Antibacterial Properties of Crude Metabolic Extracts from *Clostridium* spp. Isolated from Cattle Yards Against Some Clinical Bacterial Isolates in Benin City

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UNIVERSITY OF BENIN

BENIN CITY

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B.Sc (MAUTECH)

A THESIS WRITTEN IN THE DEPARTMENT OF MICROBIOLOGY AND SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (M.Sc.) IN ENVIRONMENTAL AND PUBLIC HEALTH MICROBIOLOGY, UNIVERSITY OF BENIN, BENIN CITY.

AUGUST, 2025.

CERTIFICATION

We certify that this work was carried out by **Molkale LAUSHUGNO (Mrs)** in the Department of Microbiology, University of Benin, Benin City.

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CERTIFICATION OF THESIS

We attest and declare that the thesis titled **Antibacterial Properties of Crude Metabolic Extracts from *Clostridium* spp. Isolated from Cattle Yards Against Some Clinical Bacterial Isolates in Benin City** has successfully passed the anti-plagiarism test and does not violate any copy right regulation.

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Dr. (Mrs). I.B. Idemudia **Date**
(Supervisor)

DEDICATION

This project work is dedicated to God Almighty, who has made it possible for me to be alive till this day and has provided for me and sustained me.

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ABSTRACT

The soil is a reservoir for various clostridial pathogens, with agricultural soils representing a major source of contamination for overlying crops and grazing livestock. Indigenous bacteria in the soil environment can produce a wide range of solvents and metabolites more efficiently. This study therefore was aimed at determining the antibacterial potential of crude metabolic extracts from *Clostridium* spp. isolated from cattle yards in Benin City, Nigeria against clinically relevant bacterial isolates.

Soil samples were collected in triplicates each at 0-5 cm depth using soil Auger from cattle farmlands in Eyaen, Aduwawa 1, Aduwawa 2 and Dumez in Benin City Nigeria. Clinical isolates were collected from Medical Microbiology Laboratory, University of Benin Teaching Hospital, Benin City Nigeria. The physicochemical parameters of the soil were analysed using standard methods. Minerals and heavy metals were determined using atomic absorption spectrophotometry. Bacteria were isolated using dilution technique and cultured on appropriate culture media followed by *in-vitro* screening for virulent factors. Isolated pathogens were subsequently assessed for antibiotic resistance to common antibiotics and resistance to crude metabolites using modified Kirby-Bauer disc diffusion method, while *Clostridium* species were further identified using molecular technique.

The phosphorus, magnesium, iron, zinc and copper where above the Federal Ministry of Environment (FMEnv) acceptable limit while electrical conductivity was below and potassium within FMEnv acceptable limit. The total *Clostridium* counts of soil samples ranged from $5.13\pm0.02 \times 10^3$cfu/g - $5.53\pm0.10 \times 10^3$cfu/g. The *Clostridium* isolates identified from the soil samples were *Clostridium sporogens, Clostridium butyricum, Clostridium bolteae, Clostridium septicum,* and *Clostridium perfringens. Staphylococcus aureus* was found to exhibit positive characteristics for gelatinase, lipase, DNAse and hemolysin production. *C. perfringens*s and *C. butyricum* had the highest zone of inhibition against

Escherichia coli (1.07±0.03 mm) and *Staphylococcus aureus* (1.07±0.03 mm), while *C. perfringens* had the least zone of inhibition against *P. aeruginosa* (0.10±0.00 mm). *Enterobacter aerogenes* had the highest percentage antibiotic resistance (50%) which was against amoxicillin, followed by *Pseudomonas aeruginosa* (42.1%) against sparfloxacin. *Clostridium* species exhibit varying degrees of antibacterial activity against specific bacterial isolates which prove its potential therapeutic applications. Therefore, *Clostridium* species can be used commercially for the production of antibiotics after purification and proper standardization.

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

The soil is a reservoir for various clostridial pathogens, with agricultural soils representing a major source of contamination for overlying crops and grazing livestock. Understanding the prevalence and behaviour of pathogens in these soils is fundamental to ascertaining and mitigating the risk of disease from agroecosystems (Pahalagedara *et al*., 2020). Indigenous bacteria in the soil environment can produce a wide range of solvents and metabolites more efficiently. The genus *Clostridium* is a, Gram-positive anaerobic bacteria. Species of *Clostridium* inhabit soils and the intestinal tract of animals, including humans (Maczulak, 2011). This genus includes several significant human pathogens, including the causative agents of botulism and tetanus. It was formerly included as an important cause of diarrhea, Clostridioides difficile, which was reclassified into the *Clostridium* genus in 2016 (Dieterle *et al*., 2019)

The genus *Clostridium* can be differentiated from endospore forming genus *Bacillus* by its obligate anaerobic growth, the shape of endospores and the lack of catalase. Species of *Desulfotomaculum* form similar endospores and can be distinguished by their requirement for sulfur (Maczulak, 2011). Glycolysis and fermentation of pyruvic acid by *Clostridium* yield the end products butyric acid, butanol, acetone, isopropanol, and carbon dioxide (Tortora *et al*., 2010). There is a commercially available polymerase chain reaction (PCR) test kit (Bactotype) for the detection of *Clostridium perfringens* and other pathogenic bacteria (Willems *et al*., 2007). *Clostridium perfringens* is a Gram-positive, rod-shaped, anaerobic, spore-forming pathogenic bacterium of the genus *Clostridium* (Kiu and Hall, 2018). *Clostridium perfringens* is ever-present in nature and can be found as normal component of decaying vegetation, marine sediment, the intestinal tract of humans and other vertebrates,

insects, and soil. It has the shortest reported generation time of any organism at 6.3 minutes in thioglycolate medium (Ryan and Ray, 2004).

Clostridium perfringens is one of the most common causes of food poisoning in the United States, alongside norovirus, *Salmonella, Campylobacter* and *Staphylococcus aureus*. However, it can sometimes be ingested and cause no harm (Juckett *et al*., 2008).

Clostridium species are readily found inhabiting soils and intestinal tracts. *Clostridium* species are also normal inhabitant of the healthy lower reproductive tract of females (Hoffman, 2012).

The main species responsible for disease in humans are *Clostridium botulinum* which produce botulinum toxin in food or wounds and causes botulism. This same toxin is known as Botox and is used in cosmetic surgery to paralyze facial muscles to reduce the signs of aging. *Clostridium perfringens* causes a wide range of symptoms, from food poisoning to cellulitis, fasciitis, necrotic enteritis and gas gangrene (Kiu and Hall, 2018; Kiu *et al*., 2019). *Clostridium botulinum* type G is difficult to recognize in mixed cultures because of its lack of the lipase marker and its low toxigenicity. Unlike all other types of *C. botlulinum*, type G does not produce the enzyme lipase, but produces only small amounts of toxin in pure culture. Its toxin is initiated by trypsin, as is the toxin from the nonproteolytic strains of types B and F and all strains of type E. Usually, a culture procedure employing a spore selection technique is used for isolating *C. botulinum*. One of the major disadvantage is that *C. botulinum* has never been established in any food poisoning outbreak or in any food may be due to a lack of suitable detection methods (Tracanna *et al*., 2017). The use of a fluorescent-antibody technique and an enzyme-linked immunosorbent assay (ELISA) to detect and identify this toxin type in feacal specimens and enrichment cultures has been described previously (Lincke *et al*., 2010; Pidot *et al*., 2014; Schieferdecker *et al*., 2019).

Clostridium spp., ubiquitously present in extreme, complex and dynamic environments such as soil and human and animal gut have received limited attention in bioactive compound discovery. Conceivably, their secondary metabolism has been evolved giving rise to various adaptative mechanisms to survive and proliferate in challenging environments (Lincke *et al*., 2010). Therefore, the synthesis of bioactive secondary metabolites, including antimicrobials, may assist the survival of *Clostridium* spp. in challenging and stressed environments. Pathogenicity of this genus has received more interest, even though most of *Clostridium* spp. are saprophytes and not involved in a disease process (Liu, 2011). Recent genome mining studies have identified the genetic potential of *Clostridium* spp. for antimicrobial biosynthesis (Tracanna *et al*., 2017). Analysis of publicly available genomes revealed the presence of biosynthesis gene clusters (BGCs) tentatively encoding for non-ribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) in the members of the genus *Clostridium* (Letzel *et al*., 2013). This emphasizes the prospect of novel antimicrobial discovery from soil *Clostridium* species.

The global spread of antimicrobial resistant bacteria and the emerging consumer trend for natural food preservatives emphasized the need of exploring novel and natural antimicrobial compounds (D'Andrea *et al*., 2019). In this context, microbial diversity in many environments has been a great interest to explore new antimicrobials (Mullis *et al*., 2019). It is therefore, necessary to undertake a study for the indentification of solvent producing clostridia from soil environment within Benin metropolis, Nigeria.

1.2 Aim and Objectives

The aim of this research was to determine the antibacterial potential of crude metabolic extracts from *Clostridium* spp. isolated from cattle yards in Benin City, Nigeria against clinically relevant bacterial.

The specific research objectives were to:

i. determine the physicochemical characteristics of the soil samples obtained from the selected cattle yards
ii. Isolate, enumerate, and identify the *Clostridium* spp. using biochemical and molecular methods.
iii. determine the phenotypic virulence properties of the clinical bacterial isolates used in the study
iv. determine the antibacterial activities of the crude metabolic extracts of *Clostridium* spp. against the clinical bacterial isolates
v. determine the antibacterial sensitivity pattern of the clinical bacterial isolates

CHAPTER TWO

LITERATURE REVIEW

2.1 The Soil

Soil is the mixture of minerals organic matter, gases, liquids and a myriad of organisms that can support plant life. It is a natural body that exists as part of the pedosphere and it performs four important functions: it is a medium for plant growth; it is a means of water storage, supply and purification; it is a modifier of the atmosphere; and it is a habitat for organisms that take part in decomposition and creation of a habitat for other organisms (Kekane *et al*., 2015).

Soil is considered the "skin of the earth" with interlaces between the lithosphere, hydrosphere, atmosphere and biosphere (Chesworth, 2008). Soil consists of a solid phase (minerals and organic matter) as well as a porous phase that holds gases and water (James, 2006; Voroney, 2006). Accordingly, soils are often treated as a three-state system (James, 2006). Soil is the end product of the influence of the climate, relief (elevation, orientation, and slope of terrain), biotic activities (organisms), and parent materials (original minerals) interacting over time (Gilluly, 1975). Soil continually undergoes development by way of numerous physical, chemical and biological processes, which include weathering with associated erosion.

Most soils have a density between I and 2 g/cm^3 (pedosphere.com). Little of the soil of planet Earth is older than the Pleistocene and none is older than the Cenozoic, (Boul *et al.*, 1973) although fossilized soils are preserved from as far back as the Archean (Retallack, 2008).

Of these, bacteria and fungi play key roles in maintaining a healthy soil. They act as decomposers that break down organic materials to produce detritus and other breakdown products. Soil detritivores, like earthworms, ingest detritus and decompose it. Saprotrophs well represented by fungi and bacteria, extract soluble nutrients from delitro. The ants (macrofaunas) help by breaking down in the same way but they also provide the motion part

as they move in their armies. Also the rodents, wood-caters help the soil to the more absorbent

2.2 Characteristics Of Soil

Soils vary widely with regard to geology, hydrology, climate, fertility and other physical attributes. The most important physical and chemical property of the soil is determined by the composition of soil, organic matter and fraction of soil (Owabor and Ogunbor, 2007).

The physical properties of soils, in order of decreasing importance, are texture, structure. density, porosity, consistency, temperature, colour and resistivity. Most of these determine the aeration of the soil and the ability of water to infiltrate and to be held in the soil. Soil texture is determined by the relative proportion of the three kinds of soil particles, called soil separates: sand, silt, and clay. Larger soil structures called 'peds are created from the separates when iron oxides, carbonates, clay, and silica with the organic constituent humus, coat particles and cause them to adhere into larger, relatively stable secondary structures. Soil density, particularly bulk density, is a measure of soil compaction. Soil porosity consists of the part of the soil volume occupied by gases and water. Soil consistency is the ability of soil to stick together. Soil temperature and colour are self-defining, lesistivi1y refers to the resistance to conduction of electric currents and affects the rate of corrosion of metal and concrete structures. The properties may vary through the depth of a soil profile.

Raj and Bhagan (2013) analysed the fluoride concentration and some other important physicochemical parameters of 51 surface soil samples and 51 underground water samples of ten fluorotic areas of Agastheeswaram Union, South India. In all the fluorotic areas the surface soil samples were having fluoride levels greater than the underground water samples. The fluoride concentration in the soil was ranging between 2 to 3.5 ppm and in the water samples it was ranging between 1.3 to 2.7 ppm. Both the levels were found to be above the permissible limit. Other parameters such as pH, alkalinity, total hardness, calcium,

magnesium, chloride, salinity and sodium were also measured. Alkalinity and pH were found to be higher than the permissible limit in all the soil and water samples at various seasons. Finally, it was predicted that leaching of minerals from the soil is responsible for the high fluoride content in water samples and this in turn is responsible for the prevalence of fluorosis in the study area.

Saroj and Dilip (2014) carried out work on the study of physicochemical parameters like pH, specific conductivity, chloride, total alkalinity, calcium, magnesium nitrate, sulphate, phosphate sodium and potassium from July, 2008 to June, 2009. During the study year fluctuation was observed in several parameters. Investigation results showed that the soli alkaline throughout the study year. The productivity of an ecosystem depends upon the quality of soil. Some parameters were above permissible limit and some below the permissible limit which affects the quality and productivity of pond soil.

Osakwe (2014) studied that the physicochemical properties of soils from natural flood disaster affected areas of the Isoko Region of Delta State, Nigeria, were investigated. The results indicated that there was an overall reduction in soil pH (5.425± 0.313), phosphorus (7.47±6.34 mgkg-1), and nitrate (0.34±0.07 mgkg-1) contents as well as exchangeable calcium 1.97±0.31 mgkg-1 potassium (0.09±0.01 mgkg-1), and effective cation exchange capacity (5.076±1.532 (cmolkg-1) and related parameters with 3.87±0.21, 77.57±5.83 and 7.99±2.72 for Base Exchange Capacity, Base Saturation and Soil Buffering capacity respectively. There was, however increased in the values of exchangeable magnesium (1.50±0.25 mgkg-1), exchangeable sodium (0.28±0.004 mgkg-1) and the exchangeable acidity with the values 0.43+0.08 and 0.42±1.02 mgkg-1 for hydrogen and aluminium respectively. There was no appreciable change in the values of Total Organic Carbon (0.40±0.096%), Total Nitrogen (0.025±0.035%) and Sulphate (0.10±0.02 mgkg-1). The overall results indicate that the flood increased soil acidity and decreased the ability of the

soils to adsorb metals, but did not have an appreciable effect on the biodegradable and compostable materials. Government should be proactive and devise measures to prevent further flood disaster in the country.

2.2.1 Texture

The mineral components of soil are sand, silt and clay, and their relative proportions determine a soils texture. Properties that are influenced by soil texture, include porosity, permeability, infiltration, shrink-swell, water-holding capacity, and susceptibility to erosion. In the illustrated USDA textural classification triangle, the only soil in which neither sand, silt nor clay predominates is called "loam'. While even pure sand, silt or clay may be considered a soil, from the perspective of food production a loam soil with a small amount of organic material is considered ideal. The mineral constituents of a loam soil might be 40% sand. 40% silt and the balance 20% clay by weight. Soil texture affects soil behaviour, in particular its retention capacity for nutrients and water (Brown, 2003).

Sand is the most stable of the mineral components of soil; it consists of rock fragments, primarily quartz particles, ranging in size from 2.0 to 0.05 mm (0.0787 to 0.0020 in) in diameter. Silt ranges in size from 0.05 to 0.002 mm (0.002 to 0.00008 in). Clay cannot be resolved by optical microscopes as its particles are 0.002 mm (7.9x 10 in) or less in diameter (Kellogg, 1957; Soil, 1957).. In medium-textured soils, clay is often washed downward through the soil profile and accumulates in the subsoil.

2.2.2 Structure

The clumping of the soil textural components of sand, silt and clay forms aggregates and te further association of those aggregates into larger units forms soil structures called peds. The adhesion of the soil textural components by organic substances, iron oxides, carbonates, clays, and silica, and the breakage of those aggregates due to expansion-contraction, and wetting-drying cycles, shape soil into distinct geometric forms. These peds evolve into units which

may have various shapes, sizes and degrees of development. A soil clod, however, is not a ped but rather a mass of soil that results from mechanical disturbance. The soil structure affects aeration, water movement, conduction of hear, plant root growth and resistance to erosion. Water has the strongest effect on soil structure due to its solution and precipitation of minerals and its effect on plant growth.

Soil structure often gives cities to its texture, organic matter content, biological activity, past soil evolution, human use, and the chemical and mineralogical conditions under which the soil formed. While texture is defined by the mineral component of a soil and is an innate property of the soil that does not change with agricultural activities, soil structure can be improved or destroyed by the choice and timing of farming practices.

2.2.3 Density

Density is the weight per unit volume of an object. Particle density is equal to the mass of solid particles divided by the volume of solid particles - it is the density of only the mineral particles that make up a soil i.e. it excludes pore space and organic material. Soil particle density is typically 2.60 to 2.75 grams per cm and is usually unchanging for a given soil. Soil particle density is lower for soils with high organic matter content, and is higher for soils with high Fe-oxides content. Soil bulk density is equal to the dry mass of the soil divided by the volume of the sol i.e. It includes air space and organic materials of the soil volume. A high bulk density is indicative of either soil compaction or high sand content. The bulk density of cultivated loam is about 1:1 to 1.4 g/cm^3 (for comparison water is 1.0 g/cm) (Woldeyohannis *et al*., 2022). Soil bulk density is highly variable for a given soil. A lower hulk density by itself does not indicate suitability for plant growth due to the influence of soil texture and structure. Soil bulk density is inherently always less than the soil particle density

2.2.4 Porosity

Pore space: is that part of the hulk volume that is not occupied by either mineral or organic matter but is open space occupied by either gases or water. Ideally, the total pore space should he 50% of the soil volume. The gas space is needed to supply oxygen to organisms decomposing organic matter, humus, and plant roots. Pore space also allows the movement and storage of water and dissolved nutrients. This property of soils effectively compartmentalizes the soil pore space such that many organisms are not in direct competition with one another, which may explain not only the large number of species present, but the fact that functionally redundant organisms (organisms with the same ecological niche) can co-exist within the same soil (Donahue *et al*., 2017).

2.2.5 Oxygen

Oxygen is another important parameter because it determines the bacterial pattern of dissimilatory and energy yielding process. Microbial utilization of aliphatic, cyclic and aromatic hydrocarbons by bacteria and fungi required electron sink. In the initial attack molecular oxygen used as electron sink. In the subsequent steps too, oxygen is the most common electron sink. In the absence of molecular oxygen, further biodegradation of partially oxygenated intermediates may be supported by nitrate or sulphate reduction. Little or no hydrocarbon metabolism occurs in strictly anoxic sediments (Dibble and Bartha, 1976).

2.2.6 Temperature

Soil temperature depends on the ratio of the energy absorbed to that lost. Soil has a temperature range between -20 to 60°C. Soil temperature regulates seed germination, plant and root growth and the availability of nutrients. Below 50 cm (20 in). soil temperature seldom changes and can be approximated by adding 1.8°C (2°F) to the mean annual air temperature. Soil temperature has important seasonal monthly and daily variations. Fluctuations in soil temperature are much lower with increasing soil depth. 1-leavy mulching

(a type of soil cover) can slow the warming of soil, and, at the same time, reduce fluctuations in surface temperature (Hillel, 1998)

2.2.7 Soil moisture

Soil moisture is another important parameter in determining the rate of biodegradation of petroleum compounds. Microbes live in the interstitial water of soil pores and the lower amount of water, the smaller the number of microbes and thus, slow removal rate through biodegradation (Dibble and Bartha, 1976).

2.2.8 Acidity or alkalinity

The acidity (pH) of the soil is an important soil parameter. Soil pH can be highly variable, ranging from 2.5 in mine spoils to 11 in alkaline deserts. Most heterotrophic bacteria favour a pH 7.0 but fungi being more tolerant to acidic conditions. Therefore, extremes pH of soils would be have a negative influence on the ability of microbial populations to degrade hydrocarbons. (Verstraete *et al.*, 1975) reported that a doubling rate of biodegradation of gasoline in an acidic (pH 4.5) soil by adjusting the pH to 7.4. Rates dropped significantly, however, when the pH was further raised to 8.5. Similarly, Dibble and Bartha (1976) observed an optimal pH of 7.8, in the range 5.0 to 7.8 for the mineralization of oily sludge in soil.

2.2.9 Colour

Soil colour is often the first impression one has when viewing soil. Striking colours and contrasting patterns are especially noticeable. In general, colour is determined by the organic matter content drainage conditions, and degree of oxidation. Soil color, while easily discerned, has little use in predicting soil characteristics. it is of' use in distinguishing boundaries within a soil profile determining the origin of a soils parent material, as an indication ol' wetness and waterlogged conditions, and as a qualitative means of measuring organic, salt and carbonate contents of soils (Shields *et, al.,* 1968)

Soil colour is primarily influenced by soil mineralogy. Many soil colours are due to various iron minerals. The development and distribution of colour in a soil profile result from chemical and biological weathering, especially redox reactions. As the primary minerals in soil parent material weather, the elements combine into new and colourful compounds. Iron forms secondary minerals of a yellow or red colour, organic matter decomposes into black and brown compounds, and manganese, sulfur and nitrogen can form black mineral deposits. These pigments can produce various colour patterns within a soil Aerobic conditions produce uniform or gradual colour changes, while reducing environments (anaerobic) result in rapid colour flow with complex. mottled patterns and points of colour concentration.

2.2.10 Resistivity

Soil resistivity is a measure of a soils ability to retard the conduction of an electric current. The he electrical resistivity of soil can affect the rate of' galvanic corrosion of metallic structures in contact with the soil. Higher moisture content or increased electrolyte concentration can lower resistivity and increase conductivity, thereby increasing the rate of corrosion (Donahue *et al.,* 1977). Soil resistivity values typically range from about 2 to 1000 fm. but more extreme values are not unusual (Edwards, 1998).

2.2.11 Electrical conductivity

Electrical conductivity is also a very important property of the soil, it is used to check the quality of the soil. It is a measure of ions present in solution (Tale and Ingole, 2015). The electrical conductivity of a soil solution increases with the increased concentration of ions. Electrical conductivity is a very quick, simple and inexpensive method to check health of soils. It is a measure of ions present in solution. The electrical conductivity of a soil solution increases with the increased concentration of ions.

2.2.12 Nitrogen

Nitrogen is the most critical element obtained by plants from the soil and is a bottleneck in plant growth (Solanki and Chavda, 2012). About 80% of the atmosphere is nitrogen gas. Nitrogen gas diffuses into water where it can be “fixed” (converted) by blue-green algae to ammonia for algal use. Nitrogen can also enter lakes and streams as inorganic nitrogen and ammonia. Because nitrogen can enter aquatic systems in many forms, there is an abundant supply of available nitrogen in these systems.

2.2.13 Phosphorus

Phosphorus is a most important element present in every living cell (Tale and Ingole, 2015). It is one of the most important micronutrient essential for plant growth. Phosphorus most often limits nutrients remains present in plant nuclei and act as an energy storage

2.2.14 Potassium

Potassium plays an important role in different physiological processes of plants, it is one of the important element for the development of the plant (Solanki and Chavda, 2012). It is involved in many plant metabolism reactions, ranging from lignin and cellulose used for the formation of cellular structural components, for regulation of photosynthesis and production of plant sugars that are used for various plant metabolic needs.

2.2.15 Soil organic matter

It is also a valuable property of soil. If the soil is poor in organic matter, then it enhances the process of soil erosion (Tale and Ingole, 2015). If the soil organic matter is present in soil, then this soil is useful for the agricultural practices. Organic matter may be added in the soil in the form of animal manures, compost, etc. The presence of the higher content of organic matter in the soil can be another possible reason for lowering of the pH. Soil organic matter content has decreased from surface to subsoil due to levelling.

2.3 Antibacterial Potential of Bacteria Isolated from Soil Samples

Antibiotic resistance in bacterial strains is taking root throughout the population and poses a serious public health challenge, which greatly demands pursuing new antibiotics or investigating new antimicrobial compounds (Cars *et al*., 2008). During the last few decades, much work has been documented, including the research on the production of new antibiotics from diverse strains of microorganisms and plants (Luepke *et al*., 2017). These microbial species and their population size depend on environmental factors such as soil texture, nutrient availability, moisture, and flora-covered soil (Laxminarayan and Chaudhury, 2016). Bacteria produce and use antibiotics in their natural habitats as protective substances to destroy the invasion of other bacterial species. The function of these antibiotics is not only protective but they also play a vital role as signaling molecules to communicate among the cells in the bacterial population (Gebreyohannes *et al*., 2013).

It is presumed from the past that natural products play a vital role in antibiotic discovery and their development (Marshall and Levy, 2011). There is a dire need to explore novel antimicrobial compounds with significant potential to kill or control a wide range of microorganisms. Antibiotics are one of the essential pillars of present-time medications. Still, unfortunately, the commonly used antibiotics lose their efficacy against several pathogenic strains and there is demand to replace old antibiotics with new ones (Santajit and Indrawattana, 2016; Bull and Stach, 2007). Microorganisms, having the ability to produce bioactive secondary metabolites, show distinct structures and biological activities. Some species of soil microflora also produce these bioactive metabolites, which are used as antibiotics (Laxminarayan and Chaudhury, 2016). Several other significant types of research have also been reported to isolate bacteria from the soil with novel antimicrobial agents (Pavunraj *et al*., 2017; Yunus *et al*., 2016).

Public health professionals recently had great difficulty dealing with multidrugresistant bacterial pathogens; therefore, MDR infections are more dangerous than nonresistant bacterial pathogens. Particularly, the frequency of resistance developed in bacterial pathogens acts as a secondary infection in various life-threatening conditions such as cancer, surgical procedures, transplantation, etc., and influences the treatment impact of modern medications (Nike *et al*., 2013). As the development of MDR strains is quite rapid, it is obvious that very limited therapeutic agents are available to treat these pathogens effectively (Dezfully and Ramanayaka, 2015).

Sadiqi *et al*. (2022) reported that *Brevibacillus formosus, Bacillus subtilis* and *Paenibacillus dendritiformis* isolated from soil samples posses antibacterial activities against multidrug-resistant strains (MDRS) such as *Acinetobacter baumannii* (ATCC 19606), Methicillin-resistant *Staphylococcus aureus* (MRSA) (BAA-1683), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (BAA-2108), *Staphylococcus aureus* (ATCC 292013), *Escherichia coli* (ATCC25922) and *Salmonella typhi* (ATCC 14028)

2.4 Sporulation Regulation in Solventogenic Clostridia

In response to changes in the environment, some bacteria produce spores to survive under unfavorable conditions. Depending on the formation mechanism and the structure, different spore types can be found in the environment (Paul *et al*. 2019). The spores, formed by Firmicutes, called endospores (Dürre 2014; Johnson 2019), are the most resilient. Endospores can survive harsh treatments such as high temperatures, the presence of oxygen (for anaerobic bacteria), desiccation, lysozyme incubation, ionizing radiation, and chemical solvents. The most studied sporulating bacteria belong to the *Bacillus* and *Clostridium* genera. The sporulation process was first described in Bacillus (Dawes and Mandelstam 1970), the model organism among spore formers, and the main features of its sporulation process are conserved in the *Clostridium* genus. Still, substantial differences in the spore morphology and

sporulation initiation have been demonstrated between the two genera and within the *Clostridium* genus (Al-Hinai *et al*., 2015; Dürre, 2014).

A sporulation model for the solventogenic clostridia (AlHinai *et al.,* 2015) has been developed thanks to studies in *C. acetobutylicum* ATCC 824. Few studies were done on sporulation in other solventogenic clostridia strains to confirm the universality of this model. Although solventogenic clostridia are often presented as a homogenous group of bacteria, based on the first phylogenic studies on the *Clostridium* genus, this is not the case. Several strains were renamed and reclassified since 2000, and recent phylogenic studies (CruzMorales *et al*., 2019; Yu *et al.,* 2019) show that *C. beijerinckii* and *C. acetobutylicum* even belong to two different clades. Out of the seventeen clades dividing the *Clostridium* genus "sensu stricto," solventogenic clostridia can be found in two groups, one harboring *C. acetobutylicum* and *C. pasteurianum* and another consisting of *C. beijerinckii, C. saccharoperbutylacetonicum,* and *C. saccharobutylicum*. Phylogenetically, *C. beijerinckii* is closer to the human pathogens *C. perfringens* and *C. botulinum* E than to the model solventogenic clostridia *C. acetobutylicum*. In line with what has been suggested for toxin genes (CruzMorales *et al*., 2019), solventogenesis genes might have been acquired by horizontal transfer. The localization of the sol operon, harboring the essential solvent genes, on a megaplasmid in *C. acetobutylicum* contrasting with the chromosomal sol operon in other clostridia supports this hypothesis. Thus, the regulation mechanisms described for *C. acetobutylicum* might not be identical in *C. beijerinckii* or other solventogenic species (Patakova *et al.,* 2013).

2.5 The Clostridial Endospore

At the end of sporulation, an endospore is released into the environment. The endospore is highly dehydrated and organized in proteinous layers protecting the core, which hosts the DNA. Five layers surround the core: the inner membrane, the germ cell wall, the cortex, the

outer membrane, and the coat. These layers ensure a robust protection of the core against chemicals, oxygen, enzymes, and heat. In the core, the DNA is bound to small acid-soluble proteins (Sasps), ribosomes, enzymes, and DPA. The DPA content can reach up to 25% of the spore's dry weight (Paredes-Sabja *et al.,* 2014)

Spores are characterized by their size, shape, and location in cells (Dürre, 2014). There is not a typical morphology for all clostridial spores, and few studies have been done to compare the spore morphology of clostridial strains. One study (Berezina *et al.,* 2012) compared the spore morphology of the four main solventogenic species (*C. acetobutylicum, C. beijerinckii, C. saccharobutylicum* and *C. saccharoperbutylacetonicum*) and showed that they all possess an oval shape. This characteristic cannot be generalized to all solventogenic clostridia since C. tetanomorphum yields round-shape spores (Patakova *et al*., 2014). The spore's location may vary within the mother cell (Fig. 5a, b) from eccentric to terminal (Dürre, 2005)

2.6 Solvent Producing *Clostridium* spp.

Solventogenic clostridia are currently investigated for their potential to produce solvents from complex feedstock such a lignocellulosic and algal feedstocks. Pretreatment of these feedstocks is necessary to the utilization by the bacteria of the carbohydrates present. During the pretreatment, di- and monosaccharides, as well as inhibitory chemicals (salts, furfurans and phenolic compounds), are formed. Studies have been conducted to evaluate the impact of these toxic compounds on cell growth and solvent formation. Hence, few reports on their effect on sporulation can be found; still, three transcriptomic studies of *C. beijerinckii* and *C. acetobutylicum* cultures exposed to phenolic compounds detected changes in the expression of sporulation genes. Exposure to ferulic acid (Lee *et al*., 2015) and syringaldehyde (Ezeji *et al*., 2007) caused an upregulation of the late-stage sporulation genes *C. beijerinckii.* In *C. acetobutylicum*, a recent study showed through a gene coexpression network analysis (Liu *et*

al., 2020) that exposure to vanillin and p-coumaric acid disturbed the transcription of early sporulation genes (spo0A, spoIIE, spoIIP) and sporulation specific sigma factors.

Acids, various metals and minerals, vitamins, and amino acids also affect both solvent production and sporulation in clostridia (Reeve and Reid, 2016; List *et al.,* 2019; Mukherjee *et al.,* 2019; Nimbalkar *et al.,* 2019), but few studies mentioned their impact on sporulation. Mukherjee *et al.* (2019) investigated butyrate and acetate's effect on sporulation in *C. saccharobutylicum* by adding them to the medium at the start of the fermentation in different concentrations. Even though the addition of acids was not necessary for sporulation, it increased the number of spores present in the culture by 40 to 100% for concentrations between 1 and 4 g L^{-1}.

As for the impact of other media components on sporulation, one study reports that the addition of adenine in the media caused a 20-h delay in the onset of sporulation in *C. saccharoperbutylacetonicum* cultures (Kiyoshi *et al.,* 2017). It is worth noting that, depending on the species, a compound can have an opposite impact on sporulation. For example, in *C. perfringens*, iron is necessary for sporulation (Lee *et al.,* 1978), while its addition to the medium impairs sporulation in *C. sporogenes* (Mah *et al.,* 2008).

Solventogenic clostridia harbor sporulation proteins requiring metal-containing cofactors; thus, the media's metal content is expected to impact sporulation regulation. For instance, homologs of SpoIIE, SpoIIQ and CsfB, an anti-sigma factor of σE and σG, were identified in *Clostridium*. In *Bacillus*, their activity requires Mn^{2+} and Zn^{2+} respectively (Król *et al.,* 2017; Martínez-Lumbreras *et al.,* 2018). In *Bacillus*, Mn^{2+} was proven to be crucial for SpoIIE's phosphatase activity and the oligomerization of SpoIIE, and thus, asymmetric division (Król *et al*., 2017). Mn2+ has also been reported to be key for the development of heat-resistant spores in *C. botulinum* (Lenz and Vogel 2014). In *C. difficile*, Zn^{2+} is necessary for the formation of the SpoIIQ-SpoIIIAH complex, involved in engulfment and essential for the

transit of molecules between mother cell and forespore (Serrano *et al.,* 2016). Zn^{2+} was reported to promote sporulation in C. botulinum (Kihm et al. 1988) but to inhibit sporulation of C. sporogenes when the concentration in the medium exceeds 3.7 mM (Lee *et al.,* 2011). Ca^{2+} is also involved in spore formation since it forms together with dipicolinic acid (DPA), and several studies have shown that Ca2+is a crucial component for spore heat resistance (Jamroskovic *et al.,* 2016).

Studies of the transcriptome of wild-type and mutant *C. beijerinckii* cultures during fermentation indicated changes in the expression of genes involved in ion- and amino acid transport at sporulation initiation. In *C. beijerinckii* NRRL B598, sporulation initiation was concomitant with an upregulation of the genes encoding a magnesium transporter, and an upregulation of genes encoding potassium, sodium, and iron transporters was detected during stationary phase (Vasylkivska *et al.,* 2019). In cultures of the asporogenous *C. beijerinckii* ΔspoIIE strain, the expression of genes encoding iron transporters were downregulated during stationary phase, indicating a potential role of iron in sporulation (Diallo *et al.,* 2020).

2.7 Metabolite Concentration

Metabolite stress has been suggested to trigger sporulation in solventogenic clostridia (Heluane *et al.,* 2011). When grown in batch reactors, solventogenic clostridia ferment the available carbohydrates into carboxylic acids, mainly acetate and butyrate, which accumulate in the culture and cause a drop in pH.

At the onset of solventogenesis and sporulation, acids are reassimilated and converted into solvents, resulting in a rise of pH in the culture. While solvent formation enables short-term relief from the pH stress, sporulation is regarded as a long-term stress response mechanism, protecting the cells from metabolic stress and interrupting sugar degradation (Sauer *et al*., 1995). Acetate and butyrate accumulation during exponential growth is proposed to trigger both solventogenesis and sporulation (Thorn *et al*., 2013). In *C. acetobutylicum*, a peak in the

intracellular undissociated acid concentration was observed at the start of the solventogenesis (Yang *et al*., 2013). Characterization of *C. acetobutylicum* recombinants deficient in phosphotransbutyrylase (Ptb), butyrate kinase (Buk) and acetate kinase (Ack) (Kuit *et al*., 2012) suggested that instead of the concentration of undissociated acids, the intracellular concentration of butyryl phosphate (BuP) might trigger both sporulation and solventogenesis. BuP is an intermediary metabolite in the ABE metabolic pathway (Fig. 6), formed during acidogenesis during butyrate formation. Studies evaluating the intracellular concentration of BuP during batch cultivation showed that BuP was indeed accumulated in the cell (Xu *et al.,* 2018). Two peaks in the cytoplasmic BuP concentration were detected inside the cells, one at the beginning of the cultivation and a second one coinciding with solventogenesis and sporulation initiation (Zhao *et al*., 2005). It was suggested that BuP acted as a phosphate donor enabling the activation of Spo0A, the master regulator of sporulation and solventogenesis (Zhao *et al*., 2005; Kuit, 2013), but data (Xu *et al*., 2018) present another post-translational regulation mechanism: protein butyrylation.

Butanol has also been suspected of triggering sporulation (Zheng *et al.,* 2009). Even though solventogenic clostridia naturally produce butanol, it affects cell growth when its concentration exceeds 0.5% v/v in the culture (Sedlar *et al.,* 2018) and becomes lethal, around 1.5% v/v (Sedlar *et al.,* 2019). Butanol concentration being a stress factor for the cells, researchers supposed that a rise in butanol concentration would initiate sporulation before it reaches toxic concentration. However, a decrease in granulose and spore number was observed in butanol stressed *C. beijerinckii* cultures (Sedlar *et al.,* 2019). Transcriptional studies on butanol stressed cultures of *C. acetobutylicum* and *C. beijerinckii* showed no notable changes in the expression of the genes encoding the sporulation-specific sigma factors (sigF, sigE, sigG, sigK) (Tomas et al. 2004; Patakova et al. 2019). In *C. acetobutylicum* ATCC 824, no butanol-dependent impact on sporulation efficiency was

described. Instead, a decrease in the expression of genes encoding small acid-soluble proteins was observed (Tomas *et al.,* 2004; Schwarz *et al.* 2012). These proteins protect the DNA present in the spores and are crucial for their heat resistance (Leggett *et al*., 2012).

Secondary metabolites have also been reported to promote sporulation in solventogenic clostridia. Two categories of secondary metabolite biosynthesis gene clusters were identified in solventogenic clostridia (Letzel *et al.,* 2013), polyketide- and ranthipeptide biosynthesis clusters. Polyketides have been studied in *C. acetobutylicum* and *C. saccharoperbutylacetonicum*. In *C. saccharoperbutylacetonicum*, polyketides involved in sporulation initiation, solvent formation, and tolerance were detected (Kosaka *et al.,* 2007; Li *et al*., 2020). In *C. acetobutylicum*, three polyketides were detected, and the structures of two of them, clostrienose and clostrienoic acid, were solved (Herman *et al.,* 2017). In both species, the disruption of polyketide clusters decreased sporulation. In *C. beijerinckii*, polyketides might also intervene in the regulation of sporulation; the interruption of sporulation in *C. beijerinckii* affected the expression of the polyketide gene cluster (Diallo *et al.,* 2020). Recently, the role of ranthipeptides, secondary metabolites belonging to the ribosomally synthesized and post-translationally modified peptide (RiPP) superfamily, was studied in *C. beijerinckii* and *C. ljungdahlii* (Chen *et al*., 2020). In *C. beijerinckii*, the genes encoding the precursor peptide and the radical SAM protein were disrupted, and the impact on the transcriptome was evaluated by RNA sequencing. In the mutant strain, sporulation genes were strongly downregulated and the agr locus encoding the Agr quorum sensing mechanism was upregulated. Secondary metabolites seem to play an important role in the initiation of sporulation, even so the interactions between the polyketides and ranthipeptides with sporulation regulators remain to be investigated.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study location were Aduwawa 1, Aduwawa 2, Dumez and Eyaen as illustrated in figure 3.1. Aduwawa is a prominent and densely populated suburban area located in Benin City, the capital of Edo State, Nigeria. Positioned along the ever-busy Benin–Auchi Expressway, Aduwawa serves as both a residential and commercial hub within the eastern corridor of Benin City. Its strategic location makes it a gateway between Benin City and the northern parts of Edo State, such as Ekpoma, Auchi, and Etsako areas. Street markets and small retail shops are widespread, with traders dealing in foodstuffs, clothing, building materials, electronics, and household goods. It also houses majority of the cattle rank in Benin City, Nigeria.

Dumez Road is a notable area, situated within Benin City, the capital of Edo State, Nigeria. Strategically located off Sapele Road, it serves as a vital residential and commercial hub, reflecting the city's dynamic urban landscape. Dumez Road lies within the Ikpoba-Okha Local Government Area, a region known for its diverse communities and economic activities. Small trade activities include foodstuffs, clothing, building materials, abattoirs, electronics, and household goods.

Eyaen is part of the Uhunmwonde Local Government Area, but is often associated with the greater Benin metropolis due to its proximity to the city. The local economy in Eyaen is driven by a combination of agriculture, commerce, and services. Traditional markets like Eyaen Market offer local produce, food items, and other goods. In recent years, the area has experienced a rise in small-scale businesses, real estate development, and hospitality services. Its location along a major highway makes it accessible and attractive for investment, especially in the transport and retail sectors.

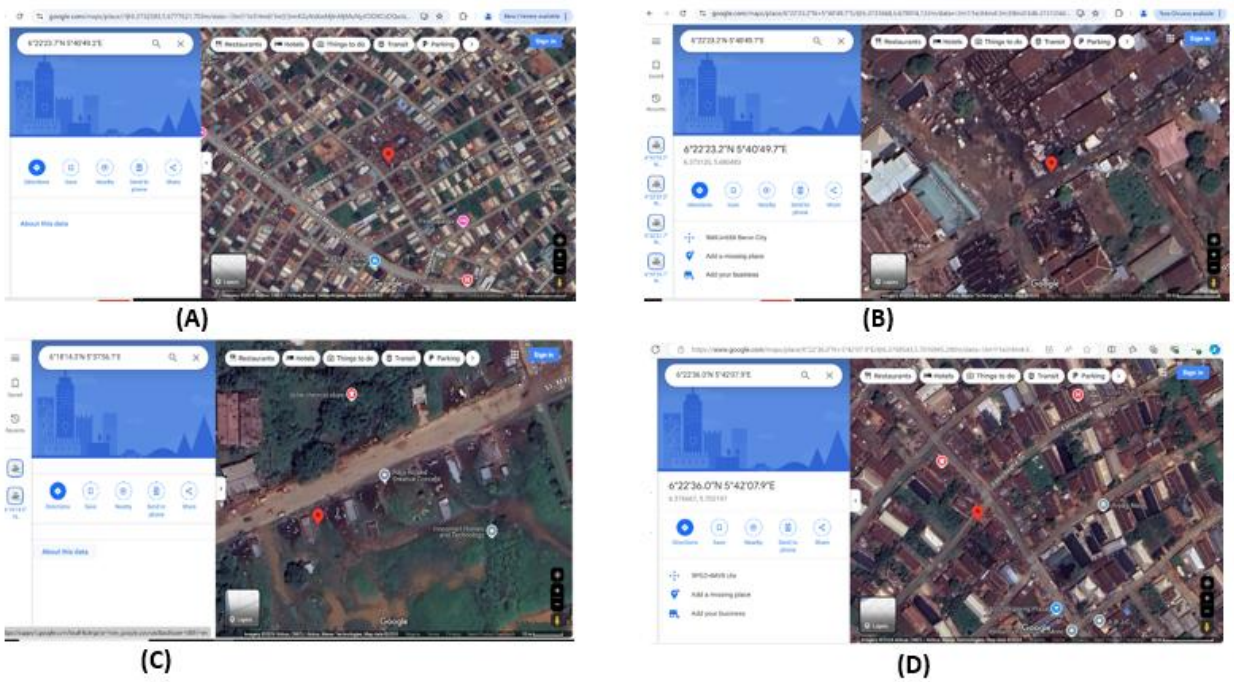

Figure 3.1: Satellite imagery of sample locations

Keys: (A) = Aduwawa 1, (B) Aduwawa 2, (C) Dumez, (D) Eyaen

3.2 Sample Collection and Preparation

Soil samples were collected in triplicates each from cattle farmlands in Eyaen, Aduwawa 1, Aduwawa 2 and Dumez in Benin City, Nigeria. Samples were collected at 0-5 cm depth using soil Auger and transferred into cellophane bags, tightly sealed with minimal air space and labelled with carbon free paper outside and stored in a cool place to prevent breaking down of organic matter. Upon collecting, the samples were transferred to a laboratory for analysis. Samples were air dried for 48 hrs, and then sieved with 2 mm mesh to remove debris, gravel and other materials prior to analysis. Clinical isolates used for the analysis were collected from Medical Microbiology Laboratory, University of Benin Teaching Hospital (UBTH), Benin City. Viable isolates were thereafter resuscitated for further analysis.

3.3 Physicochemical Analysis of the Soil Samples

The physicochemical qualities of the soil samples were determined using the methods of FEPA (1991) and the Association of Official Analytical Chemists (AOAC, 2000). The parameters determined were:

3.3.1 pH: This were determined using pH meter 3015 (Jenway, U. K.). Ten grams of the soil samples were placed in a beaker, then 10 ml of distilled water were added, and the mixture were stirred. It was allowed to stand for 30 mins. A 0.1 M phosphate buffer solution

was used to standardize the pH meter at 7.0. Then, the electrode of the pH meter was inserted into the mixture, and the pH readings were taken (FEPA, 1991) and recorded.

3.3.2 Temperature determination

The temperature was determined at the point of sample collection by dipping the bulb of mercury-in-glass thermometer into the soil suspension. As the mercury absorbs heat from the soil suspension, it expands and rises within the thermometer's narrow tube. The temperature is then read from the scale marked on the glass (FEPA, 1991)

3.3.3 Conductivity (µ/Scm)

Conductivity of the soil was determined using the standard procedure approved by AOAC (2000). The conductivity meter Thermo Fisher Scientific model Oriion Star A212 was used. The conductivity meter was powered on, and the conductivity function was activated, the instrument was then temperature-adjusted and calibrated using 0.001 M KCl solution, which produced a reading of 14.7 mS/m at 25 °C. The probe was dipped below the surface of soil samples. Time was allowed for the reading to be stabilised and the reading were recorded (FEPA, 1991, AOAC, 2000).

3.3.4 Available phosphorus (Bray-1 method)

Soil sample weighing 5 g was put into extractions cup and 30 mL of Bray-1 solution was added and stirred in a mechanical shaker for 5 mins. It was then filtered into a reagent bottle and 1 mL of extract (aliquot) was pipetted into a 50 mL volumetric flask, 6 mL of distilled water was added with 2 mL of colour developing reagent and mixed well. Subsequently, 1 mL of ascorbic acid solution was also added. It was left for 10 mins for the colour to develop. The solution was measured at 650 nm in visible-range spectrophotometer (Olsen, 1982). A graph of absorbance versus of phosphorus was read and recorded in parts per million (ppm) using the standard curve to calculate.

3.3.5 Nitrate (mg/kg)

This was determined by the colorimetric method using phenolsulphonic acid. A 5kg of the sample was weighed in a porcelain dish and evaporated at 108oC a Gallenkamp water bath. The dry residue was then dissolved by adding 2 ml of phenolsulphonic acid, followed by 20 ml of distilled water and 7 ml of concentrated ammonia solution until a maximum yellow colour developed. The absorbance was then read at a wave length of 410 nm using the Milton Roy spectronic 21D spectrophotometer. The cuvette with the sample solution was inserted into the spectrophotometer's sample holder. The instrument passed light of 410 nm wavelength through the sample and measured how much light was absorbed. The resulting absorbance value was displayed on the spectrophotometer screen.

3.3.6 Calcium

Two (2) grams of the soil sample was treated by 0.1 2,2-Dimethylvaleroyl chloride (NHCl); the volume of CO_2 from pure calcium carbonate and samples were recorded manually on a sheet of paper. Then, the percent calcium was calculated according to Horváth *et al*. (2005).

3.3.7 Potasium (K)

A solutions of 100 ppm was prepared from 1000 ppm stock solution potasiun chloride (KCl) by taking out 10 ml from stock solution and diluting it up to 100 ml using distilled water. Five grams of the soil sample was taken in a 100 ml beaker and 50 ml of ammonium acetate was added in it. The solution was mixed properly for 20 mins and then filtered with a Whatman filter paper to get clear solution. The flame photometer was adjusted to 100% by using higher concentration (10 ppm solution) after which the readings of K coucnentration was noted and recorded (AOAC, 2000)

3.3.8 Magnesium

Magnesium was extracted from the soil samples by mixing 10 ml of ammonium acetate with a 1 gram of the air-dried soil into a volumetric flask and shakened for 5 mins. Whatman filter

paper was then used to filter the solution. The filtrate was analyzed with an atomic emmission spectrometer. The absorbance was then read at a wave length of 410 nm. The results were reported in parts per million (ppm) (Kowalenko, 2008)

3.3.9 Sodium

A 100 ppm was prepared from 1000 ppm stock solution of sodium chloride (NaCl) by pipetting out 10 ml from stock solution and then diluting it with distilled water up to 100 ml. Five grams of the soil samples was taken in 100 ml beaker and 50 ml ammonium acetate was added in it. The solution was mixed properly for 20 mins and then filtered to get clear solution. The flame photometer was adjusted to 100% by using higher concentration solution (10 ppm solution) after which the readings of NA coucnentration was noted and recorded. (AOAC, 2000)

3.3.10 Determination of soil lead (Pb)

Lead (Pb) in the soil samples were determined according to the method of Ideriah *et al*. (2007). Soil sample (0.2 g) was weighed into a beaker and 6 ml freshly prepared aqua-regia (1:3) HNO_3: HCl respectively was added and allowed to stand overnight and then placed in a digestion block for 30 mins. The soil sample was allowed to cool and then filtered into a 100 mL volumetric flask with distilled water. The filtrate was analyzed for selected Pb using Perkin-Elmer Analyst 300 atomic absorption spectrophotometer (AAS).

The Perkin-Elmer Analyst 300 Atomic Absorption Spectrophotometer was calibrated with lead standards of known concentration to create a calibration curve. The instrument was set to the appropriate wavelength specific for lead (around 283.3 nm). The digested sample solution was aspirated into the AAS flame or graphite furnace, where lead atoms absorb light at the set wavelength. The instrument measures the amount of light absorbed, which is proportional to the concentration of lead in the sample (Ideriah *et al*., 2007)

3.3.11 Determination of trace element (Zinc)

Dried sieved soil sample at 5 g was digested with HNO_3-HCl according to United States Environmental Protection Agency (USEPA) method 3050B to extract the Zn. An aliquot solution of 6.0 µg of the zinc was measured with a measuring cylinder into a 125 ml separating funnel, 1 ml of 5,7-diiodooxine and 5 ml of ammonium acetate were added and diluted to 50 ml with water. This solution was shaken with 10 ml of toluene for 1 min. The concentration of Zn was measured by Perkin-Elmer Analyst 300 atomic absorption spectrophotometer (AAS) (USEPA, 1986).

3.4 Sterilization of Materials

Glassware such as Petri dishes were washed, drained and dried. They were wrapped with aluminum foil and sterilized in hot air oven at 160oC for 1 hr. After sterilization, the glassware was allowed to cool to about 40oC before use. Aseptic working environment was achieved with use of Bursen burner (Cheesbrough, 2006).

When the Bunsen burner is lit near the workspace, the heat causes the surrounding air to rise rapidly. This rising column of hot air acts as a barrier, preventing contamination from airborne microorganisms and dust entering the immediate work area. Additionally, the flame itself can be used to sterilize tools such as inoculation loops or forceps by passing them through the flame before and after use. Together, these effects help maintain aseptic conditions during microbiological procedures by minimizing the introduction of contaminants.

3.5 Media Preparation

Media for microbiological analyses were prepared according to the manufacturer's specifications.

3.5.1 Nutrient agar

Twenty-eight grams (28 g) of nutrient agar was dissolved in 1000 ml of distilled water in a conical flask corked with cotton wool and foil paper and allowed to dissolve in 1000 ml of distilled water in a conical flask. The medium was placed in an autoclave to sterilize it for 15 mins at 121 °C. After sterilization, the flask was allowed to cool before use. (Cheesbrough, 2000).

3.5.2 Reinforced Clostridial Medium (RCM) agar

A 51.0 g of Reinforced Clostridial Medium agar (MAC) was weighed and poured into a serial conical flask. One thousand milligram (1000 ml) of distilled water was added, and the mixture was sterilized at 121°C for 15 mins at 15 psi in the autoclave. It was poured into Petri dishes in 20 ml amounts and allowed to set at room temperature (Cheesbrough, 2000).

3.5.3 Mueller-Hinton agar

Mueller-Hinton agar is a microbial growth medium that is commonly used for antibiotic susceptibility testing. The medium was prepared from already sold dehydrated powder of Mueller-Hinton agar. The preparation also followed the dissolving of 38 g of Mueller-Hinton agar in 1000 ml of distilled water inside a conical flask sealed with cotton wool and aluminum foil paper. The mixture was shaken in other to attain an almost heterogeneous solution. Sterilization was subsequently carried out using the autoclave at 121^{o}C for 15 mins. The medium was cooled to 45^{0}C and then dispensed aseptically into sterile Petri dishes.

3.6 Enumeration, Identification and Characterization of Bacterial Isolates

3.6.1 Total heterotrophic bacterial counts

Triplicate plates were prepared for each volume of sample examined. All the samples to be analyzed were vigorously shaken before preparation of dilution. Then serial 10-fold dilutions of samples were prepared in physiological saline, and 1 ml of aliquot from dilution 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were spread over nutrient agar (Himedia). Then, the plates were incubated for 24

– 48 hrs at 28 – 37 ºC before bacteriological counts were performed. Number of colonies on triplicate plates having 30-300 colonies were counted and final bacterial counts were reported as cfu/g (Cheesbrough, 2006).

3.6.2 Culture of *Clostridium* species from samples

The culture of *Clostridium* species from samples was initiated by subjecting the collected specimens to a heat shock process to eliminate non-spore-forming bacteria, while preserving the viability of the spore-forming *Clostridium* species. The samples were heated at 70°C for 10 minutes in a water bath, effectively activating the spores. After heat treatment, aliquots of the processed samples were streaked onto *Clostridium* anaerobic agar (Micromaster), a selective medium formulated to support the growth of *Clostridium* species. The inoculated agar plates were placed in an anaerobic jar equipped with an anaerogen sachet to create and maintain a strictly anaerobic environment. This setup ensured that the obligate anaerobes could grow without exposure to oxygen. The plates were incubated for 48–72 hours to allow for the development of characteristic *Clostridium* colonies. Once the colonies were well-developed, the colonies were aseptically transferred to slants prepared with anaerobic agar. These slants were stored inside an anaerobic jar with fresh anaerogen sachets to maintain an oxygen-free environment. The cultures were preserved in this anaerobic condition until ready for further use, ensuring the viability and purity of the *Clostridium* isolates for downstream experiments.

3.6.3 Morphological test Gram staining

The Gram staining techniques was carried out on the basis of the component of the cell wall. Bacterial isolates which retained the colour of the initial stain are called Gram positive, while those that do not retain the primary stain when decolorized are Gram negative. The non-retention of the stain is due to the cell composition. Gram staining reagents include crystal

violet (primary dye stain), iodine (as mordant), 70 % alcohol (decolorizer) and safranin (counter stain)

Procedures: a drop of sterile distilled water was placed on a clean grease free slide. The inoculating wire loop was flamed until red hot. The loop was allowed to cool and a small portion of the organism to be gram stained was picked and smeared in the drop of water on the slide. The slide was then air dried. It was heat fixed by passing it gently over flame. The smear was stained with 1% crystal violet for 1 min and washed with distilled water. Gram iodine was added as a mordant for one min. This was drained off and 70 % alcohol was added for 30 sec. This acted as a decolorizer. The above is termed primary staining. The slide was then rinsed with distilled water. The slide was finally flooded with counter stain, safranin for 1 min and washed off with distilled water and air dried. The slide was observed under the microscope oil immersion x l00 objective lens. The Gram positive organisms appeared purple while the Gram negative organisms appeared red (Collins *et al*., 1989; Sharma *et al.,* 2009).

3.6.4 Biochemical Tests for characterization

Catalase Test

This was used to detect the presences of the enzyme catalase which catalyses the release of oxygen from water.

Procedure: Pure culture plates of each suspected bacterial isolates were flooded with 3% hydrogen peroxide solution. The formation of gas bubble was observed which indicated the presence of the enzyme in the test culture and recorded as positive result, while colonies of pure culture with no evidence of gas formation are considered catalase negative (Cheesbrough, 2006)

Oxidase Test

This was used in the identification of the enzyme oxidase produced by microorganisms,

This test was also used mainly to differentiate between *Pseudomonas* and other Gram negative rod-shaped bacteria.

Procedure: A 24 hrs culture of each isolates was then smeared on filter paper using sterilized wire loop and one drop of the oxidase reagent (l.0% aqueous tetramethyl-B-phenelenediamine dihydrochloride) was added. A positive oxidase test was indicated by the appearance of a purple colour within 10 secs. No color change within this time was considered a negative result.

Urease Test

This test was used to show if the test organism had the ability to produce the enzyme urease which catalyze the breakdown of urea to produce ammonia. The medium employed was urea agar base.

Procedure: The sterilized medium was dispensed into McCartney bottles. Finally, the test bacterial isolates was inoculated into the medium and incubated at 37°C for 24-48 hrs. A change in colour from yellow to red- pink indicated a positive result. No color change indicated a negative result. (Cheesbrough, 2006)

Indole Production Test

This was used to determine the ability of the test bacterial isolates to produce indole from the amino acid tryptophan and it is used in the identification of members of the Enterobacteria.

Procedure: The medium used was peptone broth. A 15 grams of commercially available peptone broth was dissolved in 1litre of distilled water. The medium was then sterilized by autoclaving at 121°C for 15 mins. Thereafter, about 4 m1 of the medium was dispensed into sterile MaCartney bottles and each of the bacterial isolates was inoculated into the peptone broth. The inoculated medium was incubated at 37°C for 24-48 hrs, after which one-few drops of Kovac's reagent was added. A purple ring at junction of the two liquids indicated a positive result (Cheesbrough, 2006).

Citrate utilization Test

This test is used to determine if the test organism is able to utilize citrate as a sole source of carbon and energy for growth.

Procedure: Simmon's citrate medium was used. In the preparation, 23 g of commercially available Simmon's citrate agar was dissolve in 1 litre of distilled water and sterilized by autoclaving at 12 1°C for 15 mins. The medium was then dispensed into sterile McCartney bottles, and the test organism was inoculated into the medium and incubated at 37°C for 24 hrs. A positive result is indicated by a change in colour from green to bright blue colouration. No color change indicated a negative result. (Cheesbrough, 2006)

Coagulase Test

This test is used to test the ability of the test organism to produce the enzyme coagulase which clumps plasma. This is mainly used to differentiate between *Staphylococcus aureus* from non-pathogenic *Staphylococci* species.

Procedure: A clean microscopic slide was marked into section and distilled water placed on each section. Thereafter, inoculum from the 18-24 hrs pure culture of the test organisms was inoculated into one section and a drop of human plasma was added to each section. The slide was allowed standing for 5 secs. Clumping indicated positive result while the no clumping indicated negative result. (Cheesbrough, 2006)

Sugar fermentation test

Procedure: Peptone water was used as the basal medium; 15 g of peptone water was poured into a 100 ml flask and 100 ml of distilled water was added. Inverted Durham's tubes were then placed in the test tubes and each of the test tubes was tightly corked with cotton wool. The setups were then autoclaved at 121°C for 15 mins. After this, the test tube was allowed to cool down at about 30°C and a suitable dye was added (Bromothymol blue, 0.2%). Then, each of the McCartney bottles were incubated with the test organisms by emulsifying the

organism by the side of the bottle and tilting the bottle to allow homogeneity. The test tubes were incubated at 37°C for 24 hrs. Glucose fermentation is shown by change in colour of the solution from pale pink to dark pink or red, while the displacement of the solution in the Durham tubes inside glucose bottle indicates gas production. Negative result shows no colour changes (Sharma *et al*., 2009).

3.7 Extraction of Crude Metabolites from *Clostridium* species

The extraction of crude metabolites was carried out using modified methods. Briefly, the extraction of secondary metabolites from *Clostridium* species, was carried out using cell-free supernatant of a 48-72 hrs. culture on *Clostridium* anaerobic agar (Micromaster) and then transferring well developed culture to tryptic soy broth (TSB) (supplemented with thioglycolate) which was also kept in an anaerobic jar. Following the cultivation of the *Clostridium* species on anaerobic agar under strict anaerobic conditions using anaerogen, the colonies were transferred using a sterile loop into the prepared TSB pre-reduced with a suitable reducing thioglycollate to ensure anaerobic conditions in the liquid medium. The broth was further incubated in an anaerobic environment for another 48–72 hrs, allowing the bacteria to grow and produce metabolites. After incubation, the broth was centrifuged at 3600 rpm for 15 min. and the cell free supernatant was collected by carefully pipetting it into sterile containers. The supernatant was immediately used for evaluating the presence of antimicrobial metabolites through the assessment of antimicrobial activity against target bacterial pathogens using disk diffusion assay (Govindasamy *et al.*, 2019; Arsène *et al.*, 2023).

3.8 Determination of Antibacterial Properties of crude metabolite extract from *Clostridium* species

The antimicrobial activity of the crude metabolites was evaluated using the modified Kirby-Bauer disk diffusion method. Filter paper disks (6 mm in diameter) were prepared by punching Whatman No. 1 filter paper and sterilizing the disks in an autoclave for 15–20 mins at 121°C and 15 psi. After cooling, the sterile disks were submerged in the cell-free supernatant of the *Clostridium* culture for 2 hrs to ensure adequate absorption of the metabolites. During this time, the disks were kept in sterile conditions to prevent contamination. The tested bacterial pathogens were cultured on Mueller-Hinton agar plates, which were inoculated with bacterial suspensions standardized to 0.5 McFarland turbidity. Using sterile forceps, the metabolite-soaked disks were carefully placed on the inoculated agar surfaces, ensuring even spacing between disks. The plates were incubated at 37°C for 18–24 hrs under aerobic conditions. Following incubation, the zones of inhibition around the disks were measured to the nearest millimeter using a ruler. The entire procedure was conducted under aseptic conditions to maintain sterility and accuracy. Controls included disks soaked in sterile TSB (negative control) and, where applicable, disks containing standard antibiotics (positive control) for comparison (Boyle *et al.*, 1973; Hidayat *et al.*, 2023).

3.9 Antibiotic Susceptibility Test of clinical bacterial isolates

Antibiotics used for this study included pefloxacin (10 µg), ampicillin (30 µg), zinnacef (20 µg), ciprofloxacin (10 µg), streptomycin (30 µg), septrin (30 µg), erythromycin (10 µg), amoxicillin (30 µg), rocephin (25 µg), ofloxacin (25 µg), gentamicin (10 µg), sparfloxacin (30 µg), augmentin (30 µg) and chloramphenicol (10 µg). Disk diffusion method was used for bacteria susceptibility test against commonly used antimicrobial agents. Pure isolated colonies growing on a selective agar plate were taken, bacterial suspension were adjusted to 0.5 McFarland turbidity standards. The diluted bacterial suspension was then transferred to

prepared Mueller-Hinton agar plate using a sterile cotton swab and the plate seeded uniformly by rubbing the swab against the entire agar surface, followed by 24 hrs incubation. After the inoculums were dried, commonly used antibiotic impregnated disks were applied to the surface of the inoculated plates using sterile forceps. The plates will then be incubated aerobically at 37°C for 24 hrs. Finally, the zone of inhibition were measured including the disk diameter (CLIS, 2013)

3.10. Virulence factors of bacterial clinical isolates

In the study mentioned, ESBL-positive isolates were subjected to various tests to determine the presence of virulence factors (Sharma *et al*., 2017).

3.10.1. Hemolysin production

All isolates were cultured on sheep blood agar media as described by Pavlov *et al.* (2004); Ryan and Ray (2004). Plates were incubated at 37°C for 24 hrs and then checked for a zone of hemolysis around colonies. The results were recorded as follows: α-hemolysis (greenish zones), β-hemolysis (clear zone), or γ-hemolysis (no hemolysis).

3.10.2 Gelatinase activity

All isolates were tested for gelatin production. This was done by inoculating the isolates into bacterial inoculation tubes containing nutrient gelatin medium. The tubes were incubated for 48 hrs at 37°C. Un-inoculated tubes ran beside the inoculated ones as negative controls. At the end of the incubation period, the liquefaction of the culture medium was observed after placing the culture tube at 4^{o}C overnight.

3.10.3 Deoxyribonuclease (DNase)

The method described by Pimenta *et al*. (2008) was used. The DNase agar (containing toluidine blue and methyl green) plates were inoculated by spotting a loopful of overnight pure cultures on to the surface of the tryptone soy broth, followed by aerobic incubation at 37℃ for 48 hrs. The plates were then flooded with 0.1 % 1 N HCl. The development of a red

colour or a zone of clearing indicated a positive result. No colour change or clearing around the colonies implies a negative result.

3.10.4 Lipase Test

Lipase is an enzyme that breaks down lipids (fats). Bacteria that produce lipase can utilize lipids as a nutrient source and potentially contribute to tissue damage and pathogenesis. All forms of fats are referred to as lipids in general. Three fatty acid molecules and one glycerol molecule are ester-linked to make fats. Triacylglycerols or triglycerides are the names for simple fats. Lipase, an enzyme that decomposes simple fats into their fatty acid and glycerol components. The lipase (enzyme) is found in some bacteria. Egg yolk agar is composed of pancreatic digest of casein (15.0 g), vitamin K1 (10.0 g), sodium chloride (5.0 g), papaic digest of soybean meal (5.0 g), yeast extract (5.0 g), L-cystine (0.4 g), hemin (5.0 g), egg yolk emulsion (100 ml), and agar (20.0 g). Take a loopful of the test bacterial isolate and streak it in a straight line on the egg yolk agar plate. The plate was incubated for 24 – 48 hrs at 30°C. The appearance of an iridescent shine on the plate was then checked. A positive lipase test was identified by the immediate emergence of an iridescent sheen (oil on water), which was observed when the plate was held at an angle to a light source, immediately surrounding colonies. The absence of an iridescent sheen indicates a negative test (Tille and Forbes, 2014).

3.11 Molecular Identification of *Clostridium* spp

3.11.1 Bacteria DNA extraction

Deoxyribonucleic acid was extracted using the protocol stated by. Briefly, Single colonies grown on medium were transferred to 1.5 ml of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600 g for 5 mins.

The resulting pellets were resuspended in 520 µl of TE buffer (10 mM Tris-HCl, 1 m M EDTA, pH 8.0). Fifteen microliters of 20% SDS and 3 µl of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37ºC, 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 mins. An equal volume of chloroform: isoamyl alcohol (24:1) was added, followed by incubation on ice for 5 mins and centrifugation at 7200 g 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 hrs. Deoxyribonucleic acid was collected by centrifugation at 13000 g for 10 mins, washed with 500 µl of 70% ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µl of TE buffer. (Sambrook and Russell, 2001)

3.11.2 Polymerase Chain Reaction Analysis

Bacteria Polymerase Chain Reaction

Polymerase chain reaction sequencing preparation cocktail consisted of 10 µl of 5x GoTaq colourless reaction, 3 µl of 25 mM MgCl2, 1 µl of 10 mM of dNTPs mix, 1 µl of 10 pmol each 27F 5'- AGA GTT TGA TCM TGG CTC AG-3' and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3 units of Taq DNA polymerase (Promega, USA) made up to 42 µl with sterile distilled water 8µl DNA template. Polymerase chain reaction was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at 94°C for 5 mins; followed by a 30 cycles consisting of 94°C for 30 s, 50°C for 60s and 72°C for 1 minute 30 seconds ; and a final termination at 72°C for 10 mins. And chill at 40C.

Integrity

The integrity of the amplified gene fragment was checked on a 1% agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare

1.5 % agarose gel. The suspension was boiled in a microwave for 5 mins. The molten agarose was allowed to cool to 60°C and stained with 3 µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 mins to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 l) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4 µl of each PCR product and loaded into the wells after the 100 bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120 V for 45 mins visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100 bp molecular weight ladder that was ran alongside experimental samples in the gel.

Purification of Amplified Product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µl of Na acetate 3 M and 240 µl of 95% ethanol were added to each about 40 µl PCR amplified product in a new sterile 1.5 µl tube Eppendorf, mix thoroughly by vortexing and keep at -20°C for at least 30 mins. Centrifugation for 10 mins at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellets were washed by adding 150 µl of 70% ethanol and mix then centrifuge for 15 mins at 7500 g and 4°C. Again, remove all supernatant (invert tube on trash) and invert tube on tissue paper and let it dry in the fume hood at room temperature for 10-15 mins. then resuspend with 20 µl of sterile distilled water and kept in -20°C prior to sequencing. The purified fragment was checked on a 1.5% agarose gel ran on a voltage of 110 V for about 1hrs as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific.

Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual, while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis

3.12 Data Analysis

The data generated were analyzed by one –way ANOVA (analysis of variance) at 0.5 confidence level using Genstat 12th edition analytical package as well as Non-Parametric T. test. Differences in mean were compared by Duncan's multiple range tests (Ogbeibu, 2015).

CHAPTER FOUR

RESULTS

The findings of the physicochemical parameters of soil samples from different locations is presented in Table 4.1. The pH of the soil sample ranged from 5.64±0.28 - 6.42±0.32. Electrical conductivity (EC) of soils showed EC values which ranged from 695.00±34.75 -

873.00±43.65 (µS/cm^2), organic matter ranged from 1.46±0.07 - 4.78±0.24 (mg/kg), organic content concentrations ranged from 2.52±0.13 - 8.24±0.41(mg/kg).

Phosphorus ranged from 7.65±0.38 - 11.22±0.56 (mg/kg), nitrogen ranged from 3.49±0.17 - 5.66±0.28 (mg/kg), calcium ranged from 0.84±0.04 - 1.76±0.09 (cmol/kg). Potassium ranged from 4.18±0.21 - 8.07±0.40 (cmol/kg). Magnesium ranged from 13.75±0.69 - 21.54±1.08 (cmol/kg) while sodium values were between 0.38±0.02 - 0.61±0.03 (cmol/kg).

Iron content ranged from 453.72±22.69 - 637.84±31.89 (mg/kg), zinc ranged from 65.79±3.29 - 87.52±4.38 (mg/kg), lead concentration ranged from 1.74±0.09 - 4.69±0.23 (mg/kg) while copper concentration ranged from 21.45±1.07 - 28.75±1.44 (mg/kg).

The findings of the total anaerobic bacterial counts of soil samples from different locations is shown in Table 4.2. Bacterial counts of Dumez soil samples was 5.15 ±0.07 × 10^3cfu/g, Eyean was 5.53±0.10 × 10^3cfu/g, Aduwawa was 25.25±0.03 × 10^3cfu/g while bacterial count of Aduwawa2 soil sample was 5.13±0.02 × 10^3cfu/g. The bacteria count from the soil sample ranged from 5.13±0.02 × 10^3cfu/g (Aduwawa2) to 5.53±0.10 × 10^3cfu/g, (Eyean).

Table 4.3 shows anaerobic bacterial isolates identity based on cultural, morphological and biochemical tests in the soil samples from the selected locations. Five (5) Clostridia; *Clostridium sporogens, Clostridium bolteae, Clostridium septicum, Clostridium perfringens* and *Clostridium butyricum* were isolated from the soil samples.

Table 4.1: Physicochemical parameters of soil samples from different locations

Parameter	iu dumez	Eyean	Aduwawa	Aduwawa 2	FEPA Limit
pH	6.42±0.32[a]	5.97±0.30[a]	6.13±0.31[a]	5.64±0.28[a]	6.0-8.5
EC (µS/cm^2)	754.00±37.70[a]	873.00±43.65[a]	695.00±34.75[a]	722.00±36.10[a]	<100
OM (mg/kg)	1.46±0.07[a]	4.78±0.24[a]	3.05±0.15[a]	2.58±0.13[a]	NA
OC (mg/kg)	2.52±0.13[a]	8.24±0.41[a]	5.26±0.26[a]	4.45±0.22[a]	NA
P (mg/kg)	9.75±0.49[a]	11.22±0.56[a]	7.65±0.38[a]	10.28±0.51[a]	7-20

N(mg/kg)	3.49±0.17[a]	5.66±0.28[a]	3.68±0.18[a]	4.25±0.21[a]	NA
Ca (cmol/kg)	0.98±0.05[a]	1.25±0.06[a]	1.76±0.09[a]	0.84±0.04[a]	NA
K (cmol/kg)	5.10±0.26[a]	8.07±0.40[a]	7.90±0.40[a]	4.18±0.21[a]	0.61-0.73
Mg(cmol/kg)	14.28±0.71[a]	17.63±0.88[a]	21.54±1.08[a]	13.75±0.69[a]	0.09-0.15
Na(cmol/kg)	0.38±0.02[a]	0.61±0.03[a]	0.57±0.03[a]	0.48±0.02[a]	0.5-0.7
Fe (mg/kg)	587.10±29.36[a]	496.28±24.81[a]	637.84±31.89[a]	453.72±22.69[a]	10.0-100
Zn (mg/kg)	74.82±3.74[a]	65.79±3.29[a]	87.52±4.38[a]	68.75±3.44[a]	1.0-5.0
Pb (mg/kg)	2.87±0.14[a]	4.69±0.23[a]	2.13±0.11[a]	1.74±0.09[a]	2.00-2.50
Cu (mg/kg)	28.75±1.44[a]	25.23±1.26[a]	23.68±1.18[a]	21.45±1.07[a]	2.00-250

Keys: A= IU Dumez, B= Eyean, C= Aduwawa 1, D= Aduwawa 2

EC: Electrical conductivity, OM: organic matter, OC: Organic carbon, P: phosphorus, N: nitrogen, K: potassium, Mg: magnesium. Na: sodium, Fe: iron, Zn: Zinc, Pb: Lead, Cu: Copper

Values are expressed as Mean ± Standard Error of triplicate experiments. Mean values with similar superscript within rolls are not significantly different from each other (P>0.05). Mean values with different superscript within rolls are significantly different from each other (P<0.05).

Table 4.2: Total anaerobic bacterial counts of soil samples from different locations

Sample Codes	**Total anaerobic bacterial (× 10^3cfu/g)**
Dumez	5.15 ±0.07[a]
Eyean	5.53±0.10[a]

Aduwawa 1	5.25±0.03[a]
Aduwaw 2	5.13±0.02[a]

Values are expressed as Mean ± Standard Error of triplicate experiments. Mean values with similar superscript within column are not significantly different from each other (P>0.05). Mean values with different superscript within column are significantly different from each other (P<0.05).

Table 4.3 Cultural morphological and biochemical characteristics of bacterial isolates from soil samples

Morphological	**1**	**2**	**3**	**4**	**5**
Elevation	Flat	Raised	Flat	Flat	Raised
Margin	smooth	Smooth	smooth	smooth	smooth
Color	Cream	Cream	Cream	Cream	Cream

Shape	round	Round	round	round	round
Size	small	Small	small	small	small
Gr. diff. agar	MCC	MCC	MCC	MCC	MCC
Colour	pink	Pink	pink	pink	pink
Staining					
Gram stain	+	+	+	+	+
cell type	Rod	Rod	Rod	Rod	rod
Arrangement	disperse	disperse	disperse	disperse	disperse
Color	purple	purple	purple	purple	purple
Spore staining	+	+	+	+	+
Biochemical					
KOH String Test	-	-	-	-	-
Catalase	-	-	-	-	-
Indole	-	-	-	-	-
Citrate	-	+	-	-	+
Oxidase	-	-	-	-	-
Motility	-	-	+	-	-
Urease	+	-	+	+	-
Glucose	+	+	+	+	+
Sucrose	-	+	+	-	+
Lactose	+	+	+	+	+
Mannitol	-	+	-	-	+
Gas formation	+	-	+	+	-
H_2S formation	-	-	-	-	-
TSI (Slant/Butt) reaction	A/AG	A/A	A/A	A/AG	A/A
Esculin Hydrolysis	+	+	+	+	+
Probable Identity	***Clostridium sporogens***	***Clostridium bolteae***	***Clostridium septicum***	***Clostridium perfringens***	***C. butyricum***

Keys: + = Positive, - = Negative, A/G = Acid slant/acid butt, gas produced, A/A = Acid slant/acid butt, no gas

The cultural, morphological and biochemical characteristics of clinical isolates is presented in Table 4.4. Bacterial identified included four Gram-negative bacteria; namely *Pseudomonas aeruginosa, Enterobacter aerogenes, E. coli, Salmonella enterica* and one Gram-positive bacteria; *Staphylococcus aureus.*

Table 4.5 shows the percentage occurrence of bacterial isolates from soil samples. Out of the 16 total bacterial isolated from the soil samples, five (5) were present in Aduwawa2 soil

samples, three (3) in Eyean and four (4) in Dumez and Aduwawa soil samples respectively. *Clostridium septicum* (25%) and *Clostridium butyricum* (25%) were the most occurring bacterial isolate, followed by *Clostridium sporogens* and *Clostridium perfringens* with 18.8% occurrence, while *Clostridium bolteae* was the least occurring bacterial isolate (12.5%).

Table 4.6 shows the phenotypic virulence properties of clinical bacterial isolates. *Staphylococcus aureus* showed positive gelatinase, lipase, DNAse and hemolysin. *Pseudomonas aeruginosa* characteristics*, Staphylococcus aureus, Enterobacter aerogenes, E. coli* and *Salmonella enterica* showed gelatinase and lipase showed positive characteristics, while *Pseudomonas aeruginosa, Enterobacter aerogenes* and *Salmonella enterica* were DNAse and hemolysin negative.

The antibacterial activity of *Clostridium* species against bacterial isolates is shown in Table 4.7. Extracts from *C. perfringens* and *C. butyricum* had the highest zone of inhibition against *Escherichia coli* (1.07±0.03 mm) and *Staphylococcus aureus* (1.07±0.67), while extracts from *C. sporogens* and *C. perfringens* had the least zone of inhibition against *Pseudomonas aeruginosa* and (0.10±0.00mm) and *Escherichia coli* (0.10±0.00mm) respectively.

Table 4.4 Cultural morphological and biochemical characteristics of clinical isolates

Morphological	**1**	**2**	**3**	**4**	**5**
Elevation	Raised	Raised	Flat	Flat	raised
Margin	Entire	Smooth	Undulate	Undulate	Entire
Color	lemon	Cream	Cream	Cream	Cream
Shape	Circular	Irregular	Irregular	Irregular	Circular
Size	Medium	Small	Large	Large	Medium
Gr. diff. agar	PCA	MSA	EMB	EMB	SSA
Colour	green	Yellow	pink	green	black
Staining					

Gram stain	-	+	-	-	-
cell type	rod	Cocci	Rod	Rod	rod
Arrangement	disperse	clusters	disperse	disperse	pair/chains
Color	pink	Purple	pink	pink	pink
Spore staining	-	-	-	-	-
Biochemical					
KOH String Test	+	-	+	+	+
Catalase	+	+	+	+	+
Indole	-	-	-	+	-
Citrate	+	+	+	-	-
Oxidase	+	-	-	-	-
Motility	+	-	+	+	+
Urease	+	+	-	-	-
Glucose	-	+	+	+	+
Sucrose	-	+	+	-	-
Lactose	-	+	+	+	-
Mannitol	-	-	-	-	-
Gas formation	-	-	-	+	+
H_2S formation	-	-	-	-	+
TSI (Slant/Butt) reaction	K/K	A/A*	A/A(K*)G*	A/AG	k/AG H_2S
Esculin Hydrolysis	-	-	+	-	-
Probable Identity	***Pseudomonas aeruginosa***	***Staphylococcus aureus***	***Enterobacter aerogenes***	***E. coli***	***Salmonella enterica***

Key: + = Positive, - = Negative, TSI = triple sugar iron agar test, Gr. Diff. Agar = Growth on differential agar, KOH = Potassium hydroxide,

Table 4.5: Percentage occurrence of bacterial isolates from soil samples

Isolates	Dumez	Aduwawa1	Eyean	Aduwawa2	Sum total	%
Clostridium sporogens	1	1	0	1	3	18.8
Clostridium bolteae	0	1	0	1	2	12.5
Clostridium septicum	1	1	1	1	4	25.0
Clostridium	1	0	1	1	3	18.8

perfringens						
C. butyricum	1	1	1	1	4	25.0
Total	**4**	**4**	**3**	**5**	**16**	**100**

Table 4.6 Phenotypic virulence properties of clinical bacterial isolates

Isolates	Gelatinase	Lipase	DNAse	Hemolysin
Pseudomonas				
aeruginosa	+	+	-	-
Staphylococcus	+	+	+	+

aureus				
Enterobacter				
aerogenes	+	+	-	-
E. coli	+	+	+	-
Salmonella enterica	+	+	-	-

Keys: Present (+), Absent (-)

Table 4.7: Antibacterial activities of the crude metabolic extracts of *Clostridium* spp. against the clinical bacterial isolates

Crude metabolic extracts	Zone of inhibition (mm)				
	P. aeruginosa	***S. aureus***	***E. aerogenes***	***E. coli***	***S. enterica***
A	0.42±0.03[a]	0.21±0.09[a]	0.40±0.00[a]	0.10±0.00[a]	0.90±0.21[b]

B	0.57±0.03[a]	0.23±0.03[a]	0.37±0.03[a]	0.00±0.00[b]	0.97±0.03[b]
C	0.17±0.03[a]	0.00±0.00[b]	0.56±0.07[a]	0.57±0.12[b]	0.90±0.26[a]
D	0.10±0.00[b]	0.00±0.00[b]	0.33±0.33[a]	1.07±0.03[a]	0.37±0.03[a]
E	1.03±0.67[a]	1.07±0.67[a]	0.00±0.00[b]	0.67±0.03[b]	0.63±0.03[a]

Keys: Values are expressed as Mean ± Standard Error of triplicate experiments. Mean values with similar superscript within columns are not significantly different from each other (P>0.05). Mean values with different superscript within columns are significantly different from each other (P<0.05).

A- Extracts from *C. sporogens,* B- Extracts from *C. bolteae,* C- Extracts from *C. septicum,* D- Extracts from *C. perfringens,* E- Extracts from *C. butyricum*

The antibacterial sensitivity of clinical isolates is shown in Table 4.8. *Staphylococcus aureus* was susceptible to pefloxacin, gentamicin, ampicillin zinnacef, ciprofloxacin, streptomycin and septrin and resistant to amoxicillin, rocephin and erythromycin. *Pseudomonas aeruginosa, Enterobacter aerogenes, Escherichia coli* and *Salmonella enterica* were susceptible to septrin, chloramphenicol, sparfloxacin and pefloxacin, while *Pseudomonas aeruginosa, Enterobacter aerogenes* and *Salmonella enterica* resistant to amoxicillin, augmentin and streptomycin. Multiple drug index showed that *Enterobacter aerogenes,*

Escherichia coli and *Salmonella enterica* had the highest multiple drug resistance index of 0.4, while *Pseudomonas aeruginosa* and *Pseudomonas aeruginosa* 0.3 multiple drug resistance index respectively.

Table 4.9 shows the molecular identification of anaerobes from soil samples. *Clostridium sporogens* from Dumez had 100% identity, *Clostridium butyricum* from Eyean soil had 99.47% identity, *Clostridium bolteae* from Aduwawa soil had 99.86% identity, *Clostridium septicum* from Aduwawa2 soil had 99.94% identity, while *Clostridium perfringens* from Dumez 2 had 100.00% identity.

Agarose gel electrophoresis of bacterial isolates after extraction of DNA for further accuracy. Isolate 1 to isolate 5 are represented in the various lanes as indicated in Plate 4.1. Lane 1, Lane 2, Lane 3, Lane 4 and Lane 5 indicate the genomic DNAs of *Clostridium sporogens, Clostridium butyricum, Clostridium bolteae, Clostridium septicum* and *Clostridium perfringens* respectively.

Table 4.8 Percentage antibiotic resistance pattern of bacterial isolates

G-ve	No.	SXT	CH	SP	CPX	AM	AU	CN	PEF	OFX	St
E. coli	17	5(29.4)	7(41.2)	4(23.5)	3(17.6)	7(41.2)	2(11.8)	2(11.8)	2(11.8)	3(17.6)	5(29.4)
P. aeruginosa	19	7(36.8)	6(31.6)	8(42.1)	4(21.1)	5(26.3)	2(10.5)	2(10.5)	3(15.8)	4(21.1)	5(26.3)
E. aerogenes	6	2(33.3)	1(16.7)	1(16.7)	2(33.3)	3(50.0)	0(0.0)	0(0.0)	1(16.7)	1(16.7)	0(0.0)
S. enterica	8	1(12.5)	3(37.5)	2(25.0)	0(0.0)	4(50)	3(37.5)	1(12.5)	1(12.5)	2(25.0)	3(37.5)
G+ve		**PEF**	**CN**	**APX**	**Z**	**AM**	**Ro**	**CPX**	**St**	**SXT**	**E**
S. aureus	21	7(33.3)	5(23.8)	4(19.0)	7(33.3)	3(14.3)	7(33.3)	4(19.0)	4(19.0)	2(9.5)	5(23.8)

Keys:

PEF: Pefloxacin (10 µg), APX= Ampicillin (30 µg), Z= Zinnacef (20 µg), CPX= Ciprofloxacin (10 µg), St= Streptomycin (30 µg), SXT= Septrin (30 µg), E= Erythromycin (10 µg), AM = Amoxicillin (30 µg), R = Rocephin (25 µg), OFX = ofloxacin (25 µg), CN = Gentamicin (10 µg), SP = Sparfloxacin (30 µg), AU = Augmentin (30 µg), CH = Chloramphenicol (10 µg)

MDR = Multiple Drug Resistance.

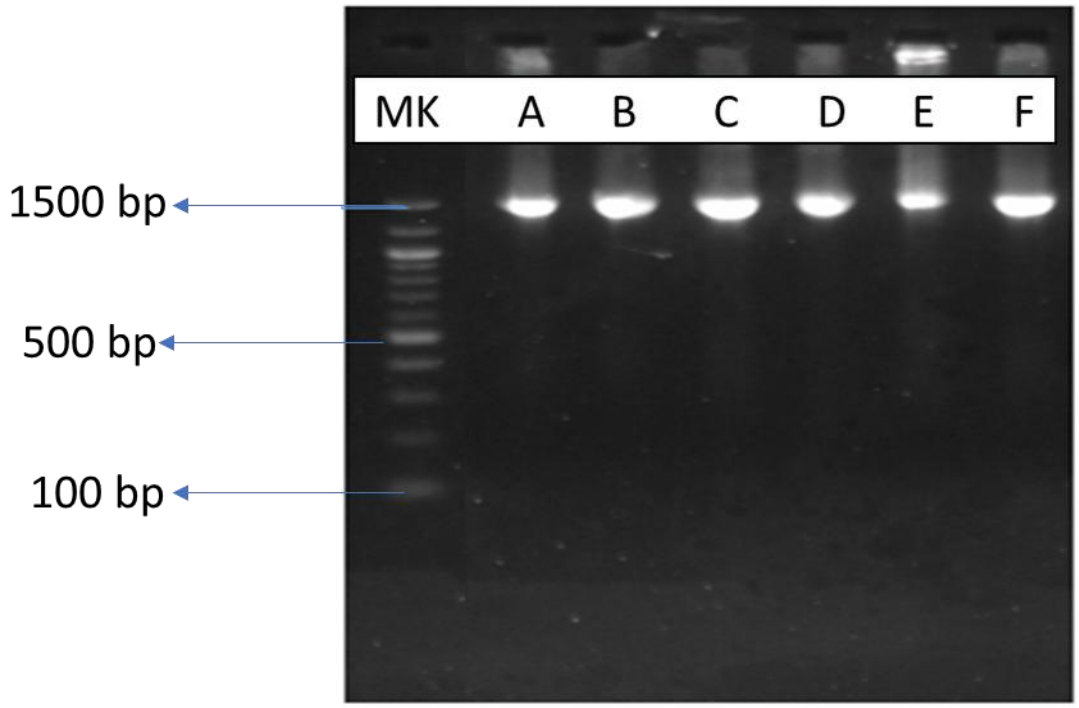

Plate 4.1: Agarose gel electrophoresis of bacterial isolates after extraction of DNA

Key: Lane MK: DNA Marker; F = Positive control

Lanes A-E = Amplified bacterial DNA. Lane A: *Clostridium sporogens,* Lane B: *Clostridium butyricum,* Lane C: *Clostridium bolteae,* Lane D: *Clostridium septicum,* Lane E: *Clostridium perfringens*

Table 4.9: Identity of *Clostridium* spp. from soil samples obtained from cattle yard

Sample code	Identity	Query cover (%)	Homology (%)	Accession number
A	*Clostridium sporogens*	100	100.00	CP011663.1
B	*Clostridium butyricum*	98	99.47	MW548182
C	*Clostridium bolteae*	99	99.86	MW548179
D	*Clostridium septicum*	100	99.94	CP034358.1
E	*Clostridium perfringens*	91	100.00	KY235305.1

Keys: A: *Clostridium* spp. from Dumez, B: *Clostridium* spp. from Eyaen, C: *Clostridium* spp. from ADUWAWA 1, D: *Clostridium* spp. from ADUWAWA 2, E: *Clostridium* spp. from Dumez

CHAPTER FIVE

DISCUSSION

Clostridia from soil environment within Benin metropolis were assessed. Near neutral pH values were obtained from soil samples from the various locations and were within FEPA desirable limits of 6.0-8.5. These values are considered normal for optimal bacterial growth in soil and corroborates with the report of Ilembayo and Kolade (2008) who reported a range of pH values of 5.96 – 8.88 for top soil samples. The metabolic activities of microorganisms in soil can often be directly linked to the pH (Hinsinger *et al.,* 2009). The pH also influences the solubility and accessibility of soil components which indirectly influence biological activity in the soil (Onojake and Osuji, 2012). The high electrical conductivity levels recorded in this study may be due to high concentration of conducting ions in soil from dissolved salts and inorganic materials (Koech, 2012; Dauda *et al*., 2016).

Generally, high bacterial counts were observed in soil samples from the location with soil samples from Eyean having the highest bacterial counts in this study. The bacteria from the soil samples was probably due to soil nutrients (Delile and Coulon, 2008). Again, the higher bacterial count in the soil samples could be attributed to more utilization of organic matter and more available nutrients in the soils (Ogugube *et al*., 2017). *Clostridium sporogens, Clostridium bolteae, Clostridium septicum, Clostridium perfringens* and *Clostridium butyricum* were isolated from the soil samples while *Pseudomonas aeruginosa, Enterobacter aerogenes, Escherichia coli, Salmonella enterica* and *Staphylococcus aureus* were identified in the clinical isolates. Most of these bacterial isolated from soil samples were not surprising as the isolates are commonly found in soil rich in organic carbon and have been reported as prominent carbon degraders by various authors (Ibrahim *et al*., 2009; Obaryori *et al*., 2012; Ismali *et al*., 2014; Obuotor *et al*., 2015;

Agarry *et al*., 2015). The abundance of these bacteria in soil could be attributed to the addition of organic wastes and catabolism of soil contaminants (Nweke and Okpokwasili, 2004).

However, among the isolated bacteria, *Clostridium septicum* and *Clostridium butyricum* had the highest occurrence (25%). *Clostridium septicum* has a wider range of metabolic activities and their ability to grow on diverse substrate and chemicals is well known, hence their high occurrence (Smith-Slatas *et al*., 2016). Once more, *Clostridium butyricum* have the ability to metabolise soil contaminants during unfavourable environmental conditions (Woo *et al*., 2011).

This study revealed the pathogenic potential of the clinical bacterial isolates. The ability of pathogenic bacteria to cause disease in a susceptible host is influenced by their ability to produce virulence factors. Virulence factors acting individually or together may induce infection depending on the host resistance. These factors compromise the host's defense mechanisms resulting in successful colonization and establishment of infection (Bushen *et al.,* 2021). All the clinical bacterial isolates in this study exhibited variable forms of virulence with *Pseudomonas aeruginosa* characteristics*, Staphylococcus aureus, Enterobacter aerogenes, E. coli* and *Salmonella enterica* showed gelatinase and lipase having positive characteristics while *Pseudomonas aeruginosa, Enterobacter aerogenes* and *Salmonella enterica* possess virulent traits by showing positive DNAse and hemolysin activity. Bacteria possessing DNase can escape from neutrophil extracellular traps (NETs), thereby helping the oganism survive (Haas *et al*., 2014). The bacteria isolated in this study are, therefore, capable of inducing infections due to the possession of virulent traits. *Staphylococcus aureus* which haemolysed red blood cells in this study (positive haemolytic activity) may cause up to one third of all bacterial infections ranging from boils and pimples to food poisoning. The ability to produce haemolysin by an organism is a measure of its pathogenicity (Sora *et al*., 2021). Organisms with heamolytic ability are able to

lyse red blood cells or other nucleated cells in the blood thereby enabling it to invade and cause disease. The serum resistant attribute of an organism is the ability of the organism to evade serum killing (Orole *et al*., 2021).

Concerning the production DNase enzyme, *Staphylococcus aureus* and *Escherichia coli* isolates were positive to this test. Kanemitsu *et al*. (2001) reported that *Staphylococcus aureus* and *Escherichia coli* were positive to DNase test. Ismail (2006) reported that the production of DNase are depending on the site of infection. Virulent traits such as hemolysin production, DNAse, lipase and gelatinase activity of bacterial isolates observed in this study also agree with the reports of various authors such as Retamal *et al*. (2022) who detected virulent factors in *Staphylococcus aureus, Enterobacter aerogenes, E. coli, Pseudomonas aeruginosa* and *Salmonella enterica* clinical isolates. Sarowska *et al*. (2022) who detected hemolysin production in *E. coli* isolated from soil samples and Gambi *et al*. (2022) that reported that *Escherichia coli* isolated from poultry showed positive gelatinase activities

Positive gelatinase production by *Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter aerogenes, E. coli* and *Salmonella enterica* observed in this study correlate with the report of Sanches *et al*. (2020) who reported a gelatinase production in *Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter aerogenes, E. coli, Salmonella enterica* and *Proteus mirabilis* isolated from soil samples.

The antibacterial activity of *Clostridium* species against bacterial isolates in this study shows that extracts from *C. perfringens* and *C. butyricum* had the highest antibacterial activity against *Escherichia coli* and *Staphylococcus aureus.* This finding is in agreement with the report of Pahalagedara *et al*. (2020) who reported that soil *Clostridium* species enriched conditioned media inhibited the growth of *Bacillus mycoides*, *Escherichia coli, Staphylococcus aureus*

and *Pseudomonas aeruginosa*. According to Tracanna *et al*. (2017) the antibacterial activity of *Clostridium* species is due to ability to produce metabolites. Letzel *et al*. (2013) linked the antibacterial activities of *Clostridium* species to the possession of high number of biosynthetic gene clusters and higher variability in cluster composition.

The antibiotic sensitivity of isolates in this study revealed that *Pseudomonas aeruginosa, Enterobacter aerogenes, Escherichia coli* and *Salmonella enterica* were susceptible to septrin, chloramphenicol, sparfloxacin and pefloxacin while *Pseudomonas aeruginosa, Enterobacter aerogenes* and *Salmonella enterica* resistant to amoxicillin, augmentin and streptomycin.. This is similar to the studies of Uwimbabazi *et al*. (2015) who reported that *Pseudomonas aureginosa, Shigella* spp *Staphylococcus aureus, Escherichia coli* and *Salmonella* spp were susceptible to septrin, sparfloxacin, augmentin amoxicillin and gentamycin. The resistance of *Pseudomonas aeruginosa, Enterobacter aerogenes* and *Salmonella enterica* to augmentin and streptomycin in this study however, is in agreement with the study of Liu *et al*. (2017).

The resistance of the bacterial isolates to few antibiotics could probably be due to chromosomally or plasmid mediated resistant genes in the bacteria genetic make-up. This could be associated with indiscriminate usage of antibiotics. This finding agrees with the work of Raja *et al*. (2018); Hala *et al*. (2015) the author's reported that *Escherichia coli, Shigella* spp., *Enterobacter aerogenes* and *Salmonella enterica* were resistant to streptomycin, septrin, ciprofloxacin, amoxicillin and ceftazidime.

In this study, *Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter aerogenes, Escherichia coli* and *Salmonella enterica* showed multiple antibiotic resistance to the tested antibiotics, especially *Enterobacter aerogenes, Escherichia coli* and *Salmonella enterica* which had the highest multiple antibiotic resistance index (0.4). This agrees with the studies of Hafsat

et al. (2015) who reported *Salmonella* spp, *Escherichia coli, Staphylococcus aureus* to be multidrug resistant to sparfloxacin, pefloxacin, augmetin, gentamycin, and zinnacef. In addition, these findings provide evidence that there is an increased emergence of antibiotic resistance. The finding is in agreement with the reports of Ndip *et al*. (2015) who found increasing emergence of antibiotic resistance in bacterial isolates wound infected patients.

Assay for soil bacterial isolates in this study revealed the presence of *Clostridium sporogens, Clostridium butyricum, Clostridium bolteae, Clostridium septicum* as well as *Clostridium perfringens* confirmed with molecular identification.

5.1 Conclusion and Recommendations

Natural products have remains as a major source of secondary metabolites. The *Clostridium* species exhibit varying degrees of antibacterial activity against specific clinical bacterial isolates. The *C. perfringens* and *C. butyricum* showed the most comprehensive antibacterial activity. The differences in the antibacterial activities can be crucial for selecting appropriate *Clostridium* species for potential therapeutic applications or further research in antibacterial treatments. Therefore, the *Clostridium* species isolated in this study can be used commercially for the production of antibiotics after purification and proper standardization following the attenuation of the virulence factors.

5.2 Contributions to Knowledge

The study has contributed to knowledge in the following ways:

I. The study revealed that *Clostridium* species exhibits varying degrees of antibacterial activity against clinically relevant bacterial isolates with *Clostridium butyricum* and *Clostridium perfringens* having the highest potential.

II. The study reveals that *Clostridium* spp with antibacterial potentials are present in cattle yards

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APPENDIX I

CULTURE MEDIA

Nutrient agar

Beef extract	3.0 g
Agar No.2	12.0 g
Peptone	5.0 g
Sodium chloride	8.0 g
Distilled water	1000 ml

Reinforced Clostridial Medium (RCM) agar

Meat Extract	10 g/l
Peptone	10 g/l
Yeast Extract	3 g/l
D(+)-Glucose	5 g/l
Starch	1 g/l
NaCl	5 g/l
C2H3NaO2	3 g/l
L-Cysteinium Chloride	0.5 g/l
Agar-Agar	0.5 g/l

Mueller Hinton Agar

Beef Extract	2 g
Acid Hydrolysate of Casein	17.5 g
Starch	1.5 g
Agar	17 g

Final pH 7.3 ± 0.1 at 25°C

APPENDIX 2

Seq1> *Clostridium* butyricum

AGGGGGGNCTTACCATGCAAGTCGAGCGATGAAGCTCCTTCGGGAGTGGATTAGCGGCGGACGGGTGAGTA
ACACGTGGGTAACCTGCCTCATAGAGGGGAATAGCCTTTCGAAAGGAAGATTAATACCGCATAAGATTGTAGT
ACCGCATGGTACAGCAATTAAAGGAGTAATCCGCTATGAGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGG
TAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCG
TGAGTGATGACGGTCTTCGGATTGTAAAGCTCTGTCTTTAGGGACGATAATGACGGTACCTAAGGAGGAAGCC
ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAA
GGGAGCGTAGGTGGATATTTAAGTGGGATGTGAAATACCCGGGCTTAACCTGGGTGCTGCATTCCAAACTGG
ATATCTAGAGTGCAGGAGAGGAAAGGAGAATTCCTAGTGTAGCGGTGAAATGCGTAGAGATTAGGAAGAATA
CCAGTGGCGAAGGCGCCTTTCTGGACTGTAACTGACACTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTAGGGGTTGTCATGACCTCTGTGCCGCCGCT
AACGCATTAAGTATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCA
CAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGAATTAC
TCTGTAATGGAGGAAGCCACTTCGGTGGCAGGAAGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCTACCATTTAGTTGAGCACTCTAGCGA
GACTGCCCGGGTTAACCGGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGGCTAGGGCTACC
CANGTGCTACAATGGTCGGTACAATGAGATGCAACCTCGCGAAAGGAGCAAACTTTAAACCGATCTCAGTTCG
GATTGGAGGCTGAAACTCGCCTACTGAAGCTG

>Seq2> *Clostridium* butyricum

CCCCCGTGGGGGGCTTACCATGCAAGTCGAGCGATGAAGCTCCTTCGGGAGTGGATTAGCGGCGGACGGGTG
AGTAACACGTGGGTAACCTGCCTCATAGAGGGGAATAGCCTTTCGAAAGGAAGATTAATACCGCATAAGATTG
TAGTACCGCATGGTACAGCAATTAAAGGAGTAATCCGCTATGAGATGGACCCGCGTCGCATTAGCTAGTTGGT
GAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACG
CCGCGTGAGTGATGACGGTCTTCGGATTGTAAAGCTCTGTCTTTAGGGACGATAATGACGGTACCTAAGGAGG
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GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATACTAGGTGTAGGGGTTGTCATGACCTCTGTGCCG
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CGCACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGA
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GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTAGTTGCTACCATTTAGTTGAGCCCTCCTA
GCGAGACTGCCCGGGTTAACCGGGAGGAAGGGGGGGATGACGTCAATCTTC

>Seq3 99% identical to *Clostridium* bolteae

TAAGTAACGCGTGGATAACCTGCCTCACACTGGGGGATAACAGTTAGAAATGACTGCTAATACCGCATAAGCGCAC
AGTACCGCATGGTACAGTGTGAAAAACTCCGGTGGTGTGAGATGGATCCGCGTCTGATTAGCCAGTTGGCGGGGT
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AGAGGTAAGTGGAATTCCTAGTGTAGCGGTGAAATGCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTT
ACTGGACGATAACTGACGTTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGAATGCTAGGTGTTGGGGGGCAAAGCCCTTCGGTGCCGTCGCAAACGCACCACT

>Seq4 Lysinibacillus fusiformis

GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTTAGTT
GGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTATGACC
TGGGCTACACACGTGCTACAATGGACGATACAAACGGTTGCCAACTCGCGAGAGGGAGCTAATCCGATAAAGTCGT
TCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG
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GAGGGTGGGGATGACTCCTCGTCGTCCTGATCCTTACGGCTTCGCATGTAATCCTACCGCCATGACATATAGTCACA
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GAGTCGCATTATACTATACTAGATGTCACGCTGCAGACGATCTCCCGGCTGCACAAGAATACAAAA

>CP009225.1 *Clostridium* sporogenes
CAATTGTCAAGTTAATATAAATAATGTATATAAAGTTGTTTATATTTTGTTTACTATATGTGAACATTCTAAGTTTCTATCAAGACT
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GTTACAGGTTAGCT

> *Clostridium* septicum
ATGGATACTGAGCTTAAATTATTATGGGAAAAAACATTAAATATTATTAAAGGTGAAATGAGTGAAGTTAGCTTTAACACCTGGA
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