PREVALENCE OF ANTIBIOTIC RESISTANT ENTEROCOCCI ISOLATED FROM FOOD

BY

SALIYUK REBECCA NVAN

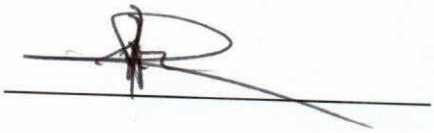
(MCB/11/0347)

BEING A RESEARCH PROJECT SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, FACULTY OF SCIENCE IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF BACHELOR SCIENCE DEGREE (B.Sc.) IN MICROBIOLOGY, FEDERAL UNIVERSITY OYE-EKITI.

OCTOBER, 2015

CERTIFICATION

This is to certify that SALIYUK REBECCA NVAN with Matric number MCB/11/0347 of the department of Microbiology, faculty of Science, Federal University Oye-Ekiti carried out this research project under the supervision of.

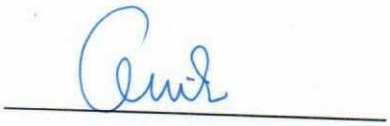


24/11/2015

DR (Mrs.) R.A.O Gabriel-Ajobiewe

Project supervisor

Date



24/11/15

Prof. Bryan Oghene

Head Of Department

Date

DEDICATION

I dedicate this project to ALMIGHTY GOD The one who was and who is to come, the lifter up of my head, who made this research possible. And also my parents whom i am forever indebted to MR/MRS ABIN SALIYUK for their parental and financial support.

ACKNOWLEDGEMENT

My sincere and profound gratitude goes to GOD ALMIGHTY for seeing me through this programme. I also want to appreciate my supervisor Dr. (Mrs.) R.A.O. Gabriel-Ajobiewe for her tireless effort in making sure i do the right things throughout this period. Special thanks goes to my Head Of Department in person of Prof. Bryan Oghene and to my lecturers in the department who have contributed one way or the other both directly and indirectly to making me who I am throughout my programme, Prof Ogundana, Dr. L. Okoror, Dr. A.O Ajayi, Dr. S.K Ojo, Dr. H.O Akinyele, Mrs. C Nwokeoma, Mr. Olufemi Bankefa, Dr. Imarhiabe and Mr. Samuel Osanyilusi MAY GOD ALMIGHTY BLESS YOU ALL.

My gratitude also goes to my father Mr. ABIN A. SALIYUK and to my mother Mrs. MARY SALIYUK for their parental guidance, and also financial provision throughout this programme by making sure i do not lack. I am forever indebted to you both. And to my siblings OLAYODE JOY and her husband OLAYODE ROTIMI, SALIYUK ESTHER, SALIYUK SARAH AND SALIYUK JAMES.

I cannot but thank someone who has always been there for me spiritually, in person of Pastor Samuel for his prayers and advice that has helped, you have always been there to listen to whatever issues i had.

Not forgetting my colleagues in the Department, Victoria, Bishop, Blessing, Joy, Daniel, Jimlas, Ganiyat, Atitebi, Funlola, Jennifer, Omolola, Francisca and to my friends in other departments Chibuchi, Timilehin, Damilola, Paul, Temitope, Elizabeth, Charity and Tunde.

TABLES OF CONTENT

LIST OF TABLES

LIST OF PLATES

ABSTRACT

Enterococci have continued to attract attention as emerging pathogen both in Hospital and the environment. Interest on their prevalence has therefore become of interest. Their resistance to multiple antibiotics was investigated from foods in Oye-Ekiti, Nigeria. From the 20 samples collected from food vendors which include: rice, beans, fufu and pounded yam were investigated for the presence of Enterococci. The isolates were assayed in-vitro against Augmentin, Amoxicillin, Erythromycin, Tetracycline, Cloxacillin, Gentamycin, Cotamoxazole, and Chloramphenicol, for sensitivity test. Three (15%) were positive for Enterococci. This study has revealed the prevalence of Enterococci strain in ready-to-eat-food in Oye-Ekiti, Nigeria. The Enterococci showed resistant trait to Augmentin, Amoxicillin, Cotamoxazole, and Cloxacillin. They do not show resistance to Gentamycin, Erythromycin, Chloramphenicol, and Tetracycline. The mere presence of Enterococci in food is indicative of poor sanitary quality of food, which may be from contaminated water. The result of this research reveals the need for proper hygiene and inspection of food outlets.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Enterococci are ubiquitous organism found as normal micro biota of the gastrointestinal tract of humans and animals. *Enterococci* have been found to colonise diverse niches and can then serve as indicators of the sanitary quality of food (Clarence *et al.*, 2009). They may also be seen in soil, water surface and in plants as well as vegetables due to feacal contamination of the environment by humans and animals (Giraffa *et al.*, 2000). *Enterococci* are used as starter culture in industries, flavour enhancer of cheese, and they are also use as probiotics (Giraffa, 2002). Their presences in some foods are unwanted and can cause food spoilage, food poisoning. The widespread of *Enterococci* in dairy product such as milk has been considered to be as a result of unhygienic conditions during milk collection and processing, as well as their resistance to pasteurization.

Recently there have been the increased interest in *Enterococci* because of their pathogenic activities especially in healthy individual and they have become more opportunistic in hospitalized patients (Saxena *et al.*, 2003). These organisms are particularly difficult to eliminate as a result of their ability to adapt to different environment, temperature and substrate. Thus, it is not a thing of surprise that antimicrobial-resistant variants of *Enterococci* have been recovered from meats, dairy products and ready-to-eat foods and even within probiotic formulations (Girrafa, 2002). *Enterococci* have been found to be involved in clinical infections such as Endocarditis, bacteraemia, urinary tract infections and neonatal sepsis (Kucerova *et al.*, 2009). *Enterococci* are also implicated in surgical complications in neonatal, Central Nervous System disorders and other infections (Abriouel *et al.*, 2009).

The main reason for the rise of nosocomial infections related to *Enterococci* is its ability to develop resistance to a wide range of antibiotics (Mundy *et al.*, 2000). Their resistance to antibiotics can be acquired or found to be an intrinsic part of them. *Enterococci* have shown intrinsic resistance to antibiotics such as: Cephalosporin, beta-lactams, sulphonamide, low levels of aminoglycosides and clindamycin, which have allowed them to acquire resistance against many commonly use antibiotics (Radu *et al.*, 2001).

The resistance of *Enterococci* is not only restricted to hospital settings, resistant strains have been carried on food which acts as reservoir of antimicrobial resistance genes. The food chain can be considered as the main train of transmission of antibiotic resistant bacteria between the animal and human population. Fermented dairy products that do not undergo heat treated before consumption also provide a vehicle for antibiotic resistance. Recent years *Enterococci* have shown resistance to vancomycin. Pressure selectively exerted by the use of antibiotics as growth promoters in food, animals appears to have created large reservoirs of transferring antibiotic resistance in various ecosystems (Aarestrup *et al.*, 2002). Clearly, there is no general conclusion on the acceptance of their presence in foodstuffs, and their role as primary pathogens is still a thing of deliberation. This research work will touch

(i) Their presence in foods;

(ii) The presence and spreading of antibiotic-resistant *Enterococci* as opportunistic pathogens in foods.

1.1.1. AIM OF STUDY

To evaluate the distribution enterococci in ready-to-eat food from food outlets from Federal University Oye-Ekiti and the Antibiotics resistance of the Enterococci specie isolated from these foods.

1.1.2. OBJECTIVES OF STUDY

The objective of this study is to get food samples from various food outlets in Federal University Oye-Ekiti and its surroundings, isolate them to get a pure strain, perform biochemical test for identification, do the antibiotic sensitivity test to determine their antibiotic resistant pattern.

1.2. LITERATURE REVIEW

1.2.1. *ENTEROCOCCI*

Bacteria of the genus *Enterococcus* or *Enterococci* (formerly the 'feacal' or Lancefield group D streptococci) are ubiquitous micro-organisms, but are predominant in the gastrointestinal tract of humans and animals. Due to their high heat tolerance and survival under adverse environmental conditions, *Enterococci* can colonise diverse niches and may then be used as indicators of the sanitary quality of food. *Enterococci* commonly occur in large numbers in vegetables, plant material and foods, mainly those of animal origin such as fermented sausages and cheeses (Abriouel *et al.*, 2008). In processed meats, *Enterococci* are generally not desirable because they cause spoilage. On the contrary, *Enterococci* have important implications in the dairy industry. They play a very important role in the development of organoleptic characteristics during the ripening of many cheeses and they have been also used as components of cheese starter cultures (Girrafa, 2002). Some *Enterococci* of food origin also share a number of useful biotechnological traits (e.g. bacteriocin production, probiotic characteristics), which led to earlier applications in fermented foods (Ogier and Serror, 2008).

Unfortunately, *Enterococci* have recently assumed major importance in clinical microbiology as well. *Enterococci* have traditionally been regarded as low-grade pathogens. However, there is no consensus on the significance of their presence in foodstuffs. Their newly accentuated ambiguity concerning the relationships of *Enterococci* with human beings is related to their enteric *habitat*, their entering the food chain, their antibiotic resistance and their possible involvement in food-borne illnesses due to the presence of virulence factors, such as the production of adhesins and aggregation substances (Kojima et al., 2010). Over the last two decades, *Enterococci* have emerged as important hospital-acquired pathogens in immune-suppressed patients and intensive-care units. The rise in hospital-acquired enterococcal infections has been in part due to the increased use of broad-spectrum antibiotics and the rising number of severely ill patients. *Enterococci* are not only intrinsically resistant to several antibiotics, but are also characterized by a potent and unique ability to exchange genetic material. In addition, selective pressure exerted by the use of antibiotics as growth promoters in food animals appears to have created large reservoirs of transferable antibiotic resistance in various ecosystems.

1.2.2. TAXONOMY

The identification of the *Enterococci* has always been problematic. Numerous enterococcal isolates, especially from an environmental source, often remain unidentified when their identification is based on phenotypic traits alone. It is difficult to unequivocally categorized isolates into one of the *Enterococcus* species by physiological tests because heterogeneity in phenotypic features is very high, regardless of the origin of the isolate (Colombo et al., 2009).

The problem with the taxonomy of *Enterococci* is generally that they are a heterogeneous group of Gram-positive cocci sharing many characteristics with the genera *Streptococcus* and *Lactococcus*. This explains why food-associated *Enterococci* have often been considered to belong to the 'lactic' microflora. More recently, other species of *Enterococci* have been proposed on the basis of

chemotaxonomic studies and phylogenetic evidence provided by 16S rDNA sequencing (Muller t.*et al.*, 2001).

1.3. PRESENCE OF THE *ENTEROCOCCI* IN FOODS

Enterococci can be readily isolated from foods, including a number of traditional fermented foods. A clear picture of the microbial ecology of these bacteria easily explains their presence in foods. *Enterococci* constitute a large proportion of the autochthonous bacteria associated with the mammalian gastrointestinal tract. Once rejected from the environment by means of human faeces or animal ejecta, they are able to colonise diverse niches because of their exceptional aptitude to resist or grow in hostile environments. Therefore, *Enterococci* are not only associated with warm-blooded animals, but they also occur in soil, surface waters and on plant and vegetables (Abriouel *et al.*, 2009). By intestinal or environmental contamination they can then colonise raw foods (e.g. milk and meat) and multiply in these materials during fermentation. They can also contaminate finished products during food processing. Therefore, many fermented foods made from meat and milk especially fermented meats and cheeses contain *Enterococci* (Colombo et al., 2009).

A wide variety of fermented meat products is produced in many parts of the world. In Europe the Italian salami are the most common, and German raw sausage which have various or multiple national and regional variants. The technology for the production of most of these products is essentially similar. Processed meats are usually salted or smoked, and for the most part eaten raw after a period of fermentation to biologically stabilize the product. When in these conditions *Enterococci*, which normally contaminate raw meats in the range of 10^2–10^4 CFU g^{-1} (Miroslav *et al.*, 2007) and are very resistant to extremes temperature, pH and salinity, tend to multiply in high numbers and act as spoilage agents in processed meats. For example, the fermented meat products salami and Landjager were found to contain *Enterococci* at numbers ranging from 10^2 to 10^5 CFU g^{-1} (Zonenschain, 2009). It was therefore suggested that a proper heat treatment during processing, such

as in the case of cooked, unfermented meats, would be necessary to eliminate *Enterococci* as spoilage microflora in fermented meat (Bhardwaj *et al.*, 2008).

Enterococci are a spoilage problem also in cooked and processed meats because they are able to survive heat involve in the processing, especially if they are initially present in high numbers most especially present is the *E. faecalis* and *E. faecium* (Hugas *et al.* 2003). In regards to this, both *E. faecalis* and *E. faecium* have been implicated in the spoilage of pasteurized canned hams (Chajęcka-Wierzchówska, 2012). Gordon and Ahmad stated that *E. faecium* can survive cooking to 68°C for 30 min during normal 'frankfurter' production Furthermore; great potential exists for recontamination with *Enterococci*, both in raw and properly cooked products, from intestinal or environmental sources. Therefore, the presence of *Enterococci* in fermented or non-fermented meat products appears unavoidable by present day applied technologies (Huys *et al.* 2004).

The presence of *Enterococci* in dairy products has long been considered an indication of insufficient sanitary conditions during the production and processing of milk. To the contrary, many authors suggest that certain strains of *Enterococci* in some cheeses may be highly desirable on the basis of their positive contribution to flavour development during the cheese ripening. This beneficial role led to the inclusion of enterococcal strains in certain starter cultures. *Enterococci* occur in a variety of cheeses, especially artisanal cheeses produced in southern Europe from raw or pasteurized milk, and in natural milk starters (Ogier and Serror, 2008). The isolation of *Enterococci* from natural milk starter cultures, which are still widely used for many Italian soft cheeses made with raw or pasteurized milk, can be explained by their thermal resistance. In fact, natural milk cultures are made by pasteurising good quality raw milk and by incubating it at 42–44°C for 12–15 h, thus promoting the natural selection of thermophilic and heat-resistant lactic acid bacteria, usually belonging to *Streptococcus thermophilus* and *Enterococcus* spp. The presence of *Enterococci* in pasteurized cheeses is generally due to recontamination after the heat treatment and to their heat resistance. The

recovery and persistence of the *Enterococci* in some cheeses during ripening can be attributed to their wide range of growth temperatures and their tolerance to pH and salt (Gordon *et al.* 1991).

1.3.1. *ENTEROCOCCI* IN CHEESE

In the traditional European cheeses manufactured in Mediterranean countries from raw or pasteurized milk *Enterococci* have been seen to be involved in it. The source of *Enterococci* in milk and in cheese is assumed to be from the faeces of dairy cows, contaminated water or milking equipment and bulk storage tanks (Gelsomino *et al.*, 2001). *E. faecalis* is considered to be the most common *Enterococcus* species in human faeces. A detailed study of raw milk cheddar cheese produced on an artisanal, family-type scale in Ireland attempted to pinpoint the sources of *Enterococci* in the faeces of dairy cows, family members, milk, curd and cheese (Gelsomino *et al.*, 2002). *E. casseliflavus* dominated among the isolates of human faeces, milk and cheese. Genotypic characterization showed that the same three clones, one of *E. faecalis* and two of *E. casseliflavus*, predominated among almost all of the milk, cheese and human feacal isolates (Gelsomino *et al.*, 2002). These clones were also isolated from bulk tanks and milking equipment. It was concluded that these clones had established themselves on the farm equipment and that this led to contamination of the milk, the curd and the cheese. The presence of these clones in the family's faeces was most likely from their consumption of the cheese (Gelsomino *et al.*, 2002). Cows' faeces were demonstrated not to be the source of *Enterococci* in the cheese in a study, because only strains of *E. faecium* and *Streptococcus bovis* were isolated from the cows' faeces (Gelsomino *et al.*, 2002). According to (Andrighetto *et al.*, 2001), the original sources of the E. casseliflavus were established. *E. faecalis* or *E. faecium* mostly occur in ripened Mediterranean cheeses. Unfortunately, no similar detailed studies have been done to determine the source of *E. faecalis* or *E. faecium* strains in other artisanal-type cheese production technology. *Enterococci* with desirable technological and metabolic traits have been proposed as part of defined starter cultures for different European cheeses, this is due to their role in ripening and flavour enhancement in cheese. (Sarantinopoulous *et al.* 2002a) studied the effect of two strains of *E.*

faecium as adjunct starter cultures, as single or combination of culture on the microbiological, physicochemical and sensory characteristics of Feta cheese. It was discovered that the presence of the enterococcal starter strains affected the growth of nonstarter LAB positively, help increase the proteolytic index and free the concentration of the amino group, enhanced the water-soluble nitrogen fractions and positively affected taste, aroma, colour, structure, and the overall sensory characteristics of the cheese (Sarantinopoulous *et al.*, 2002a).

Enterococci benefit in cheeses is that many strains produce bacteriocins. Colonization of cheeses by foodborne pathogens, particularly *Listeria monocytogenes*, has in time past led to severe outbreaks of diseases. Bacteriocin produced by *Enterococci* isolated from dairy products has been investigated. Strains producing the broad-spectrum, plasmid-encoded, cyclic bacteriocin, AS-48 (González *et al.*, 2000). Have been found in raw milk and dairy products (Rodriguez *et al.*, 2000). Enterocins or starter cultures containing bacteriocin-producing *Enterococci* have been used in model studies to improve safety of the cheeses (Sarantinopoulous *et al.*, 2002b). Varying levels of success were achieved. It was suggested that rennet, $CaCl_2$ and non-*Enterococcus* starter cultures may influence bacteriocin production and hence the successful inhibition of target bacteria (Sarantinopoulous *et al.*, 2002b).

1.3.2. *ENTEROCOCCI* IN MEATS

Enterococci presence in the gastrointestinal tract of animals has led to a high potential for contamination of meat at the time of slaughter. Pig carcasses from three different slaughtering plants were shown to contain mean log counts of 10^4–10^8 *Enterococci* per 100 cm^2 of carcass surface. *E. faecium* and *E. faecalis* were the predominant species isolated. *E. faecalis* predominates all the Gram-positive coccal species isolated from chicken samples collected at poultry abattoirs. *Enterococci* were consistently isolated from beef, poultry or pig carcasses or fresh meat in studies of antibiotic resistance of *Enterococci* (Aarestrup *et al.*, 2002).*Enterococci* do not only contaminate raw meats,

they are also associated with processed meats (cooked, boiled or roasted). Heating of meats during production may produce a selective advantage on *Enterococci* because these bacteria are among the most thermo tolerant of the non-sporulating bacteria. After surviving the heat-processing step, both *E. faecalis* and *E. faecium* have been implicated in spoilage of cured meat products, such as canned hams and chub-packed luncheon meats (Martin et al, 2005).

Enterococci have also been isolated from some types of fermented sausages. Salami and Landjäger were shown to contain *Enterococci* at numbers ranging from 100 to 2.6×10^5 CFU/g. *Enterococci* has also been isolated from dry fermented sausages known as 'chorizo', produced in Spain. Production of antilisterial bacteriocins by various *Enterococci* from Spanish-style, dry fermented sausages suggest that such strains may be suitable for use as adjunct starter cultures to improve food safety (Cintas *et al.*, 2000). Use of the bacteriocin-producing strain as a starter culture was not considered, because bacteriocin production and growth of *E. faecium* are inhibited by low temperatures and the salt and pepper ingredients used in the sausage recipe (Aymerich *et al.*, 2000a). In contrast, (Caiiewaert *et al.* 2000) showed that two bacteriocin-producing strains of *E. faecium* effectively inhibited a strain of *Listeria innocua* in model Spanish-style dry fermented sausage. Therefore, it was suggested that bacteriocins could be considered as additional bio preservative hurdles for prevention of listerial growth in fermented sausages, which then indicates that *Enterococci* may be suitable when added to meat as co-culture to improve food safety (Caiiewaert *et al.*, 2000).

1.3.3. *ENTEROCOCCI* AS PROBIOTICS

Probiotics are 'a mono- or mixed culture of live microorganisms which, when consumed by animal or man, beneficially affect the host by improving the properties of the indigenous flora'. Functional effects claimed for probiotics include: inhibition of pathogenic microorganisms, strengthening of the gut mucosal barrier, anti-mutagenic and anti-carcinogenic activities, stimulation of the immune system and lowering of blood cholesterol levels Fuller, Most probiotic cultures are of intestinal

origin and belong to the genera *Bifidobacterium* and *Lactobacillus*; however, *Enterococcus* spp. are occasionally used as probiotics.

In diarrhoea treatments, *E. faecium* SF68 has been used and it is considered to be an alternative to antibiotic treatment. Several clinical studies have shown that the treatment of enteritis with *E. faecium* SF68 was a success for both adults and children. This reduces the duration of diarrhoeal symptoms and the time whereby the patient's stools become normal. Another example of *Enterococcus* probiotic is the Causido culture that consists of two strains of *S. thermophilus* and one strain of *E. faecium*. This probiotic has been claimed to be a short-term hypocholesterolaemic (Agerholm-Larsen *et al.*, 2000), their long-term reduction of LDL-cholesterol levels was not demonstrated; thus, the clinical importance of this effect is not yet determined (Lund *et al.*, 2002). *Enterococci* use as probiotics has remained a contentious issue. While the probiotic benefits of some strains are well established, the emergence of antibiotic-resistant strains of *Enterococci* and the increased association of *Enterococci* with human disease have raised concern regarding their use as probiotics. The fear that antimicrobial resistance genes or genes encoding virulence factors can be transferred to probiotic strains in the gastrointestinal tract contributes to this controversy.

1.3.4. *ENTEROCOCCI* IN FRUIT AND VEGETABLES

Vegetables have been found to contain *Enterococci*. vegetables and fruits were purchased from different grocery stores northern Georgia, which was then cultured and showed that about 47% of the vegetables and fruit were positive for *Enterococci*. He was able to show that food purchased from grocery stores are sources of *Enterococci* (McGowan et al., 2008). Enterococci are found in vegetables and plant materials because of their ability to survive heat treatments and adverse environmental conditions (Abriouel *et al.*, 2008).

1.3.5. *ENTEROCOCCI* IN COOKED FOOD

Enterococci has been found to be in cooked food such as rice, sauced meat, chickens, vegetable soups. according to (Olawale *et.al.*, 2015), little is been said or done on enterococci in ready-to-eat food such as rice, beans, meats, chicken, pounded yam etc., in their study, it reveals that enterococci are found to be prevalent and spreading in their antibiotic resistance. This fact indicates that most food outlets do not comply with Good Manufacturing Practice (GMP).

1.4. ENTEROCOCCI, EMERGING PATHOGENS

Over the last two decades, *Enterococci*, formerly viewed as organisms of minimal clinical impact, have emerged as important hospital-acquired pathogens in immunosuppressed patients and intensive care units. *Enterococci* do not possess the common virulence factors found in many other bacteria, but they have a number of other characteristics, e.g. the resistance to antimicrobial agents, that may contribute to their virulence and make them effective opportunistic pathogens. Reports of hospital-acquired infections attributed to *Enterococci* are difficult to interpret because these bacteria are generally identified in mixed cultures with other primary pathogens, such as staphylococci and other. *Enterococci* have been implicated in cases of food poisoning, e.g. by production of biogenic amines, based on their isolation in high numbers from suspect foods, but this statement still has not found direct support (Franz *et al.*, 2003).

1.4.1. CLINICAL EPIDEMIOLOGY

The occurrence of enterococcal infections has increased in recent years accounting for approximately 10% of hospital-acquired infections in the USA. *Enterococci* are now among the most common nosocomial pathogens; they have been implicated as an important cause of endocarditis, bacteraemia, urinary tract, central nervous system, intra-abdominal and pelvic infections. Epidemiological data also indicate that *E. faecalis* is the most common species among the *Enterococci* isolated from human

illnesses while *E. faecium*, which is associated with the majority of the remaining enterococcal infections, may pose a larger antibiotic-resistant threat.

Enterococci from the gut account for 5–15 and 4% of the causes of infective endocarditis and bacteraemia, respectively, whereas urinary tract infections are the most common enterococcal, hospital-acquired infections (Franz *et al.*, 2001). In addition, there is strong evidence that *Enterococci* causing bacteraemia commonly originate from the urinary tract. It was observed that in 24% of enterococcal bacteraemia, the isolate were originated from a urinary tract infection. There is an increase incidence of intra-abdominal infections caused by vancomycin-resistant *Enterococci*. *Enterococci* may also be associated with abdominal and pelvic abscess formation and sepsis (Eaton and Gasson, 2001).

1.4.2. INCIDENCE OF VIRULENCE FACTORS AMONG *ENTEROCOCCI*

Considerable progress has been made in the last few years in determining virulence factors from clinical enterococci isolates using molecular biological techniques and model animal experiments. As a result, we now have the opportunity for studying the incidence of such virulence factors among food isolates and determine whether there is a difference in virulence potential between food and medical strains. In addition, such studies may allow evaluations on whether a strain that is intended for use as a probiotic or starter culture would be safe (Aymerich *et al.*, 2000a) .

One such study compared the incidence of virulence factors among enterococci strains intentionally added to foods as starter strains, as well as food and medical isolates (Eaton and Gasson, 2001). This study showed that enterococcal virulence factors occurred among strains from all three sources, but that the incidence of virulence factors was highest for medical strains, followed by food isolates and the lowest incidence was observed for starter strains. *E. faecalis* isolates harboured multiple virulence determinants while *E. faecium* strains, with the exception of a few strains, were generally clear of virulence determinants (Eaton and Gasson, 2001). There has been a misunderstanding in the clinical

significance of *Enterococci* in human infections due to inadequate documented reports confirming their occurrence in mixed cultures (Creti et al.,2004) The increasing resistance of *Enterococci* to antibiotics and the presence of active mechanisms of gene transfer are exacerbating the increasing findings of these bacteria as nosocomial opportunists. However, the antibiotic resistance alone cannot explain the virulence of these bacteria in the absence of pathogenicity factors (Mundy et al., 2000).

1.4.2a. PRESENCE OF VIRULENCE FACTORS AND GENETIC EXCHANGE MECHANISMS

Enterococcal virulence traits include adherence to host tissue, invasion and abscess formation, resistance to and modulation of host defense mechanisms, secretion of cytolysins and other toxic products and production of plasmid-encoded pheromones. A number of genes encoding for virulence factors (especially in *E. faecalis*) have been sequenced and characterized and their effects have been shown in human and animal studies (Franz *et al.*, 2001). Recent molecular screenings of *Enterococcus* virulence determinants indicated that medical *E. faecalis* strains had more virulence determinants than did food strains, which, in turn, had more than starter strains. Multiple determinants, e.g. those involved in adherence, cytolysin and pheromone production mechanisms, were harboured mostly by *E. faecalis* and, to a lesser extent, by *E. faecium*.

(Eaton and Gasson 2001) showed that enterococcal virulence factors were present in food and medical isolates, as well as strains used as starter cultures. However, the incidence of virulence factors was higher among the medical strains, followed by food isolates and the lowest incidence was observed for starter strains. Strains of *E. faecalis* harboured multiple virulence determinants, while *E. faecium* strains were generally clear of virulence determinants (Eaton and Gasson, 2001).

1.5. ANTIBIOTIC RESISTANCE

Antibiotic resistance (ABR) is resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it. Antibiotic resistance refers specifically to the resistance to antibiotics that occurs in common bacteria that cause infections (WHO, 2015). Resistant microorganisms (including bacteria, fungi, viruses and parasites) are able to withstand attack by antimicrobial drugs, such as antibacterial drugs (e.g., antibiotics), antifungals, antivirals, and antimalarial, so that standard treatments become ineffective and infections persist, increasing the risk of spread to others (Albrich et al., 2004).

The evolution of resistant strains is a natural phenomenon that occurs when microorganisms replicate themselves erroneously or when resistant traits are exchanged between them. The use and misuse of antimicrobial drugs accelerates the emergence of drug-resistant strains. Poor infection control practices, inadequate sanitary conditions and inappropriate food-handling encourage the further spread of ABR (Goossens et al., 2005).

1.5.1. ANTIBIOTIC RESISTANCE OF *ENTEROCOCCI*

Enterococcal virulence enhanced by their frequent resistance to commonly used antibiotics. Antibiotic resistance, which can be both intrinsic and acquired, makes *Enterococci* effective opportunists in nosocomial infections (Euba *et al.*, 2009).

Enterococci shown to be intrinsically resistance to cephalosporins, lincosamides, many β-lactams and low levels of aminoglycosides. *Enterococci* may develop increased resistance to penicillin through acquisition of β-lactamases or PBP4/5 mutations. They show low affinity to most β-lactams antibiotics because of the low affinity of penicillin binding proteins (PBPs), this enable them to synthesize cell wall components in the presence of β-lactam antibiotics (Delise *et al*,2003).

High-level penicillin resistance in *E. faecium* is most commonly associated with accumulation of point mutations in the penicillin binding region of PBP5 (Rice *et al*, 2001). A variety of point mutations have been described in both *E. Faecium* and *E. Faecalis* (Donskey *et al*, 2000).

Intrinsic resistance to many antibiotics suggests that treatment of infection could be difficult. In addition to these constitutive resistances, *Enterococci* have acquired genetic determinants that confer resistance to all classes of antimicrobials, including chloramphenicol, tetracycline and glycopeptides (Martin *et al.*, 2005). The major risk related to these latter resistance traits is that they are for the most part transferable. The genes coding for all of these antibiotic-resistant traits may be transferred by pheromone-mediated, conjugative (often multi-resistant) plasmids or transposons to both *Enterococci* (Delise and Pearl, 2003).

Enterococci can also acquire resistance to antibiotics either through mutations in existing DNA or through the acquisition of new DNA.Within acquired antibiotic resistances, vancomycin-resistant *Enterococci* (VRE) are possibly the most serious concern that has recently emerged in human clinical infections (Furustrand Tafin *et al* 2011). Two distinct forms of transferable vancomycin-resistant phenotypes have been described in *Enterococci*: the VanA phenotype (associated with a high level of inducible resistance to vancomycin and cross resistance to teicoplanin) and the VanB phenotype displaying variable levels of inducible resistance only to vancomycin (Delise and Pearl, 2003).

1.5.2. ANTIBIOTIC RESISTANCE IN FOODS

The extremely high level of antibiotic resistance observed in *Enterococci* and their widespread finding in raw foods are two key elements contributing to the frequent recovery of antibiotic-resistant *Enterococci* (ARE) in both unfermented and fermented foods. ARE have been found in meat products, dairy products, ready-to-eat foods and even within enterococcal strains proposed as probiotics (Girrafa G *et al.*, 2000)

In previous studies on European cheeses, *Enterococci* present mainly belong to *E. faecalis* and *E. Faecium,* and their resistance in different proportions, to penicillin, tetracycline, chloramphenicol, erythromycin, gentamicin, lincomycin, rifampicin, fusidic acid and vancomycin were detected; a prevalence of multiple drug resistance was also observed (Teuber *et.al*, 1999). Although ARE are found in both pasteurized and, to a much higher extent, raw milk, cheeses, their presence in raw milk

Intrinsic resistance to many antibiotics suggests that treatment of infection could be difficult. In addition to these constitutive resistances, *Enterococci* have acquired genetic determinants that confer resistance to all classes of antimicrobials, including chloramphenicol, tetracycline and glycopeptides (Martin *et al.*, 2005). The major risk related to these latter resistance traits is that they are for the most part transferable. The genes coding for all of these antibiotic-resistant traits may be transferred by pheromone-mediated, conjugative (often multi-resistant) plasmids or transposons to both *Enterococci* (Delise and Pearl, 2003).

Enterococci can also acquire resistance to antibiotics either through mutations in existing DNA or through the acquisition of new DNA.Within acquired antibiotic resistances, vancomycin-resistant *Enterococci* (VRE) are possibly the most serious concern that has recently emerged in human clinical infections (Furustrand Tafin *et al* 2011). Two distinct forms of transferable vancomycin-resistant phenotypes have been described in *Enterococci*: the VanA phenotype (associated with a high level of inducible resistance to vancomycin and cross resistance to teicoplanin) and the VanB phenotype displaying variable levels of inducible resistance only to vancomycin (Delise and Pearl, 2003).

1.5.2. ANTIBIOTIC RESISTANCE IN FOODS

The extremely high level of antibiotic resistance observed in *Enterococci* and their widespread finding in raw foods are two key elements contributing to the frequent recovery of antibiotic-resistant *Enterococci* (ARE) in both unfermented and fermented foods. ARE have been found in meat products, dairy products, ready-to-eat foods and even within enterococcal strains proposed as probiotics (Girrafa G *et al.*, 2000)

In previous studies on European cheeses, *Enterococci* present mainly belong to *E. faecalis* and *E. Faecium,* and their resistance in different proportions, to penicillin, tetracycline, chloramphenicol, erythromycin, gentamicin, lincomycin, rifampicin, fusidic acid and vancomycin were detected; a prevalence of multiple drug resistance was also observed (Teuber *et.al*, 1999). Although ARE are found in both pasteurized and, to a much higher extent, raw milk, cheeses, their presence in raw milk

in recent years indicate that colonisation with VRE frequently occurs in the community, and that many animal, food and environmental reservoirs can act as community sources for VRE outside the health care setting. In this mechanism, the transport of these resistances via the food chain to humans appears most probable. Although high-level aminoglycoside resistant (HLAR) *Enterococci* are usually defined as *Enterococci* the MIC of >2000 g/ml, some investigators propose using gentamicin at a concentration of 500, or 1000 g/ml33. Whether 500, 1000 or 2000 g of gentamicin per ml is the most appropriate concentration to use for testing is undecided. Use of any one of these concentrations will probably accurately detect high level gentamicin resistance. Since enterococcal resistance to gentamicin and streptomycin occur by different mechanisms, it is important to test susceptibilities to both agents. High level gentamicin resistance is associated with a bi functional enzyme possessing acetylase (6') and phosphotransferase (2') activities (Mohanty *et al.*, 2005).

CHAPTER TWO

2.0. MATERIALS AND METHODS

2.1. MATERIALS

Glass wares; petri-dishes, test tube, syringe, wire loop, bunsen burner, antibiotics disc, incubator (GenLab, China), oven(GenLab, China), refrigerator, conical flask, beaker, measuring cylinder, Electronic weighing balance, autoclave, water bath, McCartney bottles, super bottles, cotton wool, Amikacin, Durham tube, Spatula, Forceps, Vortex (Techmel,China),Commercial antibiotic disc (Abtek biologicals, United Kingdom).

2.1. 1. Study Area

The study area picked for this research was food vendors within Federal University Oye-Ekiti and its surroundings; Iya dabira food vendor, mama blue and mercy restaurant and bar, Asana restaurant, Food Affairs. This selected food vendors has the highest number of staff, student, corpers and indigene patronage

2.1.2. Identification of sampling point

Three sampling point were selected for the study;

Iya Dabira (IYD), Mama Blue (MB), Mercy Restaurant and Bar (MBR), Asana Restaurant(ASN) and Food Affairs(FA).Foods collected include: rice, beans, pounded yam, fufu.

2.1.3. Chemical reagents used

$Bacl_2.2H_2O$, H_2SO_4, Ethanol, Crystal violet, Grams Iodine, Safranin, Hydrogen peroxide, Phenol red, Sucrose, Lactose (Eurostar scientific, United Kingdom), Galactose, Maltose, fructose (Burgoyne Burbidge Ltd, India), D-mannitol (Kemlight laboratories, India), potassium hydroxide.

2.1.4. Media used

Nutrient agar, MacConkay agar, Mueller Hinton agar, peptone water, Nutrient broth

2.2. METHODOLOGY

2.2.1. Collection of samples

Samples include foods such as; jollof rice, fried rice, fufu, ponded yam, beans, vegetable soup. The samples were collected fresh every day for its processing. Two different food samples in two batches were collected at different days.

2.2.2. Preparation of samples

Immediately after collection of the various food samples, they were processed by preparing a stock solution by weighing 10g of the food into 90ml of peptone broth, homogenization and the serial dilution in the 10^{1}-10^{10}dilution range by taking 1ml from the stock preparation to the first test tube, and from the first to the second, this is done till the tenth test tube. For each test tube a new and different syringe is used, to prevent transferring the microbial load from one dilution to the other.

2.3. PREPARATIONS OF MEDIA

MATERIALS: Nutrient agar, MacConkay agar and blood Agar

The medium used in the growth of enterococci was composted by adding Amikacin to the medium to prevent the growth of the Enterobacteriaceae family.

2.3.1. Preparation of peptone water (Biomark, India).

1gram of peptone was weigh into 1litre (1000ml) of distilled water into a conical flask, from the prepared solution 9ml was dispensed into 10 test tubes. The test tube and conical flask were then covered with cotton wool and foil paper. The solution was allowed to dissolve before sterilization using the autoclave.

2.3.2. Nutrient agar (Biomark, India).

2.52ml of nutrient agar was weighed using the weighing balance into 90ml of distilled water, the preparation was calculated according to manufactures manual, shooked vigorously for homogenized and sterilized using the Autoclave at 121rpm for 15mins. The medium was placed in the water bath

until used. The temperature of the medium was allowed to cool to 40-45^{0}c and then addition of 1ml Amikacin solution before pouring into plates.

2.3.3. Blood agar.

This is prepared from nutrient agar by adding 5ml of sheep blood to500ml of sterilized nutrient agar.

2.3.4. MacConkay agar (Biomark, India).

4.36ml of macconkay agar was weighed into 90ml of distilled water; it was allowed to properly dissolve before sterilization using the autoclave at 121° for 15min.

The medium is allowed to cool to 40^{0}c after which 1ml of dilution 2 Amikacin solution was added to the medium before pouring.

2.3.5. Preparation of the antibiotic (Amikacin sulfate, Flagship Biotech, India).

A serial dilution of the antibiotics was carried out in four test tubes containing 9ml of sterile distilled water. 1ml of Amikacin was added to 9ml of sterile distilled water aseptically by doing it close to the flame. From the first test tube, 1ml was taken to the second; from the second another 1ml was taken to the third until the fourth test tube. 1ml of the Amikacin solution from the second diluent was added to the various media, to inhibit the growth of the Enterobacteriaceae family.

2.3.6. Pour plate technique.

After preparation of dilutions, the pour plate technique was carried out in three replicate from dilution 10^5, 10^6 and 10^7 respectively. 0.2ml from the dilution each was dispense into sterile plate labelled with the dilution factors 10^{-5}, 10^{-6} and 10^{-7} respectively. Before pouring the medium into the plates, 1ml of Amikacin solution was added and immediately the media were poured into the sterile plate.

2.3.7. Preservation of isolates.

Double strength nutrient agar slant was prepared for the slant by weighing 11.76g of nutrient agar into 210ml of distilled water, constant shaking to prevent the agar from settling. The agar is poured

into McCartney bottles, sterilized in the autoclave at 121^0 for 15mins. After sterilization, the bottles containing the sterilized medium is placed in a slant position and allowed to solidify.

2.4. CHARACTERIZATION OF BACTERIA.

Presumptive identification of isolates was carried out with the following tests: observation of colony characteristics and cell morphology, Gram staining, catalase, motility and utilization of carbon sources (fructose, mannitol, lactose, sucrose, lactose, and galactose).

2.4.1. Morphological identification

Isolates were identified by phenotypic methods (Alves et al., 2004) Enterococcus spp.

2.5. BIOCHEMICAL CHARACTERIZATION.

2.5.1. Sugar fermentation test

This test was carried out to determine the type of sugar the organism will ferment. The following sugars were used by weighing 1gram (sucrose, lactose, galactose, maltose and mannitol and D-fructose) into 100ml of nutrient broth. An indicator phenol red was added to show whether the organism can ferment the sugar or not. This is indicated by the change of the phenol red indicator broth to yellow.

2.5.1a. Production of acid and gas formation from sugars

A nutrient broth containing 0.5% sugars were prepared in test tubes in which Durham tube was invertly inserted and sterilized in the autoclave. After sterilization, the broth is allowed to cool, after which the isolated organisms were inoculated into the indicator sugar broth, one tube was left uninoculated to serve as control. The test tubes were then incubated at 37^oc for 24hours, and results were recorded.

2.5.2. Gram staining technique

Gram staining of the pure isolate gotten was done. The slide is first cleaned with ethanol and passed through the flame. A sterile loop is used to transfer a loopful of sterile water onto the slide. The loop

is then flamed red hot and allow to cool before using to pick an inoculum, the inoculum is smeared properly with the water, allow to air dry after which you heat-fix. After heat-fixing, crystal violet is added and allow for 60seconds(1min) before running off with water after which grams iodine is added and allowed for another 60seconds , washed off with water, acetone alcohol is added which is left for 30seconds and then washed off with water, lastly safranin blue is added and left for 60seconds before washing off with water. The stained slide is allowed to blot dry. After drying, drop of immersion oil is added to the stained slide and viewed under the microscope under the 100x magnification.

2.5.3. Motility tests

Motility test is a test used for identification of bacteria with flagella. This is done by placing oil immersion at the edge of the depression cavity slide, and then transferring a loopful of the culture on the Centre of a clean dry coverslip placed on a flat white slab. The slide was then covered with cover slip. Quickly and carefully, the slide was inverted and the culture drop appears hanging. The culture was examined immediately for motility under the microscope by reducing the light to reduce heating effect. Low power objective was used to focus the edge of the drop after which, high power objective was used to get a clear view.

2.5.4. Catalase test

The catalase test is a tests used to detect the enzyme catalase that act as catalyst in the breakdown of hydrogen peroxide. These tests help to identify bacterial specie that reacts with hydrogen peroxide to produce oxygen. A 24hour old culture was used for this test. A drop of 3% hydrogen peroxide was placed on the slide; a loop full of the 24hour old organism was added and smeared. The presence of the enzyme catalase is indicated by the presence of gas bubbles.

2.5.5. Grams reaction

This is used to differentiate between gram positive and gram negative organisms. A loopful of 3% Potassium hydroxide (KOH) was placed on a glass slide. A loopful of a colony of organism was emulsified on the surface of the slide in the 3% potassium hydroxide. The suspension was smeared continuously for 60 seconds, after which the loop was gently pulled from the suspension. The test is said to be positive if there is stringing within the first 30seconds of mixing the bacteria in the KOH solution.

2.6. ANTIBIOTICS SENSITIVITY TESTING

The antibiotic sensitivity test was carried out using the Kirby-Bauer method of disc diffusion by seeding the surface of the Mueller Hinton medium with the test organism; this is done by preparing the organism to meet the 0.5 McFarland standards.

2.6.1. Standardization of inoculum for antibiotics susceptibility test

For standardization of the inoculum density of a susceptibility test, a barium chloride turbidity standard of 0.5 McFarland standards was used. It was prepared as follows:

0.05ml aliquot of 0.048mol/l (1.175%) Barium chloride dehydrate($Bacl_2.2H_2O$) is added to 9.95ml of 0.18mol/l H_2SO_4(1%) with constant stirring to maintain a suspension. 5ml of the barium sulphate suspension is transferred into test tube of the same size as those used in growing or diluting the bacterial inoculum. The tubes are tightly sealed and store in the dark. It is vigorously shooked before using and checked for uniform turbid appearance. In the presence of particles, the standard is replaced.

2.6.2. Preparation of inoculum

Growth methods was used

Distinct colonies from culture plates were selected, by touching the top with wire loop and then transferring the growth into a test tube containing 4ml of peptone broth.

The broth culture is then incubated at 35^0c for4hrs until it achieves or exceeds the turbidity of 0.5 McFarland standards.

The turbidity of the actively growing broth culture is adjusted with sterile peptone water to obtain turbidity optically comparable to that of 0.5 McFarland standards.

2.6.3. Inoculation of test organisms

After 15 minutes of adjusting the turbidity of the inoculum suspended, a sterile cotton swab is dipped into the adjusted suspension. The swap is rotated several times and pressed firmly on the inside wall of the tube above the fluid level, this helps to remove excess inoculum from the swab stick.

The surface of the Mueller Hinton agar plate is innoculated by streaking the swab over the entire sterile agar surfaces, by rotating the plate approximately 360^0 for each streak to ensure even distribution of the inoculum.

After some minutes of inoculation the commercial disc (Abtek biologicals, United Kingdom.) was impregnated into the inoculated medium. Positive disc containing antibiotic concentration such as Augmentin 30mg, Amoxicillin 25mg, Erythromycin 5mg, Tetracycline 10mg, cloxacilin 5mg, Gentamycin 10mg, Cotamoxazole 25mg, Chloramphenicol 30mg. Strains were classified as Resistant, Intermediate and sensitive by CLSI(2010).

Table 1: Bacteria count after 24 hours of incubation of Isolates from food of Different food Cafeterias.

Isolate code	Cfu/ml
IYD1	$3.0x10^8$
IYD2	$2.5x10^8$
MB 1	$3.5x10^8$
MB 2	$3.25x10^8$
MRB1	$2.25x10^8$
MRB 2	$3.0x10^8$
ASN1	$1.7x10^8$
ASN2	$2.5x10^8$
FA1	$1.75x10^8$
FA2	$2.25x10^8$

Key: IYD 1: Iyadabira sample 1, MB 2: Mama Blue sample 2, ASN: Asana Restaurant 1

IYD 2: Iyadabira sample 2, MB 1: Mama Blue sample 1, ASN: Asana Restaurant 2

MBR1: Mercy Restaurant and Bar1, MBR2: Mercy Restaurant and Bar

Table 2: Morphological and Microscopic Characteristics of Bacterial isolates from food cafeterias .

Isolates code	Appearance	Colour	Elevation	Surface	Edge/Margin	Cell shape	Cell arrangement
IYD1	Circular	Creamy	Raised	Smooth	Entire	Cocci	chains and pairs
IYD2	Circular	Creamy	Raised	Smooth	Regular	Rod	Clusters
MB1	Circular	Creamy	Raised	Smooth	Regular	Cocci	Chains and pairs
MB2	Circular	Creamy	Raised	Smooth	Entire	Rod	Clusters and pairs
MRB1	Circular	Creamy	Raised	Smooth	Entire	Rod	Clusters
MRB2	Circular	Creamy	Convex	Smooth	Entire	Rod	Clusters
ASN1	Circular	Creamy	Raised	Smooth	Dented	Rod	Clusters and pairs
ASN2	Circular	Creamy	Raised	Smooth	Entire	Rod	Clusters
FA1	Circular	Creamy	Raised	Smooth	Entire	Cocci	Chains
FA2	Circular	Creamy	Raised	Smooth	Entire	Rod	Pairs and singly

Key: MRB: Mercy Restaurant and Bar

MB: Mama Blue **FA: Food affairs**

IYD: Iya Dabira **ASN: Asana restaurant**

Table 3: Biochemical Characteristics of Bacterial isolates of food from different food cafeterias.

Sampling codes	IYD1	IYD2	MB1	MB2	MRB1	MRB2	ASN1	ASN2	FA1	FA2
Grams reaction 3% KOH	+	–	+	–	+	+	+	–	–	+
Grams differential staining	+	-	+	-	+	+	+	–	–	+
Catalase test	–	+	–	+	+	+	+	+	+	–
Motility test	-	+	-	-	+	-	+	+	+	–
Sugar fermentations										
sucrose	+	–	+	–	–	–	+	–	–	+
lactose	+	+	+	+	–	–	–	–	–	+
mannitol	+	+	+	+	–	+	–	+	+	+
galactose	+	–	+	–	–	+	–	+	+	+
maltose	+	+	+	+	–	–	–	–	+	+
D-fructose	+	–		–	–	–	–	–	+	+
Probable organism	*Enterococcus*	*E.coli*	*Enterococcus*	*E.coli*	*Campylobacter*	*Micrococcus*	*Campylobacter*	*E.coli*	*Salmonella*	*Enterococcus*

KEY: + =POSITIVE, -_=NEGATIVE

Table 4: Antibacterial resistance pattern of isolates from food of different food Cafeteria

ISOLATES	AUG(30mg)	AMX(25mg)	ERY(5mg)	TET(10mg)	CXC5mg	GEN(10mg)	COT(25mg)	CHL(30mg)
$IYD_{1.1}$	R	R	R	R	R	S	R	I
$IYD_{1.2}$	R	R	I	R	R	S	R	I
$IYD_{1.3}$	I	R	I	I	R	S	R	S
$IYD_{2.1}$	R	R	S	I	R	S	R	S
$IYD_{2.2}$	R	R	S	I	R	S	R	R
$MB_{1.1}$	R	R	S	I	R	S	R	S
$MB_{1.2}$	R	R	R	R	R	S	R	I
$MB_{1.3}$	R	R	S	I	R	S	R	S
$MB_{2.1}$	R	R	S	I	R	S	R	S
$MB_{2.2}$	R	R	S	I	R	S	R	S
$MB_{2.3}$	R	R	S	I	R	S	R	S
$MRB_{1.1}$	R	R	S	I	R	S	R	S
$MRB_{1.2}$	R	R	S	I	R	S	R	S
$MRB_{2.1}$	R	R	S	I	R	S	R	S
$MRB_{2.2}$	R	R	S	I	R	S	R	S
$ASN_{1.1}$	R	R	S	I	R	S	R	S
$ASN_{1.2}$	R	R	R	R	R	S	R	I
$ASN_{1.3}$	R	R	I	R	R	S	R	I
$ASN_{2.1}$	I	R	I	I	R	S	R	S
$ASN_{2.2}$	R	R	S	I	R	S	R	S
$FA_{1.1}$	R	R	S	I	R	S	R	R
$FA_{1.2}$	R	R	S	I	R	S	R	S
$FA_{2.1}$	R	R	S	I	R	S	R	S
$FA_{2.2}$	R	R	S	I	R	S	R	S

Key :< 13.0mm=Resistant(R), 13-17.0mm=Intermediate (I), >17.0mm=Sensitive(S)

Aug- Augmentin, Amx- Amoxilin, Tet- Tetracycline, Cxc- cloxacilin, Gen-Gentamicin, Cot- Cotamoxazole , Ery- Erythromycin, Chl-Chloramphenicol

Table 5: Zones of inhibition of isolates in millimeter (mm)

Isolates codes	AUG	AMX	ERY	TET	CXC	GEN	COT	CHL
$Iyd_{1.1}$	0.00mm	0.00mm	15.6mm	0.00mm	0.00mm	17.3mm	0.0mm	14.4mm
$Iyd_{1.2}$	0.00mm	0.00mm	11.0mm	0.00mm	0.00mm	22.3mm	12.0mm	10.0mm
$Iyd_{1.3}$	10.7mm	14.6mm	16.7mm	16.3mm	0.00mm	22.7mm	0.00mm	30.7mm
$Iyd_{2.1}$	0.00mm	0.0mm	29.7mm	15mm	0.00mm	25.7mm	0.00mm	33.0mm
$Iyd_{2.2}$	0.00mm	0.00mm	24.3mm	14.3mm	0.00mm	24.0mm	0.00mm	0.00mm
$MB_{1.1}$	0.00mm	0.00mm	29.0mm	14.0mm	0.00mm	23.3mm	0.00mm	27.0mm
$MB_{1.2}$	0.00mm	0.00mm	0.00mm	0.00mm	0.00mm	23.0mm	0.00mm	13.3mm
$MB_{1.3}$	0.00mm	0.00mm	29.0mm	14.0mm	0.00mm	22.7mm	0.00mm	27.3mm
$MB_{2.1}$	0.00mm	0.00mm	30.7mm	16.7mm	0.00mm	24.7mm	0.00mm	29.7mm
$MB_{2.2}$	0.00mm	0.00mm	29.3mm	15.7mm	0.00mm	22.3mm	0.00mm	30.0mm
$MB_{2.3}$	0.00mm	0.00mm	23.3mm	16.0mm	0.00mm	20.3mm	0.00mm	30.0mm
$MRB_{1.1}$	0.00mm	0.00mm	21.0mm	13.0mm	0.00mm	22.7mm	0.00mm	29.0mm
$MRB_{1.2}$	0.00mm	0.00mm	20.0mm	11.0mm	0.00mm	24.3mm	0.00mm	31.3mm
$MRB_{2.1}$	0.00mm	0.00mm	19.0mm	10.7mm	0.00mm	26.7mm	0.00mm	34.3mm
$MRB_{2.2}$	10.3mm	9.0mm	20.7mm	10.3mm	0.00mm	21.7mm	0.00mm	29.0mm
$ASN_{1.1}$	0.00mm	0.00mm	20.0mm	13.7mm	0.00mm	26.3mm	0.00mm	13.7mm
$ASN_{1.2}$	0.00mm	0.00mm	23.7mm	11.3mm	0.00mm	26.0mm	0.00mm	12.0mm
$ASN_{1.3}$	0.00mm	0.00mm	28.0mm	11.0mm	0.00mm	33.0mm	0.00mm	25.0mm
$ASN_{2.1}$	13.0mm	0.00mm	13.7mm	13.0mm	0.00mm	28.3mm	0.00mm	30.0mm
$ASN_{2.2}$	0.00mm	0.00mm	20.0mm	13.3mm	0.00mm	24.3mm	0.00mm	31.3mm
$FA_{1.1}$	0.00mm	0.00mm	20.0mm	13.0mm	0.00mm	24.3mm	0.00mm	0.00mm
$FA_{1.2}$	0.00mm	0.00mm	21.0mm	14.0mm	0.00mm	25.7mm	0.00mm	28.3mm
$FA_{2.1}$	0.00mm	0.00mm	29.7mm	14.omm	0.00mm	22.3mm	0.00mm	30.0mm
$FA_{2.2}$	0.00mm	0.00mm	30.3mm	14.7mm	0.00mm	21.3mm	0.00mm	30.0mm

Plate 1, shows the zone of inhibition of isolate $IYD_{1.1}$ to the various antibiotics. Augmentin (0.00mm), Amoxicillin (0.00mm), Erythromycin (15.6mm), Tetracycline (0.00mm), Cloxacillin (0.00mm), Gentamicin (17.3mm), Cotamoxazole (0.00mm) and Chloraphenicol (14.4mm). This shows that the isolate $IYD_{1.1}$ is resistant to Augmentin, Amoxicillin, Tetracycline, Cloxacillin and Cotamoxazole. It was susceptible to Gentamicin, and Intermediate to Chloraphenicol and Erythromycin.

Plate 2, shows that isolate $MB_{2.2}$ is resistant to Amoxicillin, Augmentin, Tetracycline, Cotamoxazole, Cloxacillin and it was sensitive to Chloraphenicol(30.0mm), Erythromycin (29.3mm) and Gentamicin (22.3mm).

Plate 3, shows that isolate MRB is resistance to Cotamoxazole, Cloxacillin, Augmentin(10.3mm), Amoxicillin(9.0mm), and sensitive to Gentamicin(21.7mm), Chloraphenicol(29.0mm), Erythromycin(20.7mm),and Intermediate to Tetracycline (10.3mm).

Plate 4, shows that isolate $ASN_{1.3}$ is sensitive to Chloraphenicol (25.0mm), Gentamicin (33.0mm), Erythromycin (28.0mm). It shows resistance to Amoxicillin, Augmentin, Cotamoxazole, and Cloxacilin and was intermediate to Tetracycline (11.0mm).

Plate 5, shows the zone of inhibition of the antibiotics to the isolates. The isolate $FA_{1.1}$ is sensitive to Gentamicin (24.3mm) and Erythromycin (20.0mm). It shows resistance to Augmentin, Amoxicillin, Cotamoxazole, Tetracycline and Cloxacilin and Intermediate to Tetracycline.

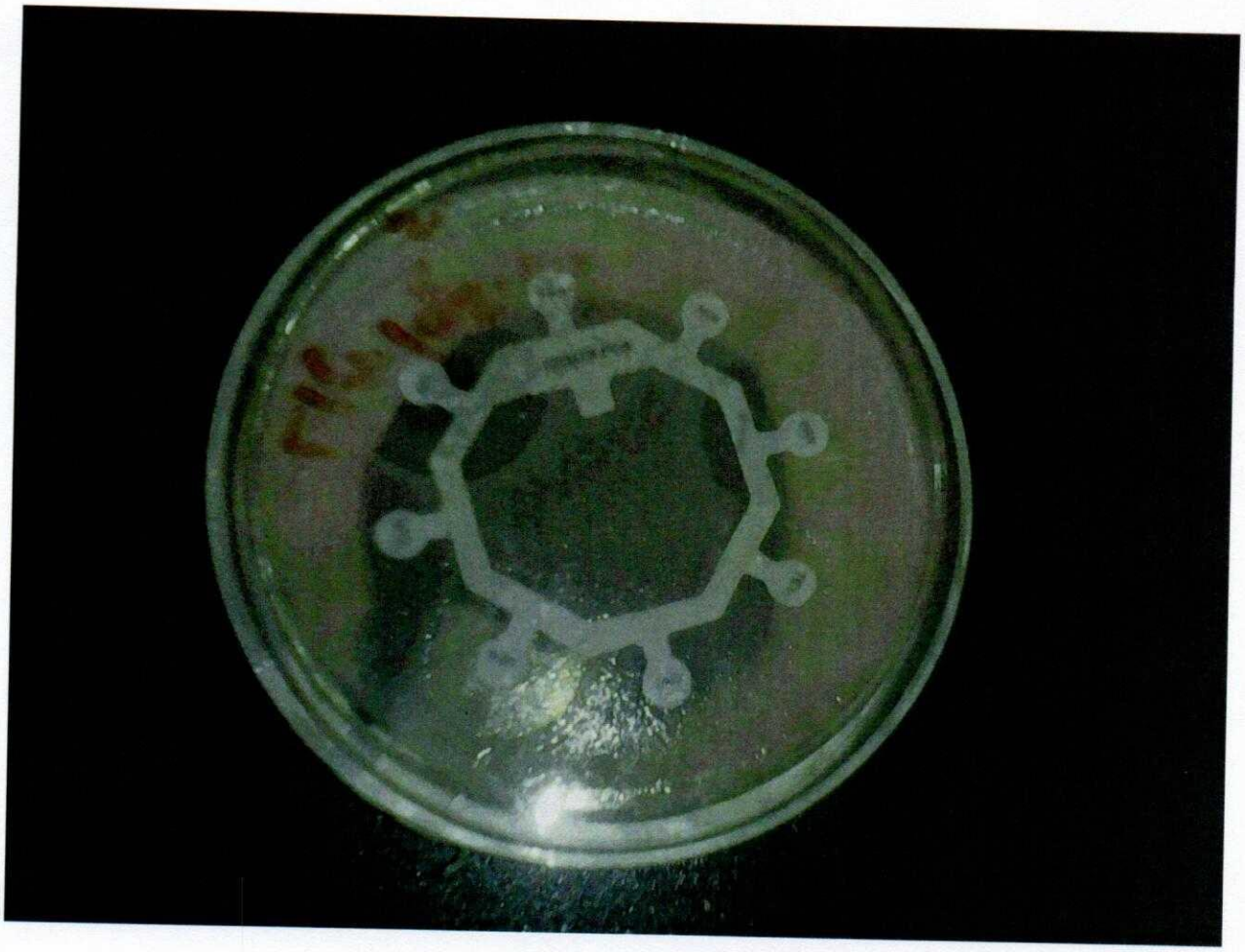


Plate 1: Showing antibiotic sensitivity of $IYD_{1.1}$

KEY: Iya dabura Isolate 1.1

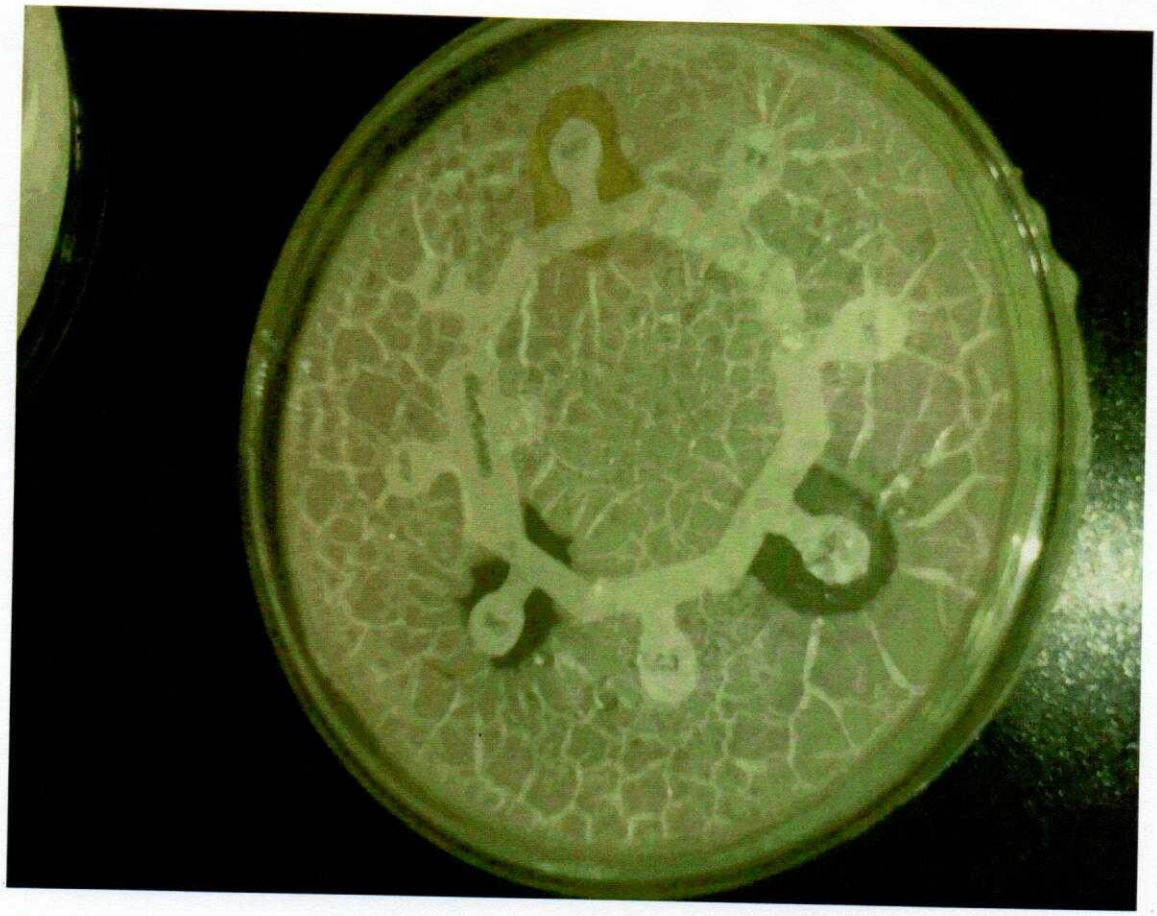


Plate 2: Showing antibiotic sensitivity of $MB_{2.2}$

KEY: MARGA BLUE Isolate 2-2

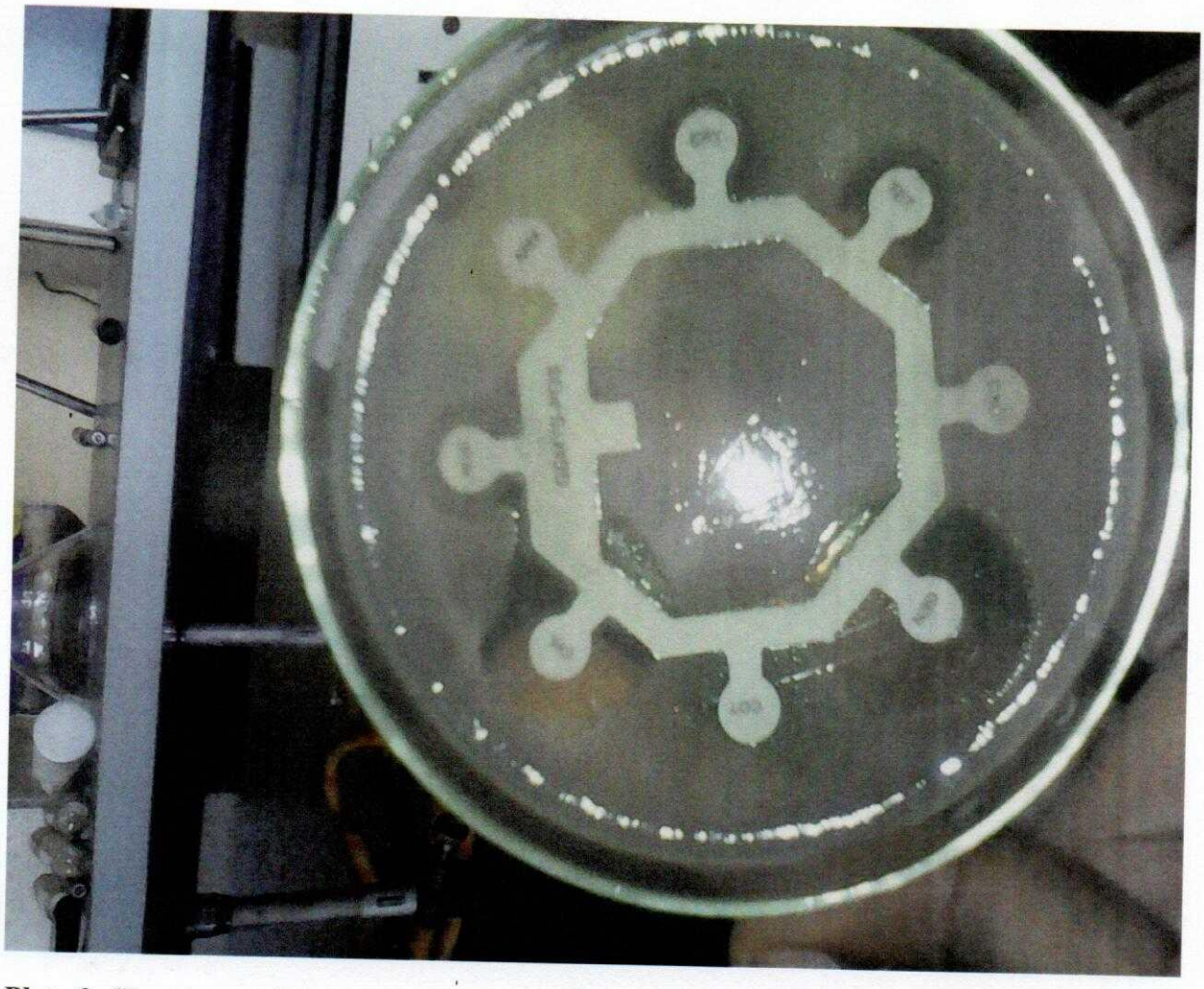


Plate 3: Showing antibiotic sensitivity of $MRB_{2.2}$

KEY: MERRY RESTAURANT AND BAR Isolate 2.2

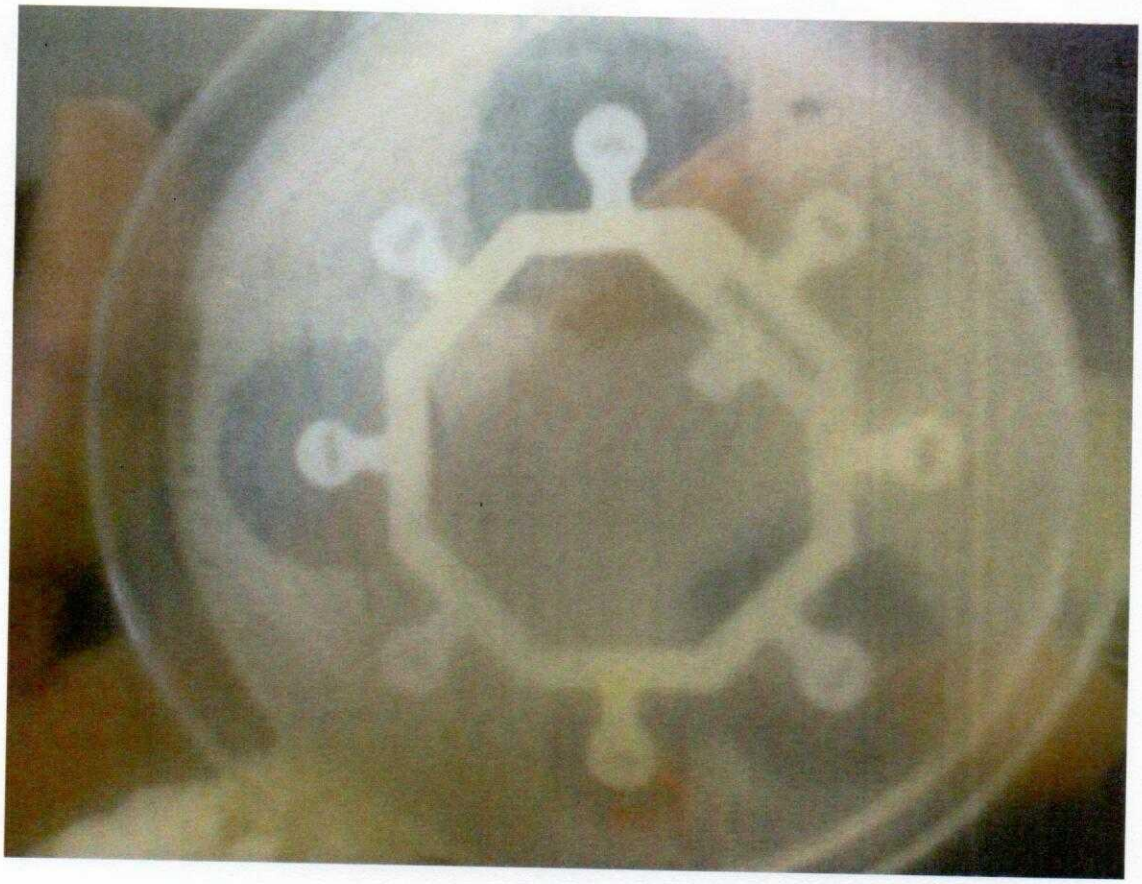


Plate 4: Showing antibiotic sensitivity of $ASN_{1.3}$

KEY: ASANSA RESTAURANT ISOLATES 1.3

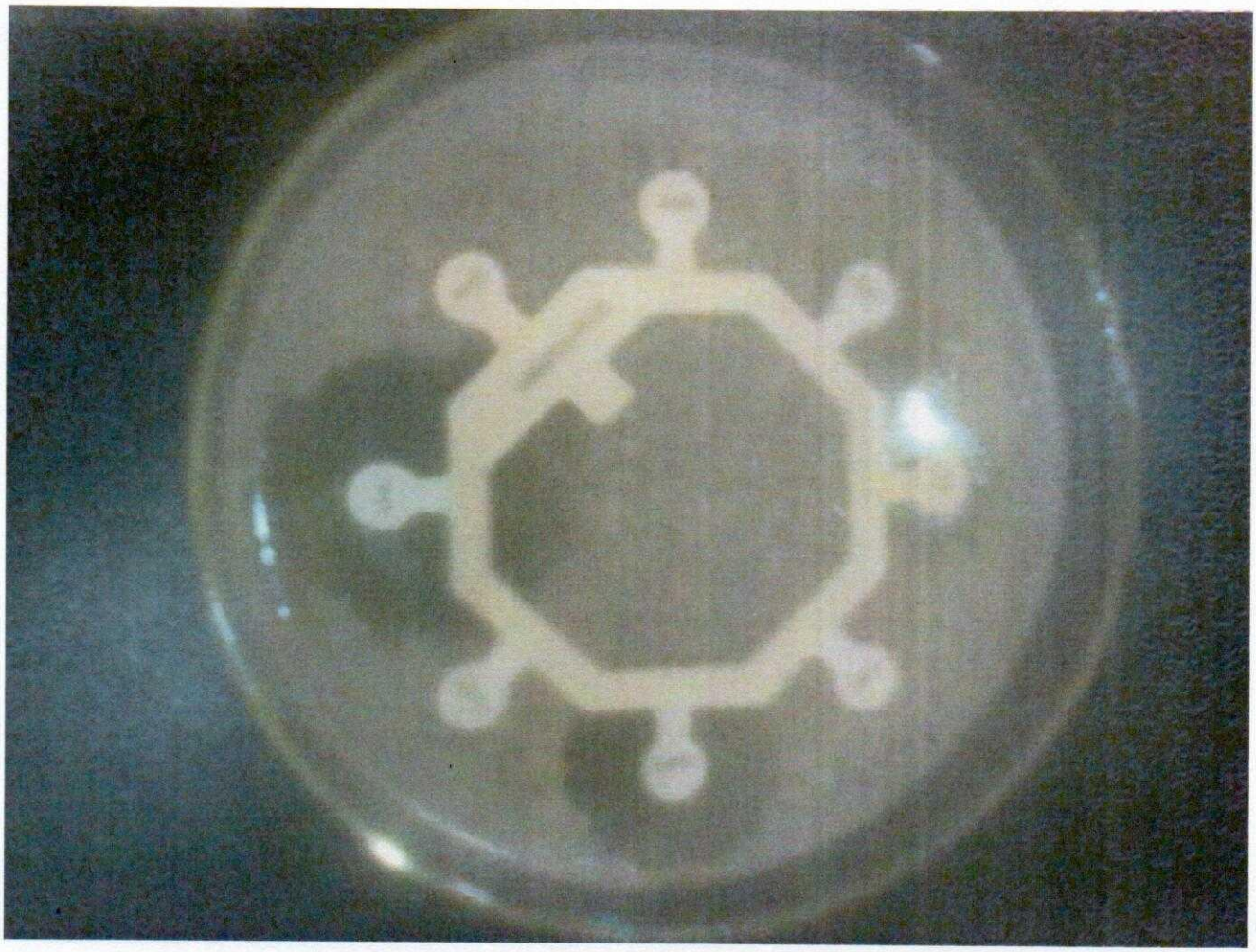


Plate 5: Showing antibiotic sensitivity of $FA_{1.1}$

KEY: FOOD AFFAIRS ISOlates 1.1

3.2. DISCUSSION

Most of the previous studies on resistance have concentrated on enterococci isolated from clinical samples and unprocessed food samples. This study investigated the prevalence of resistant strains isolated from ready-to-eat food .

Only three isolates (15%) were positive for Enterococci. Although the population of Enterococci was not quantitate within the samples, their mere presence in foods are indicator of their rise as pathogen of food, which may not necessary be major contaminants among foods from food vendors in Oye-Ekiti but may have gained entry as a result of feacal contamination . This suggest similar work by (Agboola, 2007), who reported the incidence of enterococci in clinical specimens, but not much has been said about their implication in food. According to (Famurewa *et al.*, 2003), Most incidence of Enterococci were in the palm of food handlers and table top than in the food. This was as a result of improper cleaning of the tables and poor hand sanitary , this was also in agreement with (Kousoumanis and Angelidis,2007) stating that origin of microbial contaminants include the environment, cross contamination from an infected food handler, which could be as a result of careless handling of food. (Eaton and Gasson , (2001) reported incidence of enterococci in food.(Costa-Cruz et al.,1995) reported isolation of E.faecalis in food canteens in Brazil, in His work he came to the conclusion that *Enterococcus* are parts of pathogen found in foods in Brazil. (Famurewa et al, 2003) showed that Enterococci are a rising pathogen spreading their antibiotic patterns in food, and that their presences in food are opportunistic.

The result of antibiotic resistance test showed that most strain isolated from food vendors have acquired resistance to a wide range of antibiotics. High resistance was recorded against cloxacilin followed by Augmentin, amoxicillin and Cotamoxazole. This is contrary to the report by (Olawale *et al.*, 2015) who showed that Enterococci was more resistant to erythromycin

followed by cotrimoxazole to amoxicillin, to chloramphenicol, to tetracycline to Augmentin lastly to gentamicin.(Olawale *et al.*, 2010) reported a different result to that of Famurewa, in his study he showed that Enterococci are more resistant to chloramphenicol, followed by tetracycline, then erythromycin and then ciproflaxin. This study indicates that Enterococci have acquired multiple resistances to an array of antibacterial. (Cariolato *et al.,* 2008) demonstrated only one isolate of *Enterococcus* from cheese was resistant to only two antibiotics (nalixidic acid and streptomycin). All the other ones were sensitive. The results from all studies showed that the resistance of this organism to multiple Antimicrobials has grown over time.

CHAPTER FOUR

4.0. CONCLUSION AND RECOMMENDATION

4.1. CONCLUSION

In conclusion out of Twenty (20) samples collected only three (15%) were positive for enterococci. This indicates contaminants in food. Though conclusion cannot be drown as to their involvement as food pathogen, but their mere presence indicate the likeliness of them being involve in food. They show multiple resistances to a wide array of antimibacterials.

4.2. RECOMMENDATION

I will recommend that frequent study on the involvement of these organisms in food should be carried out. The use of antibiotics should be regulated as these organisms have shown to increase in their acquisition to resistance.

REFERENCES

Aarestrup, F.M., Seyfarth, A.M., Emborg, H.D., Pedersen, K., Hendriksen, F. and Bager, F. (2002). Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistant fecal enterococci from food animals in Denmark. *Antimicrobial. Agents Chemotherapy*, 45: 2054–2059.

Abriouel, H., Omar, N.B, Molinos, A.C., Lopez, R.L., Jose Grande, M., Martinez-Viedma, P., Ortega E., Canamero M.M and Galvez, A. (2008). Comparative analysis of genetic diversity and incidence of virulence factors and antibiotic resistance among enterococcal populations from raw fruit and vegetable foods, water and soil, and clinical 265 samples. *International Journal of Food Microbiology,* 123: 38–49.

Adams, M.R. (1999). Safety of industrial lactic acid bacteria. *Journal of Biotechnology*, 68: 171–178.

Agboola, A.J. (2007). *Incidence of Nosocomial Pathogens on Hospital Beddings* B.Sc.Thesis. University of Ado-Ekiti, Nigeria. 23-24.

Agerholm-Larsen, L., Bell, M.L., Grunewald, G.K. and Aestrup, A. (2000)., The effect of a probiotic milk product on plasma cholesterol: a meta-analysis of short-term intervention studies. *European Journal of Clinical Nutrition*, **54**(11): 856-860.

Albrich, W.C., Monnet, D.L., Harbarth, S. and Monnet, D. (2004). Antibiotic selection pressure and resistance in Streptococcus pneumoniae and Streptococcus pyogenes *Emerging Infections and Disinfection,* 10 (3): 514-517.

Alves, P.I., Martin, S.M., Semedo, T., Figueiredo, J.J., Tenreiro, R. and Barreto, M.T. (2004). Comparison of phenotypic and genotypic taxonomic methods for the identification of dairy enterococci. *Antonie van Leeuwenhoek,* 85: 237-252.

Andrighetto, C., Knijff ,E., Lombardi, A., Torriani, S., Vancanneyt, M., Kersters, K., Swings, J. and Dellaglio, F. (2001). Phenotypic and genetic diversity of enterococci isolated from Italian cheeses *Journal of Dairy Reserve*, 68: 303–316.

Aymerich, T., Artigas, M.G., Garriga, M., Monfort, J.M., and Hugas, M. (2000a). Effect of sausage ingredients and additives on the production of enterocin A and enterocin B by Enterococcus *faecium CTC* 492. Optimization of *in-vitro* production and anti-listerial effect in dry-fermented sausages *Journal of Applied Microbiology,* 88: 686-694.

Bertrand, X., Mulin, B., Viel, J.F., Thouverez, M. and Talon, D. (2000). Common PFGE patterns in antibiotic-resistant Enterococcus faecalis from humans and cheeses *Food Microbiology,* 17: 543-551.

Bhardwaj, A., Malik, R.A. and Chauhan, P. (2008): Functional and safety aspects of enterococci in dairy foods *Indian Journal of Microbiol*ogy. 48, 317-325.

Caiiewaett, R., Hugas, M. and Devuyst, L. (2000). Competitiveness and bacteriocin production of Enterococci in the production of Spanish-style dry fermented sausages. *International Journal of Food Microbiology,* 57: 33-42.

Cariolato, R., Andrighetto, C. and Lombardi, A. (2008). Occurrence of Virulence Factors and Antibiotic Resistances in *Enterococcus faecalis* and *Enterococcus faecium* Collected from Dairy and Human Samples in North Italy. *Food Control*, 19: 886-892.

Chajęcka-Wierzchowska, W., Zadernowska, A., Nalepa, B. and Laniewska-Trokenheim, L. (2012). Occurrence and antibiotic resistance of enterococci in ready-to-eat food of animal origin *African Journal of Microbiology*, 6(39): 6773-6780.

Cintas, L.M., Casaus, P., Havarstein, L., Hernandez, P.E. and Nes, I.F. (2000). Biochemical and genetic characterization of enterocin P, a novel sec-dependent bacteriocin from Enterococcus faecium P13 with a broad antimicrobial spectrum *Applied and Environmental Microbiology*, **63**(11): 4321-4330.

Clarence, S.Y., Obinna, C.N. and Shalom, N.C. (2009). Assessment of bacteriological quality of ready to eat food (Meat pie) in Benin City metropolis, Nigeria *African Journal of Microbiology,* 3(6): 390-395.

CLSI (2010). Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement *(Document M100-S20-U).*

Costa-Cruz, J.M., Grdoso, M.L. and Marques, D.E. (1995). Intestinal: Parasites in School Food handlers in the city of Uberlanda, Minas Gerais, Brazil. *Review of Institute of Medical Tropics*, 37: 191-196.

Creti R., Imperi, M. and Bertuccini, L. (2004): Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *Journal of Medical Microbiology*. 53(1), 13–20.

De Jong, A., Bywater, R., Butty, P., Deroover, E., Godinho, K., Klein, U., Marion, H., Simjee, S., Smets ,K., Thomas, V., Vallé, M. and Wheadon, A. (2009): A pan-European survey of antimicrobial susceptibility towards human use antimicrobial drugs among zoonotic and commensal enteric bacteria isolated from healthy food producing animals. *Journal of Antimicrobial Chemotherapy,* 63: 733–744.

Delise, S. and Pearl, T. M. (2003). Vancomycin resistant enterococcus. A road map on how to prevent the emergence and transmission of antimicrobial resistance. *Chest,* 123: 504-518.

Donskey, C.J., Chowdhry, T.K., Hecker M.T., Hoyden C.K., Hanrahan, J.A. and Hujer A.M. (2000). Effect of antibiotic therapy on the density of vancomycin resistant enterococci in the stool of colonized patients. *New English Journal of Medicine.* 343, 1925-1932.

Eaton, T.J., and Gasson, M.J. (2001). Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates. *Applied Environmental microbiology,* 67: 1628-1635.

Endtz, H.A., Blekum, N., Braak, N., Duin, J., Kluijtmans, J. and Koeleman, J. (1996). Prevalence of vancomycin-resistant enterococci in hospital and community based patients in the Netherlands. In: Program and Abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy, Orlando, p. 37. *American Society for Microbiology,* Washington, DC.

Euba, G, Lora-Tamayo, J., Murillo, O., Pedrero, S., Cabo, J. and Verdaguer, R. (2009): Pilot study of ampicillin-ceftriaxone combination for treatment of orthopedic infections due to Enterococcus faecalis. *Antimicrobial Agents Chemotherapy,* 53: 4305–4310.

Famurewa, O., Oyagade, J.O., Femi-Ola, T.O. and Laleye, S.A. (2003). Assessment of microbial quality of ready-to-eat food retailed in Ado-Ekiti. *NISEB Journal,* 3:71-77.

Franz, C.M.A.P., Muscholl-Silberhorn, A.B., Yousif N.M.K., Vancanneyt, M., Swings, J. And Holzapfel, W.H. (2001). Incidence of virulence factors and antibiotic resistance among Enterococci isolated from food. *Applied Environmental Microbiology,* 67: 4385-4389.

Franz, C.M.A.P., Stiles, M.E., Schleifer, K.H. and Holzapfel, W.H. (2003). Enterococci in foods-a conundrum for food safety. *International journal of Food Microbiology,* 88:105-122

Furustrand, T.U., Majic, I., Zalila, B. C., Betrisey, B., Corvec, S. and Zimmerli, W. (2011) Gentamicin improves the activities of daptomycin and vancomycin against Enterococcus faecalis in vitro and in an experimental foreign-body infection model. *Antimicrobial Agents Chemotherapy,* 55: 4821–4827

Gelsomino R, Vancanneyt M, Condon S, Swings S, Cogan TM (2001).Enterococcal diversity in the environment of an Irish cheddar-type cheese making factory *International Journal of Food Microbiology*, 71:177–188.

Gelsomino, R., Vancanneyt, M., Cogan, T.M. and Swings, J. (2002). Effect of raw –milk cheese consumption on the enterococcal flora of human feaces. *Applied Environmental Microbiol*ogy, 69: 312- 319.

Giraffa, G., Olivari, A.M. and Neviani, E. (2000). Isolation of vancomycin-resistant Enterococcus faecium from Italian cheeses. *Food Microbiology,* 17: 671–677.

Giraffa, G (2002). *Enterococci* from foods. FEMS *Microbiology Review*, 26: 163-171.

Gonzalez, C., Langdon, G.M., Bruix, M., Galvez, A., Valdivia, E., Maqueda, M. and Rico, M. (2000). Bacteriocin AS 48, a microbial cyclic polypeptide structurally and functionally related to mammalian NK-lysin. *Procedure of National Academics of Science USA* 97, 11221-11226

Goossens, H., Ferech, M., Vander, S.R. and Elseviers, M. (2005). Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet,* 365: 579–587.

Gordon, C.L. and Ahmad, M.H.*(1991)* Thermal susceptibility of Streptococcus faecium strains isolated from frankfurters. *Canadian Journal of Microbiology* 37, 609–612.

Hugas, M., Garriga, M. And Aymerich, M.T. (2003). Functionality of Enterococci in meat products. *International Journal of Food Microbiology, 88: 223-233.*

Huys, G., D'Haene, K., Collard, J.M. and Swings, J. (2004). Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. *Applied Environmental Microbiology*, 70: 1555–1562.

Kojima, A., Morioka, M., Minima, M., Ishihara, K., Asai, T., Fujisawa, T., Tamura, Y. and Takahashi, T. (2010). Classification and antimicrobial susceptibilities of *Enterococcus* species isolated from apparently healthy food-producing animals in Japan. *Zoonoses and Public Health,* 57: 137–141.

Koutsoumanis, K. and Angelidis, A.S.(2007). Probabilistic modeling approach for evaluating the compliance of ready-to-eat foods with New European Union safety criteria for *Listeria monocytogenes. Applied Environmental Microbiology,* 73(15): 4996-5004.

Kucerova, K., Svobodova, H., Tuma, S., Ondrackova, I. and Plockova, M. (2009). Production of bio-amines by enterococci. *Czech Journal of Food Science,* 27: 50-52.

Lund, B., Adamsson, I. and Edlund, C. (2002). Gastrointestinal transit survival of an Enterococcus faecium probiotic strain administered with or without vancomycin *International Journal of Food Microbiology*, 77(1-2): 109-115.

Martin, B., Garriga, M., Hugas, M. and Aymerich, T.(2005). Genetic diversity and safety aspects of enterococci from slightly fermented sausages. *Journal of Applied Microbiology,* 98: 1177-1190.

McGowan-Spicer, L.L., Fedorka-Cray, P.J., Frye, J.G. and Meinersmann, R.J. (2008). Antimicrobial resistance and virulence of *Enterococcus faecalis* isolated from retail food. *Journal of Food Protection,* 71: 760–769.

Teuber, M., Meile, L. and Schwarz, F. (1999). Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek,* 76: 115–137.

WHO (2015): *"Antimicrobial resistance Fact sheet N°194"*

Zonenschain, D., Rebecchi, A. and Morelli, L. (2009). Erythromycin- and tetracycline-resistant lactobacilli in Italian fermented dry sausages. *Journal of Applied Microbiology,* 107: 1559–1568.