ISOLATION, IDENTIFICATION AND ANTIBIOGRAM OF BACTERIA FROM DUNG OF LOCAL CATTLE

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A PROJECT WORK SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF LIFE SCIENCES, UNIVERSITY OF BENIN, BENIN CITY, IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR OF SCIENCE (B.Sc) HONOURS IN MICROBIOLOGY.

MARCH, 2024.

CERTIFICATION

This is to certify that this project was carried out by Thomas Osondu Alike in the Department of Microbiology, Faculty of Life Sciences, University of Benin, under the supervision of Prof.(Mrs.) O.I. Enabulele, submitted to the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, in partial fulfillment for the requirement of the award of Bachelor of Science (B.Sc.) degree in Microbiology.

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APPROVAL

This is to certify that this project work was accepted in partial fulfillment of the requirement for the award of Bachelor of Science B.Sc. (Hons) Degree in Microbiology of the University of Benin, Benin City.

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PROF. (MRS) F.I. AKINNIBOSUN **DATE**

Head of Department

DEDICATION

I dedicate this project to God Almighty for his guidance, provision, wisdom, understanding, strength and protection during the course of this project and my brother who has taken up the burden of tertiary education.

ACKNOWLEDGEMENT

First and foremost, my deepest appreciation goes to Almighty God for the grace, wisdom and strength he bestowed upon me during the course of this project. It is by his grace, mercy and provision that this project was successfully completed. I give him all the glory.

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TABLE OF CONTENTS

TITLE PAGE i

CERTIFICATION ii

APPROVAL iii

DEDICATION iv

ACKNOWLEDGEMENT v

TABLE OF CONTENTS vi

ABSTRACT ix

CHAPTER ONE: INTRODUCTION 1

1.0 BACKGROUND OF THE STUDY 1

1.2 THE OBJECTIVES OF THE STUDY ARE TO: 2

CHAPTER TWO: LITERATURE REVIEW 3

2.0 COW DUNG 3

2.1 SOURCE OF ENERGY 4

2.2 AGRICULTURE MANAGEMENT 7

2.3 BIOREMEDIATION OF ENVIRONMENT POLLUTANTS 8

2.4 HUMAN HEALTH MANAGEMENT 9

2.5 SOURCE OF MICROBIAL ENZYMES 9

2.6 PATHOGENS ASSOCIATED WITH COW DUNG 10

2.6.1 ESCHERICHIA COLI 10

2.6.2 SALMONELLA 11

2.6.3 CAMPYLOBACTER 11

2.6.4 LISTERIA MONOCYTOGENES 12

2.6.5 YERSINIA ENTEROCOLITICA 13

2.6.6 ENTEROCOCCUS 13

2.6.7 MUNDTII 14

2.6.8 MYCOBACTERIUM 14

CHAPTER THREE: MATERIALS AND METHODS 17

3.0 STUDY AREA 17

3.1 SAMPLE COLLECTION 17

3.2 SERIAL DILUTION 17

3.4 ISOLATION AND IDENTIFICATION 18

3.5 ANTIBIOTIC SUSCEPTIBILITY TEST 18

CHAPTER FOUR: RESULTS 19

4.1 RESULT PRESENTED SHOWS THE TOTAL BACTERIA COUNT FROM COW DUNG. 19

4.2 RESULTS SHOWS THE DISTRIBUTION OF BACTERIA ISOLATES IN A SAMPLE AND SAMPLE. 21

4.3 RESULTS SHOWS ANTIBACTERIAL RESISTANCE PATTERN 23

CHAPTER FIVE 25

5.1 DISCUSSION 25

5.2 CONCLUSIONS 27

REFERENCES 28

APPENDIX 37

ABSTRACT

Cattle dung contains a lot of potentially pathogenic bacteria. Data on bacteria from local cattle (muturu) unlike its fulani relative is however scarce. This research was carried out to analyze the bacterial microflora of cattle muturu dung. A total of four stool samples received in our Laboratory were analyzed. A ten-fold serial dilution was carried out on all samples. Briefly, one (1) gram each of stool sample was weighed onto test tubes containing 9ml of sterile distilled water to make stock solutions. The tubes were homogenized after which 1ml was inoculated onto petri dish containing solidified agar and rocked for even distribution. Agar plates were incubated at 37℃ for 24hours. Bacterial counts were carried out on each of them. Identification of bacteria isolates were carried out using standard guidelines such as morphological (shape, size, arrangement, staining property, motility and spore formation) colonial (texture, elevation, margin and colour) and biochemical (catalase, oxidase, citrate utilization, urease and indole production) characteristics. Bacterial isolates recovered from cattle dung incude: *Eschericia coli*, *Salmonella* sp.,*Staphylococcus* sp., *Enterococcus* sp., *Shigella* sp., *Bacillus* sp. and *Streptococcus* sp. The antibacterial resistance pattern of bacterial isolates to routinely used antibiotics (azithromycin, levofloxacin, erythromycin, pefloxacin, gentamycin, ampicillin-cloxacillin, cefuroxime, amoxiclin, ceftriaxone and ciprofloxacin) was carried out using the Disc diffusion method. The total heterotrouphic bacterial counts ranged from 1.5×10^{3}cfu/g – 9.8×10^{5} cfu/g. Bacterial isolates showed varying resistance to the antibacterial agents used in this study. Both isolates of *Eschericia coli* were resistant to azithromycin and levofloxacin. *Bacillus* specie was resistant to erythromycin and pefloxacin while *Salmonella* sp. showed resistance to gentamycin. The presence of antibiotic resistance bacteria in local cattle dug could be of grave public health concern.

CHAPTER ONE

INTRODUCTION

1.0 Background of the Study

Cow dung is excreted by bovine animal species, which are herbivores. It consists of undigested residues of consumed matter which has passed through the cow's gastrointestinal system (Teo and Teoh, 2011).Cow dung contains 14.05-19.00 hemicellulose(%),15.31-29.00 cellulose(%) and 13.97-16.00 lignin(%) (Li *et al,2021*). It contains about 24 different minerals ranging from macro-minerals like: Nitrogen(N2), Potassium (K), Sulphur (S), Magnesium (Mg),and trace elements such as: Copper (CU),Phosphorus (PH) and Zinc (Zn).Cow dung is widely used in formation of manure which is used as bio-fertilizers, source of energy, bioremediation of environmental pollutants, human health management and source of microbial enzyme(Muhammadu *et al*, 2003). Manure of cow dung enhances the minerals of soil and also develops resistance power of plants against pests and plant diseases. Animal pathogens posing risks to animal and possibly human health include a variety of bacteria such as Salmonella spices, *Eschericia coli*, *Shigella spp.*, *Enterococuss* spp., *Staphylococuss* spp., *Streptococuss* spp., *Bacillus* spp., *micrococcuss* spp., and *Psuedomonas* spp., (Davies *et al*., 1997).some of them are endemic in commercial livestock and difficulty to eliminate from animal and production equipment. Because the path mogen in very dominant in animals, there are often present in fresh animal manure and other wastes. Therefore, the pathogens in animals manure and other wastes products pose a very big treat to human and animal health both on and off animal agriculture production equipment if the waste is not absolutely treated and contained (Crane *et al*.,1983; Graczyk *et al*., 2000).

1.2 The objectives of the study are to:

i. isolate and identify potentially pathogenic bacteria in feacal samples obtained from local cattle.

ii. determine the heterotrophic counts of bacterial isolates in feacal samples of cattles.

iii. determine the antibacterial resistance pattern of bacterial isolates from local cattles.

CHAPTER TWO

LITERATURE REVIEW

2.0 COW DUNG

Cow dung can be defined as the undigested residue of consumed food material being excreted by herbivorous bovine animal species. Being a mixture of faeces and urine in the ratio of 3:1, it mainly consists of lignin, cellulose and hemicelluloses. It also contains 24 different minerals like nitrogen, potassium, along with trace amount of sulphur, iron, magnesium, copper, cobalt and manganese. The indigenous Indian cow also contain higher amount of calcium, phosphorus, zinc and copper than the cross-breed cow (Garg and Mudgal 2007; Randhawa and Kullar 2011). Cow dung harbours a rich microbial diversity, containing different species of bacteria (*Bacillus* spp., *Corynebacterium* spp. and *LactoBacillus* spp.), protozoa and yeast (*Saccharomyces* and *Candida*) (Nene 1999; Randhawa and Kullar 2n011). Sawant *et al*. (2007) have isolated many different bacterial genera such as *Citrobacter konseri*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Kluyvera* spp., *Morgarella morganii*, *Pasteurella* spp., *Providencia alcaligenes*, *Providencia stuartii* and *Pseudomonas* spp. from cow dung.

In India, 69.9 % population resides in rural areas (The Hindu 2011), where cow (Bos indicus) is major cattle and generates 9–15 kg dung/day (Werner *et al*. 1989; Brown 2003). Waste is generally meant for discarding because it may act as a source of pollution (Pongrácz and Pohjola 2004). However, if it is used in some other process such as feedstock it may be considered as co-product (Brown 2003). People in Indian villages use cow dung for cooking purpose by direct burning. It is also used in plastering of walls and floor in rural houses for providing insulation during winter and summer. Application of smoke generated from the burnt cow dung as mosquito repellent and subsequent ash as cleaning agent for kitchen utensils is an age old

practice. Accordingly, different usage of cow dung by village peoples reflect the native knowledge associated with it. It also depict that cow plays an important role in village economy and has high socio-economic value (Dhama *et al*. 2005a).

Cow dung in India is also used as a co-product in agriculture, such as manure, biofertiliser, biopesticides, pestrepellant and as a source of energy (Dhama *et al*. 2005a). As per ayurveda, it can also act as a purifier for all the wastes in the nature (Randhwa and Khullar 2011). Therefore in India, Cow (*B*. *indicus*) is not only just milk-producing animal but also truly considered as Gomata (mother of all) and Kamdhenu (Dhama *et al*. 2005a; Jarald *et al*. 2008). Detailed study of cow dung is gaining interest around the world and few attempts have been made for utilising its potential in the field of energy production, pharmaceutical products. The review intends to highlight the possible applications of cow dung particularly in the area ranging from energy, agriculture and environment to medicine for human welfare.

2.1 SOURCE OF ENERGY

Dependence of mankind on non-renewable source of energy such as coal, oil and gases is increasing worldwide. In India, the main source of energy is coal, which accounts for 44 % of total energy consumption. Our country is now facing the shortage of coal supplies despite being the third largest coal producer in the world. According to energy information administration (EIA), our dependency on imported fossil fuels has risen to 38 % (USEIA 2014). Because of the limited availability of coal, an easily available, economical as well as environment friendly renewable source of energy is required. According to the United Nations Food and Agriculture Organisation (FAO), the animal waste on this planet produces around 55–65 % methane, which upon release in the atmosphere can affect global warming 21 times higher than the rate CO2 does.

Biogas, a mixture of different gases produced by anaerobic fermentation of organic matter from methanogenic bacteria, mainly constitutes methane (50–65 %) and CO_2 (25–45 %) (Sharma 2011). One kilogram of cow manure ncan produce 35–40 l of biogas when mixed with equal amount of water with hydraulic retention time (HRT) of 55–60 days maintained at an ambient temperature of 24–26 °C (Kalia and Singh 2004). Li *et al*. (2009) reported 67 ml/g methane yield from anaerobic digestion of cow manure, whose total and volatile solids were 23.4 and 13.8 g/l, respectively. Green bacteria such as *Pseudomonas* sp., *Azotobacter* sp. and other purple sulphur or purple non-sulphur bacteria are known to produce maximum amount of methane gas in comparison to other photosynthetic bacteria present in cow dung (Rana *et al.* 2014). The optimum production of biogas depends upon mesophilic (32–38 °C) and thermophilic (50–55 °C) temperature range (Kashyap *et al*. 2003). The inability of mesophilic microorganisms to survive in psychrophilic temperature range results in 70 % reduced production of biogas during winters in hilly areas (Kanwar and Guleri 1994). This may be due to the collapse of cell energy, outflow of intracellular substances or cell lysis of mesophiles at lower temperature (Gounot 1986). But many researchers reported a fare amount of biogas production under psychrophilic range of temperature using some modifications (Safley and Westerman 1990; Kanwar and Guleri 1994).

Cow dung is the major source of biogas or gobar gas production in India. The total population of female cows in India is 190.90 million out of which 151 million are indigenous whilst 39 million are crossbreed (Livestock Census 2012). Cow dung generated from 3–5 cattle/day can run a simple 8–10 m^3 biogas plant which is able to produce 1.5–2 m^3 biogas per day which is sufficient for the family 6–8 persons, can cook meal for 2 or 3 times or may light two lamps for 3 h or run a refrigerator for all day and can also operate a 3-KW motor generator for 1 h (Werner *et al*. 1989). A 1-m^3 biogas plant has produced 28.78 l/kg (0.028 m^3) and 32.76 l/kg (0.032 m^3) of

biogas respectively when daily feed with 22 kg of dung/m3 which is mixed with equal amount of water with 9–10 % of total solids. The maximum production of biogas from that plant is 39.00 l/kg (0.039 m3) and 40.04 l/kg (0.04 m3) respectively when operated at the temperature of 23.5 °C (Kalia and Singh 2004). On the other hand, farmer also gains 13.87 metric tons of organic fertiliser per year from the biogas plant. This co-production of biofertiliser also allow farmer to recover the initial investment for setting up of a biogas plant (Sharma 2011).

Though cow dung is solely used as the prime source for biogas production, but research continues to verify the potential of other sources for instance, addition of pig dung was found to have an enhanced effect. Mixture of cow and pig dung (60:40) showed 10 % increase in methane production as investigated by Li *et al*. (2014). Use of potato pulp and cow manure in the ratio of 20:80 also produced fair amount of methane in comparison to pure cow dung (Sanaei-Moghadam *et al*. 2014). Besides this, there are reports on comparative studies for biogas production where various feedstocks such as kitchen waste, corn waste and spent tea waste have been used along with cow dung in a ratio of 1:1 producing less average biogas after 25–30 days; however, cow dung alone produced approximately 50 % more biogas than these mixtures (Munda *et al*. 2012), thereby suggesting that other organic sources may produce biogas but cow dung still remains a potential source. In the light of above-discussed facts, biogas production can also be considered as an effective way of treating organic waste which may produce green house gases if remain untreated.

Supercapacitors are the in-between arrangement in electrochemical batteries which can store a large amount of energy that can be delivered with high power for few milliseconds (Gamby *et al*. 2001). They have high power density (103–104 W/kg), long cycle life (>106 cycles), pulse power supply, low maintenance cost, simplicity and better safety compared to secondary

batteries. The use of porous carbon as electrode material is widespread in supercapacitors. This porous carbon is synthesised by many different methods such as using silica or surfactant, aerogels, organometallic compounds, chemical activation and physical activation. All these processes are costly and consume expensive precursors and time (Lee *al. et* 2006; Fang *et al.* 2009; Kim *et al*. 2012; Yang *et al*. 2012; Bhattacharjya *et al*. 2013; Inamdar *et al*. 2013; Bhattacharjya and Sung 2014; Yang *et al*. 2014). Now focus is shifting towards natural biomass as a potential source for carbon precursors. Several natural biomasses have been explored for production of activated carbon (Demiral and Demiral 2008; Hu *et al*. 2010; Li *et al*. 2010, 2011; Wei *et al*. 2011; Xu *et al*. 2012; Biswal *et al*. 2013; Falco *et al.* 2013; Huang *et al*. 2013; Wang *et al.* 2013; Bhattacharjya and Sung 2014). Activated carbon has recently been synthesised from cow dung by a modified chemical activation method, in which partially carbonised cow dung was treated with potassium hydroxide in the ratio of 2:1. The synthesised activated carbon when tested as supercapacitor electrodes in practical showed specific capacitance of 124 F/g at 0.1 A/g and retained up to 117 F/g at 1.0 A/g current density. It is also durable for long-term operations (Yang *et al.* 2012). The synthesis of activated carbon having high surface area along with optimum micropore and mesopore volume reflects excellent electrochemical application of cow dung for supercapacitors. The literature also suggest that biological waste like cow dung can be converted into a electrode material for other energy storage and conversion systems such as Li-ion batteries and fuel cells.

2.2 AGRICULTURE MANAGEMENT

Human population is increasing worldwide giving rise to intensive farming system and unsuitable cropland management that ultimately results in reduced soil fertility (Onwudike 2010; Bedada *et al*. 2014). Extensive use of chemical fertilisers is suggested for replenishment of

nutritional deficiencies to increase crop yield. Many disadvantages of widespread use of chemical fertilisers include increase in soil acidity, mineral imbalance and soil degradation (Kang and Juo 1980; Ayoola and Makinde 2008) and even farmers nowadays do not prefer chemical fertilisers (Bedada *et al.* 2014). In composting, microorganisms decompose organic substrate aerobically into carbon dioxide, water, minerals and stabilised organic matter (Bernal *et al*. 2009; Kala *et al.* 2009; Vakili *et al*. 2015). Compost is added into the soil to improve nutrients and water-holding capacity (Arslan *et al*. 2008; Vakili *et al*. 2015).

2.3 BIOREMEDIATION OF ENVIRONMENT POLLUTANTS

Toxic chemicals find their way into the human body, plant tissue and animals through absorption (Adams *et al*. 2014). Active pharmaceutical ingredients (API) serve as a blend of various drugs that are well known to pollute the aquatic environment (Kessler 2010). Agriculture run-off also contributes towards the pollution of water bodies through which water is supplied for human consumption. Presently, in India only 10 % of total waste water is treated and rest is discharged untreated (Singh and Kohli 2012). In industrial treatment plant in Patancheru, near Hyderabad (India), 0.9 mg ciprofloxacin per gram organic matter was found downstream from common contaminated river sediment (Kristiansson *et al*. 2011; Larsson 2014). This condition is not only in India but also in China, U.S. and European countries as discharge of pharmaceuticals is also reported from these regions (Babic *et al*. 2007; Thomas et al. 2007; Fick *et al.* 2009; Kristiansson et al. 2011; Phillips *et al*. 2010; Larsson 2014). These practices are adversely affecting the environment quality which is directly related to the quality of life on earth. Discharge of these toxic compounds imparts negative effect on human health hence rejuvenation of environment is today's utmost need (Dhami *et al*. 2013; Adams *et al*. 2014).

2.4 HUMAN HEALTH MANAGEMENT

Microbial products or their derivatives can kill or inhibit the growth of susceptible pathogenic microbes (Willey *et al*. 2008). However, overuse and misuse of these antimicrobial agents have resulted in the development of resistance amongst pathogens (Aly *et al*. 2012; Sharif *et al*. 2013). At present, bacterial resistance against the antibiotics is of great concern for clinicians, public health officials and researchers as it results in substantial morbidity, mortality and increased cost of treatment (Naiemi *et al.* 2006; Abo-state *et al*. 2012; Aly *et al.* 2012; Jeyasanta *et al*. 2012; Ullah *et al.* 2012; Sharif *et al*. 2013). The pharmaceutical industries and healthcare systems of the world are continuously fighting multidrug-resistant strains of bacteria the last 50 years.

2.5 SOURCE OF MICROBIAL ENZYMES

Microbial enzymes have got immense application because microbes can easily be cultivated and their enzyme can catalyse wide variety of hydrolytic and synthetic reactions (Illavarasi 2014). Many microbial enzymes have been isolated and studied for their industrial and commercial uses. However, still there is a continuous search for the potential microorganisms that are able to synthesise industrially feasible enzymes and microbial diversity of cow dung makes it a potential source for the said purpose (Dowd *et al*. 2008). *Bacillus* spp. from cow dung is capable of producing cellulose, carboxymethyl cellulose and cellulase (Das *et al*. 2010; Sadhu *et al*. 2013; Illavarasi 2014). In case of poor enzyme production, genetically improved strains can be constructed for enhanced enzyme production. For instance, Sadhu *et al*. (2014) described that cow dung *Bacillus* spp. can be mutated with NTG to increase the cellulase production from 9.4 to 16.3 U/mg proteins.

2.6 PATHOGENS ASSOCIATED WITH COW DUNG

2.6.1 ESCHERICHIA COLI

Escherichia coli is a gram-negative, facultative anaerobic, rod-shaped and motile bacterium that exists as a normal flora in the intestinal tract of both healthy animals and humans. It serves as a reliable indicator of fecal contamination and equally indicates a possibility of the occurrence of enteropathogenic and/or toxigenic microbes found in food and water, thereby presenting with public health hazards (Tililawo et al). E. coli O157:H7 found in cattle manure has been reported as the most notorious pathogen which produces a potent toxin that can cause serious infection in humans. This strain can be called verocytotoxic (VTEC) or enterohemorrhagic (EHEC) or Shigatoxin producing (STEC) E. coli. According to Gerba and Smith. owing to the pathogen's low infective dose ascribed to as few as 10 cells and high pathogenicity, it is incriminated as the causative agent of gastrointestinal diseases (gastroenteritis). In addition, Karmali (38) highlighted that the bacterium equally causes attaching and effacing (A/E) lesions by translocating effector proteins into the host cells via a type III protein secretion system. However, cattle harboring this E. coli strain do not develop clinical disease, but serve as the main reservoir for E. coli O157:H7 (Callaway *et al*.2009) Furthermore, humans may be infected with pathogenic E. coli by way of either consuming contaminated food or water or directly by contact with livestock feces and person to person transfer (Mathews *et al* 2013). In adverse/unfavorable environmental conditions (e.g., starvation), the bacterium alters its physiological or metabolic responses in order to persist in the environment without necessarily modifying its structure like most Gram positive bacteria that form resistant spores . In addition, it is acid resistant and can equally form biofilm.

2.6.2 SALMONELLA

Salmonella species belong to the family of *Enterobacteriaceae* and are Gram negative, short, plump shaped rods, non-spore forming, motile, non-capsulated, aerobic and facultative anaerobic organisms (Godfrey and Farrel 2005). These pathogens could be found in a range of animals, including dogs, birds, cats, cattle, pigs and humans and are responsible for the infection called salmonellosis. Salmonellosis occurs via ingestion of food or water contaminated with animal feces or by direct contact with animal feces and is characterized by three major symptoms viz. septicemia, acute enteritis and chronic enteritis. However, the manifestations of salmonellosis vary depending on the species/strain and the host type (Godfrey and Farrel 2005).

Salmonella species viz. *S. Dublin*, *S. Typhimurium* and *S.* Newport constitute a major public health issue since they cause infections in both cattle and humans . More elaborately, Salmonellae have developed increasing resistance to a wide spectrum of antibiotics (i.e., multidrug resistance, MDR) traditionally employed in the treatment of salmonellosis. These MDR strains are vital because of their resistance to available antibiotics, which are critical in the treatment of bacterial infections. However, the resistance could result in high death rates and also present an opportunity for epidemic outbreaks that may be difficult to manage (Nagshetty *et al* 2010)

2.6.3 CAMPYLOBACTER

Campylobacter species are most commonly found in the intestinal tracts of animals such as cattle, pigs, chickens, wild-living mammals and birds (Baserisalehi *et al,* 2007). They are known to cause a wide variety of disorders in cattle, sheep, and pigs. For example, Salihu and coworkers (Balihu *et al,* 2009) mentioned that *Campylobacter jejuni* and *Campylobacter fetus* caused

abortion, stillbirths and birth of weak lambs in sheep at some stage in late pregnancy. Similarly, when transmitted to humans when consuming undercooked agricultural food products and contaminated water (Jaderlund *et al*, 2011), it causes an infection called campylobacteriosis, which is a self-limited and sporadic illness (Adebkule *et al*, 2009,Inglis 2010). In addition, *C. jejuni* and *Campylobacter coli* are the two most important species associated with human bacterial gastroenteritis worldwide (Sheppard 2009, Roskosz *et al*, 2014)

2.6.4 LISTERIA MONOCYTOGENES

Listeria monocytogenes is a Gram-positive, facultative, intracellular, rod-shaped bacterium incriminated as the causative agent of a severe food borne illness in humans contacted through the fecal-oral route and characterized by localized cutaneous infection. Dairy cows serve as the main reservoir and high levels of the pathogen occurs in animals fed with improperly fermented silage contaminated by growth on manure-fertilized soils. Animal listeriosis is manifested as encephalitis, meningitis, septicemia and abortions. The organism's ability to withstand heat, freezing and refrigeration temperatures have been a call for concern in the food industries since raw foods, unpasteurized milk, raw vegetables, raw and cooked poultry and refrigerated foods could serve as vehicles of transmission (Santomm 2012 and Azizoglu 2009). This characteristic is contradictory since it is a non-spore former. Notwithstanding, it can grow over a wide range of environmental conditions (pH and temperature) thus a longer survival duration in the environment (Uzeh and Adepoju 2013)

2.6.5 YERSINIA ENTEROCOLITICA

Yersinia enterocolitica, a member of the genus *Yersinia* is a Gram-negative coco *Bacillus* spp.belonging to the family of *Enterobacteriaceae*. It is unsporulated, non-capsulated and lives as an intestinal flora in many species of wild and domestic animals, including cattle as well as humans (Johannessen *et al*, 2012). It causes yersiniosis in both animals and humans manifested as lymphadenitis, acute enterocolitis, nosodum erythema, septicemia, poliartritis and even death. According to Tirzui and colleagues the cases of yersiniosis infections throughout the world are under reported and the prevalence/incidence varies from country to country owing to the availability of materials, laboratories and engagement of specialists. The species enterocolitica presents with several serotypes, however, Y. enterocolitica O:9 demonstrate cross reactivity of its smooth liposaccharide with that of Brucella, which masks the diagnosis of brucellosis caused by the latter bacterium (Chenaise *et al*,2012).. Its indirect route of transmission is mainly oral ingestion of contaminated foods of animal and plant origins (e.g., pork, beef, lamb, unpasteurized milk, raw milk, vegetables, ice creams and seafoods) as well as contaminated water (Bonardi *et al*, 2007, Graves *et al*, 2009).

2.6.6 ENTEROCOCCUS

Enterococcus species represent a subgroup of the group D fecal *Streptococcus* spp. and a a coccus, they are spherical in shape and occur either singly, in pairs or as short chains(Graves *et al*, 2009). They are Gram-positive, facultative anaerobic, lactic-acid producing bacteria that live as commensal bacteria in the gastrointestinal tract of humans and animals.Members of this subgroup include *Enterococcus faecalis*, *Enterococcccus faecium*, Enterococcus *gallinarum* or *Enterococcus casseliflavus*, *Enterococcus mundtii* and *Enterococcus avium*, which exhibit

significant differences in the incidence of virulence factors, antibiotic resistance genes and distribution in fresh and dry cattle manure((Graves *et al*, 2009, Eaton and Gasson 2001, Franz *et al*, 2001, Weaver *et al*, 2005).

2.6.7 MUNDTII

Mundtii is the species most commonly reported in cattle manure. Generally, the enterococci are considered avirulent and harmless; however, *E. faecalis* and *E. faecium* are notable opportunistic pathogens causing nosocomial infections in humans. According to the United States Environmental Protection Agency recommendations, enterococci and *E. coli* are the two bacteria regarded as indicator organisms used to determine the sanitary quality of recreational waters across the globe.

2.6.8 MYCOBACTERIUM

Mycobacterium species are acid-fast bacteria reported to be found in cattle manure. They can survive in store manure/environment for long duration since they can withstand fluctuations in temperature and pH, dehydration and exposure to sunlight. This predisposition is ascribed to their acid fast characteristic established by the high lipid and wax content of the mycobacterial cell wall. In addition, the cell wall components confer upon these bacteria the propensity to repel or not to absorb water causing them to demonstrate less susceptibility to some chemical disinfectants (Russel, 1996).

Mycobacterium avium subspecies paratuberculosis or *M. paratuberculosis* is the primary species reported in manure and is the causative agent of Johne's disease (paratuberculosis), a chronic disease of the intestine. It is equally linked to Crohn's disease (a chronic inflammatory bowel disease) in humans. Following infection of the herd, the pathogen can be shed in feces for a long

time prior to the physical manifestations of signs and symptoms associated with the disease; in this way, the condition creates great economic loss (to the producer or dairy industries) noticeable as shortened animal's lifespan, reduced milk production, reduced carcass value and poor reproductive performance. However, *M. bovis* or *M. tuberculosis* could be excreted through feces only by a small fraction of *tuberculous* animals and can be transferred through aerosolized manure emanating from tank agitation and or land application of slurry.

Bacillus and *Clostridium* species are spore forming bacteria that are commonly found in cattle manure. They belong to the classes *Bacilli* and *Clostridia* respectively, but both belong to the phylum Firmicutes (Girija *et al*, 1996). They have the tendency to form spores and persist in that resistant and dormant state when growth conditions are unfavorable but revert to vegetative cells through germination as growth conditions becomes favorable. As spores, they are insensitive to heat, desiccation and disinfectant; consequently, these bacteria were detected in cattle manure though in reduced numbers after pasteurization at 70 °C for hours in a study conducted by Marañón and colleagues (Maranon *et al*, 2006).

Bacillus species identified so far in manure entailed *B. anthracis*, *B. cereus*, *B. subtilis*, and *B. thuringiensis*, which are Gram-positive rods, aerobic spore formers and are mostly harmless and can persist for over years in the soil. The most peculiar of these species is *B. anthracis* that causes anthrax (a life threatening and dreaded disease) especially the pulmonary form through the inhalation of the bacterial spores (Bravata *et al*, 2007). The class *Clostridia* consists of Gram-positive anaerobic spore-forming bacteria that are ubiquitous in the gastrointestinal tract (Girija *et al*, 2013) and can be divided into two groups based on their ability to invade and multiply within living tissues. *Clostridium tetani* and *Clostridium botulinum* belong to the first group with little or no ability to invade and multiply in living tissues; however, their pathogenicity is

manifested by the production of powerful toxins. The second and larger group constitutes *Clostridium chauvoei*, *Clostridium septicum*, *Clostridium haemolyticum*, *Clostridium sordellii*, *Clostridium perfringens*, *Clostridium difficile,* and *Clostridium spiroforme* that are able to invade and multiply in living tissues or the intestines of the host animal. In addition, they produce less potent toxins in comparison to the former group. Based on the individual species, some are responsible for diseases, including mastitis, blackleg, hemoglobinuria, malignant edema and infant botulism in cattle and humans, respectively (Williams, 2014).

In a nutshell, it is remarkable that infections caused by the above-mentioned pathogens could be prevented or controlled through the implementation of stringent sanitation policies and appropriate hygiene measures by food producers and better personal hygienic practices by individuals.

CHAPTER THREE

MATERIALS AND METHODS

3.0 STUDY AREA

This study was carried out at the Laboratory Department of Microbiology, Faculty of Life Science, University of Benin.

3.1 SAMPLE COLLECTION

A total of four (4) cow dung samples were used for this study. They were a kind gift from Dr. A. S. Aziegbemhin.

3.2 SERIAL DILUTION

The total heterotrophic bacterial counts were determined by serial dilution. Briefly, One (1) gram each of cow dung was weighed into 9ml of sterile normal saline and homogenized to form the stock solution. From the stock solution, 1 ml was transferred into test tube labelled 10^{-1} and mixed. Using a new sterile pipette each time, 1ml of 10^{-1} was transferred to another tube labelled 10^{-2}. The same procedure was repeated until 10^{-5}. One (1) ml from tube 10^{-5} was discarded. This was repeated for all sample (Cheesbrough, 2006).

3.3 ENUMERATION OF BACTERIA

One (1) ml each of serially diluted samples from tubes 10-4 and 10-5 were pipetted onto sterile agar plate. Twenty (20) ml each molten Nutrient Agar (HiMedia, India) at 45 ^{o}C were aseptically dispensed onto the agar plate and gently rocked. The plates were allowed to solidify and incubated upside down at 37 ^{o} C overnight. Colonies formed on the plates were counted accordingly (Cheesbrough, 2006).

3.4 ISOLATION AND IDENTIFICATION

Identification was carried out using standard microbiological procedures such as: cultural characteristics(size, shape, arrangement, staining characteristics, mortility and spore formation) colonial characteristics (texture, elevation, margin and colour) biochemical characteristics(catalase, oxidase, citrate utilization, urease and indole production) were interpreted to determine the presumptive nomenclature of the bacteria isolates using Bergey's Manual of Determinative Bacteriology and ABIS online-advanced bacterial identification system (Sorescu and Stoica, 2021). The pure identified bacterial isolates were used for antibiotic susceptibility testing.

3.5 ANTIBIOTIC SUSCEPTIBILITY TEST

The antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion Bacterial suspensions equivalent to 0.5 MacFarland standard were aseptically spread on solidified Mueller-Hinton agar (Himedia, India) using sterile cotton swab sticks. Inoculated plates were allowed to dry. Afterwards, the antibiotic multi-discs at a spatial orientation of 16 mm (distance between discs) were aseptically placed on the inoculum using sterile forceps. The antibiotics used in this study and their concentrations were azithromycin (12 µg), levofloxacin (20 µg), erythromycin (10 µg), gentamicin (10 µg), pefloxacin (10 µg), ampicillin-cloxacillin (30 µg), Cefuroxime (20 µg), amoxicillin (30 µg), ceftriaxone-sodium (25 µg) and ciprofloxacin (10 µg) (Abtek, UK). The results of the diameters of the zones of inhibition were interpreted by comparing them with the Clinical Laboratory Standards Institute standards (CLSI, 2012) and each isolate was recorded as resistant, intermediate or susceptible to the various antibiotics. Multidrug resistance among the isolates was defined as resistance to $\geq$ 3 classes of antibiotics.

CHAPTER FOUR

RESULTS

Table 1 shows the total bacterial count from cow dung.

A total of four (4) samples of cow dung was analyzed. The count range of 1.5×10^{3}cfu/g-9.8×10^{5}cfu/g. The highest count was obtained in sample C while sample B had the lowest count.

Table 1: Total Heterotrophic Bacterial Count of Cattle Dung

Sample	10^{-4}	10^{-5}
A	1.82×10^{5}	3.1×10^{4}
B	1.5×10^{3}	1.4×10^{4}
C	1.5×10^{3}	9.8×10^{5}
D	1.5×10^{3}	1.25×10^{5}

Table 1 shows the distribution of bacteria isolates in cattle dung

Streptococcus sp., *Escherichia coli*, *Salmonella* sp., *Enterococcus* sp., *Shigella* sp., *Bacillus* sp., and *Staphylococcus* sp.

Table 2: Distribution of bacterial isolates in samples

Isolate	A	B	C	D
Strept. Sp.	4(100)	2(50)	0(0)	1(25)
E. coli	0(0)	1(25)	0(0)	0(0)
Enterococcus sp.	0(0)	1(25)	3(75)	1(25)
Salmonella sp.	0(0)	0(0)	1(25)	0(0)
Shigella sp.	0(0)	0(0)	0(0)	1(25)
Bacillus sp	0(0)	0(0)	0(0)	1(25)
Staph. Sp.	0(0)	1(25)	0(0)	0(0)

4.3 Results shows antibacterial resistance pattern.

100% effective against the pathogens isolated while *Shigella* and *Staphylococcus* are sensitive to all the antibacterial agent.

Table 3: Antibacterial Resistance of Bacterial Isolates from Local Cattle Dung

Isolates	n (%)	Antibacterial Agents									
		AZ	LEV	E	PEF	CN	APX	Z	AM	R	CPX
Streptococcus sp.	6 (60)	0(0)	0(0)	1(16.67)	0(0)	1(16.67)	3(50)	2(33.33)	2(33.33)	1(16.67)	0(0)
E.coli sp.	2(20)	1(100)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Enterococcus sp.	1(10)	0(0)	0(0)	1(20)	0(0)	0(0)	1(20)	2(40)	0(0)	0(0)	0(0)
Salmonella sp.	1(10)	0(0)	0(0)	0(0)	0(0)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)
Bacillus sp.	1(10)	0(0)	0(0)	1(100)	1(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Shigella sp.	1(10)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
Staphylococcus sp.	1(10)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

KEY: AZ: Azithromycin, LEV: Levofloxacin, E: Erythromycin, PEP: Pefloxacin, GN: Gentamycin, APX: Apicicillin-cloxacillin, Z: Cefuroxime, AM: Amoxicillin, R:Ceftriaxone-sodium, CPX: Ciprofloxacin.

CHAPTER FIVE

5.1 DISCUSSION

Cattle dung serves as a reservoir for most potentially pathogenic microorganisms. These animals are not confined but allowed to roam. There is possibility of pathogens finding their way into the environment. Data on feacal bacteria from local cattle (muturu) unlike its fulani relative is however scarce. The current study isolated and characterized bacteria from local cattle muturu dung with a view to determining their pathogenic attributes and antibacterial resistance pattern. Results from this study showed the mean heterotrophic bacterial counts from local cattle muturu ranged between $1.5x10^{3}$cfu/g-$9.8x10^{5}$cfu/g. This is in agreement with the research of Daniella and Gabriele (2009). This could be attributed to influence of diet, similarity in bacterial composition of the digestive tract as well the time of analysis. However, Rosel *et al* (2013) recorded a higher range variation in the heterotrophic counts. This may be attributed to various environmental factor and time of analysis.

The bacteria isolated from cattle dung in this study include Streptococcus spp., *Escherichia coli*, *enterococcus* sp., *Salmonella* sp., *Shigella* sp., *Bacillus* sp. and *Staphylococcus* sp. However, there are relatively few work on the isolation of bacteria from local cattle dung. *Streptococcus* sp.and *Bacillus* sp. were predominant while *Shigella* sp. being the least. This is in agreement with the work of Funoso and Folakemi who isolated *Escherichia coli* and *Shigella* sp., *Streptococcus* sp. and *Escherichia coli* were predominant apparently due to their ability to survive in the gastrointestinal tract of cattles. Another reason for the predominance could be due to their ability to form biofilm. Biofilm is essential for resistance to adverse environmental condition by bacteria. However the attributes behind the least occurance of *Shigella* sp. could be

because of the acidic nature of the intestine of ruminant animals. The antibacterial resistance pattern showed that *Bacillus* sp. was 100% resistant to erythromycin and perfloxacin. Which is in concordance to the research work of Abu and Wondikom (2018). This may be attributed to the presence of resistance determinants on plasmids with similar selective makers or as a result of independence simultaneous development of resistant to different agents. Similarly, *Eschericia coli* was 100% resistant to azithromycin and levofloxacin . This is concordance to the research work of Abu and wondikom (2018). Reasons behind this could be as the result of resistance developed of time using the antibiotics in treatment of an infection caused by *Eschericia coli* pathogens. The antibacterial resistance conducted shows that *staphylococcus* sp., *Streptococcus* sp. and *Salmonella* sp. showed resistance amoxicillin, ampicillin-cloxacillin and penicillin. They were however not resistant to ciprofloxacin. Ciprofloxacin was proven to be the most effective against Gram positive and Gram negative isolated analzed in the sample of study. This is in agreement with the works of Funoso and Folakemi (2013) and Abu and Wondikom (2018) .Reasons for this resistance could be as a result could be as a result of a result of wide spectrum of the antibiotics. Futhermore, *Salmonella* sp. was 100% resistance to gentamycin. This is also in aggreament to the work of Funoso and Folakemi (2013), which recorded 66.7% resistant of *Salmonella* sp. to gentamycin antibiotics. Reasons behind this could be that *Salmonella* sp. Produced an efflux pumps extruding the antibiotics from bacterial cell wall or that it has developed mutation in its genes that allows it to resist the effect of the antibacterial agents. *Bacillus* sp. and *Eschericia coli* showed the highest resistant rate. This is also in alignment when the work of Fabienne *et al* (2014).This could be as a result of genetic mutation or environmental factor which has greatly contributed to their resistant effect. Resistance of antibiotics has increased to the high rate of infections of local cattle pathogens. Their resistance

could be as a result of enzymatic inactivation, modification of antimicrobial agents alteration of the target sites or expulsion of antibacterial agent effect through efflux pump.

5.2 CONCLUSION

Cow dung host a wide variety of microorganisms. Exploitation of cow dung microflora can contribute significantly in sustainable agriculture and energy requirements. Due to the high bacterial bio burden and drug resistant bacteria in cattle dung, it is suspected that they may find their way into the environment. Local cattle should be confined to avoid transmission of resistant pathogens through their dung.

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APPENDIX

APPENDIX I

Table1: Cultural characteristics, Morphological characteristics and Biochemical characteristics of bacterial isolates from cow dung.

APPENDIX II

MORPHOLOGICAL IDENTIFICATION

Gram Staining

A thin smear was prepared on a clean microscope slide. The smear was stained with crystal violent for 60 seconds and rapidly washed off with distilled water for 5 seconds and cover the smear with Lugol's iodine for 60 seconds and washed off with distilled water. Decolorized using 90% ethanol and washed off immediately with distilled water. Smear was counter stained with safranin reagent for 60 seconds and then washed off with distilled water. The slide was blot dry using paper towel.

The stained cell were examined under microscope with oil immersion using only 100x objective lens. Gram positive cells stain purple or blue while Gram negative cells stain pink or red.

BIOCHEMICAL TEST

Oxidase Test

Procedure: place a piece of filter paper in a clean petri dish and add 2 or 3 drops of freshly prepared oxidase reagent. Using a piece of stick or glass rod, remove a colony of the test organism and Smear it on the filter paper. Positive colonies turn bluish - purple.

Catalase Test

Procedure: 1ml of hydrogen peroxide solution was poured in a test tube. A sterile glass rod was used to collect or remove several colonies of the test organism and immersed. In the hydrogen peroxide solution, the test tube was observed for immediate bubbling of glass which indicate a positive reaction. Equation of reaction: $2H202 \longrightarrow H20+O2$

Indole Test

Procedure: place a piece of filter paper in a clean petri dish add 2or 3 drops of freshly prepared indole reagent. Using a sterile swab stick, pick a colony of the test organism and Smear it on a filter paper. Positive colonies turns greenish.

Citrate Utilization

Procedure: prepare slants of Simmon Citrate Agar in a test tubes as recommend by the manufacturer (store at 2-8°c), using a sterile inoculating loop, the colony is inoculation on the slant and incubated at 35°c for 24 hours. A blue color indicate positive reaction.

Sugar fermentation and production of gases using Triple sugar iron Agar (TSI)

TSI was prepared following manufacturer's instruction and prepared media was placed in a test tube and kept in a slant positive for it to solidify. The slant and butt of the media was inoculated with the test bacterium using a sterile swab stick and it was incubated for 18-24 hours. The results were read on the basis of acid or alkaline production in the slant or butt region of the tube and gas production was confirmed by the presence of crack or air bubbles in the slant or butt region. Moreso, production of hydrogen sulphide was confirmed by the blackening of the medium. A prepared laboratory chat was used for result interpretation in line with microbiological standards as well as other biochemical test carried out on the isolates to confirm or ascertain their identity.

MEDIA PREPARATION

Nutrient Agar 28g of powdered nutrient Agar were dissolved in 1000ml of distilled water and allowed to soak for 10mins and then sterilized with an autoclave for 15 minutes at 121°c and cooled at water bath at the temperature of 45-55°c and pour into petri dishes.

Eosin Methylene Blue

37.5g of powdered EMB Agar was dissolved in 1000ml of distilled water. Shake to homogenized, sterilize using 121°c for 151mintues. Allow to cool at 45-55°c before dispensing into different petri dishes.

Simmons Citrate Agar

24g of powdered SCA was dissolved in 100ml of distilled water, shake for 2 minutes. Dispense into a test tube by adding 5ml and sterilize by autoclaving at 121^0C for 15 minutes. The media was incubated at 37^oc for 48hours.