BACTERIOLOGICAL QUALITY AND INCIDENCE OF SALMONELLA SPECIES FROM RAW AND FERMENTED MILK IN FIKA LOCAL GOVERNMENT AREA YOBE STATE, NIGERIA

BY

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**A DISSERTATION SUBMITTED TO THE SCHOOL OF POST GRADUATE STUDIES,
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DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, FACULTY OF VETERINARY MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA

AUGUST, 2018

DECLARATION

I hereby declare that the work in this dissertation titled "Bacteriological quality and incidence of *Salmonella* species from raw and fermented milk in Fika local government area Yobe state, Nigeria" was performed by me in the Department of Veterinary Public Health and Preventive Medicine, under the supervision of Dr. M. K. Lawan and Prof. C. N. Kwanashie. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation has been previously presented for another degree or diploma at any institution.

Dauda Ezra Babale _____ _____

Signature Date

CERTIFICATION

This dissertation titled "Bacteriological quality and incidence of *Salmonella* species from raw and fermented milk in Fika local government area Yobe state, Nigeria" by **Dauda Ezra Babale** meets the regulations governing the award of the degree of Masters of Veterinary Public Health and Preventive Medicine of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Dr. M. K. Lawan Chairman, Supervisory Committee	Signature	Date
Prof. C. N. Kwanashie Member, Supervisory Committee	Signature	Date
Prof. M. Bello Head of Department	Signature	Date
Prof. S. Z. Abubakar Dean, School of Postgraduate Studies	Signature	Date

DEDICATION

This work is dedicated to my parents Mr. and Mrs. Dauda Babale, for their prayers and support throughout the course of my study.

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First of all, I wish to express my deep gratitude to God almighty who gave me life, health, strength and ability to finish my thesis.

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ABBREVIATIONS

BG	Brilliant Green agar
BIS	Bismuth Sulphite agar
BPW	Buffered Peptone Water
CDC	Centers for Disease Control and Prevention
CFU	Coliform Forming Units
DNA	Deoxyribonucleic Acid
H	Flagella antigen
KW	Kaffmann White scheme
L. G. A.	Local Government Area
MKTTn	Muller-Kauffmann Tetrathionate- Novobiocin broth
MR-VP	Methyl Red Voges Proskauer broth
MSRV	Modified Semisolid Rappaport Vasiliadis
NAFDAC	National Agency for Food and Drug Administration and Control
O	Somatic antigen
PCR	Polymerase Chain Reaction
RVS	Rappaport Vasiliadis Soy broth
SC	Cystine broth
SD	Standard deviation
SIM	Sulfide, Indole, and Motility medium
Spp	Species
TAPC	Total Aerobic Plate Count
TSI	Triple Sugar Iron Agar
TT	Tetrathionate Broth
UHT	Ultra high temperature

USAID	United States Agency for International Development
Vi	Capsular Polysaccharide Antigen
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate agar

ABSTRACT

Salmonellosis is one of the most important food borne diseases worldwide, transmitted through a wide range of animal products including milk and milk products. An investigation was conducted to determine the bacteriological quality, prevalence and antibiogram of *Salmonella* species from raw and fermented cow milk. A total of 344 samples were collected from three selected major markets and Fulani herdsmen settlements in Fika local government area, comprising of 167 raw milk and 177 fermented milk samples respectively. The samples were analyzed for total aerobic plate count (TAPC). Isolation and characterization of *Salmonella* was done using cultural, conventional biochemical tests and Microbact 24E, after which antibiotic susceptibility of each isolate was determined by Kirby Bauer disk diffusion method. *Salmonella* isolates were screened for *inv*A virulence gene using polymerase chain reaction (PCR). The TAPC of both raw and fermented milk ranged between 5.1 $\log_{10}$ CFU/ml to 7.4 $\log_{10}$ CFU/ml, with mean and standard deviation of 5.82±2.06 $\log_{10}$ CFU/ml and 6.62±1.10 $\log_{10}$ CFU/ml respectively. Overall *Salmonella* prevalence of 1.7% was obtained from the samples; of which higher prevalence of 2.3% was obtained from raw milk samples while fermented milk had 1.1%. All of the isolates were susceptible to enrofloxacin and Gentamycin (100%) while none of the isolates was susceptible to Amoxycillin Clavulinic acid, Cefotaxime, ticarcillin and Chloramphenicol. *Salmonella* isolates were also susceptible to Imipenem (83.4%), Ciprofloxacin (83.4) and Sulfamethoxazole (50%). All the isolates of *Salmonella* exhibited varying resistance pattern, they were resistant to at least five antimicrobial agents tested, while one isolate was resistant to nine antimicrobial agents. Three isolates (two from raw milk and one from fermented milk) were positive for *inv*A gene. The study has established high Total Aerobic Plate Counts with minimum counts exceeding the acceptable limit of 5.7 Log_{10} CFU/ml and the occurrence of multidrug resistant *Salmonella* species in fresh and fermented milk samples. Therefore, adequate hygiene should be maintained at milking points and markets.

TABLE OF CONTENTS

CONTENTS PAGE

LIST OF TABLES

LIST OF FIGURE

LIST OF PLATE

LIST OF APPENDICES

CHAPTER ONE

1.0 INTRODUCTION

1.1 Introduction

Salmonellae are Gram negative, short, plump, rod shaped, non-spore forming, noncapsulated, aerobic and facultative anaerobic organisms that is classified under the family *Enterobacteriaceae*, (Manyi-Loh *et al*., 2016). The genus *Salmonella* consists of two species: *Salmonella enterica*, and *Salmonella bongori*. The *Salmonella enterica* is subdivided into six subspecies enterica (I), salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV) and indica (VI). There are more than 2541 serotypes majority of which belong to the *Salmonella enterica* subspecies enterica (Percival *et al*., 2004; Pui *et al*., 2011).

Salmonellosis is one of the most common food-borne bacterial diseases worldwide (Forshell and Wierup, 2006). There have been increased outbreaks of human salmonellosis in most parts of the world resulting from animal infections (Forshell and Wierup, 2006). Foods of animal sources like raw meats, milk and milk products, and eggs have been incriminated as sources of infection to humans (Brodsky *et al.*, 2002). Other sources of infection are water, soil, and vegetables contaminated with animal feces (Ekdahl *et al.*, 2005).

Milk is an opaque white liquid produced by the mammary glands of mammals that provides primary source of nutrition for young mammals (William and Ruth, 2005; Olatunji *et al*., 2012). Exact components of raw milk vary by species, but it contains significant amounts of saturated fat, protein and calcium as well as vitamins (Donovan, 2006). Cow's milk has a pH ranging from 6.4 to 6.8, making it slightly acidic (William and

Ruth, 2005). Milk is an essential first food for man, for countless of generations. It has formed an important part of man's diet not only for the infant, but in many societies throughout life (Komorowski and Early, 1992). Milk provides excellent medium for growth of both pathogenic and spoilage microorganisms (Lawan *et al*., 2012; Seema, 2015).

Mixed fermentations take place in milk as a result of the activities of natural flora present in the milk or from the environment (Parmjit, 2011). Fermented milk products are made in Asia, Africa, the Middle East, and in Northern and Eastern Europe (Savadogo *et al*., 2004). In Nigeria and especially in the Northern part, fermented milk is produced and consumed as supplement to main meals in homes and to supplement the economy of the family (Maduka *et al.*, 2014).

Milk and milk products provide a favorable environment for microbial growth and thus gets contaminated easily (Norman and Gravani, 2006). It has been documented that the bacteria associated with locally fermented milk products include lactic acid bacteria, *Escherichia coli* O157:H7, coliforms, *Salmonella* spp., *Campylobacter* spp., *Listeria* spp., and other pathogenic species (Gilmour and Rowe, 1990).

Pathogenic bacteria that have become resistant to antibiotic drug therapy have increased the problems of public health all over the world, and it is an increasing global health threat (Levy, 2001).

1.2 Statement of Research Problem

The incidence of food-borne infections is of global concern and these infections are mainly caused by enteric microorganisms, especially bacteria (CDC, 2005; WHO, 2007; Addis and Sisay, 2015); such as *Escherichia coli* O157:H7, coliforms, *Salmonella* spp,

Campylobacter spp and *Listeria* spp. Salmonellosis is one of the major causes of gastroenteritis affecting over 16 million people worldwide annually, with 500,000 to 600,000 of these cases proving to be fatal (Pang *et al*., 1998; USAID 2003; Ohud *et al*., 2012).

Many food-borne pathogens have been isolated from milk and milk products in some African countries such as Burkina Faso (Savadogo *et al*., 2004), South Africa (Beukes *et al.*, 2001), Ghana (Akabanda *et al*., 2010), Tanzania (Lubote *et al*., 2014), Algeria (Chenouf *et al*., 2016), and Ethiopia (Liyuwork *et al*., 2013). The presence of pathogenic bacteria in milk such as *Salmonella* spp, *Brucella* spp, *Mycobacterium bovis, Listeria monocytogenes* and *Campylobacter jejuni* can render raw and processed milk products unsafe for human consumption (Guerra *et al*., 2003; Nanu *et al*., 2007). In different parts of Nigeria, several investigations have demonstrated the presence of *Salmonella* in milk and fermented milk products. *Salmonella* has been isolated from fermented milk and milk products in Maiduguri and Bida (Ogbonna, 2011; Maduka *et al.,* 2013; Abdullahi and Mohammed, 2015). Other areas in the country where *Salmonella* spp has been isolated from milk and milk products include Kanam, Keffi and Zaria (Karshima *et al.,* 2013; Makwin *et al.,* 2014; Tamba *et al.,* 2016). It is doubtful if there is any safety measure or quality control to ascertain safety and wholesomeness of milk and fermented milk vended in Fika L. G. A. by any regulatory agency.

Milking and milk handling practices in informal sector are done commonly without observing hygienic practices. It is a common practice to vend milk in inappropriate containers and storage equipment (Kurwijila *et al.*, 2006; Oluwale *et al.,* 2017). Exposure of milk products to potential microbial contamination during processing, storage and

transportation without basic sanitary practices can result spoilage, decrease its shelf life and render it unfit for human consumption (Lu *et al.,* 2013). Food safety is a major concern with street foods as these foods are generally prepared and sold under unhygienic market conditions, with limited access to sanitary services and garbage disposal facilities (Rheinländer *et al*., 2008; Okojie and Isah, 2014). Fermented milk is produced in homes where it safety is not considered, however it is sold to all interested people as food (Omotosho, *et al.,* 2013). It is a common practice in the Fika L. G. A. to find people eating and drinking beverages at market places and patronizing mobile food vendors and safety of such food are not ascertained.

There has been increased reports of outbreaks of human salmonellosis in most parts of the world, resulting from animal infections, consumption of milk product, contaminated environment and facilities with animal faeces (De-Buyer *et al*., 2001; Forshell and Wierup, 2006; Pedro and Boris, 2001). Salmonellosis is often associated with gastroenteritis, particularly in children, the elderly, and immunocompromised, and can lead to invasive and focal infections that can be severe (Hohmann, 2001). Chronic carriage of *Salmonella* infection has been documented in children under the age of five, leading to prolonged duration of excretion and infection with different *Salmonella* and including resistant strains (Corrado *et al.,* 1992; Gabrielle and Nigel, 2013). *Salmonella* related diseases have been documented by some hospital-based studies conducted in different parts of Nigeria (Akinyemi *et al.,* 2000). Kayode *et al.* (2010) isolated *Salmonella* from humans in Ibadan, and Ifeanyi *et al.,* (2013) documented the presence of *Salmonella* in children in Abuja.

The emergence of multidrug-resistant *Salmonella* has been on the increase and has become a major health concern (Alcaine *et al.,* 2007; Francesca *et al.,* 2015). One of the contributing

factors for the widespread dissemination of multi drug resistant bacteria has been the indiscriminate prophylactic and therapeutic use of antimicrobial agents in food animals (Angulo *et al.,* 2004). Antimicrobial use in animal production systems has long been suspected to be a cause of the emergence and dissemination of antimicrobial resistant *Salmonella* (Alexander *et al.,* 2009). The relatively high level of resistance to antimicrobial agents constitutes a major threat to Public Health as it may spread bacterial resistance among the populace who come in contact with milk products (Okpalugo *et al*., 2008).

1.3 Justification

Poor market sanitation is an intractable problem in Nigeria and has contributed to the spread of infectious diseases and environmental degradation (Abejegah *et al.,* 2013). Most fermented milk vendors do not have permanent building for selling fermented milk. They are sold in open market where flies and dust particles can serve as sources of contamination. Therefore, there is need to assess the microbial quality of fermented milk sold at the markets.

In Nigeria and especially in the Northern part, fermented milk of various types are produced and consumed as supplement to main meals in homes and even for sale (Maduka *et al.,* 2014); most of the products are not subjected to quality control (Bertu *et al.,* 2010). Since there is little or no quality control during production and handling in informal sector, there is potential risk of contamination by zoonotic pathogens and adulterants which may be unsafe for human consumption.

Several studies in Nigeria have documented the occurrence of *Salmonella* in milk and milk products (Olatunji *et al*., 2012; Karshima *et al*., 2013; Laba and Udonsek, 2013) with prevalences of 8.7%, 4% and 17.06% from raw milk in Kanam, Ilorin and Gwagwalada

respectively. Tamba *et al*. (2016) documented a prevalence of 6.4% from raw milk and fermented milk in Zaria. However, there is paucity of information regarding bacteriological quality of raw and fermented milk In Fika Local Government Area.

The data obtained and analyzed may contribute in designing an effective control and prevention of *Salmonella* infection.

1.4 Aim and Objectives

1.4.1 Aim of the Study

The aim of the study was to evaluate the bacteriological quality and antibiogram of *Salmonella* identified from raw and fermented milk samples from Fika Local Government Area (L. G. A.) of Yobe state.

1.4.2 Objectives of the Study

1. To determine the total aerobic plate count of raw and fermented milk samples from Fika L. G. A. Yobe State.
2. To determine the occurrence of *Salmonella* of raw and locally fermented milk (nono) sold in Fika L. G. A. Yobe state.
3. To detect the presence of *inv*A gene from isolated *Salmonella* spp by PCR.
4. To determine the anti-biograms of *Salmonella* isolates from raw and locally fermented milk.

1.5 Research Questions

1. What is the estimated bacterial load of raw and fermented milk samples in Fika L. G. A Yobe State?
2. Are *Salmonella* species present in raw and locally fermented milk in Fika L. G. A. Yobe state?

3. Do the *Salmonella* specie isolates harbor *inv*A gene?
4. What are the anti-biograms of the *Salmonella* isolates?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Taxonomy

Salmonella is the genus of bacteria belonging to the family Enterobacteriaceae (Guthrie., 1991), composed of gram negative rod shaped, facultative anaerobic, catalase-positive, oxidase-negative (OIE, 2006), Although most members of this genus are motile by peritrichous flagella, a few non-flagellated variants such as *Salmonella enterica* subsp. *enterica* serovar Gallinarum and *Salmonella* Pullorum from poultry are non-motile (D'Aoust *et al*., 2001; Farmer III, 2003; Agbaje *et al.,* 2011). Salmonellae are chemoorganotrophic with ability to metabolize nutrients by both respiratory and fermentative pathways (D'Aoust *et al*., 2001). The genus was officially named after the American pathologist Daniel Elmer Salmon. His colleague, T. Smith, first isolated the bacterium from porcine intestine in 1884 as a common cause of hog cholera and designated the types train *Bacillus cholerae suis* (Lin-Hui Su and Cheng-Hsun Chiu, 2007). Later the name was changed to *Salmonella cholerae-suis* by J. Lignières in 1900. The nomenclature of *Salmonella* has undergone many changes within the past decades (Euzéby., 1999; Brenner *et al*., 2000; Tindall *et al*., 2005).

The genus *Salmonella* contains two species; *S. enterica* and *S. bongori*, which was formerly subspecies V. Six subspecies, are differentiated within *S. enterica* based on their biochemical and genomic characteristics, a Roman numeral and a name are used for the designation of these six subspecies as described by (Lamas *et al.,* 2018):

i. *enterica* - I
ii. *salamae* – II

iii. *arizonae* – IIIa

iv. *diarizonae* – IIIb

v. *houtenae* – IV

vi. *indica* – V

With regard to food safety *S. enterica* subsp. *enterica* is the subspecies of most concern because the strains within these serogroups are known to cause 99% of *Salmonella* infections in humans (Brenner *et al.*, 2000; Bell and Kyriakides, 2002).

2.2 Classification of *Salmonella*

Scientific classification

Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gamma Proteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Salmonella*
Species	*S. bongori* and *S. enterica*

The family *enterobacteriaceae* to which *Salmonella* belongs consists of five different genera namely: *Salmonellae, Escherichiae, Klebsiellae, Proteae* and *Edwardsiellae* (Tindall *et al*, 2005).

Taxonomy of *Salmonella* is complicated and classification of its species has been controversial for several years. However, the taxonomy of this genus is now established on a scientific basis and a corresponding nomenclature has been proposed by the World Health Organization (WHO) collaborating centre for references and research on *Salmonella*

(Lin-Hui Su and Cheng-Hsun Chiul, 2007). The genus *Salmonella* is divided into 5 biochemically distinct subgenera: the various motile and non-host adapted serotypes of subgenus 1 are often referred to as paratyphoid *salmonella*. Because the degree of genetic relatedness among the *salmonella* is so great, researchers suggest that the genus actually consists of only a single species (Tindall *et al*., 2005; Edwards and Ewing, 1986). However, the names of individual serotypes remain in common usage to facilitate diagnostic classification and epidemiological analysis.

Table 2.1 Species and subspecies in the *Salmonella* genus **(**Lamas *et al*., 2018**)**

Salmonella species	Subspecies	Number of Serovars
S. enterica	*enterica*	1531
	salamae	505
	arizonae	99
	diarizonae	336
	houtenae	73
	indica	13
S. bongori		22
Total		**2579**

Table adapted from Lamas *et al*. (2018).

2.3 Serological Identification of *Salmonella*

Serological identification of *Salmonella* serovars was first proposed by White and expanded by Kauffman (Le Minor and Poppof, 1987). The list of 2,501 *Salmonella* serotypes is maintained and annually updated by the World Health organization (WHO) with Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (Brenner *et al.,* 2000). The Kauffman- White scheme (KW) is based on the antigenic structure of *Salmonella* serotypes (Helmuth, 2000). The antigenic properties and variations of the O (surface polysaccharide) and H (flagellar) antigens from each serovar are summarized and described in what is known as the antigenic formulae.

The nomenclature of the genus *Salmonella* has evolved from the initial one serotype-one species concept proposed by Kauffmann on the basis of the of O (somatic) and H (flagellar) antigens, a capsular polysaccharide, the Vi antigen is present on *Salmonella* Typhi and few other serovars of *Salmonella*, including *Salmonella* Dublin (Mortimer *et al*., 2004; CIDRAP, 2006; Wattiau *et al.,* 2008).

The structure of each microbial cell is dependent on a variety of antigenic molecules, which are at the time dependent of many determinant groups (chemical groups). Thus it is the chemical makeup and the arrangement of these determinant groups that assign the immunological specificity of the antigen (Guthrie, 1991).

The cross absorption of antisera is used to reveal the antigenic structure of *Salmonella* (Helmuth, 2000). The composition and structure of polysaccharides, which constitute a part of the structure of the cell surface, allow for recognition and differentiation of O antigens (Guthrie, 1991). In the KW scheme O antigens are indicated in brackets when they are easily modified by mutation, otherwise they are underlined when these factors are determined by bacteriophages or plasmids (Helmuth, 2000). H antigens are present in the flagellar, they are composed of protein subunits called flagellin that are typically diphasic and thought to help the bacteria to survive host immune responses (Helmuth, 2000). A capsular polysaccharide is found in some serovars (Typhi, Paratyphi C and Dublin) is termed “The virulence (Vi) antigen”. This factor first needs to be heated at 100 °C for 60 min to remove the capsule; otherwise it would not be agglutinable with anti-O antiserum (Helmuth, 2000).

Serological typing of *Salmonella enterica* serovars requires, over 150 O and H antigens and more than 250 antisera (Cai *et al*., 2005; Wattiau *et al.,* 2008). The problem with this

conventional method is that it is laborious, time consuming, and cannot differentiate within serovars (Nashwa *et al*., 2009). It also depends on the availability of hundreds of antisera, needs highly trained personnel, consumes high volumes of reagents, and a minimum of three days is required to identify a serotype (Alvarez *et al.*, 2004; Cai *et al*., 2005; Yoshida *et al*., 2007).

2.4 *Salmonella*: Disease and Pathogenesis

Salmonella are well-known pathogens, highly adaptive and potentially pathogenic for humans and animals. *Salmonella* infections are capable of producing serious infections that are often foodborne and present as gastroenteritis, enteric fever, and bacteremia or septicemia conditions (Guthrie, 1991; Monteville and Matthews, 2008). *Salmonella* Typhi and *Salmonella* Paratyphi colonize only in humans, so they can be acquired only from close contact with a person who has typhoid fever, from a chronic carrier, or from water or food contaminated by human feaces **(**Tindall *et al*., 2005**),** causing enteric fever (Guthrie, 1991). The period of incubation for this infection ranges from 8 to 28 days and the common symptoms include fever, diarrhoea, abdominal pain, and headache (Monteville and Matthews, 2008). When the infection is due to the consumption of a food item contaminated with non-typhoid *Salmonella* strains, the disease is often self-limiting in healthy individuals. Symptoms appear 6 to 72 hours after ingestion, diarrhoea, nausea, vomiting and abdominal pain disappear within 2 to 4 days (Pedro and Boris, 2001). The treatment is based more on fluid and electrolyte replacement than on antibiotic use. Infections caused by nontyphoid *Salmonella* serotypes can also evolve into systemic infections followed by chronic conditions (Emmerson and Jones, 2003).

While certain serovars of *Salmonella enterica* cause disease in humans and a variety of animals, other serovars are highly restricted to a specific host. *Salmonella* spp causes gastroenteritis, enteric fever and typhoid fever **(**Fluit, 2005; Shu-Kee *et al*., 2015**).** The outcome of *Salmonella* infections is determined by the host and the status of the bacterium. Whereas, age, genetic and environmental factors mainly determine the status of the host, the status of the bacterium is determined by virulence factors (Alphons and Jaap, 2005**).** Young children, the elderly and patients with chronic illnesses or immunocompromised systems are particularly susceptible to salmonellosis (Bell and Kyriakides., 2002). Infective dose of *Salmonella* bacterium required to overcome host defenses and cause disease varies, usually about 10^6 to 10^8 CFU is needed (Humphrey, 2000; 2006). It has been reported that lower numbers of *S. enterica* may be capable of causing outbreaks, especially in cases involving foods with a high fat content (Humphrey, 2000; Bell and Kyriakides., 2002; Jay *et al.*, 2003; Humphrey, 2006).

2.4.1 Salmonellosis in Cattle and Sheep

Salmonellosis affects cattle worldwide and is most commonly associated with the serovars Dublin and Typhimurium (Murray, 1994; Stevenson and Hughes, 1988; Wallis, 2006). *S.* Typhimurium is considered a broad-range host and hence, is frequently isolated from both cattle and humans. While *S.* Dublin is capable of causing serious disease in humans, it is a host adapted serovar. It commonly causes gastroenteritis and/or septicaemia in cattle but is rarely isolated from humans (Murray, 1994; Wallis, 2006). Cattle are useful as a model for studying *Salmonella* enteropathogenesis: they are a natural target host for *Salmonella* bacteria and its virulence mechanisms are similar to those in humans (Wallis and Galyov, 2000; Zhang *et al.*, 2003). Several studies conducted on the virulence factors involved in

the intestinal colonization and inflammation (Jones *et al.*, 1998; Watson *et al.*, 1998; Libby *et al.*, 1997; Wallis *et al.*, 1995; 2007; Wallis, 2006; Morgan *et al.*, 2004; Zhang *et al.*, 2002), and development of systemic disease (Wallis *et al.*, 1995; Libby *et al.*, 1997; Wallis, 2006) in cattle have allowed researchers to gain a better understanding of *Salmonella* pathogenesis in animals and humans.

Ovine salmonellosis has been associated with *Salmonella* serovars Typhumurium, Dublin and Abortusovis (Murray, 1994; Uzzau *et al.*, 2001). *Salmonella* Abortusovis is an ovine restricted serovar, causing abortions and stillbirths in pregnant ewes and mortality in neonates (Rubino *et al.*, 1993; Uzzau *et al.*, 2001; Cagiola *et al*., 2007). It has been reported that *S.* Abortusovis is able to invade ovine intestinal mucosa in a SPI1-dependent manner but does not cause enteritis, leading to the suggestion of the serovar being involved in dissemination and persistence at systemic disease sites (Uzzau *et al.*, 2001). In addition, mutations in the virulence factors of this serovar have shown a reduced virulence in the mouse model (Rubino *et al.*, 1993; Uzzau *et al.*, 2000).

2. 4.2 Salmonellosis in humans

Salmonellosis occurs both in sporadic cases and outbreaks affecting a family or several hundreds or thousands of people in a population (Pedro and Boris, 2001); *Salmonella* serotypes affecting humans can be divided into three groups that cause distinctive clinical syndromes, typhoid fever, bacteremia and enteritis (Lawrence, 1999). The non-typhoid *Salmonella* serotypes can cause different manifestations in humans, including acute gastroenteritis, bacteremia, and extraintestinal localized infections involving many organs (Cai *et al*., 2005). Within *Salmonella enterica* subspecies I (*Salmonella enterica* subspecies enterica), the most common O-antigen serogroups are A, B, C1, C2, D and E. Strains

within these serogroups cause approximately 99% of *Salmonella* infections in humans and warm-blooded animals. Serotypes in other subspecies are usually isolated from cold-blooded animals and the environment but rarely from humans (Brenner *et al.,* 2000).

Following ingestion of contaminated food or water, the pathogenesis of both typhoid and *Salmonella* enteritis begins with the intestinal phase, while only typhoid progresses to a systemic phase (Yan *et al*., 2003). Transmission of this disease within the human population is generally a result of poor sanitation, consumption of contaminated food and water (Bertrand *et al*., 2008). The broad host-range *Salmonella* serovars are prevalent within warm-blooded animal populations that make up the human food supply, and bacterial transmission generally results from consumption of raw or undercooked food products (Brown *et al*., 2005).

The vast majority of *Salmonella* infections are transmitted from animals to humans through food and occasionally from person to person through the fecal-oral route (Nesa *et al*., 2011). In general, *Salmonella* cause one or more of four broad clinical syndromes such as gastro-enteritis, enteric fever, septicemia with associated focal lesions, and asymptomatic long-term carriage (Dworkin *et al.*, 2001). The convalescent carrier may shed salmonellae for several weeks and, more rarely, for a few months (Pedro and Boris, 2001).

2.5 *Salmonella* in Foods

Salmonellosis is one of the main infectious causes of enteric disease in human being worldwide, and most cases are more likely to be related to food products of animal origin (Mejia *et al*., 2006).

A period of temperature abuse which allows the *Salmonella* spp. to grow in food and/or inadequate or absence of final heat treatment are common factors contributing to outbreaks

of *Salmonella* infection (Barakat, 2011). Meat, poultry, egg, dairy products, and fruits and vegetables are primary transmission vehicles; they may be undercooked, allowing the *Salmonella* strains to survive, or they may cross-contaminate other foods consumed without further cooking (Barakat, 2011). Presence of *Salmonella* spp. in fresh raw products can vary widely (Harris *et al*., 2003).

Research on *Salmonella* frequency in different countries is extensive, and *Salmonella* serotypes have been isolated in a variety of foods (Barakat, 2011). Poultry and egg products have long been recognized as an important *Salmonella* source (Skov *et al*., 1999). *Salmonella* can contaminate eggs on the shell or internally, and egg shells are much more frequently contaminated than the white and yolk. Furthermore, egg surface contamination is associated with many different serotypes, while infection of the white and yolk is primarily associated with *S*. Enteritidis (Barakat, 2011). Dallal *et al*. (2010) isolated *Salmonella* from Chicken in Iran, (Freitas *et al*., 2010) from Poultry carcass in Brazil.

In Nigeria, the study carried out by (Anejo-Okopi *et al*., 2016) indicates that, 20 out of 120 meat samples analyzed were positive for *Salmonella* in Jos Metropolis. Out of a total of 435 beef and meat products samples screened for *Salmonella* in Zaria, 10 were positive for *Salmonella* (Tafida *et al*., 2013). Obi and Ike, (2015) analyzed 300 cloacal swab samples in Nsukka, and bacteriological characterization revealed 12 strains of *Salmonella*.

Pasteurization effectively kills *Salmonella* in milk, but consumption of unpasteurized milk and milk products is a well-documented risk factor for salmonellosis in humans (McEwes *et al*., 1988). Inadequately pasteurized milk as well as post-pasteurization contamination of milk and milk products is recognized sources of human disease. Mohammed and Abdullahi

(2015), (Tamba *et al*., 2016) and (Ogbonna, 2011) have isolated *Salmonella* from milk and milk product in Sokoto, Zaria and Maiduguri respectively.

Salmonella is among the most worrisome of the pathogenic microorganisms found in minimally-processed fresh produce (Heaton *et al*., 2008; CDC, 2009). *Salmonella* spp. are often isolated during routine surveys of produce such as lettuce, cauliflower, sprouts, mustard cress, endive and spinach (Thunberg *et al*., 2002) unpasteurized juices and fresh salad fruits and vegetables (CDC, 2009). In Nigeria *Salmonella* has been isolated from vegetables in Port Harcourt by (Odu and Okomuda 2013), Sokoto (Bagudo *et al*., 2014), and in Maiduguri (Raufu *et al*., 2014).

2.6 Salmonellosis and Public Health

Salmonellosis is an important global public health problem causing substantial morbidity. In spite of the improvement in hygiene, food processing, education of food handlers and information to the consumers, food borne diseases still dominate as the most important public health problem in most countries (Barakat, 2011). There are reports of foodborne salmonellosis in humans since the 19th century, caused by the ingestion of products such as bovine meat, poultry, eggs, milk, milk product, seafood, and fresh produce contaminated with the organism (Barrow, 1993; Carrique-Mas and Davies, 2008; Foley *et al*., 2008). Many foods, particularly those of animal origin, have been identified as vehicles for transmission of these pathogens to human beings and spreading them to the processing and kitchen environment (Solari *et al*., 2003).

In developed countries food is recognized as the most frequently implicated vehicle of transmission of microorganisms and causes heavy financial burden on health care systems (Jong and Ekdahl, 2006). International trade and transport of live animals and foodstuffs of

animal's origin increased not only the dissemination of *Salmonella* and contaminants in foodstuffs, but also lead to outbreaks in humans (Plym and Wierup, 2006). Globalization, commercialization and distribution make it possible for a contaminated foodstuff to affect the health of people in several countries at the same time (Tauxe *et al*., 2010). It is one of the most problematic zoonosis in terms of public health all over the world because of the high endemicity and its difficulty to control (Antunes *et al*., 2003) and the resulting significant morbidity and mortality rates (Cardoso *et al*., 2002).

2.7 Use of antibiotics and resistance

Microbial resistance is related to strains of microorganisms that are able to multiply in the presence of concentrations of antimicrobial compounds even higher than those given as therapeutic doses to humans (Barakat, 2011). The irrational and widespread use of antibiotics in developing countries results from availability of these drugs causing inappropriate use and ultimately resulting in steady increase in antibiotic resistance (Islam *et al*., 2010).

Despite the emergence of newer antibacterial drugs, enteric fever has remained a major challenge in health sector (Zaki and Karande, 2011). The rapid emergence of multidrug resistance *Salmonella* due to wide application of antibiotics in clinical practices has caused great difficulties in medical treatment (Sehra *et al*., 2013). The emergence of multidrug-resistant *Salmonella* Typhi strains spread worldwide, resulting in high rates of morbidity and mortality (Zaki and Karande, 2011).

In veterinary medicine, antimicrobial agents are used in therapy, metaphylaxis, prophylaxis, and as growth promoters (Scharwz *et al*., 2001). Veterinary use of antimicrobials agent is considered a key factor in the emergence of antimicrobial-resistant

Salmonella, and multiple resistance to antimicrobials is more commonly observed in isolates from food animals than from human clinical cases (Hong *et al*., 2016). Use of antimicrobials in food production is considered one of a major factor increasing emergence of antimicrobial resistant bacteria (Landers *et al*., 2012). *Salmonella* contained varying metabolic characteristics, levels of virulence, and multi-drug resistance genes that complicate treatment in areas that resistance are prevalent (Deng *et al*., 2003; Ugboko and Nandita, 2014). The emergence of such resistance in zoonotic pathogens poses a pressing threat to public health, as they may be transferred to humans through the food chain (Fernández *et al*., 2018)

2.8 Culture and isolation of *Salmonella*

The conventional technique for the detection of the *Salmonella* microorganism includes the following steps: pre-enrichment, selective enrichment, isolation and selection, biochemical characterization, and final confirmation. This technique requires at least four days for a negative result and six to seven days for the identification and confirmation of positive samples (Soumet *et al.*, 1997).

Injured Enterobacteriaceae have been enriched using non-selective buffered peptone water (BPW), pre-enrichment is necessary to permit the detection of low numbers of *Salmonella* or injured *Salmonella* (ISO, 2002).

Cultures from non-selective pre-enrichment media are typically inoculated into secondary selective enrichment broths such as Selenite Cystine broth (SC), Rappaport Vasilliadis Soy broth (RVS), Tetrathionate Broth (TT), or Mueller-Kauffmann Tetrathionate- Novobiocin broth (MKTTn) and incubated at elevated temperatures 37°C for 18-24 hours before stab culturing on 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 (Zee, 1994) for clinical laboratory diagnosis.

The suspected colonies of *Salmonella* on the above media are subjected to the following biochemical tests.

2.9 Biochemical tests

Salmonella Conventional biochemical including; urease test, triple sugar iron (TSI) slant reaction, indole test, Citrate utilization test using Simmon's citrate agar, motility test and carbohydrates fermentation tests (xylose, lactose, sucrose), according to (Barrow and Feltham, 2003). Typical *Salmonella* reaction are Indole negative, Methyl red positive, Voges-Proskauer (VP) negative, Citrate positive, urease negative, motile in Motility medium, produces H_2S, oxidase negative, ferment glucose and mannitol but fail to ferments sucrose and lactose (USAID, 2003).

2.10 Polymerase Chain Reaction (PCR) for *Salmonella*

The *inv* A gene of *Salmonella* contain sequences unique to this genus and has been proved to be a suitable polymerase chain reaction (PCR) target with potential diagnostic application (Jamshidi *et al*., 2008). The gene is an invasion gene conserved among *Salmonella* serotypes (Oliveira *et al*., 2003). Primers to target this region are used which through complimentary base pairing anneal to the *inv* A target sequences. DNA strands are copied and amplified with the presence of *Taq* polymerase (Van der Zee and Huis, 2000).

After DNA extraction, forward and reverse primers, *Taq* polymerase, dNTPs, $MgCl_2$, and water are mixed and put in a thermal cycler to run the PCR. The three steps of

denaturation, annealing and elongation are repeated in about 40 cycles. Analysis of the amplified DNA is done with agarose gel electrophoresis (Al-Gallas *et al*., 2002)

2.11 Prevention and control of *Salmonella*

Management and biosecurity measures that reduce the risk of *Salmonella* infections in cattle according to NADIS, (2018) include:

1. Avoid introducing potentially infected animals by maintaining a closed herd. Quarantine all introduced stock for at least four weeks.
2. Source new stock from other farms with high health status and not markets.
3. Avoid shared bulls and communal grazing areas.
4. Isolate sick animals in dedicated isolation boxes and not calving boxes.
5. Minimize fecal contamination of feedstuffs, feeding containers, water troughs and equipment.
6. Scrape manure and remove organic debris from the herds.
7. Maintain good fences to prevent straying of neighboring stock.
8. Ensure that milk from ill cows (or cows that have been in contact with such cows) is not fed to calves.
9. Control rodents, birds and feral cat populations.
10. Only spread slurry on arable land wherever possible. Leave all grazing land at least three weeks after spreading slurry.
11. Insist visitors have clean boots and disinfect before entering and leaving the farm premises.

2.12 Milk

Milk may be defined as the secretion of the female mammal used for the feeding of her young, and has been described as close to being nature's perfect food (Ensminger, 1993; Nickerson, 1999). Milk is an essential first food for man, for countless of generations, it has formed an important part of man's diet not only for the infant, but in many societies throughout life. (Komorowski and Early, 1992).

Raw or processed milk has been reported as a good medium that supports the growth of several bacteria with resultant spoilage of the product or infections/intoxications in consumers (Murinda *et al*., 2004; Oliver *et al*., 2005). Microbes may gain entry into raw milk directly from dairy cows experiencing subclinical or clinical mastitis (Rodojcic-Prodaova and Necev, 1991), from the farm environment particularly the water source and utensils used for the storage of milk on farm or during transportation (Murphy and Boor, 2000). Microorganisms in milk have been observed to undergo rapid multiplication at high ambient temperatures (Jayarao and Henning, 2001; Gillespie *et al*., 2005; Hussein and Sakuma, 2005). A number of bacteria including *S. aureus*, *Escherichia coli* and *Salmonella spp* have been recovered from raw milk (De Buyser *et al*., 2001) and some of these have been determined to be pathogenic and toxigenic, and implicated in milk-borne diseases (Maguire *et al*., 1992; De Buyser *et al*., 2001).

Milk is highly valued and has been reported to provide essential nutrients in higher amounts than other staple foods (Oyawoye *et al*., 1997). Milk has been reported to be utilized in the production of at least 400 different fermented products all over the world (Prescott *et al*., 2008). Health complications associated with consumption of inadequately pasteurized milk products include serious infections with antibiotics therapeutic failure due

to antibiotic resistance development. Antibiotics reportedly used to treat infectious disease have been implicated in the development of multiple antibiotic resistances thereby rendering the antibiotic treatment ineffective (Johnston *et al*., 1983; Devriese *et al.,* 1997). When low doses of antibiotics are used, they inhibit the growth of susceptible bacteria while resistant bacteria thrive and grow such as in the presence of tetracycline (Eichner and Gravitz, 1999).

Pasteurization requires heating milk to a specific temperature for a minimum period of time, and then quickly cooling it back down to refrigeration temperatures 4°C (De Buyser *et al*., 2001). Pasteurization has been regarded as an effective method to eliminate bacterial pathogens in milk however, post-pasteurization contamination is possible usually through unhygienic practices of workers, or the use of unsterilized containers or post-pasteurization equipment (Leedom, 2006; Lejeune and Rajala- Schultz, 2009; Oliver *et al*., 2005). The risk of microbial transmission also occurs via dairy workers at all points during milk processing, the equipment and practices on the farm (Leedom, 2006). So, while pasteurization can reduce microbial contamination, it does not ensure that milk is sterile throughout the supply chain (Lejeune and Rajala-Schultz, 2009).

2.13 Milk composition and nutritive value

Milk is defined as the secretion of the mammary gland of mammals used for the feeding of their young, and has been described as close to being nature's perfect food (Ensminger, 1993; Nickerson, 1999). The substances in milk have been reported to provide both energy and materials necessary for growth and maintenance of health. Bovine milk is commonly consumed by majority throughout the world, however, in some regions goat's milk or sheep milk may be more commonly used (Dogan *et al*., 2002). Fresh milk is neutral or

slightly alkaline but on souring becomes acid because of the lactic acid formed by bacterial action on lactose. It has a water content of 88% and 12% of solids which constitute 3.5% fats, 0.6% salts, 4.8% carbohydrate, and 3.1% protein (Pape-Zambito *et al*., 2007). It has a wide range of nutritional benefits and supplies a variety of nutrients including protein, vitamins, minerals (especially calcium), fat and carbohydrate (Medhammar, 2012).

2.13.1 Milk Fat

Milk fat being an animal fat, is characterized as being fatty acids, its typically contains 70% saturated fatty acids SFA, 25% monounsaturated fatty acids MFA, and 5% polyunsaturated fatty acids PFA (Grummer, 1991). These are required by the human body for normal metabolism and growth. Short (C2 to C6) and medium chain (C8 to C12) fatty acids account for about 12% of the fatty acids of milk and being more readily digested (Lee and Gerrior, 2002). They do not contribute to the elevation of blood lipids nor are they deposited in adipose tissue (Lee and Gerrior, 2002).

2.13.2 Proteins

Milk is generally considered an important protein source in the human diet, supplying approximately 32 g protein/l. Its protein fraction can be divided into soluble and insoluble proteins. Soluble proteins, named whey proteins, represent 20% of milk protein fraction, whereas the insoluble, namely caseins, represent 80% (Haug *et al*., 2007; Severin and Wenshui, 2005). Both are classified as high-quality proteins considering human amino acid requirements, digestibility, and bioavailability. In fact, milk proteins are frequently considered the best protein source taking in to account the essential amino acid score and protein-digestibility corrected amino acid score (Schaafsma, 2000; Boye *et al*., 2012). The amino acid profile is quite different between the two fractions: Whey is especially rich in

branched chain amino acids, i.e., leucine, isoleucine, and valine as well as lysine, whereas casein has a higher proportion of histidine, methionine, and phenylalanine (Tang *et al*., 2009; Furst and Stehle, 2004). Apart from the high-quality and biological value, milk proteins and several bioactive peptides resulting from their enzymatic hydrolysis have shown multiple biological roles that could exert a protective action in human health. These main biological actions include antibacterial, antiviral, antifungal, antioxidant, antihypertensive, antimicrobial, antithrombotic, opioid, and immunomodulatory roles, in addition to improving absorption of other nutrients (Mills *et al*., 2011).

2.13.3 Carbohydrate

The major carbohydrate in milk of most mammals is lactose, usually called milk sugar. It is water soluble occurring as a soluble molecule in milk. Lactose with the exception of water is, at about 4.6%, the principal component of milk; however, it is the less important of the solids both nutritionally and commercially. Lactose consists of two molecules, D-glucose and D-galactose and is digested or broken down into these constituents by the enzyme lactase (Mustapha *et al*., 1997). Lactose is a useful source of dietary energy. However, adults in certain racial groups lose the ability to digest it and suffer discomfort and other symptoms of digestive upset as a result of consuming substantial quantities of dairy products containing lactose, a condition called lactose intolerance (Vesa *et al*., 2000).

2.13.4 Minerals

Many trace elements essential for health and growth, are present in milk. Sodium, calcium, potassium and phosphorus account for about 4% by weight of the fat-free human body. Some of the trace minerals are, zinc, cobalt, iodine and iron (Gaucheron, 2005; Zamberlin *et al*., 2012). Minerals in milk provide constancy of osmotic pressure. This property can

prevent the depression of freezing point temperature. The amount of minerals in milk can provide the recommended daily allowance for calcium and phosphorus (Zamberlin *et al*., 2012). These minerals are widely recognized as important factor for bone development and growth of children (Okolo *et al*., 2000).

2.13.5 Vitamins

The milk vitamin profile includes liposoluble (A, D, E) and hydrosoluble vitamins (B complex and vitamin C) (Haug *et al*., 2007; Gaucheron, 2011). The concentrations of fat-soluble vitamins in milk depend on milk fat content, thus low-fat and skim milk varieties have lower amounts of A, D, and E vitamins. In some countries, skim milk is fortified with A and D vitamins to improve its nutritional richness. Vitamin A is especially important in growth, development, immunity, and eye health. Its content in milk depends mainly on fat amount, but also on factors like animal feed and season (Gaucheron, 2011). Whole milk is generally considered a good vitamin A source, supplying around 172 mg/100 g; however, vitamin A content in skim or fat-free milk can be as low as 102 mg/100 g and 5 mg/100 g, respectively (Lindmark *et al*., 2003; Schönfeldt *et al*., 2012). Thus, several countries have chosen to fortify fat-reduced milk products to improve nutritional status and reduced vitamin A deficiency, especially in children (Miller and Welch, 2013). Despite being globally considered a good source, milk itself does not present considerable amounts of vitamin D except when fortified. Previous studies have reported values within 5 and 35 IU/L (Leerbeck and Søndergaard, 1980), which is in accordance with reference nutritional tables. Commercially available whole milk, to which vitamin D is added, presents within 40 to 51 IU/100 g (Lindmark *et al*., 2003; Schönfeldt *et al*., 2012). Recently, vitamin D started to deserve more attention as a polyvalent micronutrient considering some attributed

protective actions. Studies have suggested vitamin D has anticarcinogenic (Krishnan and Feldman, 2011; Mamede *et al.*, 2011). cardioprotective (Pate and Zhan, 2012), and immunomodulatory (Chun *et al*., 2011). Effects and nevertheless is crucial in calcium absorption, thus in bone mass formation, and can be determinant in the prevention of osteoporosis (Sadat-Ali *et al*., 2011).

2.14 Sources of bacterial contamination in milk products

The isolation of pathogenic and coliform bacteria from milk indicates that milk may be contaminated from udder of animals, utensils used for milking or the water used as well as handlers (Bonfoh *et al.,* 2003). The number and types of microorganisms present in milk and dairy products depend on the microbial quality of milk used, heat treatment of milk, the conditions in which the products are manufactured, the temperatures and duration of storage, feeding of the animals, season, area, general sanitation in the environment, quality of starter cultures, occurrence of phages and quality of water (Robinson and Tamime, 2002), and the main sources of contamination are the dairy cattle handlers and dairy equipment (Zeinhom and Latef, 2014).

2.15 Bacteriological quality of milk

Milk has been considered as one of the most important primary foods, however, several bacterial pathogens have been detected in milk including enterohaemorragic *Escherichia coli*, *Staphylococcus aureus*, *Salmonella species*, and *Yersinia enterocolitica* (Gulmez and Guven, 2003; CDC, 2003; Mazurek *et al*., 2004; Tekinşen and Özdemir, 2006). Bacterial pathogens from milk also include psychrotrophic microorganisms, mainly belonging to the genus *Pseudomonas*, that are responsible for the spoilage of milk and dairy products owing to their ability to produce heat-resistant proteolytic and lipolytic enzymes at chill

temperatures (Gilmour and Rowe, 1990). Their enzymes can withstand heat treatments of pasteurization and ultrahigh temperature treatments (UHT) (Lopez-Fandino *et al*., 1993; Koka and Weimer, 2001). These pathogens have been linked to livestock, feed, and storage environment (Marco and Wells-Bennik, 2008). The bacteriological quality of milk is strictly related to the management practice, such as equipment and environmental hygiene, cow wellness, packaging and handling (Little *et al*., 2008).

The incidence of milk-borne infections has markedly increased over the last 20 years, with nearly a quarter of the population at higher risk for illness today (Oliver *et al*., 2005). Milk borne disease surveillance began in the US in the early 1900s in response to morbidity caused by milk-transmitted typhoid fever and infantile diarrhoea (Cliver, 1990). From 1998 to 2005, the Centers for Disease Control and Prevention of the United State America (CDC) identified 45 outbreaks of milk-borne illnesses that involved unpasteurized milk, or cheese made from unpasteurized milk, accounting for 1.007 illnesses, 104 hospitalizations, and two deaths (CDC, 2003).

Many of these milk borne diseases that historically caused significant mortality and morbidity were largely eradicated in the industrialized world as a result of sanitation and pasteurization, disease control efforts in animals and other measures (Tauxe, 2002). Although many milk borne infections are controlled, the burden of emerging bacterial pathogens remains substantial.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Fika Local Government Area of Yobe state which is located in the Northeastern region of Nigeria, within latitudes 10^{o} 17' 07", 00" N and longitudes 11^{o} 18' 29" E (Figure 1) and a total land area of 2208 km^2. It has an estimated population of 136,736. The main occupation of people living in Fika Local Government Area includes; farming, livestock rearing and trading (NPC, 2006).

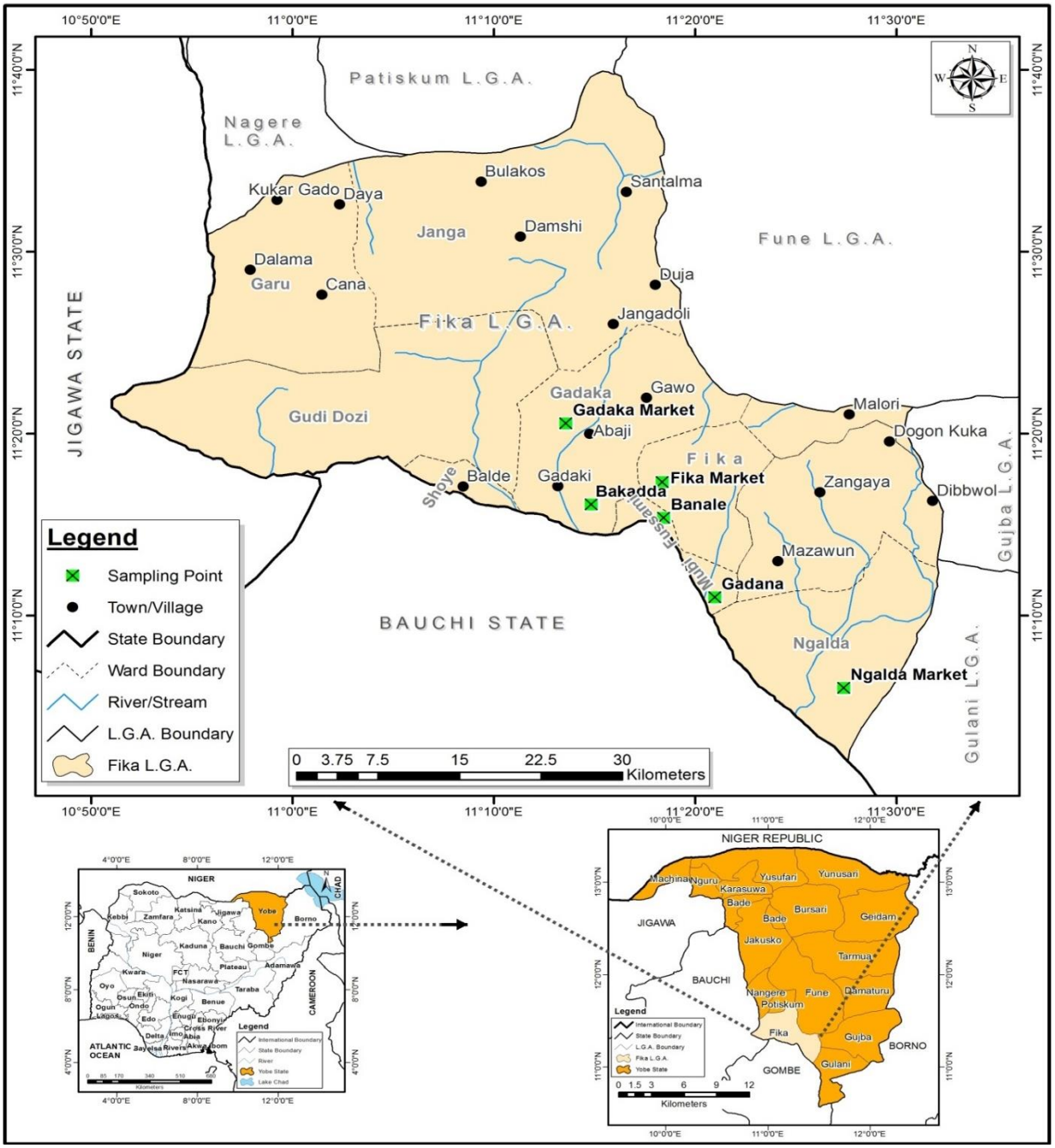

Figure 1: Map of Fika L.G.A. showing Sampling Point
(Source: Google Earth Map Pro 4.0, 2014)

3.2 Study Design

Cross sectional study was used for this work. Fermented milk and raw milk samples were collected from three selected major markets and Fulani settlements in Fika local government area.

3.3 Sample size

Sample size was determined using the formula as described by Thrusfield (1997).

$$N = \frac{z^2 pq}{d^2}$$

Where

N= Sample size

Z= Standard deviation at 95% confidence interval (1.96)

P= Prevalence 9.8% for raw milk and 7.6% for fermented milk was obtained from (Ogbonna, 2011).

q= 1- *p*

d= level of significance = 0.05

$N = \frac{1.96^2 \times 0.098\ (1-0.098)}{0.05^2}$

= 136 samples of raw milk.

$N = \frac{1.96^2 \times 0.076\ (1-0.076)}{0.05^2}$

= 108 samples of fermented milk.

A total of 344 samples were collected, comprising of 167 raw milk and 177 fermented milk samples to increase the precision.

3.4 Samples collection

A total of 344 of both raw and fermented milk samples were collected for the study. Fermented milk (Nono) samples were purchased from the 3 selected markets. The markets were; Ngalda, Fika and Gadaka markets; these are the major markets in the sampling area where fermented milk are being sold in large quantity. Fresh cow milk samples were collected from Fulani settlements between the hours 6:00AM and 8:00AM. Twenty ml of raw and fermented milk samples were aseptically collected into sterile sample bottles and labeled. Out of the 20ml collected, 1 ml was transferred to 5 ml of buffered peptone water (BPW) (Oxoid,UK) for *Salmonella* isolation and labeled accordingly while the remaining 19 ml was used for pH determination and total aerobic plate count determination. The samples were stored in ice pack container and immediately transported to the Bacterial Zoonoses Laboratory of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University Zaria for analysis.

3.5 Sample Processing and Laboratory Procedure

3.5.1 Determination of pH

The pH of each milk sample was measured using a pH meter as described by Ogbonna *et al.* (2011).

3.5.2 Total Aerobic Plate Count determination

Standard plate counts method was used to determine the total aerobic plate counts of raw and fermented milk samples. Ten-fold serial dilutions of each sample were carried out using 1ml of the sample and 9 ml of sterile physiological saline solution. By spread plate method, 0.1 ml of 10^5 dilution factor was inoculated into nutrient agar for total aerobic plate counts as described (Sandears, 2012; Lawan *et al.*, 2011). After inoculation, the plates

were incubated at 37 ^{0}C for 24 hours. Colonies on nutrient agar plate were enumerated and expressed in $\log_{10}$ colony forming unit (CFU/ml).

3.5.3 Isolation of *Salmonella* spp

Samples in BPW were incubated at 37^{0}C for 24 hours for pre-enrichment of *Salmonella* species. One ml of the pre-enriched culture was transferred into 10 ml of Rappaport Vassiliadis (RV) broth, which was incubated at 37ºC for 24 hours for selective enrichment. A loopful of enriched broth was subculture on Xylose-Lysine Deoxycholate (XLD) Agar. The plates were incubated at 37^{0}C for 24 hours. Any colony that appeared pinkish with or without dark centers on XLD agar was suspected to be *Salmonella* (Menghistu *et al*., 2011). Suspected positive samples were subculture on Nutrients Agar slants for further tests.

3.5.5 Biochemical characterization of *Salmonella* spp

All the suspected *Salmonella* colonies on XLD agar were subjected to conventional biochemical tests as previously described (Barrow and Feltham, 2003; Cheesbrough, 2003). One characteristic colony appearing pinkish with or without dark centers on XLD was picked and inoculated into Triple Sugar Iron (TSI), Sulfide, Indole and Motility medium (SIM) and urea agar. Colonies that gave reactions typical of *Salmonella* in all or most of the test substrates were considered to belong to the genus *Salmonella.*

3.5.5.1 Triple-sugar iron Agar Test (TSI): The medium contains three (3) sugars namely: glucose, lactose and sucrose. The pH indicator is phenol red, and detection system for hydrogen sulphide (H_2S) production is included. This medium was prepared as agar slope and the test organism was inoculated by stabbing the medium with the aid of sterilized straight wire loop, and the surface of slope is inoculated by streaking and then incubated at

37^{o}C for 24 hours, after which observation was made. Gas production was determined by cracking of the medium, formation of H_2S was determine by the blackening of the whole buffer or a streak of ring of blackening at the slant butt junction, glucose fermentation was determine by the yellowing of the butt. The red or dark pink coloration of the media in slant or in butt was considered as alkaline reaction. The fermentation of lactose or sucrose or both was determined by the yellowing of both the butt and the slant and the motility was determined by observing the line inoculation; sharply defined line of inoculation indicating positive motility (Cappuccino and Sherman, 1992; Tuhin *et al*., 2013).

3.5.5.2 Urea medium

Urea medium screens out urease-producing organisms (e.g *Klebsiella* and *Proteus*). Urea agar is inoculated heavily over the entire surface of the slant. Loosen caps before incubating overnight at 35°–37°C. Urease-positive cultures produce an alkaline reaction in the medium, evidenced by a pinkish-red color. Urease-negative organisms do not change the colour of the medium, which is a pale yellowish-pink. *Salmonella spp* is urea negative, therefore the colour of the medium do not change (Cappuccino and Sherman, 1992; USAID, 2003).

3.5.5.3 Indole test

Two ml of peptone water was inoculated with 5 ml of bacterial culture and incubated for 48 hours. Kovac's reagent (0.5 ml) was added and mixed thoroughly. The tube was then allowed to stand for a while. The appearance of red colour on the whole medium was considered as a positive test for the production of indole by the organisms. *Salmonella spp* do not produced red colour, it is indole negative organism (Tuhin *et al*., 2013).

3.5.5.4 Citrate Utilization Test

This was carried out by inoculating the test organism in test tube containing Simon's citrate medium and this was incubated for 24 to 72 hours. Turbidity and blue colour indicates positive for citrate utilization test and no color change indicates negative test (Cappuccino and Sherman, 1992; Cheesbrough, 2003).

3.5.5.5 MR-VP Test

Five millilitres (5ml) of MRVP broth was inoculated with the test organism and incubated for 48-72 hour at 37^{o}C after which, one milliliter of the broth was transferred into a small tube. Some small quantity (2-3 drops) of methyl red test was added. A red colour on the addition of the indicator signified a positive methyl red test while yellow colour signified a negative test. To the rest of the broth in the original tube some drops (five) of 4% Potassium hydroxide (KHO) were added followed by some (fifteen) drops of 5% α-naphtol in ethanol. The test tube (sealed with cotton plug) was shaken and place in a sloping position. The development of a red colour starting from the liquid- air interface within 1 hour indicated a VP positive test while no color change indicated a VP negative test (Tuhin *et al*., 2013).

3.5.5.6 Motility Test

Motility agar was prepared and inoculated with a sterile Pasteur loop making a single stab about 1-2 cm down into the medium. The motility will be examined after incubation at 37 ^{0}C for 24 hour. If the isolates are positive for motility, there was presence of diffuse growth (appearing as colouring of the medium) away from the line of inoculation. All *Salmonella* species are motile except *Salmonella* Gallinarum and *Salmonella* Pullorum (Cheesbrough, 2002).

3.5.6 Microbact 24E Gram-Negative Bacillus (GNB) Rapid Identification System

Presumptive *Salmonella* isolate following the biochemical characterization were subjected to Microbact 24E tests. One colony of each isolate was selected after subculture on XLD and emulsified in 3ml of peptone water and incubated at 37^{o} C for 24 hours. Using sterile pipette, one drop of the suspension in peptone water culture was mixed with 2.5ml sterile normal saline solution (0.85%). The test was carried out and interpreted as recommended by manufacturer (Oxoid Limited Wade Road, Basingstoke Hants, RG24 8PW, UK).

Four digit codes were obtained and fed into the computer identification software. This gave the probable identity of the organism tested in percentage. The Microbact software permits a 75% cut-off point for a probable identification. All tested organisms that gave a score less than 75% probability was not accepted as *Salmonella.*

3.6 Detection of *inv*A Gene by PCR Method

3.6.1 DNA extraction and amplification

Positive *Salmonella* isolates obtained from biochemical tests were used for PCR. The isolates were subculture on nutrient Agar. A colony of each *Salmonella* isolates on nutrient agar plate was picked and suspended in 400 µl of lysis buffer to which 25 µl of proteinase K was added. The suspension was incubated at 65 ^{0}C was vortexed at 20 minutes interval for 1 hour, after which 400 µl of phenol chloroform was added to each suspension, vortexed and centrifuged at 13000 revolution per minutes (rpm) for10 minutes. Supernatants were collected into fresh sterile tube and 400 µl of chloroform was added. The suspension was vortexed and centrifuged at 14000 (rpm) for 5 minutes. The supernatants were then collected in fresh, sterile tubes and 1000 µl of absolute ethanol was added followed by 40 µl of 3 molar acetate. These were kept overnight at -20 ^{0}C. The

incubated suspension was centrifuged at 14000 rpm for 10 minutes and the supernatants discarded. 400 µl of 70% ethanol was then added to each pellet centrifuging for 5 min at 14,000 rpm. The supernatant were discarded and each pellet were allowed to air-dry, after which 50 µl of ultra-pure water was added.

PCR was carried out using Table 3.1 sequence. Reaction with these primers was carried out in a 50 micro litres amplification mixture consisting of 25 microlitres of PCR Master Mixture (Genei, Bangalore), 2 micro litres of each primer, 19 micro litres of molecular grade water and 2 micro litres of extraction for each isolate used in the reaction. Amplifications were carried out in an Eppendorf's Master cycler Gradient using the following cycling condition; Initial denaturation at 94^{O}C for 5 min, followed by 30 cycles with denaturation at 94^{0}C for 1 min, annealing at 60^{0}C for 2 min, extension at 72^{0}C for 2 min with a final extension at 72^{0}C for 5 min.

PCR amplicons were separated by 1.5% agarose gel electrophoresis at 80volt, stained with ethidium bromide and visualized by UV illumination and photographed.

Table 3.1 *Salmonella* specific primers, S139 and S141 used for the amplification of *inv*A gene.

Gene	Primer	Reference
*inv*A	forward primers 5´ GTG AAA TTA TCG CCA CGT TCG GGC AA 3´ reverse primers 5´ TCATCG CAC CGT CAAAGG AAC C -3´	Rahn *et al*., 1992

3.7 Antibiotic sensitivity test

Susceptibility testing was performed using Kirby-Bauer disk diffusion method (CLSI, 2016). A culture of each *Salmonella* isolates was prepared in Tryptic Soy broth (TSB) and incubated at 37°C for 18 hours.

Dry sterile plates of prepared Mueller Hinton's agar were inoculated with 0.5 McFarland standard inoculum of 18 hours culture test *Salmonella* isolates. Sterile swabs was dipped into the broth culture with the excess broth drained by pressing on the inner side of the tube; and used to streak the Mueller Hinton agar in three directions until the entire surface was streaked. After inoculation, plates were allowed to dry in incubator at 37°C before placing the sensitivity multi-disc, of various antibiotics aseptically. The antimicrobial drugs used included Ciprofloxacin (5 µg), Chloramphenicol (30 µg), Tetracycline (30 µg), Imipenem (10 µg), Ampicillin (10 µg), Enrofloxacin (5 µg), Sulfamethoxazole (25 µg), Ticarcillin (85 µg), Gentamicin (30 µg), Cefotaxime (30 µg), Cefixime (5 µg) and Amoxycillin-clavulanic acid (30 µg) using disk diffusion method, following incubation The zones of inhibition was measured to the nearest millimeter as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2016).

3.8 Data Analysis

Results obtained from total aerobic counts were converted to $\log_{10}$ (CFU/ml) and expressed as logarithmic mean and standard deviation. Chi-square was used to test for association between occurrences of *Salmonella* from the different markets sampled using Statistical Package for Social Science (SPSS) version 17.0. P value ≤ 0.05 was considered significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Total Aerobic Plate Count and pH of the Samples Collected

The mean and standard deviation for raw and fermented milk samples were 5.82±2.06 and 6.62±1.10 respectively. A mean total aerobic plate counts for both raw and fermented milk samples ranged from 1 to 7.4 log $_{10}$ CFU/ml.

Table 4.2 shows mean total aerobic plate counts of raw milk samples based on sampling location. The mean ranged between 5.46 and 6.10 log $_{10}$ CFU/ml; Banale recorded the highest mean value 6.10 log $_{10}$ CFU/ml while Gadana had the lowest mean value 5.46 log $_{10}$ CFU/ml.

The mean total aerobic plate count of fermented milk samples is presented in Table 4.3, with mean ranges of 6.60 and 6.52; there was no statistically significance difference between locations.

The results shown in Table 4.2 revealed the mean pH value of raw milk samples. The mean pH value of samples from Banale and Bakadda was 6.41 while that of Gadana was 6.44. Table 4.3 shows the mean pH value of fermented milk samples. Ngalda had a mean pH value was 3.73, Fika was 3.58 and that of Gadaka was 3.54.

The pH of raw milk samples from different location ranged between 6.0 - 6.8, while that of fermented milk samples from markets were between 6.0 – 6.8 as presented in Table 4.1. There was significance difference between pH and samples types P value = < 0.05.

Table 4.1 Mean Total Aerobic Plate Counts and pH of Raw and Fermented milk

Sample types	Log_{10} CFU/ml (Mean ± SD)	pH (Mean ± SD)	Chi-square	p-value
Raw milk	5.82±2.06	6.42± 0.20	2.056	0.085
Fermented milk	6.62±1.10	3.64±0.44		

Table 4.2 Mean Total Aerobic Plate Counts and pH of Raw Milk Samples Collected from Selected Herdsmen Settlements.

Sampling location	**No of samples examined**	**TAPC Mean± SD (CFU/ml)**	**pH (Mean± SD)**
Banale	60	6.10± 1.75	6.41±0.22
Gadana	49	5.46± 2.67	6.41±0.18
Bakadda	58	5.87± 1.68	6.44±0.20
P values for TAPC and pH are		0.249 and	0.673 respectively

Table 4.3 Mean Total Aerobic Plate Count and pH of Fermented Milk Samples collected from Selected Markets.

Sampling location	**No of samples examined**	**TAPC Mean± SD (CFU/ml)**	**pH (Mean± SD)**
Ngalda	60	6.60± 1.05	3.73±0.02
Fika	55	6.78± 0.59	3.58±0.08
Gadaka	62	6.52± 1.43	3.59±0.07
P values for TAPC and pH are		0.477 and	0.133 respectively

4.2 Microbact Identification of the Isolates

Table 4.4 shows the *Salmonella* spp distribution from raw and fermented milk according to Microbact 24E tests. Total number of 344 samples was collected for both raw and fermented milk. 6 samples were identified positive for *Salmonella* spp using Microbact 24E kit out of 28 samples that were *Salmonella* spp suspects based on conventional biochemical tests. The prevalence of 2.3% and 1.2% were recorded for raw and fermented milk respectively based on Microbact 24E test as presented in table 4.4.

A total number of 167 of raw milk samples were collected, out of which 4 (2.3%) were positive *Salmonella* spp based on Microbact tests. Banale has the highest number of *Salmonella* spp with 10 (16.7%) while Gadana and Bakadda have 6 (3.3%) and 8 (3.2%) Table 4.4. Two 2 (1.1%) isolates were positive for *Salmonella* spp based on Microbact tests out of 177 fermented milk samples tested.

Table 4.4 Distribution of *Salmonella* spp Identified from raw and fermented milk.

Type of milk	No of sample	No of positive (%) Microbact 24E	Fisher's Exact test	P value
Raw milk	167	4 (2.3)	0.803	0.437
Fermented milk	177	2 (1.1)		
Total	344	6 (1.7)		

Table 4.5 Microbact 24E Identification of *Salmonella* spp Isolated from Raw milk Samples Collected from Selected Herdsmen Settlements.

Location	No of sample collected	No of positive (%) Microbat
Banale	60	2 (3.3)
Gadana	49	0 (0.0)
Bakadda	58	2 (3.2)
Total	167	4 (2.3)

Table 4.6 Microbact 24E Identification of *Salmonella* spp Isolated from locally fermented milk Samples Collected from Selected Markets.

Location	No of sample collected	No of positive (%) Microbact 24E
Ngalda	60	1 (1.7)
Fika	55	1 (2.0)
Gadaka	62	0 (0.0)
Total	177	2 (1.1)

4.3 Identification of *Salmonella* species

Six isolates were identified as *Salmonella* species using Microbact 24E kit as presented in Table 4.7. Isolate BnR41 and BkR24 from raw milk were identified as *Salmonella salamae,* and *Salmonella indica* respectively, while BnR52 and BkR49 from raw milk were *Salmonella diarizonae.* The remaining two isolates NF05and FF22 from fermented milk were identified as *Salmonella diarizonae*.

4.4 Other Bacteria identified

Eight other species of pathogenic bacteria were identified using Microbact 24E, these are: *Acinetobacter baumannii, Klebsiella* spp*, Serratia* spp*, Citrobacter* spp*, Proteus mirabilis, Xanthomonas maltophilia, Escherichia coli* and *Hafnia alvei* Table 4.7.

Table 4.7 *Salmonella* specie and other microorganisms identified using Microbact 24E

Isolates number	Source	Organism Identified by Microbact 24E
GnR49	Raw milk	*Serratia liquefaciens*
BkR57	Raw milk	*Serratia liquefaciens*
GnR35	Raw milk	*Serratia liquefaciens*
GnR13	Raw milk	*Serratia odorifera bio gp* 1
GnR12	Raw milk	*Klebsiella ornithinolytica*
GnR51	Raw milk	*Klebsiella cryocrescens*
BkR11	Raw milk	*Klebsiella oxytica*
BnR59	Raw milk	*Klebsiella ozaenae*
BkR21	Raw milk	*Klebsiella oxytica*
BnR57	Raw milk	*Acinetobacter baumannii*
BnR20	Raw milk	*Acinetobacter baumannii*
BkR59	Raw milk	*Acinetobacter baumannii*
BkR41	Raw milk	*Acinetobacter baumannii*
BkR19	Raw milk	*Acinetobacter baumannii*
BnR27	Raw milk	*Acinetobacter baumannii*
BnR50	Raw milk	*Escherichia coli*
BnR38	Raw milk	*Proteus mirabilis*
BnR41	Raw milk	*Salmonella salamae*
BkR24	Raw milk	*Salmonella indica*
BnR52	Raw milk	*Salmonella diarizonae*
BkR49	Raw milk	*Salmonella diarizonae*
GnR47	Raw milk	*Hafnia alvei*
GnR02	Raw milk	*Xanthomonas moltophilia*
BnR18	Raw milk	*Citrobacter diversus*
FF25	Fermented milk	*Citrobacter amalonaticus*
FF14	Fermented milk	*Citrobacter braakii*
FF22	Fermented milk	*Salmonella diarizonae*
NF05	Fermented milk	*Salmonella diarizonae*

Table 4.8 Bacteria isolated from raw and fermented milk from the sampling area

S/NO	Organisms identified	Frequency	Number of percentage (%)
1	*Salmonella* spp	6	21.4
2	*Acinetobacter* spp	6	21.4
3	*Klebsiella* spp	5	17.9
4	*Serratia* spp	4	14.2
5	*Citrobacter* spp	3	10.7
6	*Proteus mirabilis*	1	3.6
7	*Xanthomonas maltophilia*	1	3.6
8	*Escherichia coli*	1	3.6
9	*Hafnia alvei*	1	3.6
Total		28	100

4.5 Result of PCR for *Salmonella*

Plate 1 shows the PCR amplification of *inv*A gene. Three isolates were positive for *Salmonella inv*A gene. NF 05 from fermented milk, BnR 52 and BkR 24 isolates from raw milk produced *inv*A gene bands of 284 bp that is consistent with the positive control band used.

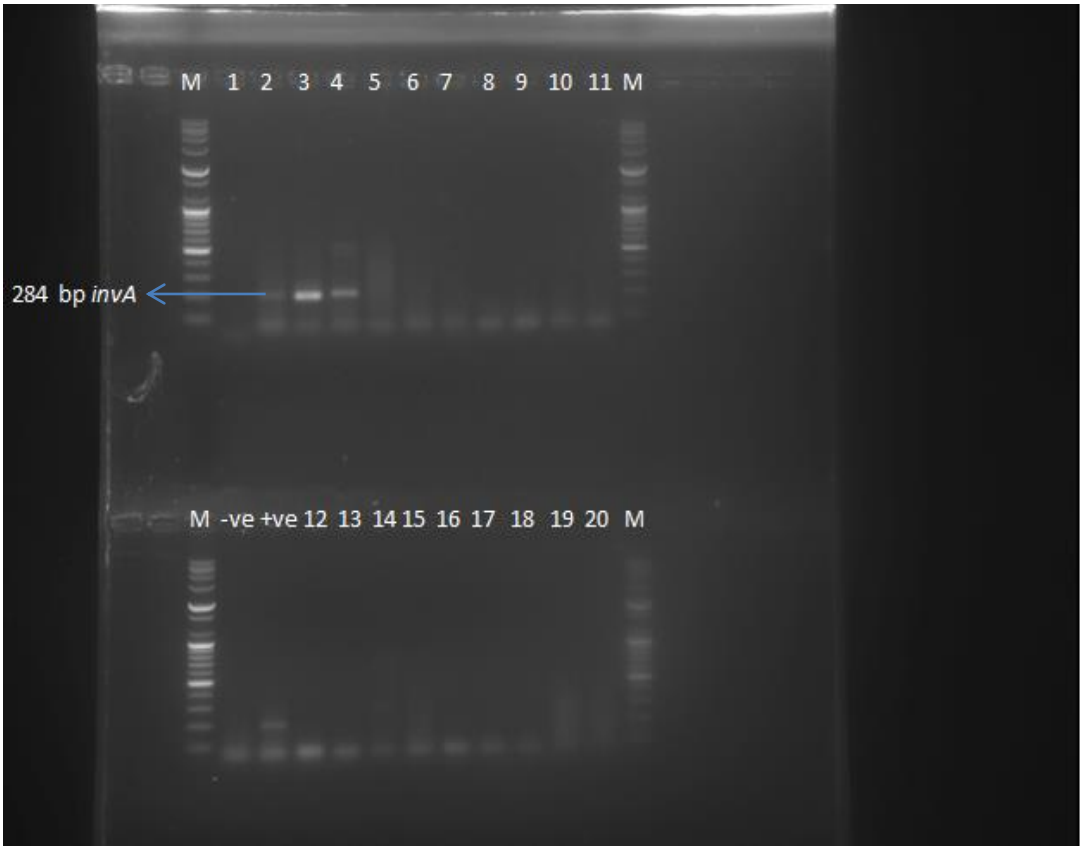

Plate 1: An agarose gel showing amplicons of *inv*A gene from *Salmonella* isolates. M; marker, -ve; *Salmonella inv*A negative control, +ve; *Salmonella inv*A positive control, 2; NF 05 fermented milk from Ngalda, 3; BnR 52 raw milk from Banale, and 4; BkR 24 raw milk from Bakadda.

4.6 Antibiotic Susceptibility Testing

Table 4.9 shows result for susceptibility tests of *Salmonella* isolates. Twelve (12) antibacterial agents were used for susceptibility testing of 6 *Salmonella* isolates. All of the isolates were resistant to cefotaxime, ticarcillin and chloramphenicol 100% while all the isolates were susceptible to enrofloxacin and gentamicin 100%. The result also showed high resistance to ampicillin and cefixime. One *Salmonella* isolates was resistance to imipenem Table 4.11.

Other bacterial isolates showed high frequency of resistance to ticarcillin, cefixime, amoxicillin clavulinic acid, tetracycline and ampicillin. There was 100% susceptibility against enrofloxcin among other bacterial isolates, however, imipenem was observed to be ineffective against one of the bacterial isolate obtained Table 4.12.

The resistance patterns of 6 tested *Salmonella* isolates varied.

Table 4.9 Antibiotic Resistance Patterns of *Salmonella* isolates from raw and fermented milk

Isolates	Source	Antibiotics Resistance Pattern	Multi Drug Resistance (MAR) index
Salmonella diarizonae	Raw milk	AMP, AMC, CTX, TIM, C.	0.41
Salmonella salamae	Raw milk	TE, AMP, CFM, AMC, CTX, TIM, C.	0.58
Salmonella diarizonae	Raw milk	TE, AMP, CFM, XST, AMC, CTX, TIM, C.	0.66
Salmonella indica	Raw milk	TE, CFM, XST, AMC, CTX, TIM, C.	0.58
Salmonella diarizonae	Fermented milk	CIP, AMP, CFM, XST, AMC, IPM, CTX, TIM, C.	0.75
Salmonella diarizonae	Fermented milk	TE, AMP, CFM, AMC, CTX, TIM, C.	0.58

CIP; Ciprofloxacin, C; Chloramphenicol, TE; Tetracycline, IPM; Imipenem, AMP; Ampicillin, ENT; Enrofloxacin, XST; Sulfamethoxazole, TIM; Ticarcillin, CN; Gentamicin, CTX; Cefotaxime, CFM; Cefixime, AMC; Amoxicillin-clavulanic acid.

Table 4.10 Antibiotic Resistance Patterns of other Bacterial isolates from raw and fermented milk.

Isolate	Antibiotics Resistance Pattern	Multi Drug Resistance (MAR) index
Citrobacter amalonaticus	CIP, TE, AMP, CFM, XST, AMC, C, CN, TIM.	0.75
Xanthomonas maltophilia	CIP, TE, AMP, CFM, XST, AMC, C, TIM.	0.67
Escherichia coli	CIP, TE, AMP, CFM, AMC, CTX, C, TIM.	0.67
Serratia liquefaciens	TE, AMP, CFM, XST, AMC, C, TIM.	0.58
Acinetobacter baumannii	TE, AMP, CFM, XST, AMC, CTX, TIM.	0.58
Citrobacter braakii	TE, AMP, CFM, AMC, CTX, C, TIM.	0.58
Serratia liquefaciens	AMP, CFM, XST, AMC, CTX, TIM.	0.5
Acinetobacter baumannii	TE, CFM, XST, AMC, CTX, TIM.	0.5
Klebsiella cryocrescens	TE, AMP, CFM, AMC, CTX, TIM.	0.5
Acinetobacter baumannii	AMP, CFM, XST, AMC, CN, TIM.	0.5
Proteus mirabilis	TE, AMP, CFM, XST, AMC, TIM.	0.5
Acinetobacter baumannii	TE, AMP, CFM, AMC, C, TIM.	0.5
Citrobacter diversus	TE, AMP, CFM, XST, AMC, TIM.	0.5
Serratia liquefaciens	TE, AMP, CFM, XST, AMC, TIM.	0.5
Acinetobacter baumannii	TE, AMP, CFM, XST, AMC, TIM.	0.5
Acinetobacter baumannii	TE, AMP, CFM, AMC, C, TIM.	0.5
Klebsiella oxytica	CIP, AMP, AMC, IPM, TIM.	0.42
Klebsiella ozaenae	TE, CFM, XST, AMC, TIM.	0.42
Serratia odorifera bio gp 1	CIP, AMP, CFM, XST, TIM.	0.42
Hafnia alvei	AMP, CFM, XST, AMC, TIM.	0.42
Klebsiella ornithinolytica	CFM, AMC, C, TIM.	0.33
Klebsiella oxytica	CFM, AMC, CTX, TIM.	0.33

CIP; Ciprofloxacin, C; Chloramphenicol, TE; Tetracycline, IPM; Imipenem, AMP; Ampicillin, ENT; Enrofloxacin, XST; Sulfamethoxazole, TIM; Ticarcillin, CN; Gentamicin, CTX; Cefotaxime, CFM; Cefixime, AMC; Amoxycillin-clavulanic acid.

Table 4.11 Antibiotic Susceptibility of *Salmonella* isolates from raw and fermented milk samples

Antibiotic agent (Potency)	No (%) of *Salmonella* isolates exhibiting resistance.
Ciprofloxacin (CIP 5 μg)	1 (16.6)
Tetracycline (TE 30 μg)	4 (66.6)
Ampicillin (AMP 10 μg)	5 (83.3)
Cefixime (CFM 5 μg)	5 (83.3)
Sulfamethoxazole (XST 25 μg)	3 (50)
Amoxycillin-clavulanic acid (AMC 30 μg)	6 (100)
Imipenem (IPM 10 μg)	1 (16.6)
Cefotaxime (CTX 30 μg)	6 (100)
Ticarcillin (TIM 85 μg)	6 (100)
Chloramphenicol (C 30 μg)	6 (100)
Enrofloxacin (ENT 5 μg)	0 (0)
Gentamicin (CN 30 μg)	0 (0)

Table 4.12 Antibiotic Susceptibility of other Bacterial isolates from raw and fermented milk samples

Antibiotic agent (Potency)	No (%) of other bacteria isolates exhibiting resistance.
Ciprofloxacin (CIP 5 μg)	5 (22.7)
Chloramphenical (C 30 μg)	8 (36.3)
Tetracycline (TE 30 μg)	15 (68.1)
Imipenem (IPM 10 μg)	1 (4.5)
Ampicillin (AMP 10 μg)	15 (68.1)
Enrofloxacin (ENT 5 μg)	0 (0)
Sulfamethoxazole (XST 25 μg)	14 (63.6)
Ticarcillin (TIM 85 μg)	22 (100)
Gentamicin (CN 30 μg)	2 (9.0)
Cefotaxime (CTX 30 μg)	7 (31.8)
Cefixime (CFM 5 μg)	21 (95.4)
Amoxycillin-clavulanic acid (AMC 30 μg)	20 (90.9)

CHAPTER FIVE

5.0 DISCUSSION

The result of this study revealed that there was bacteriological contamination of both raw and fermented milk. The mean value of Total Aerobic Plate Counts (TAPC) of raw milk and fermented milk was higher than acceptable minimum counts of 5.7 Log_{10} CFU/ml (European Food Safety Authority, 2004). Unhygienic milking environment and unclean milking equipment may be the reason for the high TAPC obtained. The study area is located along the streams which are the sources of water for human and animal consumption, using of such water for processing of milk may be a source of contamination. Unhygienic milking practice, lack of potable water for processing the milk and unhygienic milking environment as well as possible faecal contamination of raw milk (Karshima *et al.*, 2013). Contamination during processing, utensils, and selling environment may also account for the higher TAPC of fermented milk. The presence of starter culture in fermented milk may also be the reason for high TAPC in the fermented milk. Often, not all fermented milk sent to the markets are sold on the same day in most developing countries, such unsold milk are taken back to the markets without consideration for preservation and safety. This implies that, consumption of such products may be of public health hazard to consumer. Abid *et al*., (2009) reported that counts greater than 5.7 Log_{10} CFU/ml for raw milk and milk products indicates a serious fault in hygiene during the production line. This study disagrees with the work of Lawan *et al.,* (2012) who reported higher value before pasteurization (7 – 8.9) Log_{10} and lower value of TAPC after pasteurization (3.7 – 4.20) Log_{10} respectively. The mean TAPC of both raw and fermented milk from different

sampling locations is above the European Union permissible value for milk which is 5.7 Log_{10} cfu/ml (European Food Safety Authority, 2004).

The pH range of raw milk was 6.0-6.8 with mean pH value of 6.42 and that of fermented milk was 2.8-4.6 with mean pH value of 3.64. The pH values obtained from raw milk samples were not within the normal pH range indicating that there were contaminations or bacterial growth in the milk. Fresh cow milk has a pH value that ranges between 6.6 and 6.8 (FAO, 1999). The pH ranges of fermented milk 2.8-4.6 obtained in this study may be due to the activities of the lactic acid bacteria, starter culture used, and duration of fermentation. According to a study carried out previously (Koutsoumanis *et al*., 2004), the minimum pH value that permitted the growth of *Salmonella* was 3.94, this may be another reason for low prevalence of *Salmonella* from fermented milk samples obtained in this study. Ogbonno *et al*. (2011) and Adesokan *et al*. (2011) reported the pH of 3.8-5.6 and 5.51-6.21 respectively for fermented milk.

The prevalence of *Salmonella* in this study was found to be 1.7% based on Microbact 24E. Milk contamination with microbes could originate from many sources like feed, soil, faeces and the milking cow itself (Gleeson *et al.,* 2013). Other possible sources of milk contamination may be utensils and water used during milking, collection and processing of milk, the use of previously fermented products as starter culture may also be a source of contamination. This presents a risk to the health of the public, especially the consumers of these milk products. The overall prevalence of 1.7 % recorded in this study is lower than 4.0% prevalence obtained by Tamba *et al.,* (2016) in Zaria. Karshima *et al.* (2013) reported the prevalence of 6.4% in Kanam while Markwin *et al.* (2014) recorded the prevalence of

17.5% in Keffi. However, (Mhone *et al*., 2012) reported 0.0% prevalence of *Salmonella* spp from raw and processed cow milk in a study carried out in Zimbabwe.

The prevalence of *Salmonella* from this study was higher in raw milk (2.3%) than in locally fermented milk which has the prevalence of (1.1%). Low pH and effect of fermentation in fermented milk could be the cause of variation in prevalence between raw and fermented milk because fermented milk is not a suitable environment for the growth of *Salmonella* spp and spoilages microorganisms (Kim *et al*., 2018). The pH of fermented foods is reduced to values of 4 or less due to the presence of acids formed during the fermentation process, thus inhibiting the growth of pathogenic and spoilage organisms (Sanni, 1993; Mensah, 1997). Higher prevalence of *Salmonella* spp in raw milk was reported (Tamba *et al.,* 2016; Karshima *et al.,* 2013) but in the work carried out by Markwin *et al.* (2014) a lower prevalence in raw milk than in fermented milk was reported. Location, hygienic practices, the availability of potable water and good management practices may be the reasons of these variations.

Out of six *Salmonella* spp were identified, three isolates that haboured *inv* A gene were identified by Microbact as *Salmonella diarizonae* and *Salmonella indica* from raw milk and *Salmonella diarizonae* from fermented milk. *Salmonella arizonae* is mainly isolated from cold-blooded animals or the environment but rarely from mammalian sources including humans (Abdullahi, 2010; Pui *et al.*, 2011). Bovine species is particularly susceptible to *Salmonella* infection (Siham and Taha, 2009).

The results obtained is of public health importance due to the presence of *Salmonella* species and other pathogenic microorganism that may be potential sources of food borne infections and some related diseases for the consumers of these products in the sampling

areas. Microbial contamination of milk is multi-factorial originating from sources like the air, feed, soil, faeces and the milking cow itself. Other possible sources of milk contamination include poor sanitary conditions of the milkers hands, clothings, utensils and water used in the collection and processing of milk. In a study conducted in Cairo, Hassan *et al*. (2015) reported that unsanitary conditions associated with the handling of the milk in the market, limited knowledge on the hygienic production of milk and unavailability of cooling facilities during production, handling and transportation of milk are other factors contributing to high bacterial load in milk and milk product. The presence of flies and poor sanitary environment where fermented milk is being sold could also contaminate the products.

The invasion gene, *inv*A was detected in 3 out of the twenty of *Salmonella* isolates tested in this study. This gene is responsible for *Salmonella* virulence especially the invasion of the host cells by the bacteria. Implication of detecting the gene from the *Salmonella* species isolated is that, the organisms are potentially pathogenic and will be able to penetrate host epithelia cells, causing infection. It is present in all invasive strains of *Salmonella* (Galán, 1996). The absence of the gene in the confirmed *Salmonella* isolates may lead to lack of invasiveness. Some authors reported the presence of *inv*A gene in almost all of the *Salmonella* isolates tested. This study is at variance with the reports of Tafida *et al*. (2013) and Nashwa *et al*. (2009) who reported the presence of *inv*A gene in all of the *Salmonella* isolates tested. However, Oludairo *et al*. (2013) reported that five out of eight *Salmonella* isolates were positive for *inv*A gene while (Bacci *et al*., 2006) detected *inv*A gene in 62 out of the 63 strains of *Salmonella* isolates screened.

Antibiograms assay of the *Salmonella* isolates from this study revealed varying levels of resistance to the antibiotics tested, but all isolates were resistant to at least five antibiotics. *Salmonella* isolated from this study were highly resistant to six antibiotics namely; Ticarcillin, Cefotaxime, Chloramphenicol, Amoxycillin clavulinic acid, Cefixime and Ampicillin. Equally, the antimicrobial resistance profiles revealed susceptibility to four antibiotics used these are; Enrofloxacin, Gentamicin, Imipenem and Ciprofloxacin as represented in Table 4.11. This agrees with previous studies carried out by (Tamba *et al.*, 2016) who in similar study in Zaria showed that significant number of *Salmonella* isolates were resistant to ampicillin and amoxicillin. The highest levels of susceptibility in this study were detected in Enrofloxacin, Gentamicin, Imipenem and Ciprofloxacin. This is in agreement with (Bata *et al*., 2016) who reported that, the *Salmonella* isolates tested were susceptible to chloramphenicol, Gentamicin and ciprofloxacin. The situation of antibiotic resistance is more complex and difficult, because *Salmonella* and bacterial pathogens are not routinely cultured and their resistance to commonly employed antibacterial both in public health and veterinary practices is rarely determined (Muktar *et* al., 2015). One of the *Salmonella* isolate in this study was resistant to Imipenem. Carbapenems possess broad spectrum antibacterial activity and are considered one of the most reliable drugs for treating bacterial infections. The emergence and spread of resistance to Carbapenems antibiotics constitute a major public health concern as there may be transmission of resistance strain to humans through food chain. Antimicrobial resistant foodborne pathogens are considered to be acquired primarily through consumption of contaminated food of animal origin or water (Mead *et al*., 1999). Implication of this investigation is that antimicrobial resistant strains of pathogenic bacteria may colonize the human population

through consumption of contaminated cow milk products sold locally at the study area and this can lead to failures in chemotherapy among the human consumers of these products.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The results of this study has established that, there is high total aerobic plate counts in both raw and fermented milk; 5.82 Log_{10} CFU/ml and 6.62 Log_{10} CFU/ml respectively, which is more than the permissible value 5.7 Log_{10} CFU/ml (European Food Safety Authority, 2004). This is capable of endangering human health when consumed which may lead to food borne illness.

The study established the overall *Salmonella* prevalence of 1.7 % from both raw and fermented milk samples, with 2.3% from raw milk and 1.1 % from fermented milk in Fika L.G.A. Their presence in raw and fermented milk suggests that consumption of any of these products has potential health risk to the consumers in the study area.

The invasion gene *inv*A was detected in three *Salmonella* species isolated in this study. *Salmonella* spp from raw and fermented milk may be transferred to the consumer. Antibiograms of the bacterial isolates revealed varying levels of resistance to the antibiotics tested which may cause health hazard and may spread bacterial resistance among the populace. However, the isolates were generally susceptible to Enrofloxacin and Gentamicin.

6.2 Recommendations

The following recommendations should be considered based on the findings of this study:

1. There is need for public awareness to always keep the animal housing and milking environment clean and the need to wash and disinfect the udder before milking.

2. Milkers should practice personal hygiene by using clean cloths and washing their hands regularly with potable water before milking.
3. Raw milk should be properly pasteurized before consumption or processing into other products including fermented milk.
4. Bacteriological examination of milk and milk products should be carried out periodically so as to assess their suitability for human consumption.
5. Government agencies like National Agency for Food and Drug Administration and Control (NAFDAC) to assess the indiscriminate use of antibiotics by animal owners.
6. Continuous studies should be initiated and to monitor the link between the *Salmonella* species and their resistance pattern between food animals and human.
7. Further studies should be carried out in the study area so as to identify the *Salmonella* spp in milk, milk products and other foods using more sensitive and faster techniques in order to reveal the true prevalence of foodborne diseases particularly salmonellosis.

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APPENDICES

Appendix I: The mean pH of raw milk samples based on sample location

S/No	Location	No of sample collected	pH mean±SD
1	Banale	60	6.41±0.22a
2	Gadana	49	6.44±0.18a
3	Bakadda	58	6.41±0.20a
Total		167	

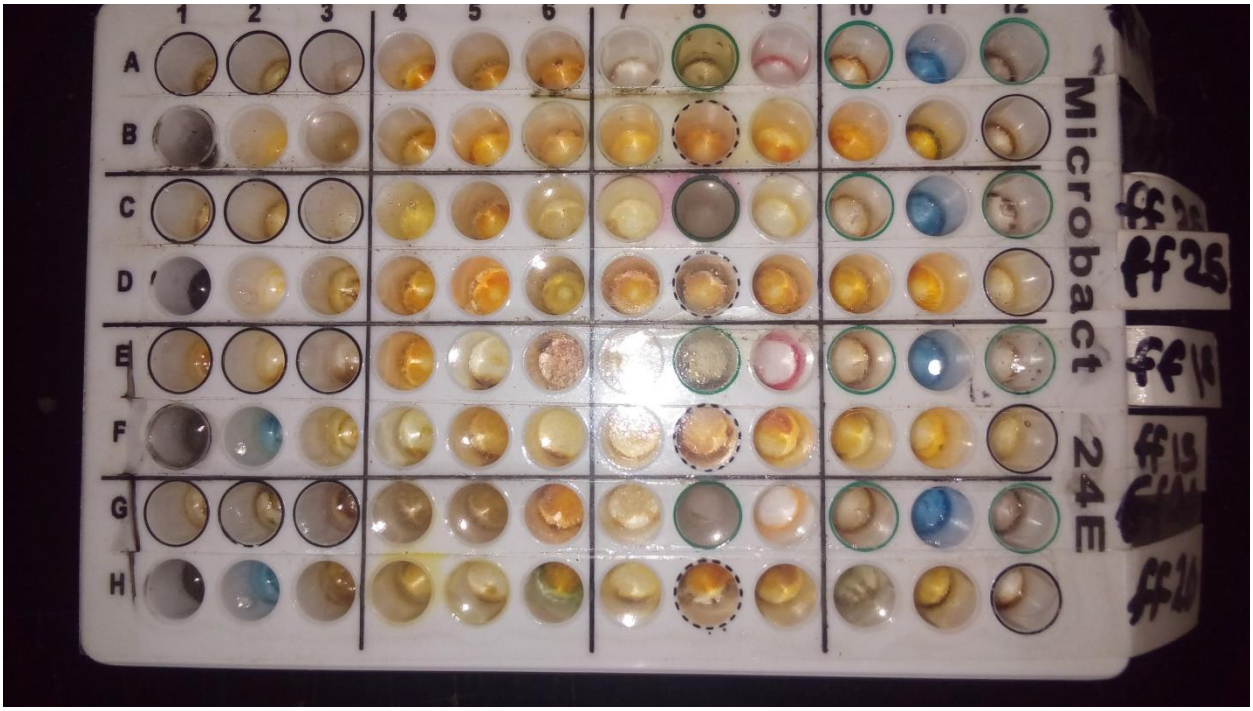
Appendix II: The mean pH of fermented milk samples based on sample location

S/No	Location	No of sample collected	pH mean±SD
1	Ngalda	60	3.73±0.02a
2	Fika	55	3.58±0.08a
2	Gadaka	62	3.59±0.07a
Total		177	

Appendix III: Mean pH of raw and fermented milk samples collected from Fika local government area

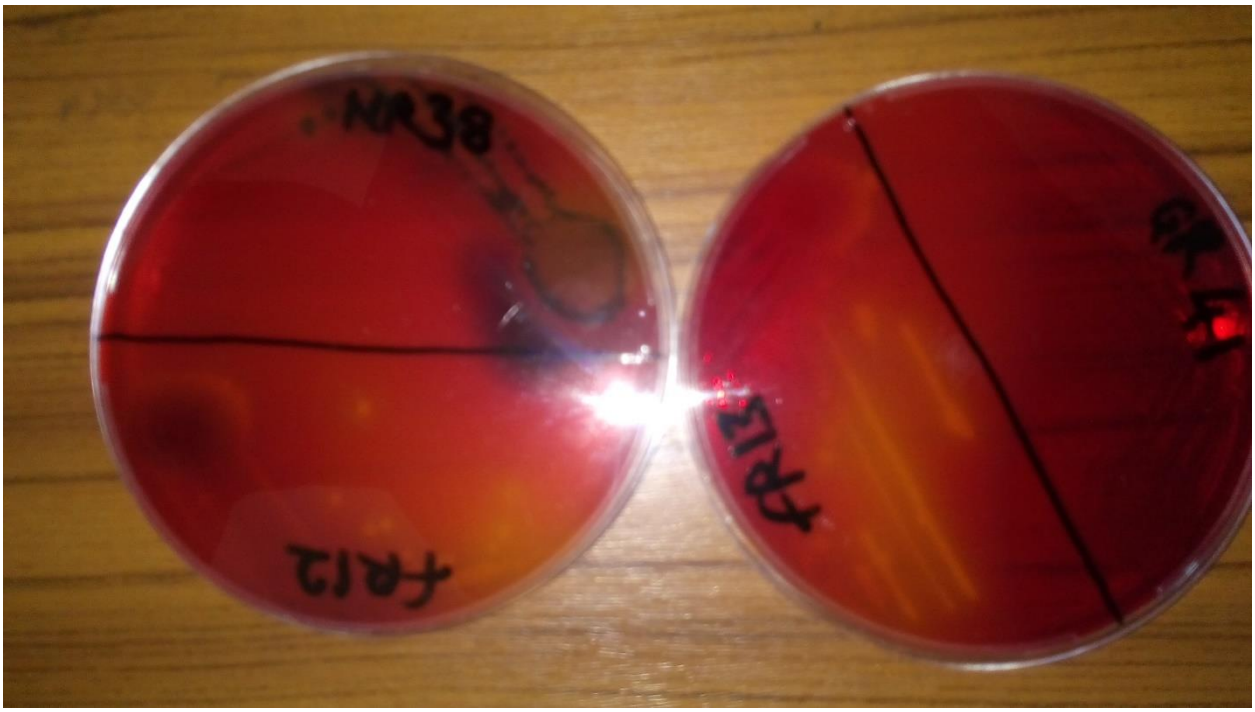
Sample type	Range	pH value (mean±SD)
Raw milk	6.0 – 6.8	6.42±0.20
Fermented milk	2.8 – 4.6	3.64±0.44

Appendix IV: Microbacts 24E following inoculation of suspected *Salmonella* isolates



Appendix V: Disc dispenser

Appendix VI: XLD agar Inoculated with fermented milk samples



Appendix VII: Total aerobic plate count and pH of raw and fermented milk samples

SAMPLE NO	COUNT ON NA Log10	pH
NF01	5.47712	3.6
NF02	7.07918	3.8
NF03	7.14613	3.7
NF04	7.26717	3.8
NF05	6.97772	3.8
NF06	7.04139	3.9
NF07	6.81291	3.8
NF08	6.8451	3.8
NF09	6.14613	3.7
NF10	5.77815	3.6
NF11	7	3.6
NF12	5.69897	3.5
NF13	7.35218	3.7
NF14	6.07918	3.7
NF15	7.35218	3.8
NF16	6.57978	3.6
NF17	7.41497	3.9
NF18	5.90309	3.8
NF19	6.60206	3.8
NF20	6.69897	3.9
NF21	6.5682	3.8
NF22	7.34242	3.8
NF23	5.8451	3.9
NF24	7	3.3
NF25	6.95424	3.7
NF26	7.39794	3.8
NF27	7.30103	3.6
NF28	6.90309	3.7
NF29	5.95424	3.6
NF30	6.27875	3.6
NF31	7.30103	4
NF32	5.47712	3.9
NF33	6.47712	3.9
NF34	7	3.9
NF35	6	3.6
NF36	6.04139	3.8

NF37	7.41497	3.8
NF38	7.43136	3.5
NF39	7.39794	3.7
NF40	6.5563	3.8
NF41	5.60206	3.8
NF42	7.38021	3.8
NF43	6.77815	3.8
NF44	7.14613	3.6
NF45	7.30103	3.7
NF46	0	3.7
NF47	6	3.7
NF48	6.72428	3.5
NF49	7.17609	3.9
NF50	6	3.6
NF51	6.95424	3.5
NF52	7.30103	3.6
NF53	7.0607	3.8
NF54	7.39794	3.9
NF55	6.54407	3.9
NF56	6.34242	3.6
NF57	6.90309	3.7
NF58	5.60206	3.6
NF59	6.44716	3.6
NF60	7.43136	3.8
GnR01	5.60206	6.4
GnR02	7.23045	6.2
GnR03	6.90309	6.7
GnR04	7.20412	6.3
GnR05	6.69897	6.6
GnR06	6.5563	6.6
GnR07	7.30103	6.7
GnR08	7.14613	6.4
GnR09	7	6.6
GnR10	0	6.3
GnR11	0	6.5
GnR12	7.38917	6.3
GnR13	7.43136	6.5
GnR14	0	6.4
GnR15	7.17609	6.4

GnR16	0	6.2
GnR17	7.07918	6.4
GnR18	5.69897	6.5
GnR19	0	6.7
GnR20	0	6.6
GnR21	0	6.5
GnR22	7.07918	6.2
GnR23	6.5563	6.3
GnR24	6.53148	6.5
GnR25	0	6.4
GnR26	0	6.4
GnR27	5.60206	6.4
GnR28	5.30103	6.7
GnR29	5.47712	6.5
GnR30	6.36173	6.2
GnR31	5.47712	6.4
GnR32	7.04139	6.5
GnR33	6.41497	6.3
GnR34	6	6.2
GnR35	7.30103	6.2
GnR36	7.25527	6.5
GnR37	6.63347	6.6
GnR38	6.69897	6.4
GnR39	5.60206	6.5
GnR40	7.41497	6
GnR41	0	6.8
GnR42	5.90309	6.3
GnR43	6.8451	6.4
GnR44	7.39794	6
GnR45	6.60206	6.3
GnR46	7.41497	6.6
GnR47	6.25527	6.4
GnR48	7.39794	6.3
GnR49	7	6.3
BnR01	6.64345	6.6
BnR02	0	6.2
BnR03	5.60206	6
BnR04	7.23045	6.3
BnR05	5.30103	6.2

BnR06	7.32222	6.3
BnR07	0	6
BnR08	7.07918	6.4
BnR09	7	6.4
BnR10	6.77815	6.4
BnR11	6.30103	6.2
BnR12	6.96379	6.4
BnR13	6.47712	6.5
BnR14	6.60206	6.7
BnR15	6.74036	6.3
BnR16	7.41497	6.8
BnR17	6	6.1
BnR18	6	6.4
BnR19	6.69897	6.7
BnR20	6.38021	6.7
BnR21	0	6.2
BnR22	5.47712	6.8
BnR23	0	6.3
BnR24	5	6.4
BnR25	6.17609	6.4
BnR26	5.8451	6.8
BnR27	7.17609	6.5
BnR28	6.41497	6
BnR29	6.54407	6.5
BnR30	6.60206	6.3
BnR31	5.77815	6.2
BnR32	6.51851	6.5
BnR33	6.30103	6.2
BnR34	6.38021	6.7
BnR35	7.43136	6.3
BnR36	6.92942	6.1
BnR37	6.11394	6.7
BnR38	6.30103	6.6
BnR39	6.17609	6.3
BnR40	6.60206	6.4
BnR41	6.65321	6.5
BnR42	6.30103	6.7
BnR43	6.14613	6.1
BnR44	6.60206	6.5

BnR45	7.44716	6.8
BnR46	7.32222	6.7
BnR47	7.38021	6.3
BnR48	7.19033	6.8
BnR49	7.39794	6.2
BnR50	5.77815	6.5
BnR51	6.80618	6.5
BnR52	6.20412	6.3
BnR53	7.42325	6.4
BnR54	5.69897	6.1
BnR55	6.66276	6.5
BnR56	7.23045	6.2
BnR57	5.69897	6.5
BnR58	7.43136	6.3
BnR59	6	6.4
BnR60	6	6.2
BkR01	6.50515	6.4
BkR02	7.43136	6.8
BkR03	5.30103	6.5
BkR04	5.30103	6.5
BkR05	5.69897	6.5
BkR06	5.30103	6.7
BkR07	7.41497	6.4
BkR08	5.60206	6.2
BkR09	5.47712	6.5
BkR10	0	6.5
BkR11	5.60206	6.3
BkR12	6	6.2
BkR13	6.25527	6.3
BkR14	6.14613	6.4
BkR15	7	6.4
BkR16	0	6.2
BkR17	6.53148	6.4
BkR18	6.65321	6.7
BkR19	7.30103	6.6
BkR20	6.32222	6
BkR21	7	6.7
BkR22	5	6.5
BkR23	5.30103	6.7

BkR24	5.95424	6.1
BkR25	6.5563	6.4
BkR26	6.77815	6.4
BkR27	6.07918	6.6
BkR28	5.60206	6.4
BkR29	7.07918	6.3
BkR30	7.39794	6.5
BkR31	6	6.4
BkR32	6.30103	6.1
BkR33	6.07918	6.8
BkR34	0	6
BkR35	6.30103	6.1
BkR36	5.47712	6.7
BkR37	6.39794	6
BkR38	6.62325	6.3
BkR39	7.17609	6.4
BkR40	5.77815	6.4
BkR41	6.47712	6.3
BkR42	7.25527	6.2
BkR43	5.77815	6.6
BkR44	7.44716	6.6
BkR45	5.8451	6.2
BkR46	5.8451	6.6
BkR47	7.04139	6.4
BkR48	6.04139	6.3
BkR49	7.17609	6.5
BkR50	6.81291	6.3
BkR51	6.69897	6.6
BkR52	6	6.5
BkR53	5.90309	6.6
BkR54	6.49136	6.6
BkR55	0	6.2
BkR56	5.90309	6.3
BkR57	6.47712	6
BkR58	6	6.3
GF01	6	4.6
GF02	7	3.2
GF03	7.07918	3.4
GF04	6.07918	3.5

GF05	7.33244	4.5
GF06	5.30103	2.9
GF07	7.40824	4
GF08	7.11394	4.4
GF09	5.69897	4.4
GF10	7.30103	2.9
GF11	5.47712	3.1
GF12	6.77815	2.9
GF13	6.8451	3.4
GF14	5.30103	3.8
GF15	7.44716	4.3
GF16	7.44716	3
GF17	7.30103	3.7
GF18	6.38021	2.9
GF19	6.64345	3.4
GF20	7.17609	3.2
GF21	6.64345	3.2
GF22	0	4.4
GF23	6.74036	3.4
GF24	5.69897	4.1
GF25	7.41497	3.5
GF26	7.38021	3.7
GF27	7.38021	3.8
GF28	5.60206	4.3
GF29	7.43136	3.4
GF30	7.14613	3
GF31	7.17609	3.1
GF32	7.14613	3.2
GF33	7.07918	3.6
GF34	7.11394	2.8
GF35	5.47712	2.8
GF36	7.42325	4.1
GF37	5.8451	3.4
GF38	7.43136	4
GF39	0	2.9
GF40	7	4.2
GF41	7.02938	3.9
GF42	7.34242	3.2
GF43	6.76343	3.2

GF44	6.5563	3.3
GF45	7.17609	3.9
GF46	7	4.6
GF47	6.95424	3.6
GF48	7.35218	3.7
GF49	6.92942	4
GF50	7.44716	3.1
GF51	5.90309	4
GF52	5	3.7
GF53	7.32222	4.3
GF54	7.38917	3.2
GF55	5.60206	3.2
GF56	7.43136	3
GF57	5.47712	4.1
GF58	7.13988	4
GF59	6.95424	3.6
GF60	7.35218	3.7
GF61	7.17609	4
GF62	7	3.1
FF01	7	3.9
FF02	5.60206	2.8
FF03	5.14613	2.9
FF04	7.38021	2.8
FF05	7.43136	4.2
FF06	6.68124	3.9
FF07	6.44716	2.9
FF08	5.77815	3.4
FF09	6.47712	3
FF10	6.86923	3.7
FF11	7.17609	3.4
FF12	7.09691	3.2
FF13	7.40654	3.4
FF14	7.43136	4.1
FF15	6.97772	3
FF16	7	2.9
FF17	6	3.9
FF18	7.39794	3.9
FF19	7.06446	4
FF20	5.8451	3

FF21	6.25527	4.6
FF22	6.64345	4.2
FF23	6.5563	4.1
FF24	6.86923	3.1
FF25	7.38021	4.2
FF26	7.07918	3.7
FF27	6.43136	3
FF28	6.5563	4.1
FF29	5.47712	4.4
FF30	7.43136	2.9
FF31	6.23045	3.4
FF32	6.97313	4
FF33	7.38021	3.5
FF34	6.77815	4.6
FF35	7.43136	3
FF36	6.95424	4.2
FF37	6.87506	3.6
FF38	7.44716	3
FF39	6.30103	3.4
FF40	6.14613	4.4
FF41	6	3.4
FF42	6.39794	2.8
FF43	7.07918	2.9
FF44	7.44716	3.8
FF45	7.30103	4.1
FF46	7.39794	3.7
FF47	7.43136	3.7
FF48	6.68124	4.1
FF49	6.8451	3.1
FF50	6.77815	3.5
FF51	7.43136	4.6
FF52	6.30103	3
FF53	6.14613	4.2
FF54	7.43136	3.6
FF55	6.95424	3.7