacteria and harbor a great ability to spread (Pierce *et al.,* 2017). MBL-producing bacteria are regarded as the most important environmental pathogens, and further spread of them in the healthcare settings will pose a serious global threat in the future. Therefore, active surveillance is needed to detect the prevalence and incidence of MBL-producing bacteria in the environment and help prevent the spread of these organisms (Beresford and Maley, 2019).

The worldwide distribution of *E. coli* harboring metallo-β-lactamases (MBLs) and AmpC β-lactamases (AmpC) is a serious threat, and due to MBL production, carbapenem resistance is progressively spreading among clinical isolates of *E. coli* (Tewari *et al.,* 2018).Treatment of infections has been complicated by the emergence of multidrug-resistant (MDR) strains of *E. coli* (Aghil *et al.,* 2021).Food-producing animals have been identified as the primary source of antibiotic-resistant pathogenic bacteria within the Enterobacteriaceae family across multiple countries (Dey *et al.,* 2023; Sobur *et al.,* 2019; Ejikeugwu *et al.,* 2016). Among the most encountered members of Enterobacteriaceae in this category are *Escherichia coli*, *Klebsiella* species, *Shigella* species, and *Salmonella* species, owing to their role in causing diseases in livestock (de Souza *et al.,* 2023; Ugbo *et al.,* 2023). Limited information is available on the prevalence of MBLs in MDR *Escherichia coli* from animal at Ebonyi State, knowing that Ebonyi people depend much on donkey meat as their source of meat and protein. If not checkmated periodically, this could lead to the spread of MDR *Escherichia coli* harboring MBLs in the environment. Thus, investigating metallo-beta-lactamases (MBLs) in this bacterium is of utmost importance to address, manage the emergence and spread of antibiotic-resistant strains among food-producing animals, it is also a strong critical measure for safeguarding both animal and human health. Thus, the need for studying the occurrences of metallo-beta-lactamase in multidrug resistance *Escherichia coli* isolated from donkey slaughter market.

MATERIALS AND METHODS

Study area: This study was conducted in a popular donkey market (Nkwo Jaki) at Ezzamgbo in Ohaukwu Local Government Area of Ebonyi State. Ohaukwu Local Government Area has an estimated population of 196,000 (NPC 2006) with three major clans namely; Ezzangbo, Ngbo, Effium and covers an estimated area of 252 km^2. The area lies within latitudes 6^0 3' N to 6^0 50' N and longitudes 7^0 80' E to 8^0 00' E with climatic conditions such as rainy season (March-October) and dry season (October-February).

Sample collection and processing: Exactly 75 samples (25 each from knifes, tables and slabs) were taken aseptically from donkey abattoir at Ezzamgbo, Ohaukwu Ebonyi

State using sterile swab stick. The sterile swab sticks were used to collect the samples from the donkeys slaughter equipment by rotating at an angle of 180ºC. The swab sticks were returned to their respective containers and labeled properly. The samples were immediately transported to the Applied Microbiology Laboratory of Ebonyi State University, Abakaliki for bacteriological analysis. Briefly, each of the collected (knifes, tables and slabs) samples were inserted into 5 ml of freshly prepared nutrient broth and the tubes were loosely covered with cotton wool. The tubes were arranged on test tube rack and were incubated at 37ºC for 18-24 hours. Bacterial growth was suspected by the presence of turbidity or cloudiness in the tubes after incubation. Tubes that showed turbidity were further sub-cultured onto solid culture media plates for the isolation of the primary bacterium (Ejikeugwu *et al*., 2018).

Isolation and identification of Escherichia coli: The bacterial colonies obtained were further inoculated on freshly prepared Eosin Methylene Blue Agar (EMBA) (HiMedia M317) and MacConkey Agar (MCA) (HiMedia MH081) media and incubated at 37°C for 18-24 hours for the purpose of isolation of *E. coli.* Further identification of *E. coli* was done using Gram staining and other standard microbiological methods which includes the biochemical test; Triple Sugar Iron Agar (TSIA) (HiMedia M021): Simmons Citrate Agar (SCA) (HiMedia M099), IMVIC media, such as Sulfide Indole Motility (SIM) (HiMedia M181): Methyl red (MR): Voges-Proskauer (VP) (Merck; 105712) (Yanestria *et al*., 2022).

Antibiotic susceptibility testing: Susceptibility testing was done on Mueller Hinton agar plates (Oxoid, UK) using the Kirby-Bauer disk diffusion method as per the criteria of Clinical Laboratory Standard Institute (CLSI, 2020). The different classes of antibiotics disk which includes Fluoroqinolone (CIP) ciprofloxacin; 5µg, Macrolides (E) erythromycin; 30µg, Tetracycline (TE) tetracycline; 30 µg, Beta-lactams (AMP) ampicillins; 30µg, and Aminoglycosides (CN) gentamycin; 500µg. All the antibiotic disks were procured from Oxoid limited (Oxoid, UK). A loopful of the test organism (adjusted to 0.5 McFarland turbidity standards) was streaked on freshly prepared Muller-Hinton agar plates; and the plates were allowed to stand for 15 minutes. The antibiotic disks were placed at a distance of 30mm apart from each other and 15mm away from the edge of the plate and the susceptibility plates were incubated at 37ºC for 24 hours (Ugbo *et al*., 2023; CLSI, 2020). The zones of inhibition diameter were measured according to the CLSI criteria.

Multiple antimicrobial resistance index (MARI): The MAR index for a single isolate was calculated as the number of antibiotics to which an isolate is resistant to (**a**) divided by the total number of antibiotics tested against the isolate (**b**) (Ejikeugwu *et al*., 2017).

Screening for the presence of metallo-betalactamase (MBL): All the multidrug resistance *Escherichia coli* isolates were screened for the production of MBL by determining their susceptibility to any of the carbapenems including imipenem (IPM) (10 µg), meropenem (MEM) (10 µg), ertapenem (ETP) (10 µg) (Dey *et al*., 2023). The Kirby-Bauer disk diffusion technique were used, and each of the antibiotics disk were placed at a distance of 20 mm apart and the plates were incubated at 37ºC for 18-24hours. MBL enzyme-producing isolates were suspected when the test organism(s) showed reduced susceptibility to any of the tested antibiotics. The isolates showing inhibition zone diameter (IZD) of ≤ 23 mm were suspected to produce MBL and these isolates were subjected to phenotypic confirmation test according to the method of (Ejikeugwu *et al*., 2016).

Phenotypic detection of metallo β-lactamase (MBLs): The multidrug resistance *Escherichia coli* isolates found to be resistant to imipenem or meropenem as identified in the screening test were subjected to phenotypic studies for the presence of metallo β-lactamase (MBL)

using modified Hodge test method. The pure culture of the multidrug resistance *Escherichia coli* isolates were adjusted to 0.5 McFarland turbidity standards and aseptically swabbed on Mueller-Hinton (MH) agar plates. The standard antibiotic disks of imipenem (10 µg) and meropenem (10 µg) impregnated with EDTA (1 µg) were aseptically placed on MH agar plates. Additionally, supplementary imipenem (10 µg) and meropenem (10 µg) disks without EDTA were also placed alongside with the antibiotic disks impregnated with the chelating agent (EDTA) at a distance of 20 mm away from each other. The chelating agents were initially tested on the test bacteria prior to the phenotypic assay to confirm there were no inhibitory effect on the test organisms. All the plates were incubated at 37°C for 18-24 hours and zone of inhibition were recorded after incubation. A difference of ≥ 7 mm between the zones of inhibition of any of the carbapenem disks with or without the chelating agents infers metallo-betalactamase production according to (Bajracharya *et al*., 2023).

RESULTS

E. coli isolates were isolated from 30(40.0%) samples out of the 75 donkey slaughter equipment samples analyzed. The result revealed that slab harbored highest number of *E. coli* isolates 10(40.0%), followed by table 12(48.0%) and knife had the least occurrence of *E. coli* 8(32.0%) table (Table 1). The *E. coli* isolated from the slaughter equipment presented different kinds of resistance to the tested antibiotics; tetracycline (40.0%-80.0%), gentamicin (25.0%-30.0%), erythromycin (40.0%-50.0%), ampicillin (70.0%-75.0%) and ciprofloxacin (20.0%-37.5%). The most effective antibiotics against the tested *E. coli* isolates were ciprofloxacin (80.0%) and gentamicin (75.0%) (Table 2). The test revealed that 46.7% of *E. coli* isolates were MDR. Some of the *E. coli* isolates were approximately totally resistant to ampicillin (AMP), gentamycin (CN), ciprofloxacin (CIP), tetracycline (TE), erythromycin (E) and presented three different MDR patterns which includes; TE-CN-E-AMP-CIP, TE-E-AMP-CIP and TE-CN-E-AMP (Table 3). Out of the 14 MDR *E. coli* isolates, knife had 3 (37.5%), table had 6 (50.0%) and slab harbored 5 (50.0%). However, five (35.7%) MDR *E. coli* isolates were confirmed to produce metallo β-lactamase (MBLs), knife 1 (33.3%), table 2 (33.3%) and slab 2 (40.0%) (Table 4).

Table 1: Distribution of *E. coli* isolates from donkey slaughter equipment

Sample/Source	No of Samples	No Positive for *E. coli*	Percentage (%)
Knife	25	8	32.0
Table	25	12	48.0
Slab	25	10	40.0
Total	75	30	40.0

Table 2: Antimicrobial susceptibility profile of *E. coli* isolated from donkey slaughter equipment

SOURCE	TE		CN		E		AMP		CIP	
	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)
Knife	4(50.0)	4(50.0)	6(75.0)	2(25.0)	4(50.0)	4(50.0)	2(25.0)	6(75.0)	5(62.5)	3(37.5)
Table	7(58.3)	5(41.7)	9(75.0)	3(25.0)	7(58.3)	5(41.7)	3(25.0)	9(75.0)	8(66.7)	4(33.3)
Slab	2(20.0)	8(80.0)	7(70.0)	3(30.0)	6(60.0)	4(40.0)	3(30.0)	7(70.0)	8(80.0)	2(20.0)

Key: E = erythromycin, CIP = ciprofloxacin, AMP = ampicillin, CN = gentamicin, TE = tetracycline

Table 3: Multidrug resistance patterns of *E. coli* isolates from donkey slaughter equipment

Isolates Code	Isolates	Number of antibiotics that isolates were resistant to (a)	Total number of antibiotics tested (b)	MAR index (a/b)
K 13	*E. coli*	TE-E-AMP-CIP	5	0.80
K 24	*E. coli*	TE-CN-E-AMP-CIP	5	1.00
T 4	*E. coli*	TE-CIP-E-AMP	5	0.80
T 16	*E. coli*	TE-E-AMP-CN-CIP	5	1.00
T 21	*E. coli*	TE-E-AMP-CIP	5	0.80
T23	*E. coli*	TE-CN-E-AMP	5	0.80
S 2	*E. coli*	TE-E-AMP-CN-CIP	5	1.00
S 9	*E. coli*	TE-CN-E-AMP-CIP	5	1.00
S 14	*E. coli*	TE-E-AMP-CIP	5	0.80
Average MDR index				8/9 (0.88)

Key: K = Knife; T = Table; S = Slab; CIP = ciprofloxacin; E = erythromycin; TE = tetracycline; AMP = ampicillin; and CN = gentamycin

Table 4: Prevalence of MDR-*Escherichia coli* producing metallo β-lactamase (MBLs)

Sample/Source	No of *E. coli* screened	No of MDR-*E. coli* suspected for MBL (%)	No of MDR-*E. coli* +ve for MBL (%)	No of MDR-*E. coli* -ve for MBL (%)
Knife	8	3 (37.5)	1 (33.3)	2 (66.7)
Table	12	6 (50.0)	2 (33.3)	4 (66.7)
Slab	10	5 (50.0)	2 (40.0)	3 (60.0)
Total	30	14 (46.7)	5 (35.7)	9 (64.3)

DISCUSSION

The global emergence and fast spread of pathogenic microorganisms showing multidrug resistance to antimicrobial agents is a serious public health threat. *Enterobacteriaceae* especially *Escherichia coli* producing metallo beta-lactamase (MBLs) is one of the mechanisms associated with severe bacterial infections in clinical setting. Multidrug resistant properties of these microorganisms have limited and complicated treatment options. This study recorded presence of *E. coli* in donkey slaughter equipment with high level of prevalence at percentage of 40.0. *E. coli* isolates (5.2%) has been reported by previous researcher on slaughtered donkey from slaughter house in Kaduna State, Nigeria (Esonu *et al*., 2022), but their report was very low when compared to the observation of the current study and this could be attributed to the differences in the study areas. Another study revealed a significant prevalence of *E. coli* strains (cattle- 88.7%), (chicken- 81%), (swine- 89.5%) and raising concerns about potential transmission to humans through contaminated food animals (Eyitayo *et al*., 2018). A total prevalence of 12.0% *E. coli* was reported from animal harvested for human consumption in Ethiopia. The detection of *E. coli* from animal origin raises concerns about the potential for contamination of meat products and the transmission of this pathogen to consumers (Adanech and Temesgen, 2018). *E. coli* is a diverse group of bacteria which is a normal flora of in the gastrointestinal tract of animals which are harmless, but can be pathogenic in case of disease condition in the animals. Thus, in the context of food safety, the presence of pathogenic *E. coli* on slaughter equipment is a critical issue that requires attention to prevent food-borne illnesses (Yanestria *et al*., 2022; Adanech and Temesgen, 2018). Another study on the microbial contamination of cattle carcasses and slaughter observed the presence of *Enterobacteriaceae*, emphasizing the importance of maintaining hygienic practices to prevent contamination (Pierluigi

et al., 2016). Several other studies have also investigated the presence of *E. coli* in animal such as goat, sheep, cattle, donkey, chickens slaughter environments and reported a great level of prevalence (Eltai *et al*., 2020; Esonu *et al*., 2022; Ugbo *et al*., 2023). The identification of *E. coli* on animals slaughter equipment underscores the importance of implementing rigorous hygiene practices and sanitation measures in abattoirs to minimize the risk of contamination.

The *E. coli* isolates recovered from the slaughter equipment presented different degrees of resistance to the tested antibiotic classes; tetracycline (40.0% to 80.0%), gentamicin (25.0% to 30.0%), erythromycin (40.0% to 50.0%), ampicillin (70.0% to 75.0%) and ciprofloxacin (20.0% to 37.5%). High level of resistance to tetracycline, ampicillin as reported in this study is in accordance with the observation of Mahmoodi *et al.* (2020) who reported *E. coli* resistance to tetracycline as (17.5% to 42.3%), ampicillin (24.6% to 64.7%), low level of resistance to ciprofloxacin (5.2% to 25.4%) and gentamicin (1.9% to 6.5%). Ejikeugwu *et al.* (2016) reported similar level of resistance on *E. coli* isolates from abattoir environment, where they observed that ciprofloxacin showed resistance of 28.1% and gentamicin (18.8%).High level of resistance has been reported on *E. coli* isolates by previous researcher to ciprofloxacin (66.1%) (Nazmul *et al*., 2019). A study on pathogenic *E. coli* isolates from chicken meat in Bharatpur, Chitwan reported lower resistance level to ciprofloxacin 16.67% and gentamicin (33.3%) (Bajracharya *et al*., 2023) and is in line the findings of this study which reported resistance to ciprofloxacin to be (20.0%) and gentamicin (25.0%).Out of the thirty *E. coli* isolates identified from donkey slaughters equipment, 14 (46.7%) were recorded to present multidrug resistance. Multidrug resistance *E. coli* (MDR *E. coli*) isolates in this study showed resistance to four and five different classes of antibiotics with three major different patterns which includes; TE-CN-E-AMP-CIP, TE-E-AMP-CIP and TE-CN-E-AMP. Similar findings of antibiotic resistance pattern includes; ATM-CIP-CN-E, ATM-CIP-C-E, ATM-CN-E, ATM-CIP-E, CIP-C-E were reported on multidrug resistance avian pathogenic *E. coli* (MDR-APEC) isolated from ducks on wet markets in Surabaya (Kendek *et al*., 2024). Multidrug resistance *E. coli* isolates was reported to show resistance to three (CTX, TZP, IMP (19.0%)); four (ATM, MEM, CIP, CN (23.0%)); five (MEM, AMC, FEP, AMP, CEX (15.0%)) and six (AMC, CTX, ATM, IMP, CIP, CEX (5.0%)) different classes of antibiotics (Nazmul *et al.,* 2019. Previous study on multidrug resistance *E. coli* from animal source observed that isolates were resistance to three and five different classes of antibiotic; amoxicillin (AMOX), doxycycline (DO), cotrimoxazole (COT) (41.61%); amoxicillin (AMOX), doxycycline, (DO), cotrimoxazole (COT), azithromycin (AZM) (25.0%) and amoxicillin (AMOX), doxycycline (DO), cotrimoxazole (COT), azithromycin (AZM), gentamicin (GEN) (16.6%) (Bajracharya *et al*., 2023). The detection of MDR *E. coli* in slaughter equipment showing resistance to three and five different classes of antibiotics suggests that those classes of antibiotics may have be abused as growth promoters, and during treatment of infections in the animals. This study revealed the occurrences of metallo β-lactamase (MBLs) in MDR- *E. coli* to be 5 (35.7%); knife had 1 (33.3%), table harbored 2 (33.3%) and slab had2 (40.0%). The study done on *E. coli* isolated from animals which includes cow and cloacae swabs of poultry birds revealed the presence of MBLs phenotypically to be 39.9% and 45.7% respectively (Ejikeugwu *et al.,* 2017) and is in agreement with 35.7% of MBLs report in this present study. The prevalence of MBL-producing *E. coli* isolates in this study is in accordance with the observation of the occurrence of MBL-producing *E. coli* isolates (Chouchani *et al*., 2011). Metallo β-lactamase (MBLs) producing *E. coli* (5.0%) was reported from animal (chicken) (Dey *et al*., 2023), and 66.0% in clinical (Mahmoodi *et al*., 2020).

Another study from animal reported similar prevalence of *E. coli* isolates positive for MBL production in India (Chakraborty *et al*., 2010). The presence of MBLs in MDR *E. coli* is a significant concern as it has been linked to numerous outbreaks of foodborne illnesses in humans and this observation highlights a potential foodborne public health hazard (Madec *et al*., 2017) thus, the need for regular screening of MBLs from animal. The MBL-producing *E. coli* isolates can carry mobile genetic elements with great ability to spread in the environment (Mahmoodi *et al*., 2020). This study establishes early detection of MBL-producing *E. coli* isolates particularly their reservoir to help in maintaining suitable antimicrobial therapies.

CONCLUSION

Occurrence of MBLs in MDR *E. coli* was reported in donkey slaughter equipment to be 35.7% which is alarming and these suggest serious public health threat if not controlled. The identification of MDR *E. coli* on slaughter equipment revealed the need for stringent hygiene practices in abattoirs to mitigate contamination risks and ensure consumer safety. However, the encouragement of periodical research and accurate detection of MBL- production in MDR *E. coli* isolates from animal source, abattoir environment and clinical samples is of utmost public health importance due to its multidrug resistant properties harbored by these organisms to safeguarding both animal and public health.

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Assessing the Phytochemical Profile and Antimicrobial Efficacy of *Allium sativum* Against Some Bacterial Pathogens

Unegbu V. N.[1*] Nwachukwu N. O.[2] and Okoronkwo C. U.[3]

1. Department of Microbiology, University of Agriculture and Environmental Sciences, Umuagwo Imo State, Nigeria
2. Department of Medical Laboratory Science, Abia State University Uturu, Abia State, Nigeria
3. Department of Microbiology, Abia State University Uturu, Abia State, Nigeria

* Corresponding author: donval4u@yahoo.com

Abstract: This research was carried out to examine the phytochemical composition and antibacterial efficacy of garlic against some bacteria pathogens. A standardized phytochemical investigation was done qualitatively utilizing benchmark protocols. The garlic extract's ability to combat bacterial growth was evaluated using the agar well diffusion assay. Potency of the *Allium sativum* extracts was investigated by determining minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values via micro dilution methodology against selected bacteria pathogens. Phytochemical analysis of the garlic extracts revealed many bioactive constituents, including saponins, flavonoids, tannins, alkaloids, terpenoids, glycosides, anthraquinolones and steroids; which are found diverse levels of presence across various extracts The antimicrobial efficacy of *Allium sativum* extracts (aqueous and ethanol) was evaluated at potency from 25 to 200 milligram/ml, resulting in diameters of inhibition size 10-22 millimeter (*S. aureus*), 9-21 millimeter (*E. coli*). Garlic extracts MIC against *Staphylococcus aureus* ranged from 25 milligram/milliliter (ethanol) to 50 milligram/milliliter aqueous while *E. coli* had a uniform MIC of 25 milligram/milliliter for both extracts. Both *S. aureus* and *E. coli* exhibited MBC values of 50 milligram/milliliter (aqueous extract) and 25 milligram/milliliter (ethanol extract), indicating ethanol extract's enhanced bactericidal potency. The observed antibacterial effects of garlic extracts are likely due to the synergistic action of some of its bioactive compounds; supporting its potential use as a natural antimicrobial agent. The ethanol extract of garlic exhibited superior antimicrobial activity (11-22 mm) compared to the aqueous extract (9-21 mm); displaying concentration-dependent inhibition. Therefore, it is advisable to conduct a thorough structural elucidation of garlic bioactive constituents to assess the actual potency among various phytochemicals discovered in it.

Key word: Ethanol extracts, aqueous extracts, *Staphylococcus aureus, Escherichia coli, Allium sativum*

INTRODUCTION

Medicinal plants are gaining significant attention. This is because they offer a promising alternative to synthetic medications, boasting affordability, minimal side effects, and greater patient acceptability with a high therapeutic potential (Sanusi *et al*., 2017). The growing interest in Ayurvedic medicine, which utilizes around 420 plant species – including garlic – is a prime example of the growing interest in natural health solutions for man's own health benefits (Islam *et al*., 2014).

Phytochemicals present in herbaceous plants comprises tannins, flavonoids, terpenoids, alkaloids, saponins and steroids etc. (Shuaibu, *et al.,* 2019). Medicinal plants harbor bioactive compounds with unique phytochemical properties which varies among different plant species. This unique property exhibited by medicinal plant is as a result of their biodiversity and capacity to induce significant physiological effects in humans (Shuaibu *et al*., 2019).

Across Nigerian cultures and dialects, garlic is referred to as 'Ata ile' in Yoruba, 'Galiki' in Igbo, and 'Tafarnuwa' in Hausa (Fadiji, 2019). It boasts of ancient medicinal uses with broad applications to include antibacterial properties and effectiveness in managing arthrosclerosis, cancer and diabetes (Toledano *et al*., 2019).

Research has shown garlic to have cardiovascular benefits, lowering pressure and blood plasma cholesterol, while also preventing platelet aggregation (Toledano *et al*., 2019). Its bioactive constituents, alliin and alliinase, drive these benefits, supporting traditional uses for garlic in treating various ailments like dental issues

and wounds, viral infections, as well as spiritual ailments (Kim *et al*., 2017).

Escherichia coli is a rod-shaped gram negative bacterium that is found in the gut of warm blooded animals. *Staphylococcus aureus* is a non-motile, facultative anaerobes, gram-positive and circular shaped bacterium which is a topical commensal microorganism of the human body, frequently isolated from the skin and nasopharynx (Unegbu *et al.,* 2020).

Antibiotic resistance poses a significant challenge, prompting researchers worldwide to exploring innovative solutions, including the screening of medicinal plants (Unegbu *et al.,* 2019). This quest seeks to uncover novel, effective, and safer therapeutic agents capable of combating infectious diseases (Unegbu *et al.,* 2019). Therefore, the aim of this research was to assess the phytochemical profile and antimicrobial efficacy of *Allium sativum* extracts against some bacteria pathogens.

MATERIALS AND METHODS

Preparation of plant samples: Wholesome garlic bulbs, bought from the open market and certified by a plant biologist were processed in the laboratory by washing, slicing, air-drying, and pulverization into powder using sterile equipment, and stored in a transparent glass bottle for subsequent use (Unegbu *et al.,* 2020).

Collection and confirmation of the model bacteria: Model cultures of *E. coli* and *S. aureus* were sourced at Nnamdi Azikiwe University's Department of Microbiology, Anambra State, Nigeria. Their identities were confirmed through standard microbiological techniques and subsequently preserved at 4°C.on slants containing nutrient agar (CLSI, 2014).

Biochemical characterization of the model bacteria E. coli: For isolation and confirmation, the culture of *E coli* was plated on Eosine Methylene Blue agar and incubated for a period of 24 hrs. Colonies with a distinct green metallic sheen indicated a positive result; and confirmed by sub-culturing onto Chromagar *E. coli* medium (Oxoid, Basingstoke, UK), where they exhibited a blue/violet appearance (CLSI guidelines (2014).

Staphylococcus aureus: Following a positive catalase test result, *S. aureus* was grown on blood agar and incubated overnight 37°C. The isolated colonies were then inoculated onto Mannitol salt agar (MSA) for 24 hours; and a smooth, yellow, circular colonies appearance on MSA showed a positive result, according to CLSI protocols (2014).

Standardization of the model organism: A 24hours old growth broth of *E. coli and S. aureus* were standardized by inoculating the model bacteria into 5 ml of sterile nutrient rich broth and incubating for 2 hours. The turbidity of the broth was calibrated to align to 0.5 McFarland standard, indicating optimal growth (Garba *et al*., 2013).

Synthesis of aqueous and ethanol garlic extracts: Preparation of garlic extracts was done following established protocols as described by Garba, *et al.* (2013).

Preparation of aqueous extract: Garlic powder (10g) was infused in 100ml of distilled water and allowed to steep overnight. The solution was filtered using Whatman filter paper, and the resulting filtrate immediately vaporized by the use of a water bath. The residue was stored in universal sterile container at 4°C until there is need for it (CLSI, 2014).

Preparation of ethanol extract: Garlic powder (10 g) was soaked overnight in 100ml ethanol (95%) and resulting solution filtered to remove solids. The resulting solvent was evaporated; and yielded the garlic extract, which was stored at 4°C (CLSI, 2014).

Extract dilution: The already prepared extracts of garlic (aqueous and ethanol) were diluted with distilled water to a desired concentrations (200 to 25 mg/ml), as established by CLSI (2014).

Sterility test of garlic extract: Both garlic extracts was tested (sterility and contamination) following the protocol outlined by Unegbu *et al.* (2019). A 1-ml sample of each extract was transferred to

N.A plates aseptically and incubated overnight at 37°C. The lack of growth on the plates verified the extracts' sterility.

Qualitative phytochemical analysis of the extracts: A phytochemical screening was conducted to identify various bioactive constituents like tannins, flavonoids, saponins, glycosides, carbohydrates, alkaloids, cardiac glycosides, phenols, phlobatannins, steroids anthraquinones and terpenoids, using the method as outlined by Valentine *et al.,*. (2020).

Antimicrobial efficacy of extract of Allium sativum bulbs: The antimicrobial activity of garlic was analyzed using the in vitro diffusion technique, according to the method of Shuaibu *et al.* (2019). Standardized bacterial cultures (5 millimeters) were evenly introduced and spread into inoculated nutrient agar plates. Six wells (5 mm diameter) were created, and 0.5 ml of garlic extract (250, 100, 50, and 25 mg/ml) were dispensed into each well. Gentamicin (20 mg/ml) was used to indicate positive control while distilled water was used to indicate negative control. The perforated plates were incubated overnight at 37°C and the resulting zones of inhibitions were measured with the help of a meter rule.

Evaluation of minimum inhibitory concentration of the extracts: The MIC of *Allium sativum* extracts against the test organisms was evaluated using the broth dilution assay. Nutrient broth (5 millimeters) was mixed with 5 millimeters of each extract, serially diluted to achieve concentrations of 200-25 mg/ml, and inoculated with a loopful of the test organisms. After 24-hour incubation at 37°C, the lowest concentration inhibiting growth was recorded as the MIC (Shuaibu *et al.,* 2019).

Evaluation of minimum bactericidal concentration (MBC) of extracts: Through sub cultivation of 1 ml of bacterial culture from MIC tubes with no visible growth NA plates, and subsequent overnight incubation (37°C), the MBC was determined. The MBC was identified as the lowest concentration of the sample that inhibited growth upon sub-culturing (Shuaibu *et al.,* 2019).

Statistical analysis: Through the use of one way ANOVA, data were analyzed; and a p-value of ≤ 0.05 was considered significant with the use of SPSS software version 21.0.

RESULTS

Table 1 showed the plant based secondary metabolite screening of the aqueous and ethanol samples of *Allium sativum* bulbs. It showed the existence of different phytochemical constituents like tannin, saponin, terpenoid, alkaloids, flavonoid, steroid and glycoside at different concentrations.

Table 2 illustrates the antibacterial efficacy of *Allium sativum* aqueous and ethanol sample against the two model bacteria. *Allium sativum* aqueous extract exhibited zone of inhibition diameters of 21, 19, 14, and 10 mm against *S. aureus* at concentrations (200, 100, 50, and 25 mg/ml0 respectively. The ethanol extract of *Allium sativum* demonstrated zone of inhibition diameters of 22, 20, 16, and 11 mm against *S. aureus* at concentrations of (200, 100, 50, and 25 mg/ml) respectively. The antibacterial efficacy of aqueous and ethanol samples to *E. coli* is also presented in Table 2.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of garlic bulb samples on the model bacteria are summarized in table 3. For *S. aureus,* MIC & MBC of the aqueous extract were both 50 mg/ml. In contrast, MIC & MBC of aqueous sample against *E. coli* were 25 mg/ml and 50 mg/ml, respectively. MIC & MBC figures of ethanol extract on *S. aureus* and *E. coli* are also summarized in Table 3.

Table 1: Photoactive compounds of ethanol extracts (EE) and aqueous (AE) extracts garlic

Photoactive compounds	AE	EE
Carbohydrate	+	-
Terpenoid	+	++
Tannin	+++	++
Flavonoids	++	+++
Saponins	++	+
Alkaloids	++	+++
Glycosides	+	++
Steroids	+	+++
Anthraquinone	+	+
Phlobatannis	-	-
Phenol	-	-

Key: +++ = high concentration; ++ = moderate concentration; + = trace concentration; - = zero concentration

Table 2: Antibacterial efficacy of garlic bulb extracts against the two model bacteria

	diameter zone of inhibition (millimeter) of extract concentrations						
Isolate	200	100	50	25	+C	-C	Extracts
S. aureus	21± 0.02	19± 0.01	14± 0.05	10± 0.03	17±0.01	0	AE
S. aureus	22± 0.04	20± 0.01	16± 0.02	11± 0.04	17± 0.01	0	EE
E. coli	18± 0.05	15± 0.03	11± 0.04	9± 0.01	18± 0.01	0	AE
E. coli	21± 0.02	17± 0.01	14± 0.03	11± 0.04	18± 0.01	0	EE
P level (0.05)	***	***	***	***	***	***	

Key: +C: reference standard (Gentamicin, 20 mg/ml); -C: baseline control (Sterile water); *** = significant difference at $p \leq 0.05$

Table 3: MIC and MBC values of aqueous and ethanol samples of garlic against the model bacteria

	Extracts concentration in milligram/milliliter		
Isolates	MIC	MBC	Extracts
S. aureus	50 ± 0.02	50± 0.01	AE
S. aureus	25 ± 0.02	25± 0.01	EE
E. coli	25 ± 0.04	50 ± 0.02	AE
E. coli	25 ± 0.04	25± 0.03	EE
p level (0.05)	***	***	

Key: *** = significant difference at $p \leq 0.05$

DISCUSSION

The findings from the phytochemical constituents of the extracts confirmed the existence of rich bioactive agents like steroids, tannins, saponins, alkaloids, flavonoids, terpenoids and glycosides in various concentrations of garlic extracts. This was similar to studies conducted in different part of Nigeria (Akintobi *et al.,* 2013; Fadiji, 2019). The observed antimicrobial efficacy of garlic extracts can be attributed to the presence of phytochemicals such as alkaloids, flavonoids, saponins and steroids, which have been previously reported to exhibit antimicrobial properties (Akintobi *et al*., 2013).

Alkaloids, such as quinine, ephedrine, morphine, strychnine, and nicotine, possess established therapeutic benefits, including anesthetic, cardio-protective, and anti-inflammatory effects, and are commonly used in clinical settings (Hyunjoo, 2018).

According to Hyunjoo (2018), flavonoids exhibit a range of biological properties, including cytotoxic, antimicrobial, anti-inflammatory and antitumor activities, but their most notable characteristic is their

potent antioxidant capacity. Saponins exhibit a broad spectrum of biological activities, including antiprotozoal, antioxidant, antimollusk and antinutrient properties, as well as inducing hypoglycemia, and demonstrating antifungal and antiviral effects (Fadiji, 2019). Similarly, steroids exhibit analgesic and central nervous system activities (Garba *et al.,* 2013).

The bacterial species tested exhibited varying susceptibility to garlic extracts derived from different organic solvents, with ethanol extracts demonstrating significantly higher antimicrobial activity relative to aqueous extracts. Valentine *et al.* (2020) highlighted the constraints of using water as a solvent in seeking innovative antimicrobial agents, citing its ineffectiveness in extracting non-polar compounds.

The antimicrobial efficacy of garlic sample was higher in ethanol extracts than aqueous extracts, inhibiting both model bacteria in a dose-dependent manner. This variation is attributed to differences in phytochemical content between the extracts (Shrestha *et al.,* 2016). This finding was similar to the work of Martins *et al.* (2016).

This research showed that ethanolic extracts of garlic exhibited significantly enhanced zones of inhibition than aqueous extracts at all tested concentrations, illustrating their potential as a novel, natural antimicrobial agent. Comparable results were reported by Szychowski *et al.* (2018). This study revealed varying effective concentrations against both model bacteria used in this research work. Notably, the ethanol extracts exhibited low MIC and MBC values, which suggest potent antibacterial activity, supporting the findings of Elzein *et al.* (2018). The differences in MBC values obtained for garlic extract against *E. coli* and *S. aureus* suggest variations in phytochemical properties, leading to distinct inhibitory and bactericidal effects on the test organisms, outperforming standard antibiotics (Abubakar and Usman, 2016).

The variation in antimicrobial effects among the extracts can be further explained by differences in their chemical composition, as noted by Abiola *et al.* (2017). This corroborates the work of El-Hamidi and El-Shami (2015) and Abiola *et al.* (2017), which showed that garlic extracts exhibit potent bactericidal effects, outperforming those of standard antibiotics.

CONCLUSION

The observed antibacterial effects of garlic extracts are likely due to the synergistic action of secondary metabolites, including tannins, flavonoids, saponins, terpenoids, glycosides, steroids, alkaloids, and anthraquinones; supporting its potential use as a natural antimicrobial agent. Therefore, it is advisable to conduct a thorough structural elucidation of garlic bioactive constituents to reveal the real bactericidal activities of various phytochemicals discovered in it. In addition, pharmaceutical companies should channel more resources to the developments of novel drugs which has garlic as its natural origin.

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