RISK ASSESSMENT AND ANTIBIOTICS RESISTANCE OF *E.Coli* AND *Salmonella* SPP IN ABATTOIR WASTEWATER IN BENIN CITY.

BY

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UNIVERSITY OF BENIN

BENIN CITY

EDO STATE

NOVEMBER, 2022.

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A PROJECT 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 (HONOURS) IN MICROBIOLOGY

NOVEMBER, 2022.

CERTIFICATION

We certify that this project work was carried out by MOMOH ELIZABETH in partial fulfillment of the requirement for the award of Bachelor of Science (B.Sc.) Degree in Microbiology.

Dr (Mrs) R. Adams — Date

(Project Supervisor)

APPROVAL

This project work is accepted in partial fulfillment for the award of Bachelor of Science, B.Sc. (Hons.) in the Department of Microbiology, University of Benin, Benin City.

_____ _____

Professor (Mrs.) F.I. AKINIBOSUN DATE

(Head of Department)

DEDICATION

This project is dedicated to the Almighty God, my helper who has favoured me during the course of this project.

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I would like to acknowledge God almighty for life, His unending love, guidance, good health, sound mind and the success of this research work. I would also like to acknowledge my parents, late Mr & Mrs Momoh Ogbeifun for their moral upbringing and also the family of Mr Emmanuel Ogbeifun for their financial support, mentorship and moral support, which is the reason I pull through every day. My profound gratitude goes to my supervisor Dr (Mrs) R. Adams and my senior colleague Mr Daniel Itoro for their assistance and support. I can't forget my then and now roomates especially Dr Mrs Vikky Frank and my fellow finals students, i say thank you.

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ABSTRACT

The research was carried out to evaluate the risk assessment and antibiotic resistant of *E.coli* and *Salmonella spp* in abattoir waste water in Benin city. Standard bacteriological methods were used to enumerate the total bacterial count (*E.coli* and *Salmonella spp*) of abattoir waste water using pour plate methods after serial dilution. The bacterial isolates were characterized and identified using morphological and biochemical methods and sugar fermentation test. The percentage distribution and frequency of the isolates were evaluated using statistical method. From the result the total *Escherichia coli* colony count in cfu/ml ranges from 0.7 x 10^3 – 0.8 x 10^3 (cfu/ml) while that of *Salmonella spp* ranges from 0.7 x 10^3 – 0.8 x 10^3 (cfu/ml). Following the cultural, morphological and biochemical characteristics of bacteria from abattoir waste water samples, the result revealed that *Salmonella enterica*, 2 *Escherichia coli* strains, *Salmonella spp*, and *Salmonella typhi*. The antibiotic sensitivity test revealed that all isolates found in this study were multiple antibiotic resistant bacteria. The multiple antibiotic resistant index of these isolates ranges from 0.5 to 0.525 as against the recommended 0.2 by WHO.

CHAPTER ONE

1.0 INTRODUCTION

According to Mittal (2004) and Adeyemi-Ale (2014) waste generated at abattoirs pose a serious threat to the environment because of direct discharges of wastewaters into the ecosystems which most times are not effectively treated. These wastes are high in organics and fats (Asibor 2017). These result to the destruction of primary producers in the water. Biodegradable organic matter in receiving waters create high competition for oxygen within the ecosystem leading to high levels of BOD and a reduction in dissolved oxygen (DO), which is detrimental to aquatic life and also affects sediments and surrounding soil (Ojegunle and Lateef 2017). In a typical Nigerian abattoir, the surrounding land is often marshy due to improper channelling of wastewater arising from the dressing of the slaughtered animals and washings at the lairage (Asibor 2017). Most Nigerian abattoirs are situated close to surface water bodies in order to have access to cheap water supply needed for slaughtered animal processing and to provide a sink for the run-off from meat processing activities (Omole and Longe, 2008). In Nigeria, like in many other developing countries, discharge of untreated wastes into the environment is a major environmental issue. Compromised water quality and poor sanitary conditions of abattoirs in the livestock sector have added in no small way to the lotic water system (Adeyemo *et al*, 2002).

The continuous drive to increase meat production to meet the protein needs of the population is usually associated with some pollution problems (Hinton *et al*., 2000). The pollution problems include air, water, food and soil pollution (Ezeoha and Ungwuishiwu, 2011). One type of waste that is of great concern to both urban and rural areas in Nigeria is the abattoir or slaughterhouse waste (Ezeoha and Ungwuishiwu, 2011). Abattoir is any premises used for or in connection with slaughter of animals whose meat is intended for human consumption, and include a slaughter house, but does not include a place situated on a farm (Abattoir Acts,

1985). Abattoirs all over the world are known to, directly or indirectly, pollute environment through the various processes (Neboh *et al*., 2013). This is because less than 1% of the world's fresh water about 0.007% of the overall water on earth is readily accessible for direct human use (UNESCO, 2006). Abattoirs are usually located near water bodies in order to gain access to water for processing (Neboh *et al*., 2013). Yola abattoir is not an exception, as it is located near River Chouchi (Akindawa *et al*., 2009). Large amounts of waste water used by the abattoir drains into the surrounding environment (Amisu *et al*., 2003). These wastes typically contain fat, grease, hairs, feathers, manure, grit, undigested feed, blood, bones (Nafarnda *et al*., 2006; Osibanjo and Adie, 2007). Organic and inorganic solids, and salts and chemicals added during processing operations (Rabah *et al*., 2010). urine and aborted foetus (Akindawa *et al.*, 2009).

In ruminant animals, the first stomach or paunch contains undigested materials called paunch manure, which contains long hairs, whole grains and large fragments (Akindawa *et al*.,2009). The excreta poses undigested feed, mostly cellulose-fibre, undigested protein, excess nitrogen from digested protein, residues from digested fluids, waste minerals, worn-out cells from intestinal linings, mucus, bacteria and foreign matter such as dirt consumed, calcium (Ca), magnesium (Mg), iron (Fe), phosphorus (P), sodium (Na) et cetera (Neboh *et al*., 2013). These could increase the levels of nitrogen (N), phosphorus (P) and total solids in receiving environments considerably (Omole and Longe, 2008) or introduce certain elements such as iron (Fe), lead (Pb), zinc (Zn) and calcium (Ca). Present in minute quantity, and make them the leading chemicals, thus, altering the physicochemical nature of the soil (Tortora *et al*., 2007). Some of these chemicals may be toxic to the microbial, floral and faunal community of the soil (Rabah *et al*., 2008). The resultant consequences could be the degradation of soil fertility due to accumulation of certain nutrients and heavy metals that may lead to low productivity in the surrounding farm lands, in addition to the damages and destruction of

aquatic lives (Rabah *et al*., 2008). Over the years Nigeria has witnessed an increased in population which resulted to increased in agricultural production with increased in cultivable area of production.

Abattoir effluent is complex in composition and may exert an effect on the environment. Abattoir waste comprises of organic and inorganic solids, blood, fat, long hair, faeces, undigested feed materials, large fragments of plants, worn out cells, intestinal lining, mucus, water, chemicals mostly cellulose-fibre, calcium, magnesium, iron, phosphorus, sodium, ash and microorganisms etc. (Ubwa, *et al*., 2013, Ezeoha and Ugwuishiwu, 2011). Waste effluent could alter the physicochemical properties of the soil (Rabah *et al*., 2008). The indiscriminate discharge of waste waterinto the soil could cause certain elements (example, phosphorus and calcium etc.) previously absent or present in minute quantities to be introduced leading to the increase in the content of these chemical elements and thus altering the physicochemical nature of the soil (Tyagi *al*., 2013).Continuous discharge of abattoir effluent into the soil could fix or complex plant nutrients thereby making them unavailable for plant use thus affecting the fertility of the soil which may lead to low productivity in the surrounding farmlands (Rabah *et al*., 2008).

Meat has been part of human diets for most of mankind's existence. Beef, is a high source of protein, which contains all the essential amino acids and iron. The iron found in meat is in the form of heme iron, which is better absorbed than the non-heme iron found in plants (Zahedi *et al*., 2018). Through the ages, a need existed for controlling the activities of producers, processors and manufacturers of food intended for human consumption. This need led to the development of food laws in South Africa (Department of Health, 2006). Furthermore, public awareness of meat associated health hazards, such as heart attacks (Eryuruk *et al*., 2018) and fatal *E. coli* bacterial infections (Ethelberg, Smith, Torpdahl, Lisby, Boel, Jensen, Nielsen and Mølbalk , 2009) led to the development of stringent legislative requirements to ensure the

supply of safe food products. In the 1960s, there was a concern about poor hygiene at South African abattoirs. This inadequacy was addressed by creating the Abattoir Commission under the Abattoir Industry Act 54, 1976. This commission then developed hygiene norms and standards for abattoirs and became the only body with the authority to approve applications for building new abattoirs in South Africa (Department of Agriculture, 2007). As a result, the red meat industry became extensively regulated by the Abattoir Commission.

The meat industry was characterized by the following: restrictions on the movement of meat between different areas, compulsory auctioning of carcasses according to grade and mass in controlled areas and supply control via permits and quotas ((Rabah *et al*., 2010). The South African meat industry has gone through many regulatory changes since 1994 (Red Meat Abattoir Association, 2006). 2 In South Africa, abattoirs are graded A, B, C, D or E according to throughput (number of animals slaughtered per day). For example, Grade A abattoirs have a throughput > 100 slaughter units, while grade E abattoirs have a throughput of one to eight slaughter units per day (DoA, 2007).

To ensure standardized hygiene practices at all abattoirs, the DoA introduced the Hygiene Assessment System (HAS) in 1999. HAS is used to quantify the hygiene standards at abattoirs, against national standards. The Gauteng Provincial DoA, Directorate: Veterinary Public Health (GPDoA: VPH) introduced a rating scheme, Abattoir Hygiene Rating Scheme (AHRS) in 2003. Although participation in AHRS is voluntary, abattoirs that achieve high ratings receive special certificates and are made known to the public. AHRS ratings range from five stars (90 and above), four stars (75 to 89) and three stars (60 to 74). Abattoirs that score a rating below 60 do not receive any awards (DoA, 2003). The significance of a higher rating is the assurance that the products derived from such an abattoir are of high quality, with a longer shelf life from a hygiene point of view (RMAA, 2004). A rating awarded to an abattoir is valid for twelve months, after which the rating is reassessed. South African

abattoirs that export meat and meat products to the European Union are, in addition to HAS, are required to implement the HACCP system, based on European Union requirements (Umaru *et a*l., 2018). The DoA allocates resident official state veterinarians to these abattoirs and the importing countries(including Nigeria) periodically carry out physical audits of the HACCP plans of these abattoirs.

1.1 Aim and Objective Of Study

The aim and objective of this study is to ascertain the presence of bacteria (*E. Coli* and *Salmonella spp*). From the disposal of abattoir effluent in Benin City and also evaluate the distribution of multidrug resistant bacteria(*E. Coli* and *Salmonella spp*) isolated from abattoir wastes in Benin city.

CHAPTER TWO:

2.0 LITERATURE REVIEW

2.1 Abattoir Pathogen

Escherichia coli were first isolated by a German paediatrician, Theodore Esherich, in 1884 from faeces of human neonates (Khan *et al*., 2016). The bacterium was then called Bacterium coli commune. Most *E. coli* strains are harmless commensals, however, some strains are pathogenic and cause diarrhoeal diseases. *E. coli* strains that cause diarrhoeal illness are categorized into specific groups based on virulence properties, mechanisms of pathogenicity, clinical syndromes and distinct O:H serogroups. These categories include enteropathogenic *E. coli* strains (EPEC), enteroinvasive *E. coli* strains (EIEC), diffuse adhering *E. coli* strains (DAEC), enteroaggregative *E. coli* strains (EAEC) and enterohaemorrhagic *E. coli* strains (EHEC). *E. coli* O157:H7 and many *E. coli* produce verotoxins, hence they have been named VT-producing *E. coli* (VTEC). The first confirmed isolation of *E. coli* O157:H7 in the United States of America (USA) was in 1975 from a Californian woman with bloody diarrhoea, while the first reported isolation of the pathogen from cattle was in Argentina in 1977, while the bacterium was first identified as a human pathogen in 1982 (Fernandez, 2008). The spread of *E. coli* O157:H7 in North America coincided with the importation of infected cattle from Argentina, where the rates of human infection were previously about three times higher than those found in North America (McMicheal *et al*., 2011). *E. coli* O157:H7 is usually a harmless bacterial strain present in the intestines of cattle. Probably, the potentially lethal variant of *E. coli* O157:H7 evolved in Argentinian cattle reservoir when the harmless variant of the bacterium acquired a gene from the deadly Shigella bacterium through a viral agent, resulting in the lethal 8 form (Neboh *et al*., 2013). The resultant strain soon became prevalent in key cattle-growing regions throughout North America. However, for a new bacterial strain to become an infectious disease, it has to be able to survive the media through which it is transmitted to humans (Akindawa *et al.*, 2009). In the Walkerton, Canada waterborne

outbreak of 2000, which affected more than 2,300 residents and resulted in seven deaths (Howaeth *et al.,* 2000), the pathogen had travelled from the intestines of cattle, through the surface water pathways, through the soils into ground water pathways and through the constructed drinking water system to be ultimately consumed by humans, where the pathogen survived the hosts' gastric acid defences (Bridges *et al*.,2001). Contaminated food and water cause 700,000 deaths in Africa annually (Khan et al., 2001).

2.2 Associated Foodborne Outbreaks

E. coli O157:H7 outbreaks have been reported most frequently in developed countries and in over 30 countries on six continents (Boyle, Swerdlom & Griffith, 1995; Chapman, 1994; Griffith & Tauxe, 1991; Mead and Griffith 1998; Nataro and Kaper, 1996; Water, Sharp & Dev, 1991). In Africa, *E. coli* O157:H7 associated outbreaks have been reported in South Africa (Browning *et al*., 1990), Swaziland (Isaäcson *et al*., 1993) and in the Central African Republic (Germanii, Soro, Vohito, Morel and Morvan, 1997), Kenya (Bryant et al.,2003), Gabon (Presterl, Zwick, Reichmann, Aichelburg, Winkler and Kremsner, 2003, Nigeria (Bello and Oyedemi *et al.*, 2009) and the Ivory Coast (Dadie, Karou, Adom, Kette and Dosso, 2000). The outbreak that occurred in Swaziland in November 1992 was the largest recorded in Africa (Longe *et al*., 2017). The infection rate was 42% of 778 screened residents (Raji *et al*., 2006), many of whom had bloody diarrhoea and abdominal pains which distinguished these patients from those who had cholera. Symptomic cholera infection almost always manifests as profuse watery diarrhoea (Eslami *et al*., 2017). The major problem with *E. coli* O157:H7 is that it is not detected by the usual methods used to isolate and identify traditional enteric bacterial pathogens. 9 Moreover, microbiology laboratories in many African countries do not routinely test *E. coli* O157:H7. Hence, many infections may go unrecognized (Iroha *et al*., 2016). Iroha *et al*. (2016) suggested three public health measures (educating the public on dangers of eating undercooked meat, increasing awareness among

clinicians of *E. coli* O157:H7 infection and mandating case reporting) to control infections associated with *E. coli* O157:H7 in Africa. The impact of gastroenteritis is dramatic for developing countries where an estimated 2.5–3.2 million children

2.3 Meat Spoilage

Meat is one of the most perishable foods and its composition is ideal for growth of a wide range of spoilage bacteria. The composition of the meat spoilage flora is greatly influenced by the storage conditions such as temperature and the type of packaging material (Nkansah *et al. (*2019),). Spoilage of aerobically stored meat is dominated by Pseudomonas spp. (Mayr *et al*. 2003). Mayr *et al*. (2003) 13 observed 83 to 100% *Pseudomonas spp*. on aerobically stored meat after 11 days of cold storage. In another study, Ercolini, Russo, Torrier, Masi and Villian (2006) studied microbial spoilage under three different conditions of modified atmosphere packaging, air, (ii) 60% oxygen and 40% carbon dioxed (MAP 1) and (iii) 20% oxygen and 40% carbon dioxed (MAP2) and low storage temperature (5 °C). Spoilage of meat, which varied according to the packaging conditions, occurred between 7 and 14 days of storage. *Pseudomonas spp*. were identified as acting during beef storage in air, while *Pseudomonas spp* and *L. sakei* were the main species found during storage using MAP3. When the numbers of pseudomonads reach around 100 million per gram, at the meat surface, they produce a putrid odour and slime forms (Food Science Australia, 2003). The main defects in meat are off-odours, and offflavours, but discolouration and gas production also occur (Borch *et al*., 1996). The shelf life of meat is considerably increased by vacuum packaging. When oxygen impearmable packaging is used, the growth of gram–positive, mostly LAB is favoured because of increased carbon dioxed levels and lowered oxidative–reduction potential (Borch *et al*., 1996). LAB grow slowly at low temperature. LAB could grow to 10–100 million per gram after about 6 weeks of storage, but do not produce signs of

spoilage. After 14–16 weeks, bitter or liver-like flavours develop (Food Science Australia, 2003).

2.4 Sources and Control of Contamination of Meat And Meat Products

Red meat animals can be infected or carry a wide range of microorganisms, which are potentially pathogenic for man. The most important of these are zoonotic bacteria, principally pathogenic serotypes of *E. coli*, such as O157:H7, *Salmonella and Camplylobacter* spp. (Rahab *et ai., (*2008). However, the presence of Salmonella or Campylobacter on food does not necessarily mean that infection will result. Many Salmonella serotypes common in food animals have not been extensively implicated in human infection, probably because they are less virulent compared to serotypes like *S. enteritidis and S. typhimurium*, or maybe they do not 14 survive in the food chain (Humphrey and Jørgensen, 2006). Risks associated with the consumption of contaminated meat and meat products can be reduced by implementing systematic controls from farm-to-fork. At farm level, strategies including the application of probiotics in animals and diet management may reduce the shedding of *E. coli* O157:H7 by slaughter animals.

Animals presented for slaughter Slaughter animals may arrive at an abattoir positive for different pathogens. Humane treatment of food animals impacts on meat safety and should receive increased attention worldwide (Gradin, 2006). Animal stressing may damage meat quality and lead to more contamination and cross contamination with pathogens due to resultant increased pathogen shedding. Excretion levels of pathogens such as *E. coli* O157:H7 can be higher after transportation, which is associated with stress in animal hosts, leading to the spread of faeces containing high levels of pathogenic organisms on the live animal hide, with subsequent contamination of carcasses during slaughter (Sofos, 2008). Furthermore, during hide stripping, some bacteria originating from the animal hide become suspended in the abattoir atmosphere. This contaminated air may come into contact with food products, i.e.

carcasses, containers, equipment and other food contact surfaces during processing, where they may adhere strongly (Reheem and Movenikeji, 2008). Nevertheless, the major source of carcass contamination is contact with the skin during hide removal or contamination by spillage of stomach contents during evisceration (Humphrey and Jørgensen, 2006). Meat safety can be assured through the development of an integrated control of pathogens, including pre-slaughter strategies, post slaughter or during processing in the plant, at retail and food services and at home (Sofos, 2008). Therefore, the target of control pre-slaughter should be to minimize sources, levels, access and transfer of contamination to animals and produce. Challenges at pre-slaughter include the diagnosis of animals as carriers of pathogenic organisms. Diagnosing cattle on the farm or the feedlot as being "infected" by pathogenic bacteria is difficult because pathogenic bacteria often have little or no 15 effect on the health or production efficacy of animals. Therefore such animals are asymptomatic (Sofos, 2008). (Sofos, 2008). observed that all experimental animals inoculated with *E. coli* O157:H7 in their study remained clinically healthy, with no evidence of diarrhoea. Cattle have been found to be insensitive to the deleterious effects of the toxins produced by *E. coli* O157:H7 and other EHEC (Pruimboom-Brees, Morgan, Ackermann, Nystrom, Samuel, Cornick & Moon, 2000). Detection of *E. coli* O157:H7 is also complicated by the fact that faecal shedding can be sporadic, with an animal testing positive for EHEC one day, but not again for several days or even weeks (Sofos, 2008).

2.5 Abattoir waste

The abattoir industry is one of the industries that contributes to the problem of possible foodborne disease and potential health hazards associated with food especially meat, by improper handling of condemned material (Adeyemi and Adeyemo, 2007). Abattoir waste is defined as waste or waste water from an abattoir which could consist of pollutants such as animal faeces, blood, fat, animal trimmings, condemned organs and carcasses, paunch

content and urine (Department of water Affairs (DWAF), 2001), together with inedible organs. Inedible offal includes skin, ears, gall bladder, foetuses, hair, hooves, snouts and horns (DoA, 2007). Abattoir waste material carries a high level of microorganisms that may cause disease in humans and animals, such as Salmonella and Escherichia coli bacteria (Brown, 2006). According to South African legislation (DoA, 2007; DWAF, 2002), abattoir waste has to undergo pre-treatment before it can be disposed of. The Red Meat Regulations of 2004 (DoA, 2007) stipulate that any condemned material must be disposed of by means of a total incineration, denaturing and burial at secure sites. The burial sites have to be approved by the Provincial Executive Officer and the local government, wherein access should be controlled to avoid illegal removal of condemned material. Furthermore, in cases where condemned material is sterilised, the sterilization facility has to be registered. However, enforcement of these regulations is questionable because raw abattoir waste including animal carcasses has 16 been found on landfill sites in some provinces of South Africa (Makana and Municipality, 2004). Roberts, de Jager and Blight (2009) demonstrated that the majority of the abattoirs in their study did not adhere to the legislation. For example, in cases where condemned material was disposed of by burying, it was evident that it had not been burnt before burying as a legislative requirement. Furthermore, incineration was not properly achieved because in most cases both abattoir managers and workers failed to adhere to the required temperature for proper incineration of abattoir waste. Moreover, in cases where municipal landfill site was used, it was found that access was not controlled. South Africa is an upper-middle-income country (Federation of International Trade Associations (FITA), 2010). Despite its wealth, the experience of the majority of South African households is either one of outright poverty or of continued vulnerability to becoming poor (Agbola, 2003). This situation forces poor communities to seek any possible food source which includes landfill sites, dumping grounds and disposable sites used by abattoirs (Derbyshire, 2003).

This situation creates an opportunity for human beings (scavengers) living off refuse sites to consume condemned abattoir products and carcasses, which could result in significant health problems leading to possible disease and death (Omole and Longe, 2008). Hepburn, McRae and Ogden (2002) demonstrated that *E. coli* survives in abattoir waste products stored at a low temperature and increases in numbers at abusive storage temperature. In addition, Avery *et al*. (2009) showed that heat treatment (60 and 50 °C for 10 min) of abattoir waste that has been artificially inoculated with *E. coli* O157:H7 did not achieve a complete kill of the pathogen. Therefore, it is imperative for the South African meat industry to develop strategies and mechanisms that may ensure proper handling of abattoir waste. In addition, governmental policing strategies need to be improved.

On-farm control strategies On-farm pathogen reduction programmes contribute to the control of food safety problems by reducing both the probability of pathogen presence and environmental 17 pollution. Such control measures directly minimize water and produce contamination and direct animal-human transmission of pathogens (Sofos, 2008). Different synergetic strategies can be used to reduce the levels of pathogens in live animals including the use of probacterial and antipathogen strategies and dietary and management strategies.

Use of probiotics in animals

Probiotics are defined as commensal (harmless or beneficial) bacteria used to reduce pathogenic bacteria in the gut (Mittal, 2004). Probiotics beneficially affect the host by improving its microbial balance. Such commensal organisms in the gut can be competitive or antagonistic to foodborne pathogenic bacteria. Probacterial strategies can be categorized into two groups: 1) the introduction of a “normal” (non-pathogen containing) intestinal microbial population (probiotics) or 2) providing a limiting substrate (prebiotic) that is not digestible by the host animal but which may allow an already existing microbial population to expand its

niche in the gastrointestinal population. The concept of addition of an exogenous bacterial population to the intestinal tract is called Competitive Exclusion (CE). The CE culture may be composed of a single or multiple strains of a single bacterium, or even several different species of bacteria. The CE culture will limit the population of pathogenic bacteria by competing for limited nutrients, by binding sites along the gut epithelium or by producing toxic compounds (Sofos, 2008). (Sofos, 2008). carried out a study in which experimental animals were inoculated with probiotic bacteria and the control animals were not. The authors used *E. coli* strains, excluding *E. coli* O157:H7 for their probiotic population. (Rabah *et al*., 2010). detected *E. coli* O157:H7 in rumen fluid of experimental animals for an average of 14 days post-inoculation with probiotic bacteria, whereas from the rumen fluid of control animals *E. coli* O157:H7 was detected for 26 days. (Rabah *et al*., 2010). concluded that treatment of cattle with probiotic bacteria can reduce the level of carriage and faecal shedding of *E. coli* O157:H7 and may thereby reduce environmental contamination 18 with *E. coli* O157:H7. In another study, Brashears *et al*. (2003) reported the effective use of L. acidophilus culture in the diet of finishing feedlot animals in decreasing *E. coli* O157:H7 shedding by more than 50%. Faecal shedding of *E. coli* O157:H7 is directly correlated with levels of carcass contamination (Elder *et al*., 2000), emphasizing that the live animal is a critical link in the production chain.

2.6 Control measures at abattoir level

Animal coats are a significant source of microbial contamination of the carcass (Hudson, Mead and Hinton, 1998). Many countries apply a subjective ordinal visual rating system to food animals presented for slaughter, categorizing animals by degree of coat cleanliness (Small *et al*., 2005). Animals assessed as clean are normally processed for human consumption. However, research on cleanliness of the animal coat has demonstrated that visually clean cattle often carry pathogens, for example, *E. coli* O157:H7 and *Salmonella spp*

(Avery *et al*. 2002; Small *et al*., 2002) confirming that hides should always be considered as posing a serious risk to meat safety. All operations during slaughter and dressing of food animals that involve penetration of the skin such as penetrative stunning of animals, also in South Africa, carry a risk of introducing pathogenic bacteria from the skin onto edible parts of the animal as penetrative stunning pistols are not sterilized between animals (Buncic, McKinstry *et al*. 2005). Buncic *et al*. (2002) demonstrated in their study investigating the role of the penetrative pistol in spreading microbial contamination to edible parts and the abattoir environment that microbial contamination association with penetrative stunning can spread from the brain to the edible parts of the same animal, including muscles via the blood circulation. In addition, Buncic *et al*. (2002) observed that positive detection of the marker organisms in their study varied between individual animals, between types of tissues/organs and between types of the marker organism. The authors then speculated that the variation could have been due to factors including: (a) differences in stunning-associated damage of the brain blood vessels between individual animals, which could cause different counts of the bacteria to enter the blood circulation, (b) differences in post-stunning rate and/or duration of the heart activity between individual animals, which could cause different transfer of bacteria through the animal bodies, and (c) difference in volume/mass of blood, tissue 22 and organs between individual animals, which could result in different "dilution" factors for the counts of the marker organisms. Blood and liver were most commonly contaminated (in 90% of animals), followed by the lungs and spleen (in 80% of animals), deep muscle (in 20% animals) and on the carcass surface (in 50% of animals). In this study penetrative stunning was also positively linked to the spread of contamination to the environment. The marker organisms were present in protective clothing samples collected from the slaughtermen conducting the stunning of animals and samples collected from the pelt (leg, breast and shoulder) of the stunned animals. Buncic *et al*. (2002) further demonstrated that penetrative

stunning could spread contamination to subsequently stunned animals if equipment was not cleaned and sterilized between animals. Small *et al*. (2005) evaluated the effects of pre-skinning hide decontamination on carcass contamination. They recorded a positive correlation between the microbial loads on skinned carcasses with those on the hide of the same animal. They concluded that pre-skin hide decontamination would reduce overall microbial loads introduced into the slaughter line environment and onto the dressed carcasses, and hence, improve meat quality and safety.

2.7 Effect of line speed on carcass hygiene

According to (Sofos, 2008). line speed may have serious implications in relation to carcass contamination. The faster the line operates, the more opportunities there are for mistakes to be made and hence for more contamination to occur. The relationship between line speed and carcass contamination is influenced by a large number of factors including operator fatigue, knife skills, length of working day, levels of boredom and the presence or absence of proper management structures such as HACCP. The most important aspect is whether or not the operatives have sufficient time to carry out their jobs. In some countries, the speed line is regulated by the number of carcasses that an inspector can examine in an hour (Rabah et al., 2010).

The hygienic status of dressed carcasses is largely dependent upon the general slaughterhouse hygiene and the skills of the workers revealed that 89% of workers at the abattoir where they conducted their study had no training in safe food handling, and as a result, personal hygiene standards were also found to be low (Sofos, 2008). Furthermore, (Sofos, 2008). showed that the incidence of coagulasepositive staphylococcus (CPS) at one of the abattoirs in their study increased by 33.5% after evisceration compared to counts enumerated before evisceration. This increase corresponded to the heavy contamination of the hands of workers performing

the evisceration task. Desmarchelier *et al*. (1999) found that the hands of 75% of workers at trimming of visible contamination step were contaminated with CPS. Therefore, Desmarchelier *et al*. (1999) concluded that workers' hands could have been a source of carcass contamination with CPS, hence a large increase in counts after chilling for 72 hours. The significance of workers' contribution to carcass contamination was also illustrated by Wagude (1999) who observed a great improvement in the microbiological quality of beef after training workers on sanitation, personal hygiene and hand washing techniques.

2.7.1 Hygiene Management Systems At Abattoirs

Cattle slaughter operations, such as bleeding, dressing and evisceration expose sterile muscle to microbiological contamination that is present on the skin, in the digestive tract and in the environment (Sofos *et al*., 1999). With a view to reducing the risks associated with the presence of food pathogens on carcasses, the need to achieve standardized control systems, and the desire to access international markets, the South African meat industry approached the government for assistance. The DoA co-ordinated a task team representing South African Meat Industries Company (SAMIC), RMAA and GPDoA: VPH. This task team developed the 24 Hygiene Assessment System (HAS) based on the United Kingdom's evaluation/audit system. HAS became a mandatory requirement for all registered abattoirs in 2000 upon its inclusion in the Meat Safety Act 40 of 2000 (RMAA, 2008).

2.7.2 Hygiene Assessment System

In the South African context, HAS is a tool, that is used in conjunction with the hygiene management system (HMS) to improve hygiene standards at abattoirs. The assessments are carried out using a HAS form by hygiene managers (HM) at abattoirs and verified by provincial veterinary public health (VPH) inspectors. Has is a visual inspection of monitoring the 10 criteria on the HAS form to assess compliance to Meat Safety Act 40, 2000 (DoA, 2009). In South Africa, all animals presented for slaughter at any registered abattoir are

examined before slaughter, anter mortem inspection. Antermortem is the first line of defence in protecting the public from potentially harmful meat products by accepting and allowing for slaughter only those animals that are healthy and capable of being converted into wholesome products for consumers (Ojegunle and Lateef, 2017). HAS monitors hygiene and animal welfare status of all registered abattoirs in South Africa to maintain uniform standards of hygiene performance (DoA, 2009). Abattoir operatives at dehiding and evisceration are subjected to training offered by RMAA to ensure that bacterial populations from the hide and intestinal contents are prevented from being transferred onto animal carcasses. Some South African abattoirs that export meat and meat products to the European Union member states, in addition to the use of HAS, have also implemented the Hazard Analysis Critical Control Point (HACCP) system.

2.7.3 Hazard Analysis Critical Control Point

HACCP was jointly developed in the USA by the Pillsbury Corporation and the United States Army Laboratories as a system that would provide a degree of certainty that food was free from pathogens and toxins (Sofos, 2008). HACCP identifies the potential hazards (physical, chemical or microbiological) in the process and then designs the process and control systems to minimize the risks. The implementation of an HACCP system is based on seven principles: conduct a hazard analysis; identify the Critical Control Points (CCP); establish the critical limits; establish monitoring systems; establish corrective action; establish documentation concerning all procedures and records appropriate to these principles and their application; and establish verification procedures (Codex Alimentarius Commission, 2002). HACCP is a preventative control system wherein hazards are identified, critical control points (CCPs) are determined and the methods for control and compliance are clearly specified (Kinsella, Sheridan & Rowe, 2006). International Standards Organization (ISO) 22000 and most other HACCP guides, specify that there are other prerequisites necessary before HACCP plans

should be developed, including appropriate sanitation and hygienic practices and assembly of a multidisciplinary HACCP team, identification of products , process flow diagram, and controls already practiced. The decision tree technique should be used to identify CCPs followed by the prescription of corrective measures that should be implemented to control biological hazards. Misidentification of CCPs in a HACCP plan may render the prescribed standard operating procedures ineffective, resulting in an HACCP system that may give variable and inadequate control over microbiological conditions of raw meat (Bryant *et al*.,2003). Finally, the implementation of HACCP systems at abattoirs has to be preceded by the establishment of microbiological data specific to the abattoir for the objective assessment of risks. Wagude (1999) compared the levels of bacterial contamination before and after the implementation of HACCP at a South African abattoir. After the implementation of HACCP, the author reported a reduction in the incidence of *Staphylococcus aureus* and *Salmonella spp*. after the carcass splitting step and further reductions in S. aureus count after chilling. 27

However, total bacterial counts and *Escherichia coli* counts remained similar after the implementation of HACCP. The author attributed most contamination of carcasses to a lack of training of abattoir operatives. Wagude (1999) reported that the unhygienic practices were aggravated by the link between the number of animals processed and the renumeration of operatives that resulted in the line speed being high. A foodborne outbreak in the USA in late 1992, which was linked to the consumption of undercooked beef patties, triggered the introduction of stringent regulatory requirements by the United States Department of Agriculture (Eustace and Vanderlinde, 1999). The Pathogen Reduction Final Rule, gazetted in 1996, required the implementation by all meat and poultry processing plants of sanitation standard operating procedures, the adaptation of HACCP programmes and sampling of carcasses for generic *E. coli* and *Salmonella* for HACCP verification as part of the Rule. Furthermore, performance standards for those organisms were established for most slaughter

classes. Although the final rule does not mandate decontamination treatments (acid washes, chemical dehairing, steam vacuuming, trimming, hot water spray washes and steam pasteurization), a large number of USA meat packers use these decontamination interventions. According to Smulders and Greer (1998), one of the advantages of the application of organic acids is that residual antimicrobial activity is demonstrable over extended periods of storage. Contrary to the USA, the European Union advocates strict control of processing hygiene, to ensure the safety of meat and meat products. The European Union authorities view the inclusion of decontamination intervention as a way of masking evidence of inadequate hygienic processes (Eustace & Vanderlinde, 1999).

As a result, exporting countries to the European Union, including South Africa could not use chemical decontamination interventions during animal slaughter, dressing and processing of primal cuts. However, according to the European Directive EC/471/2001, all red meat slaughterhouses within the European Community are obliged to operate according to HACCP principles (European Union, 2001). As a result of food crises and international trade requirements, the international community has had to review its food safety laws, with the sole intention of providing the highest 28 level of protection of human health (Hugas & Tsigarida, 2008). Similarly, at European level, Regulation No. 853/2004, which was brought into force in 2006, permits the use of substances other than potable or clean water to remove microbial surface contamination from foods of animal origin. However, the European Commission authorises the use of such substances only after the European Food Safety Authority (EFSA) has provided chemical and microbiological risk assessments.

2.7.4 Common Control Measures Used At Has Alone And Has And Haccp Abattoirs

Hot water sanitation of slaughter equipment

One common practice at most meat facilities, including those in South Africa, is to sanitize meat-cutting equipment (knives, neck splitters, bung tiers and saws) by dipping it into

containers of hot water (82 °C) adjacent to processing lines to reduce the carcass-to-carcass spread of pathogenic and spoilage bacteria. Gill and McGinnis (2004) demonstrated the potential of tools used for carcass dressing to contaminate carcasses during slaughter and dressing. However, the presence of organic materials on slaughtering equipment reduces the antimicrobial activity of hot water. Hot water tends to coagulate protein, which allows organic material to adhere to equipment surfaces and leads to a greater difficulty in removing meat residues. Taormina and Dorsa (2007) found that brief (1 s) dip treatments of slaughter equipment had limited efficacy, compared to longer immersion time (5 s). Trimming of visible contamination During animal slaughter, contaminated carcasses are transferred from a processing to a detaining rail where visible contamination is removed a procedure called trimming. Trimming is an on-line process used to remove excess fat, small faecal spots and smears from beef (Peng *et al.,* 2020). Trimming, which removes enteric pathogens associated with the contaminating matter (Peng *et al*., 2020), is followed by visual inspection to ensure that contamination has been adequately removed after which the trimmed carcasses are returned to the processing line. Since, in South Africa, decontamination treatments such as acid washes are not used visible contamination is removed by trimming, followed by cold-water washing. Subsequently, carcasses are sent to the coolers. Gill and Landers (2004) documented the effectiveness of the trimming of visibly contaminated carcasses on the reduction of both total bacterial counts and of *E. coli* counts on beef carcasses. Steam vacuuming Steam vacuuming uses steam and/or hot water to loosen soil and kill bacteria. Then the application of a vacuum removes the wastewater and contaminants (Meat Industry Services, 2006). Steam vacuuming equipment is a hand-held device consisting of a vacuum wand with a hot spray nozzle delivering water at 88–94 °C to the carcass surface under pressure while simultaneously vacuuming the area (Mims *et al.,* 2004). Steam continually sanitises the device (Bolton *et al*., 2001). According to Sofos, (2008), steam vacuuming

reduced the mean aerobic plate counts (APC) and total coliform counts (TCC) by 0.72 and 0.26 log10 cfu/cm2 respectively, from carcasses which did not have visible faecal contamination. A combination of knife trimming and steam vacuuming showed a higher reduction of the mean APC and TCC from visibly contaminated carcasses, by 1.38 log10 cfu/cm² and 1.59 log10 cfu/cm² , respectively. 30 The effectiveness of steam vacuuming depends on the diligence of application of the operative and the operational status of the equipment. For example, Bolton *et al*. (2001) observed increased reduction in APC after the operatives had been sufficiently trained in operating the steam-vacuuming device. Moreover, the curvature of some surfaces may make proper contact with the vacuum head difficult which can reduce the effectiveness of the treatment. Furthermore, at least 10 s is required for a pasteurising effect and operatives on-line may not have sufficient time for the job (Bolton *et al*., 2001). Bolton *et al*. (2001) recommended critical limits for steam vacuuming systems.

2.7.5 Competition between Spoilage and Food Pathogens during Beef Storage

Meat is a complex food ecosystem of which the chemical and physical properties can allow the colonization and development of a great number and variety of organisms (Ojegunle and Lateef, 2008). Bacterial attachment is probably the first step in the contamination of solid surfaces. Attachment is generally considered to be a two-stage process. The first stage is reversible attachment that occurs when the bacteria are trapped in a water film on the contact surface. The second stage is irreversible attachment that occurs as the bacteria form a more permanent physical attachment (Ezeoha and Ungwuishiwu, 2011). Benito *et al*. (1996) demonstrated that attachment of LAB to beef muscle was influenced by both the immersion time and the cell concentration in the adhesion medium. Interestingly, with *E. coli* O157:H7, attachment to beef surfaces was rapid, occurring within the first few minutes of incubation with little increase in attachment occurring when exposure times were extended (Sheridan, 2007). Bacterial surface structures that assist their adhesion to surfaces include outer

membrane proteins, capsular polysaccharides, lipopolysaccharides, curli, nonhemagglutinating pili, fibrillae and flagella. However, Chen, Rossman and Pawar (2007) did not observe major differences in attachment by curliated and noncurliated cells of *E. coli* O157:H7 to beef.

2.8 Factors influencing growth of bacteria on meat

The shelf life of meat depends on the number and type of bacteria initially present and their further growth in the ecological conditions applied during storage, particularly the storage temperature, pH and gaseous atmosphere (Russo *et al*., 2006). Meat stored aerobically at chill temperatures is dominated by *Pseudomonas spp*. (Asibor 2017), while the typical microbiota of vacuum-packed fresh meat products stored at chill temperatures consists of LAB and Enterobacteriaceae at levels of 108 and 106 log10 cfu/g, respectively (Sun and Ockerman, 2004). When different microbial species live in the same environment they certainly influence each other and in the case of meat, the interactions between microbial groups during storage play an important role in both the development and prevention of spoilage. This antagonistic activity is defined as antibiosis. Russo *et al*. (2006) demonstrated antagonistic activity of LAB against Brochothrix thermosphacta in vitro at 5 °C for 7 days. The authors concluded that the LAB antagonistic activity was not due to bacteriocins but rather to the effect of decreased pH and competition for substrate, as substrate competition and antagonism are important in the selection of microflora in any given ecological niche (Russo et al., 2006).

2.8.1 Bacterial Interactions on Meat

Competition between microflora is an effective way to preserve food and guard against pathogenic microbial growth (Sun and Ockerman, 2004). The inhibition of pathogens can be due to the production and excretion of substances that are inhibitory or lethal to other microbial cells, competition for attachment/adhesion sites, rendering the environment

unfavourable/undesirable to other microorganisms, competition for oxygen and other nutrients or combinations of the above (Amisi *et al*., (2003)). Jay (1996) suggested that low levels of background organisms do not have much influence on pathogens. Jay (1996) hypothesized that increasing levels of background flora may inhibit the survival of pathogenic microorganisms on meat due to microbial interactions among these microorganisms. Jay (1996) suggested protecting carcasses with appropriate mixtures of harmless bacteria, which may compete by occupying attachment sites, thereby reducing colonization by pathogens.

2.8.2 Influence of Storage Conditions of Meat on Its Microbiota

Meat is a good support of bacterial growth due to its composition: 75% water and many different metabolites such as aminoacids, peptides, nucleotides and sugars (Liu and Haynes, 2011). It is, however, a relatively poor source of sugar for bacteria, but an important source of proteins (Sun and Ockerman 2004). The types and proportion of bacteria growing on meat during storage result from the type of contamination introduced by the processing of meat and from the physico-chemical factors applied during storage. These factors (temperature, pH, nutrient and composition of the atmosphere) constitute hurdles that play a crucial role in the activity and growth of microorganisms. Following attachment, packaging influences the microflora of meat during storage (Macián *et al* ., 2007).

2.8.3 Microbiota of aerobically chilled stored beef

In aerobically stored meats, milk, fish and vegetables, members of the Pseudomonas genus are the microorganisms that rapidly dominate the flora. Although the species that grow on different food products are often different they share important common features (). When bacteria coexist within a given ecological niche, substrate competition and antagonism are important in the selection of the resultant microflora (Gram, 1993). Gram (1993) demonstrated the antibacterial activities of 209 *Pseudomonas* strains isolated from spoiled

and fresh fish in agar assays against organisms including food pathogens. The antimicrobial activity was more pronounced in strains that produced siderophores. However, the addition of iron to the substrate resulted in the elimination of antibacterial activity of two-thirds of those strains. Therefore, the inhibitory activity of those strains may have been siderophoremediated competition for iron.

In another study suggested that the sequestering of iron by *Pseudomonas spp.* was an advantage to other bacteria that were able to utilize the siderophores. For example, Gram (1993) observed dense growth of L. 36 monocytogenes and *S. aureus* around the wells containing *Pseudomonas spp.* Gram (1993) then suggested that antagonistic *Pseudomonas spp.* could have created a more advantageous nutritional composition, either through the supply of iron (Asibor 2017) or by increasing the availability of low-molecular weight nutrients (Asibor 2017). In addition, even among the *Pseudomonas* species competition exists. For example, *Pseudomonas fragi* does not synthesize pyoverdine (the green fluorescent pigment) but is able to synthesize its receptors on the outer membrane. This feature enables *P. fragi* to compete for sidorephore produced by the growth of other *Pseudomonas spp.* (Labadie, 1999).

2.9 Effect of storage temperature on the competitive activity of P. fluorescens on beef

Low storage temperature increases the lag phases of both spoilage and pathogenic bacteria on meat (Sun and Ockerman 2004). Sun and Ockerman (2004) investigated the influence of different levels of P. fluorescens on the growth of *E. coli* O157:H7 when co-cultured on sterile meat. These authors used different inoculation levels (zero, 2 and 4 cfu/ml) for both bacteria and incubated the inoculated samples under 4 and 25 °C for 14 days and 30 h, respectively. Under low temperature storage (4 °C), Sun and Ockerman (2004) observed an expected interaction between storage time and the *P. fluorescens* level in the inoculum. *P. fluorescens* count increased as storage period increased. The capacity of P. fluorescens to

grow faster than their competitors at temperatures below 2 and 15 °C is due to their ability to utilize glucose, amino acids and lactic acid (Sheridan, 2007). However, Sun and Ockerman (2004) also observed a slow growth rate by *Pseudomonas fluorescens.*

CHAPTER THREE

3.0 MATERIALS AND METHOD

3.1 Collection of samples

Abattoir wastewater were collected from Ikpoba slope slaughter house. The samples were transported aseptically to Microbiology Laboratory, University of Benin, Benin City for microbiological analysis.

3.2 Preparation and Sterilization of Culture Media

All culture media was prepared according to the manufacturer's instructions. Sterilization will be at 121ºC at 15psi for 15 min unless otherwise stated by manufacturer.

3.2.1 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 will be the placed in an autoclave to sterilize it for 15 minutes at 121 °C. After sterilization, the flask will be allowed to cool.

3.2.2 Eosine-methylene Blue

Eosine-methylene blue agar was prepared by dissolving 500ml of Eosin is a dye in 250mls of distilled water and boil to completely dissolve agar. The autoclave will be sterilized for 15 minutes at 15 psi (121ºC). It will be then cool to 60ºC and before pouring into sterile Petri dishes.

3.2.3 Preparation of Salmonella agar

63.02g of salmonella and shigella 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 minutes at 121 °C. After sterilization, the flask will be allowed to cool.

3.3 Enumeration and isolation of total heterotrophic bacterial and count

10^5 serial dilution of the samples were prepared aseptically in sterile physiological saline. An aliquot of 0.1 ml was inoculated using the pour plating technique. . Tryptone soy agar (supplemented with fluconazole) was used for enumeration. Plates were incubated at 37±2ºC for 24 hours. The number of colony forming unit per milliliter (cfu/ml) was calculated using the formula below:

$$\frac{cfu}{ml} = \frac{number\ of\ colonies\ x\ dilution\ fold/series}{volume\ of\ inoculum}$$

(Willey *et al.,* 2008)

3.3.1 Phenotypic identification of Bacteria from samples

Several tests such as Gram reaction, catalase, urease, indole, oxidase, sugar fermentation, citrate utilization, respective reaction on triple sugar iron agar tests were carried out to presumptively identify bacterial isolates (Okafor *et al*., 2020).

3.4 Morphological identification

3.4.1 Gram staining:

This test was done to confirm the cell type of the bacteria to be used. Gram staining techniques was used for differentiation between Gram-positive and Gram-negative bacteria. Organisms that retain the primary stain are called Gram positive while those that do not retain the primary stain when decolourized are called Gram negative. The non-retention of the stain is due to the cell composition. The Gram stain procedure is as follows:

A smear of the bacteria isolate was made on grease free slide and heat fix by passing over flame. The smear was flooded with crystal violet which is the primary stain for 1min then washed with distilled water. Subsequently the slides were flooded with Lugol's iodine solution for 30sec and then washed off with distilled water. 95% alcohol was used for decolorization for 10sec and immediately washed off with distilled water. Finally, the smear was counter stained with saffranin for 1min and washed off. The slides were allowed to air

dry before observing under the microscope using an oil immersion objective lens of ×100 magnifications to view the slides.

3.5 Biochemical identification

Biochemical test was carried out so as to help in the identification of the bacteria isolates as phenotypic (cultural) characteristics is not sufficient. The various biochemical test carried out are shown below;

3.5.1 Oxidase test

This is mainly used to differentiate between *Pseudomonas* from other Gram-negative rods. Oxidase test was carried out to identify bacteria species that will produce cytochtome oxidase enzyme. *Staphylococcus aureus* and *Escherichia coli* which are Gram-positive and Gram-negative respectively were employed as control. A piece of filter paper using sterilized wire loop 2-3 drops of freshly prepared oxidase reagent (1% aqueous tetramethyl-3-phenyl nediamine dichloride) was added. A positive oxidase test is indicated by purple colouration within 10 seconds.

3.5.2 Urease test.

This is used to test organisms that have the ability to produce the enzyme urease which catalyzes the breakdown of urea to produce ammonia. The test is usually used to differentiate organisms like *Proteus mirabilis* from other non-urease positive organism. A sterilized medium was dispensed into test tubes aseptically and the test bacteria isolated were inoculated into the medium and incubated at 37 ºC for 24 hours. A change in colour from yellow to red-pink confirmed the presence of urease.

3.5.3 Indole production test

This test was used to determine which of the isolates has the ability to split indole from tryptophan present in peptone water. The test is usually used in differentiating Gram-negative Bacilli especially those of enterobacteriaceae. Five grams of commercially available peptone broth was dissolved in 1litre of distilled water. The medium was then sterilized by

autoclaving at 121 degree centigrade for 15 minutes. The 4 ml of the medium was dispensed into sterile test tube and each of the bacterial isolates was inoculated into the peptone broth. The inoculated media was incubated at 37 ºC for 24 hours after which few drops of KOVAC reagent was added. KOVAC reagents consist of 150 ml of amylalcohol, 10 g dimethylamino benzaldehyde and 150 ml of concentrated hydrochloric acid. Positive test was indicated by the red colouration that occurs immediately at the upper part of the test tube.

3.5.4 Citrate utilization test

This test is used to identify which of the isolate can utilize citrate as the sole source of carbon for metabolism. The medium used for this test is Simon`s citrate agar. In the preparation, 22 g of commercially available Simon's citrate agar was dissolved in a litre of distilled water and sterilized by autoclaving at 121 ºC for 15 minutes. The medium is dispensed into test tubes and the test organism was inoculated by stabling the medium on the tubes using sterile straight inoculation wire containing culture. The tubes were incubated at 37 ºC for about 24 hours. Positive result is indicated by a change in colour from green to bright blue colouration.

3.5.5 Catalase test

This is a test to detect the presence or absence of catalase enzyme. The catalase enzyme catalyses the breakdowns of hydrogen peroxide to release free oxygen gas and the formation of water. A few drops of freshly prepared 3% hydrogen peroxide were added onto the bacterial isolates smeared on a slide. The production of gas bubble indicated catalase enzyme positive.

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

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

TSI was prepared following manufacturer's instruction and the prepared media was placed in a test tube and kept in a slant position for it to solidify. The slant and butt of the medium was inoculated with the test bacterium using a sterile loop 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 but region. More so, 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 standard protocol as well as other biochemical tests carried out on the isolates to confirm or ascertain their identity.

3.6 Antibiotic susceptibility test

The bacteria isolates were subjected to commonly used antibiotics in Nigeria using Kirby Bauer agar disc diffusion technique as described by Aromolaran and Badejo (2014) and Akinyemi *et al*. (2005). Pure colonies of bacterial isolates were streaked on sterile Muller Hinton agar (MHA) plates and incubated at 37^{o} C for 24 h. The bacterial cells were harvested into sterile normal saline solution and standardized using 0.5 McFarland standards. The cultured cells were introduced on the surface of sterile MHA using sterile swab sticks and multi-disc antibiotics were placed on the culture media aseptically and incubated at 37^{o} C for 24 h. The antibiotic discs used were ceftazidime (30µg), cefuroxime (30µg), gentamicin (10µg), ciprofloxacin (5µg), ofloxacin (5µg), nitrofurantoin (300µg), ampicillin (10µg), and augmentin (30µg). The diameter of the zones of inhibition around each disc was measured after the incubation period and recorded

3.7 Pathogenicity test (DNase)

The extracellular protease activity of the isolates will be assayed on DNase plates supplemented with 1% casein (v/v). The density of this suspension was adjusted to 0. 5 McFarland standard, which is the equivalent of 1. 5 x 10^8 cells/mL. 1 ml sample of this suspension was inoculated on TSA plates supplemented with 1% casein and incubated at 37oC for 24 to 48h. Zone of clearance due to casein hydrolysis was considered a positive result while no clearance was considered a negative result.

3.8 Multiple Antibiotics Resistance (MAR) Indexing of Isolates

The multidrug resistance of the bacterial isolates was analyzed using the multiple antibiotic resistance (MAR) index. The multiple antibiotic resistance (MAR) index was defined as a/b where 'a' represent the number of antibiotics to which the isolate was resistant to and 'b' the number of antibiotics to which the isolate was exposed (Chitanand *et al*. 2010).

3.9 Data Analysis

The data were analysed using the SPSS package version 21.0. All data are mean of three replicates. The mean, range and standard deviation of each parameter was determined. The means were separated using Duncan's Multiple Range test (SPSS, 2010).

CHAPTER FOUR

4.0 RESULTS

This work was aimed at assessing the microbial risk of *Escherichia coli* and *Salmonella spp* in abattoir waste water. From the result the total Escherichia coli colony count in Log_{10} cfu/ml ranges from 0.7×10^3- 0.8×10^3 (cfu/ml) while that of Salmonella sp ranges from 0.7×10^3 – 0.8×10^3 (cfu/ml). detailed results of this study is represented in figure 1, 2, 3 and table 1 and below.

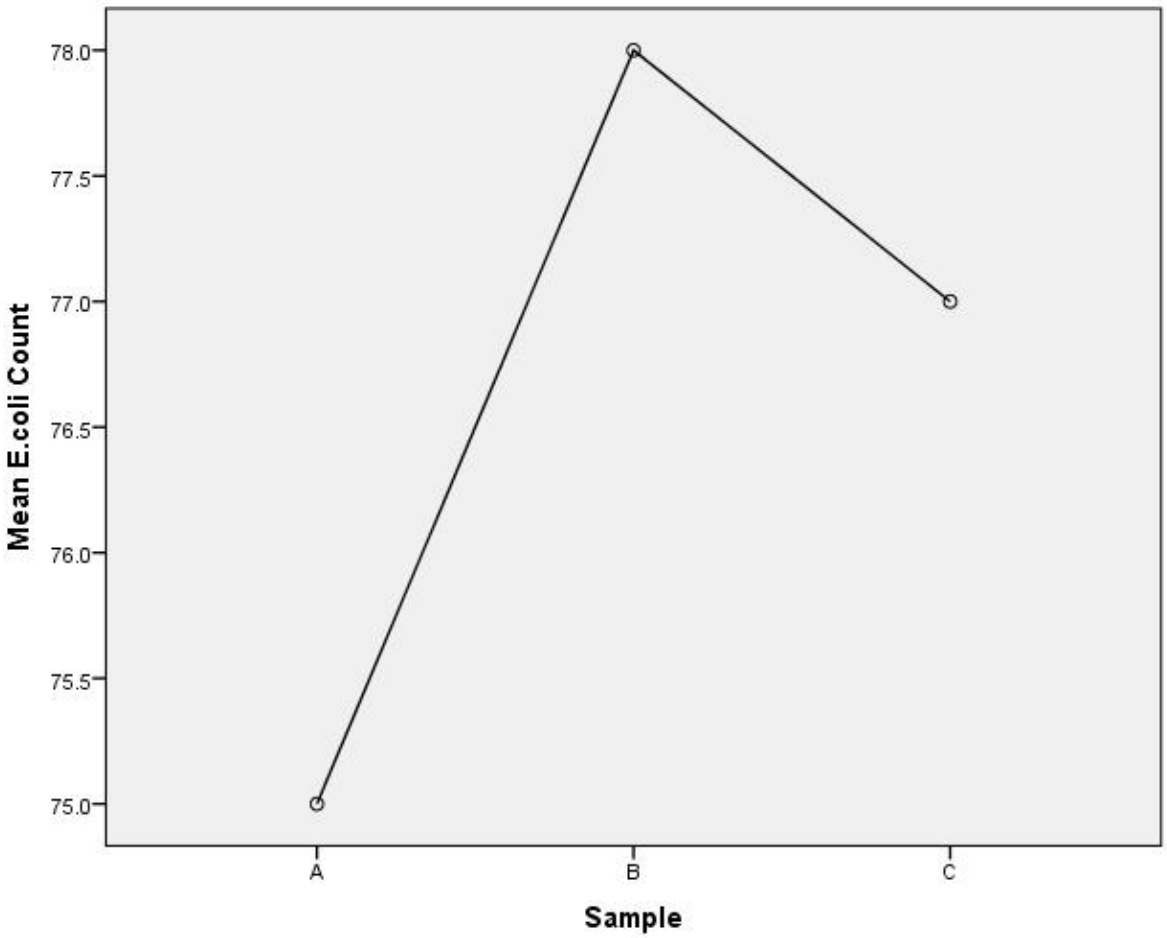

Figure 4.1: Total *Escherichia coli* count in cfu/ml of abattoir samples

One-way ANOVA showed that there is no significant difference in *E.coli* counts in the three samples (P>0.05).

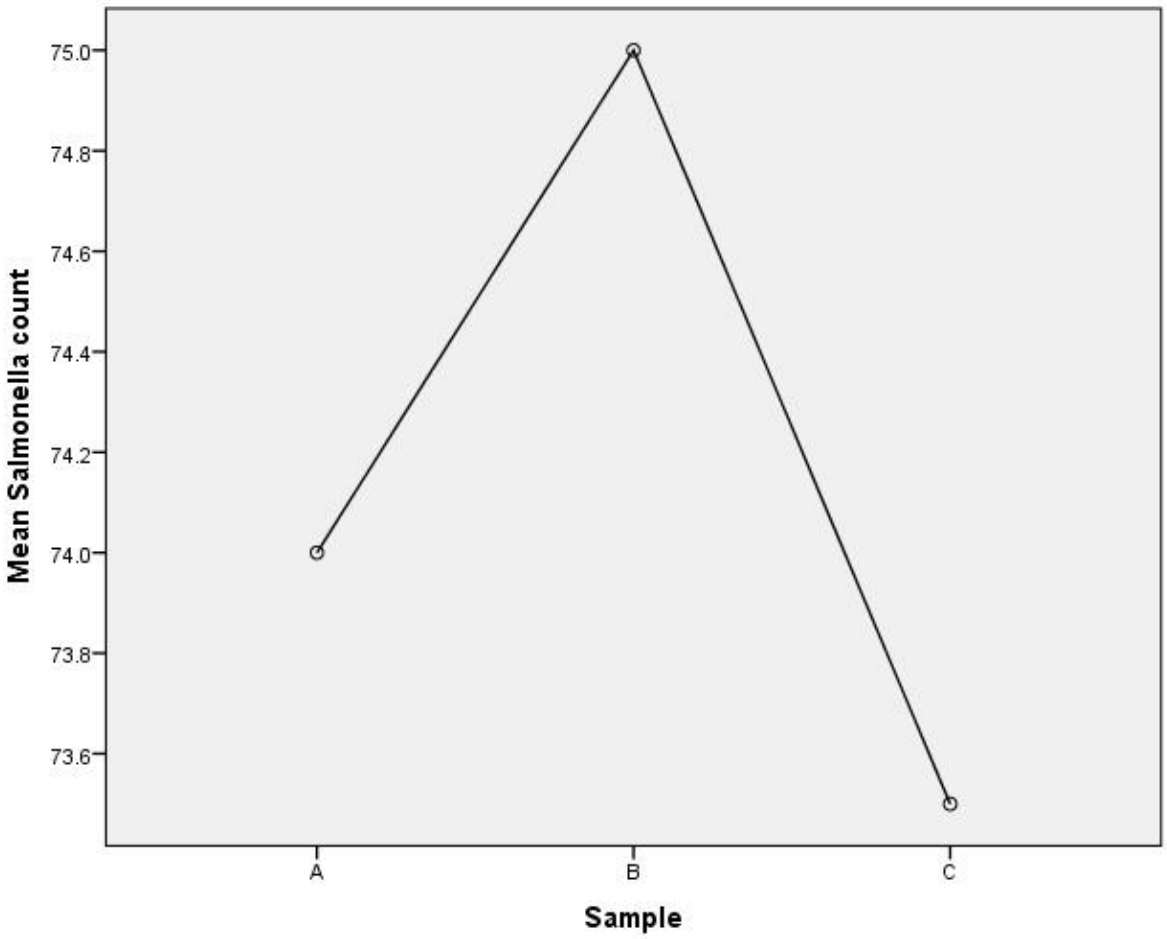

Figure 2: Total *Salmonella spp* count in cfu/ml of abattoir samples

One-way ANOVA showed that there is no significant difference in Salmonella count in the three samples (P>0.05).

Table 4.1: cultural and morphological characteristics of isolates

Cultural characteristics	1	2	3	4	5
Colour	Cream	Cream	Golden yellow	Cream	Cream
Shape	Circular	Circular	Circular	Circular	Circular
Elevation	Convex	Convex	Convex	Convex	Convex
Margin	Entire	Entire	Entire	Entire	Entire
Size	Small	Small	Small	Small	Small
Morphological characteristics					
KOH	+	+	+	+	+
Gram stain	-	-	-	-	-
Cell morphology	Rod	Rod	Rod	Rod	Rod
Cell arrangement	Single	Single	Single	Single	Single
Biochemical characteristics					
Catalase	+	+	+	+	+
Coagulase	-	-	+		
Indole	+	-	-	-	-
Oxidase	-	-	-	-	-
Citrate	-	-	-	-	-
Urease	-	-	-	-	-
H2S production	-	+	-	+	-
Glucose	+	+	+	+	+

Lactose	+	-	+	+	+
Sucrose	-	-	+	+	-
Mannitol	+	+	+	+	+
Gr. Diff.	Green metallic Sheen (EMB)	Black (SSA)	Dark yellow (SSA)	Black (SSA)	Pink (EMB)
Identity	*E. coli*	*Salmonella* typhi	*Salmonella* sp.	*Salmonella enterica* .	*E. coli*

Table 4.2. Phenotypic virulence determinants of bacterial isolates obtained from samples

Bacterial Isolates	DNase	Hemolysin	Gelatinase
Escherichia coli	+	β	-
Salmonella typhi	-	γ	-
Salmonella enterica	+	β	-
Salmonella sp	+	γ	-
Escherichia coli	+	β	-

Key: + = positive (present) - = negative (absent)

Table 4. 3: Antimicrobial Susceptibility Test

ISOLATES	CS	CIP	GEN	E	TE	M	CD	AG
E.coli	0(R)	17(S)	22(S)	0(R)	0(R)	0(R)	9(R)	12(I)
Salmonella typhi	0(R)	14(S)	15(S)	8(R)	0(R)	7(R)	11(I)	14(S)
Salmonella sp	0(R)	19(S)	15(S)	10(I)	9(R)	7(R)	0(R)	15(S)
Salmonella enterica	0(R)	16(S)	14(S)	0(R)	0(R)	0(R)	0(R)	14(S)
E.coli	0(R)	17(S)	20(S)	0(R)	0(R)	0(R)	9(R)	12(I)

KEY

- CS: COLISTIN
- CIP: CIPROFLOXACIN
- GEN: GENTAMICIN
- E: ERYTHROMYCIN
- TE: TETRACYCLIN
- M: METRONIDAZOLE
- CD: CLINDAMYCIN
- AG: AUGMENTIN
- S: SUSCEPTIBLE
- R: RESISTANT

Table 4.4: Multiple antibiotic resistant index

ISOLATES	MARI	Antibiotics to which the antibiotics are resistant
E.coli	0.625	CS, E, TE, M, CD
Salmonella typhi	0.5	CS, E, TE, M.
Salmonella sp	0.5	CS, M, CD, TE.
Salmonella enterica	0.625	CS, E, TE, M, CD
E.coli	0.625	CS, E, TE, M, CD

KEY

- CS: COLISTIN
- CIP: CIPROFLOXACIN
- GEN: GENTAMICIN
- E: ERYTHROMYCIN
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- M: METRONIDAZOLE
- CD: CLINDAMYCIN
- AG: AUGMENTIN

CHAPTER FIVE

5.0 DISCUSSION

This study was aimed at assessing antibiotic resistant *Escherichia coli and Salmonella* sp in abattoir waste water in Benin city. Potential health risks from waterborne pathogens can exist in water contaminated by abattoir effluents, runoff from feedlots, dairy farms, grazed pastures, fallow and sod amended with poultry litter, grassland treated with dairy manure, and sewage sludge treated land. Such contamination of water bodies from abattoir wastes could be a major source of environmental and public health hazards. Wastes from slaughterhouses/abattoirs are known to habour fat, grease, hair, feathers, flesh, manure, grit and undigested feed, blood, bones and process water which are typifications for high organic level (Neboh *et al*., 2013). From the result the total Escherichia coli colony count in cfu/ml ranges from 0.7×10^3 - 0.8×10^3 (cfu/ml) while that of Salmonella sp ranges from 0.7×10^3 – 0.8×10^3 (cfu/ml). this study was in agreement with Cheesbrough (2006) who analysed heterotrophic bacterial contamination in abattoir waste water. The microorganisms of interest in this study were *Escherichia coli* and *Salmonella* sp. However five strains of these bacteria were isolated, which include *Salmonella enterica*, 2 *Escherichia coli* strains, *salmonella* sp, and *Salmonella typhi*. This microbes were also among those isolated by Adesemoye *et al*.(2006).

The presence of *Escherichia coli* and *Salmonella* species proves that there is human fecal pollution of these areas. The isolation of these Coliforms shows a current human contaminations of the sampling points and is of great Public Health concern (Khan *et al*., 2016). The presence of this isolates around the abattoir vicinity could be due to the presence of hydrocarbons within the abattoir. This observation supports the report by Zahedi *et al.* (2018). The presence of pathogenic microorganisms especially *E. coli* and *Salmonella* can lead to the transmission of water borne diseases such as, Typhoid fever, Cholera, food poisoning and gastroenteritis (Mittal, 2004).

High level of contamination of the abattoir wastewater as revealed in this study, further confirmed the dangers associated with discharging untreated wastewater to the environment, thus the need for adequate treatment to ensure decontamination. We use some of the words of Omole *et al* (2008) to submit that sustainability in food production (in this case – meat production) should be given priority of place since it intertwines with public health and economic development.

These values are higher than the WHO accepted limit for microbial contamination for any surface sample which should not exceed 1.20×10^6 CFU/ml or 7.12 $\log_{10}$ cfu/ml (Rabah *et al*., 2010). The wastewater could eventually percolate into surrounding surface and ground waters which poses danger to those working in the abattoirs and those living around them as available water sources close to them become contaminated by the effluents. The result obtained from this study is consistent with previous studies done by other authors such as Adesemoye *et al*. (2006) and Rabah *et al*. (2010), who reported similar high count in the range of ($\times 10^7$ CFU/g) bacteria from soil samples contaminated with wastewater at Agege and Ojo in Lagos and also, in Sokoto abattoir, respectively in Nigeria.

The presence of these microorganisms is worrisome mainly because these microorganisms are opportunistic human pathogens and even though may not infect healthy humans but may infect immune-compromised individuals (Longe and Omole 2008). Similar findings were reported by Akindewa *et al*. (2020) who reported the isolation of *Escherichia coli, Clostridium* spp., and Salmonella spp. among other organisms from Aba River as a result of contamination from abattoir effluents. Also, Mittle (2004) reported the isolation of similar organisms from water contaminated with fecal material in Jimeta-Yola, Nigeria. The presence of *Escherichia coli* and *Salmonella* in this study give credence to human fecal contamination of these sites. The isolation of *E. coli* and other coliforms is an indication of

recent human contamination of the sampling points, and is of great public health concern (Umaru *et al*., 2018).

From the pathogenicity test conducted, all isolates except *Salmonella typhi* were positive to DNase test, *Salmonella typhi* and *Salmonella sp* were also gamma hemolysis isolates while others were beta hemolysis. All isolates from this study were gelatinase negative, however the positive result of most of the isolates to virulence test revealed its pathogenicity and risk to animals and humans as well. This study was similar to (Nwanta *et al., 2010*). pathogens cause major disease on exposure by human. (Onuoha *et al*., 2016). The emergence of antimicrobial resistant bacteria increases in environment where antimicrobials are indiscriminately used in the public. In Nigeria and other developing countries, acquired bacterial resistance to antimicrobial agents is common and the complex socio-economic and behavioral factors associated with this phenomenon include abuse of antibiotics among other complex factor. The antibiotic sensitivity test in this study revealed that all isolates were susceptible to only two antibiotics namely ciprofloxacin and gentamicin and partially augmentin but were resistance to collistin, erythromycin, metronidazole, tetracyclin and clindamycin. The spread of multiple antibiotic resistant pathogenic bacteria have been recognised by the World Organization for Animal Health (WOAH), the Food and Agriculture Organization (FAO) and the Bacteriological and Physicochemical Analysis of Waste Water SULE *et al*.(2016) as a serious global human and animal health problem. The development of bacterial antimicrobial resistance is neither an unexpected nor a new phenomenon. It is, however, an increasingly troublesome situation because of the frequency with which new emerging resistance phenotypes are occurring among many bacterial pathogens and even commensal organisms (Zahedi *et al*., 2018).

Rabah *et al*. (2009) who recorded that isolates demonstrated resistance to a wide range of antimicrobial agents. The possible reason may be due to difference in time, variation of

environment, and the type of contaminated effluents. Multiple bacterial resistances to drugs had earlier been reported in aquaculture environments by other workers across the globe (Nwanta *et al*., 2010). Iroha *et al*. (2016) had reported up to six different resistance pattern and resistance to (two or more drugs) in 93% of tested isolates. Resistance to multiple antibiotics can lead to occurrence of newly emerging resistant bacteria which may be transmitted to consumers causing infection that are difficult to treat. The fact that antimicrobial resistant genes are common in environment and play an important role for bacterial survival, the prevalence of multi-drug resistance bacteria in abattoir effluent is probably due to a multitude of biological as well as ecological factors. Because they are multi-drug resistant implies that there is possibility of these bacteria to harbor plasmids with several genes conferring resistance to a broad array of antibiotics. These suggest that there is high chance of spreading these pathogens and the associated resistant genes to humans and animals.

5.1 CONCLUSION

Going by international standard, any water contaminated to this level is neither good for domestic use nor is it supposed to be discharged directly into the environment without treatment. Urgent steps must be taken now in other to avoid a health crisis in the foreseeable future. This is because heavy metals accumulate in the body for a long time before constituting health risks. This study has so far shown that the sanitary and hygiene conditions of the abattoir are far from ideal and the discharged untreated effluent contains antibiotic resistant bacteria that could impact on public health.

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