MOLECULAR CHARACTERISATION AND ANTIBIOGRAM OF *STAPHYLOCOCCUS AUREUS* AND *SALMONELLA* SPP. ISOLATED FROM MILK AND MILK PRODUCTS IN PARTS OF KADUNA STATE, NIGERIA

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BY

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA

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DEPARTMENT OF MICROBIOLOGY,
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FEBRUARY, 2017

DECLARATION

I declare that the work in this thesis entitled, **'MOLECULAR CHARACTERISATION AND ANTIBIOGRAM OF *STAPHYLOCOCCUS AUREUS* AND *SALMONELLA* SPP. ISOLATED FROM MILK AND MILK PRODUCTS IN PARTS OF KADUNA STATE, NIGERIA'**, has been carried out by me in the Department of Microbiology, Faculty of Science, Ahmadu Bello University, Zaria, Nigeria. The information derived from the literature has been duly acknowledged in the text and list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other institution.

Ngozi Ogbeyalu OKPO _____ _____

Signature Date

CERTIFICATION

This thesis entitled 'MOLECULAR CHARACTERIZATION AND ANTIBIOGRAM OF *STAPHYLOCOCCUS AUREUS* AND *SALMONELLA* SPP. ISOLATED FROM MILK AND MILK PRODUCTS IN PARTS OF KADUNA STATE**,** NIGERIA', by Ngozi Ogbeyalu OKPO meets the regulations governing the award of degree of Doctor of Philosophy of the Ahmadu Bello University, and is approved for its contributions to knowledge and literary presentation.

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Prof. K. Bala Dean, School of Postgraduate Studies	_____ Signature	_____ Date

DEDICATION

This thesis is dedicated to my mother Lolo Martha Izeghe Elendu (of blessed memory) and all women in the field of science.

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ABSTRACT

Milk and milk products play an important role in food borne diseases. Three hundred and twenty (320) samples comprising of eighty (80) each of fresh milk, Nono, Manshanu and Kindrimo obtained from four local government areas (Giwa, Kaduna North, Chikun and Soba) in Kaduna State, were examined. The chemical compositions of samples, bacteriological and molecular characteristics of isolates were carried out using standard methods. Results of the chemical characteristics of the samples revealed that no significant ($P \leq 0.05$) difference existed in the pH and acidity of fresh milk and milk products samples from the selected locations. Mean pH values of samples ranged from 3.36 ± 0.23 to 6.43 ±0.27 while mean acidity values was 0.47 ± 0.13 to 3.28 ± 0.49. Acidity values for other milk products; Nono (1.87 ± 0.80), Manshanu (3.28 ± 0.49) and Kindrimo (1.78 ± 0.97) were significantly different from fresh milk (0.47 ± 0.13). Mean values of the proximate composition of the samples revealed that their total solids contents were within the recommended (>12.5%) WHO/FAO standard. Results also showed that milk products except fresh milk had low ash and protein contents compared to WHO/FAO standards of 0.90 ± 0.10 and 4.00 ± 0.10 for ash and protein respectively. Mean total viable counts for samples ranged from $\log_{10}$3.90 ± 3.18 cfu/ml (Manshanu) to $\log_{10}$ 6.04 ± 5.04 cfu/ml (Nono). Mean counts obtained from Manshanu were significantly different from that obtained from other products, though there was no significant difference at ($p \leq 0.05$) between the total viable counts obtained from Nono ($\log_{10}$ 6.04 ± 5.04 cfu/ml) and Kindrimo ($\log_{10}$5.95 ± 4.89 cfu/ml). Prevalence of potential pathogenic bacteria was 42.19% (135) while *Acinetobacter haemolyticus* and *Salmonella pullorum* had the least percentage occurrence of 0.7% and *Staphylococcus* spp. had the highest percentage occurrence of 41.48% in the samples. Antibiogram of bacterial isolates revealed high activity for gentamicin (100%), Ciprofloxacin (100%) and chloramphenicol (96.55%). Resistance was shown (65.52%) to tetracycline a commonly used antibiotics reflecting a public health concern. Most resistant isolates had a multiple antibiotics index of 0.2 (35%) with a least multiple antibiotics resistance index of 0.5 (5%). The study also revealed the occurrence and expression of classical Staphylococcal enterotoxin A gene 17.9% (19), B 17.9% (5), C 10.7% (3) and D gene 7.1% (2) by enterotoxin producing strains of *S.*

*aureus*in the fresh milk and milk products. Molecular screening for virulence genes in the *S. aureus* and *Salmonella pullorum* isolates showed 35% (7) *S. aureus* strains to harbour genes for enterotoxin A with B 5% (1). The M*ec A* gene was detected in 55% (11) of the *S. aureus* strains while in the *Salmonella pullorum* isolate neither the *Inv A* nor the *Sal Stn* gene were detected. Findings of this research shows that the nutritional content of the fresh milk and milk products were of appreciable nutritional status. However the high total viable counts and high presence of a wide range of microorganisms, particularly enteric pathogens and enterotoxigenic strains of *S. aureus* suggests that fresh milk and milk products might be an important source of food-borne infection and intoxication.

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ABBREVIATIONS, DEFINITIONS, GLOSSARY AND SYMBOLS

'Fura' Millet cereal in balls made from millet flour which has been spiced with ginger, cloves and sometimes pepper.

'Nono' locally fermented milk/skimmed.

'Kindrimo' a viscous full fat (or partially skimmed) traditionally fermented milk.

'Manshanu' milk fat

'Madara' fresh milk/raw unfermented milk.

CDC Centres for disease control

CFU Colony forming unit

CPS Coagulase positive Staphylococci

ELISA Enzyme linked immunosorbent assay

GMP Good manufacturing practices

MAR multiple antibiotic resistance

MARI multiple antibiotic resistance index

NTS Non- typhoidal salmonella

PCR Polymerase chain reaction

Stn Salmonella enterotoxin

SE Staphylococcal enterotoxin

SEA Staphylococcal enterotoxin A

SEB Staphylococcal enterotoxin B

SEC Staphylococcal enterotoxin C

SED	Staphylococcal enterotoxin D
SFP	Staphylococcal food poisoning
SFPO	Staphylococcal food poisoning outbreak
TSI	Triple sugar Iron

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Food-borne infections are common and constitute an important health and economic burden globally (Nazzal *et al.,* 2012). Through microbial activity alone, approximately one-fourth of the world's food supply could be lost (Varga, 2007).

Milk has been described as a nearly perfect food because it contains the essential nutrients required by the body in appropriate proportions (Karshima *et al*., 2013). It is an aqueous colloidal suspension of proteins, fats, and carbohydrates that contains numerous vitamins and minerals (Ogbolu *et al.,* 2014). Milk has distinct physical, chemical and biological characteristics, which justify its high quality for consumption (Hemalatha and Shanti, 2010). These characteristics present a favorable environment for the multiplication of various bacteria and an efficient vehicle for transmission of diseases to humans (Donkor *et al*., 2007). Milk can contain Pathogenic bacteria, such as *Salmonella* spp.,*Staphylococcus aureus,Escherichia coli,Mycobacteriumtuberculosis,Listeria* spp.and *Brucella* spp. (Bereda *et al.,*2014). However, the presence of these pathogenic bacteria in milk and milk products has emerged as a major public health concern (Lingathurai and Vellathurai,2010).

Salmonella food infection is one of the most common and widely distributed diseases in the world, estimated to cause 1.3 billion cases of gastroenteritis and three million deaths worldwide (Ohud *et al.,*2012). Concern about *Salmonella spp.* has increased since its first isolation because of its effects on health, food and economy of man (Esona *et al.,* 2004,

Mushtaq-ul-Hassan *et al.,* 2008; Razzaque *et al.,* 2009). Enteric infections caused by species of *Salmonella* have also great Zoonotic importance chiefly due to their ubiquitous distribution, the growing number of serotypes, wide host spectrum, complex pathogenesis and the complicated epidemiological chain involving animals, man and the environment (El-Gazzar and Marth; 1992). *Salmonella* continues to be a major concern for the dairy industry because this has caused outbreaks of illness and have been isolated from various dairy products in the market place (El-Gazzar and Marth, 1992).

Staphylococcus aureus is one of the most common causes of food borne-acquired infections, causing a wide variety of infections, from simple abscesses to fatal sepsis, as well as endocarditis, meningitis and toxinoses including food poisoning and toxic shock syndrome (Onasanya *et al.,* 2003).

Staphylococcal food borne intoxication is one of the most common forms of bacterial food-borne disease in many countries (Balaban and Rasooly, 2000). This type of food-borne intoxication, whose major symptoms are vomiting and diarrhoea, occur within 30minutes - 8 h after ingestion of heat-stable staphylococcal enterotoxins (SEs) preformed in food by enterotoxigenic strains of Coagulase-positive staphylococci (cps), mainly *S. aureus* (Le Loir *et al*., 2003). *Staphylococcus aureus* are Gram -positive, facultative anaerobic cocci. Major virulence factors of *S.aureus* include Staphylococcal enterotoxins (SEs) that cause both food poisoning and toxic shock syndrome (Orwin *et al.,* 2003).*Staphylococcus aureus* enterotoxins are ranked as one of the most prevalent worldwide causes of gastroentereritis (Boerema *et al*., 2006). Dairy foods are frequently contaminated with Staphylococci (Umoh *et al.,* 1990[a], Imani *et al*., 2007). *Staphylococcus aureus* have been reported in

fermented milk products (Fura and Maishanu) (Umoh *et al.,* 1990[b]) and in 'Fura - da Nono', a cereal based ready - to - eat food prepared from sorghum or millet (Umoh, 1990[b]).

The genes responsible for toxins produced by *S.aureus* can be detected rapidly by the polymerase chain reaction (PCR). This is a technique for the *in vitro* amplification of specific segments of DNA; it is a sensitive and specific identification method which targets these *S.aureus* genes (Johnson *et.al.,* 1991; Wilson *et.al.,* 1991; Tsen and Chen, 1992; Becker *et al.,* 1998 and Wang *et.al.,* 2002).

Antimicrobial resistance is the reduced susceptibility of pathogenic bacteria to one or more of the antimicrobial agents administered in clinical medicine (ECDC, 2013). Antimicrobial resistance is on the rise in most countries across the globe. The result is that some serious bacterial infections are becoming difficult to treat and therefore, causing more disease, prolonged and severe disease, which in turn leads to higher healthcare costs and reduced quality of life thereby making antimicrobial resistance to constitute a serious danger to public health (Cohen, 2006).

1.2 Statement of Research Problem

Food-borne diseases remain a threat despite advances in medical technology. Milk is a good substrate for *S. aureus* growth and among the foods implicated in staphylococcal food poisoning (SFP), milk and dairy products play an important role, since enterotoxigenic strains of *S. aureus* have been frequently isolated in them (Normanno *et al.,* 2007; Jorgensen *et al.,* 2005[b]). Raw milk, other milk products such as Nono (locally fermented milk), Kindrimo and Manshanu (fat from milk) are often commonly hawked and consumed

on many Northern Nigeria streets (Umoh *et al*., 1990[b]). These products due to their processing and distribution practices often are exposed to conditions which may permit growth of contaminating organisms and possible toxin producers (Ezeonu and Ezurike, 2007). Currently, there is also limited information regarding the prevalence of classical enterotoxins of *S. aureus* in fresh milk and milk products in Nigeria.

Milk had also been implicated in enteric infections and non–typhoid salmonellosis *(*Umoh *et al*., 1990[a]; Akhtar *et al.,* 2010).It is well known that *Salmonella* is a major food-borne pathogen and that it can cause severe clinical symptoms, such as acute gastroenteritis (Boyle *et al.,* 2007). Chopra *et al.* (1999) in previous studies had identified and characterized the activities of several virulence factors in *Salmonella*, but the molecular mechanisms by which many of them contribute to the development of acute gastroenteritis is still unclear.

Data and statistics are available on food poisoning outbreaks from countries in Europe, North America, South America and Asia. However, there is a lack of information on food poisoning outbreaks in general (*Salmonella* and Staphylococcal food poisoning inclusive) in African countries, Nigeria inclusive. Surveillance and reports of *Salmonella* spp.and Staphylococci in foods are limited, making it difficult to estimate the true situation (Esona *et al.,* 2004).

An accurate, appropriately collated antibiogram represents integral resource for health care providers. However this is lacking when assessing bacterial resistance and guidance in antimicrobial selection and formulary decisions. The use of a well produced and

disseminated antibiogram improves overall patient outcomes for those patients requiring antimicrobial therapy (Eiland *et al.,* 2008).

1.3 Justification

Milk is considered a nutritious food because it contains several important nutrients including proteins and vitamins. Equally, it can be a vehicle for several pathogenic bacteria such as S*taphylococcus aureus* and *Salmonella* spp. (Esona *et al.,* 2004; Jorgensen *et al.,* 2005[a]; Rall *et al*. 2008). Nigeria like other developing countries has its dairy industry facing major problem of low demand for raw milk, partly because of public health concerns over its safety and quality. Traditional dairy products such as Fresh Milk ('Madara'), 'Nono', 'Kindrimo' and 'Manshanu' are still produced in small batches, in unhygienic conditions and also hawked without permission from appropriate authorities. Presently, it is difficult to ascertain the extent to which these indigenous dairy products contribute to food poisoning outbreaks. The extent of risk posed by consumption of raw milk and milk products in the country is not well documented. The physicochemical analysis of these products will help ascertain the products' safety and quality. However, if efforts are made to practice good manufacturing practices (GMP) it would not only protect public health, but also stimulate growth of the dairy industry in Nigeria.

There is a widespread belief that staphylococcal food poisoning (sfp) is very common in Nigeria (Adesiyun, 1984), but there is no accurate record to support this and also limiting are reports of *Salmonella* in food and animals. In Nigeria, there exists limited reports and

documentation of food poisoning outbreaks of *Staphylococcus aureus* and *salmonella spp.*, the degree of contamination and possible enterotoxin and cytotoxin production.

Multidrug resistance by various bacteria against the most commonly prescribed antibiotics poses concern among health care providers and the general populace (Mbuh *et al., 2*008). Consequently also of limited reports are the antibiotic sensitivity patterns of bacterial pathogens associated with these dairy products. Hence, if these needs stated above are met then the objectives of this research would have been achieved.

1.4 Aim of the Study

The aim of this research was to isolate, characterize and determine the antibiogram of S*taphylococcus aureus* and *Salmonella* spp. isolated from fresh milk and milk products consumed in parts of Kaduna State.

1.5 Specific Objectives

The specific objectives of this work were to:

1. determine the chemical composition of fresh milk and milk products sampled.
2. evaluate the bacteriological quality and detect *Staphylococcus aureus* and *Salmonella* spp. in the fresh milk and milk products.
3. determine the antibiogram of the isolates obtained.
4. determine the enterotoxin production by the isolates, phenotypically
5. detect the presence of virulence genes in each of the isolates.
6. detect the presence of *Mec A* gene in enterotoxigenic *Staphylococcus aureus* isolates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Food Borne Pathogens and Diseases

Microbiological quality of food indicates the amount of microbial contaminants it has, a high level of contamination indicates low quality of food storage and its handling more likely to transmit diseases (Oranusi *et al.,* 2013). Safe food is a basic human right despite many foods are frequently contaminated with naturally occurring pathogenic microorganisms (Oranusi *et al.,* 2013). Such pathogens cannot be detected organoleptically (seen, smelt or tasted), but can cause disease of varying severity, including death especially if the way they are conserved during the time prior for sales provides conditions for these microorganisms to grow and reach considerable levels of contamination (Singh *et al*., 2014). Food-borne illness is a major international health problem (Shehabi and Haddadin, 1982; Mensah *et al.,* 2002). Over 200 known diseases are transmitted through eating food contaminated by a variety of agents including bacteria, parasite, viruses, and fungi (Oliver *et al.,* 2005). Each year, millions of persons become ill from food-borne diseases, though many cases are not reported (CDC, 1999).

2.2 Milk and Milk Products

Milk is an aqueous colloidal suspension of proteins, fats, and carbohydrates that contain numerous vitamins and minerals (Ogbolu *et al.,* 2014). Milk and its products form part of the human diet all over the world. The nutritive value of milk is widely documented (Huth *et al.,* 2006). In Northern Nigeria and many other parts of Africa, milk and milk products are traditional staple food commodities for the nomadic population (Okeke *et al*., 2012).

Raw milk and Fermented milk of various types are produced and consumed as supplement to normal meals in homes and even for sale (Maduka *et al.,* 2013). Many farm families consume raw milk simply because it is a traditional practice and less expensive to buying pasteurized retailed milk (Hegarty *et al*., 2002). They are recognized by the elites as natural balanced diet and so are increasingly consumed by the urban populace in the continent (Okeke *et al.,* 2012).Increased consumption of low fat dairy products have been linked with lower incidence of chronic disease, such as cancer, hypertension, osteoporosis (Huth *et al*., 2006) and atherosclerosis (Nettleton *et al.,* 2008). Milk may also protect against oxidative stress (Hunter *et al*., 2012) as it contains antioxidant compounds, like whey and casein proteins, vitamin A, C and E (Zulueta *et al*., 2009).

2.4 Composition of Milk

Milk constitutes majorly of water, fat, protein, carbohydrate (in the form of lactose) and mineral matter or ash. Milk composition varies depending on the type of feeds given to the animals, type of animal and breed (Marth and Steel, 1998). Water constitutes 80 - 87% of milk. Milk fat is present in milk in the form of small globules dispersed in milk plasma. Milk fat is a mixture of different fatty acid esters called triglycerides. Triglycerides (triacylglycerols) account for 97-98% of the total fat in cows' milk. The triglycerides are made of glycerol esterified with fatty acid chains with 4 – 20 carbon atoms (O'Connor and Tripathi, 1995; Marth and Steel, 1998).

There are two distinct types of proteins in milk, casein and whey proteins or globulins. Caseins make up over 80 – 86 % of the total protein content (O'Connor and Tripathi, 1995). They can be further divided into five groups – the alphas1, alphas2, beta, gamma,

and Kappa caseins. Caseins do not have an organized structure, thus they cannot be denatured by heating (Jensen, 1995). Whey is the milk serum proteins. When casein is precipitated out of skim milk, whey is left over. Casein are the curds and the liquid left over is the whey. Whey proteins are globular proteins which may be denatured when heated above temperature above 65^{o}C. The major components of whey are beta lactoglobulin and alpha lactalbumin (Jensen, 1995).

Lactose is the main carbohydrate of milk. It is formed by the union of one molecule of D-galactose (engaged by its semi-acetyl function) and one molecule of D-glucose (committed by its hydroxyl 4 position). It has a β-galactoside 1, 4 bond (which is hydrolyzed by a β-galactosidase) and is a 4-D-glucopyranosyl-β-D-galactopyranose. Although lactose is a sugar, it does not have a sweet flavor. Its concentration varies slightly in milk (4.5 to 5.2 g / 100 g) contrary to the concentration of fat; concentration of lactose cannot be easily modified by feeding (Fillion, 2006). It is used as substrate during the fermentation of milk by lactic acid bacteria. This differs in the fermented products such as yoghurt and cheese (Guetouache *et al.*, 2014). It plays a role in fermented milk production. The amount of lactic acid produced by lactic acid bacteria in a fermented milk product depends not only on the bacterium itself (the bacterial strain more less active) and operating parameters, but also on the available amount of lactic acid bacteria (Fillion, 2006).

2.5 Nigerian Indigenous Milk Products:

In Nigeria, mainly the Fulanis produce milk locally and the excess milk is processed into some indigenous fermented products for preservation (Akinyele *et al.*, 1999). 'Nono',

‘Kindrimo’ and ‘Manshanu’ are some of these fermented dairy products produced in Nigeria.

Nono is a general name used for locally fermented cow milk and it is widely consumed in many African countries and forms a major part of the staple food in Northern Nigeria (Obande and Azua, 2013). It is an opaque white to milky coloured liquid food drink got from fermented raw milk. It is ahealthy 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’ (Obande and Azua, 2013). Nono is the Fulani word for cow’s milk sold by the Fulani women, hence mostly available in the northern part of Nigeria (Yahuza, 2001). They are produced mainly by the nomadic Hausa/ Fulani cattle rearers (Adesokan *et al.,* 2011). The preparation includes pouring fresh milk directly obtained from a cow into a properly washed semi-dried calabash and kept wide open in the sun for approximately two hours to facilitate isolation of the fat layer. Some quantity of overnight fermented milk is added therefore, to serve as a starter culture and the inoculated fresh milk is left overnight at room temperature for fermentation to get sour milk known as “Kindirmo”. This is followed by the addition of large volume of water to the curdled sour milk which when stirred with a T-shaped stick to a liquid of fine consistency, gives rise to “Nono”. The most common product often mixed with nono is called “Fura” (a dumping made of millet or maize) to make a preparation called fura da nono. ‘Nono is rich in protein, essential amino acids, phosphorous and vitamins (Nebedum and Obiakor, 2007).

'Kindrimo' is full fat or partially skimmed milk (Okeke *et al*., 2012). 'Kindrimo' is produced by milking the cow into a container. This is followed by the addition of small quantity of already fermented yoghurt or milk and is covered for 24 hours to ferment (Maduka *et al.*, 2013). 'Manshanu' is fat from milk. Consumers have a strong preference for these traditionally processed milk products due to their satisfying nature and also its high protein content (Obande and Azua, 2013). It could also be due to its affordability in comparison with imported, processed milk products whose prices are higher.

2.6 Microbiological Quality of Milk and Milk Products

Spoilage in milk occurs as the change of flavour, undesirable coagulation of milk proteins, and the increased concentration of free fatty and amino acids. In addition, depending on the type of dairy product, the atypical texture and proportion of certain undesirable organic compounds are occurred (McPhee and Griffihs, 2002; Cempírkova and Mikulova, 2009). The spoilageand reduced quality of milk and milk products can be both the consequence of the presence of live organisms and/or their thermostable enzymes (Koka and Weimer, 2001). Spoilage microorganisms such psychrotophs have significant negative effects on yields, affects the suitability of milk for the production of other milk products, as well as limits the shelf life of milk products (Causin, 1982).

The majority of psychrotrophic bacteria that cause milk and milk products spoilage are not pathogenic. However, certain species of these bacteria have the ability to produce toxins and/or show resistance to antibiotics and therefore must be considered as opportunistic human pathogenic bacteria (Munsch-Alatossava and Alatossava, 2006; Hemalatha and Banu, 2010).

The EU standard for raw milk quality requires that the total count of mesophilic aerobic bacteria is lower than 30,000 cfu mL^{-1}, and the count of psychrotrophic bacteria is lower than 5,000 cfu mL^{-1}. The average standard plate counts (SPC) for can and bulk milk are ~700,000 bacteria/mL and ~100,000 bacteria/mL respectively, depending on temperature and handling conditions. The microbial standards for grade "A" raw milk are 100,000 bacteria/mL, for individual producer milk, 300,000 bacteria/mL, as commingled milk, 75,000 cells/mL as somatic cell count (SCC) (PMO, 2001). In that sense, any increase of psychrotophs in the microbial population of raw milk will negatively influence the product quality to a certain extent and, indirectly, will reduce the incomes.

2.7 Bacterial Contaminants of Milk and Milk Products

The distinctive microbial populations in raw milk at the time of processing has a significant role to play on shelf life, organoleptic quality, spoilage and out comes of the raw milk, processed milk as well as on the other dairy products (Samarzija *et al*.,2012).

Unfortunately, cold and extended storage of raw milk, as a common practice in dairy sector today, encourages the growth of psychrotrophic bacteria (Samarzija *et al*., 2012). Psychrotrophic bacteria are the most commonly isolated organisms which caused the spoilage of the heat treated milk and dairy products as the result of post-pasteurization contamination of the products (Larsen and Jorgensen, 1997; Eneroth *et al.,* 2000; Santana *et al.,* 2004). Psychrotrophic bacteria are ubiquitous in nature, primarily in water and soil, including vegetation. A small number of psychrotrophic bacteria can also be present in the air. Their optimal metabolic activity is expressed at temperatures between 20 and 30 °C. However, they can grow and multiply at low temperatures through an enrichment of

polyunsaturated fatty acid in their membrane lipids. The wide distribution of these bacteria indicates a remarkable degree of their physiological and genetic adaptability (Spier *et al*., 2000).

Psychrotrophic bacteria isolated from cooled milk belong to Gram-negative and Gram-positive genera and are taxonomically classified into seven classes. *Gammaprotobacteria, Bacilli* and *Actinobacteria* are the dominant classes containing between 19 and 21 species, while *Alphaproteobacteria, Betaproteobacteria*, *Flavobacteria* and *Sphingobacteria* are the four less significant classes (Hantsis- Zacharov and Halpern, 2007).

A significant number of bacterial strains isolated from cooled milk belong to the genera: *Bacillus, Stenotrophomonas, Acinetobacter, Pseudomonas* and the species *Burkholderia cepacia* (previously called *Pseudomonas cepacia*) and some of them are considered potentially pathogenic bacteria. Namely, these bacterial species are associated with infections in humans and animals, particularly in cases of immune-repression, and they show pronounced resistance to antibiotics (Foght *et al.,* 1996; Svensson *et al.,* 2005; Munsch-Alatossava and Alatossava, 2005; Beena *et al.,* 2011). The Gram-positive psychrotrophic bacteria isolated from raw milk include following genera: *Bacillus*, *Clostridium, Corynebacterium, Microbacterium, Micrococcus, Arthobacter, Streptococcus, Staphylococus* and *Lactobacillus*. The presence of *B. cereus* in: 52 % of ice cream samples, 29 % powdered milk samples, 17 % of fermented milk samples and 2 % of pasteurized milk samples had been reported by Wong *et al.* (1988a). *Bacillus* spp. are today considered the main microbial causes for the spoilage of milk and milk products, and the main reason for significant economic losses in the dairy industry (Brown, 2000; Samarzija *et al.*,2012).

2.7.1 Effects of psychrotrophic bacteria on milk quality

The negative impacts of psychrotrophic bacteria on the quality of milk and dairy products are unquestionable. It is estimated that modern dairy industry has losses of up to 30 % due to spoilage and reduced product quality caused by psychrotrophic bacteria (Garbutt, 1997; Randolph, 2006). Psychrotrophic bacteria are found everywhere in nature, primarily in water and soil, including vegetation. A small number of psychrotrophic bacteria can also be present in the air. Their optimal metabolic activity is expressed at temperatures between 20 and 30 °C. However, they can grow and multiply at low temperatures through an enrichment of polyunsaturated fatty acid in their membrane lipids (Schinik, 1999).

In line with other quality aspects, such as suitability of milk for the production of dairy products, psychrotophs have a significant negative effects on yields as well as on limiting shelf life of dairy products (Causin, 1982).

Bacillus spp. (with the predominance of the species *B. cereus*) isolated from milk expresses both proteolytic and lipolytic activities, and approximately in 80 % of cases also a phospholipolytic activity (Matta and Punj, 1999). The most commonly isolated species among the bacteria belongingto genus Bacillus, from raw, heat treated milk and dairy products are; *B. stearothermophilus*, *B. licheniformis*, *B. coagulans*, *B. cereus*, *B. subtilis* and *B. Circulans.* These thermoresistant psychrotrophic aerobic or facultative anaerobic bacteria have spores that are activated immediately after the heat treatment of milk by forming vegetative forms. The vegetative cells of *Bacillus* spp. and those of *Pseudomonas* spp. have capacity to form broad spectrum of thermostable extracellular and intracellular hydrolytic enzymes. These enzymes have the ability to break down the major constituents

of milk; milk fat, protein and lecithin (Dogan and Boor, 2003; Chen *et al*., 2003; 2004). It had also been reported that proteolysis caused by psychrotrophic bacteria has a negative effect on the milk products flavour, which has been described as bitter, foreign, unclean, fruity, yeasty or metallic (Ran *et al.,* 1988). Milk fat are also hydrolysed through the activity of the bacterial lipases of psychrotrophic bacteria, releasing free fatty acids causing primarily changes in product flavour that is described as rancid, unclean, soapy or bitter. The lipolytic flavours defects are particularly seen in cream, butter, cheese and sterilised (UHT) milk (Stead, 1986; Champagne *et al.,* 1994). Some bacterial contaminants in the dairy industry such as *B. subtilis*, *B. cereus* (facultative anaerobic bacteria) and *P. fluorescens* (aerobic bacteria) are capable of jointly forming biofilm (Constantin, 2009). These biofilms formed can be a difficult to resolve causing long-lasting source of permanent product contamination with bacteria that cause spoilage and/or can be opportunistic pathogenic (Sillankorva *et al.,* 2008; Simooes *et al.,* 2010).

2.8 Sources of Contamination in Milk and Milk Products

Spoilage bacteria are not part of the natural microbial population of the udder, and therefore their presence in raw milk is exclusively the result of milk contamination after milking (Munsch-Alatossava *et al*., 2005).

The most commonly stated sources of Gram-negative psychrotrophic bacteria are: residual water in milking machines, milk pipelines or coolers, dirty udders and teats, inadequate cleaning of surfaces of dairy equipment for reception, transport and storage of milk, and biofilm (Santana *et al.,* 2004; Simooes *et al.*, 2010). A high correlation with incidence of *Bacillus* spp. in raw milk was confirmed by Lukaseva *et al*. (2001), who explained this as

being due to changes in meals and poor udder hygiene during milking, and not due to a seasonal influence as earlier opined by Sutherland and Murdoch, (1994).Milk when cooled and held at approximately 4 °C prior to processing, causes the changes in microbial population. Under those conditions, the dominant Gram-positive bacteria are replaced by Gram-negative and Gram-positive psychrotrophic bacteria. These bacteria in most of the cases have considerable negative effects on the quality of milk as well as dairy products (Lafarge *et al*., 2004). Among bacterial species that are associated with milk and dairy products, *Pseudomonas* spp. and *Bacillus* spp. are the most common isolated organisms in raw or heat treated milk at the time of spoilage (Mc Phee and Griffiths, 2002). Contamination of the cow's teats with soil can be a potential source of spores of *B. cereus* in raw milk though largely regionally specific (Samarzija *et a*l., 2012). The level of contamination of Bacillus spp. in milk is $\geq 10^6$ cfu mL-1. Filling machines in the dairy industry represent the most common site for product recontamination by psychrotophic bacteria.

2.9 Microbial Analysis of Milk and Milk Products

Pathogenic bacteria have been a major concern to the public all over the world. Various bacteria of public health concern such as *Salmonella* spp. *Listeria monocytogenes, Campylobacter jejuni, Yersinia enterocolitica*, pathogenic strains of *Escherichia coli* and enterotoxigenic strains of *Staphylococcus aureus* may be found in milk and dairy products (Varga, 2007). Because of the specific make-up of milk it is impossible to avoid contamination of milk with micro-organisms therefore the microbial content of milk is a major feature in determining its quality (Karmen and Slavia, 2008). It is presumed that

differences in feeding and housing strategies of cows may influence the microbial quality of milk (Coorevits *et al*., 2008). Bacterial contamination of raw milk can come from different sources: air, milking equipment, feed, soil, faeces and grass (Coorevits *et al*., 2008). The number and types of micro-organisms in milk immediately after milking are affected by factors such as animal and equipment cleanliness, season, feed and animal health (Karmen and Slavia, 2008). It is therefore important and emphatic to microbiologically examine milk and dairy foods. These analyses are however critical for the assessment of quality and safety conformation with standards and specifications and regulatory compliance (Donkor, 2007).

Microbial analysis of milk and milk products includes tests such as total bacterial count, yeasts, moulds and Coliform estimation (Bereda *et al.,* 2014). These parameters are useful to measure its sanitary quality and most grading of milk is on the basis of some method for estimating numbers of Bacteria.

2.10 The Genus *Salmonella*

Salmonella first came into prominence in the 1880's, soon after the isolation of the "hog cholera bacillus "by Salmon and Smith (Steele, 1963). The genus name was given to the group in honour of Dr. D.E Salmon and the first organism of the group isolated was named *Salmonellacholerae-suis*. During the next two decades, Salmonellae were found by numerous investigators in a variety of diseases in animals (Steele, 1963). *Salmonella abortus-equi* was isolated in 1893 from vagina of an aborting mare by Kilbourne whilethe causative organism of "fowl typhoid", *Salmonella gallinarum* was found in 1889 (Steele,

1963). A few years later, the "mouse typhoid bacillus"which was later given the name *Salmonella typhimurium*, was isolated (Steele, 1963).

Salmonella spp. are facultatively anaerobic gram negative rod-shape bacteria belonging to the family Enterobacteriaceae, with diameters 0.7 - 1.5µm and lengths 2-3µm (D'Aoust *et al.*, 2007). The genus comprises more than 2,600 serovars. Although members of this genus are motile by peritrichous flagella, non flagellated variants, such as *Salmonella* serovar *Pullorum* and *Salmonella* serovar *Gallinarum* and non-motile strains resulting from dyfuntional flagella do occur (D'Aoust *et al.*, 2007). The growth and survival properties of each serotype differ. Once the bacteria are in the body, the incubation time is only about 8 - 48h. The bacteria multiply and invade the intestinal mucosa, where they produce enterotoxins and cytotoxins that destroy the epithelial cells. Salmonellae are generally not heat resistant and normally grow at 35^{o}C (EL-Gazzar and Marth; 1992; D'Aoust and Purvis 1998). *Salmonella* can grow at much lower temperatures, provided that the incubation time is suitably extended (EL-Gazzar and Marth, 1992).

Salmonellae catabolize D-glucose and other carbohydrates with the production of acid and gas. Salmonellae are oxidase negative and catalase positive, grow on citrate as a sole carbon source, generally produce hydrogen sulfide,de carboxylate lysine and ornithine,and do not hydrolyze urea.A typical *Salmonella* isolate would produce acid and gas from glucose in triple sugar iron(TSI) agar medium and would not utilize lactose or sucrose in TSI or differential plating media,such as brilliant green,xylose lysine desoxychocolate,and Hekton enteric agars (Andrews *et al.*, 1995; D'Aoust and Purvis, 1998).

2.10.1 Epidemiology of *Salmonella*

A majority of *Salmonella* cases are never linked to outbreaks.According to CDC reports 2008, *Salmonella* isolates from outbreaks comprised only 5.4% of the total *Salmonella* cases identified; the remaining 94.6% of cases were sporadic with no known connection to other cases. *Salmonella typhi* and *Salmonella paratyphi* are rarely encountered outside endemic countries or in returned travellers (Haeusler and Curtis, 2013). Other serovars of *Salmonella* responsible for non-typhoid Salmonellosis are distributed worldwide (Majowicz*et al.*, 2010). These have an estimated 93.8 million episodes and 155,000 deaths each year (Majowicz*et al.*, 2010). Salm*onella Typhimurium* and *Salmonella* ser.Enteritidis account for nearly 80% of all human isolates reported globally (Vieira-Pinto*et al*., 2005). *Salmonella* spp. is estimated to cause about 1.4 million non-typhoidal infections in humans per year in the United States. Non-typhoidal *Salmonella* is one of the most reported causes of food-borne infections (Lynch and Tauxe 2009). It has been estimated that 95% of these cases were due to consumption of contaminated food (Mead *et al*., 1999). Food products of animal origin (contaminated meat, poultry, eggs, and dairy products) are considered the most commonly identified source of *Salmonella* infection (Tansel *et al*., 2003 and Bruun *et al.*, 2009). CDC estimates that the true incidence of *Salmonella* infections in the United state is about 38-fold greater than the case actually reported (CDC, 2008).

The infection is typically not fatal, with about 15,000 cases annually requiring hospitalization and over 400 deaths (Voetsch *et al*., 2004). Onyango *et al*. (2009), reports that about 80% of deaths due to *Salmonella* are found in Africa, while in London *Salmonella* remains the second most commonly reported gastrointestinal infection after *Campylobacter.*

2.10.2 *Salmonella* serovars/serotypes

Serotyping is a useful epidemiological tool in identifying circulating serotype and to characterize out breaks (Odumeru and Leon-Velarde, 2012). The antigenic formula of LeMinor and Popoff (2001) is a standard method for naming the serovars. Serotyping is normally undertaken at reference laboratory and is rarely perfomed in routine food or clinical laboratories.

Reclassification of serovars of *Salmonella*in a new sub-specie group have emerged over the decade using new DNA-based typing methods such as random amplified polymorphic DNA (RAPD) technique, ribotyping, pulsed filled gell electrophoresis (PFGE) and multilocus enzyme electrophoresis (MLEE) (Reeves *et al.,* 1989, Baumler *et al.,* 1998*).* These new analytical methods have provided more information on the relatedness of different strains (Doyle *et al.,* 2009).

The international Kauffmann-White scheme for designating *Salmonella* serotype was adopted by the Centres for Disease control (CDC, 2003) in order to better coordinate and compare information among countries. There are now two species of *Salmonella: Salmonella enteric and Salmonella bongori* (LeMinor and Popoff 1987, Reeves *et al.,* 1989; Boyd *et al.,* 1996; Grimont and Weill, 2007). Members of *S. enteric* spp. are divided into seven sub-specie groups (I, II, IIIa, IIIb, IV, VI and VII). Group I include serovars causing disease in humans and other warm blooded animals such as *S. enteric,* Sv Typhi, Paratyphi, Sendi, Typhimurium, Enteritidis, Cholerasis, Dublin, Gallinarum, Pullorum and Abortusovis (Baumler *et al.,* 1998).

Groups II-VII includes *S. enteric* serovars frequently isolated from cold blooded vertebrate (Baumler *et al.,* 1998). Groups V includes *S. bongori* (Boyd *et al.,* 1996). This numbers assigned to these groups refers to the O and H- antigen on the surfaces of the cell on different serotypes (Grimont and Weill, 2007) according to the Kauffmann and White scheme.

2.10.3 Important *Salmonella* serotypes

The importance of *Salmonella* serotypes is measured by the severity of disease (Wollin, 2007). Serotypes from salmonelosis cases were examined to determine if they were associated with hospitalization, invasive disease or fatalities. Wollin, 2007 reported *S.* Typhimurium, *S. enteritidis*, and *S.* Newport to be the most frequently encountered during a survey of salmonellosis cases in Europe, 1996-2006, while *S.* Javiana and *S.* Heidelberg to had caused the highest rate of invasive diseases and hospitalization in Michigan (1995-2001). *Salmonella Dublin, S. Muenster* and *S. choleraesuis* were reported to have accounted for the highest case fertility rate. *Salmonella* Typhi is the most virulent salmonella and still causes often water borne outbreak in developing countries (Luwis *et al.,* 2005). Such large outbreaks do not occur in more developed countries with chlorinated drinking water, adequate sewage treatment facilities and pasteurized milk (Olsen *et al.,* 2003). *Salmonella enteritidis* is the most frequently reported serotypes from Africa, Asia and Latin America with *S.* Typhimuriumand *S.* Typhi ranking second and third in those regions (Galamis *et al.,* 2006). Reen *et al.,* 2005 have reported *S.* Typhimuriumamong serovars that is commonly associated with human salmonellosis in most European countries and in the United States. Although greater than 2500 serovars of *S. enteric* have

been identified, most human infections are caused by a limited number of serovars (Hendriksen *et al.,* 2011). In most developed countries, S. Enterica serovars Typhimurium and Enteritidis are the most commonly reported causes of human salmonellosis. Other serovars appear to be more prevalence in specific regions such as *Salmonella* servovar Stanley and *Salmonella* serovar Weltevreden in South East Asia (Olsen *et al.,* 2001, Herikstad *et al.,* 2004). Strain types and serovars in human and animal population may follow the introduction of the strain in international travel, human migration, food, animal feed and livestock trade (Crump *et al.,* 2002).

2.10.4 Salmonellosis

Salmonella infections lead to a variety of diseases known as Salmonellosis. *Salmonella* infection can be divided into two major groups of clinical importance: Typhoidal salmonelosis (*Salmonella typhi* and *Salmonella paratyphi*) and non-typhoidal salmonellosis (all *Salmonella* serovars other than *Salmonella typhi* and *Salmonella paratyphi*) (Scallan *et al.,* 2011, CDC 2011). Many non-typhoidal *salmonella* (NTS)/ salmonellosis serovar infections results in diarrhoea diseases, abdominal pain and in some cases, vomiting and fever. Others include; bacteraemia, extra intestinal faecal infection in infants and more serious complication among the elderly and immunocompromised adults (Goldberg and Rabin, 1988; Miller *et al.,* 1995; Gordon, 2008). Salmonellosis also includes several clinical manifestations such as gastro enteritis (food poisoning), enteric fever and carrier state complication (Rubin and Weinstein, 1977; Goldberg and Rubin, 1988; Rumeu *et al.,* 1997).

The infected asymptomatic host carries viable bacteria for long period of time up to several months or years (Buchwald and Blaser, 1984). The carrier state is an important cause of food borne salmonellosis as it leads to spreading the pathogen into farms, food handlers and consumers (Garcia-del, 2000). People become infected after eating food or drinking beverages that had been handled by people who were infected or by drinking water that had been contaminated by sewage containing bacterium (Carol *et al.,* 1989, WHO, 2008) with the exception of typhoid and paratyphoid fever, cases of Salmonellosis generally involve mild to moderate symptoms of gastro enteritis lasting for about 5 days (CDC, 2008). Intestinal salmonellosis typically resolves in 5-7 days and does not require treatment with antibiotics. However, bacteraemia occurs in 3-10% reported culture confirmed cases. This is particularly common among patients at the extreme of age and those who are immunocompromised (Glynn *et al.,* 1998*;* Hohmann, 2001). *Salmonella* may also cause urinary tract infections and some times migrate out of the intestine and cause septicaemia and reatic arthritis (Wilson and Whitehead, 2006; Rohekar *et al.,* 2008 and Ternhag *et al.,* 2008). However, there is a major problem in the treatment of cases of non- typhoidal *Salmonella* (Su *et al.,* 2001; Chiu *et al.,* 2002). Growing antimicrobial resistance may have added to the difficulty in treating patients with such infections and thus may have leadi to increased morbidity and mortality (Mead *et al.,* 1999).

2.10.5 Reservoirs of *Salmonella*

Salmonella naturally live in the intestines of humans and other animals, therefore faecal material is the ultimate source of these bacteria (Foley and lynne, 2008). *Salmonella* are often pathogenic for humans or animals when acquired through faecal oral route (Jawetz *et*

al., 2007). A wide variety of animals, particularly food animals, have been identified as reservoirs for non- typhoidal *Salmonella* (Uzzau *et al*., 2000, Cray *et al.,* 2004 and Pires*et al*., 2009). Most serotypes are not host-specific, but a few species are restricted to one kind of animal such as *S. Pullorum* in chickens and *S. tyhi* (causative agent of typhoid fever) in humans (CDC, 2008). However, not all animal sources are farm animals, many pets (cats and other animals) and wild animals have been reported as vehicles for Salmonellosis (Foley and Lynne, 2008). In recent years a variety of reptilian pets and some mammals have been found to carry Salmonellae and pass them on to their human caretakers (Schroter *et al*., 2004, Mermin *et al.,* 2004). Aquaria housing tropical fish have also been vehicles for human Salmonellosis (Mermin *et al.,* 2004). Salmonellae are present in a variety of wild animals including birds and rodents that may transport these bacteria around and between farms and human environments (Hall and Saito; 2008). Several *Salmonella* serotypes have been isolated from flies and beetles on farms and poultry rearing facilities (Olsen and Hammack, 2000 and Bailey *et al.,* 2001). This is usually via faecal deposits which could have been transported to foods, feed and drinking water for farm animals. However, according to Majowicz *et al.* (2010) around 86% of Salmonellosis is transmitted globally through food.

2.10.6 *Salmonella* in milk and Milk products

The genus *Salmonella* is ever present in the environment and is one of the most common causes of food borne infections worldwide (Beal *et al*., 2004). Unhygienic food continues to be a cause of major concern in humans, leading to about 10% to 15% of acute gastroenteritis (Chen *et al*., 2000). In United Kingdom and European countries there is

increased testing, surveillance and investigation by regulating authorities, food manufacturers and public health laboratories to minimize *Salmonella* food poisoning (Chen *et al*., 2000).

The outbreaks of human Salmonellesis have been associated with *Salmonella* (De Buyer *et al*., 2001). Results shows that *Salmonella* spp. is one of the most etiologic agents responsible for several outbreaks associated with the consumption of raw milk (De Buyer *et al*., 2001). Also several outbreaks of salmonellosis have been traced to dried milk products (EFSA, 2004). These outbreaks of salmonellosis traced to dried milk products have occurred in different countries (Gelosa, 1994; Usera *et al*., 1996; Threlfall *et al*., 1998; Bornemann *et al*., 2002). Previous studies had also reported on prevalence of *Salmonella* in raw milk (Sandgren *et al.,* 2008). Salmonellae cause enteric infection characterized mainly by gastroenteritis on humans and other animals worldwide, and sometimes in severe cases it can result in systemic infection and even death. In Tanzania, Lubote *et al.* (2014) had reported a 28% prevalence of *Salmonella* in raw milk which was higher than 20% obtained in Ethiopia (Tadesse and Dabassa, 2012) and 8.7% in Nigeria (Karshima *et al.,* 2013). However, relatively higher prevalence of 70% had been obtained in India (Pant *et al.,* 2013). Incidence of *Salmonella* in raw milk using different methods and frequency of detection was reported to range 0.17 to 28.6% (Kaushik *et al.,* 2014). In all the presence of *Salmonella* in raw milk indicates faecal contamination due to poor animal husbandry and hygienic practices, inappropriate transportation and storage facilities, lack of cooling systems and use of unsafe water.

2.10.7 Pathogenesis of *Salmonella*

Salmonella a food borne pathogen causes severe clinical manifestations, including acute gastroenteritis and typhoid fever (Boyle *et al*., 2007). Advances had been made in the understanding of the pathogenesis of intestinal infections caused by *Salmonella* spp. (Peterson, 1985). Enterotoxigenicity has been recognized as one of the important pathological attributes of diarrhoea inducing bacteria (Rahmann *et al*., 2000). *Salmonella* serotypes, which are known for their association with gastroenteritis and diarrhoea in man and animals, have also been shown to produce enterotoxin (Rahmann *et al.,* 2000). Although the serovars of *Salmonellaenterica* have been shown to carry *Salmonella* enterotoxin (*Stn*) gene, only a limited number of these strains have been found phenotypically to produce enterotoxin *(Stn*) when cultured by conventional methods (Dinjus *et al*., 1997). *Salmonella* enterotoxin (*Stn*) gene is specially distributed in *Salmonella* spp. irrespective of their serotypes (Dinjus *et al*., 1997, Makino*et al*., 1999, Moore *et al.,* 2007, Lee *et al.,* 2009). This indicates that the *Stn* gene might be useful for the identification or detection of *Salmonella* and *Stn* might be involved in functions unique to *Salmonella* (Chopra *et al.,* 1999). Various authors (Dinjus *et al.,* 1997, Makino *et al*., 1999, Moore *et al.,* 2007, Lee *et al.,* 2009) had proposed that *Stn* is a *Salmonella* virulence factor and is responsible for the enterotoxicity of *Salmonella.* Nakano *et al,* (2012) had opined that biological activities of *Stn*were important to *Salmonella* virulence, especially acute gastroenteritis. However, the contribution of *Stn* to virulence is variable in each *Salmonella* strain and the molecular mechanism of *Stn* has not been elucidated (Nakano *et al.,* 2012). Several enterotoxic factors had been isolated from either *Salmonella* culture or broken cells of *Salmonella*, which could be responsible for the diarrhoea in *Salmonella*

Gastroenteritis (Rumeu *et al.*, 1996). It had been opined by Penigrahi *et al*, (1987) that *Salmonella* enterotoxin may be responsible at least in part, for the loss of fluid and electrolytes from the small intestine during infection with *Salmonella* Typhimurium. Rahman *et al*., (2000) reported that expression of *Stn* gene needed an induction signal from epithelials cells. This shows however that *Salmonella* cells as well as other enteric bacteria recognize host environment signals from intestinal epithelial cells to begin rapid growth, hence contribute to its capacity to initiate pathogenic processes. It is clear that Salmonellae colonize and invade the intestinal mucosa and cause mucosal inflammation, fluid secretion and tissue damage (Penigrahis *et al.*, 1987). *Salmonella* spp. produces heat-labile enterotoxin that alters fluid and electrolyte secretion in the intestine (Peterson *et al.*, 1984). Though it appears that the *Stn* gene is common in the enterobacteria, *Salmonella* spp. heat-labile *stn* have structural relatedness to cholera toxin (CT) (Chopra *et al*, 1987). In addition to the discovering of the enterotoxin, another heat-stable toxic substance produced by *S.enteritidis* and *S.*Typhyimurium had been reported (Koupal and Deibel, 1975; Koo and Peterson, 1982; Reitmeyer *et al.*, 1986). This toxic substance was characterized as an outer bound cytoxin that inhibits protein synthesis in cultured cells as well as in intestinal mucosa (Koo and Peterson 1983, Koo *et al.*, 1984; Reitmeyer *et al*., 1986, Ashkenazi *et al*., 1988). Other cytotoxic products of *salmonella* had been detected in L-forms of this bacterium (Kita *et al.*, 1993). Two genes *Cyx* and *Sly* A, had been reported by Libby *et al*., 1990 and 1994 to express haemolytic activity when cloned in *E.coli*, but not in their original *Salmonella* hosts. However the deduced sequence of *Salmolysin*, the product of *Sly* A gene, showed significant homology with regulatory proteins (Dehoux and Cossart, 1995).

Salmonellae are often pathogenic for humans or animals when acquired by the oral route. They are transmitted from animals and animal products to humans, where they cause enteritis, systemic infection, and enteric fever (Pelczar *et al*., 2001).

More than 2,500 serotypes of *Salmonella* exist, characterized by different combinations of O and H antigens (Pelczar *et al*; 2001). All of which are pathogenic for humans and often for animals (Pelczar *et al*., 2001). However, only some of these serotypes have been frequentlyassociated with food-borne illnesses. *Salmonella* is the second most dominant bacterial cause of food-bornegastroenteritis worldwide (Odumeru and Leon-Velarde, 2012). Often, most people who suffer from *Salmonella* infections have temporary gastroenteritis, which usually does not require treatment. However, when infection becomes invasive, antimicrobial treatment is mandatory. Symptoms generally occur 8 to 72 hours after ingestion of the pathogen and can last 3 to 5 days. Children, the elderly and immunocompromised individuals are the most susceptibleto salmonellosis infections. The annual economic cost due to food-borne *Salmonella* infections in the United States alone is estimated at $2.4 billion, with an estimated 1.4 million cases of salmonellosis and more than 500 deaths annually (Odumeru and Leon-Velarde, 2012).

2.10.8 Detection and isolation of *Salmonella*

Generally, detection methods are based on physiological and biochemical markers of theorganism (Williams, 1981). Cultural methods are based on nutrient acquisition, biochemical characteristics, and metabolic products unique to *Salmonella* spp. (Odumeru and Leon-Velarde, 2012). More rapid immunological and molecular screening methods of detection have been devised to detect cell surface markers and nucleic acids, respectively.

2.10.8.1. Culture methods of identification and isolation

Culture based methods are still the most widely used detection techniques and remain the gold standard for the detection of *Salmonella* due to their selectivity and sensitivity (Odumeru and Leon-Velarde, 2012). Depending on the approach, standard culture methods typically require 5–7 days to obtain a result as they rely on the ability of *Salmonella* to multiply to visible colonies, which can then be characterized by performing additional biochemical and or serological tests. Due to their widespread use, numerous and varied bacteriological media (selective enrichment broths and selective agar plates) are applied to best monitor for *Salmonella* in food and food ingredients. The media may contain inhibitors in order to stop or delay the growth of non-target organisms, or particular substrates that only the target bacteria can degrade, or that confer a particular colour to the growing colonies (Manafi, 2000). Cultural methods typically involve the enrichment of a portion of the food sample to recover sub-lethally injured cells due to heat, cold, acid, or osmotic shock (Sandel *et al.,* 2003; Gracias and McKillip, 2004) in a non-selective pre-enrichment media, such as Buffered Peptone Water (BPW), and to increase the number of target cells as these are generally not uniformly distributed in foods, typically occur in low numbers, and may be present in a mixed microbial population. Next, primary enrichment cultures are then inoculated into secondary selective enrichment broths, such as Selenite Cystine broth (SC), Rappaport Vasiliadis Soy broth (RVS), Tetrathionate Broth (TT), or Muller-Kauffmann Tetrathionate-Novobiocin broth (MKTTn) and incubated at elevated temperatures (37°C or 42°C for 18-24 hours) before being struck onto selective agars such as Xylose Lysine Deoxycholate agar (XLD agar), Bismuth Sulphite agar (BIS), Brilliant Green agar (BG) with or without the addition of sulfadiazine or sulfapyridine (BGS),

modified semisolid Rappaport Vasiliadis (MSRV), Salmonella Shigella Agar, or Hektoen Enteric agar (Odumeru and Leon-Velarde, 2012).

Typical *Salmonella* colonies based on morphology and or indicative biochemical reactions on selective agars are then cultured onto non-selective media prior to confirmatory testing. There are well-established confirmations and identification procedures for *Salmonella*. Preliminary identification is traditionally performed using classical biochemical andSerological tests. Key biochemical tests include the fermentation of glucose, negative ureasereaction, lysine decarboxylase, negative indole test, H_2S production, and fermentation ofdulcitol. Serological confirmation tests typically utilize polyvalent antisera for flagellar (H) and somatic (O) antigens. Isolates with a typical biochemical profile, which agglutinate withboth H and O antisera, are identified as *Salmonella* species. Where results are inconclusive, itmay be necessary to perform additional biochemical tests. Positive isolates are often sent forfurther serotyping to identify the serovar using specific antisera as per the Kauffman-White (KW) typing scheme recognizing 46 O antigens, and 119 H antigens, thereby permitting thecharacterization of 2,541 serotypes (Shipp and Rowe, 1980). Serotyping is a usefulepidemiological tool in identifying circulating serotypes and to characterize outbreaks. The antigenic formulae of Le Minor and Popoff (2001) are a standard method for naming theserovars. Although standard culture methods are excessively time-consuming, there is potential for further improvements, and thus many attempts have been made to maximize their efficiency by introducing new technologies, making reliability of detection more convenient, user friendly, as well as by reducing the costs of materials and labour (Weenk, 1992; De Boer and Beumer, 1999). For example, biochemical confirmatory tests may be easily replaced by commercial

identification kits such as the API 20E (BioMérieux) or other commercially available bacterial identification kits. The detection of sub-lethally damaged cells is of utmost importance as these may still pose a risk to human health and may lead to false negative results. The recovery of injured bacteria are based on overlay methods such as tryptic soy agar (TSA) overlaid on XLD selective agar (Kang and Fung, 2000) and other approaches also include the development of single enrichment broths where multiple step enrichments are usually required.

Apart from cultural methods more rapid and immunological and molecular screening methods of detection have been devised. Immunological based methods include, Rapid agglutination assays, enzyme-linked immunosorbent assays (ELISA) and Lateral flow immunoassays. Molecular methods include; Polymerase chain reaction(PCR),multiplex PCR, Reversed transcriptase PCR(RT-PCR), Nucleic acid hybridization and phage based detection methods (Mullis *et al.*,1986; Chan *et al.*,1988; Hill and Lampel, 1990; Hill and Keaster, 1991, Masters *et al*., 1994; Cheesebrough and Donnelly, 1996; Chamberlain *et al.*,1998; Espy *et al.*, 2006; Hagens and Loessner, 2007).

2.11 The Staphylococci

The name *Staphylococcus* (staphyle, bunch of grapes) was introduced by Ogston (1883) for the groupmicrococci causing inflammation and suppuration. He was the first to differentiate two kinds of pyogenic cocci: one arranged in cocci: one arranged in groups or masses was called"*Staphylococcus*" and another arranged in chainswas namedBillroth's *Streptococcus* (Gotz *et al.*, 2006).

Staphylococci are Gram-positive bacteria, with diameters of 0.5 – 1.5 μm and characterised by individual cocci, which divide in more than one plane to form grape-like clusters. To date, there are 32 species and eight sub-species in the genus *Staphylococcus*, many of which preferentially colonise the human body (Kloos and Bannerman, 1994), however *Staphylococcus aureus* and *Staphylococcus epidermidis* are the two most characterised and studied strains. The staphylococci are non-motile, non-spore forming facultative anaerobes that grow by aerobic respiration or by fermentation. Most species have a relative complex nutritional requirement, however; in general they require an organic source of nitrogen, supplied by 5 to 12 essential amino acids, e.g. arginine, valine, and B vitamins, including thiamine and nicotinamide (Kloos and Schleifer, 1986; Wilkinson, 1997). Members of this genus are catalase-positive and oxidase-negative, distinguishing them from the genus streptococci, which are catalase-negative, and have a different cell wall composition to staphylococci (Wilkinson, 1997). Staphylococci are tolerant to high concentrations of salt (Wilkinson, 1997) and show resistance to heat (Kloos and Lambe, 1991). Pathogenic staphylococci are commonly identified by their ability to produce coagulase, and thus clot blood (Kloos and Musselwhite, 1975). This distinguishes the coagulase positive strains, *S. aureus* (a human pathogen), and *S. intermedius* and *S. hyicus* (two animal pathogens), from the other staphylococcal species such as *S. epidermidis*, that are coagulase-negative (CoNS).

2.11.1 The Taxonomy of Staphylococci

In the 1986 edition of Bergey's manual of systematic Bacteriology, the family *Micrococcaceae* include four genera: *Planococcus*, *Micrococcus*, *Stomatococcus* and

Staphylococcus (Bergan and Kocur, 1986; Kocur, 1986). Subsequent genetic studies (e.g DNA-ribosomal RNA hybridization 16s rRNA sequencing) and chemotaxonomic analyses (e.g, cell-wall composition, cellular fatty acids) documented the diversity of these microorganisms and established that these four genera should not be combined into a single family. However in the new edition of Bergey's manual of systematic Bacteriology, the Staphylococci are in the phylum firmicutes and comprises Genus 1 in Family v ('Staphylococcaceae'), order 1 (Bacillales^{AL}) in class III 'Bacilli'.Family v also includes *Gemella*, *Macrococcus*, and *Salinicoccus* (Garity and Hott, 2000).

2.11.2 *Staphylococcus aureus*

The species of Staphylococci named *aureus*, refers to those that the colonies (often) have a golden colour when grown on solid media, whilst Coagulase negative staphylococci form pale, translucent, white colonies (Howard and Kloos, 1987). This organism is a Gram-positive, coccus-shaped bacterium. *Staphylococcus aureus* is commonly carried asymptomatically on human skin, typically around the nose and nails. Occasionally the organism enters the bloodstream to cause invasive disease (Fan *et al.*, 2009). It is also a significant cause of a wide range of infectious diseases in humans. *Staphylococcus aureus* often causes life-threatening deep seated infections like bacteraemia, endocarditis and pneumonia (Kanafani and Fowler, 2006). Food poisoning occurs when food containing preformed enterotoxins made by the organism is ingested. The disease is characterized by symptoms including nausea, vomiting, abdominal cramps, and diarrhoea lasting from 24 to 48 h and the complete recovery usually occurs within a few days (Pinto *et al.*, 2005).

Staphylococcus aureus can grow between 7 ^{o}C and 45 ^{o}C. Its optimal temperature range is around 35-39 ^{o}C. Generally the organism grows best at a neutral pH, however it can grow between ranges of pH 4-9. It can grow in both aerobic and anaerobic conditions (better in the presence of oxygen) and can survive in highly desiccated environments.

There are up to 21 different enterotoxins that this organism can produce. These may all be able to cause a wide range of illnesses in humans. Food poisoning is attributed to around 11 of these toxins; SES SEA to SEE and only SEH, SEG and SEI (McLauchlin *et al.*, 2000, Omoe *et al.*, 2002 and Bukowski *et al.*, 2010) have been proven to induce gastroenteric syndrome. However the most prevalent toxin associated with food poisoning is SEA (Klotz *et al.*, 2003). *Staphylococcus aureus* is readily killed by cooking; however its toxins are very heat resistant for example the Decimal reduction time of enterotoxin B at 149^{o}C 30 is 100 min (NZFSA, 2001). This is extremely problematic, as cooking times may not extend this long, suggesting that viable toxin may survive and cause food poisoning. The toxins are also highly stable and resist most proteolytic enzymes, such as pepsin or trypsin, and thus keep their activity in the digestive tract after ingestion (Loir *et al.*, 2003).

2.11.3 Reservoirs of *Staphylococcus aureus*

Staphylococcus aureus belongs to the normal flora found on the skin and mucous membranes of mammals and birds. This bacterium can be disseminated in the environment of its hosts and survives for long periods in these areas (Hennekinne *et al*., 2011). Several biotypes isolated from different hosts (human, poultry, cattle and sheep/goat) have been described within *S. aureus* species demonstrating the close adaptation of the bacterium to

its host. They were identified according to four biochemical tests (Staphylokinase, ß-haemolysin production, coagulation of bovine plasma and growth type on crystal violet agar) following the simplified biotyping scheme described by Devriese, 1984. *Staphylococcus aureus* biotyping has traced or estimated the origin of this organism in various food products and in food-poisoning out breaks (Hennekinne *et al*., 2003; Kerouanton *et al.,* 2007). Pulsed-field electrophoresis(PFGE) and Stapylococcal protein A gene (Spa) typing have been used singularly or in association to trace *S.aureus* strains from small and large ruminants while studying food poisoning outbreaks by various authors (Shimizu *et al.*,2000; Wei and Chiou, 2002; Kerouanton *et al*., 2007; Ostyn *et al.*, 2010).

2.11.4 Staphylococcal food poisoning/intoxication

Staphylococcal food poisoning (SFP) is a common cause of food borne illness worldwide (Asao *et al.*, 2003, Pillsbury *et al.*, 2013). Any food that provides a convenient medium for the growth of coagulase positive Staphylococcus may be involved in Staphylococcal food poisoning outbreaks (SFPO) (Hennekinne *et al.,* 2011). Foods frequently involved differ widely from one country to another, probably due to differing food habits (Le Loir *et al*., 2003). Staphylococcal food poisoning occurs following ingestion of staphylococcal enterotoxins which are heat resistant. These heat –stable enterotoxins are produced in food following contamination by staphylococci (Pillsbury *et al*., 2013). Staphylococcal enterotoxins (SEs) are produced by enterotoxigenic strains of coagulase-positive staphylococci (CPS), mainly *Staphylococcus aureus* (Jablonski and Bohach, 1997) and very occasionally by other staphylococci species such as Staphylococcus intermedius (Genigeorgis, 1989; Khambaty *et al*., 1994). Foods including sliced meat, meat products,

salads, pastries, custards, and raw milk and cheese products present a particular contamination risk (Heymam, 2008). The prerequisite of Staphylococcal Food Poisoning (SFP) is that food or one of its ingredients is contaminated with an enterotoxigenic strain of *Staphylococcus* spp. Moreover, to induce SFP, conditions for staphylococci growth and enterotoxin production are needed. Such large population of staphylococci to meet these conditions is indicative of unhygienic food handling procedures and temperature abuse over a period of time to allow for bacterial growth (Hennekinne *et al.,* 2012). Sources of *S.aureus* include the food handlers either through skin infection on uncovered hands or arms, or via coughing or sneezing over food that is not subjected to further cooking (Pillsbury *et al*., 2013). This is usually followed by growth of the organism and subsequent toxin production. For the bacteria to grow to sufficient numbers, the contaminated food must be left in temperature conditions where the bacteria are able to proliferate. In summary, five conditions are required to induce SFP. These conditions include; (1) a source containing enterotoxin-producing staphylococci: raw materials, healthy or infected carrier, (2) transfer of staphylococci from source to food, e.g., unclean food preparation tools because of poor hygiene practices, (3) food composition with favorable physicochemical characteristics for *S. aureus* growth and toxinogenesis, (4) favorable temperature and sufficient time for bacterial growth and toxin production, and (5) ingestion of food containing sufficient amounts of toxin to provoke symptoms (Asao *et al*., 2003).

Staphylococcus aureus produces pre-formed toxins that have an emetic and diarrheal effect (Hennekinne *et al*., 2012). However, Staphylococcal Food Poisoning is a short-term disease and usually results in full recovery, doctors do not take it very seriously, especially

when the outbreak affects only a few people. However, such outbreaks should be reported to appropriate authorities (De Buyser *et al.,* 2001).

2.11.5 *Staphylococcus aureus* in milk and milk products

Milk and milk products are common vehicles of staphylococcal food poisoning (De Buyser *et al.*, 2001; Jorgensen *et al*., 2005[a]). *Staphylococcus aureus due* to its good growth in milk with respect to the conditions of intrinsic and extrinsic factors, presents a potential or even actual threat to human health. Not only the production of the surface factors, degradative enzymes and toxins, but also the production of heat-stable enterotoxins (SEA-SEV) makes it such a dangerous microorganism (Ote *et al*., 2011). The presence of food born pathogens is due to direct contact with contaminated sources in the dairy environment, and the excretion in the udder of the infected animal (Oliver *et al*., 2005). *Staphylococcus aureus* is recognized worldwide as a pathogen causing intramamary infections in dairy cows; hence it is of economic significance to dairy farms as it reduces milk quality and production (Karahan *et al*., 2009). The presence of enterotoxigenic *S. aureus* in foods generally represents a potential danger for consumers if they had a chance to multiply up to 10^6cfu/ml or cfu/g (Medvedova *et al.,* 2014). The bovine mammary gland can be a significant reservoir of enterotoxigenic strains of *Staphylococcus aureus* (Tondo *et al.,* 2000). The skin and mucosa membranes of milking animals are also important reservoirs, especially if mastitis occurs in the herd. In the case of an infected udder, *S. aureus* can contaminate milk during milking in a density ranging from 1–8 log CFU/ml, mostly about 4 log CFU/ml (Scherrer *et al*. 2004; Boynukara *et al.*, 2008).

Several studies had also suggested that the transfer of *S. aureus* between humans and cows is also possible (Roberson *et al*., 1994; Zadoks *et al*., 2002; Vyletelova *et al.,* 2011). The organism finds its way into milk through hands (infected wounds, skin lesions) or by coughing and sneezing (Asperger and Zangerl, 2003). The lack of proper hygienic measures during milk processing would also increase the counts of *S. aureus*. Therefore *S. aureus* can also contaminate heat-treated milk and can be subsequently present in cheeses prepared from both raw and pasteurised milk (Medvedova *et al.*, 2014).

2.11.6 Toxin Production in *Staphylococcus aureus*

Staphylococcus aureus produces a wide variety of toxic proteins, including the staphylococcal enterotoxins (SEs) A-E and G-J, the toxic shock syndrome toxin-1 (TSST-1), and the exfoliative toxins (ETs) A and B (Lina *et al*., 1997). These toxins were initially described as being responsible for specific acute staphylococcal toxemia syndromes, such as toxic shock syndrome (TSS) and staphylococcal scarlet fever (SSF) (both due to TSST-1, SEB, and SEC), scalded skin syndrome (SSS, due to the ETs), and staphylococcal food poisoning (due to the SEs) (Arbuthnott *et al.*,1990, Lina *et al.*, 1997).

Staphylococcus aureus produces up to 15 different enterotoxins. Food poisoning is attributed to the around eight of these toxins; SEs SEA to SEE and only SEH, SEG and SEI (McLauchlin, *et al.,* 2000, and Omoe, *et al.,* 2002), which have been proven to induce gastroenteric syndrome. However the most prevalent toxin associated with food poisoning is SEA (Klotz, *et al.*, 2003).

The majority of the toxins produced by this organism have similar three dimensional structures; however some differ slightly in that they have small additional loops. The most notable of these is a cysteine loop structure present in many of the Staphylococcal enterotoxins (SE). This loop is thought to be important for emetic activity in SEs. This is because some of the SE's identified without these loops have a severely lower emetic activity (Orwin, *et al.,* 2003).

The structure of the SE is also very similar to those that cause Toxic Shock Syndrome, which is a very serious condition caused by *Staphylococcus aureus.* The enterotoxins are produced when the food contaminated with *Staphylococcus aureus* is not stored at low or high enough temperatures allowing it to proliferate. Toxins produced are heat stable. They can survive a reheating process. They are also highly stable and resist most proteolytic enzymes, such as pepsin or trypsin, and thus keep their activity in the digestive tract after ingestion (Loir, *et al.,* 2003). The infective dose required to induce staphylococcal food poisoning in humans is estimated to be around 0.1 μg and it may vary with patient sensitivity (Evenson, *et al*., 1988).

Various methods had been developed for detecting the production of staphylococcal enterotoxins (SEs) from *S. aureus.* These methods include immunoassay, e.g., enzyme-linked immunosorbent assay (ELISA), immunodiffusion, radioimmunoassay, and latex agglutination. However, since there is DNA sequence information available for all described SEs, Polymerase chain reaction (PCR) offers alternative method for detection of these genes (Gilligan *et al*., 2000; Mclauchlin *et al*., 2000 and Omoe *et al.,* 2002).

2.11.7 Virulence in *Staphylococcus aureus*

Several virulence factors have been implicated in the pathogenesis of *S. aureus* strains and are described in literature (Kerouanton *et al*., 2007; Normanno *et al.,* 2007; Vancraeynest *et al*., 2007). These include; thermonuclease, hyaluronidase, lipases and hemolysins (Sandel andMcKillip, 2004; Kuroda *et al.*, 2007), which are involved in tissue invasion of the host cells. Perhaps the most notable virulence factors associated with this microorganism are the heat-stable enterotoxins (SEs), that cause the sporadic food-poisoning syndrome or food borne outbreaks, and the toxic shock syndrome toxin 1 (TSST-1), which diminishes the immune response of a colonized host (Martin *et al*., 2003; Sandel and McKillip, 2004; Kerouanton *et al.,* 2007; Vancraeynest *et al*., 2007). It has also been opined by Jochman *et al.* (2013), that Virulence factors of *S. aureus* may modulate the host immune response and affect the clinical course of infection. On the other hand, most of the severe *S. aureus* infections are due to the cumulative effects of several virulence determinants (Nejma *et al*., 2006).

Virulence factors in bacteria may be divided in three functional categories: Factors that mediate adhesion of bacteria to host cells; those that produce tissue damage; and those that protect the bacteria against the host's immune extracellular matrix, such as fibrinogen, fibronectin and collagen allow the bacteria to colonize damaged tissues (Visai *et al*., 2000). Clumping factor A (Clfa) is considered one of most important adhesion factors and has been identified as a virulence factor in an endocarditis model (McDevitt *et al*., 1995). *Staphylococcus aureus* produces a variety of exoproteins that contribute to its ability to colonize mammary gland (Salasia *et al.,* 2004), with five of them being different

membrane-damaging toxins, four hemolysins (alpha-, beta-, gamma-, and delta-hemolysin) and leucocidin. Beta and alpha hemolysins are the most important in pathogenesis of the intramamalian infections (Park *et al.*, 2004).

Adhesion to epithelial cells is the initial step in the process of infection by *S. aureus* (Cifrain *et al*., 1994), binding to various components of the hemolysins that contribute to their ability to colonize and cause disease in mammalian hosts. Hemolysin is recognized as potential virulence factor of *S.aureus.* Hemolysin toxins that act on cell membranes include alpha-toxin, beta-toxin, delta-toxin, among others (Wang *et al*., 2011). Alpha hemolysin is the most studied of the *S. aureus* cytotoxins (Dinges *et al.,* 2000). Beta toxin is a sphingomyelinase expressed by most strains isolated from bovine intra-mammary infections, but rarely by human isolates (Cifrain *et al*., 1996). The beta-toxin is an Mg2+-dependent sphingomyelinase C, which degrades sphingomyelin in the outer phospholipid layer of the membrane (Linehan *et al.,* 2003). It has been reported that both toxins increase *S. aureus* adherence to mammary epithelial cells (Cifrain *et al.,* 1996, Caiazza and O'Toole 2003). The capsule has been shown to promote *S. aureus* virulence in several animal infection models (Sordelli *et al.,* 2000). Capsulated *S. aureus* strains are more resistant to phagocytosis than non-capsulated strains, and allow the bacteria to remain in the infected hosts (Thakker *et al.,* 1998).

2.11.8 Detection and isolation of *Staphylococcus aureus* from foods

Staphylococcus aureus has been confirmed to be the causative agent of many cases of severe food poisoning; therefore, the presence of *S. aureus* or its enterotoxins in foods is of major concern and usually indicates poor sanitation. The ideal identification of *S. aureus*

clinical isolates requires a battery of tests and this is costly in resource limited settings. Though there is no single test that can guarantee reliable results for the identification of *S. aureus*, Kateete *et al*. (2010) had proposed tube DNase and Coagulase as better option in resource limited setting such as in developing countries. Phenotypic tests are the core methods in the diagnosis of staphylococcal infections in developing countries. In these tests, coagulase tests are usually confirmatory for *S. aureus (*Bello and Qahtani, 2006; Mugalu *et al*., 2006). Coagulase testing is performed using the slide coagulase Test (SCT) or the tube coagulase Test (TCT) methods (Jernigan *et al*., 1996). Cultural methods for the isolation of *S. aureus* include the use of special media such as Manitol salt agar. Mannitol salt agar (MSA) has been in use since 1945 as a selective medium for the isolation of pathogenic staphylococci. The media was found to be valuable for use with specific specimens such as sputum from patients with cystic fibrosis (Sparham, 1978). Mannitol salt agar (MSA) was first investigated as a medium for susceptibility testing in 1985 (Lally *et al*., 1985). Its value as a screening medium for MRSA had been explored in several studies (La Zonby and Starzyk, 1986; Melino *et al.*, 1996). It has been further assessed as a primary isolation medium for recovery of MRSA when containing 6 mg of oxacillin per ml (Van Enk and Thompson, 1992). Manitol salt agar is regarded as an important medium for the isolation of *S. aureus* from water, milk, the skin, respiratory tract secretions, and the nose (Webster, 1992; Sabbour and El Zanfy, 1997), although inhibitory concentrations of beta-lactam compounds had been reported to result in failure of *S. aureus* growth on MSA (Marchese *et al.*, 1995). The salt concentration in the medium is known to be of great importance for *S. aureus* (Kampf *et al.*, 1998). Molecular assays includes Polymerase

chain reaction (PCR). Polymerase chain reaction (PCR)-amplification of the *nuc* gene is performed as a gold standard during the isolation of *S. aureus* (Kateete *et al*., 2010).

2.12 Antibiotics Resistance in Bacteria

The valuable life spans of most antibiotics used in treating bacterial infections have been diminished by the development of resistant strains. It appears that the emergence of antimicrobial resistant bacteria is inevitable to most new drug and it is recognized as a major problem in the treatment of microbial infections in hospitals and in the community (Yoneyama and Katsumata, 2006). Antibiotic resistance has remained the most common problem of diseases in Africa (Okeke *et al.,* 2005). The global problem of antimicrobial resistance is particularly pressing in developing countries, where the infectious diseaseburden is high.Here cost constraints prevent the widespread application of newer, more expensiveagents (Okeke *et al.,* 2005). Studies from different parts of Nigeria have observed temporal trends in the prevalence ofresistance among enteric organisms and have shown increasing prevalence in the previous years (Okeke *et al.,* 2000). Growing antimicrobial resistance may add to the difficulty in treating patients with such infections, thus leading to increased morbidity and mortality (Mead *et al.,* 1999).

Antimicrobial resistance is the reduced susceptibility of pathogenic bacteria to one or more of the antimicrobial agents administered in clinical medicine (Cohen, 2006). Antibiotics are traditionally defined as natural compounds, produced by microorganisms, with selective antibacterial activity that does not have any strong effects on human cells. Their mechanism of action is either through killing the bacteria (bactericidal effect) or by inhibiting bacterial growth (bacteriostatic effect) (Davison *et al.,* 2000). Although

microorganisms use a combination of mechanisms in developing resistance however it is possible to identify a dominant mechanism (Lambert, 2002; Schweizer, 2003).

Resistance can be acquired as a result of genetic events causingalterations in the pre-existing bacterial genome such aspoint mutations and gene amplifications. The other major mechanism is horizontal gene transfer between bacteria bothwithin and between species, where transposons, integrons orplasmids are introduced into an organism. The successive introduction of new antibiotics has catalysed the accumulation of resistance mechanisms that travel between microbes, creating clones with multiresistant properties (Livermore, 2003; Livermore, 2004).

Bacterial resistance can be defined either genotypically – thebacteria carry certain resistance elements; phenotypically –the bacteria can survive and grow above a certain level ofantibiotic in the laboratory; or clinically – the bacteria areable to multiply in humans in the presence of drug concentrations achievable during therapy (Livermore, 2003; Livermore, 2004). The relationship between antibiotic use and resistance is complex; a major driving factor for antibiotic resistance is antibiotic use/abuse both within medicine and veterinary medicine (Yah *et al.,* 2006*;* Yah *et al*., 2008).

Multiple antibiotic resistance (MAR) in bacteria is most commonly associated with the presence of plasmids which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype (Daini *et al.,* 2005). In many areas of the world, multidrug resistance with more than 50% rates of resistance to the commonly used antimicrobial agents had been reported (Chiu *et al.,* 2002). Multiple drug resistance (MDR) in bacteria

causes mortality and morbidity, complicates treatment and increases health - care outlay (Alagumaruthanayagam *et al*., 2009).

2.12.1 *Mec A* Gene

Staphylococcus aureus is considered as one of the most common bacteria in the clinical practice which has shown an increasing incidence of antibiotic resistance over the past decade (Lindsley *et al*., 2008; Mohanasoundaram and Lalitha 2008). The *mec-A* gene, is a genetic element found in meticillin-resistant *Staphylococcus aureus* (MRSA) strains. It encodes an additional penicillin binding protein PBP2a, which has a low affinity to all ß-lactam antibiotics and is responsible for the meticillin resistance of MRSA strains (Lindsley *et al.,* 2008; Khosravi *et al.,* 2012). The first case of meticillin-resistant *Staphylococcus aureus* (MRSA) was reported in the early 1960, which has become an increasing problem in health care centers and communities (Akpaka *et al*., 2007). It is assumed that methicillin-resistance encoding gene has evolved in coagulase-negative staphylococci (CNS) (Archer *et al*., 1994; Barbier *et al*., 2010).

Methicillin-resistant *S. aureus* are divided into three groups according to their epidemiological and genetic characteristics: hospital acquired (HA-MRSA), community-associated (CA-MRSA), and livestock-associated (LA-MRSA). The groups differ in sensitivity to antibiotics, in the location and size of the chromosomal cassette (SCC*mec*), and in the presence of Panton Valentine leukocidin gene (PVL) (Huber *et al*. 2010). Kwon *et al.* (2005) in their work had demonstrated that MRSA isolated from bovine milk in Korea were not genetically correlated with other CA-MRSA of human origin.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study area is some parts of Kaduna state. Kaduna State occupies an area of approximately 48,483.2 km^2 in the Northern guinea Savannah zone. It has an annual rainfall of 1000 to1250mm and temperature ranges of 17^0C - 33^0C. According to Ahmadu Bello University, Zaria master plan, (2000) the state lies between longitude 30^0E and latitude $11^0$30N. There are two marked seasons in the state; dry windy season (Harmattan) and the wet rainy season.

The state has a predominant Hausa/Fulani population in the Kaduna North and Kaduna central senatorial district. The main livelihood of its people is agriculture which includes cash crops, food crops and animal husbandry (Ahmadu Bello University Master Plan, 2000).

3.2 Study Design

3.2.1 Sampling Method

The sampling method employed was convenience sampling (non-probability sampling). Sampling units were selected based on the availability or easy access of fresh milk and milk products.

3.2.2 Sampling Locations

The study area was made up of four (4) local government areas; Giwa, Kaduna North, Soba and Chikun local government areas. These constitute the sampling frame. They are shown in Figure 3.1.

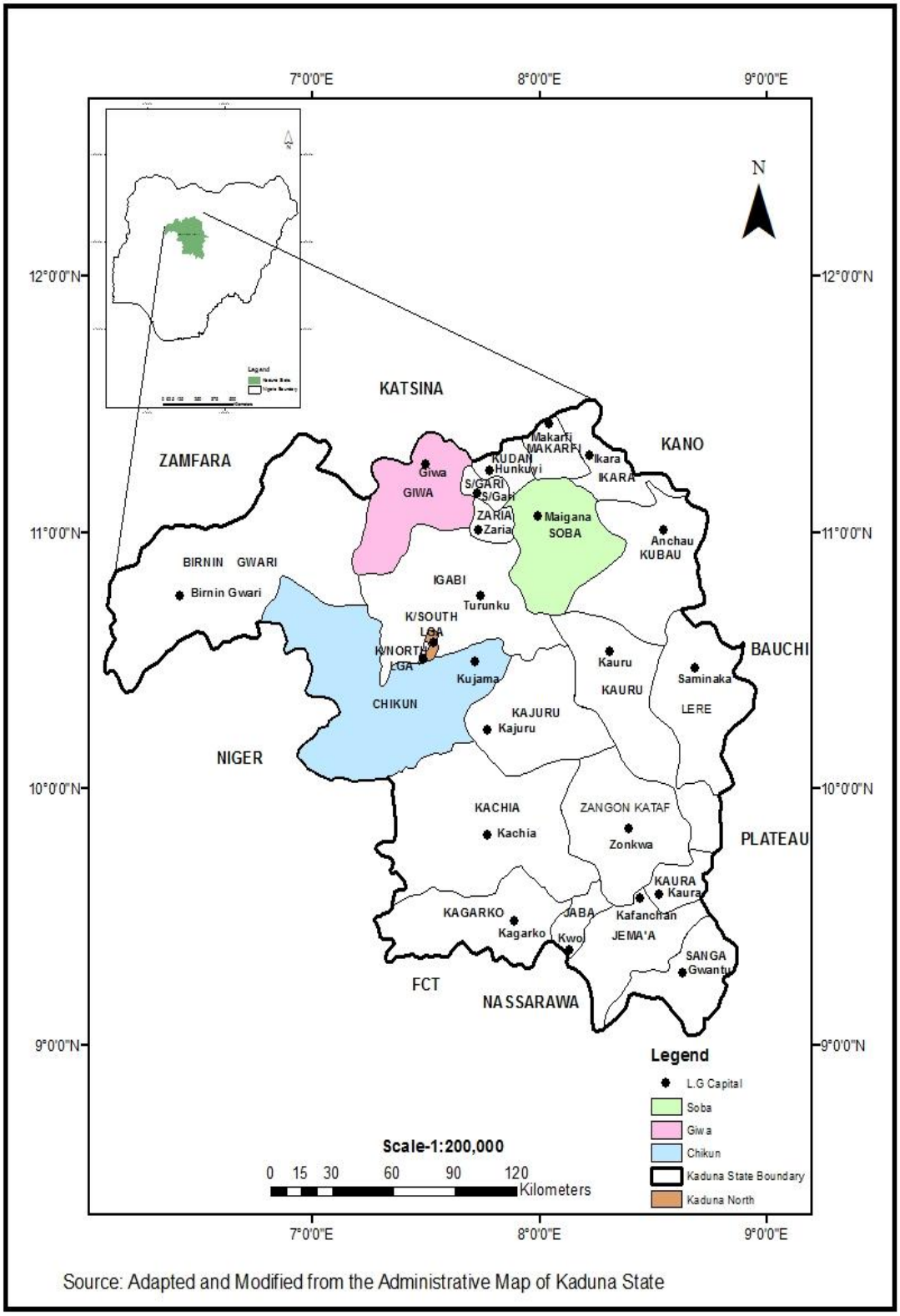
Target sampling points included; Farm Steads or Fulani settlements for Fresh milk, while other samples (Manshanu, Nono and Kindrimo) were obtained from motor parks and main markets in the various locations. The motor parks included, Kawo Motor Park in Kaduna North, Soba Motor Park in Soba local government area. Farm steads/Fulani settlements wereUngwan Fulani Jabawa (Giwa), Ungwan Rimi settlements (Kaduna North), Ungwan Fulani Chikun and Soba, while the markets included; Giwa market (Tuesday) and Ungwan Rimi market, Kaduna.

3.3 Sample Collection

A total of 320 Fresh milk and milk products samples, comprising of 80 samples each of Fresh milk, Nono, Manshanu and Kindrimo were collected.

The samples were placed in sterile containers in ice-packed coolers and taken to the Laboratory, Department of Microbiology, Ahmadu Bello University Zaria, for evaluation.

A.



Source: Adapted and Modified from the Administrative Map of Kaduna State

Figure 3.1: Map of Kaduna state showing sampling sites.

3.4 Chemical Analyses of Samples

The fresh milk and milk products were analyzed for pH, total titratable acidity; moisture, ash, total solids, crude protein and lipid content using standard methods.

3.4.1 Determination of Chemical Composition of samples

3.4.1.1 pH

The pH was determined by the method described by Akpakpunam and Safa- Dedeh, (1995), using pH meter, Cybersan, model 500. The pH meter was standardized by dipping it into sterile water and in buffers of known pH (4 and 7). Ten (10) ml of the respective samples were dissolved in 100ml of distilled water. Themixture was allowed to equilibrate for 3minutes at room temperature. The pH was then determined by inserting the electrode of the pH meter in the sample. The result displayed on the pH meter was recorded.

3.4.1.2 Total titratable acidity (As lactic acid)

Total titratable acidity (TTA) for fresh milk, Nono and Kindrimo were determined by the method according to AOAC, (2013). Acidity for Manshanu was determined by the method described by Abdulla *et al.* (2015). Ten (10) g of the sample was melted; the fat was dissolved in a well mixed neutral solvent consisting of 25 ml diethyl ether and 25 ml 95% ethanol. It was then titrated with aqueous 0.1 N NaOH using 1 ml of phenolphthalein indicator solution with continuous shaking until a pink colour that persisted for at least 15

Sec was obtained for complete neutralization. The test was done in triplicate. The titratable acidity expressed as % lactic acid and calculated using the following formula:

$$\% \text{ Titratable Acidity (Lactic Acid)} = \frac{\text{Titre x chemical factor x 100}}{\text{Weight/Volume of sample}}$$

$$= \frac{\text{Titrex 0.009 x100}}{\text{Weight/Volume}}$$

Where: Chemical factor = 0.009

3.4.2 Proximate Analyses of Samples

3.4.2.1 Moisture content

The moisture content was determined by oven method as described by AOAC (2013). In this process, 3ml of the sample was dried in a hot air oven at 100^{o}C for 24 hours. The loss in weight was determined and recorded as the moisture content and expressed as;

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

Where;

W_1 = Initial weight of the sample

W_2 = Weight of the dried sample.

3.4.2.2 Ash content

Ash content was determined by the direct heating method as contained in AOAC (2013). In this method, 3ml each of the samples was measured into a crucible of known weight. The sample was first ignited and transferred into a muffle furnace, set at 550^0C. The sample was left in the furnace for 8 h to ensure proper ashing. It was then cooled in a desiccator and weight of the ash determined. The % ash content was calculated as;

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

Where;

W_1 = Initial weight of the sample

W_2 = Weight of the dried sample.

3.4.2.3 Total solids

Total Solids was determined as described by AOAC (2013). Three (3) ml of the sample was weighed into a dry Petri dish of a known weight. The total portion was pre-dried for 25 minutes on water bath and then dried for 3 h at 100^oC in a hot air oven. The Total Solid of sample is the weight of the dried sample residue and calculated as:

$$\% \text{ Total Solid} = \frac{W_2 - W_1 \times 100}{W_1 - W}$$

Where: W = Weight of the dish

W_1 = Weight of dish and sample test portion
W_2 = Weight of dish and dry sample.

3.4.2.4 Crude Protein Content

Macro kjeldahl method as described by Onyeike and Osuji (2003) was used to determine the crude protein content. Exactly 3.0ml of defatted sample, in an ash less filter paper was dropped into 300ml Kjeldahl flask. Twenty five (25) ml of H_2SO_4 and 3g of digesting catalyst was also dropped into the Kjeldahl flask. The flask was then transferred to the Kjedahl digestion apparatus. The sample was digested until a clear green colour was obtained. The digest was cooled and diluted to 100ml with distilled water.

For distillation, 200ml of the diluted digest was measured into a 500ml kjeldahl flask containing antibumping chips and 40% NaOH was slowly added by the side of the flask. A 250ml conical flask containing a mixture of 50ml of 20% Boric acid with indicator was used to trap the ammonia liberated. Distillation was then done by placing the conical flask and Kjeldahl flask on the distillation apparatus. These were heated and the distillate (NH_3) was collected into boric acid solution. On complete distillation, the distillate was then titrated with 0.1M HCl. A blank was run through along with the sample. The % crude protein was calculated as follows:

$$\% N = \frac{14 \times M \times V_t \times T_v - T_b \times 100}{\text{Weight of sample (ml)} \times V_a}$$

Where sample weight = (3g)

M = Actual molarity of Acid (0.1)

T_v= Titre Volume of HCl used for sample.

T_b = Total Volume of acid used to titrate the blank.

V_t = Total volume of diluted digest

V_a= Aliquot volume distilled

% Crude protein = % Nitrogen × 6.38 (specific factor for milk)

3.4.2.5 Lipid content

The soxhlet solvent extraction method described by AOAC (2013) was used to determine the lipidcontent. In this method 3ml of the sample was weighed into a round bottom flask of known weight, containing few anti-bumping chips. Three hundred (300) ml of petroleum ether (40 – 60^0C) was poured into the flask and fitted with soxhlet extraction unit for extraction. The unit was set to heat and the heating rate was adjusted until the solvent was refluxing at a steady rate. The extraction was carried out for 6 hours. The solvent was recovered and the oil was dried in the oven at 70^0C one hour. The round bottom flask containing the oil was cooled in the desiccator and weighed.

The flask was reweighed and the percentage lipid was calculated as;

$$\% \text{ Lipid} = \frac{\text{Weight of extracted lipid}}{\text{Weight of sample used.}} \times 100$$

3.4.2.6 Lactose Content

Lactose content was determined by subtracting the lipid, protein and total ash percentages from the percentage of the total solids (O'Mahoney, 1988):

% Lactose = % Total Solid – % (lipid + protein + ash)

3.5 Bacteriological Analyses

3.5.1 Aerobic mesophilic plate count

Aerobic Mesophilic plate count was carried out as described by Umoh *et al*, (1990[b]). Into 90 mls sterile 0.1% buffered peptone water, 10 mls of fresh milk, Nono and Kindrimo were placed and mixed thoroughly. Appropriate 10-fold dilutions of fresh milk, Nono and Kindrimo were pour-plated in duplicate into Nutrient agar plates for determination of Aerobic Mesophilic colony forming units. Dilutions for Nono and Kindrimo were 10^{-5} and 10^{-6} respectively while those of fresh milk were10^{-2} and 10^{-3}. Manshanu was melted in a water bath set at 45^0C and 1ml from 10^{-2} and 10^{-3}were also pour-plated with nutrient agar. The nutrient agar plates were incubated at 37^0C for 24h. Plates having 30-300 colonies were counted using a colony counter and counts recorded.

3.5.2 Isolation of *Staphylococcus aureus*

This was done as described by Imanifooladi*et al.* (2010).Each fresh milk and milk product sample was diluted in the ratio 1/100 in normal saline. From each solution produced, 1ml was transferred to 9mls cooked meat media culture with 9% NaCl and incubated at 37^0C for 48 h. In the second phase, 0.1ml from each previously cultured medium is then transferred to Baird-Parker agar (BPA) and incubated for 24 h. Black colonies with transparent zone on Baird-Parker agar were considered as presumptive *Staphylococcus* species. They were picked and stored on nutrient agar slants for further confirmation tests.

3.6 Characterization of Presumptive *Stappylococcus aureus* Isolates

3.6.1 Gram Reaction

Presumptive *Staphylococcus* species stored on nutrient agar slants were examined microscopically following the method as described by Cheesebrough, (2009).

From freshly prepared culture, a colony was picked and emulsified in sterile distilled water, a thin preparation made on a slide and heat fixed. The fixed smear was covered with crystal violet stain for 60 Sec. The stain was rapidly washed off with clean water. The water was tipped off and the smear covered with Lugol's iodine for 60 Sec. The iodine was washed off with clean water. Rapid decolourization was done with acetone-alcohol and washed immediately with clean water. The smear was covered with Safranine stain for 60 Sec. The Stain was washed off with clean water and air dried. Morphology and Gram reactions of the isolates were determined by use of a microscope (CETI-Belgium).

All selected colonies on Brain Heart infusion agar slants that were Gram positive cocci in clusters were subjected to some biochemical tests following standard procedures as described by Cheesbrough (2009) and included the following;

3.6.2 Coagulase Production (Slide Test)

A drop of distilled water was placed on each end of a slide. A colony of the test organismwas emulsified in each of the drops to make two thick suspensions. A loopful of plasma was added to one of the suspensions and mixed gently while no plasma was added to the second to serve as control. Clumping of the organisms within 10 seconds was taken

as a positive result for Coagulase production. However,no clumping within 10 seconds indicated no bound Coagulase.

3.6.3 Catalase Test

A portion of colony from a 24 h old Nutrient Agar culture was placed in a drop of hydrogen peroxide on a clean glass slide. An immediate, rapid bubbling was read as a positive reaction for the production of the enzyme catalase by the organism. The absence of bubbles was read as a negative result.

3.6.4 Fermentation of Glucose and Mannitol

Presumptive *Staphylococcus aureus* was screened for growth on Mannitol salt agar (MSA). Mannitol salt agar (Merck) was prepared according to themanufacturer's instructions. A single colony was picked from pure colonies of Gram positive cocci in clusters, catalase and coagulasepositive staphylococci and streaked on the MSA plate. Fermentation of mannitol was confirmed by growth of yellow colonies on MSA, surroundedby yellow zones after 24 h of incubation at37°C, indicating a positive result.

3.7 Confirmation of Presumptive Positive Isolates using Microgen™ STAPH-ID KIT

Coagulase, Gram and catalase positive strains were biochemically identified using the Microgen™ STAPH–identification system (Microgen Bioproducts, United Kingdom). Single colonies of the presumptive isolates were tested with 12 standardized biochemical substrates following the manufacturer's instructions. The test organisms were identified by

interpreting the permutations of metabolized substrates using the microgen identification system software (MID-60).

3.8 Isolation of *Salmonella* Spp.

The isolation method for *salmonella* spp. followed a three – stage procedure as described by Esona *et al.* (2004) and Henzlar *et al.,* (1994). Pre – enrichment was done by adding 25ml of sample to 225ml of 1% buffered peptone water andincubated at37^0C for 16h. Enrichment of samples was done by incubating 1ml of milk samples in enrichment broth, selenite Fbroth (Merck) and incubated at 37^0C for 24h. The enrichment broths were streaked onto salmonella - shigella agar (SSA) (Merck) plates for isolation. The plates were incubated at 37^0C for 48h. Typical colonies were identified by Gram staining. Presumptive isolates (Gram negative rods) were subjected to series of biochemical tests. Microgen^{TM} Enterobacteriaceae GnA-Identification System was used for further biochemical distinction of the *Salmonella* spp.

3.9 Motility Test

Tubes containing 0.4% (w/v) agar (motility medium) were inoculated with presumptive Salmonella isolates. This was done by stabbing the medium with a fine needle to a depth 1-2cm short of the bottom of the tube with the test organism. The tubes were incubated at 37^0C for 24 h. Growth at the line of inoculation and diffusion of organism in the soft medium indicated motility.

3.10 Biochemical Characterization of Isolates

Presumptive isolates were subjected to biochemical tests as described by Cheesbrough, (2009) as follows;

3.10.1 Triple sugar iron agar (TSI) reactions

Triple sugar iron reactions are based on the ability of the organism to ferment lactose, sucrose and glucose and to produce hydrogen sulphide gas. Slopes of TSI agar medium were prepared as instructed by the manufacturers. The TSI agar slopes were then inoculated with the test organisms. A straight sterile needle was used to first stab the butt, while streaking the slope in a zig-zag pattern. The tubes were incubated with loose tops at 37^0C for 24 h. A yellow butt (acid production) and red-pink slope indicating the fermentation of glucose only, with or without production of gas and hydrogen sulphide (H_2S) was recorded as a positive test. Deeper red to purple (more alkaline) slant indicated the production of ammonia from the oxidative deamination of amino acids. Black colour was indicative of H_2S production.

3.10.2 Citrate utilization

Citrate utilization test was performed using Simmon's citrate agar. Slopes of the medium were prepared in bijou bottles as recommended by the manufacturer. From a saline suspension of the test organism, a sterile straight wire was used to streak the slope and then stabbed the butt. The bottles were incubated at 35^0C for up to 48 h. A bright blue colour in the medium indicated a positive citrate test.

3.10.3 Indole test

The test organisms were inoculated in bijou bottles containing 3 ml of sterile tryptone water.

The bottles were incubated at 37^0C for up to 48 h. Indole was tested for by the addition of 0.5 ml of Kovac's reagent. Formation of a red coloured ring at the surface layer within 10 minutes of addition indicated a positive result.

3.10.4 Oxidase test

Oxidase test was performed using the oxidase reagent; tetramethyl-p- phenylene diamine dihydrochloride. Few drops of dilute solution (about 1%) of the oxidase reagent were used to wet a piece of filter paper. A bit of growth of the test organism from nutrient agar medium was smeared on the wet piece of filter paper with sterile plastic stick. The development of an intense purple colour by the bacterial cells in the smears within 10 seconds indicated a positive oxidase test.

3.11 Confirmation of Presumptive Isolates using Microgen^{TM} GnA + B ID System

Presumptive oxidase negative isolates were further identified using microgen^{TM} GnA – ID system following the manufacturer's instructions. Dehydrated substrates in microwells containing the test strips were reconstituted with a saline suspension of the organisms. The wells containing saline suspension of the test organism were incubated for up to 24 h at 37^0C. After incubation, on addition of specific reagents, the colour changes in the individual metabolized substrates were recorded. The permutation of metabolized

substrates were interpreted using the identification system software (MID-60) provided by the manufacturer to identify the test organisms.

3.12 Determination of Antibiogram of Isolates

Antibiotic susceptibility tests for isolates of *Staphylococcus aureus* and *Salmonella* spp. were performed according to the Kirby- Bauer method as cited in the evaluation methods of the Clinical and Laboratory standards Institutes (CLSI) (CLSI, 2014). Isolates grown on nutrient agar overnight were suspended in 2ml sterile normal saline (0.9% sodium chloride solution). A turbidity equivalent was prepared by comparing with a 0.5 MacFarland standard. Bacterial suspensions of 0.1ml were spread on plates of sterile Mueller-Hinton agar with the help of a sterile cotton swab to form a smooth bacterial lawn. The inocula were allowed to dry for 5 minutes. Commercially prepared standard antibiotic susceptibility test discs impregnated with known agent and strength were then dispensed on the agar surface. Within 15 minutes of application of the discs, plates were incubated overnight at 37^0C. *Staphylococcus aureus* ATCC 6538 was used as standard strain for quality control (for *S.aureus* isolates). Characterization of strains as susceptible, intermediate or resistant was based on the size of inhibition zone around the disc compared with the interpretation standards provided by the manufacturers. Antimicrobial drugs used were Tetracycline (30µg), Trimethoprim/Sulfamethoxazole (25µg), chloramphenicol (30µg), erythromycin (15µg), gentamicin (10µg), Amoxicillin/Clavulanic acid (30µg), Cefoxitin (30µg), Ciprofloxacin (5µg) and Vancomycin (30µg) (Vancomycin was used only for *S.aureus* isolates).

3.12.1 Multiple antibiotic resistance (MAR) and multiple antibiotic resistance index (MARI) of *Staphylococcus aureus* and *Salmonella* spp.

Multiple antibiotic resistance (MAR) for this study is defined as resistance of isolate to three or more antibiotics (Lambert, 2002 and Osundiya *et al.*, 2013).

Multiple antibiotic resistance index (MARI) was calculated according to Furtula *et al.*, (2013) as the ratio of number of antibiotics to which an organism is resistant, to the total number of antibiotics to which an organism is exposed to. The following equation was used,

$$MAR_{index} = a/bc$$

Where 'a' represents number of antibiotics to which isolates were resistant, 'b'represents the number of antibiotics to which isolates were exposed, and 'c' represents the number of isolates per sample.

3.13 Enterotoxin Assay in Culture Fluids

Production of Staphylococcal enterotoxins (SEs); SEA, SEB, SEC and SED in culture fluids of *S.aureus* isolates was evaluated using Staphylococcal Enterotoxin Test Reversed Passive Latex Agglutination (SET-RPLA) (TD900, Oxoid) as recommended by the manufacturers.

The strains to be tested were grown in 10 ml of tryptone soy broth (CM0219B, Oxoid) for 16–18 h at 37^0C with shaking aerobically. At the end of incubation, bacterial cells were harvested by centrifuging at 900g for 20 minutes in refrigerated centrifuge. The supernatant was tested for the presence and typing of Staphylococcal enterotoxins.

In each test well, 25µl of the latex reagents sensitized with antisera to SEA, SEB, SEC and SED and the controls were mixed with supernatant dilutions while performing a doubling dilution on each row. The content of each well was mixed by agitation by hand and covered with a lid to avoid evaporation. The plates were left undisturbed on a vibration free surface for 24h at room temperature. Soluble antigens of bacterial toxins were detected judging agglutination patterns of each. These were compared to the positive and negative controls.

3.13 Genotypic Screening for SEA, SEB, SEC and SED, MecA, Sal En and, *InvA genes*for *Staphylococcus aureus* and *Salmonella* spp.

3.13.1 Primer design

The oligonucleotide primer sequence used for the PCR analyses were obtained from Hsieh*et al, (2008) for* Staphylococcus enterotoxins; *SEA, SEB, SEC* and *SED* genes. *MecA* gene primers were obtained from Lee, (2003). Salmonella *inv A*primer design was obtained from Ferretti *et al,* (2001) and *Sal En* (enterotoxin) was from Murugkar *et al,* (2003). All primers for *S. aureus* (*SEA-SED* and *Mec A*) were manufactured by (Integrated DNA Technologies Limited, Belgium) While *Inv A* and *Sal En* gene primers were designed by Inquaba Biotech South Africa. The Primer sequences and the corresponding sequence locations of the oligonucleotide primers used in this study are as shown in the Table 3.1.

Table 3.1: Primers, Nucleotide Sequences and Expected amplicon sizes of target genes of bacterial isolates:

Primer	Nucleotide Sequence (5'-3')	Expected Amplicon Size(bp)	Reference
SEA-A2	5'-ATTAACCGAAGGTTCTGTAGA-3'		Hsieh *et al.*,2008
SEA-U1	5'-TTGCGTAAAAAGTCTGAATT-3'	582	
SEB-B2	5'- TTTTTCTTTGTCGTAAGATAA-3'		Hsieh *et al.*,2008
SEB-U1	5'-CCAACGTTTTAGCAGAGAAG-3'	732	
SEC-C2	5'-TAAGTTCCCATTACAAAGTG-3'		Hsieh *et al.*,2008
SEC-U1	5'-CCAACGTTTTAGCAGAGAAG-3'	403	
SED-D2	5'-TAATGCTATATCTTATAGGG-3'		Hsieh *et al.*,2008
SED-U1	5'-TTGCGTAAAAAGTCTGAATT-3'	251	
Mec-A2	5'- AGTTCTGCAGTACCGGATTTGC-3'		Lee,2003
Mec-A1	5'-AAAATCGATGGTAAAGGTTGGC -3'	533	
Sal En-2	5'-ATTCGTAACCCGCTCTCGTCC-3'		Murugkar *et al.*, 2003.
Sal En-1	5'-TTGTGTCGTTATCACTGGCAA-3'	617	
Inv A-2	5'-TCCCGGCAGAGTTCCCATT-3'		Ferretti *et al.*, 2001.
InvA-1	5'-GCTGCGCGCGAACGGCGAAG-3'	389	

Key: *SEA-D= Staphylococcus aureus Enterotoxin A-D, Inv A*=Salmonella invasive gene, *Sal En* = Salmonella Enterotoxin, *Mec A*= Methicillin-resistance component (oxacillin/methicillin resistance gene).

3.14 Cell Preparation for DNA Extraction

The preparation of the cell of isolates was carried out using the method by Dubey, (2009). Single colonies were picked from freshly streaked isolates on Salmonella Shigella Agar (for *Salmonella*) and Manitol Salt Agar (for *S. aureus*) and inoculated into 5ml Luria and Bertani (LB)* broth medium. These were incubated overnight at 37^{o}C for 18 h. Bacterial cells were then harvested by centrifugation at 4^{o}C, 8,000rpm (6800 x g) in a refrigerated microcentrifuge for 30 seconds in eppendorff tubes. The supernatants were decanted and cells harvested.

*Luria and Bertani broth media was prepared by dissolving;

Peptone (10g), NaCl (5g), IN NaoH (10ml), Yeast extract (5g), in 1 L of distilled water at pH 7.0 adjusted with NaOH solution and sterilized at 121^{o}C for 15 minutes.

3.15 Extraction of DNA from Isolates

Genomic DNA extraction was carried out with zuppy^{TM} Genomic DNA extraction Kit (Inqaba Biotech, South Africa) using the protocol described by the manufacturer.

Into a 1.5 ml micro centrifuge tube an overnight culture was added. This was spun for 2 minutes at 10,000g, discarding the supernatant. This was followed by the addition of 400µl Genomic lysis buffer (blue), incubated at 45^{o}C for 30 minutes and spun for 2 minutes at 10,000rpm. The lysate was transferred to a spin column and centrifuged at 10,000g for 2minutes. The flow through obtained was discarded and 400µm of DNA wash buffer I (zyppy^{TM}) ^{i} was added. The resultant was centrifuged at 10,000g for 2 minutes and the flow through discarded. Four hundred micro litre (400µl) of DNA wash buffer II

(zyppy™) [ii] was then added and centrifuged at 10,000g for 2 minutes.The flow through was discarded and the empty spin column was spun for 2 minutes. The spin column was transferred to a 1.5ml eppenduff tube and 50µl of DNA elution buffer (zyppy™) [iii]added. This was incubated at room temperature for 5 minutes. Centrifugation was then carried out at 10,000 x g for 5 minutes to elute the genomic DNA.

i. Zuppy™ wash buffer I (with 100% ethanol).
ii. Wash buffer II (with 100% ethanol).
iii. Zuppy™ elution buffer (10mM Tris-HCL, P^H 8.5 and 0.1mM EDTA).

3.15.1 Agarose gel electrophoresis of the DNA

To ascertain that the DNA was actually extracted, the eluent was subjected to agarose gel electrophoresis. A combination of 1% agarose gel[1] was used to resolve the genomic DNA. This combination was heated in a 250ml beaker flask in a microwave for 2 minutes until the agarose has been dissolved. Twenty (20) µl of ethidiubm bromide, (5.0mg/ml) was added as dye to the agarose gel, and mixed. The gel was poured onto a mini horizontal gel electrophoresis tank and the casting combs were inserted at the red bands to ensure easy view of the wells. The gel was allowed to set for 30 minutes and after which the genomic DNA was filled into the wells.

The TAE electrophoresis buffer (1x) was then loaded onto the wells of the gel. The mini horizontal electrophoresis gel was set up, covered and connected running from cathode (-) to anode (+). Electrophoresis was carried out at 72 volts for 40 minutes to allow easy separation of the samples based on molecular weight. At the completion of the electrophoresis, the gel was removed from the buffer (Tris) and viewed under a trans-

illuminator UV light of wavelength 302 nm. The band pattern of the DNA fragments were then photographed with a Polaroid camera and documented using an electrophoresis gel documentation system. The size was compared to the standard DNA molecular weight marker used. The DNA molecular weight marker used was the 1kb ladder.

1% agaose gel combination:

1.0g agarose in 2mls of 10x concentration of Tris acetate ethylene diamine tetra acetate (TAE), 98mls distilled water.

3.16 PCR Amplification of Target Genes of Isolates

PCR amplification was done using the Gene AMP, PCR system 9700 (applied Biosystem). The PCR reactions were performed in reaction buffer (10x), Mgcl (magnesium chloride) (4mM) in a total volume of 25µl. This contained; 1µl (≈1ng) of template DNA, 20 pM each of the primers, Inv A-U, Inv A-R, Sal En - U, Sal En – E, SA-U, SA-A, SA-U, SA-B, SA-U,SA-C,SA-U, SA-D, Mec A-U, Mec A-R, separately. One (1) unit of Taq polymerase and 0.2mM of mixed deoxynucleotide tri-phosphate were added to each set. A negative control set contained the PCR product without the DNA template of the respective samples. PCR was performed under the following conditions: initial denaturation at 95^{o}C for 5minutes. Subsequently followed by 35cycles of 94^{o}C for 30 seconds, this was followed by 50 - 60^{o}C step-up for 30 seconds. A 52 - 42^{o}C touchdown was run for 30 seconds. Initial extension was at 72^{o}C for 30 seconds while final extension of 10 minutes was at 72^{o}C.

3.16.1 Agarose gel electrophoresis of the PCR products

Ten micro litres (10µl) of the PCR products were loaded into wells of 10% agarose gel containing ethidium bromide. One hundred (100) bp DNA ladder was loaded along sides with the PCR products. Electrophoresis was carried out in Tris Acetate EDTA buffer (1 x concentration) at 72mv for 40 minutes until bands were separated. The gel was removed at the completion of electrophoresis and the band pattern viewed on a UV trans-illuminator light of wavelength 302nm. Photographs of bands were taken using a gel documenting machine (Enduro^{TM} GDS; labnet). The sizes were assessed and estimated from the molecular sizes of the DNA ladder against their migration distance.

*I x Tris buffer contains:

Tris 40mM, Acetic Acid 20mM, EDTA 1Mm, pH 8.0

Ethidium bromide (20ml x 50 x TAE) and 40 µl of 10µg/ml ethidium bromide per litre.

3.17 Statistical Analysis

The significant differences between means of values obtained for chemical analyses and total viable counts of samples were calculated by a one-way analysis of variance (ANOVA). The Duncan Multiple Ratio Test and Tukey Test were used to separate means. Significant differences were at ($p \leq 0.05$).

All statistical analyses were conducted using SPSS 20.0 software (SPSS Inc., Chicago, IL). Results were also presented in charts and tables.

CHAPTER FOUR

4.0 RESULTS

4.1 Chemical Composition of Milk and Milk Products

Results of chemical composition of samples are displayed in Table 4.1. pH values ranged from 3.36 ± 0.23 - 6.43 ± 0.27. Fresh milk pH values were in the range, 6.17 ± 1.15 - 6.80 ± 1.17. Nono, Kindrimo and Manshanu pH ranged from, 3.63 ± 0.07 - 4.45 ± 0.07, 3.11 ± 0.08 to 3.62 ± 0.02 and 3.07 ± 0.04 - 3.94 ± 0.03 respectively. Manshanu had the least pH value of 3.36 ± 0.23 with fresh milk having the highest value of 6.43 ± 0.23. There was significant difference at $P \leq 0.05$.

Acidity of the fresh milk and milk products samples ranged from 0.47 ± 0.13 - 3.28 ± 0.49 as revealed in Table 4.2. The acidity for fresh milk and Nono samples ranged from 0.30 ± 0.01 - 0.60 ± 0.03 and 0.96 ± 0.072 - 2.72 ± 0.23 respectively. The acidity ranges for Manshanu and Kindrimo were 2.80 ± 0.04 - 4.02 ± 0.04 and 0.78 ± 0.02 – 3.20 ± 0.51 respectively. Fresh milk had the least acidity (0.47 ± 0.13) with Manshanu having the highest of 3.28 ± 0.49. Significant difference existed among the sample types at $P \leq 0.05$.

Mean values of pH of samples from selected locations were compared in Table 4.1. There were significant differences at ($p \leq 0.05$) in the pH of Nono, Manshanu and Kindrimo samples from selected locations. There was no significant difference ($p \leq 0.05$) in the fresh milk samples from selected locations.Mean values for acidity from selected locations (Table 4.2) revealed significant differences at ($p \leq 0.05$) in the acidity of Nono, Manshanu and Kindrimo samples from selected locations while no significant difference ($p \leq 0.05$) existed in the fresh milk samples.

Table 4.1: Mean (± SD) pH of Samples from Selected Locations.

	Sample Type			
Location	Fresh Milk	Nono	Manshanu	Kindrimo
Giwa	6.80 ± 0.04^{a}	4.45 ± 0.07^{c}	3.17 ± 0.01^{a}	3.94 ± 0.025^{b}
K/North	6.173 ± 0.07^{a}	3.63 ± 0.07^{a}	3.53 ± 0.06^{b}	3.79 ± 0.07^{b}
Chikun	6.53 ± 0.04^{a}	4.43 ± 0.11^{c}	3.11 ± 0.08^{a}	3.85 ± 0.09^{b}
Soba	6.22 ± 0.09^{a}	3.90 ± 0.08^{b}	3.62 ± 0.02^{b}	3.07 ± 0.04^{a}
Mean	6.43 ± 0.27^{c}	4.10 ± 0.37^{b}	3.36 ± 0.23^{a}	3.66 ± 0.37^{a}
P- Value	0.874	0.000	0.000	0.000

Means were separated with Turkey Test, *significance exists at ($P \leq 0.05$). Means with same superscripts in the same row are not significantly different at ($P \leq 0.05$).

Key: SD = Mean standard deviation, K/North = Kaduna North.

Table 4.2: Mean (± SD) Acidity of Samples from Selected Locations.

	Sample Type			
Location	Fresh Milk	Nono	Manshanu	Kindrimo
Giwa	0.30 ± 0.01^{a}	1.30 ± 0.05^{a}	2.80 ± 0.04^{a}	1.29 ± 0.03^{ab}
K/North	0.57 ± 0.08^{a}	2.72 ± 0.23^{b}	3.32 ± 0.09^{c}	3.20 ± 0.51^{c}
Chikun	0.60 ± 0.04^{a}	0.96 ± 0.07^{a}	2.98 ± 0.09^{b}	1.83 ± 0.10^{b}
Soba	0.40 ± 0.02^{a}	2.50 ± 0.10^{b}	4.02 ± 0.04^{d}	0.78 ± 0.02^{a}
Mean	0.47 ± 0.13^{a}	1.87 ± 0.80^{b}	3.28 ± 0.49^{c}	1.78 ± 0.97^{b}
P- Value	0.160	0.000	0.000	0.000

Means were separated with Turkey Test, *significance exists at ($P \leq 0.05$). Means with same superscripts in the same row are not significantly different at ($P \leq 0.05$).

Key: SD = Mean standard deviation, K/North = Kaduna North.

4.2 Proximate Composition of Samples

Table 4.3 reveals the proximate composition of fresh milk and milk product samples. Mean scores ranged from; total solids 13.40 ± 1.30 – 19.78 ± 2.81, protein 0.19 ± 0.03 – 3.26 ± 0.07, lipid 2.62 ± 0.32 – 5.20 ± 0.35, ash 0.55 ± 0.07 – 0.89 ± 0.07 and lactose 7.813 ± 1.81 – 13.81 ± 2.78. Significance differences at ($p \leq 0.05$) existed in the total solids, moisture, ash, lactose and protein.

The proximate composition for samples from selected locations is as shown in Table 4.4, for fresh milk. There were no significant differences (at $p \leq 0.05$) in the proximate composition of samples from the different locations.

Table 4.5 reveals the proximate composition for Nono samples. There were no significant differences (at $p \leq 0.05$) for moisture, total solids and lactose content in Nono samples, while there existed significant differences (at $p \leq 0.05$) in protein, lipid and ash contents.

Table 4.6 displays the proximate composition of Manshanu. Significance existed (at $p \leq 0.05$) in all tested proximate compositions for this product.

The proximate composition for Kindrimo samples is shown in Table 4.7. In the Kindrimo samples significance existed (at $p \leq 0.05$) in all tested proximate compositions.

Table 4.3: Mean Values (±SD) for Proximate Composition of Samples.

	Sample Type				
Variable	Fresh Milk	Nono	Manshanu	Kindrimo	P-Value
Moisture	86.85±1.16[b]	85.45±0.94[ab]	74.26± 21.29[a]	86.04±1.06[b]	0.018
Ash content	0.89 ± 0.07[c]	0.71 ± 0.08[b]	0.58 ± 0.07[a]	0.61 ± 0.10[a]	0.000
Total Solids	13.40±1.30[a]	14.55± 0.94[a]	19.78 ± 2.81[b]	13.88±0.94[a]	0.000
Protein	3.26 ± 0.07[b]	0.25 ± 0.14[a]	0.19 ± 0.03[a]	0.26 ± 0.07[a]	0.000
Lipid	3.59 ± 0.56[b]	2.62 ± 0.32[a]	5.20 ± 0.35[c]	3.75± 0.61[b]	0.000
Lactose	7.31 ± 1.00[a]	10.93± 0.89[b]	13.81± 2.78[c]	9.26 ± 1.50[a]	0.000

Means were separated with Turkey Test, *significance exists at ($P \leq 0.05$). Means with same superscripts in the same row are not significantly different at ($P \leq 0.05$).

Key: SD = Mean standard deviation.

Table 4.4: Mean Values (±SD) for Proximate Composition of Fresh Milk Samples from Selected Locations.

Location	Moisture	Ash	T/Solids	Lipid	Protein	Lactose
Giwa	88.0±0.6[a]	0.73 ± 0.02[a]	12.00 ± 0.01[a]	3.47 ± 0.08[a]	3.29 ±0.005[a]	7.68 ± 1.29[a]
K/North	86.60±0.12[a]	0.70 ± 0.02[a]	14.40 ± 0.12 [a]	3.20 ± 0.02[a]	3.26 ± 0.003[a]	7.21 ± 0.24[a]
Chikun	85.30± 0.29[a]	0.71 ± 0.01[a]	14.70 ± 0.29 [a]	3.20 ± 0.02[a]	3.17 ± 0.01[a]	8.08 ± 0.71[a]
Soba	87.50± 0.18[a]	0.65 ± 0.03[a]	12.50 ± 0.18 [a]	4.50 ± 0.02[a]	3.34 ± 0.02[a]	6.26 ± 0.71[a]
Mean	86.85±1.16	0.89 ± 0.07	13.40 ± 1.30	3.59 ± 0.56	3.26 ± 0.07	7.31 ± 1.00
P-Value	0.475	0.518	0.171	0.688	0.520	0.1161

Means were separated by Turkey Test, *significance exists at ($P \leq 0.05$). Means with same superscripts in the same column are not significantly different at ($P \leq 0.05$).

Key: SD = Standard deviation.

Table 4.5: Mean Values (±SD) for Proximate Composition of Nono Samples from the Selected Locations.

	Parameter					
Location	Moisture	Ash	Total solids	Protein	Lipid	Lactose
Giwa	86.10±1.80[a]	0.78±0.03[b]	13.90±1.80[a]	0.07±0.01[a]	2.10±0.01[a]	10.75±1.88[a]
K/North	84.80±0.19[a]	0.67±0.02[a]	15.20±0.19[a]	0.34±0.05[c]	2.78±0.02[b]	11.41±0.28[a]
Chikun	85.22±0.08[a]	0.77±0.04[b]	14.78±0.08[a]	0.41±0.002[d]	2.78±0.01[b]	10.82±0.13[a]
Soba	85.45±0.94[a]	0.60±0.02[a]	14.32±0.41[a]	0.17±0.01[b]	2.83±0.02[c]	10.72±0.46[a]
Mean	85.45 ± 0.94	0.71 ± 0.08	14.55± 0.94	0.25 ± 0.14	2.62 ± 0.32	10.93 ± 0.89
P-Value	0.376	0.000	0.403	0.000	0.000	0.802

Means were separated by Turkey Test, *significance exists at (P ≤0.05). Means with same superscripts in the same column are not significantly different at (P≤0.05).

Key: SD = Standard deviation.

Table 4.6: Mean Values (±SD) for Proximate Composition of Manshanu from Selected Locations.

	Parameters					
Location	Moisture	Ash	Total solids	Protein	Lipid	Lactose
Giwa	78.10±0.12^{a}	0.5±0.02^{a}	21.71±0.43^{c}	0.22±0.002^{c}	5.75±0.02^{c}	15.24±0.43^{bc}
K/North	77.70±0.74^{a}	0.67±0.01^{d}	22.30±1.11^{c}	0.15±0.002^{a}	4.93±0.04^{a}	16.53±1.17^{c}
Chikun	80.50±0.63^{b}	0.55±0.01^{b}	19.50±0.63^{b}	0.17±0.03^{abc}	4.93±0.02^{a}	13.85±0.63^{b}
Soba	84.40±0.05^{c}	0.61±0.02^{c}	15.60±0.05^{a}	0.20±0.002^{bc}	5.20±0.03^{b}	9.62±0.06^{a}
Mean	74.26 ± 21.29	0.58 ± 2.81	19.78 ± 2.81	0.19± 0.03	5.20 ± 0.35	13.81 ± 2.78
P-Value	0.000	0.000	0.000	0.002	0.000	0.000

Means were separated by Turkey Test, *significance exists at ($P \leq 0.05$). Means with same superscripts in the same column are not significantly different at ($P \leq 0.05$).

Key: SD = Standard deviation, K/North=Kaduna North.

Table 4.7: Mean Values (±SD) for Proximate Composition of Kindrimo from Selected Locations.

	Parameters					
Location	Moisture	Ash	Total solids	Protein	Lipid	Lactose
Giwa	87.20±0.86^{c}	0.69±0.05^{b}	12.80±0.86^{a}	0.31±0.004^{c}	4.74±0.01^{c}	7.06±0.80^{a}
K/N	84.67±0.41^{a}	0.65±0.03^{b}	15.00±0.21^{c}	0.22±0.002^{b}	3.33±0.02^{a}	10.80±0.20^{c}
Chikun	86.50±0.11^{bc}	0.65±0.01^{b}	13.50±0.11^{ab}	0.34±0.03^{c}	3.33±0.02^{a}	9.18±0.17^{b}
Soba	85.80±0.07^{ab}	0.45±0.01^{a}	14.20±0.07^{bc}	0.17±0.02	3.60±0.02^{b}	9.98±0.08^{bc}
Mean	86.04 ± 1.06	0.61 ± 0.10	13.88 ± 0.94	0.26 ± 0.07	3.75 ± 0.61	9.26 ± 1.50
P-Value	0.001	0.000	0.002	0.000	0.000	0.000

Means were separated by Turkey Test, *significance exists at (P ≤0.05). Means with same superscripts in the same column are not significantly different at (P≤0.05).

Key: SD = Standard deviation, K/N=Kaduna North.

4.3 Mean Total Viable Counts of Samples

Mean Total viable counts of fresh milk and milk products from various locations is presented in Table 4.8. Mean total viable counts for fresh milk ranged from 4.64 ± 3.50 to 5.87 ± 5.10 (log_{10}Cfu/ml). Giwa had the highest mean counts and were significantly different at p≤ (0.05) from all other locations. The least mean counts (4.64 ± 3.5log_{10}Cfu/ml) were obtained from Kaduna North L.G.A.

Mean total viable counts for Nono ranged from 5.79 ± 4.69 to 6.24 ± 5.56 (log_{10}Cfu/ml). Chikun had the highest mean counts and were significantly different at p≤ (0.05) from those from Giwa and Chikun. Least mean counts (5.79 ± 4.96 (log_{10}Cfu/ml) were obtained from Soba.

Mean total viable countsfor Kindrimo ranged from 5.42 ± 5.09 to 6.14 ± 5.00(log_{10}Cfu/ml). Chikun had the highest mean counts and were significantly different at p≤ (0.05) from those from Giwa, Chikun and Soba. The least mean counts (5.42 ± 5.09log_{10}Cfu/ml) were obtained from Soba.

Average means of total viable counts of Manshanu from various locations ranged from 3.69± 2.88 to 4.91± 3.01(log_{10}Cfu/ml). Chikun had the highest mean counts with Kaduna North having the least (3.69± 2.88 log_{10}Cfu/ml).There was no significant difference at p≤ (0.05) among the various locations.

Mean total viable counts of samples from various locations ranged from 3.90 ± 3.18 to 6.04 ± 5.04 (log_{10}Cfu/ml). Least mean counts were obtained from Manshanu, while Nono had the highest.

Table 4.8: Total Viable Counts of Fresh Milk and Milk Products from Selected Locations.

Sample Location		Sample Type			
		Fresh Milk	Nono	Manshanu	Kindrimo
	Number examined	Mean±SEM (log_{10}Cfu/ml)	Mean±SEM (log_{10}Cfu/ml)	Mean±SEM (log_{10}Cfu/ml)	Mean±SEM (log_{10}Cfu/ml)
Giwa	20	5.87 ± 5.10^{b}	6.14 ± 5.00^{b}	3.82 ± 3.62^{a}	6.07 ± 5.11^{c}
Kaduna North	20	4.64 ± 3.50^{a}	5.80 ± 4.77^{a}	3.69 ± 2.88^{a}	5.86 ± 5.13^{b}
Chikun	20	4.87 ± 4.25^{a}	6.24 ± 5.56^{b}	4.91 ± 3.01^{a}	6.14 ± 5.00^{c}
Soba	20	5.24 ± 4.21^{a}	5.79 ± 4.96^{a}	4.09 ± 3.58^{a}	5.42 ± 5.09^{a}
Mean		5.41 ± 4.65	6.04 ± 5.04	3.90 ± 3.18	5.95 ± 4.89
P-value		0.000*	0.000*	0.319	0.000*

Duncan Multiple Ratio Test. Mean values with different superscripts in same column are significantly different at $p \leq (0.05)$.

*= Significance exists at $p \leq (0.05)$.

Key: SEM = Standard error mean, Cfu = Colony forming unit.

4.4 Frequency of Occurrence of Potential Pathogenic Isolates from Samples

The biochemical characteristics of presumptive *Staphylococcus* spp. and *Salmonella* spp.isolates are displayed in Appendices I and II. *Staphylococcus aureus* isolateswere catalase and Coagulase positive with fermentation of mannitol while other *Staphylococcus* spp. were negative. *Salmonella* spp. isolates had acid (yellow) butt with gas formation on TSI agar differenciating them from all other Enterobacteriaceae.

Appendices III and IV reveal the confirmation of the biochemical characteristics of *Staphylococcus* species, *Salmonella* and other Enterobacteriaceae using microgen^{TM} Staphylococcus-ID and Enterobacteriaceae GNA-ID system.

Table 4.9 gives a frequency of occurrence in relation to the total number of organism isolated from samples. A total of 45 Enterobacteriaceae species, 34 *Bacillus* species and 56 *Staphylococcus* species were isolated.

The percentage distribution of bacteria isolates from fresh milk and milk product samples is revealed in Figure 4.1. Fresh milk had the highest percentage contamination (37.5%) with Bacillus species accounting for most (17.5%) of its contamination. Kindrimo was the least contaminated, accounting for 9.6% of the total bacterial contamination.

The occurrence of *Staphylococcus aureus* and *Salmonella* spp. in fresh milk and milk products showed that out of a total of 320 fresh milk and milk products examined, 28 *S.aureus* strains were isolated from the different products. Manshanu had the highest frequency of 9 (32.1%) of occurrence of *S.aureus*. Kindrimo had the least frequency of 5 (17.9%). One sample of Nono was contaminated with *Salmonella* (Table 4.10).

Percentage occurrence of *S. aureus* and *Salmonella* spp. in the different sampling locations is presented in Table 4.11. Kaduna North sample had the highest occurrence of *S. aureus* 9 (32.05%). Chikun and Soba had the least occurrence of 5 (17.9%). A total of 28 *S. aureus* isolates were obtained from all the locations. *Salmonella pollurum was* found in only Chikun local Government area.

Table 4.9: Frequency of occurrence of Potential Pathogenic Bacterial species Isolated from fresh Milk and Milk Products.

Bacterial isolates	Frequency	Percentage (%)
Bacillus species	34	25.2
Staphylococcus aureus	28	20.7
Staphylococcus chromogens	04	3.0
Staphylococcus capitis	01	0.7
Staphylococcus haemolyticus	03	2.2
Staphylococcus hyicus	06	4.4
Staphylococcus xylosus	14	10.4
Pantoea agglomerans	04	3.0
Escherichia coli	03	2.2
Acetobacter haemolyticus	01	0.7
Salmonella Pullorum	01	0.7
Proteus vulgaris	02	1.5
Proteus mirabilis	20	14.8
Acinetobacter lwoffi	03	2.2
Providencia stuartii	06	4.4
Providencia rettgeri	02	1.5
Citrobacter diversus	01	0.7
Hafnia alvei	02	1.5
Total	135	100

Key: n = 135

Table 4.10: Frequency of Occurrence of *S.aureus* and *Salmonella* spp. in Milk and Milk Products.

Sample Type	No. of Samples tested	No.(%)positive *S.aureus*	No.(%) positive *Salmonella*	Total% *(S.aureus* and *Salmonella* Spp.)
Fresh Milk	80	8(28.6)	0(0)	8(27.6)
Nono	80	6(21.4)	1(1.3)	7(24.1)
Manshanu	80	9(32.1)	0	9(31.0)
Kindrimo	80	5(17.9)	0	5(17.2)
Total	320	28(100.0)	1	29(100.0)

Key: % = Percentage.

Table 4.11: Occurrence of *S.aureus* and *Salmonella* in the Different Sampling Locations.

Location	No. tested	No.(%)positive *S.aureus*	No.(%)positive *Salmonella*	Total % *(S.aureus* and *Salmonella* Spp.)
Giwa	80	9(32.1)	0	8(31.0)
K/North	80	9(32.1)	0	9(31.0)
Chikun	80	5(17.9)	1(1.3)	6(20.7)
Soba	80	5(17.9)	0	5(17.2)
Total	320	28(100.0)	1(1.3)	29(100.0)

Key: K/North = Kaduna North.

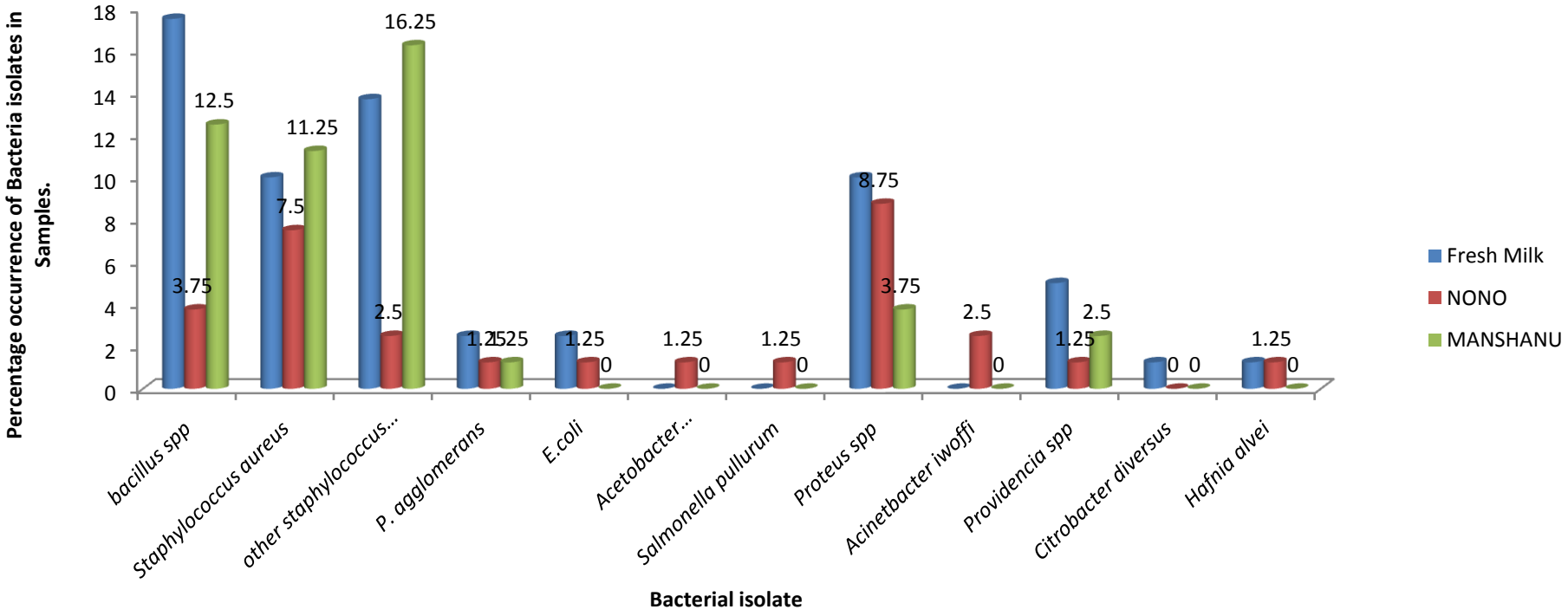


Figure 4.1: Distribution of Bacterial Isolates from Fresh Milk and Milk Products

4.5 Antibiotic Susceptibility of Isolates

A total of 28 *S.aureus* isolates were tested against nine (9) antibiotics (Table 4.12). The isolates had 50.0% resistance to Amoxicillin-Clavulanic acid and were highly resistant to Cefoxitin at 71.4%. Resistance to Vancomycin and Erythromycin were at 60.7%. The isolates showed a 64.3% resistance to Tetracycline, 100% susceptibility for Gentamicin and Ciprofloxacin with a 94.4% susceptibility to Chloramphenicol. There was a 75% susceptibility to Sulfonamides and Trimethroprim while intermediate patterns of 14.3% and 3.6% were shown for Tetracyclin and Chloramphenicol respectively.

The antibiogram of *Salmonella pullorum* isolate is shown in Table 4.13. The isolate showed resistance to Amoxicillin-Clavulanic acid, Cefoxitin, Tetracycline and Erythromycin. Sensitivity was shown for Gentamicin, Ciprofloxacin and Chloramphenicol.

A total of 20 (68.97%) bacterial isolates exhibited resistance to multiple antibiotics (Table 4.14). Isolates exhibited multiple antibiotics resistance patterns of 3(24.14%) to 6(13.79%) antibiotics combinations.

Distribution of multiple antibiotics resistance among sample types (Figure 4.2) ranged from 10% (Kindrimo) to 35% (manshanu).

Figure 4.3 displays the distribution of multiple antibiotics resistance in the different locations. The highest occurrence was observed in Giwa (40%) with Soba (10%) having the least (Figure 4.3).

Percentage occurrence of multiple antibiotic resistance indices is shown in Figure 4.4. The multiple antibiotic resistance indices ranged from 5% occurrence of 0.5 - 0.2 and 0.3 which had 35% occurrence, each (Figure 4.4).

Table 4.12: Antibiogram of *S.aureus* Isolates from Fresh Milk and Milk Products.

Drug Family	Antibacterial Agent	Disk Concentration (µg)	Resistant n(%)	Intermediate n(%)	Susceptible n(%)
Penicillin	Amoxicillin-Clavulanic acid (Aug)	30	14(50)	0	14(50)
Cephalosporins	Cefoxitin (Fox)	30	20(71.4)	0	8(28.6)
Aminoglycosides	Gentamicin (GN)	10	0	0	28(100)
Tetracycline	Tetracycline (TE)	30	18(64.3)	4(14.3)	6(21.4)
Fluoroquinolones	Ciprofloxacin(CIP)	5	0	0	28(100)
Sulfonamides	Sulfonamides and Trimethroprim (SXT)	25	7(25)	0	21(75.0)
Phenicols	Chloramphenicol (C)	30	0	1(3.6)	27(96.4)
Glycopeptides	Vancomycin (VA)	30	17(60.7)	0	11(39.3)
Macrolides	Erythromycin (E)	15	17(60.7)	0	11(39.3)

Key: n= number, %= Percentage.

Table 4.13: Antibiogram of *Salmonella pullorum* isolated from fresh milk and milk products.

Antibiotics Tested	Disc concentration (µg)	Sensitivity Pattern		
		Resistant	Intermediate	Susceptible
		R	I	S
Amoxicillin-Clavulanic acid(Aug)	30	R	-	-
Cefoxitin (Fox)	30	R	-	-
Gentamicin (GN)	10	-	-	S
Tetracycline (TE)	30	R	-	-
Ciprofloxacin(CIP)	5	-	-	S
Sulfonamides and Trimethroprim(SXT)	25	-	I	-
Chloramphenicol(C)	30	-	-	S
Erythromycin (E)	15	R	-	-
Total(n)		4	1	3

Table 4.14: Multiple Antibiotic Resistance Pattern of Bacterial Isolates from fresh milk and milk products.

No. of Antibiotics	Resistance Pattern	No. of Bacterial isolate with Pattern		
		S.aureus (%)	*Salmonella pullorum* (%)	MARI
3	TE,E,Fox; Aug,SxT,Fox; TE,E,AUG; VA,E,Fox, F,AUG,Fox	7(24.14)	-	0.3
4	VA,TE,AUG,Fox; TE,E,AUG,Fox, VA,TE,Fox,AUG (2) VA,TE,E,Fox(2), VA,TE,E,Fox	6(20.69)	1(3.45)	0.4
5	VA,TE,E,AUG,Fox (2), VA.TE,E,Fox,SxT	2(6.90)	-	0.6
6	VA,TE,SxT,E,AUG,Fox, VA,TE,E,Fox,SxT,AUG, VA,TE,SxT,E,AUG,Fox, VA,TE,SxT,E,AUG,Fox	4(13.79)	-	0.7
Total (%)		19(65.52)	1(3.45)	20(68.97)

Key: Aug= Amoxicillin-Clavulanic acid; Fox= Cefoxitin; CN= Gentamicin; TE= Tetracyclin; Cip= Ciprofloxacin; SXT= Sulfonamides and Trimethroprim; C= Chloramphenicol; VA= Vancomycin; E= Erythromycin. Percentage = %, where **n**=29(Total number of Bacterial isolates), MARI= multiple antibiotic resistance index.

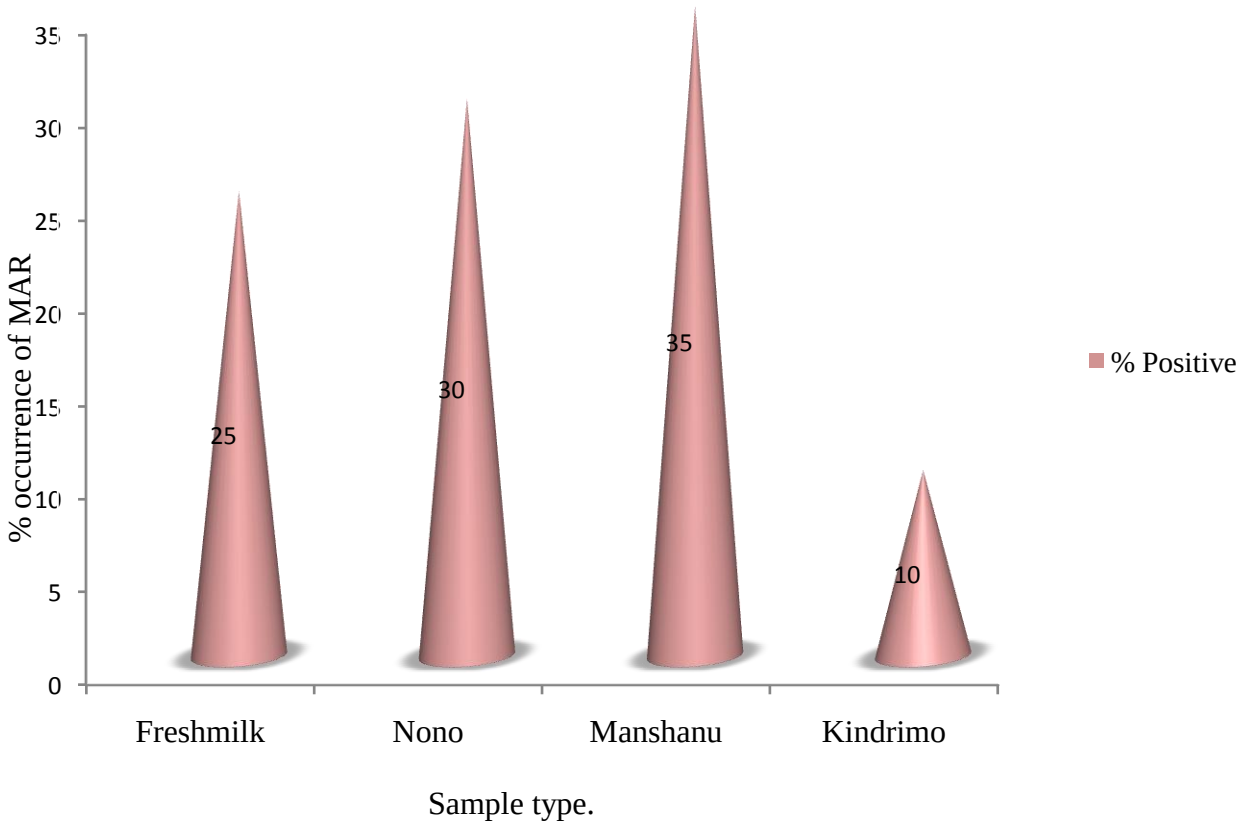


Figure 4.2: Occurrence of Multiple Antibiotics Resistance Indices in Fresh Milk and Milk Products.

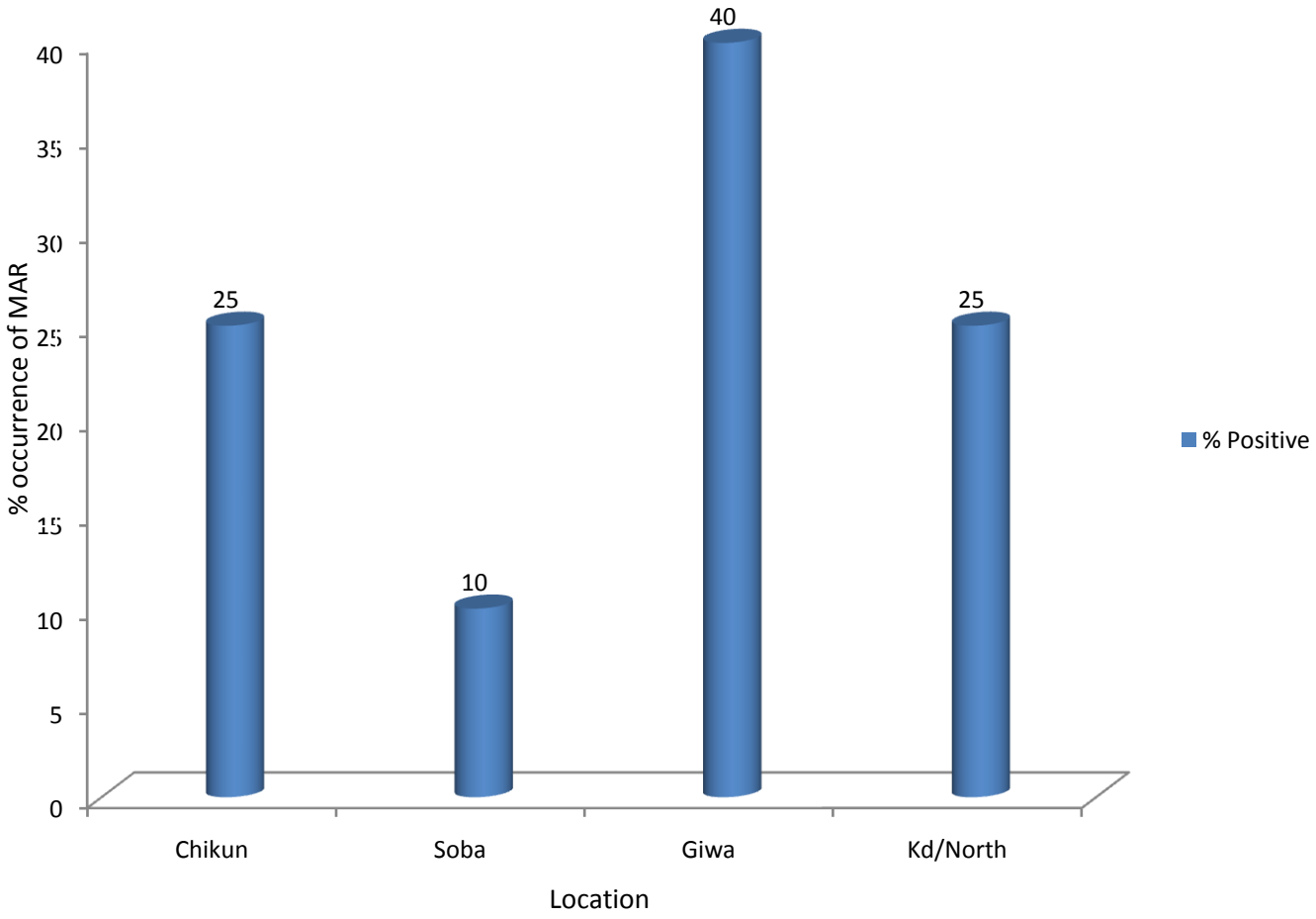


Figure 4.3: Distribution of MAR Isolates in Selected Locations.

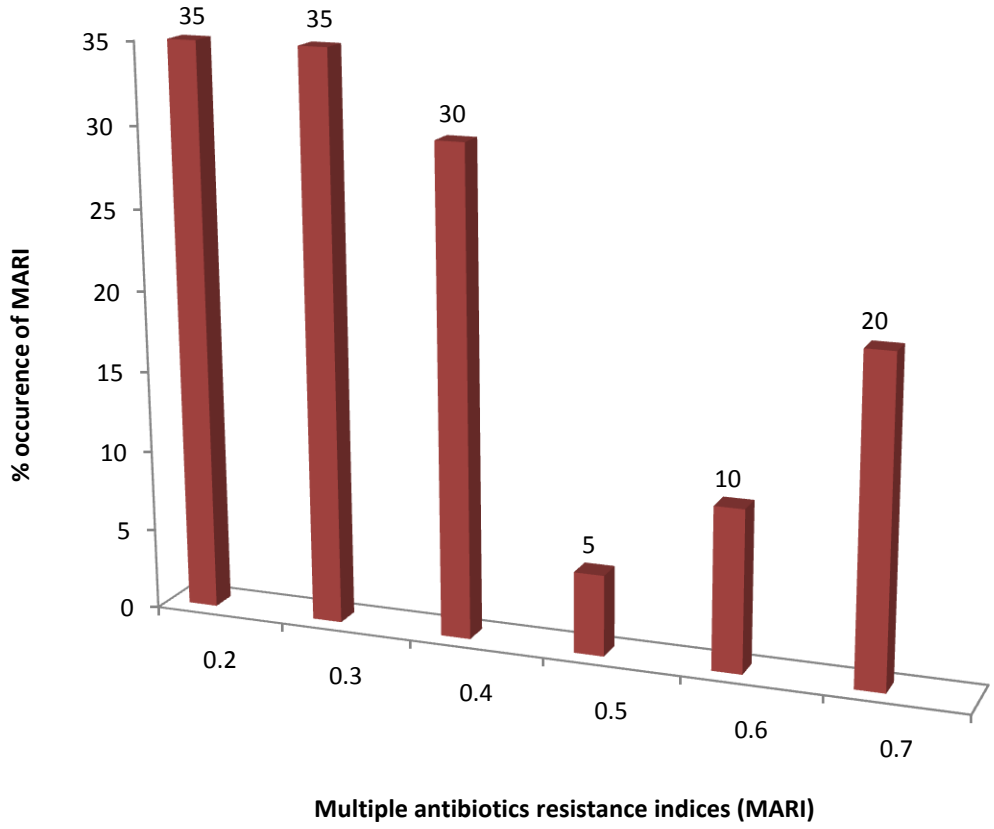


Figure 4.4: Percentage occurrence of Multiple Antibiotics Resistance Indices (MARI) among bacterial isolates.

4.6 Expression of Staphylococcal Enterotoxin in Samples

Table 4.15; shows the expression of Staphylococcal enterotoxin in fresh milk and milk Products. SEAs were most expressed (19) 67.9%, while SEDs were least expressed. There were combinations of SEAB (4) 14.6%, SEAC (1) 3.6%, SEAD (1) 3.6%, SEABC (1) 3.6% and SEABD (1) 3.6%.

Frequency of Staphylococcal enterotoxin expression in fresh milk and milk products is presented in Figure 4.5. Staphylococcal enterotoxins were mostly expressed in Manshanu (35%).

Occurrence of enterotoxigenic *S.aureus* in the different sampling locations is displayed in Figure 4.6. Kaduna North had highest occurrence of 7 (35%), with Chikun having the least 3 (15%) occurrence.

Table 4.15: Frequency of expression of staphylococcal enterotoxin in fresh milk and milk products.

Source of *S.aureus*	No. Tested	Enterotoxin Type.												
		SE(A)	SE(B)	SE(C)	SE(D)	SE(AB)	SE(AC)	SE(AD)	SE(BC)	SE(BD)	SE(ABC)	SE(ABD)	SE(ABCD)
F/milk	8	6	2	1	1	2	-	1	-	-	-	-	-
Manshanu	9	7	1	1	-	-	-	-	-	-	1	-	-
Nono	6	3	1	1	-	2	1	-	-	-	-	-	-
Kindrimo	5	3	1	-	1	-	-	-	-	-	-	1	-
Total(n)	(28)	(19)	(5)	(3)	(2)	(4)	(1)	(1)	(0)	(0)	(1)	(1)	(0)
%	100%	67.9%	17.9%	10.7%	7.1%	14.3%	3.6%	3.6%			3.6%	3.6%	

Key: n= Number, %= Percentage, SE= staphylococcal enterotoxin, f/milk= fresh milk.

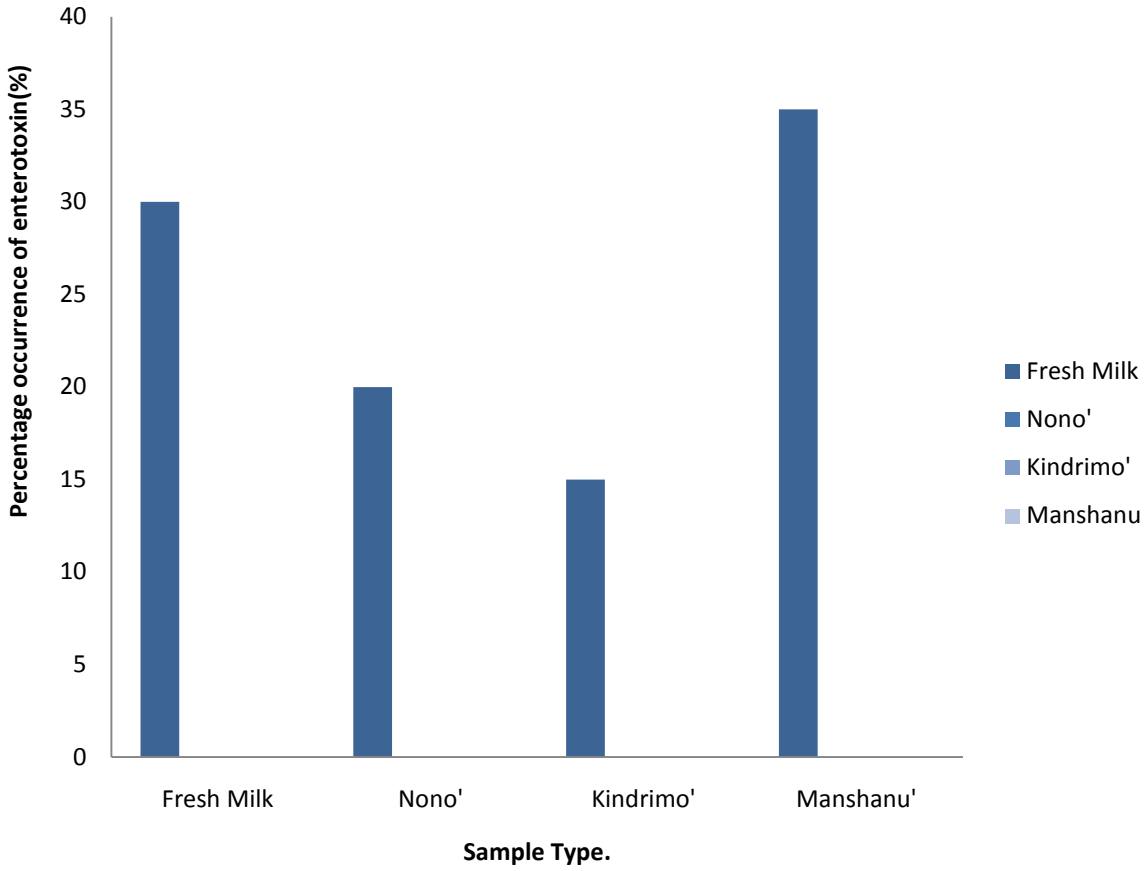


Figure 4.5: Frequency of Occurrence of Staphylococcal Enterotoxin in Fresh Milk and Milk samples.

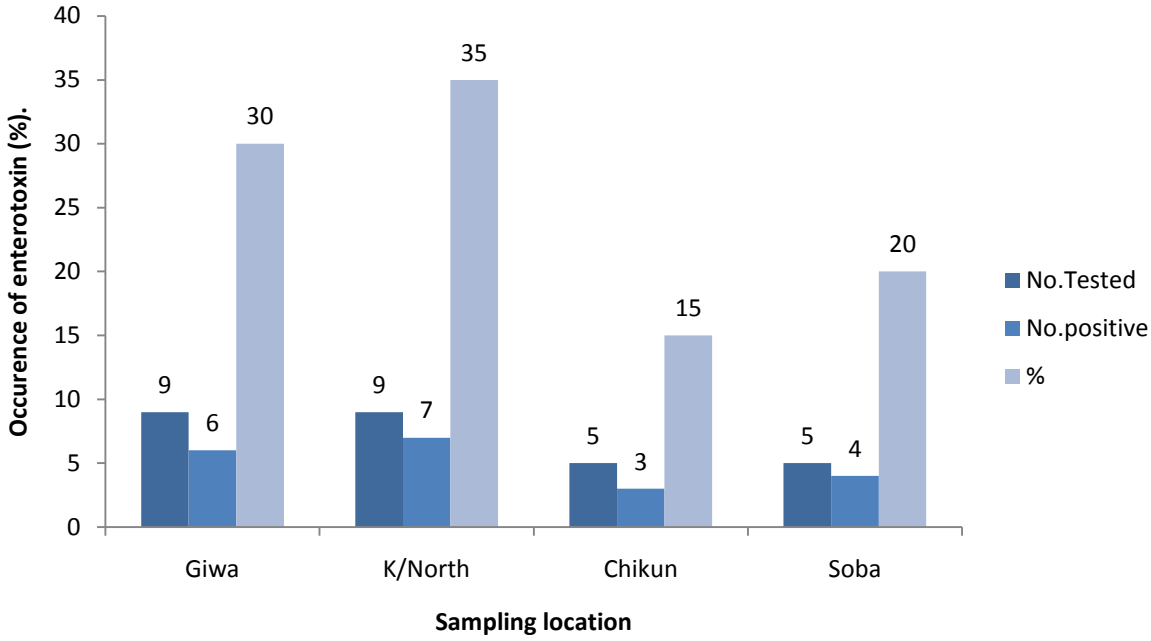


Figure 4.6: Distribution of Enterotoxigenic *S.aureus* in the Different Sampling Locations.

Key: K/North = Kaduna North.

4.7 Polymerase Chain Reaction (PCR) Amplification of Target Genes

Agarose gel photo of extracted DNA of isolates is displayed in plate I.The distribution of *Mec A* and Staphylococcal Enterotoxin (SE) genes among Fresh milk and milk products is displayed in Table 4.16. Fresh milk, Nono and Kindrimo had 10% occurrence of *Mec A* and *SE* gene. Manshanu had the highest occurrence of both genes, *SE* (20%) and *Mec A* (20%). Nono and Manshanu had co-occurrence of *SE* and *Mec A* genes of 1 (5%) and 2 (10%) respectively.

Polymerase chain reaction (PCR) amplification of *Sal-En* and *Inv A* genes in *Salmonella pullorum*isolate is shown in plate I1. The expected amplicon sizes were *Inv A* -389bp and 617bp for *Sal-En.* There were no amplifications at the expected base pairs for *Sal*-En and *Sal Inv A* genes respectively.

Amplification of *SEA*, *SEB*, *SEC* and *SED* genes are shown in Plate III. A total of 7 (35%) had positive amplification of 582bp for *SEA* while only 1 (5.0%) isolate was amplified at 732bp for SEB gene.

Polymerase chain reaction (PCR) amplification of *Mec A* gene gave positive amplification of 533 bp for 11 (55%) *S.aureus* isolates as revealed in Plate IV.

Table 4.16: Distribution of *Mec A* and *SE* genes in samples.

Sample Type	No. Examined	No. Positive *SE* (%)	No. Positive *Mec A* (%)	No. Co-positive *SE* and *Mec A* (%)
Fresh milk	6	2(10)	3(15)	-
Nono	4	1(5)	2(10)	1(5)
Manshanu	7	4(20)	4(20)	2(10)
Kindrimo	3	-	2(10)	-
Total (%)	20	7 (35)	11(55)	3(15)

Key: n= 20.

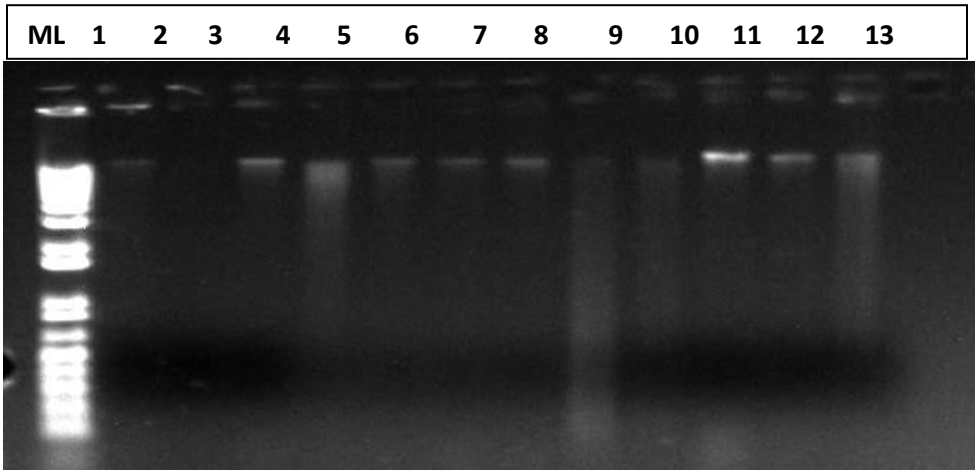


Plate 1: Agarose gel photo of extracted DNA of isolates.

ML = 100 bp DNA ladder, Lanes 1-13 = isolates.

L1 L2 L3 L4 ML L5 L6

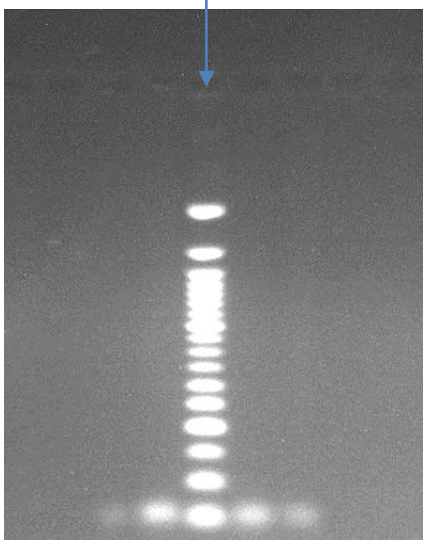


Plate 1I: Multiplex PCR amplification products of *Sal-En* and *Inv A* genes.

(Expected amplicon sizes; *Inv A*=389bp, *Sal-En*=617bp).

ML= Molecular ladder.

L1= Negative control, L2, L3, L4,L5 and L6= test organism (*Salmonella pullorum* fromNono).

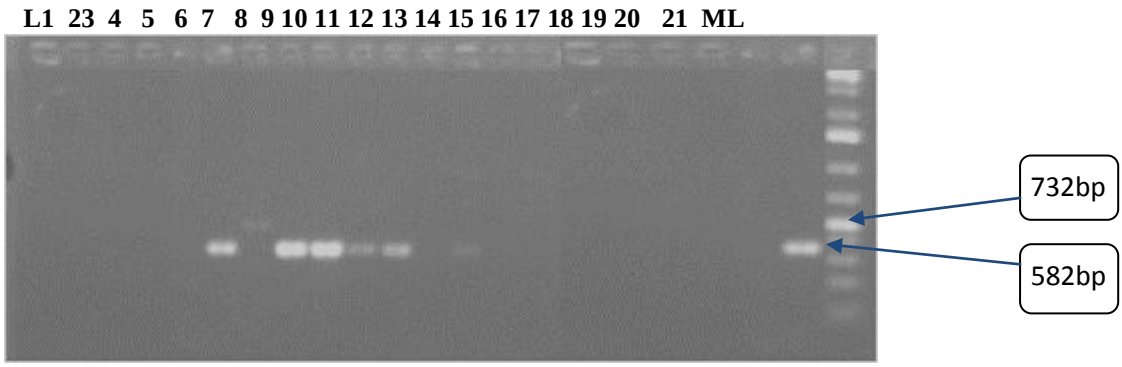


Plate III: Multiplex PCR amplification products of *SEA, SEB, SEC* and *SED* genes.

ML= Molecular ladder.L1= Negative control, L2 - L21= Samples (test organism, *S.aureus* isolates from fresh milk and milk products).L6, L8, L9, L10, L11, L13 and L21 positive amplification of 582 bp for *SEA*.L7= Positive amplification of 732bp for *SEB* gene.

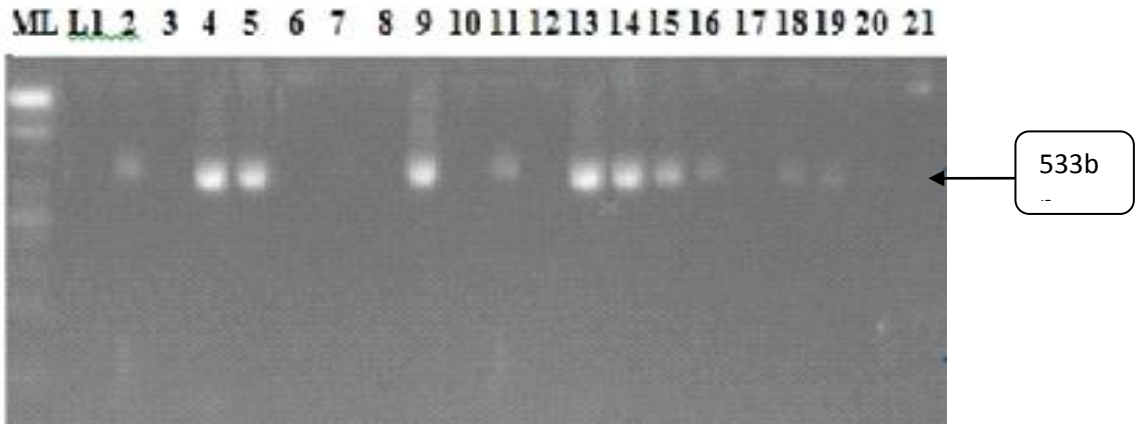


Plate IV: PCR amplification products of *Mec A* gene.

ML= Molecular Ladder, L= Lane, L1= Negative control,L2- L21= test organisms (*S. aureus* isolates fresh milk and milk products). L2, L4, L5, L9, L11, L13, L14, L15, L16, L18 and L19=Positive amplification of 533bp for *MecA* gene.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Chemical Composition of Milk and Milk Products

Milk pH gives an indication of milk hygiene and fresh milk should not be <6.6 or >6.8. The pH of raw milk therefore plays an important role as it has an effect on the distribution and growth rate of microorganisms in the milk. According to O'Connor (1995), pH values higher than 6.8 indicates mastitis milk and pH values below 6.6 indicates acidity increase of milk due to bacterial multiplication. Food and Agricultural Organization (FAO), (1999) standard for fresh milk is pH 6.50 ± 0.30, pH of fresh milk samples from Chikun (6.53 ± 1.33) were within this standard range while those from Soba (6.22 ± 1.26) and Kaduna North (6.17 ± 1.15) (Table 4.1) were not, indicating probable bacterial growth in the samples. Fresh milk pH values from Giwa (6.80 ± 1.17) were within the range reported by other researchers (Rehman and Salaria, 2005; Obi and Ikenebomeh 2007; Lingathurai *et al.,* 2009; Fulya, 2011; Ogbonna *et al*., 2012 and Egwaikhide *et al.,* 2014).

The production of acid in milk is normally termed 'souring' and the sour taste of such milk is due to lactic acid production. The percentage of acid present in milk is a rough indicator of its age and the manner in which it has been handled (Gemechu *et al*. 2015). Acidity in fresh milk samples from the study areas was relatively high. Normal fresh milk has an apparent acidity of 0.14 to 0.16% as lactic acid (O'Connor, 1995). High titratable acidity obtained suggests bacterial contamination and subsequent multiplication during handling and transportation. Acidity of the other milk products (Nono, Manshanu and Kindrimo)

were significantly different (at $p \leq 0.05$) (Table 4.2) from fresh milk, indicating lactic acid fermentation. Nono and Kindrimo were not significantly different (at $p \leq 0.05$) from each other, indicating product similarity. Also the titratable acidity ranges from 0.47 to 3.28 showed that as the pH of the samples reduced the titratable acidity increased. Similar trends in acidity results was obtained by other researchers which revealed lactic acid fermentation (Obi and Ikenebomeh, 2007; Egwaikhide *et al.,* 2014).

5.2 Proximate Composition

Proximate composition of fresh milk and milk products showed that Manshanu had the highest total solid content with fresh milk having the least (Table 4.3). However all fresh milk and milk products samples had total solid content values higher than the minimal standard content of 12.75 ± 2.50 (FAO,1999) and also European Union established quality standards for total solids content of cow milk of not less than 12.5% (FAO/WHO, 2007). These showed fresh milk and milk products samples to be within the recommended standards. Different values for total solid content of raw milk samples have also been reported by different scholars. The variation could be due to difference in breed, feeding and management practices which have important effects on milk composition and quality (O'Connor, 1995). Total solid percentage values were similar with that obtained by Kra *et al.* (2013).

Fresh milk samples had the highest mean percentage value for protein (3.26 ± 0.07) with Manshanu having the least (0.19 ± 0.03) as revealed in Table 4.3. The low protein content in other milk products could have been due to protein degradation. All fresh milk samples

were below the FAO/WHO, (2007) standard of 4.00 ± 0.10 for protein in fresh milk but had protein values which could serve as nitrogen source for microorganisms.

Results of the study showed Manshanu to have had the highest lipid content (Table 4.3). This is not surprising because Manshanu is milk fat. The study revealed a decline in lipid content during fermentation into Nono or Kindrimo. Kindrimo had higher lipid content than Nono. Nono is usually defatted during processing. Difference in lipid content in Nono and Kindrimo from various locations could have arisen due to variation in process methodology. It has been reported in previous works that high lipid content increases the total solid (Neitz and Robertson, 1991). Similar trends were observed in this study; high lipid content leading to high total solids. Lipid plays an important role in improving the consistency and keeping quality of fermented milk products. It also provides twice as much energy as same quantity of carbohydrate and protein (Ehirim and Onyeneke, 2013).

Ash content of the samples differed significantly ($p \leq 0.05$) from each other (Table 4.3). Fresh milk had the highest ash content mean value (0.89 ± 0.07) with Manshanu having the least (0.58 ± 0.07). The standard FAO/WHO, 2007 standard lies at 0.90 ± 0.10. The ash values are an index of mineral content and were relatively low in the samples. The mean lactose values were low (7.31 ± 1.0 – 13.81 ± 2.78). The low lactose values could be attributed to the process of fermentation which converts carbohydrate, basically lactose to lactic acid (Ehirim and Ndimantang, 2004; Egwaikhide *et al*., 2014). Manshanu, Nono and Kindrimo (the fermented products) had higher percentages of lactose. This may have arisen due to the low level of ash content or storage at ambient temperature, which could be attributed to utilization of available carbohydrate (lactose) and other organic matters by

spoilage microorganisms leading to a decrease in proportion of available carbohydrate and inorganic ash content, more over low ash content is known to aid in preservation of fermented foods (Nahar *et al.,* 2007).

Lactose values differed significantly ($p \leq 0.05$) among sample types and location. The lactose content significant difference might be due to the action of lactose hydrolyzing enzymes produced by microorganisms as a result of storage temperature variation of the samples.

5.3 Mean Total Viable Counts of Samples

Bacteriological quality of fresh milk, Nono, Kindirmo and Manshanu revealed that the total viable counts (Table 4.8) were not in conformity with the standard as recommended (APHA, 2001). Grade A raw milk ($<10^5$cfu/ml) and Grade B (milk from local producers) ($<10^6$cfu/ml). The high total count for fresh milk (Log_{10}5.41cfu/ml) and Nono (Log_{10} 6.04cfu/ml) obtained in thisstudy showed that these values are high for fresh milk and milk products. Manshanu (Log_{10}3.90 cfu/ml) and Kindirmo (Log_{10}5.95 cfu/ml) had values within the standard range. Abid *et al.* (2009) reported that counts greater than 10^3cfu/ml for raw milk indicates a serious fault in hygiene during the production line. Another worker reported total bacterial count in fresh milk to be $12.5x10^6$cfu/ml (Lingathurai and Vellathural, 2010) in Pakistan and India. The possible sources of contaminating organisms associated with the products in this study could be the use of previously fermented Nonoas starter culture (back slopping) and the useof unclean water for processing. The contaminating organisms could also be introduced viamicro flora adhering to the calabash, spoons, and bowls used by the producers. Also the high microbial contamination of the

samples used in this study could be due to the state of health of the milked animals. High microbial contamination in milk may be due to clinical andsubclinical state of the cattle as organisms of milk and milk products may get into milk eitherdirectly from udder and or indirectly through infected body discharge of the animal which may find its way into the milk (Egbere, 2008; Ogbonna *et al.,* 2012).

The high counts of microorganismsin milk during the presentstudy also indicated that those microorganismsnot only contaminate milk butalso multiply and grow on it. This might be due to the fact that the milk is a good nutritive medium for thegrowth of the microorganisms, especially with poor sanitary procedures and the lack of the cooling facilities. These conditions are further aggravated by the tropical hot climate (Pourhassan and Taravat, 2011).

5.4 Occurrence of Potential Pathogenic Bacterial Isolates from Samples

Most of the organisms identified were enterobacteria as displayed in Table 4.9, Figure 4.1 and Appendices I - IV. Enterobacteria are common inhabitants of the intestinal tract of various domestic animals including cow, and are commonly found in cow dung which was observed to be abundant at the milking environments, and therefore easy contamination ofthe milk as a result of poor sanitation of the milking environments. *Bacillus* and *Staphylococcus* are associated with food borne intoxication through production of enterotoxins, and the main agents involved here are *Bacillus cereus* and *Staphylococcus aureus* (both had high frequency of occurrence) (Ryser,1998). *Bacillus* spp. may have been introduced into milk from the soil, cattle feed, milking equipment, and the udder, while *Staphylococcus* spp.could come from the udder or skin of humans. *Bacillus spp*. has been

implicated as the main reason for significant economic losses in the dairy industry (Brown, 2000). *Bacillus* spp. such as B. *licheniformis, B. cereus, B. subtilis, B. Mycoides,* and *B.megaterium* are said to be able to produce spores which can survive pasteurization (Ledenbach and Marshal, 2009). Similarly, the enterotoxin produced by*Staphylococcus aureus* and some Coagulase negative Staphylococci (CNS) are also heat stable and can also survive pasteurization (Ledenbach and Marshal,2009). Apart from *Escherichia coli,* which has some toxin producing strains, the other food related pathogens identified in the milk samples such as *Proteus* spp. hardly produce toxin, neither do they form spores. *Proteus* spp. though isolated, their presence in milk sample is less frequent. However, being coliforms they may have occurred in milk samples probably as contaminants. Other bacteria species isolated include, *Acinetobacter, Enterobacter, Providencia*, *Citrobacter* and *Hafnia. Acinetobacter* species are known to cause ropiness of milk and secretion of extracellular enzymes both at psychrophilic and mesophilic temperatures (Uzeh *et al*., 2006).

The results also indicated *Staphylococcus aureus* as the most prevalent bacteria being implicated. The isolation of *Staphylococcus aureus* is of public health significance since it is said to be a commonly recovered pathogen in outbreaks of food poisoning due to milk and milk product (Junaidu *et al*., 2011).

The high frequency of occurrence of *S. aureus* in the four locations might refer to improper public health measurement, sanitary and poor cleaning of people concerned with milk marketing. In addition, the primitive system oftransportation and marketing practiced in these locations may also be predisposing factors. *Staphylococcus aureus* may originate

from mastitic animals or human sources (Akram *et al.*, 2013; Oliver *et al*., 2005). Kaduna North had the highest occurrence of *S. aureus*. This may have been due to the primitive systems of storage, transportation and marketing practices at the sampling locations which were the motor parks and the market places. Obviously, the quantities of *S. aureus* in food products are related to these factors.

The presence of *Salmonella* in Nono is in close agreement with that of Junaidu *et al*. (2011) who isolated 2(2.17%) in clinically mastitic cow and 2.6% overall prevalence reported by Van Kessel *et al.* (2004) from bulk tank milk. This was however higher than 0.0% prevalence reported by Mhone *et al.* (2012). The presence of this organism may indicate fecal contamination of milk but more importantly an indicator for poor sanitary practice during milking. De- Buyser (2001) had reported that *Salmonella* is one of the most etiological agents implicated in several outbreaks associated with the consumption of raw milk and milk products. All salmonellae are of public health concern having the ability to produce infection ranging from a mild self limiting form of gastroenteritis to septicemia and life threatening typhoid fever (Oliver *et al.*, 2005). Thus, their presence in raw milk samples poses health risks to consumers that consumed them without any heat treatment. The *Salmonella* spp. isolated was *S. pullorum. S. pullorum* is of the *Salmonella* serovar *Gallinarum* and is responsible for fowl typhoid and Pullorum disease of breeding flocks (Proux *et al.,* 2002). Although *S. pullorum* are considered to be adapted to birds, a few infections have been reported in mammals (Anderson *et al.,* 2006). *S. pullorum* occasionally causes acute, self limiting enteritis in people who eat massively contaminated food (CFSPH, 2009) hence *S. pollurum* is not considered to be a serious public health concern.

5.5 Antibiogram and Multidrug Resistance

Isolates of both genera demonstrated high levels of susceptibility to Gentamycin, Ciprofloxacin and Chloramphenicol Tables 4.12 and 4.13. This may have implications on the effectiveness of the local and probably antibiotics resistance control programs. The resistance to moderate resistance to Penicillin (Cefoxitin) by the *Salmonella* and *S.aureus* isolates respectively corroborates documented increasing penicillinase-producing β-lactamases among organisms. The high performance of some of these antibiotics (Gentamicin, Ciprofloxacin and Chloramphenicol) could also be due to their molecular sizes a factor which enhances their solubility in diluents thus promoting their penetration power through cell wall into the cytoplasm of the target microorganism. This finding is in line with Maillard (2002) and Poole (2002) who respectively opined that the high efficacy of antibiotics may be traced to their molecular sizes. The present study revealed a high resistance to tetracycline (65.52%) by the bacterial isolates. Tetracyclines are broad-spectrum antibiotics that are active against erlichias, rickettsias, anaplasmas and *Mycoplasma* as well as some protozoa (Prescott, 2000). Tetracycline is presently usedwidely in animal husbandry and human clinical medicine because of its low toxicity and broad spectrum of activity (Kim *et al.,* 2006) which makes it affect a broad range ofbacterial species. These potentialshave increased its opportunityto develop resistance in diverse populations (Steinman *et al.,* 2003). This trend of resistance is therefore a matter of concern for humans, for livestock disease management and production in general owning to the existing emergence of bacterial strains resistant to other major antibiotics. The use of antibiotics in food animals had been reported to enhance the spread of bacteria resistant to antibiotics via the food to humans and cause human infection (Phillips *et al.,* 2004). A

similar trend of resistance has been reported by Oluyege *et al*. (2009), who reported various organisms' resistance to antimicrobials, including oxytetracycline in cooked food sold on a south-western university campus in Nigeria. The presence of *tet*^{r}gene, responsible for resistance in tetracycline had also been reported in food animal production environment in Nigeria by Adelowo and Fagede, (2008). More also, this determinant gene (tet^{r}) is located on transferable plasmids and are moved between unrelated species and genera by conjugation (Chopra and Roberts, 2001; Roberts, 2005).

Multidrug resistance for this study is defined as resistance of isolate to three or more antibiotics (Lambert, 2002 and Osundiya *et al.,* 2013). MAR index was calculated as the ratio of number of antibiotics to which an organism is resistant to total number of antibiotics to which an organism is exposed (Furtula *et al.,* 2013). MAR index values greater than 0.2 indicates high risk source of contamination where antibiotics are often used (Furtula *et al.,* 2013). Multiple antibiotic resistance (MAR) in bacteria is most commonly associated with the presence of plasmids which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype (Daini *et al.,* 2005).

Giwa location had the highest percentage occurrence of MAR (40%) (Fig.4.3) suggesting area of high and indiscriminate usage of antibiotics (Furtula *et al.,* 2013). This observation in Giwa suggests that the high antibiotic consumption rate in the area may have arisen by no other factor other than increases in animal population. Similar trends of increased livestock-antibiotics usage had been reported by Adesokan *et al. (*2015). Recent researches by Van Boeckel *et al.* (2014) and Adesokan *et al.* (2015) while surveying global antibiotics consumption and antimicrobial usage in livestock animals in south-western Nigeria

respectively have shown substantial increase in antibiotics consumption in humans and livestock in developing countries. This is a matter of concern for livestock disease management and livestock production in general owning to the existing emergence of bacterial strains resistant to major antibiotics. This pattern of antibiotic consumption is usually due to lack of regulatory control in the sales of veterinary drugs in most developing countries. The high percentage occurrence of multiple antibiotics resistance (MAR) index (68.97%) among the bacterial isolates in this study might have arisen due to common practices such as self medication and over the counter usage of antibiotics. In addition to this is continued administration of antibiotics repeatedly against infections that appear non-responsive to the normal dose given earlier (Ezenduka *et al.,* 2011). The residues of antibiotics have been reported in tissues of food animals and their products (Kabir *et al*. 2004; Adesokan *et al*. 2013). Another probable reason for high percentage occurrence of multiple antibiotics resistance (MAR) in *S.aureus* isolates could be as opined by Gundogan *et al.* (2006) that *S. aureus* strains are known to be frequently resistant to antibiotic therapy due to their capacity to produce an exopolysaccharide barrier and because of their location within microabscesses, which limit the action of drugs.

Manshanu had higher percentage occurrence of MAR, *Mec A* and *SE* genes than other fresh milk and milk products obviously because the product had the highest number of *S.aureus* occurrence.

5.6 Expression of Staphylococcal Enterotoxin in Samples

Enterotoxin A was detected to be mostly expressed by *S. aureus* in this study (Table 4.15). It is known that SEs are similar in structural and biological properties but differ in amounts

produced and in the mechanismsof gene regulation (Klotz *et al*., 2003). SEA is produced throughout the log phase of growth, while SEB, SEC, and SED are produced in greater quantities during the transition from the exponential to the stationary phase of growth (Zhang and Stewart, 2000). However, in accordance with the instructions of the manufacturer, SET-RPLAis performed with overnight liquid cultures. These cultures are in the stationary phase. This factor might explainwhy more strains are found positive for SEA using SET-RPLA. The locations of the enterotoxin genes are also different. SEA is carried on a prophage (Betley and Mekalanos, 1985), and SED is carried by a plasmid. The gene for SEC is localized on pathogenicity islands (Zhang and Stewart, 2000). The SEB gene (*ent B*) is also located on a chromosome (Novick *et al.*, 2003). It is also known that the transcription of SEB, SEC and SED is subject to regulation by the accessory gene regulator (*agr*) two-component regulatory system, which controls most of the staphylococcal exoprotein virulence factors (Novick *et al*., 2001), and that thetranscription of SEA is not regulated by the *agr* system (Zhang and Stewart, 2000). SET-RPLA is based on the *agr* system; hence more of SEA was produced.

Manshanu and fresh milk were mostly contaminated by enterotoxigenic strains of *S. aureus. Staphylococcus aureus* is quite sensitive to microbial competition. This feature has been particularly well studied in fermented food products. Genigeorgis (1989) demonstrated that the higher the concentration of competing microorganisms in milk, the lower the rate of *S. aureus* growth and SE production. Competition with lactic acid bacteria has been reported in other researches on cheese (Otero *et al*., 1988; Vernozy-Rozand *et al.,* 1998) and fermented sausage production (Sameshima *et al*., 1998). The effect of lactic acid starter is mainly due to lactic acid production, lowered pH, oxygen

peroxide production, competition for nutrients and is sometimes due to the synthesis of antimicrobial substances, such as bacteriocins (Le Loir, 2003). Manshanu and fresh milk had initial lower counts than Nono and Kindrimo suggesting reason for their higher production of enterotoxin.

The variations of staphylococcal isolates in their ability to produce enterotoxins depend on the source (Bennet, 1986). Human interference and the dairy food producers also determine the level of contamination of dairy products. Manshanu was more contaminated with enterotoxigenic strains of *S. aureus* suggesting more handling. Manshanu is usually dished out with bare hands by the local producers and retailers, hence the more the handling, the more contamination.

5.7 PCR Amplification of Target Genes

5.7.1 PCR amplification of *Sal-En* and *Inv A* genes

The genes, *Salmonella* enterotoxin *Sal-En or Stn* and the *Inv A* gene were absent in the *Salmonella* isolate, *Salmonella pullorum. S. pullorum* is of *S. enterica* subsp. enterica, serovar Gallinarium biovar Pullorum.*Salmonella* enterotoxin*(Stn)* is said to be widely distributed among the *Salmonella* serovars irrespective of their serotypes and source of isolation (Moore and Feist, 2007; Lee *et al.*, 2009). *Salmonella* enterotoxin (*Stn)*is opined to be involved in the maintenance of membrane composition and integrity (Nakano *et al.*, 2012), though it is proposed not to be associated with the virulence of *Salmonella* (Invasion, survival in macrophages, cytotoxic activity and enterotoxicity) by some researchers (Watson *et al.*, 1998; Wallis *et al.*, 1999). The *Inv A* is also one of the genes

that assist the organism in expressing its virulence in the host cells. *Inv A* genes are involved in adhesion and invasion alongside other genes such as *Sef* genes (Murugkar *et al.,* 2003).

The virulence determinants of *Salmonella enterica* are clustered into characteristic genomic regions known as SPIs (S*almonella* pathogenicity islands), while certain genes located on large plasmids are also known to be important in the virulence of this bacterium (Marcus *et al.,* 2000 and Hensel, 2004). During the process of *Salmonella* infection, theseinvasion genesare required for bacterial entry into host cells (Mills *et al*., 1995). These genes are present in all invasive strains of Salmonella (Galan, 1996). Variations reportedly exist among serotypes with respect to the presence or absence of SPIs, a feature predicted to be responsible for host adaptation, expansion of host range and differences in severity of infections by different serotypes (Bueno *et al.,* 2004 and Saroj *et al.,* 2008). *Salmonella pullorum* is known to cause Fowl typhoid in birds though afew infections have been reported in mammals (Anderson *et al.,* 2006).

5.7.2 PCR amplification of *SEA, SEB, SEC* and *SED* genes

The frequency of enterotoxin-encoding genes in *S. aureus* from fresh milk and milk products observed in the present study (28.57%) is an indication of the probability of milk serving as source of food poisoning by *S.aureus* when not properly stored. This was similar to 31.1% reported by Imanifooladi *et al*. (2010) in *S. aureus* from domestic dairy products in Iran. High frequencies of enterotoxin-encoding genes have been reported in *S. aureus* from bovine origin in studies carried out in Brazil (Rall *et al.* 2008) and in France (Rosec and Gigaud, 2002). The PCR results showed a clear difference with the Reversed

Passive Latex Agglutination (RPLA) assay results, in contrast with other researchers (Mclauchin *et al.,* 2000; Jorgensen *et al*., 2005; Anvari *et al.,* 2008 and Imanifooladi *et al.,* 2010). In this study, only one isolate (5.0%) was able to be detected genotypically for the SEB gene which is an indication of differences between the genotypical and phenotypical methods. This trend is similar to that observed by Imanifooladi *et al.* (2010). It had been stated that under the best conditions, 40% to 50% of *S.aureus* isolates with SEB gene are capable of enterotoxin production (Najera-Sanchez, 2003).

On the whole, only isolates harbouring a single gene were detected, but positive isolates harbouring two or more genes have been reported in *S. aureus* from goat (Scherrer *et al*. 2004, Lyra *et al*. 2013) and bovine milk (Rall *et al*. 2008).The occurrence of multiple genes in *S. aureus* is considered rare (Jorgensen *et al., 2005*), which can explain the negative results obtained for *SEC and SED* genes in this study. Some conditions are said to be necessary for *S. aureus* to produce enterotoxins throughout the logarithmic phase of growth or during the transition from the exponenential to the stationary phase (Otero *et al.,* 1990; Betly and Mekalanos, 1998). It has been stated that the production of Staphylococcal enterotoxins (SEs) may depend on the host environment and this may play a role in the adaptation of *S. aureus* to the host. Therefore, the expression of enterotoxin genes depends on factors such as the origin and identity of the bacterial isolate and the host environment of the bacterial isolate (Imanifooladi, 2010). The host plays an important role in assisting adaptation between the bacteria and their surrounding environment. Bacteria isolated from cows mostly produce SEA and SED (Morandi *et al.,* 2007; Normanno *et al*., 2005) while most bacteria isolated from skin and human wounds produce SEB (Imanifooladi, 2007). The results showed the phenotypical methods to be sensitive, unlike what have been

previously reported by Imanifooladi, (2010). Techniques based on DNA such as PCR are able to determine the presence of enterotoxin genes but cannot demonstrate their expression. The polymerase chain reaction (PCR) is a technique for the *in vitro* amplification of specific segments of DNA. It offers a rapid, sensitive and specific identification method for the genes responsible for toxins produced by *S.aureus* (Johnson *et al.,* 1991; Wilson *et al.,* 1991; Tsen and Chen, 1992; Becker *et al.,* 1998 and Wang *et al.,* 2002).

5.7.3 PCR Amplification of *Mec A* Gene

Methicillin-resistant *S. aureus* (MRSA) includes *S. aureus* that has acquired a gene, called *MEC* A, giving them resistance to methicillin and essentially to all other beta-lactam antibiotics (Lee, 2003, Ito *et al.,* 2014). The study revealed a total of 11(55%) isolates with this gene. The presence of *mec* A gene might have occurred as a result of inappropriate antibiotics usage (Buyukcangaz *et al.,* 2013). This is not under estimated in the formation of bacterial resistance and in multidrug resistant strains (Chambers, 1997).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The following conclusions are therefore made from this research;

i. The nutritional content of the fresh milk and milk products showed that they were of appreciable nutritional status especially in the ash (mineral) and protein content for fresh milk. The lactose and lipid contents in the fresh milk and milk products were above the recommended standards and hence were good substrates for bacterial growth and multiplication.

ii. The high total viable counts of microorganism and the presence of a wide range of organisms, particularly enteric pathogens isolated from the fresh milk and milk products showed that these informally marketed fresh milk and milk products in the four Local government areas could be an important source of food borne infection. This therefore might create health hazards for consumers of these fresh milk and milk products, particularly as the transfer of pathogens via such food chain is very well documented.

iii. Antibiogram of bacterial isolates revealed high performance of some of the antibiotics used such as gentamycin, ciprofloxacin and chloramphenicol while resistance was exhibited for tetracycline, a commonly used antibiotics thereby posing a public health concern.

iv. The study also revealed the occurrence and expression of the classical Staphylococcal enterotoxin by enterotoxin producing strains of *Staphylococcus*

aureus in the fresh milk and milk products, a serious public health threat for consumers of these local dairy products.

v. It can also be concluded that fresh milk and milk products harbour enterotoxigenic strains of *S. aureus*. The genes encoding two (2) of the classic genes (*SEA* and *SEB*) were detected.

vi. The co-occurrence of enterotoxin genes and *Mec A* gene (an antibiotics resistance gene) in the tested *S. aureus* isolates were also detected. This indicates that the pathogenic potential of *S. aureus* may be greater than previously thought in fresh milk and milk products. The results also showed that Staphylococcal enterotoxic genes have a broad distribution worldwide and also show differences in the geographical and host distribution of these genes.

6.2 Recommendations

From the findings of this research the following recommendations are therefore made;

i. An important source of microbial contamination of the milk is faecal pollution probably from cow dung. There is then need for instituting effective control measures to protect public health. This includes mandatory milk pasteurization by traders and improved hygienic handling of the commodity during milking and ensuring milking is not done on cow dung.

ii. There is also the need to carry out a similar study with raw milk not sold through informal chains to assess the health risk. Such milk is usually produced by farms of the Universities, Research institutes and Ministries of Agriculture in Nigeria.

iii. There is need for educational and extension programs for consumers, producers and users about the risk of consumption of raw milk in order to evaluate and correct the situation.

iv. The main obstacle for researchers working to identify solutions to antimicrobial resistance is lack of data, especially on drug use. There is therefore need for a more robust data on the types and quantities of antibiotics sold for use in humans and food-producing animals. This would include the purpose of their use; disease treatment, disease prevention or growth promotion (for animals) and the method used to administer them. This will allow researchers to determine how the uses of antibiotics affect or bring about the emergence of antibiotic-resistant bacteria.

v. The recent identification of new Staphylococcal enterotoxins has considerably increased the perceived frequency of enterotoxigenic staphylococci isolates. This has therefore created need for further studies to confirm the expression of these new enterotoxins by *S.aureus*, and to assess their significance for food borne disease, for Nigerian cases.

These findings therefore indicate the need for regular monitoring of fresh milk and milk products essential for this pathogen to prevent public health problems.

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APPENDICES

Appendix I: Biochemical Characteristics of Presumptive *Staphylococcus* spp. Isolates**.**

Serial number	Isolate code	Coagulase Production	Catalase Test	Fermentation of glucose and mannitol	Inference
1	CHk10	+	+	+	*S.aureus*
2	CHk7	+	+	+	*S.aureus*
3	CHN14	+	+	+	*S.aureus*
4	CHN11	+	+	+	*S.aureus*
5	CHN9	+	+	+	*S.aureus*
6	CHMn6	-	+	-	*Staphylococcus* spp.
7	SBMd8	-	+	-	*Staphylococcus* spp.
8	SBN13	+	+	-	*S.aureus*
9	SBMd11	-	+	-	*Staphylococcus* spp.
10	SBMd9	-	+	-	*Staphylococcus* spp.
11	SBN12	-	+	-	*Staphylococcus* spp.
12	SBMn3	-	+	-	*Staphylococcus* spp.
13	SBMd1	-	+	-	*Staphylococcus* spp.
14	SBMd20	+	+	-	*S.aureus*
15	SBMd9	+	+	+	*S.aureus*
16	SBMd4	+	+	-	*S.aureus*
17	SBMd3	-	+	-	*Staphylococcus* spp.
18	SBk3	-	+	-	*Staphylococcus* spp
19	SBMd8	+	+	-	*S.aureus*
20	SBMd16	-	+	+	*Staphylococcus* spp
21	SBMd17	-	+	+	*Staphylococcus* spp
22	GWMn20	+	+	-	*Staphylococcus* spp
23	GWN19	+	+	-	*Staphylococcus* spp
24	GWMd15	-	+	-	*Staphylococcus* spp
25	GWk19	+	+	-	*Staphylococcus* spp
26	GWMn19	+	+	-	*Staphylococcus* spp

27	GWMn16	+	+	-	*Staphylococcus* spp
28	GWMn17	+	+	-	*Staphylococcus* spp
29	GWMd15	+	+	+	*S.aureus*
30	GWMd14	+	-	-	*Staphylococcus* spp
31	GWMn11	+	+	-	*Staphylococcus* spp
32	GWMd19	+	-	-	*Staphylococcus* spp
33	GWMn5	-	-	+	*Staphylococcus* spp
34	GWMn18	-	+	+	*Staphylococcus* spp
35	GWMn20	-	-	-	*Staphylococcus* spp
36	GWMn7	+	-	-	*Staphylococcus* spp
37	GWMn14	-	-	-	*Staphylococcus* spp
38	GWMn8	-	-	-	*Staphylococcus* spp
39	GWMn13	+	-	-	*Staphylococcus* spp
40	KdN6	-	-	-	*Staphylococcus* spp
41	Kdk6	+	-	-	*Staphylococcus* spp
42	KdMn3	-	+	-	*Staphylococcus* spp
43	KdMn7	-	+	-	*Staphylococcus* spp
44	KdMn4	+	-	-	*Staphylococcus* spp
45	KdMd11	+	+	-	*S.aureus*
46	KdMd3	+	-	-	*Staphylococcus* spp
47	KdMd13	+	+	+	*S.aureus*
48	KdMd1	-	-	-	*Staphylococcus* spp
49	KdMn12	+	+	-	*Staphylococcus* spp
50	KdK8	+	-	-	*Staphylococcus* spp
51	KdMn5	-	-	-	*Staphylococcus* spp
52	KdMd10	-	-	-	*Staphylococcus* spp
53	KdMn7	+	+	-	*S.aureus*
54	KdK9	+	-	-	*S.aureus*
55	KdN12	+	+	-	*S.aureus*
56	KdMn8	-	-	-	*Staphylococcus* spp

Appendix II: Biochemical Characteristics of Presumptive *Salmonella* spp. Isolates**.**

Serial Number	Isolate Code	TSI			Indole Test	Oxidase	Citrate	Motility	Inference
		Slant	Butt	H2S					
1	CN8	NC	AG	+	-	-	+	+	*Salmonella enteritidis*
2	CN6	A	AG	+	-	-	+	-	*Proteus* spp.
3	SMd19	A	AG	+	+	-	+	+	*Proteus* spp.
4	SK15	A	AG	+	-	-	-	-	*Proteus* spp.
5	SK16	A	AG	+	-	-	-	+	*Proteus* spp.
6	SMd2	A	AG	-	-	-	+	+	*E. coli*

Key: AG = Acid (yellow) and gas formation. A = Acid (yellow), NC = no change, + = hydrogen sulphide (black), - = no hydrogen sulphide (no black colouration).

Appendix III: Biochemical characterization of Enterobacteriaceae isolates using Microgen^{TM} Enterobacteriaceae GNA- ID System.

Biochemical reaction	*Salmonella*	*P. stuartii*	*C. diversus*	*H. alvei*	*P. vulgaris*	*P.mirabilis*	*A.haemolyticus*	*A. lwofi*	*E.coli (In)*
Lysine	+	-	-	+	-	-	+	-	+
Ornithine	-	-	+	+	-	+	+	-	+
H_2S	+	-	+	-	+	+	-	-	-
Glucose	+	+	+	-	+	+	-	-	+
Mannitol	+	-	-	-	-	-	-	-	+
Xylose	-	-	+	+	-	+	-	-	+
ONPG	-	-	+	+	+	-	-	+	-
Indole	-	-	+	-	-	-	-	-	+
Urease	+	+	+	-	+	+	-	-	-
V.P	-	-	-	+	-	+	-	+	-
Citrate	+	+	+	-	-	-	+	-	-
TDA	-	+	-	+	+	+	-	-	-

Key: = + Positive,- Negative,E.coli(In)=*Escherichia coli(inactive),* A.lwofi= *Acinetobacter lwofi, A.haemolyticus=Acetobacter haemolyticus*, *P.mirabilis= Proteus mirabilis, P.vulgaris= Proteus vulgaris, H.alvei=Hafnia alvei, P. Stuartii=Providencia stuartii.C. diversus= Citrobacter diversus.*

Appendix IV: Biochemical characteristics of *Staphylococcus* species isolated from milk and milk products using Microgen Staphylococcus-ID system.

B/c Rxn	*S.aureus*	*S.chromogens*	*S. hyicus*	*S.xylosus*	*S.capitis*	*S.haemolyticus*
LAT	+	-	+	-	-	-
CPG	+	+	+	+	-	+
NIT	+	+	-	-	-	+
SUC	-	-	-	-	-	+
TRE	+	+	-	-	-	+
MAN	+	-	-	-	-	+
NAG	+	+	-	+	-	+
MNS	+	-	-	+	-	+
TUR	-	-	-	-	-	+
PHO	+	+	+	+	+	+
βGL	+	+	+	+	-	+
βGN	-	-	+	+	-	+
NIT						
URE	+	+	-	+	-	-
ARG	+	+	+	-	+	+
PYR	-	-	-	+	-	-

Key: + Positive, - negative. B/Ch rxn= Biochemical reaction. LAT=index agglutination test, CPG=colony pigmentation,NIT =nitrate, SUC=sucrose, TRE=trehalose, MAN=Mannitol ,NAG=N-acetylglucosamine,MNS=mannose, TUR=turanose, PHO=alkaline phosphate, βGL=β-glucosidase, βGN=β-glucoronidase,URE=urease,ARG=arginine,PYR=pynolidonyl arylamidase.

Appendix V: Antibiotic Disk Interpretative Criteria and Quality Control

Antibiotic	CODE	CLS, Interpretation Criteria	S≥	I Diameter (mm)	R≤
Tetracycline	TE	Enterobacteriaceae	15	12 – 14	11
		Staphylococcus spp.	19	15 – 18	14
Trimethoprim/ sulfamethoxazole	SXT	Enterobacteriaceae	16	11 – 15	10
		Staphylococcus spp.	16	11 – 15	10
Gentamicin	CN	Enterobacteriaceae	15	13 – 14	12
		Staphylococcus spp.	15	13 – 14	12
Erythromycin	E	*Staphylococcus* spp.	23	14 – 22	13
Amoxicillin/ clavulanic acid	AUG	Enterobacteriaceae	18	14 – 17	13
		Staphylococcus spp.	20	--- --	19
Chroramphenicol	C	Enterobacteriaceae	18	13 – 17	12
		Staphylococcus spp.	18	13 – 17	12
Cefoxitin	FOX	Enterobacteriaceae	18	15 – 17	14
		S. aureus	22	--- --	12
Ciprofloxacin	CIP	Enterobacteriaceae	21	16 – 20	15
		Staphylococcus spp.	21	16 – 20	15
vancomycin	VA	*Staphylococcus* spp.	17	15 – 16	14

Appendix VI: REAGENTS AND MEDIA

(a) Oxidase reagent

To make 10ml

Tetremethyl – p – phenylenediamineDihydrochloride ----------0.1g

Distilled water ----------10ml

(Prepared immediately before use)

(b) Kovac's Reagent.

P. Dimethylamino Benzaldehyde ----------10g

Concentrated Hydrocloric Acid (HCL) ----------50ml

Gently add the acid and store in a refrigerator.

(c) Mannitol Salt Agar

Approximate Formula* Per Liter Purified Water

Beef Extract .....1.0 g

D-Mannitol…….....10.0 g

Pancreatic Digest of Casein .....5.0 g

Phenol Red…….....0.025 g

Peptic Digest of Animal Tissue .....5.0 g

Agar .....15.0 g

Sodium Chloride .....75.0 g

*Adjusted and/or supplemented as required to meet performance criteria.

(d) Cooked meat medium

Heart muscle----------454.0

Peptone----------10.0

Lab-Lemco powder----------10.0

sodium chloride----------5.0

glucose ----------2.0

pH 7.2 ± 0.2 at 25^0C.

(e) Mueller- Hinton Agar

gm/litre

Beef,dehydrated infusion from ---------- 300.0

Casein hydrolysate ---------- 17.5

Starch ----------1.5

Agar ----------17.0

pH 7.4 ± 0.2

(f) Nutrient Agar

gm/litre

'Lab-Lemco' powder ----------1.0

Yeast extract ----------2.0

Peptone ----------5.0

Sodium chloride ----------5.0

Agar ----------15.0

pH 7.4 ± 0.2

(g)Peptone Water

gm/litre

Peptone ----------10.0

Sodium chloride ----------5.0

pH 7.2 ± 0.2

(h) Selenite F Broth

gm/litre

Peptone from casein ---------- 5.0

L (-) Cystine ----------0.01

Lactose ----------4.0

Phosphate buffer ----------10.0

Sodium hydrogen selenite ----------4.0

pH 7.0 ± 0.2

(i)Brain heart Infusion Agar

gm/litre

Calf brain infusion solids ---------- 12.5

Beefheart Infusion solids ----------5.0

Proteose peptone ----------10.0

Solid chloride ---------- 5.0

Glucose ---------- 2.0

Di- sodium phosphate ----------2.5

Agar ----------10.0

pH 7.4 ± 0.2

(j) Simmons citrate Agar

gm/litre

Magnesium Sulphate ----------0.2

Ammonium dihydrogen phosphate ---------- 0.2

Sodium ammonium phosphate ---------- 0.8

Sodium citrate, tribasic ---------- -2.0

Sodium chloride ----------5.0

Bromothymol blue ----------0.08

Agar ----------15.0

pH 7.0 ± 0.2

(k) Salmonella shigella Agar

gm/litre

'Lab- Lemco' powder ----------5.0

Peptone ---------- 5.0

Lactose ----------10.0

Bile salts ---------- 8.5

Sodium citrate ---------- 10.0

Sodium thiosulphate ---------- 8.5

Ferric citrate ---------- 1.0

Brilliant green ---------- 0.00033

Neutral red ---------- 0.025

Agar ----------15.0

pH 7.0 ± 0.2

(l)Baird – Parker agar

gm/litre

Casein Peptone ---------- 10

Meat extract ---------- ----5

Yeast extract ---------- ---1

Lithium chloride ---------- 5

Glycine ----------12

Sodium pyruvate ---------- 10

Agar ----------15

pH 6.8 ± 0.2

(m)Triple sugar iron Agar

gm/litre

'Lab-lemco' powder ---------- 3.0

Yeast extract ---------- 3.0

Peptone ---------- 20.0

Sodium chloride ---------- 5.0

Lactose ---------- 10.0

Sucrose ---------- 10.0

Glucose ---------- 1.0

Ferric citrate ---------- 0.3

Sodium thiosulphate ---------- 0.3

Phenol red ----------0.024

Agar ---------- 12.0

pH 7.4 ± 0.2