# MOLECULAR CHARACTERIZATION OF METHICILLIN RESISTANT *Staphylococcus aureus* (MRSA) ISOLATED FROM NONO AND YOGHURT CONSUMED IN KADUNA METROPOLIS, KADUNA STATE, NIGERIA.

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**ZARIA, NIGERIA**

**JUNE, 2016**

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**DEPARTMENT OF MICROBIOLOGY,**
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**AHMADU BELLO UNIVERSITY,**
**ZARIA, NIGERIA.**

**JUNE, 2016**

## DECLARATION

Ihereby declare that the work in this dissertation entitled '**Molecular Characterization of Methicillin Resistant *Staphylococcus aureus* (MRSA) isolated from Nono and Yoghurt Consumed in Kaduna Metropolis,Kaduna, Nigeria'**, hasbeen carried out by me in the Department of Microbiology, Faculty of Science, Ahmadu Bello University, Zaria, Nigeria, under the careful supervision of Prof. I.O. Abdullahi and Prof C.M.Z.Whong. The information derived from the literature has been duly acknowledged in the text and a list of references provided.

| | | |
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## CERTIFICATION

This dissertation entitled "**MOLECULAR CHARACTERIZATION OF METHICILLIN RESISTANT *Staphylococcus aureus* (MRSA) ISOLATED FROM NONO AND YOGHURT CONSUMED IN KADUNA METROPOLIS, KADUNA,NIGERIA"**by **FAUSAT ADEOLA ABDULRAHMAN,** (MSc/Sci/3403/2011-2012) meets the regulations governing the award of degree of Master of Science,Ahmadu Bello University Zaria and is approved for its contributions to knowledge and literary presentation.

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## DEDICATION

This dissertation is dedicated to my Husband Dr. M.K.A. Abdulrahman and my lovely daughters; Aminat, Shakeerat,Fatihat and my little ‘Dr’ Maryam Abdulrahman for their Prayers and Perseverance.

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## ABSTRACT

Three hundred and eighty four (384) milk samples (nono and yoghurt) were purchased from different selling points/vendors/hawkers and were evaluated for the occurrence of*S.aureus*.The physicochemical and proximate analysis (pH, total titrable acidity, moisture content, protein content, fat content,ash content,) were determined.The results of the chemical analysis showed that for nono, the pH ranged from 3.56-3.61, total titratable acidity: 0.71-1.72, moisture content: 82.23-83.87%, total protein content: 1.47-1.58 %, fat content: 0.35-0.38 %, ash content: 0.30-0.31% a. For yoghurt, the pH ranged from 3.76-3.82, total titratable acidity: 1.10-1.15, moisture content: 77.76-80.06%, total protein content: 3.38-3.40%, fat content: 1.76-1.79% and ash content: 0.68-0.70% from all the study areas. Out of the 384 Nono and Yoghurt samples examined,104 samples were contaminated with *S.aureus*. Out of the 104 isolates of *S.aureus*thatwere isolated and identified,88 were coagulase positive*S. aureus*.The Microgen Staph ID kit (Oxoid) was utilised to further confirm the characteristics of the 60 isolates of the coagulase positive*S.aureus*.The kit confirmed 43 isolates as*S.aureus* and the remaining 17 as other species of*S.aureus* which included; *S.intermedius,S. chromogenes,S.hyicus,S.cohnii subsp.urealyticum and S.heamolyticus*respectively.Twenty of the coagulase positive *S.aureus* strains were also investigated for staphylococcal enterotoxins (SE);SEA,SEB,SEC and SED. A total of 19 (95%) of the isolates were found to produce one or more staphylococcal enterotoxin (SEs). Nineteen of the isolates produced only one type of the SEs with SEA being the predominant toxins. The antibacterial susceptibility testing was carried out on 43 isolates of*S. aureus*. The results showed resistance to Oxacillin 43(100%),Tetracycline10(23.3%),Cefoxitin8(18.6%),Clindamycin10(23.3%),Erythromycin 1(2.3%),Vancomycin 1(2.3%), Chloramphenicol 1(2.3%).All the isolates were

susceptible to Ciprofloxacin.Most of the*Staphylococcus aureus*isolates tested exhibited multiple antibiotics resistance (MAR).Twenty (20) isolates of*S.aureus*representative of the study area were selected for genotyping by polymerase chain reaction (PCR) to detect the *mecA* gene.Only 1 of the 20 isolates was genotypically confirmed as MRSA positive. The results indicated the presence of *S.aureus* in the milk samples, antimicrobial resistance strains of*S.aureus,* enterotoxigenicstrains of*S. aureus* and presence of MRSA in the milk samples which are of public health importance. As all these can contribute to foodborne intoxication which can lead to food poisoning. Therefore there is need for better control of the spread of the antibiotics resistant strains as well as milk samples contamination sources. There should be proper pasteurisation of raw milk and fermentation of milk to help eliminate MRSA before human consumption.

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# CHAPTER ONE

## 1.0 INTRODUCTION

### 1.1 Background

Milk is a food of outstanding interest and has been taken by humans since the earliest prehistoric times and still forms the basis of most nation's economics (Egwaikhide *et al*., 2014). It is designed by nature to be a complete food for young animals and of high nutritional values. The principal constituents of milk include fat, protein,total solid, lactose, ash and in addition to this, it contains several hundred minor constituents which include milk fat, vitamins, metal ion and flavor compounds, which have a major impact on the nutritional and sensory properties of milk and dairy products (Egwaikhide *et al*., 2014). Milk is a highly valuable food, but raw milk contains and favour growth of many microorganisms (Helena *et al.*, 2010). Milk and its derivatives are considered as major sources of *Staphylococcus aureus* infection in man and it may be present in milk and other dairy products (Capuro *et al.*, 2010) which serve an excellent medium for many kinds of microorganisms.

Dairy products constitute an important group of food that was known to contain energy and nutrients such as fat, protein, carbohydrates, and calcium. They also make an important contribution to vitamin intake (Igbabul *et al*., 2013). Yoghurt is a fermented milk product whose typical flavor (sour taste) is attributable to the production of lactic acid, acetylaldehyde, acetic acid and diacetyl from carbohydrates by the fermenting organism.

It is one of the most popular fermented products widely consumed all over the world and is more nutritious than many other fermented milk products because it contains a high level of milk solids in addition to nutrients developed during fermentation process,

and its sensory attributes have a large effect on consumer acceptability (Saint-eve *et al.*, 2008). The flavour, texture and aroma of yoghurt vary depending on the country of origin as well as other factors like raw material formulation and manufacturing process. It is obtained by lactic acid fermentation of milk by the action of a starter culture containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus* and it can be in the form of a highly viscous liquid whereas in other countries, it takes the form of a softer gel. Yoghurt is produced in drinking form and can be frozen or blended with other ingredient to create for example mouse type products, sorbet yoghurt ice cream, or other form of dairy desert (Igbabul *et al*., 2013).

Nono is a Nigeria milk food similar to yoghurt and other fermented milk products that is traditionally produced and consumed particularly by the Hausas and Fulanis in northern Nigeria, it is a product of spontaneous fermentation caused by the introduction of starter culture into fresh milk, raw milk, unskimmed milk (Manshanu). Nono is produced in contaminated environment by hand milking of cow into large calabashes (Kworra), that is a crude cultured with subsequent skimming to remove the surface cream that become the fulanis butter (manshanu) which is a byproduct of preparation of milk cereal based food known as fura da nono (Obi and Ikenebomeh, 2007). Although acclaimed traditionally to have a better nutritional values than the unfermented milk, nono is basically kept unrefrigerated and its acceptability depends on the texture, flavor and taste which are in turn dependent upon the inherent microbial constituents. Nono being a crude cultured whole milk whose fermentation is brought about by a number of bacterial species from various sources that contaminate the fresh milk. During milk production, microbial contamination generally occurs from three main sources; from within the udder, from the exterior of the udder and from the surface of milk handling

and storage equipment, but the surrounding air, feed, soil, faeces and grass are possible sources of contaminations (Solomon *et al*., 2013).

*Staphylococcus aureus* is one of the major bacterial pathogens which causes clinical infection and food poisoning cases (Yu- Cheng *et al.,* 2008).

*Staphylococcus aureus* is an opportunistic human pathogen which is considered as the third most common pathogen causing food poisoning in the world is involved in a wide variety of diseases in humans and animals, and its pathogenicity is mainly related to a combination of toxin-mediated virulence, invasive capacity, and antibiotic resistance (Argudin *et al.,* 2010). It is also considered as a major foodborne pathogen (Hennekinne *et al.,* 2010). Some strains are able to produce enterotoxins within foodstuff, causing Staphylococcus food poisoning (SFP), (Argudin *et al.,* 2010) and Staphylococcus food poisoning is caused by the ingestion of food that contains enterotoxins (Scherrer *et al.,* 2004) which is one of the most prevalent causes of gastroenteritis worldwide.

It is also considered as a versatile pathogen in humans and animals which is responsible for a diverse spectrum of diseases ranging from minor skin infections to life threatening diseases, such as pneumonia and meningitis (Jangra *et al.,* 2010) and is also one of the most important agents of food poisoning globally (Genigeorgis, 1989).

The source of *Staphylococcus aureus* in raw milk is from cows with mastitis, from handlers or from poor hygiene, but when found in milk high levels of contamination can be reached quickly under favourable conditions. Its presence in foods can be a risk to human health, causing a public health problem, as these bacteria produce toxins that can cause toxic food infections (Quintana *et al.,* 2006). It can gain access to milk either by direct excretion from udder with clinical or sub clinical Staphylococcal mastitis or

by contamination from the environment during handling of raw milk (Scherrer *et al.,* 2004, Jorgensen *et al.,* 2005). When the udder is infected, *Staphylococcus aureus* may be excreted through milk in variable numbers up to $10^8$ cfu/ml (Asperger *et al.,* 2003).

It is well documented that *Staphylococcus aureus* produces several virulence factors, such as exoproteins and various cell surface protein that contribute to the pathogenicity of this organism. Most of these virulence factors have been well characterized (Baba *et al.,* 2002).

## 1.2 Statement of the Research Problem

Human illnesses from milk borne pathogens is usually associated with the consumption of raw milk or milk products (nono, yoghurt) and for the past 20 years, foodborne illnesses from dairy products consumption had been predominantly associated with *Salmonella enterica, Staphylococcus aureus, Listeria monocytogenes, Campylobacter jejuni,* and *Escherichia coli* 0157:H7 (CDC Division of Bacterial and Mycotic Diseases, 2005).

Contamination of dairy products with *Staphylococcus aureus* (*S. aureus*) bacteria may influence considerably their harmlessness, decrease their shelf quality and endanger the health of consumers.

Extensive therapeutic use of antimicrobials or with their administration as growth promoters in animal production has led to the development of resistance both in human and animals (Normanno *et al.,* 2007).

The emergence of bacteria resistant to most of the commonly used antibiotics and the occurrence of antibiotic-resistance bacteria in food of animal origin is a major public

hralth threat and of utmost importance to public health workers, veterinarians, food microbiologists and those interested in bacterial zoonosis (Khan *et al.*, 2001).

In the last decade, Antibiotics resistance in bacteria has caused increased concerns about public health as *S. aureus* can be characterized by single or multiple antibiotics resistance which represent a major threat to public health (Perreira *et al.*, 2009).

However,the emergence of resistance to multiple antibiotics among *S. aureus* has created breaking news for health practitioners and researchers as Hossein *et al.* (2010) and Khan *et al.* (2001) reported that there is high incidence of multiple antibiotic-resistant (MAR) pathogenic bacteria such as *S. aureus* in unpasteurized and pasteurized packaged milk. These resistant bacteria can be transferred to humans through the consumption of milk, thus rendering the resultant food-related infections difficult to treat.

Methicillin resistance is of particular relevance because it is conferred by the present gene (*Mec A*), which encodes production of an altered penicillin binding protein (PBP) (PBP2a or PBP2') that has low affinity for all beta-lactam antimicrobials (penicillin, cephalosporins, carbapenems) (Kwon *et al.*, 2005), and *Mec A* gene is considered as a useful molecular marker of methicillin resistance in all Staphylococci.

Until now, methicillin resistant *Staphylococcus aureus* (MRSA) strains were only rarely detected in food, as its transmission by food consumption has not been investigated thoroughly. Although, the presence or occurrence of MRSA in food may leads to food intoxication causing food poisoning.

### 1.3 Justification of the Research Problem

Milk borne illnesses have been noticed since early days in the dairy industriesand human illnesses from milk borne pathogens are usually associated with consumption of raw milk or products made from raw milk such as yoghurt and nono.

Milk which is mostly consumed raw or unpasteurized (as in the case of nono) in the study area may have some public health implications, as it serves as a medium or vehicle for the transmission of bacterial diseases which include salmonellosis, cholera, streptococcal infection, brucellosis, staphylococcus food poisoning (CDC,2011). Although, milk has an outstanding nutritional quality which make it an excellent medium for bacterial growth but when consumed without pasteurization can be an important source of bacterial infection.

African data on *Staphylococcus aureus* particularly antibiotics susceptibility are limited (Ghebremedhin *et al*., 2009), although MRSA has disseminated in African countries. Between 1996 and 1997, the prevalence of MRSA determination in eight African countries were relatively high. In Nigeria, Kenya, Cameroun (21-60%) and were below 10% in Tunisia and Algeria (Olonitola *et al.*, 2007; Okon *et al*., 2009; Ghebremedhin, 2009; Fadeyi *et al.*, 2010; Nwankwo *et al.*, 2010).

Currently, little information is available on the molecular characterization of strains of *S. aureus* associated with nono and yoghurt particularly in the study area. This information will provide an insight into the molecular aspect of *S. aureus* and also to detect putative virulence factors that are associated with the *S. aureus* infection. There is also limited information on staphylococcal enterotoxins (SEs) and methicillin resistance strains of *S. aureus* associated with nono and yoghurt.

## 1.4 Aim of the Study

This study was aimed at isolating and molecularly characterizing, *S. aureus* strains isolated from nono and yoghurt samples in Kaduna Metropolis, Kaduna State.

## 1.5 Specific Objectives

The Specific objectives of this work were to:

1. Determine the Physicochemical and Proximate composition of nono and yoghurt samples.
2. Determine the total viable counts of the organism in nono and yoghurt samples.
3. Isolate and characterize *Staphylococcus aureus* from nono and yoghurt samples.
4. Determine enterotoxin production by *Staphylococcus aureus* isolates using Staphylococcal Enterotoxin Rapid Passive Latex Agglutination (SET-RPLA) test kit.
5. Determine the antibacterial susceptibility patterns of the *Staphylococcus aureus* isolates.
6. Detect Mec A gene in the *Staphylococcus aureus* isolates using PCR techniques.

# CHAPTER TWO

## 2.0 LITERATURE REVIEW

### 2.1 Milk and Milk Products

Milk is one of the greatest blessings that are given to Humans by Nature. Milk is considered a complete and nutritious food; not only for the new-born mammal and for the human beings, but it is considered as a good medium for many microorganisms. Raw untreated milk is still used by large number of farm families and workers and by a growing segment of the general population who believe that the milk is not only safe but also imparts beneficial health effects that are destroyed by pasteurization (LeJeune and Rajala-Schultz, 2009). For this reason, utilization of both raw untreated milk and raw milk cheeses has frequently been associated with foodborne illness. Zoonotic bacterial agent's presence in raw milk is of great public health and economic significance.

In Africa, the level of cultural awareness among farmers about the importance of economic and public health from zoonotic diseases is low, and this increases the effort required to control these diseases (Munyeme, *et al*. 2010). Raw milk is usually colonized by a variety of many zoonotic pathogens such as *Campylobacter jejuni*, Enterohaemorragic *Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, Staphylococcus aureus* and *Yersinia enterocolitica* therefore; they represent an important source of foodborne pathogens. These pathogens in milk have been linked to the environment in the farm, mixing clean milk with mastitis milk and from livestock (Marco & Wells-Bennik, 2008).

The natural raw milk obtained from the mammary gland of healthy animal is usually with low microbial load and the application of all hygienic measures during milking prevents milk from contaminating as well. The bacteria can access to the milk through colonization of the teat canal or an infected udder (clinical and subclinical mastitis) or gets contaminated from milk utensils or water supply used (Hayes*et al.,* 2001). The presence of bacteria in milk has many undesirable effects on the quality and safety of milk and its products (Szteyn *et al.,* 2005). Milk contaminated by high levels of bacteria usually becomes unsuitable for further processing (Nanu *et al.* 2007).

Yoghurt is made by the controlled thermoduric fermentation of pasteurized non-fat or low-fat milk, carried out around $45^0$C (Prescott *et al.,* 2008). It is probably the most popular fermented milk product in the United States and in Nigeria too (Nwagu and Amadi, 2010; Prescott *et al.,* 2008). It is produced both commercially and by individuals, using yoghurt-making kits. Commercial starter culture consisting of *Lactobacillus bulgaricus* and *Streptococcus thermophiles* are used in the fermentation process of yoghurt production (Prescott *et al.,* 2008). Nono is produced by essentially illiterate Fulani's in villages with poor knowledge of shelf-life, product safety, sanitation and aseptic milking techniques, handlers of this products may unknowingly introduce pathogenic micro organisms into the products (Godwin and Emmanuel, 2013). These products do not undergo further processing before being sold for consumption; these foods may become potential sources of illness to their consumers (Uzeh *et al.,* 2006).

Many dairy products, even those made from pasteurized milk, have been associated with food-borne diseases. This is partly because milk and milk products provide favourable media for microbial growth. Different categories of microorganisms

including fungi, bacteria, rickettsia and viruses could be found in milk since the udder of the animal could harbour organisms while others come as contaminants due to poor handling (Ogbonna, 2011).

Most handlers or sellers of nono are street peddlers. Often, not all nono brought to the market by peddlers are sold the same day and in most developing countries, such unsold ones are reintroduced into the market with no further treatment or proper preservation. Thus, pathogenic organisms that might have gained access into the product have enough time to multiply and produce harmful metabolites (Ogbonna, 2011).

### 2.1.1 Composition of milk

Milk is an essential first food for man, forming an important part of man's diet both for infants and adults. The majority of milk consumed throughout the world is bovine milk, although in some regions, milk from goat, ewe or mare may be more commonly used (Godwin and Emmanuel, 2013). Milk is a major source of dietary energy, protein and fat, contributing on average 134 kcal of energy/capita per day, 8 g of protein/capita per day and 7.3 g of fat/capita per day in 2009 (FAOSTAT, 2012). Milk contains carbohydrate in form of lactose, fat, vitamins and chloride, inorganic phosphate and citrate (Komorowski and Early, 1992).

Water is the main component in all milk, ranging from an average of 68 percent in reindeer milk to 91 percent in donkey milk. The main carbohydrate is lactose, which is involved in the intestinal absorption of calcium, magnesium and phosphorus, and the utilization of vitamin D (Campbell and Marshall, 1975, cited in Park *et al.,* 2007). Lactose also provides a ready source of energy for the neonate, providing 30 percent of

the energy in bovine milk, nearly 40 percent in human milk and 53–66 percent in equine milk (Fox, 2008).

Cow milk accounted for 83 percent of global milk production in 2010 (FAOSTAT, 2012). Cow milk contains more protein and minerals, especially calcium and phosphorus, than human milk. Cow milk contains more protein than doe's human milk, but human milk contains more lactose, resulting in comparable energy contents. Cow milk and human milk differ in the amounts of various proteins they contain. Human milk does not contain β-lactoglobulin, one of the main proteins associated with cow milk allergy.12 Levels of water-soluble vitamins in human milk reflect maternal levels and depend on the mother's diet, but these vitamins are synthesized within the body of the cow and levels are not diet-dependent in cow milk

Milk composition is affected by various factors, including stage of lactation, breed differences, number of calving (parity), seasonal variations, age and health of animal, feed and management effects including number of milking per day and herd size (Jenkins and McGuire, 2006). The influence of animal feed on milk composition has been, and continues to be, the focus of many studies. Milk can be modified to improve its nutrient value and sensory quality by changing the animal's diet (Slots *et al.,* 2009; Wiking *et al.,* 2010).

None of the milk is a source of iron, which is the main reason why animal milk are not recommended in the complementary feeding of infants less than 12 months old. In addition, feeding animal milk to infants can lead to intestinal bleeding and loss of iron, as has been shown on studies on cow milk (Ziegler, 2007). The high calcium and casein contents in most milk also inhibit the absorption of dietary nonheme iron (Ziegler, 2007).

The consumers' expectation of processed milk is that it should have the typical appearance of milk, be free from extraneous matter, and should have a clean and slightly sweet taste, with no abnormal odours or taints (Godwin and Emmanuel, 2013). Also, apart from the microbiological quality of raw milk being of great importance in regard to product and food safety, raw milk should be unadulterated and free of taints, antibiotics, blood and visible sediment (Godwin and Emmanuel, 2013).

Very little raw milk (i.e., "milk which has not been heated beyond 40°C or undergone any treatment that has an equivalent effect") is now drunk (FAO and WHO, 2009). The quality of milk is determined by aspects of composition and hygiene. Due to its complex biochemical composition and high water activity milk serves as an excellent culture medium for the growth and multiplication of many kinds of microorganisms (Soomro *et al.,* 2002). Therefore in the processing of milk, some of them may produce undesirable effects and some micro-organisms produce food infections which can either carry the pathogens that will increase the likelihood of infection of the consumer`s food (Soomro *et al.,* 2002).

### 2.1.2 Nono

'Nono' is the Hausa name for fermented milk which is sold along with butter, (maishanu) a by- product of its production (Omotosho *et al.,* 2013). Nono also called 'nunu' by some tribes in Nigeria, contained good quantities of amino acid, calcium, phosphorous and vitamin A, C, E and B complex (Nebedum and Obiakor, 2007). Nono is a general name used for locally fermented cow milk and it is widely consumed in many African countries, including Nigeria (Uzeh *et al.,* 2006). Predominantly, nono is being prepared by the nomadic Fulani/Hausa cattle herdsmen who control over 80% of Nigeria's cattle production. Consumption of nono is limited to the Fulani/hausa

indigenes (Obi and Ikenebomeh, 2007, Adesokan *et al.*, 2011), since non indigene see their preparation as apparently unhygienic and has poor shelf life (Yahuza, 2011). It is gaining wider acceptance nowadays. It is an opaque white to milky coloured liquid food drink gotten from fermented raw milk. It is a healthy food whose consumption transverses the Saharan tribes of West African Sub-region extending to the inhabitants of the Mediterranean region and also the Middle East. In the Middle East, it is called 'dahi' or 'lassi' (Nahar *et al.,* 2007).

It is a crude cultured whole milk whose fermentation may be brought about by a number of bacterial species from various sources that contaminate the fresh milk (Atanda and Ikenebomeh, 1990). 'Nono' is locally produced in homes, especially in villages where shelf-life and safety of the product is not considered (Omotosho *et al.,* 2013). 'Nono' is however sold to all interested people as food. 'Nono' is produced from non-pasteurized cow milk collected in a calabash and allowed to ferment naturally for 24 h (Olasupo *et al.,* 1996).

Fresh cow milk is unfermented, raw cow milk collected fresh, from the cow's udder (Godwin and Emmanuel, 2013). Consumers have a strong preference for these traditionally produced and processed milk products due to the satisfying nature and also its high protein content (Belewu and Aina, 2005). It could also be due to its affordability in comparison with imported, processed milk products whose prices are higher.

Nono has been claimed to be highly contaminated with such organisms like *Staphylococcus aureus, Escherichia coli, Bacillus aureus, Pseudomonas aeroginosa*, due to the gross unhygienic methods of preparation and handling (Obi and Ikenebomeh, 2007).

### 2.1.3 Yoghurt

Yoghurt is a semi solid fermented milk product which originated in Mesopotamia thousands of years ago. Evidence has shown that these people had domesticated goat and sheep around 5000 B.C. The milk from these animals was stored in goured and in the warm climate.It naturally formed curd which was an early form of yoghurt (Omola *et al*., 2014). One legend tells that yoghurt was born by miracle of nature. Microorganism of various kinds happened to land in a pitcher of milk that belong to a Turkish norms. The result was what the turks called 'Yogurut'. The name yogurut was supposedly introduced in the $8^{th}$ century and was changed in the $11^{th}$ century to the current version, yoghurt (Omola *et al*., 2014). In the early years of milk fermentation, milk was simply allowed to ferment by its normal microbiota but the actual process was not completely understood, but culture could be maintained by inoculating fresh milk with fermented milk (Kerr and Mcttale, 2001). Today lactic acid-producing microorganisms are added to milk to decrease the pH of the milk and produce many different fermented milk products (Guarne *et al*., 2005). Yoghurt is made by the controlled thermoduric fermentation of pasteurized non fat or low fat milk, carried out around $45^{o}$C (Prescott, 2008). Yoghurt has been around for many years.It is only recently (within the last 30-40 yrs) that it has become popular, today it plays a major role in many different world (origins). It is probably the most popular fermented milk product in the United State and Nigeria (Nwagu and Amadi, 2010; Prescott, 2008). It is produced both commercially and industrially using yoghurt making kits. Commercial Starter culture consisting of *Lactobacillus bulgaricus and Streptococcus thermophilus* are used in the fermentation process of yoghurt (Prescott, 2008).

Increase in the demand for yoghurt in Nigeria in recent times has led to proliferation of different kinds of yoghurt, spoilage and pathogenic microorganisms are capable of being a source of illness and sickness for the large populace.

## 2.2 The Genus Staphylococcus

Rosenbach in 1884 was given the credit for the name Staphylococcus and the species name *Staphylococcus aureus* (Ryan and Ray, 2004). *Staphylococcus aureus* is coined from the Greek and Latin words (Greek.Staphyle=bunch of grapes; and Latin. Coccus=spherical bacterium, aureus=golden) or golden staph (as pronounced 'staff') is the most common species of *Staphylococcus* bacteria causing infections in humans and animals (Ryan and Ray, 2004)

Taxonomically, *Staphylococcus aureus* is placed into:

Kingdom-Bacteriae

Phyllum - (Division) XIII. Firmicutes (Lat:firmus=strong, cutis=skin, referring to the cell wall).

Class I. - Bacilli

Order I. - Bacillales

Family VIII - Staphylococcaceae

Genus - *Staphylococcus*

Species - *Staphylococcus aureus* (Bergey's Manual, 2001).

### 2.2.1 Biology of staphylococci

*Staphylococcus aureus* is the most significant human pathogen among the *Staphylococci* (Sandel *et al.,* 2003). The staphylococci group are gram positive, spherical cocci that are widely distributed in the environment and on the integuments of man and animals. They seldom form chains but occur singly or irregular aggregations or grape-like clusters, because the cells divide in three perpendicular manner on solid medium. The staphylococci are non motile and do not form spores. They are catalase positive, oxidasenegative, and are facultative anaerobes, but most seem to prefer an aerobic condition and only very few are strictly anaerobic. Nearly all strains of *S. aureus* produce the enzyme coagulase (Madigan and Martinko, 2005). There are eleven capsular polysaccharides serotypes that have been described for *S. aureus* (Katherine and Lee, 2004) and the most common in clinical isolates are types 5 and 8. The main capsular components are N-acetylaminouronic acids and N-acetylfucosamine.

Staphylococcal bacteriophages were among the first to be demonstrated, and this has led to the establishment of a bacteriophage typing method for epidemiologic studies. Most pathogenic strains belong to phage groups II and III, but all are capable of causing disease.

The cell wall of staphylococcal is 50 percent peptidoglycan by weight, and the peptidoglycan consists of alternating polysaccharides subunits of N-acetylglucosamine and N-acetylmuramic acid with 1,4β linkages. The peptidoglycan chains are cross-linked by tetrapeptide chains bound to N-acetylmuramic acid by a pentaglycine bridge specific for *S. aureus* (Madigan and Martinko, 2005).

### 2.2.2 Growth and physiological properties of *S.aureus*

On agar plates, the staphylococci form round, opaque, glistering colonies and grows at an optimum temperature of $37^0$C, though it can grow at any temperature between $10^0$C and $46^0$C, The species is a facultative anaerobes whose growth is enhanced in the presence of $O_2$ and $CO_2$. The Strains of *Staphylococcus aureus* are versatile because they can digest proteins and lipids, and ferment a variety of sugars. This species is considered among the most resistant of all non-spore forming pathogens with well developed capacities to withstand high salt (7.5% - 10%), extreme pH, and high temperatures (up to $60^0$C for 60 minutes). Colonial variants of the staphylococci are mucoid, smooth and rough. In addition, staphylococci form a G-type (gonidial - type) colony that is a very small, dwarf variant of normal sized colonies. It is often obtained in the presence of antibiotics or increased salt concentrations and it grows more slowly than normal cocci. Its rate of growth is influenced by carbon dioxide tension. While it has some similarity to L forms, apparently its cell walls are intact. Some staphylococci produce pigment and such pigment forms optimally at or below body temperature and aerobically on enriched medium containing fatty acids and carbohydrates (glucose, Galactose, mannose, mannitol or trehalose). On the other hand, arabinose and rhamnose have no relation to pigment production. Pigment is not produced in liquid culture or anaerobically. *Staphylococcus aureus* typically produce golden yellow pigment, while *Staphylococcus epidermidis* are composed of at least two carotenoids, sarcinaxanthine and B-carotene (Sulaiman, 2011).

## 2.3 Species of Staphylococcus

Approximately 40 different species of staphylococci have been identified to date (Lindsay, 2008). Staphylococcus 91X/A01/138, *S. arlettae, S. aureus, S. auricularis, S.*

*caprae, S. carnosus, S. chromogenes, S. cohnii, S. condiment, S. croceolyticus, S. delphini, S. epidermidis, S. equorum, S. faecalis, S. felis, S. fleuretitii, S. gallinarum, S. haemolyticus, S. hominis, S. hyicus, S. intermedius, S. kloosii, S. lentus, S. lugdunensis, S. lutrae, S. lyticans, S. muscae, S. nepalensis, S. pasteuri, S. pettenkoferi, S. piscifermentants, S. pseudintermedius, S. roseus, S. saccharolyticus, S. saprophyticus, S. schleiferi, S. sciuri, S. simulans, S. succinus, S. vitulinus, S. warneri, S. xylosus*

### 2.3.1 Some of the pathogenic staphylococcal species of human and animal

#### *2.3.1.1 Staphylococcus intermedius*

Isolates that were traditionally identified as *S. intermedius* colonize the skin, hair coat and particularly mucocutaneous sites such as the nose, mouth and anus of dogs and other animals including horses, mink and pigeons (Allaker *et al.*, 1992a; 1992b).

Dogs with skin problems are commonly diagnosed with superficial pyodermo (also known as superficial bacterial folliculitis), and *S. intermedius* is regarded as the primary pathogen isolated from pyoderma cases. In fact, superficial bacterial follicullitis is the most frequent skin disease in dogs after flea allergy dermatitis (Gross *et al.*, 2005). Superficial bacterial pyoderma is a skin infection of the follicle (follicullitis) and the main clinical feature is the formation of follicular pustules which have an erythematous base and are filled with white or yellow purulent material. Canine cutaneous staphylococcal infections often occur as secondary infections to predisposing factors such as atopic dermatitis (AD), flea allergy dermatitis, demodicosis or hypothyroidism (Gross *et al.*, 2005). *S. intermedius* is also the primary pathogen of deep pyoderma which may develop from superficial pyoderma, impetigo and superficial spreading pyoderma. *S. intermedius* is not usually isolated from humans and seems to show a host-specificity to canine corneocytes (Simou *et al.*, 2005a).

However, transmission between humans and their pets can occur (Guardabassi *et al.,* 2004), and *S. intermedius* can be regarded as a true zoonotic pathogen (Tanner *et al.,* 2000). A common route of invasive infection in humans is through dog bite wounds and several instances of life-threatening infections of humans have been reported (Talan *et al.,* 2003; Pottumarthy *et al.,* 2004).

*2.3.1.2Staphylococcus epidermidis*

*Staphylococcus epidermidis* is an opportunistic pathogen whose ability to persist and multiply in a variety or environments results in a wide spectrum of disease in both humans and animals. As reported by Sulaiman, 2011 that in humans, *S. epidermidis* infection is one of the most devastating complications of prosthetic join surgery.

However, recent studies have reported the increasing frequency of *S. epidermidis* intramammary infections of cows, sheep and goats. *S. epidermidis* infections are often milder than those produced by *S. aureus*, but can result in significant loss of milk production. For example, one study showed that *S. epidermidis* infections caused an 8.7% loss in milk production from a 305-day milk yield total (Sulaiman, 2011). The population genetics of *S. epidermidis* are not well understood, but recently a MLST system was developed which has identified some genetic diversity between *S. epidermidis* strain (Thomas *et al.,* 2007). The study did not analyse the relationship between human and animal strains but the ability of *S. epidermidis* to cause chronic mastitis is well recognized and very little is known about the virulence factor that contribute to pathogenesis. Interestingly the bap gene that encodes the biofilm associated protein was found in a number of different staphylococcal species including *S. epidermidis, S. chromogenes, S. xylosus, S. hyicus, and S. simulans.* The bap genes from coagulase negative staphylococci are involved in biofilm formation, similar to *S.*

*aureus* (Tormo *et al.,* 2005). As such Bap may contribute to the intramammary pathogenesis of *S. epidermidis*

*2.3.1.3Staphylococcus scuiri*

As reported by Chen *et al.* (2007) from a publication, that there is a highly pathogenic strain of *S. scuiri* HBXX06 that caused an acute form of exudative epidermidis in piglets in both natural and experimental conditions and this suggested that the isolates HBXX06, was a new emerging pathogen for exudative epidermidis in pigs.

Members of the *S. scuiri* group are widely distributed in nature and can be isolated from variety of animals and products of animal origin as well as from humans (Stepanovic *et al.,* 2003; Shittu *et al.,* 2004), but most of them are non-pathogenic to animals. However, they are important human pathogens responsible for episodes of ondocarditis, septic shock, pertitonitis, urinary tract infection, wound infection sand pelvic inflammatory disease (Chen *et al.,* 2007).

Currently, little information is known regarding the pathogenicity of *S. scuiri* in animals, and it has also been reported that *S. scuiri* can be associated with mastitis in ruminants such as goats and cows (Rahman *et al.,* 2005).

*2.3.1.4 Staphylococcus hyicus*

*Staphylococcus hyicus* is generally recognized as the causative agent of skin diseases exudative epidermitis in pigs, while other species of *Staphylococcus* may also be involved in this disease

*Staphylococcus hyicus* is associated with the skin disease exudative epidermitis in pigs, also called Greasy Pig Disease. This organism has also been occasionally isolated from

cow with mastitis, horses with dermatitis, and chickens with exudative dermatitis or tenosynovitis (Sulaiman, 2011). In all cases, productionof several exfoliative toxins, have been involved in the pathogenesis of the disease. Using the skin disease exudative epidermitis in pigs as a model of infection, the exfoliative toxins responsible for the characteristic lesions associated with the disease have been identified, cloned and purified from strains in Japan and Denmark (Ahrens and Andresen, 2004).

## 2.4 Identification of the Genus Staphylococcus

Members of the genus *Staphylococcus* have historically been classified and differentiated on the basis of a variety of phenotypic characteristics such as morphology and biochemical reactions. Pigment was the first criterion used to identify *Staphylococcal* species, and in 1884, Rosenbach identified members of the genus *Staphylococcus* based on the colour of colonies. Rosenbach called Staphylococci forming orange-yellow colonies *S. aureus* (or *S. pyogenes aureus*), while staphylococci forming white colonies were named *S. albus* (or *S. pyogenes albus*) (Sulaiman, 2011). It was not until almost 45 years later (1930) that another characteristic was described for differentiation between staphylococci, the coagulase test investigating the ability of staphylococcal species to clot blood plasma (Kloos, 1980). This test resulted in the separation of staphylococci into two groups, the coagulase positive staphylococci (CPS) (formally *S. aureus*) and coagulase negative staphylococci (CNS). Later on, during the 1960s, Baird-Parker conducted some of the most comprehensive of a large number of isolates of staphylococci, and proposed a sub-division into six sub-groups I-VI. *S. aureus* was placed in sub-group I, and sub-groups II-VI contained different types of CNS.These sub-group were later called the *S. epidermidis* group. However, during the following years other approaches were used to identify members of the genus

*Staphylococcus*, which resulted in the discovery and naming of new species and subspecies of CPS, but mainly of CNS. Based on the these characteristics used in those studies, 37 species and 17 subspecies were identified in the genus *Staphylococcus* (Bannerman and Peacock, 2007).

### 2.4.1 Identification of coagulase positive *Staphylococci* (CPS)

In the Manual of Clinical Microbiology 5th edition, there are six species of CPS other than *S. aureus*that are listed, e.g. *S. deiphini, S. intermedius, S. lutrae, S. pseudointermedius, S. schleferi subs. Coagulans*, and the coagulase variable *S. hyicus* (Bannerman and Peacock, 2007). *S. aureus* being pathogenic to the human and animal, is considered the most important of the CPS species whereas *S. hyicus* is less diagnosed and *S. intermedius* are frequently diagnosed in human (Griffeth *et al.,* 2008; Saadatian-Elahi *et al.,* 2008). In bovine mastitis, *S. aureus* is also considered to be the most relevant CPS, as *S. hyicus and S. intermedius* have only rarely been identified (Roberson *et al.,* 1990, Rampone *et al.,* 1993). However, few studies have investigated the prevalence of those CPS in mastitis, and today all bovine CPS diagnosed in routine laboratories are considered as *S. aureus* (Hogan *et al.,* 1999). When species identification of CPS is attempted, phenotypic and genotypic methods have been used.

### 2.4.2 Identification of coagulase negative *Staphylococci* (CNS)

In humans and animals, CNS has mainly been considered to be saprophytic and only rarely pathogenic. Currently, 30 species and subspecies of CNS are listed (Bannerman and Peacock, 2007), some of these species are recognized as potential pathogens to both human and animals (Layer *et al.,* 2006). The species that most frequently cause diseases in humans are *S. epidermidis, S. haemolyticus, and S. saprophyticus* (Cunha Mde *et al.,* 2004), while *S. stimulans, S. chromogenes, S. hyicus* and *S.*

*epidermidis*seem to be the most common in bovine mastitis (Luthje and Schwarz, 2006; Taponen *et al.,* 2006). The identification of CNS is, however, complicated by the heterogeneity of this group of bacteria. Therefore, a large number of biochemical tests are needed for phenotypic differentiation between CNS species (Martineau *et al.,* 2001; Bannerman and Peacock, 2007). Identification of CNS can, however be made using genotypic methods.

*2.4.2.1 Phenotypic Methods*

Different procedures involving a varying number of biochemical tests have been proposed

(Thorberg and Barnstorm, 2000, Bannerman, 2003; Cunha Mde *et al*., 2004 Bannerman and Peacock, 2007) for identification of CNS isolates from humans and animals. These procedures are mostly not suitable for routine use as they are laborious due to the large number of biochemical tests required. Moreover, several tests are rather time-consuming. In addition, some of the procedures were not able to identify some of the most important CNS (Thorberg and Barnstorm, 2000; Cunha Mde *et al.*, 2004; Bannerman and Peacock, 2007). For routine identification of CNS isolates, ease of testing and time needed for the tests are critical variables. Different commercial identification systems have been developed to identify members of the genus *Staphylococcus* and in particular those of the CNS group. Approximately, twenty different commercial systems are available (Bascomb and Manafi, 1998) and approximately six of them have been tested on CNS isolates from animals (Ruegg, 2008). Most commercial identification systsems are based on a limited number of biochemical reactions, and were mainly developed for identification of human isolates. The accuracy, i.e. the level of agreement between the commercial system and a given

reference method, reported for the commercial systems varied ranging from 11% to 99% (Watts and Yancey, 1994; Bascomb and Manafi, 1998). Thecolonial morphology on blood agar, pigmentation and hemolytic are not sufficient for species identification of CPS (Bannerman and Peacock, 2007). There are, however, a few tests that have used with some success for differentiation between *S. aureus. S. hyicus* and *S. intermedius*, e.g. P-agar supplemented with acriflavin, anaerobic fermentation of mannitol, production of accretion form glucose, and expression of B-galactosidase (Devriese *et al.*, 2005) Recently,conventional tests failed to distinguish between *S.aureus,S.intermedius* and *S. pseudintermedius* (Sasaki *et al.,* 2007b). Several commercial identification systems have been used to improve the identification accuracy of Coagulase Positive Staphylococci (CPS) (Watts and Yancey, 1994; Bascomb and Manafi, 1998). However, the differentiation accuracy varied markedly (38%-91%) when both human and animal isolates were tested (Bascomb and Manafi, 1998).

*2.4.2.2 Genotypic methods*

Genotypic methods use DNA as the basis for Coagulase Negative Staphylococci (CNS) identification.Generally, such methods have higher discriminatory power, reproducibility and typeability than conventional phenotypic methods (Stepan *et al.*, 2004).

Various other DNA based methods such as 16S rRNA sequencing (Becker *et al.*, 2004), ribotying (Svec *et al.*, 2004), PCR – restriction fragment length polymorphism analysis (Becker *et al.*, 2004). Amplified length polymorphism fingerprinting (AFLP) (Taponen *et al.*, 2007), internal transcribed spacer PCR (Fujitu *et al.*, 2005), tRNA intergenic spacer length polymorphism analysis (Maes *et al.*, 1997) and whole –

genome DNA-DNA hybirdization analysis (Svec *et al.,* 2004) have also been described for species identification of the genus *Staphylococcus*. None of these methods is today considered suitable for routine use due to problem with differentiation between some Staphylococci, and interpretation of results, due to being too time consuming and expensive. In the last decade, several genes, e.g. 16SrRNA, 23SrRNA, nuc, coa and tuf, have been used for identification of *S. aureus* and other clinically important CPS using PCR-based methods. Among those methods, real-time PCR is increasingly adopted for diagnose of CPS in humans due to its high sensititivity and specificity, and low time-consumption (Riffon *et al.,* 2001; Epsy *et al.,* 2006). For example, a PCR targeting the nuc gene is now becoming established in clinical micorbiology laboratories for detection of *S. aureus* in blood cultures in humans (Hogg *et al.,* 2008) similarly, Graber *et al.* (2007) developed a PCR test that allows specific detection of low concentrations of *S. aureus* in bovine milk samples. However, less encouraging results have been achieved when trying to identify CPS other than *S. aureus* (Takahashi *et al.,* 1999). Other gene targets have also been proposed such as sodA and hsp60 (also called cpn60 pr groEL). They have been reported to be more divergent than 16SSrRNA genes (Devriese *et al.,* 2005) in staphylococcal identification, having sufficient discriminative power to differentiate between *S. delphini, S. intermedius, and S. pseudintermedius* (Sasaki *et al.,* 2007a). They are used to a lesser extent as reviewed by Stephan *et al.* (2001).

## 2.5 Various Typing Techniques for *S. aureus*

Various phenotypic method such as phage typing, and multi-locus enzyme electrophoresis (MLEE) have been used to differentiate between strains of *Staphylococcus aureus*. Phage typing have several draw backs, which confine its use to

relatively few reference laboratories (Bannerman *et al*., 1995). For example, when use in an outbreak situation, the main disadvantage was the high proportion of isolates which were non-typeable (Weller, 2000). Even though MLEE patterns are relatively easy to read and interpret, the facts that strains comparisonis difficult (application of sophisticated algorithms and computer software are needed), the technique is labour intensive, and that unrelated isolates were mistakenly included when epidemiologically linked *S. aureus* were evaluated have confined MLEE to the research laboratory (Tenover *et al.,* 1994). Among other genotypic methods that have been use to differentiate between *S. aureus* strains are Pulsed-Field Gel Electrophoresis (PFGE), Ribotyping, Binary-Typing (BT), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD) and Multilocus Sequence Typing (MLST).

Today, PFGE is considered to be the 'gold standard', especially when typing bovine *S. aureus* (Stepan *et al.,* 2004) strains due to its excellent typeability, reproducibility (intra-and inter-laboratory), discriminatory power and easy interpretation (Zadoks *et al.,* 2002) and the costs involved are also moderate (Olive and Bean, 1999). The main disadvantage of PFGE is that it is time consuming as it takes several days before the result is achieved.

Among other genotypic methods used is ribotyping which exhibits excellent reproducibility and stability, but has lower discriminatory power than other techniques (Tenover *et al., 1*994). Although, it is also time consuming and technically complicated, ribotyping has not been widely adopted for typing *S. aureus* (Weller, 2000). Binary-typing is a relatively new robust technique, with discriminatory power superior to that of PFGE, and will possibly become a powerful tool for strain

characterization of bovine *S. aureus*, but is so far not widely use (Weller, 2000; Zadoks *et al.,* 2000; Stepan *et al.,* 2004). The disadvantage of this method is the time needed to complete the process (Van Leeuwen *et al.,* 1999). Amplified fragment length polymorphism fingerprinting is another method used which is easy to interpret, has high discriminatory power, good inter-laboratory reproducibility and results are obtained in two days (Olive and Bean, 1999). However, the costs are substantial, which may be prohibitive for many laboratories. Multilocus sequence typing provides a new approach to molecular epidemiology making it possible to track global spread of bacteria (Turner and Feil, 2007). The mobile SCCmec elements, are classified by analysis of their cassette chromosomes recombines (ccr) and *mec A* gene complexes (Ito *et al.,* 2001). SCCmec types also differ with regards to their acquisition of resistance determinants acquired as the result of integration of plasmids and transposes (Ito *et al.,* 2001). At least 5 SCC mec types (1-V), varying in size from~ 20kb to 68kb have been identified. The advantages of MLST are its high discriminatory power, and reproducibility, and results can be compared between studies and between laboratories. However, it has the disadvantage of being expensive and highly work demanding as reviewed by Stepan *et al.* (2004).

## 2.6 *Staphylococcus aureus* in Animals

*Staphylococcus aureus* colonizes a wide variety of members of the animal kingdom, including mammals, reptiles, and birds. In addition to the natural hosts identified, infection models for this clinically important bacterial species have been established in lower organisms, such as insects (Drosophila melanogaster) and worms (Ceanorhabditis elegans) (Garcia-lara *et al.,* 2005). Although, a normal flora of skin and mucous membrane, *S. aureus* can cause a variety of different animal infections

including tick- association pyaemia in lambs (Webster and Mitchell, 1989), staphylococcus in rabbits (Hermans *et al*., 2003), oedematous and necrotic dermatitis, septicaemia, abscesses and chondronecrosis in chickens (Mcnamee *et al.,* 1999), and pneumonia and osteomyelitis complex in turkeys (Alfonso and Barnes, 2006). However, the most economically important *S. aureus* infections of animals are intra-mammary infections (IMI) of lactating ruminants often leading to chronic mastitis (Miles *et al*., 1992). Because these types of infection is very difficult to eradicate with antibiotic thereapy, a premature culling of animals, involving substantial production losses, is often the only efficient strategy for control. The ability of *S.aureus* to cause disease is due to a combination of virulence factors, including toxins, adhesives, secreted exo-proteins and biofilm- related factors. Thus, its ability to cause infections in humans and animals raises the important question of whether *S. aureus* strains are capable of causing infections of different host species or are largely adapted to infect a single host only as population genetic studies have been employed to address this question.

## 2.7 Population Structure of *S. aureus* from Humans and Animals

### 2.7.1 Population structure of *S. aureus* from human and animals infections

Several studies have examined the diversity among natural populations of *S. aureus* infecting humans and animals (Van Leeuwen *et al.,* 2005). Important insights have been obtained from studies using Multilocus enzyme electrophoresis (MLEE) and more recently, Multilocus sequence typing (MLST). For example, in a classic population genetic study, MLEE was used to construct a framework of over 2000 isolates of *S.aureus.*

### 2.7.2 Sub-typing of *S. aureus*

It has been recognized that *S. aureus* contains a lot of different strains. Therefore, sub-typing of *S. aureus* isolates may be a necessary procedure when investigating outbreaks of disease, sources and routes of infection and virulence factors. Over the last decade, various phenotypic and genotypic methods have been developed and explored for their efficacy in differentiation between *S. aureus* strains.

## 2.8 Human *Staphylococcus aureus* Disease

*Staphylococcus aureus* is an important pathogen due to a combination of toxin-mediated virulence factors, invasiveness and antibiotic resistance. This bacterium is a significant cause of nosocomial infections, as well as community – acquired disease (Wenzel *et al.,* 1991). Some *S. aureus* strains are able to produce staphylococcal enterotoxins (SEs) and are the causative of *S. aureus*, it can grow in a wide variety of foods. This characteristic plus their ecological niche, can easily explain their incidence in foodstuffs that require manipulation during production, including fermented food products, such as cheese, yoghurt, nono etc. Although, *S aureus* contamination can be readily avoided by heat treatment of food, it remains a major cause of food borne disease because it can contaminate food products during preparation and processing. Staphylococcal food poisoning, rely on on single type of virulence factor, the staphylococcal enterotoxins. The symptoms of staphylococcal food poisoning are abdominal cramps, nausea, vomiting, sometimes followed by diarrhea (never diarrhea alone). The onset of symptoms is rapid (from 30 min to 8h) and usually spontaneous remission is observed after 24h (Loir *et al.,* 2003). The cell wall peptidoglycan and teichoic acid contributes to the pathogenicity of *Staphylococci* (Prescott, 2008). The pathogenicity of *Staphylococcus aureus* is due to its numerous virulence factors. It is

present as a normal flora of human beings and colonizes skin, but may become pathogenic and result in minor skin infections and abscesses, to life-threatening disease such as pneumonia, meningitis, endocarditis, toxic shock syndrome (TSS), septicaemia, mastitis, phlebitis, urinary tract infections, and osteomyelitis (Bhatia, 2009). Humans are the natural reservoir of *S. aureus* as 30 to 50 percent of healthy adults are colonized (Sulaiman, 2011). Both methicillin sensitive and methicillin resistant isolates are persistent colonizers (Wenszel and Perl, 1995) and persons colonized with *S. aureus* are at increased risk for subsequent infections (Wenzel *et al.,* 1995). Rates of Staphylococcal colonization are high among patients with type 1 diabetes (Tuazon and Shegren, 1974), surgical patients (Kutymans *et al.,* 1995), and patients with the acquired immunodeficiency syndrome (Weinke *et al.,* 1992). Patients with qualitative or quantitative defects in leukocyte function are also increased risk for staphylococcal diseases (Waldvogel, 1995).

### 2.8.1 Colonization of mammary gland

Studies have shown that it is possible to induce experimental *S. aureus* intramammary infection by intrammary infusion of a low number of bacteria (<100cfu) through the teat canal (Sulaiman, 2011). These observations demonstrate that milk can support the growth of *S. aureus*, since it can hydrolyse casein and ferment lactose. The natural defence mechanisms of the mammary gland against infection are inefficient to prevent survival of the agents. Bacterial surface properties play a major role in the host bacterium relationship (Sulaiman, 2011).

### 2.8.2 Virulence factors of staphylococci

*S. aureus* exhibits a great variety of virulence factors with no single toxin or enzyme completely responsible for the virulence seen in the species as seen in Table 2.1

(Kathleen and Barrychess, 2008). As reported by Haveri *et al*. (2007) that *Staphylococcus aureus* is able to produce a wide range of extracellular toxins and virulence factors that contributed to the disease and among the virulence factors of *S. aureus* antibiotics resistance plays an important role.

*Staphylococcus aureus* has a record of developing resistance quickly and successfully to antibiotics and its importance as a nosocomial and community acquired pathogen has become a global health concern, due to its remarkable capability of evolving different mechanism of resistance to most antimicrobial agents (Nwankwo and Nasiru, 2011).

*S. aureus* has a diverse arsenal of components and products that contributes to the pathogenesis of the infection. These components and products have overlapping roles and can act either alone or in concert. Staphylococci are known to produce microbial surface components recognizing adhesive matrix modules (MSCRAMMs), which comprise the main adhesives of the organism and include collagen-binding protein, fibronectin binding proteins, fibrinogen-binding protein, elastin binding protein, clumping factor, and the matrix adhesion factor. As many as twelve other surface proteins may cotain membrane anchor domains and potentially qualify as MSCRAMMs.

Among the secreted staphylococcal virulence factor are the enterotoxins (SEA to SEE and SEG to SEQ), other toxin like exfoliative toxin A and B, toxic shock syndrome toxin, and the exotoxin-like protein family. *Staphylococcus aureus* can also express several cell wall-associated proteins with binding capacity for various host proteins, such as fibronectin, fibrinogen and collagen. In addition, *S. aureus* might express extracellular, antiphagocytic capsular polysaccharides (Baba *et al.*, 2002).

Table 2.1: Major Virulence factors of *Staphylococcus aureus*

| **Name** | Enzyme/Toxin | Effect |
|---|---|---|
| Coagulase | Enzymes | Coagulates blood plasma |
| Hyaluronidase | Enzymes | Digest connective tissue of the host |
| Staphylokinase | Enzymes | Digest blood clots |
| Lipase | Enzymes | Digest oils, allowing bacteria to easily colonize the skin |
| Penicillinase | Enzymes | Inactivates penicillin rendering the bacterium resistant |
| Hemolysin and B, (,8) | Toxin | Lyses red blood cells |
| Leukocidin | Toxin | Lyse neutrophils and macrophages |
| Exfoliative Toxins (A, B) | Toxin | Cause desquarnation of the skin |
| Toxic shock syndrome Toxin | Toxin | Induces fever, vomiting rash, organ, damage |

*2.8.2.1 Role of toxins*

*Staphylococcus aureu*s can produce four different haemotytic toxins:α, β, γ, and δ toxins. Two (α and β) toxins appear to play a major role in the virulence of *S. aureus*. Alpha toxin, which is produced by 20% to about 50% of strains from bovine IMI (Sutra and Pourtel, 1994), is a cytolysis that binds to cell membranes and forms hexameric pores leading to cell death as a consequence of rapid egress of cytoplasmic components (Bhakdi and Tranum, 1991). The α toxin is the most important in terms of biological affects on its lyse RBC while also causing leukocytes, renal tissues and both skeletal and heart muscle tranum, (Kathleen and Barrychess, 2008). The β toxin, a types C sphingomyelinase, is produced by 75 100% of these strains (Jonsson *et al.*, 1991).

*2.8.2.2. Role of adhesins*

The first step in the colonization of the mammary gland by *S. aureus* seems to be adhesion to epithelial cells. The in-vitro adhesion of *S. aureus* to bovine mammary epithelial cells has been demonstrated (Opdebeck *et al.*, 1988). This phenomenon varies with strains and with origin of the cells. The attachment of *S. aureus* to epithelial cells of lactiferous ducts has been confirmed in vivo by microscopic examination of sections of experimentally infected mammary glands (Gudding *et al.*, 1984). The mechanism of *S. aureus* to mammary epihelilal cells is not well understood. Two types of interactions could be involved; non specific physicochemical interactions and specific interactions between bacteria cell-wall associated receptors and host components (Sutra and Pourtel, 1994).

*2.8.2.3 Expression of antiphagocylic factors by S. aureus*

The ability of *S. aureus* to avoid opsonins presents in normal serum is an important factor in the success of infection. Antibodies that recognized cell- surface components such as teichoic acid, peptidoglycan and surface- associated proteins are present in most sera. *S. aureus* expresses surface – associated anti- opsonic proteins and a polysaccharide capsule that can both interfere with the deposition of antibodies and complement formation by classical and alternate pathways, or with their access to neutrophil complement receptor and fc receptor.

Therefore, efficient phagocytosis by neutrophils that requires recognition of bound complement and antibodies are compromised (Foster, 2005).

*2.8.2.4 Role of extracellular enzymes and coagulase*

Pathogenic *S. aureus* typically produces coagulase, an enzyme that clots plasma. The precise importance of coagulase to the disease process remains uncertain. It may be that coagulase causes fibrin to be deposited around staphylococcal cells fibrin can stop the action of host defenses such as phagocytoses,or it may promotes staphylococcal adherence to tissues. Because 97% of all human isolates of *S. aureus* produces this enzyme its presence is considered the most diagnostics species characteristics (Kathleen and Barrychess, 2008).

*S. aureus*, produces numerous extracullar enzymes (Sulaiman, 2011) including hyaluronidase, phosphatase, nuclease, lipase, catalase, staphylokinase and proteases, which have been implicated in the pathogenesis of bovine IMI. The ability of *S. aureus* to multiply rapidly in milk is probably a major component of its virulence. Enzymes could allow *S. aureus* to use milk substrates for their metabolism and consequently, to

adapt to mild and grow (Anderson, 1976). However, the involvement of staphylococcal enzymes in the alteration of mammary tissues remains to be demonstrated clearly.

### 2.8.3 Staphylococcal food borne diseases

*Staphylococcal aureus* is a common cause of food borne illness that is not covered in some epidemiologic surveillance programmes. Some strains of *S. aureus* causes staphylococcal food poisoning through production of heat- labile staphylococcal toxins. All individuals are believed to be susceptible to these types of bacterial intoxication. However, the intensity of the symptoms vary, relating to the amount of contaminated food ingested and susceptibility of the individual to the toxin. Onset of staphylococcal food poisoning symptoms ranges form 30 minutes to seven hours (the mean is two to four hours) after consumption of foods containing preformed toxins. Nausea, vomiting abdominal cramps, prostration and diarrhea are common symptoms. Typical foods associated with *S. aureus* outbreaks include high- products that are mishandled during processing or preparation e.g fermented meats, cheese, cream filled bakery products, cooked poultry, sea product and dry pasta. Time and temperature control to prevent the growth of the organisms is the primary control measure of *S. aureus*. Personal hygiene is also important to help prevent contamination from food handlers. It is important to note that heating after toxins is formed is not an effective control method (ICMSF, 1996).

#### *2.8.3.1 Staphylococcal food poisoning and enterotoxin*

Diseases associated with toxin production by *S.aureus* are food intoxication, scalded skin syndrome and toxic shock syndrome (TSS) (Kathleen and Barrychess, 2008). An estimated 30% of all cases of food borne illnesses world wide are caused by Staphylococci (Kadariya *et al*., 2014). The Bacterium *Staphylococcus aureus* causes

20% to 40% of cases of foodborne illness each year.Staphylococcal food poisoning (SFP) is an intoxication resulting from the ingestion of food or beverages containing preformed enterotoxins (SEs) (Mc lauchlin *et al*., 2000).

Enterotoxinsproduced by certain Strains are responsible for a common type of food poisoning in the United States. Enterotoxins of certain strains of coagulase positive *S.aureus* are the agent of staphylococcal food poisoning and are heat stable (inactivation require $100^0$C for at least 30 minutes). It is caused by ingestion of improperly prepared, stored or cooked food and the illness is also associated with food such as Custards, sauces, cream pastries, processed meats, chicken salad etc that have been contaminated during preparation and left unrefrigerated for a few hours.The severity of the illness depends on the amount of food ingested, the amount of toxin in the ingested food and general health of the victims. ,

Enterotoxins are short extracellular proteins that are water soluble and are most commonly described as very stable, and are resistant to heat as well as degrading enzymes (Le Loir *et al*., 2003). Toxins can be formed at optimum temp of $35^0$C - $37^0$C and resist boiling for 30 minutes or more (Park, 2002). Because staphylococci produce heat stable enterotoxins, the food can be extensively and properly cooked, killing the bacteria without destroying the toxin. Thirteen different enterotoxins have been identified, enterotoxin A, B, C1, C2, D, are E are the most common.Enterotoxin A and B are super antigens (Prescott, 2008). To date, 21SEs or enterotoxin like protein (SEIS) have been identified and designated SEA to SEIV (Schlievert *et al*., 2007; Thomas *et al*., 2007), while SEs are the toxin that induce emesis, the related SEIS either lack emethic activity or have not yet been tested for this (Lina *et al*., 2004). The genes encoding the different enterotoxins are caused and disseminated by different mobile

genetic elements like prophages, plasmids, pathogenecity islands (SaPIs), enterotoxigenic cluster (egc), and staphylococcus Cassette chromosome (Scc) (Zheng and Stewart, 2001).

Typical symptoms of SFP includes; severe abdominal pain, cramps, diarrhea explosive vomiting and nausea. The onset of symptoms is rapid usually 1 – 8 hrs) and of short duration usually less than 24hrs. The mortality rate of staphylococcal food poisoning is negligible among healthy individuals.

## 2.9 Antimicrobial Resistance in *Staphylococcus aureus*

### 2.9.1 Antimicrobial medications

The development of Salvarsan by Paul Ehrlich in 1910 was the first document example of a chemical used successfully as an antimicrobial medication (Nester *et al.,* 2001). Antimicrobials are chemotherapeutic agents that are effective in treating microbial infections. Antibiotics are antimicrobial chemicals naturally produced by particular micro-organisms. Most modern antibiotics come from micro-organisms that normally reside in the soil, these include species of the bacteria *Streptomyces* and *Bacillus* and the eukaryotic fungi penicillium and cephalosporium (Nester *et al.,* 2001).

### 2.9.2 Antimicrobial activity

Antibacterial agents can be grouped by their mode of action, i.e. their ability to inhibit the synthesis of the cell wall, cell membrane, proteins and nucleic acids of bacteria (Cheesbrough, 2000).

The Mode of Action of Antimicrobial Agents as described by Cheesbrough, (2000) are;

1. **Inhibitors of Bacterial Cell Wall Synthesis**

a. Penicillins (Beta-Lactam agents). Examples are Benzylpenicillin (Penicillin G). Phenoxymethyl (penicillin V), Ampicillin, Amoxycillin, Flucloxacillin, Cloxacillin, Carbenicillin, Tricarcillin, Azlocillin, Piperacillin and Mecillinam.

b. Cephalosporin (Beta-Lactam agents); examples are, cepharadine, cefuroxime, ceftazidime, cefotaxime.

c. Glycopeptides: examples are; Vancomycin and Teicoplanin.

2. **Inhibitors of Protein Synthesis**

a. Aminoglycosides: Gentamicin, Tobramycin, Kanamycin, Streptomycin, Amikacin and Netilmicin

b. Macrolides; Erythromycin

c. Lincosamides; Clindamycin

d Chloramphenicol

e. Tetracyclines; Tetracycline and Doxycycline

3. **Inhibitors of Bacterial Nucleic Acid Synthesis**

a. Quinolones; Nalidixic acid and fluroquinolones (Ciprofloxacin, Norfloxacin,

b. Sulphonamides and Trimethoprim; Co-trimoxazole, Sulphadimidine, Trimethoprim, and Sulphadoxine.

c. Metronidazole

The β-Lactam drugs irreversibly inhibit enzymes involved in the final steps of cell wall synthesis. They have been extensively studied because they are such important antimicrobial agents, having very few side effects other than allergic reactions. Their properties such as acid stability, spectrum of activity, and half-life can be modified by chemically altering their structure. The different β-lactam drugs vary in their spectrum of activity. Some are more active against Gram-positive bacterial, where as others are more active against Gram-negative bacteria (Nester *et al.*, 2001). Some bacteria can resist the effects of certain β-lactam drugs by synthesizing an enzyme called a β-lactamase that breaks the critical β-lactam ring, destroying the activity of the antibiotic. Just as there are many β-lactam drugs, there are various β-lactamases, these differ in the range of drugs they destroy. A β-lactamase that was originally detected in some strains of staphylococci only inactivates member of the penicillin family to reflect this fact, it is often called a penicllinase, in contrast some of the β-Lactamases drugs. The extended spectrum β-lactam inactivates on both the penicillins and the cephalosporins. As a whole, gram-negative bacteria can produce a much more extensive array of β-lactamases than Gram-positive organisms (Nester *et al.*, 2001).

### 2.9.3 Antimicrobial resistance

Antimicrobial resistance has now become a public health concern and several investigation have been conducted to study the antimicrobial resistance pattern of *S. aureus* and it has been shown that the organism is resistant to β-lactam antibiotics, aminoglycoside and macrolides. *S. aureus* strains carry a wide variety of multi-drug resistant genes on plasmids, which can be exchanged and spread among different species of Staphylococci (Chigbu and Ezeronye, 2003). The development of resistance is multifactorial namely:

I, Overuse and misuse of antimicrobials by doctors and health personnel;

ii. Poor compliance

iv. Counterfeit drugs

v. Poor control of antimicrobials in hospitals

vi. Poor standard of hygiene-personal and environment in hospitals

vii. Use of antibiotics in animal husbandry, horticulture and aquaculture\

viii. International travel and trade (Lucas and Gilles 2003).

The situation is made to be more difficult in developing countries like Nigeria where antimicrobial drugs are readily available to consumers across the counter with or without prescription from a medical practitioner. Such practice has led to misuse of antimicrobial drugs with the associated high prevalence of drug resistance among *Staphylococci* (Chigbu and Ezeronye, 2003).

### 2.9.4 Factors responsible for spread of resistance to *S. aureus*

In early 1970s, physicians were finally forced to abandon their belief that, given the vast array of effective antimicrobial agents, virtually all bacterial infections were treatable. Their optimism was shaken by the emergence of resistance to multiple antibiotics among pathogens such as *Staphylococcus aureus, Streptococcus pneumonia, Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* (Lowry, 2003). The evolution of increasingly antimicrobial – resistant bacterial species stems from a multitude of factors that include the widespread and sometimes in appropriate use of antimicrobials, the extensive use of these agents as growth enhancers in animal feeds

and, with the increase in national and international travel, the relative ease with which antimicrobial – resistant bacteria cross geographic barriers (Swartz, 1997). The issue of antibiotic resistance is of major concern among the Gram-positive bacteria, pnuemococci, enterococci, and staphylococci. Multidrug resistance is now the norm among these pathogens. *S. aureus* is perhaps the pathogen of greatest concern because of its intrinsic virulence, its ability to cause a diverse array of live threatening infections, and its capacity to adapt to different environmental conditions. As rapidly as new antibiotics are introduced, staphylococci have developed efficient mechanisms to neutralize them (Waldvogel 2000).

### 2.9.5 Mechanisms of *Staphylococcus aureus* resistance to antimicrobials

There are three major mechanisms by which bacteria have become resistant to antimicrobial agents: enzymatic inactivation as well as reduced intracellular accumulation of the antimicrobials, but also protection, alteration or replacement of the cellular target sites (Schwarz and Noble, 1999). A bacterium is considered to be resistant to an antimicrobial agent when the concentration of the antimicrobial agent at the site of infection is not sufficiently high to either inhibit replication of the bacterium or even kill it (Schwarz and Chalsus – Dancla, 2000). This definition clearly shows that antimicrobial resistance is not solely a microbiological problem, but also includes pharmacological, pharmaco-kinetic and clinical aspects. Antimicrobial resistance is a highly flexible property bacteria and the resistance mechanism. Up to six different mechanisms have been described as conferring resistance to the same antimicrobial agent. Some resistance mechanisms are distributed among a wide variety of bacteria, while others appear to be specific for certain bacterial species. In contrast to genes or

species – specific intrinsic resistance properties which are mainly based on either the lack of or the inaccessibility of the target site for the antimicrobial agents.

Acquired resistance represents a strain – specific property which may be based on mutations in certain chromosomal "house-keeping" genes which act as target for antimicrobial agents. These mutations often have little or no influence on the biological activity of the gene products, but render them insensitive to the inhibitory activities of the respective antimicrobial agents (Arcangioli *et al.,* 1999). Acquired resistance, however, is more often associated with the acquisition of mobile genetic elements that carry one or more resistance genes (Arcangioli *et al*., 1999). Such resistance genes code for protein which do not usually have a known function in physiological cell metabolism, but mediate resistance to either single antimicrobial substances or members of the same class of substance. They can also mediate resistance members of different classes of antimicrobials which, however, have same target site within the bacterial cell. In the case of multidrug transporter systems which export toxic metabolite from the cell, resistance to structurally and functionally different antibiotics, often in addition to hydrophobic actions, detergents or nucleic acid binding compounds, has been largely observed (Putman *et al.,* 2000).

*2.9.5.1 Resistance to β-lactams*

Staphylococcal resistance to penicillin is mediated by blaz, the genes that encode β-lactamase. The predominantly extracellular enzyme, synthesized when staphylococci are exposed to β-lactam ring, rendering the β-lactam inactive. Blaz is under the control of two adjacent regulatory genes, the anti-repressor bla*R1* and the repressor bla*I*1 (Kernodle, 2000). The signaling pathway responsible for β-lactamase synthesis requires sequential cleavage of the regulatory proteins BlaR I.Following exposure to β-lactams,

BlaRI, a transmembrane sensor-transducer protein, cleaves itself (Zhang, *et al.,* 2001). Zhang *et a*l. (2001) hypothesize that the cleaved protein functions as a protease that cleaves the repressor BlaI, directly or indirectly (an additional protein, BlaR2, may be involved in this pathway) and allows Blaz to synthesize the enzyme.

*2.9.5.2 Resistance to tetracycline*

Several different mechanisms of tetracycline resistance have been described so far, among which active efflux and ribosome protection are the most prevalent mechanisms among gram-positive and gram negative pathogens (Robert, 1996). The energy – dependent efflux of tetracycline is mediated by at least two types of transmembrane proteins (TCS), both of which exchange a proton for tetracycline-cation complex. On the basis of hybridization experiments, at least 14 different classes can be differentiated (Levy *et al.,* 1999). Among them, the most intensively studied classes are A, B, C, D, H, K, and L. the genes tet(k) and tet (L) are mainly present in gram-positive bacteria and code for a protein which consists of 14 transmenbrane regions. Their Tc-inducible expression is regulated by a mechanism known as "translational attenuation". Genes of classes K and L are frequently found on small plasmids which in rare cases may be integrated into other plasmids or into the chromosomal DNA, but may also undergo interplasmic recombination with other resistance plamids. Tc-inducible expression of these tet genes based on the binding of tetracycline-Mg complex to the tet repressor protein which, in the absence of tetractycline, blocks transcription of the tet structural gene (Roberts, 1996).

*2.9.5.3 Resistance to methicillin*

Methicillin resistance requires the presence of the chromosomally localized *mec A* gene (Kernodle, 2000). *mec A* is responsible for synthesis of penicillin – binding protein 2a

(PBP2A; also called PBP2') a 78-KDa protein (Hartman and Tomasz, 1984). PBPS are membrane-bound enzymes that catalyze the tranpeptidation reaction that is necessary for cross-linkage of peptidoglycan chains (Ghuysen, 1994). PBP2a substitutes for the other PBPs and because of its low affinity for all β-lactam antibiotics, and it enables staphylococci to survive exposure to high concentration of these agents. Thus, resistance to Methicillin confers resistance to β-lactam agents, including cephalosporins. PBP2a differs from other PBPs in that its active site blocks binding of all β-lactams but allows the tranpeptidation reaction to preceed (Lim and Strynadka, 2002). One pecularity of Methicillin-resistant *Staphylococcus aureus* (MRSA) is their heterogeneous expression of resistance, whereby individual strains can generate subpopulations with different degrees of higher resistance. There is also a remarkable variability in resistance levels between different MRSA strain lineages, ranging from oxacillin minimal inhibitory concentration (MIC) values of >0.5 to over 1000mg/1 (McCallum *et al.,* 2010). In the absence of *mec A, S. aureus* mutants with decreased susceptibility to beta-lactams can be selected for growth on increasing drug concentration. This type of resistance is however generally costly to the cell. Laboratory – generated mutants grow slower and have changes in the amount and affinity of the resident PBPs, in addition to other undetermined mutations (Berger-Bachi *et al.,* 2002). Clinical isolates that lack mecA but borderline oxacillin resistance phenotypes carry amino acid substitutions in the transpeptidase domain of PBP2 which are responsible for the increased resistance (Nadarajah *et al.,* 2006).

*2.9.5.4 Resistance to aminoglycosides*

Resistance to amino glycoside is mainly based on enzymatic inactivation by amino glycoside – modifying enzymes (Show *et al.,* 1993). Moreover decreased uptake of

amino glycoside and chromosomal mutation conferring high-level resistance to streptomycin have also been described (Quintiliani *et al.,* 1999). Enzymatic inactivation of amino glycosides is conferred by N-acetyltranferases, o-adenyltransferases or o-phosphotransferases (Show *et al.,* 1993). Most of these enzymes exhibit specific substrate spectrums. However, several other enzymes have been identified, some of which are part of integrons/gene cassette (Recchia and Hall, 1995). Four classes of N – acetyltransferases (AACs) are known which acetylate the amino groups at positions 1-, 3-, 21- and 61 (Wright, 1999).

*2.9.5.5 Resistance to macrolides, lincosamides and streptogramins*

The mechanisms involved in resistance to macrolides, lincosamides and streptogramins so far observed among gram-positive bacteria include target modification, active efflux and enzymatic inactivation (Leclereq and Courvalin, 1991a; 1991b).

Target modification by Rrna methylases has been detected in a wide variety of gram positive bacteria and it is commonly due to expression of a plasmid or transposon – borne erm gene where gene product demethylates a specific adenine residue (A2058) in a conserved region of the 23SrRNA (Leclereq and Courvalin, 1991b). This methylation confers resistance to macrolides, lincosamides and B-compound of streptogramins (MLSb antibiotics). Enzymatic inactivation of MLS antibiotics is mediated by a number of different enzymes, each of which displays a narrow substrate spectrum (Leclereq and courvalin, 1991b; Roberts *et al*., 1999).

*2.9.5.6 Resistance to chloramphenicol*

Chloramphenicol resistance in gram positive bacteria is mainly due to enzymatic inactivation (Alekshun and Levy, 2000). Efflux systems which confer either only

chloramphenicol resistance or combined resistance to chloramphenicol have also been described (Cloekaert *et al.,* 2000; Keyes *et al.,* 2000).

*2.9.5.7 Resistance to quinolones*

Resistance to quinolones results from the stepwise acquisition or chromosomal mutations. The confluence of high bacterial density, the likely pre-existence or resistant subpopulations and sometimes limited quinolones concentrations achieved at sites of staphylococcal infections creates an environment that fosters selection of resistance (Tomasz, 1994). The quinolones act on DNA gyrase, which relieves DNA supercoiling, and topoisomerase IV, which separates concatenated DNA strands. Amino acid changes in critical regions of the enzyme – DNA complex (quinolones resistance determining region (QRDR) reduce quinolones affinity for both of its targets.

The Par C subunit GrIA is *S. aureus* of topoisomerase IV and GYr A subunit in gyrase are the sites most prone to resistance mutation and the primary drug target in staphylococci (Hooper, 2002).

*2.9.2.8 Resistance to vancomycin*

Two forms of *S. aureus* resistance to Vancomycin have been identified (Walsh and Howe, 2002). One form has been identified in the Vancomycin Intermediate *Staphylococcus aureus* (VISA) strains, which have MICs to vancomycin of 8-16/MI (Hiramatsu, 1997a). A pre-VISA stage of resistance, heterogeneously resistant, has also been identified. The heteroresistant strains remain susceptible to vancomycin contain resistant subpopulations. It is hypothesized that, on exposure to vancomycin, the VISA isolates are selected from the vancomycin-resistant subpopulations (Hiramatsu, 1997b). The reduced susceptibility of vancomycin appears to result from changes in

peptidoglycan biosynthesis. The VISA strains are notable for the additional quantities of synthesized peptidoglycan that result in irregularly shaped, thickened cells walls.

The second form of vacomycin resistance has resulted from the probable conjugal transfer of the van operon from vancomycin-resistant *E. faecalis*. Showsh *et al*. (2001) reported that the enterococcal plasmid containing van A also encodes a sex pheromone that is synthesized by *S. aureus* suggesting a potential facilitator or conjugal transfer. The Vancomycin Resistant *Staphylococcus aureus* (VRSA) demonstrate complete vancomycin resistance, with MICs of ≥128μ/ml. Resistance in these isolates is caused by alteration of terminal peptide to D-Alanine-D-Lactose instead of D-Alanine-D-Alanine. Synthesis of D-Alanine-D-Lactose occurs only with exposure to low concentrations of vancomycin. As a result, the additional biosynthetic demands are limited and the VRSA strain is ecologically fit (Gonzalez-Zorn and Courvalin, 2003). The resistance of these to both B-lactams and glycopeptides increase the likelihood that VRSA strains will rapidly become more prevalent.

*2.9.5.9 Resistance to Sulfonamide and Trimethoprim*

Sulfonamides and trimethoprim block different enzymatic steps in tetrahydrofolate biosynthesis. Sulfonamides are structural analogues of p-aminobenzoic acid and competitively inhibits the enzymes dihydropteric acid synthetase (DHPS) while trimethprim competitively inhibits the enzymes dihydrofolate reductase (DHFR).While some bacteria are intrinsically resistant, acquired resistance may be due to chromosomal mutations or to plasmid – encoded DHPS or DHFR enzymes which are resistant to sulfonamides and trimethoprim, respectively (Elwell and Fling, 1989; Quintilian *et al.,* 1999).

## 2.10 Methicilin-Resistant *Staphylococcus aureus* of Animals

The first Methicillin-resistant *Staphylococcus aureus* (MRSA) strain of human origin was identified in the UK in 1961 but the first major outbreak in the USA was reported in 1968 (Sulaiman, 2011). Subsequently it was shown that MRSA strains had acquired a gene (*mec A*) encoding and altered penicillin-binding protein which was responsible for Methicillin resistance. MRSA strains are now ubiquitous worldwide and are a significant healthcare problem (Appelbaum, 2006). A combination of lateral gene transfer of the *mec A* gene into distinct phylogenetic lines of *S. aureus*, and clonal dissemination, has been responsible for the emergence and spread of MRSA strains (Fitzgerald *et al.,* 2001b; Enright *et al.*, 2002). The first isolation of MRSA from a non-human source was from milk of a cow and several isolates of bovine MRSA has been identified since then, but with low frequency (Lee, 2003; JuhaszKaszanyitzky *et al.,* 2007; Moon *et al.,* 2007). Subsequently, MRSA has been found in a wide variety of domestic species including dogs (Guardabassi *et al.,* 2004), cats (Bender *et al.,* 2005), horses (Shimizu *et al.,* 1997) pig (Huijsdens *et al.,* 2006), and chickens (Lee, 2003). Several reports have indicated an increase in the number of MRSA infections in dogs, cats and horses in recent years (Lee, 2003; Baptiste *et al.,* 2005; Loeffler *et al.,* 2005). The possibility that companion animal could act ac the source of zoonotic infections of humans caused by MRSA is of interest. MRSA clones isolated from dogs and cats share similar genetic backgrounds to human nosocomial MRSA suggesting that the common strains spread from humans to animals rather than the emergence of new MRSA clones (Baptiste *et al.,* 2005).

Although some host – specific and tissue-specific genes have been identified which contribute to disease pathogenesis in ruminants, it is likely that combinations of

virulence factors and allelic variation of orthologous genes play an important role in host adaptation. Current genome sequencing projects in various laboratories will allow examination of the genetic basis for the host – specific pathogenesis of *S. aureus* (Sung and Lindsay, 2007)

### 2.10.1 Molecular epidemiology of methicillin resistance

The mechanism of resistance to Methicillin was uncovered in 1981 with the identification of reduced – affinity penicillin G – binding proteins in MRSA (Sulaiman, 2011). The altered penicillin G binding protein, (PBP2) retains effective transpeptidase activity while having reduced affinity for penicillin and other availableβ lactams antibiotics. PBP2a exhibit both a reduced rate- constant for acylation byβ lactams and elevated dissociation constants (Fuda *et al.,* 2004). These 2 factors, acting together, prevent acylation of PBP2a and thus result in βlactams resistance (Fuda *et al.,* 2004).

PBP2a is encoded by the *mec A* gene. The mobile *mec A* gene complex is comprised of mec A together with its regulator genes, *mec 1* and *mec R*, and resides within a genomic island, the staphylococcal cassette chromosome mec (SCCmec ) that constitute 1% - 2% of the ~ 2.9 million bp *S. aureus* chromosome (Chambers, 2001). SCC mec also contains the insertion sequence, IS431mec, as well as recombinases necessary for site specific integration and excision. Some SCCmec types also contain various additional genetic elements such as Tn554 (which encodes resistance to Macrolides, Clindamycin and Streptogramin B) and PT181 (which encodes resistance to tetracycline (Chambers, 2001).

### 2.10.2 Molecular evolutionary history

Although PFGE is commonly used to determine the relatedness of isolates for epidemiologic purpose, this method is insufficiently discriminatory for evolutionary studies (Enright *et al.,* 2000). The overall genetic background of *S. aureus* isolates has been unambiguously determined through multilocus sequence typing genes (Hiramatsu *et al.,* 2004). The mobile Sccmec elements, on the other hand, are classified by analysis of their cassette chromosomes recombinase (ccr) and mec A gene complexes (Ito *et al.,* 2001). SCCmec types also differ with regard to their acquisition of resistance determinants acquired as the result of integration of plasmids and transponsons (Ito *et al.,* 2001). Al least 5 SCCmec types (1 - V), varying in size from – 20kb to 68kb have been identified (Table 2.2).The smallest of these – SCCmec types I, IV, and V contain only recombinase genes and the structural and regulatory genes for resistance to Methicillin and lack the transposable element and genes encoding resistance to non β-lactam antibiotic carried by types II and III (Max *et al.,* 2002; Ito *et al.,* 2004). SCCmec types I – IV contain alleles of ccrA and ccrB, whereas type V, which has uptill date been identified in a small number of Australian CA-MRSA isolates, contains a novel ccr designated ccrC (O'Brien *et al.,* 2004). Two possible additional SCCmec have recently been identified among Australian CA-MRSA strains (O'Brien *et al.,* 2004).

**Table 2.2: Characteristics of Staphylococcal chromosome mec (SCCmec types I – V)**

| SCCmec | SCCmec Size (kb) | Other Antibiotic Resistant Genes on SCCmec | Origin of *S. aureus* Carrying SCCmec | Presence of PVL in *S. aureus* isolates carrying the SCCmec |
|---|---|---|---|---|
| I | 34 | | Hospital | Infrequent |
| II | 53 | PUB110(aad)[a]Tn554(ermA)[c] | Hospital | Infrequent |
| III | 67 | PUB110(aaD)[b]Pt181(tetk)[d] | Hospital | Infrequent |
| IV | 21-41 | | Communitiy | Infrequent |
| V | 28 | | Communitiy | Unknown |

NOTE: Data adapted from Wisplinghoff *et al.*(2003) and Burine *et al.*(2000).

PVL: Panton – Valentine Leukocidin

[a]In general, <5% of *S. aureus* strains that carry SCCmec types I- III also carry the pvl gene; with some exceptions, 40% of *S. aureus* strains that carry SCCmec types IV also carry the pvl gene

[b]Encodes resistance to tobramaycin and kanamycin

[c]Encodes resistance to macrolide – lincosamide – streptogramin antibiotic

[d]Encodes resistance to tetracycline.

### 2.10.3 MRSA of humans in Nigeria

A recent study by Fadeyi *et al.,* (2010), at the University of IIorin Teaching Hospital (UITH) Nigeria, reported that out of 198 health workers of the critical care unit of a Nigeria hospital, 104 had MRSA. In Nigeria, the prevalence of MRSA was 34.7% of all staphylococcal isolates of which approximately 13.7% were recovered from patients in the intensive care unit (Taiwo *et al.,* 2004). Other studies in Nigeria indicated that MRSA is not uncommon in our environment (Kesah *et al.,* 2003; Adesida*et al,* 2005; Taiwo *et al.,* 2005; Azeez – Akande *et al.,* 2008;Nwankwo *et al*., 2010).

# CHAPTER THREE

## 3.0 MATERIALS AND METHODS

### 3.1 Study Area

The study was carried out in Kaduna metropolis, Kaduna which comprises of Kawo (Kaduna north), Bakin ruwa (Kaduna south), and Tudun Wada (Kaduna south), Kaduna State. Kaduna state is located at the center of northern Nigeria with the coordinates 10°31′N 7°26′E 10.517°N 7.433°E. The state shares boundaries with Niger state to the west, Zamfara, Katsina and Kano state to the North, Bauchi and Plateau state to the east and FCT Abuja and Nassarawa states to the south (Kaduna state, 2010) (Fig 3.1). Kaduna is the state capital and the large city with very high agricultural activities.

Agriculture and animal rearing namely cattle, sheep, goat and rearing are one of the major occupations of people of Kaduna. Agriculture being the main occupation with about 80% of the people engaged in farming, Cash and Food crops that are cultivated and produced include:- cotton, groundnut, maize, tobacco, beans, guinea, corn, millet, ginger etc.

#### 3.1.1 Sample size

A Sample Size of 384 were collected and it comprises both nono (192) and yoghurt (192) samples which were used for this study and it was based on the prevalence of 52.4% for *Staphylococcus aureus* reported by Gali*et al*., 2013), in a study conducted in both Zaria and Kaduna.

The sample size was determined by this equation / formula $N=\frac{PqZ^2}{d}$

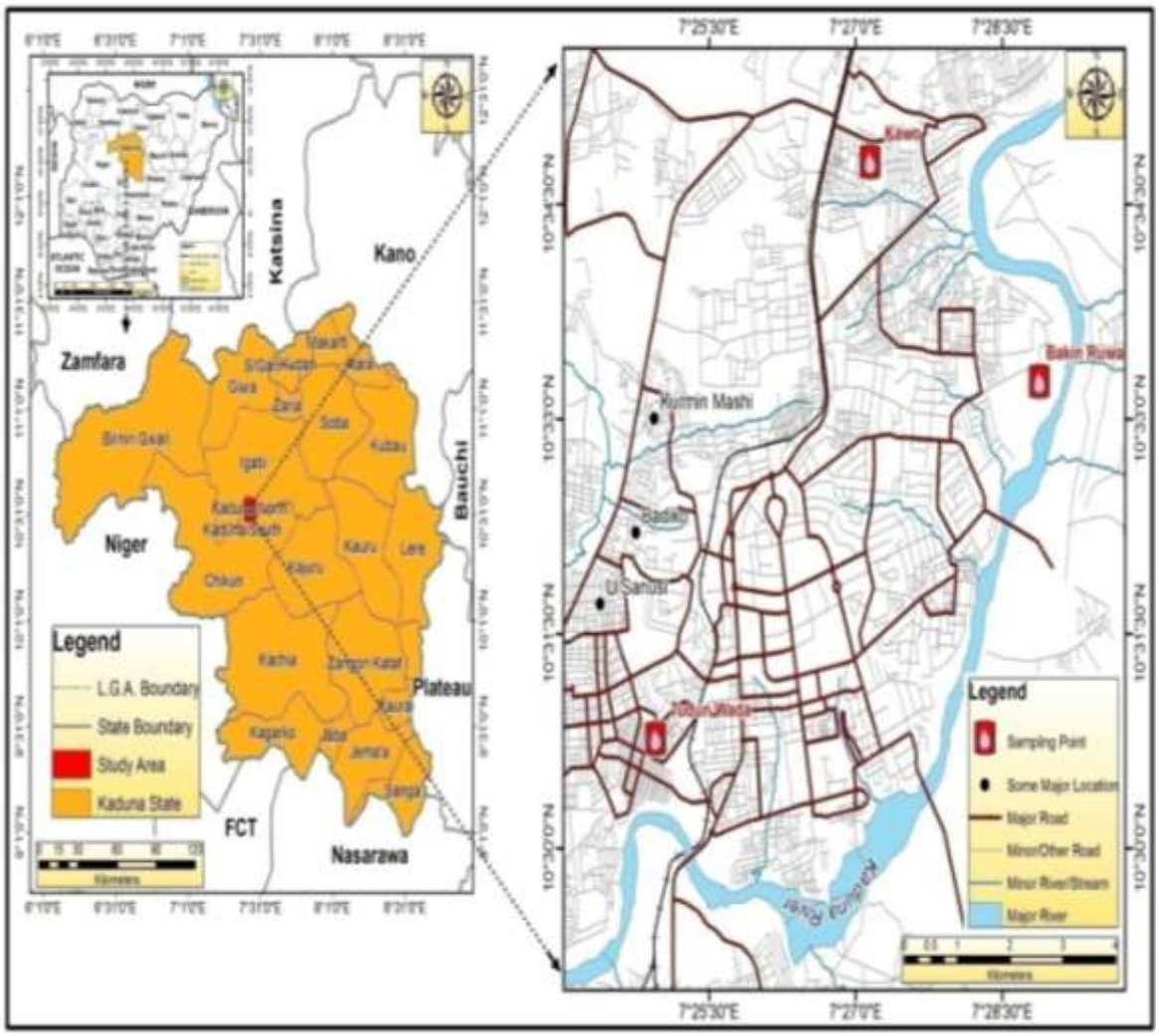



**Figure 3.1: Map of Kaduna showingSampling Points**

**(Source: Modified from the Administrative Map of Kaduna State)**

Where N= sample size, Z= statistics for a levels of 95%, confidence interval =1.96 P= prevalence rate from the previous studies, d= levels of significance (allowable error) 5% = 0.05, Q = I – P

P= $\frac{195}{372}$ X100 = 52.4% (Prevalence rate from the previous studies)

$$N = \frac{1.96^2 \times 0.524 \times 1 - 0.524}{(0.05)^2} = 383.22 = 384$$

3.1.2 Collection of samples

Three hundred and eighty four (384) milk samples were collected of which One hundred Ninety two (192) samples of locally fermented milk (nono ) were purchased randomly into a sterile and chilling container from different selling points/ hawkers in the selected study area and transported without delay to the laboratory for proximate, physicochemical and microbiological analysis while one hundred and Ninety two (192) samples of yoghurt, comprising both sachet and bottled types of yoghurt of different volumes and brands were also purchased randmonly form different selling points/ hawkers in the study area as well.

## 3.2 Physicochemical and Proximate Analysis of the Samples (Nono and Yoghurt).

The Fermented milk samples (nono and Yoghurt) were analysed for pH, total titrable acidity, moisture content, total protein, fats and ash. Standard procedure was used according to AOAC, (2005).

### 3.2.1 pH determination

The pH of the sample was determined using a pH meter (Cybersan pH meter, model 500). The electrode of the pH meter was standardized by diping it into sterile water after which two different buffers (4 and 7) was used. The set electrode was then used for the various samples by inserting the electrode of the pH meter in the sample, then the results displayed and readings were recorded.

### 3.2.2 Total titratable acidity determination

This was determined by the method described by AOAC (2005). Ten (10) ml of the samples was dissolved in distilled water and mixed thoroughly. One (1)ml of phenolphthalein indicator was introduced into 10ml of the samples (mixed solution). It was titrated against standard sodium hydroxide solution until pink colour persist for 10 secs for complete neutralization, and the reading was taken and recorded.

Mathemathically,Total Titratable Acidity can be expressed as;

$$\frac{\text{Molecular weight of Lactic acid x Titratable acidity}}{\text{Volumeof sample}} \text{ x } \frac{100}{1}$$

### 3.2.3 Moisture content determination

The moisture content was determined by Oven method as described by AOAC (2005). In this process, Two(2)g of the sample was dried in a hot air oven for 24 hours at $106^0$C.The lost in weight was determined and recorded as moisture content and expressed as;

% moisture = $\frac{W_1 - W_2}{W_1}$ x 100% where $W_1$=initial weight of the sample,$W_2$=weight of the dried sample.

### 3.2.4 Crude protein content determination

The macro Kjeldah method as described by AOAC (2005) was used to determine the crude protein content. Two grams (2g) of the sample was introduced into the digestion flask. Ten (10) g of copper sulphate and sodium sulphate in the ratio of 3:1 and 25ml of concentrated sulphuric acid was added to the digestion flask. The flask was placed into digestion block infume cupboard and heated until frothing ceased, given a clear and light blue colouration. The mixture was allow to cool and was diluted with distilled water until it reached 25ml of volumentric flask. Ten(10)ml of mixture was poured into the distillation apparatus and 10ml of 40% sodium hydroxide was added. The released ammonia byboric acid was allowed to continue until 10ml of boric acid is treated with 0.02ml of hydrochloric acid until the green colour change to purple. The nitrogen in the sample was then determined. The percentage nitrogen of the sample was calculated as:

% nitrogen = molar mass of $N_2$ x acid conc (0.02ml) x volume made x titre value x 100,

### 3.2.5 Crude fat content determination

The Soxhlet Solvent extraction method as described by AOAC (2005) was used to determine the fat content. In this method, 2g of the sample was weighed into flat bottom flask of 500ml with the extractor mounted on it. The thimble was held half way into the extractor and the weight of the sample was carefully transferred into the thimble and the thimble was plugged with cotton wool.The extraction was carried out at the temperature of 40-60$^0$C for 8 hours. The solvent was removed by evaporation and the remaining apart of the flask was dried in the oven at 80$^0$C for 30 minutes and was finally cooled in dessicator. The flask was re weighed and the percentage fat was calculated as;

$$\% \text{ fat} = \frac{\text{weight of extracted fat}}{\text{weight of sample used}} \times 100\%$$

3.2.6 Ash content determination

The as content was determined by the direct heating method as contain in AOAC (2005). In this method, 2g each of the samples was measured into a crucible of known weight, the sample was burnt into ash in a muffle furnace for 3 hours at $550^0$C. It was then cooled in dessicator and the weight of the ash was finally determined. The ash% content was calculated as;

$$\% \text{ ash} = \frac{W_1 - W_2}{W_2} \times 100\%$$

Where: $W_1$=initial weight of the sample,$W_2$=weight of the dried sample.

## 3.3 Isolation, Enumeration and Characterization of *S. aureus*

3.3.1 Serial dilution

Samples of nono contained in airtight sterile bottle were shaken vigorously to ensure homogenuity. Ten fold serial dilution was carried out aseptically according to the methods of Fawole and Oso (2005). A serial dilution was carried out aseptically by pipetting 10mls of nono and yoghurt respectively into 90mls of peptone water blank, the dilution from $10^{-2}$ and $10^{-3}$ for yoghurt and $10^{-3}$ and $10^{-5}$ for nono were used. Then 1ml of the dilution was pipetted into appropriately labeled sterile Petri dishes with the appropriate media (nutrient agar) poured into each plate and gently swirled for proper mixture of the medium and sample. The plates were allowed to solidify before incubated at $37^0$C for 24 hours.

3.3.2 Determination of total viable count in the nono and yoghurt samples

This was carried out using the nutrient agar by the spread plate techniques from the composite nono and yoghurt samples.

One (1)ml of the samples was inoculated on the nutrient agar plates and spread over aseptically on the surface of the agar medium using a bent glass rod after flame sterilization. A serial dilution was done for both nono and yoghurt as described above. The plates were inverted and incubated at 37$^{o}$C for 24 hours. Colonies formed were counted and recorded as total viable count

and Colony forming unit/ ml (Cfu/ml) is calculated as

$$\text{CFU} = \frac{\text{Number of colony counted} \times \text{dilution factor}}{\text{volume inoculated}}$$

### 3.4. Isolation and Identification of *Staphylococcus aureus*.

All the samples were inoculated on mannitol salt agar medium by pipetting One (1) ml of the samplesand spread over aseptically on the agar medium using a bent glass rod after flame sterilization and incubated at 37$^{o}$C for 24 hours. Discrete colonies that were isolatedare reinnoculated onto Mannitol Salt Agar in order to obtain pure culture of isolates. These pure isolates were then kept at 4$^{0}$C for identification purpose (Michaylova, 2007, Benson 2005). Colonies showing a yellowish pigmentation suspected to be staphylococci were picked up, subcultured on mannitol salt agar plates and isolation and identification of *S. aureus* was performed using the following test reactions:- Gram staining reaction, catalase test, coagulate test, growth on mannitol salt agar, DNase test. Typical isolates of *S. aureus* from the tests above were kept on nutrient agar slant for further study.

### 3.4.1 Gram staining procedures

A smear of the isolates was made on a grease free glass slides and then heat fixed, and left to dry. The fixed smear was covered with crystal violet for one minute and rinsed rapidly with clean water. The smear was covered with Lugols iodine for one minute and also washed off with water. It was then decolorized rapidly with ethanol for few seconds and rinsed with water and lastly it was then counter stained with Saffranine dye for 30 secs and rinsed with water. Finally, the smear was placed on the draining rack for drying, thereafter the smear was examined microscopically. First with x40 objective to obtain the level of distribution of material and then withoil immersion x100 objective to observe the microscopic appearance of the bacteria (Cheesebrough, 2000).

### 3.4.2 Catalase tests

This was carried out by placing 2-3ml of Hydrogen peroxide in a test tube, with a sterile wooden application or plastic rod, some colonies of the organisms were picked and immersed into the Hydrogen peroxide solution. Appearance of gas bubbling and effervescence are indication of a positive result (Cheesebrough, 2000).

### 3.4.3 Coagulase test

A drop of distilled water was placed on two separate slides. The isolates were emulsified on each of the drops, forming thick suspensions. A loopful of plasma was added to the suspensions and mixed gently and then clumping was observed.The presence of clumping within ten second was recorded as positive for *Staphylococcus aureus* (Cheesebrough, 2000).

### 3.4.4 DNAse (Deoxyribonuclease) activity

*S. aureus* isolates were streaked or spot inoculate on the surface of a DNASE Agar base and incubated at 37$^{o}$C for 24 hours. After incubation the plates were flooded with 1N-HCl Excess acid were tipped off, positive result showed a clear zone around the colony (Cheesebrough, 2000).

## 3.5 Confirmation of *S. aureus* using Microgen 12S *Staphlococcus* Identification Systems

Sixty (60) isolates were selected at random from the three study area and subjected to further characterization using Microgen staphylococci 12S identification system test, for the confirmation of the *Staphylococci* species.

The test was conducted according to the instruction of the kit manufacture (Oxoid, Ltd, Basingstokes and U.K). Briefly, a single colony from a 24 hours culture picked and emulsified in staphylococci suspending medium supplied in the kit to a homogenous suspension using a sterile bacteriological loop. Using a sterile pipette, 3-4 drops (approximately 100 µl) of the bacterial suspension was added to the wells of each test strip and incubated at 37$^{o}$C for 18-24 hours. For purity check, 1 drop of the bacterial suspension (*S. aureus*) was inoculated at the same temperature and time as the strip. After incubation, the results were read and recorded on to the Microgen $^{TM}$ Staph ID report form provided in the kit. Interpretation of the result was done using the colour chart provided in the kit. Once the results have been entered on the report form, each block of three reactions were into a numerical value. Three numbers were added together to obtain 5 digit of Microgen $^{TM}$ Staph code which was then entered into the computer aided software for identification.

### 3.6Detection of Staphylococcal Enterotoxins (SES)

Twenty (20) isolates were investigated for SEs production using a staphylococcal enterotoxin A,B,C,D detection kit by reverse passive latex agglutination according to Manufacturer's instructions (Oxoid Ltd, Basingstoke,UK) as described below;

3.6.1 Production of enterotoxins in culture fluids

Colonies from isolated *S.aureus* were inoculatedonto Tryptose soy broth and incubated at $37^0$C for 20 hours with shaking. The suspension was centrifuged at 900g for 20 minutes at $4^0$C. The supernatant was retained for assay of toxin content.

3.6.2 Assay method

A V-bottom microtitre plate was arranged such that, each row will have eight wells. Each sample needed the use of five rows. 25µl of the diluents provided in the kit was added to each well in the five rows: 25µl of the test sample was added in the first well of each of the rows and a doubling dilution with 25µl was conducted to the seventh well.25µl of the Latex sensitized enterotoxins A,B,C,D were added to row 1,2,3 and 4 respectively. To the $5^{th}$ row, 25µl of the latex control was added. The plates were rotated to enable mixing the contents of the wells and incubated at $37^0$C for 24 hours. Agglutination was observed against a black background.

### 3.7 Antibacterial Susceptibility Testing

Antibacterial susceptibility test was conducted on 43 isolates of *S. aureus* against 8 antimicrobial agents following the Kirby Bauer disk diffusion method institute (CLSI) (CLSI, 2006). The microbial agents and concentration used were; Erythromycin are (5µg), Ciprofloxacin (5µg), Chloramphenicol (30µg), Clindamycin (2µg), Tetracycline

(30μg),Cefoxitin (30μg), Oxacillin (1μg), and Vancomycin (30μg). Each isolates to be tested was cultured in tryptose soy broth to a 0.5 macfarland standard and incubated at $37^0$C for 6hours. The isolates were than swabbed onto Mueller Hinton Agar, using sterile cotton swabs before application of the antimicrobial discs. The respective plate was seeded uniformly by rubbing the swab against the entire agar surface. Each antibacterial impregnated disk was applied onto the surface of the inoculated plate, using a disk dispenser (Oxoid, Basing stoke, U.S). The plates were incubated at $37^0$C for 18 hours. The interpretation of the growth of inhibition zones and classification of isolates as susceptible intermediate and resistant followed the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Those isolates that were resistant to methicillin or oxacllin (ZI ≤ 14mm) were regarded as Methicillin Resistant *Staphylococcus aureus* (MRSA) or Oxacillin Resistant *Staphylococcus aureus* (ORSA) according to (CLSI) while those with zone of inhibition (ZI≥ 4mm) were termed susceptible *Staphylococcus aureus* **ATCC 25923** was used as a positive control.

3.7.1 Multiple antibiotics resistance index (mar):

Multiple Antibiotics Resistance (MAR) index was determined by dividing the number of antibiotics to which the isolates was resistant to by the total number of antibiotics tested (Olayinka *et al*., 2004).

$$\text{MAR Index} = = \frac{\text{Number of Antibiotics isolate is resistant to}}{\text{Total Number of Antibiotics tested .}}$$

### 3.8 Preparation of DNA using DNase Tissue Kit (QIAGEN)

Three to five colonies from a fresh culture of *S. aureus* were suspended in 180 μl of TE buffer Tris- HCI/I, 1M of ethylene diamine tetra acetic acid (EDTA)/1, pH8) in a sterile

1.5ml microphage tube. To the culture suspension, 8µlof lysostaphin (1.8ul/µl; Sigma, Steichen, Germany) was added. The mixture was mixed vigorously with a vortex mixer for 30 seconds then incubated at $37^0$C for one hour. The mixture was vortexed twice during the incubation. After the incubation, 20µl of proteinase K was added to the suspension and incubated at $56^o$C for 2 hours. After the incubation, 200µl of 100% ethanol was added and vortexes thoroughly. The lysate (suspension) was loaded in to a DNeasy filter column contained in a 2ml micro centrifuge tube and centrifuged at 11000G for 1 minute after keeping the lysate for 2 minutes. The flow through in the microcentrifuge tube was discarded; when necessary. The spin was repeated. The column was then placed in a new tube and washed by addition of 500µ1 of AW1 washing buffer. This was allowed to stand for 1 minute and spun for another minute at 11000G. The flow through was discarded. Using a new collection tube, the column was washed for the second time by addition of another 500µ1 of Buffer AW2 and stored for 1 minute, then spun for another minute at 11000G. The flow through was discarded and the same procedure was repeated using the same column to remove residual ethanol. Finally, the DNA was eluted by placing the column in a 1.5ml micro centrifuge tube and added 200µ1 of elution buffer. The buffer was dispensed directly onto silica membrane. The column was allowed to stand for 10 minutes to ensure complete elution before spinning for 15 minutes. The eluted DNA was stored at $-20^0$C

### 3.8.1 Thermal cycling procedure for various programmes

DNA amplification was carried out in a thermo cycler with a pre-cycle at $94^0$C for 4 minutes and final extension at $72^0$C for 5min were used in all programmes.

### 3.8.2 PCR master mix

A 30µl reaction mixture was used for the DNA amplification; containing 1µl of the primer (10pmol/µ1), 0.6µl of deoxynucleoside triphosphates (10mmo1/liter), 3µ1 of thermophilic buffer, 1.8µl of $MgCl_2$ (25mmol/liter), 0.1µl of Taq DNA polymerase (5U/ µ1), 20 µ1 of distilled water and 2.5µ1 to template DNA in a 200 µ1 reaction tube.

### 3.8.3 Agarose gel electrophoresis of PCR products

Twelve micro liters of PCR products mixed with 2µ1 of gel loading dye were loaded for electrophoresis in precast wells in 2% agarose gel with tris acetate-electrophoresis (TAE) buffer (0.04 mol of tris-HC1/1, 1mmo1 of EDTA/1 pH 8.0). Product size were assessed using 5 µ1 of molecular weight marker. Gels were viewed and photographed using a gel documentation system.

# CHAPTER FOUR

## 4.0 RESULTS

### 4.1 Physicochemical and Proximate analysis of Nono and Yoghurt

The physicochemical and proximate analyses of nono and yoghurt samples are presentedin Tables 4.1, 4.2 and 4.3. The mean value for the pH of nono ranged from 3.56-3.61, total titratable acidity ranged from 0.71-1.72, moisture content: 82.23-83.87%, total protein contents: 1.47-1.58%, fat content: 0.35-0.38%, ash content:0.30-0.31%, from all the study areas. For yoghurt, pH ranged from 3.76-3.82, total tritable acidity: 1.10-1.15, moisture contents: 77.76-80.06%, total protein contents: 3.38-3.40%s, fat contents: 1.76-1.79%, while the ash contents: 0.68-0.70%.

### 4.2 Total Viable (Colony) Count of the Bacteria in Nono and Yoghurt

The mean values of the total viable count in colony forming unit/ml (cfu/ml) in the nono and yoghurt samples were presented in the Table 4.4. The results showed that for nono, the counts obtained from Tudun wada was high compared to that of kawo and Bakinruwa and there was a significant different ($p \leq 0.05$) in the counts within the study areas. For yoghurt, Tudun wada recorded the highest bacteria count, followed by Bakinruwa and then Kawo.

**Table 4.1: The Physicochemical Composition of the Nono and Yoghurt Samples Used.**

| Sample Location | Physicochemical parameters | | | |
|---|---|---|---|---|
| | pH | | Total titratable Acidity | |
| | **Nono** | **Yoghurt** | **Nono** | **Yoghurt** |
| Tudun wada | 3.61±0.15 | 3.76±0.06 | 0.72±0.10 | 1.11±0.02 |
| Bakin ruwa | 3.56±0.16 | 3.79±0.03 | 1.72±0.04 | 1.10±0.08 |
| Kawo | 3.57±0.13 | 3.82±0.03 | 0.71±0.07 | 1.15±0.02 |
| Total | 3.58±0.15 | 3.79±0.02 | 1.05±0.64 | 1.12±0.02 |
| **p-value** | **0.087** | **0.565** | **0.363** | **0.140** |

ANOVA, P≤0.05, ⁎=Significant at P≤ 0.05

**Table 4.2: The Proximate Composition of the Nono Samples Used.**

| Sample Location | Proximate Parameters | | | |
|---|---|---|---|---|
| | **Moisture content (%)** | **Crude Protein content (%)** | **CrudeFat content (%)** | **Ash content (%)** |
| Tudun Wada | 82.23±0.40[b] | 1.47±0.03[b] | 0.35±0.02[a] | 0.30±0.04[a] |
| Bakin Ruwa | 82.78±0.51[b] | 1.58±0.02[a] | 0.35±0.02[a] | 0.31±0.04[a] |
| Kawo | 83.87±0.50[a] | 1.54±0.03[ab] | 0.38±0.01[a] | 0.30±0.04[a] |
| Total | 82.95±0.28 | 1.53±0.02 | 0.36±0.01 | 0.31±0.01 |
| **p-value** | **0.047*** | **0.012*** | **0.438** | **0.204** |

ANOVA, *=Significant exist at P≤0.05, (%)=Percentage.

**Table 4.3: The Proximate Composition of the Yoghurt Samples Used.**

| Sample Location | Proximate Parameters | | | |
|---|---|---|---|---|
| | **Moisture content (%)** | **Crude Protein content (%)** | **Crude Fat content (%)** | **Ash content (%)** |
| Tudun Wada | 77.76±0.66 | 3.40±0.01 | 1.79±0.02 | 0.68±0.05 |
| Bakin Ruwa | 78.68±0.75 | 3.38±0.01 | 1.79±0.03 | 0.70±0.01 |
| Kawo | 80.06±0.39 | 3.38±0.01 | 1.76±0.02 | 0.69±0.04 |
| Total | 78.83±0.36 | 3.39±0.01 | 1.78±0.01 | 0.69±0.01 |
| **p-value** | **0.032∗** | **0.224** | **0.723** | **0.091** |

ANOVA, P≤0.05, ∗=Significant at P≤0.05, (%) =Percentage.

**Table 4.4: Mean value for the Total Viable Counts in Nono and Yoghurt (Cfu/ml).**

| Sample location | Nono | Yoghurt |
|---|---|---|
| Tudun wada | $1.29\times10^{5}\pm1.56\times10^{5a}$ | $3.91\times10^{3}\pm7.76\times10^{5b}$ |
| Bakin ruwa | $7.63\times10^{4}\pm3.60\times10^{5b}$ | $1.87\times10^{3}\pm1.93\times10^{3a}$ |
| Kawo | $1.19\times10^{5}\pm9.88\times10^{5b}$ | $1.78\times10^{3}\pm1.99\times10^{3a}$ |
| **P –value** | **0.017*** | **0.018*** |

ANOVA,± SEM=Standard error mean, Cfu/ml= Colony forming unit per ml

## 4.3 Isolation and Identification of *S. aureus*

Out of the 384 milk samples of nono and yoghurt tested, 104 *Staphylococcuss aureus* were isolated. Eighty five percent (88/104) of the isolates were coagulase positive while eighty percent (83/104) of the isolates demonstrated a Dnase activity (Appendix I).

Sixty of the isolates previously identified as *S. aureus* according to the conventional techniques were subjected to Microgen Staph ID (kit) test (0xoid).

Forty three (43) isolates were confirmed as *S. aureus* (Appendix II) while seventeen (17) isolates were confirmed as other species of *Staphylococci* e.g *S. xylosus, S. intermedius, S. chromogenes, S. hyicus, S. Cohnii subsp urealyticus and S. Heamolyticus* respectively.

Also, the prevalence of *Staphylococcus aureus* in nono sample was found to be 43.8%, while that of yoghurt sample was 10.4% and the prevalence of *S. aureus* in both samples was found to be 27.1% (Table 4.5).

## 4.4 Enterotoxin Production by *S. aureus* Isolates

Out of the twenty (20) isolates tested for enterotoxins production, 19 Isolates produced only one types of SEs with SEA being the post predominant toxin. Eight (8) isolates produced all the 4 types of SEs (A,B,C, and D) each, 14 isolates produced more than one SEs and SEA and SEB was the most commonly produced (Table 4.6)

## 4.5 Antibacterial Susceptibility Test Results for the *S. aureus* isolates

The result of the antibacterial susceptibility testing showed that all the isolates were resistant to oxacillin. That is, they recorded 100% resistance to oxacillin. The isolates

were moderately resistance to Clindamycin (23.3%), Tetracycline (23.3%) and Cefoxitin (18.6%) each. Relatively low resistance was observed to Vancomycin, Erythromycin and Chloramphenicol (2.3%) respectively (Table 4.7) while no degree of resistance was observed to Ciprofloxacin.

Some of the forty three (43) *Staphylococcus aureus* strains tested exhibited Multiple Antibiotic Resistance (MAR) to the eight antibiotics tested.Eleven (11) isolates showed resistance to two antibiotics, Six (6) isolates showed resistance to three antibiotics while two (2) isolates showed resistant to five antibiotics. Multiple antibiotics resistance (MAR) were determined for the isolates. Three resistant phenotypes were obtained with varying combinations of 2, 3 and 5. The predominant MAR phenotypes were presented in Table 4.5 and Figure 4.1.

### 4.6 Molecular Characterisation of the Isolates

The identity of the strains of *Staphylococcus aureus* isolated were confirmed by PCR amplification of the gene encoding *mec A* gene with the primer sequence of Forward primer: AAAATCGATGGTAAAGGTTGGC and Backward primer: AGTTCTGCAGTACCGGATTTGC with an amplicon size of 533 bp and Plate II shows the amplicon size of the gene fragments amplified by PCR and only one of the twenty isolates was *mec A* positive.

**Table 4.5: Prevalence of *Staphylococcus aureus* isolated from Nono and Yoghurt Samples**.

| Samples | Number Examined | Number Positive | % prevalence |
|---|---|---|---|
| Nono | 192 | 84 | 43.8 |
| Yoghurt | 192 | 20 | 10.4 |
| Both | 384 | 104 | 27.1 |

%= percentage

**Table 4.6: Distribution of Staphylococcal Enterotoxin Production (SE) by *S. aureus* isolated from Nono and Yoghurt.**

| | | Enterotoxin Type | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Origin of *S. aureus* | No. tested | SE(A) | SE(B) | SE(C) | SE(D) | SE(AB) | SE(AC) | SE(AD) | SE(BC) | SE(CD) | SE(ABC) | SE(BCD) | SE(ABCD) |
| Nono | 11 | 10 | 6 | 6 | 6 | 6 | 6 | 6 | 5 | 5 | 5 | 5 | 5 |
| Yoghurt | 9 | 9 | 8 | 3 | 3 | 8 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| Total | 20 | 19 | 14 | 9 | 9 | 14 | 9 | 9 | 8 | 8 | 8 | 8 | 8 |

Key: No = Number, SE = Staphylococcal Enterotoxin

**Table 4.7: Antibacterial Susceptibility testing of *S. aureus* isolates from Nono and Yoghurt.**

| Antibiotics No tested n=43 | No Resistant (%) | No Intermediate (%) | No Susceptible (%) |
|---|---|---|---|
| Oxacillin 1µg | 43(100) | 0(0.0) | 0(0.0) |
| Tetracycline 30µg | 10(23.3) | 2(4.6) | 31(72.1) |
| Ciprofloxacin 5µg | 0(0.0) | 0(0.0) | 43(100) |
| Erythromycin 15µg | 1(2.3) | 7(16.3) | 35(81.4) |
| Chloramphenicol 30µg | 1(2.3) | 2(4.6) | 40(93.0) |
| Vancomycin 30µg | 1(2.3) | 0(0.0) | 42(97.7) |
| Cefoxitin 30µg | 8(18.6) | 0(0.0) | 34(79.1) |
| Clindamycin 2µg | 10(23.3) | 18(41.9) | 15(34.9) |

No= Number, %=Percentage

**Table 4.8: Frequency and Percentage of Multi- drug resistance pattern or Multiple antibiotics resistance (MAR) of *S. aureus* isolates to selected antimicrobial agents.**

| Number Antibiotics Combination | Frequency of Isolates | Resistance (%) | Resistant phenotype |
|---|---|---|---|
| 2 | 2 | 10.5 | FOX, OX, |
| | 6 | 31.5 | TE,FOX, |
| | 2 | 10.5 | DA, OX |
| | 1 | 5.3 | C, OX |
| 3 | 4 | 21.1 | TE, DA, C |
| | 2 | 10.5 | OX, TE, DA |
| 5 | 1 | 5.3 | DA, TE, FOX, OX, VA |
| | 1 | 5.3 | DA, TE, FOX, OX, E |
| Total | 19 | 100.0 | |

Key: FOX=Cefoxitin, OX=Oxacillin, TE=Tetracycline, DA, Clindamycin, C=Chloramphenicol, E= Erythromycin, VA=Vancomycin. % = Percentage

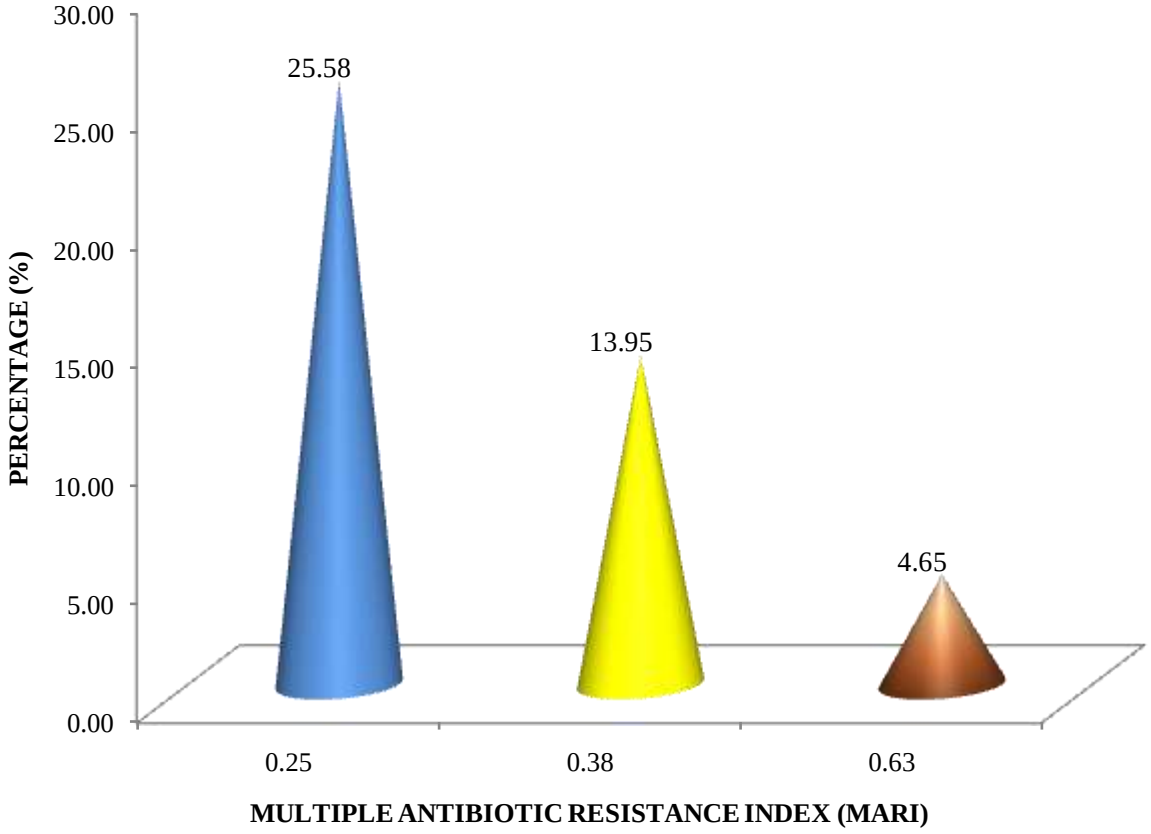



**Figure 4.1**: Multiple Antibiotics Resistance indices and the Percentage of Isolates.

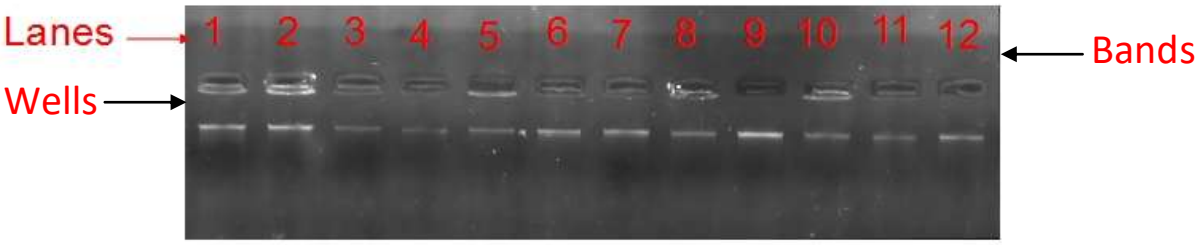



PLATE 1: Gel electrophoresis of DNA extracted from *S. aureus* isolates.

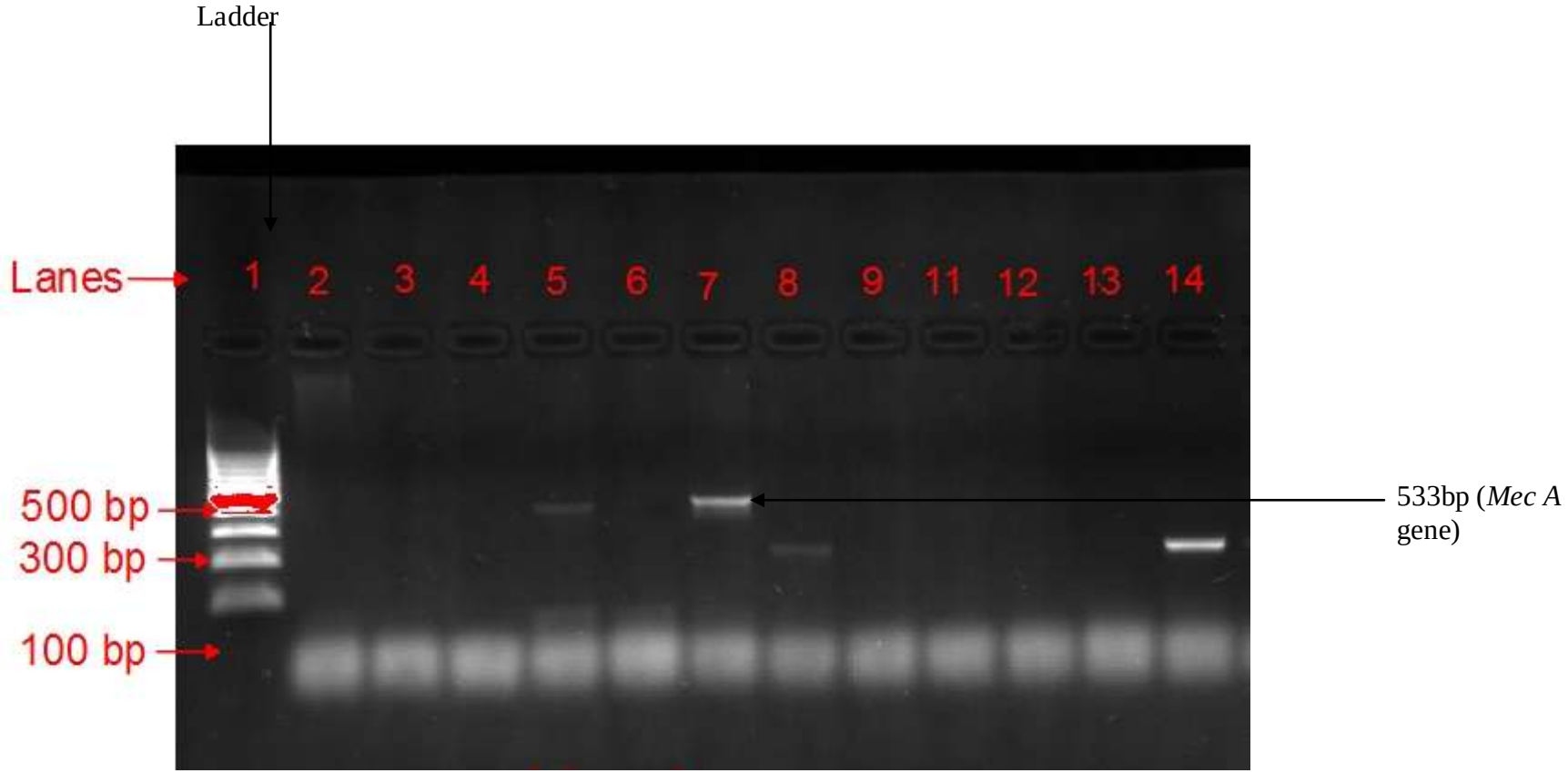



Figure 2: Amplicons of the *mec A* gene of *S. aureus* with a size of 533 bp. Lane 1 (100 bp ladder) lane 2 – 14 PCR Amplicons

# CHAPTER FIVE

## 5.0 DISCUSSION

The mean value for pH of nono obtained ranged from 3.56-3.61 while that of yoghurt ranged from 3.76-3.82. This is in aggrement with the work carried out by (Umoh), 1991 who reported pH of 3.8-4.4 for nono and pH of 3.7-4.4 for yoghurt as well as Sokolinska *et al*. (2004) also reported that the pH value of milk and milk products decreases during manufacturing process from the time it was inoculated with bacteria culture to the time it was manufactured. This is because lactic strains have the ability to ferment lactose into lactic acid thereby increasing the acidity (decrease in pH) of the fermented milk products. This is also supported by the findings of Atanda *et al*. (2006) who reported that fermentation of milk during nono production reduced the pH from 6.3 to 3.8 due to the production of organic acid. The pH of nono recorded in this study is in contrast with the pH 5.7 recorded by Obi and Ikenebomeh, (2007), 5.51-6.29 reported by Adesokan *et al*. (2011); pH 4.09-4.42 by Egwaikhide *et al*. (2014) and 3.7-6.5 reported by Okeke *et al*. (2014) while for yoghurt the pH is in contrast with the pH of 3.8-5.4 reported by El-bakri and El-Zubair (2009); pH of 4.73-5.11 by Igbabul *et al*. (2013) and pH of 5.35 by Omola *et al*. (2014). The difference in their pH could be as a result of some factors like length of fermentation and the starter culture used. The effect of length of fermentation of milk samples could results to increase in acidity of the milk samples, thereby leading tothe reduction of pH which determines the number and kinds of contaminating microorganisms present in the milk sample. The starter culture effect (Lactic acid starter culture effect) on the milk samples could lead to increase in (1) Lactic acid production which lower the pH and (2) increase in oxygen peroxide production.

The Total Titratable acidity (TTA) of nono, shows that the mean value ranged from 0.71-1.72, and that of yoghurt ranges from 1.10-1.15.The TTA obtained from the milk products might be due to the facts that titratable acidity of the raw milk increases on storage due to the action of microorganism and as fermentation progresses or continues titratable acidity of the milk products increases. Increase in TTA (That is, reduction in pH) could be attributed to accumulation of some organic acid and acetic acid which could results from the activities of some fermentative microorganisms such as Lactic acid bacteria and yeast in the milk samples. These mean values of the total titratable acidity of nono obtained in this work is higher compared to the mean value of 0.6-0.9 reported by Umoh *et al*. (1991). However, the mean value of the total titrable acidity of yogurt is in agreement with the findings of Umoh, 1991 who reported a TTA of 0.9-1.4, but in contrast with the report of Okeke *et al*. (2014) who recorded a TTA range of 0.1-0.9, Egwaikhide and Faremi, (2010) recorded a lower TTA range of 0.14-0.16 and Igbabul *et al.* (2013) who reported TTA of 0.90-0.93. However, there was no significant difference in the total titrable acidity of both samples.

The Mean value obtained for the moisture content of nono was 82.23-83.87% while that of yoghurt as 77.76-80.06%. There was a significant difference in the moisture content of both samples ($P \geq 0.05$). This is in agreement with the work of Igbabul *et al.* (*2*013), Uzeh *et al.* (2006) and Ahmad, 1994 who all stated that the maximum moisture content in yoghurt to be 84%. When there is an increase in moisture content of milk it implies increase in water activity ($a_w$) which can support microbial growth and consequently reducing the shelf life of the milk products (Ajai *et al.*,2012). Also, increase in moisture content could

affect the stability and safety of food with respect to microbial growth and proliferation, hence the milk products will require cold storage.

The Crude protein content for nono ranged from 1.47-1.58% while that of yoghurt was 3.38-3.40%. This is in agreement with the work of Umoh *et al*. (1991) who reported protein content of 1.21-1.90% in nono and 3.45% in yoghurt but disagree with the protein content of 2.73% in nono reported by Egwaikhide *et al*. (2014). The low protein content observed in nono from this study might be attributed to the practice of dilution of milk with water after fermentation by the handlers of nono to increase the volume. For yoghurt, the crude protein value obtained was lower than 4.02-6.14% that was reported by Igbabul *et al.* (2013) and 3.5% reported by Eearly, (1988). The increase in crude protein content of yoghurt could be due to some anabolic processes leading to polymer build up or due to microbial cell proliferation. There was a significant difference in the protein content of the nono samples ($p \geq 0.05$).

The mean value for the crude fat content of nono ranged from 0.35-0.38% while that of yoghurt was 1.76-1.79%.This is in agreement with the work reported by Umoh *et al.* (1991) who reported fat content of 0.08-0.68% in nono and Igbabul *et al.* (2013) who reported 1.32-3.25% in yoghurt samples and is in contrast to the work carried out by Umoh *et al*. (1991) who reported fat content of 2%, (Ladokun and Oni), 2014 that reported a high fat content of 9.76-15.02% from different milk types and Omola *et al*. (2014) who reported fat content of 1.5-2.5%. The decrease incrude fat content observed in the milk samples could be as a result of increase in fermentation time which might be attributed to increase activities of lipolytic enzymes during fermentation which also hydrolysed fat component (Triacylglycerol) into fatty acid and glycerol. Fat content are usually higher in food of

animal origin than plant origin as fat content of food is the major contributor to energy value of food. As reported by Astuti *et al*., (2000) that the fatty acid were used by some microorganisms such as moulds resulting in decrease in fermentation in the fermenting milk samples at the end of fermentation. So fat plays an important role in improving the consistency of yoghurt and also provide twice as much energy as some quantities of carbohydrate and protein (Ehirin and Onyeneke, 2013). According to FAOSTAT,2012 standard, fat content of about 0.5-10 is good but 3.0 was the best.

The mean value for the Ash content of nono was 0.30-0.31% while that of yoghurt is 0.68-0.70%. The value recorded in these findings might be due to the facts that ash content is an index of mineral content. This is in agreement with the work reported by Umoh *et al*. (1991) who reported ash content of 0.24-0.37% and 0.75% in nono and yoghurt respectively, Ladokun and Oni, 2014, who reported 0.52-0.96% in yoghurt and that of Igbabul *et al.* (2013) who reported ash content of 0.41-1.02%. But contrast with the ash content of 0.2- 1.80 by Egwaikhide *et al.* (2014). The higher ash content recorded in their findings might arise from increase in water content, increase percentage of fermentable bacteria and higher mineral content. The ash content value is said to be an index of mineral content which is needed for bone development, teeth formation and body function of the consumer (Trachoo and Misty, 1998).

Although, the total viable counts in yoghurt (1.78-3.91) $x10^3$ cfu/ml is within the acceptable limit of $10^3$-$10^4$cfm/ml in accordance to the guidelines for the microbiological quality of food samples (Gilbert *et al*., 2000). Also,$<10^5$cfu/ml of total viable counts (TVC) of yoghurt was reagarded as the marginal limit of acceptable microbiological quality of food based on standard methods of International Commission on Microbiology

Specification of Food (ICMSF,1996). A TVC of $>10^6$cfu/ml is regarded as potentially hazardous. Though, the TVC obtained for yoghurt in this study is lower than that of nono but it might be an indication of contamination of the yoghurt samples either during packaging or at the preparatory stage or during handling or poor handling of yoghurt during processing and packaging of the products. Nono being the local version of yoghurt recorded a higher count (7.63-1.29) $x10^5$ cfu/ml than yoghurt and there is a significant difference in these counts. The higher counts in nono might be due to handling of the products, processing or sanitary condition of the handlers, low level of hygiene maintained during processing and sale of the product (nono), quality of water used (for both nono and yoghurt), the utensils, exposure of nono while it is usually displayed for sale in the bowl as all these can serve as sources of contamination to the product. Also, the higher counts observed in nono might be due to the facts that the keeping time of nono is longer before consumption and this increases the acidity (as we observed in this study nono has a low pH). This means that increase in acidity determines the numbers and contaminating organisms. Other reasons for higher counts observed in nono could be as a result of the length of time of storage of raw milk used for the production of nono which might allow for microbial contaminants to multiply and increase in number thereby influencing the total viable/bacteria counts and the types of bacteria present in the bulk raw milk.

Most handlers or sellers of nono are street peddlers and often not all nono that are brought to the market by the peddlers are sold the same day, such unsold ones are reintroduced into the market with no further treatment or proper preservation. Thus, allowing pathogenic organisms to gain access into the products and have enough time to multiply and produced harmful metabolites. The street paddlers usually add left over nono of the previous day and

or previously fermented nono (back slopping) as a starter culture for the production of nono, by so doing the organisms (particularly *Staphylococcus aureus*) have enough time to multiply thereby increasing the bacteria counts in the nono.

The prevalence of *S. aureus* in nono samples (Table 4.5) was found to be 43.8% while that of the Yoghurt sample was found to be 10.4%, but the prevalence of *S. aureus* in both samples was found to be 27.1%. This is much lower than the prevalence of 52.4% reported by Gali *et al.*, 2013. The lower prevalence rate recorded in yoghurt might be attributed to the effect of pasteurization during the manufacturing process which inhibits the multiplication of organisms while higher prevalence observed in nono is indicative of poor hygienic measures during production, handling and distribution. But, the lower prevalence of *S. aureus* recorded in this study for the both samples might largely be due to improved sanitary conditions and processing of the milk products. Another study conducted in Maiduguri by Mai Siyama *et al.* (2014) also reported a lower prevalence of 17.1% of *S. aureus* isolated from Nasal and milk product samples, which is much lower than the prevalence of this study. This implies that the presence of *Staphylococcus aureus* in the milk samples particularly raw milk that are used as the major raw materials generally comes from cow with mastitis, from handlers or from deficient hygienic practices. Similar studies reported a higher prevalence of 94.5% of *S. aureus* isolated from milk and ice cream by Gundogan *et al.,* 2006 in turkey, 56% prevalence by Gundogan and AVCI, 2014 in turkey from raw milk and dairy products,75% in Bangladesh by Begum *et al* .,2007 which is higher than the prevalence of this study while 25% prevalence by Arefi *et al.,* 2013 in Iran from Iranian cheese, 25.53% by Jahan *et al.*,2015 in Bangladesh from raw cow milk are lower than the prevalence of this study. Also Ateba *et al.,* 2010 reported

prevalence of 60% and 40% from milk (both from communal and commercial farms) in South Africa which are higher than the prevalence of this study while El-Jakee *et al.,* 2008 reported a lower prevalence of 19.1% in Egypt from bovine, chicken, concentrated ration,dog and human which is lower than this study's prevalence.

In This Study, 80% of the staphylococci isolates were Dnase positive which is much higher than the study demonstrated by Sulaiman, (2011) which recorded 78% of the isolates to be Dnase positive. Although, the high specificity and sensitivity of the coagulase test (Blobe and Schlisser, 1994) has made it to be considered as a standard method for the identification of *S. aureus* but in this study 85% of the *S. aureus* were positive in the slide coagulase test which is not in agreement with the study carried out by Sulaiman, 2011 that reported a 100% of *S. aureus* being positive in the coagulase test after 4 hours of incubation. This could mean that the isolates from that study is probably of human origin. The difference might also be due to the method used or the fact that the milk samples in this study have undergone some processing.

The microgen Staph ID kit proved successful in identifying 43 isolates out of 60 (72%) of the *S. aureus* on which it was applied. The remaining 17 isolates were identified as *S. xylosus, S. intermedius, S. chromogenes, S. hyicus, S. cohnii subsp. Urealyticus and S. heamolyticus* respectively.

This discrepancy in the use of biochemical test kit and the conventional biochemical tests can be explained by the facts that conventional method/tests failed to distinguish between *S. aureus, S. intermedius* and *S. pseudintermedius* (Sasaki *et al.,* 2007).

*Staphylococcus aureus* have developed β lactams resistance worldwide, although reported prevalence rates indicates that wide variations exist regionally (De oliveira *et al.,* 2000; Normanno *et al.,* 2007). Depending on the Origin of the Sample, the prevalence of β lactams resistance strains of *S. aureus* are extremely different (Stephan *et al.,* 2001; Jorgensen *et al.*, 2005; Anderson *et al.,* 2006).

This study showed a frequency of resistance of 100%, 23.3%, 18.6%, 18.6% to Oxacillin, Tetracycline, Clindamycin, Cefoxitin respectively by *S. aureus* tested. A similar study carried out by Gali *et al.,* 2013 reported 100% resistance of the isolates to Oxacillin. Another study carried out by Sulaiman, 2011 reported 35.6% of the isolates were resistance to Oxacillin which is lower than the one reported in this study. Viera-a-mora *et al.* (2000) reported 87% resistance to oxacillin. The prevalence of resistance of the isolates to oxacillin (100%) by disc diffusion method is considered important because it is used to assay for resistance to methicillin,which is considered unstable while oxacillin is better at maintaining its activity when stored (Felten *et al.,* 2002, Tiwari *et al.,* 2009). As recommended by CLSI (2007), some of the isolates of the *Staphylococcus aureus* tested were resistant to oxacillin because it more reliably detects methicillin-resistant strains of *S.aureus* and resistant to oxacillin indicates that it is more likely to detect heteroresistant strains of *Staphylococcus aureus* and it also maintain its activity during storage better than methicillin..

Oxacillin was used to replace Methicillin in this study due to the fact that, (1) Methicillin as an antibiotics is not available during the time this research was carried out, (2) It is no longer the drug of choice for testing or treatment of *S. aureuss* infection (CLSI, 2006), (3)

Oxacillin was also used because it can assess methicillin resistance due to the unstable nature of methicillin as reported by (Akpaka *et al.,* 2006; Felten *et al.,* 2002).

Relatively low resistance (2.3%) was observed in Erythromycin, Vancomycin and Chloramphenicol respectively. The relatively low resistance observed in Vancomycin and Erythromycin may be partly due to the high cost of the drugs. Although Erythromycin is one of the frontline antimicrobial agents routinely used in human and veterinary medicine. Others include Tetracycline, Penicillin and some Quinolones. Due to the facts that Tetracycline is one of the most commonly used antibiotics in the Nigerian Livestock industry,the resistance to Tetracycline was 23.3% which is higher than that the one reported by Sulaiman, 2012 who reported 19.2%. This could mean that there was probably a moderate usage of tetracycline within the study area and the study areas differ.

Antibiotics resistance is influenced by the different practices of administering antimicrobial products (dosage, timing frequency, type and frequent changes of types) (Aerestrup, 1999, cited in Sulaiman, 2011). Also extended use and inappropriate use of antibiotics in Agriculture stock farming and in the treatment of human diseases were identified as the cause of increasing resistance to antimicrobial agents (Pesavento *et al.,* 2007) also there are some genes that are responsible for the majority of the resistance to Chloramphenicol, Tetracycline, Ampicillin, Sulfamethoxazole/Trimethoprim (although, ampicillin and sulfamethoxazole/trimethoprim were not used in this study) which are often encoded by conjugate plasmids are likely to evolve because of antibiotics misuse (Robertson *et al.,* 2003).

An absolute absence of resistance to Ciprofloxacin was observed in this study. This connotes that in the event of outbreak of Methicillin resistant *Staphylococcus aureus* (MRSA), Ciprofloxacin could be reliably effective as a drug of choice in the treatment of MRSA infections. The difference between this study and other is the observe resistance to vancomycin which is drug of choice for the treatment of MRSA but recently vancomycin or other glycopeptides intermediately resistant *S. aureus* (VISA/GISA) have emerged, although the resistance was relatively low (2.3%). This is surprising and should be of concern.

The finding in this study showed that the isolates showed multiple resistance to Oxacillin is of concern. From the 43 isolates, Eleven (11), Six (6) and Two (2) isolates were resistant to two, three, five antibiotics respectively. The multi drug resistant was found to be in agreement with that of Sharma *et al.*, 2011 who also found out that *S. aureus* was resistant to several antibiotics. Methicillin (Oxacillin) resistance *S. aureus* (MRSA/ORSA) is an important human pathogen and may produce serious infections that may result in septicaemia (Paesavento *et al.*, 2007). Infection due to this organism have been associated with increased morbidity, mortality and prolonged hospitalization (NNIS, 2003).

MRSA infections are of global health concern due to severity of the illness they may cause. The main source of the pathogen is health care associated (HA-) infections.Until now, methicillin resistant *S. aureus* strains were only rarely detected in food, but transmission of MRSA by food consumption has not been investigated thoroughly (Sulaiman, 2011).

Only one (1) out of the twenty (20) (5%) *S. aureus* strains representative of all isolates from the study area screened for *Mec A* was positive. A similar studies by Sulaiman,2011 showed that only 2 out of 20 from raw milk were mec A positive, also Gali *et al*., 2013 reported 4 out of 18 from raw milk and kindrimo were all positive to *MecA*. The *mecA* gene is the most common gene that mediate methicillin/oxacillin resistance in staphylococci and one of the peculiarity of MRSA which carry the mecA gene is its heterogenous

Expression of resistance in which an individual strain can generates subpopulation with different degree of higher resistance. This can also be explain further that the detection of methicillin/oxacillin resistance can be due to the presence of subpopulation (one susceptible and the other resistant) that may coexist within a culture of Staphylococci. Also all the cells in a culture may carry the genetic information for the resistance, but only a small number may express the resistance invitro. The paucity of local studies in MRSA in milk and milk products/foods generally makes it difficult to make any comparison and to assess the MRSA strains in milk in Nigeria.

At global level, only a few data are available on the presence of MRSA in dairy products. Recently, Hummerjohann *et al.,* 2014 identified 1 MRSA out of 623 isolates from swiss bovine milk cheese, Carfora *et al.,* 2014 also identified 3 MRSA in bovine, ovine milk and dairy products. In another studies, Kwon *et al.,* 2005 showed only 0.18% samples in unproceesed cow milk in South Korea contained MRSA. In Italy, out of the 160 *S. aureus* isolates from milk and dairy products analysed, 6(3.75%) were screened for *mecA* positive (Normanno *et al*., 2007). 1.7% in bovine milk in turkey, 0% from raw milk in Bangladesh, 0% in bulk milk in U.S.A, 1.4% in raw milk in Switzerland were also reported by (Kwon

*et al*., 2005, Huber *et al.,* 2010, Turkyilmaz *et al*., 2010). However, this study present prevalence is lower than 29.3% in raw milk in republic of Korea and 14.7% in raw goat milk in Czech republic (Lee,2003; Strastkova *et al*., 2009).

In a study conducted by Umoh *et al,* (1990a) in Zaria, a low prevalence of 19% enterotoxigenic strains of *S. aureus* from bovine mastitis milk was reported. Sulaiman, (2012) reported 61.6% of the isolates were found to produce one or more staphylococcal enterotoxin (SEs) ,while in this study 40% of the isolates were found to produce one or more staphylococcal enterotoxin (SE) which is lower than that reported by Sulaiman, 2012, but higher than the prevalence reported by Umoh *et al*. (1990).

A study carried out by Umoh reported that the incidence of enterotoxin production by *S. aureus* isolated from fermented milk products showed that out of a total of 369 samples tested, all the samples of nono contain 6.1% of the enterotoxin while 8.1% from fura. From that it was reported that human biotypes of staphylococci are more enterotoxigenic than animal biotypes. Although, isolates from milk and milk products were both of human and animal origin, raw milk had more contamination from animal origin and fermented milk from human origin. Many isolates that elaborated enterotoxin A are commonly associated with food poisoning (Umoh *et al.,* 1991b). The predominance of SEA contradicts reports from countries such as Switzerland, Brazil, and Japan where SEC-producing *S.aureus* were frequently isolated (Stephan *et al.,* 2001; de Silva *et al.,* 2005; Katsuda *et al.,* 2005). Various reports have indicated that SEA producing strains are mainly from humans, whereas SEC are from animals (Orden *et al.,* 1992, cited in Sulaiman *et al*., 2013). A review by Genigeorgis (1989) concluded that the predominant staphylococcal enterotoxin (SE) secreted by isolates from food and which are involved in staphylococcal

gastroenteritis is SEA. The determination of *S. aureus* strains able to produce several types of enterotoxins simultaneously have been reported (Joffe and Baranovics, 2006). The facts that some of the milk samples (nono and yoghurt) examined contained enterotoxigenic strains of *Staphylococcus* is of public health importance, and milk products like nono which is mostly consumed in the study area could be vehicles for milkborne intoxication. The occurrence of new types of staphylococcal enterotoxins have been reported (Zschock *et al.,* 2005) which includes SEG, SEH, SEI and SEJ.

In a study conducted by Latvia, Joffe and Baranovics (2006) reported a prevalence of 77.3% enterotoxigenic *S. aureus* strains from bovine mastitis.

In the present study, only one out of the 20 oxacillin resistant *S. aureus* isolates was mecA positive. All the 20 isolates which exhibited resistance to oxacillin by the disc diffusion methods lacked the *mec A* gene.

This is in agreement with the studies of Adesida *et al.,* 2005 who screened for mec A gene by PCR in Nigeria and detected the gene in 1.4% of hospital isolates in southern, western and north central region of Nigeria, and is also in agreement with the studies of Gali*et al.,* 2013 who screened for mec A gene and detected the gene in 4.8% of isolates from raw milk and kindrimo.

It was noted that PCR assay for the detection of MRSA do not always give indisputable results because some isolates which have been found to be mec A negative in the PCR were resistance to Oxacillin (MRSA). This agrees with the finding of Lee *et al.,* 2003 who demonstrated that some of the isolates that did not carry mecA gene were considered phenotypically resistant to oxacillin (MRSA) due to the fact that phenotypic expression of

resistance can vary depending on the cellular growth conditions. Also it was reported by Olonitola *et al.,* 2007 that some isolates that have been found to be mecA positive, were susceptible to both methicillin/oxacillin.

There are some possible mechanism of methicillin resistance which includes; (1) Staphylococci resistance to oxacillin/methicillin oocur when an isolates produced an altered penicillin binding protein PBP2a, which encoded the mecA and the resistance in MRSA is primarily mediated by mecA gene which encode for a novel PBP2a.(2) The methicillin resistance acquired by *Staphylococcus aureus* is by insertion of Staphylococcal cassette chromosomes (SCC), carrying the mecA gene into the chromosomes.(3) Acquisition of the gene encoding a modified penicillin binding protein (PBP) known as PBP2a found in MRSA and the highest level of resistance can come from mutation in the chromosomal genes.

# CHAPTER SIX

## 6.0 CONCLUSION AND RECOMMENDATIONS

### 6.1 Conclusion

The results of the chemical analysis showed that for nono, the pH ranged from 3.56-3.61, total titratable acidity: 0.71-1.72, moisture content: 82.23-83.87%, total protein content: 1.47-1.58 %, fat content: 0.35-0.38 %, ash content: 0.30-0.31% a. For yoghurt, the pH ranged from 3.76-3.82, total titratable acidity: 1.10-1.15, moisture content: 77.76-80.06%, total protein content: 3.38-3.40%, fat content: 1.76-1.79% and ash content: 0.68-0.70% from all the study areas.

The total viable count were (7.63 – 1.29) x $10^5$cfu/ml and (1.78 – 3.91) x $10^3$ cfu/ml for yoghurt and nono respectively. The total viable counts result showed that the counts is still within the acceptable limits ($10^3$-$10^4$cfu/ml) of the regulated standard (<$10^5$cfu/ml) of ICMSF standard/guidelines as in the case of yoghurt sample, and there is no acceptable microbial limit that has been established for nono.

*Staphylococcus aureus* in nono and yoghurt sample were isolated and characterised using both conventional method and Microgen Staph ID kit. The prevalence of *S. aureus* in nono was found to be 43.8%, for yoghurt was 10.4% while for both was 27.1%, and the Microgen Staph ID kit was demonstrated to be more efficient for the detection and identification of *S. aureus* from the milk samples, user friendly and seems to be better at differentiation at species level.

Nineteen (95%) of the *S. aureus* isolates tested were enterotoxin producers, and thus of public health importance. Also, the enterotoxigenic strains of these staphylococci contribute to foodborne intoxication as in the cases of food poisoning are usually due to staphylococcal enterotoxin A (SEA), which is the predominant toxin associated with food.

Most of the isolates showed multiple antimicrobial resistance (MAR). Methicillin resistant *Staphylococcu aureus* (MRSA/ORSA) has emerged in the study area and could threaten the successful treatment of the disease/infections. This is evident by the fact that all the isolates were resistant to Oxacillin by the antibiotics susceptibility testing.

The Polymerase chain reaction (PCR) techniques detected the presence of *mec A* gene in only one strain of the isolates. This implies that Nucleic acid amplification test such as PCR can be used to detect *mec A* gene which is the most common gene that mediate methicillin/oxacillin resistance in Staphylococci. However, *mec A* PCR tests will not detect novel resistance mechanism such as *mecC* or uncommon phenotypes such as borderline resistance oxacillin resistance.

**6.2 Recommendations**

1. This study also recommends proper pasteurization and fermentation of milk before human consumption and also the practise of hygienic measures during milking procedures to limit the spread to human as there could bepossibility of staphylococcal enterotoxin milk-borne intoxication.

2 Proper awareness and enforcement of safe handling, personal hygiene and good sanitation should be imposed on foodprocessors at all levels, in order to reduce the

incidence of *Staphylococcus aureus* infection through consumption of contaminated products.

3 There is need to reach out to farmers with information, education, creation of awareness on proper milking hygiene, mastitis control and guidance so that milk production, quality and safety will be improved.

4 Food safety guidelines for the pasteurization of food like milk and milk products and distribution in unopened form is being recommended and the practices of preparation and distribution of nono and other fermented products in open calabash should be discouraged.

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# APPENDICES

**APPENDIX I: Biochemical Characteristics of *S. aureus* isolates of Nono and Yoghurt Samples tested**

| | | Numbers of positive (%) | | |
|---|---|---|---|---|
| | **Test** | **Nono** | **Yoghurt** | **Both** |
| 1. | Catalase | 84 (100) | 20 (100) | 104 (100) |
| 2. | Coagulase | 68 (80) | 20 (100) | 88 (85) |
| 3. | DNase | 63 (75) | 20 (100) | 83 (80) |

**APPENDIX II: Phenotypic Typing of some of the *S. aureus* isolates from Nono and Yoghurt using Microgen Staph – ID Kits**

| Sample code | LAT | CPG | NIT (9a) | Sucrose (SUC) | Trehalose (TRE) | Manitol (MAN) | N-Acetyl Gluseamine | Manose | Turanose | Alkaline | Glucosidase | Nitrate | Urease | Arginine | PYR | Octa-Code | Organism |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| n19 | + | + | - | + | + | + | + | + | - | + | - | - | + | + | + | 67647 | *S.chromogene* |
| Tn8 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 77777 | *S.xylosus* |
| n 14 | + | + | - | + | + | + | + | + | - | + | - | + | - | + | + | 67653 | *S. haemolyticu* |
| n 15 | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - | 77764 | *S.aureus* |
| n 18 | + | + | + | + | + | + | + | + | + | + | - | - | + | + | - | 77746 | *S.aureus* |
| n 16 | + | + | - | + | + | + | + | + | - | + | + | + | + | + | - | 67676 | *S.aureus* |
| n 58 | + | + | + | + | + | + | + | + | - | + | + | + | + | - | - | 77674 | *S.aureus* |
| n10 | + | + | - | + | + | + | + | + | - | + | + | + | + | + | - | 67676 | *S.aureus* |
| n34 | + | + | - | + | + | + | + | + | - | + | - | - | - | + | - | 67642 | *S.aureus* |
| n56 | + | + | + | + | + | + | + | + | + | + | - | - | + | - | + | 77745 | *S.chromogen* |
| n25 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 77777 | *S.xylosus* |
| n21 | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | 77677 | *S.xylosus* |
| n63 | + | + | - | + | + | + | + | + | - | + | - | + | + | - | - | 67654 | *S.cohnii* subs *urealyticum* |
| n60 | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | 77775 | *S.xylosus* |
| n17 | + | + | - | + | + | + | + | + | + | + | - | - | + | + | + | 67747 | *S.aureus* |
| n84 | + | + | - | + | + | + | + | + | + | + | - | - | + | + | + | 67747 | *S.aureus* |
| n112 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | *S.xylosus* |
| n114 | + | + | - | + | + | + | + | + | - | + | - | - | - | - | - | 67640 | *S.aureus* |
| n71 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 77777 | *S.xylosus* |
| n68 | + | + | + | + | + | + | + | + | - | + | + | + | + | - | - | 77674 | *S.aureus* |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **n121** | + | + | + | + | + | + | + | + | - | + | - | + | + | - | - | **77654** | ***S.intermediu*** |
| **n78** | + | + | - | + | + | + | + | + | + | + | - | + | + | + | - | **67756** | ***S.aureus*** |
| **n94** | + | + | - | + | + | + | + | + | - | + | + | + | + | + | - | **67676** | ***S.aureus*** |
| **n105** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | **77776** | ***S.aureus*** |
| **n76** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | **77776** | ***S.aureus*** |
| **n91** | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | **77677** | ***S.aureus*** |
| **n81** | + | + | - | + | + | + | + | + | + | + | + | - | + | - | + | **67765** | ***S.aureus*** |
| **n86** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | **77777** | ***S.xylosus*** |
| **n96** | + | + | + | + | + | + | + | + | - | + | - | - | + | - | + | **77645** | ***S.chromogen*** |
| **n177** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | **77776** | ***S.aureus*** |
| **n137** | + | + | - | + | + | + | + | + | - | + | - | - | - | + | - | **67642** | ***S.aureus*** |
| **n138** | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | **77677** | ***S.xylosus*** |
| **n163** | + | + | - | + | + | + | + | + | + | + | + | + | + | - | - | **67774** | ***S.aureus*** |
| **n144** | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | **77774** | ***S.aureus*** |
| **n165** | + | + | - | + | + | + | + | + | - | + | - | + | - | + | + | **67653** | ***S.haemolyticu*** |
| **n184** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | **77776** | ***S.aureus*** |
| **n180** | + | + | - | + | + | + | + | + | - | + | + | + | + | + | - | **67676** | ***S.aureus*** |
| **n170** | + | + | - | + | + | + | + | + | - | + | + | + | + | + | - | **67676** | ***S.aureus*** |
| **n141** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | **77776** | ***S.aureus*** |
| **n172** | + | + | - | + | + | + | + | + | - | + | - | + | + | + | - | **67656** | ***S.hyicus*** |
| **y45** | + | + | - | + | + | + | + | + | + | + | - | - | - | - | - | **67740** | ***S.aureus*** |
| **y46** | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | **77744** | ***S.aureus*** |
| **y50** | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | **77744** | ***S.aureus*** |
| **y65** | + | + | + | + | + | + | + | + | + | + | + | - | + | - | - | **77764** | ***S.aureus*** |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **y71** | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | **77740** | ***S.aureus*** |
| **y79** | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | **77740** | ***S.aureus*** |
| **y80** | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | **77740** | ***S.aureus*** |
| **y85** | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | **77740** | ***S.aureus*** |
| **y91** | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | **77744** | ***S.aureus*** |
| **y100** | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | **77740** | ***S.aureus*** |
| **y101** | + | + | - | + | + | + | + | + | + | + | + | - | + | - | - | **67764** | ***S.aureus*** |
| **y116** | + | + | - | + | + | + | + | + | + | + | - | - | - | - | - | **67740** | ***S.aureus*** |
| **y111** | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | **77740** | ***S.aureus*** |
| **y120** | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | **77740** | ***S.aureus*** |
| **y124** | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | **77744** | ***S.aureus*** |
| **y130** | + | + | + | + | + | + | + | + | + | + | - | - | + | - | - | **77744** | ***S.aureus*** |
| **y140** | + | + | - | + | + | + | + | + | + | + | - | - | - | - | - | **67740** | ***S.aureus*** |
| **y150** | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | **77760** | ***S.aureus*** |
| **y166** | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | **77740** | ***S.aureus*** |
| **y177** | + | + | + | + | + | + | + | + | + | + | + | - | + | + | - | **77766** | ***S.aureus*** |

T= Tudun wada, K=Kawo, B=Bakin ruwa, n= nono, y= yoghurt

LAT=Latex Agglutination Test, NIT=Nitrate, CPG= Colony Pigmentation, PYR=Pyrrolidonyl arylamidase