PRODUCTION OF BACTERIOCIN BY LACTIC ACID BACTERIA ISOLATED FROM CHEESE TO ENCHANCE THE SHELF LIFE OF MILLET DOUGH BALL (*FURA*)

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ABSTRACT

Bacteriocin for enhancing the shelf life of food is very promising in the food industry to substitute chemical preservatives due to been generally Regarded as Safe (GRAS). Consumer's awareness over chemical free foods has led to the search for alternative method of food preservation using Lactic acid bacteria. The aim of this study was to enhance the shelf life of millet dough ball (f*ura*) using bacteriocin producing bacteria isolated from fermented cheese. Two strains of lactic acid bacteria (LAB) of the genera *Lactobacillus* specie were isolated from cheese with mean count ranged from $2.3 \times 10^7 \pm 2.0 \times 10^6$ - $1.9 \times 10^7 \pm 2.3 \times 10^6$. The orgarnisms were identify as organisms as *Lactobacillus plantarum* strain AAAbd and *Lactobacillus bulgaricus* strain MN94590. They were tested for antibacterial activity against *Staphylococcus aureus* and *Salmonella* specie using agar well diffusion method, zones of inhibition were measured ranged from 6±0.00 to 12±2.00 Lactic acid bacteria strain were isolated and purified using ammonium sulphate precipitation, trichloroacetic acid (TC) precipitation and ultrafiltration studies to produce bacteriocin. Bacteriocins activity was determined at 2, 4, 6, 8, 10, and 12 pH range, storage conditions at 4 °C, 10 °C and 37 °C and heat temperatures at 60 °C, 100 °C and 121 °C for 15 min. Assessment of the biopreservative potential of bacteriocin on millet dough ball (*fura*) showed that the shelf life of millet dough ball (*fura*) was extended for 13 and 11 days on addition of bacteriocin produced by *Lactobacillus plantarum* strain AAAbd (plantaricin) and *Lactobacillus bulgaricus* strain MN945906 (bulgarican) respectively, while the initial shelf life of millet dough ball (*fura)* without bacteriocin (control) had a shelf life of 5 days, at 4° C. Proximate composition of millet dough ball (*fura*) sample such as Moisture content (54.36±0.09 – 53.21±0.02) of bacteriocin inoculated fura showed a significant difference ($P<0.05$) compared with *fura* without bacteriocin (control). This study suggest the use of bacteriocins from Lactic acid bacteria as a natural preservative in *fura* has shown to be effective against foodborne pathogens.

TABLE OF CONTENTS

Contents **Page**

Cover Page		
Title Page		ii
Declaration		iii
Certification		iv
Dedication		v
Acknowledgements		vi
Abstract		viii
Table of Contents		xi
List of Abbreviation		xv
List of Tables		xvi
List of Figures		xvii
List of Plates		xviii
List of Appendices		xix
CHAPTER ONE		
1.0	**INTRODUCTION**	1
1.1	Background to the Study	1
1.2	Statement of the Research Problem	4
1.3	Justification for the Study	5
1.4	Aim and objectives of the Study	5
CHAPTER TWO		
2.0	**LITERATURE REVIEW**	7
2.1	Fermentation	7
2.2	The role of fermentation in food	8

2.2.1 Flavour enhancement 8

2.2.2 Nutritional Quality 8

2.2.3 Preservative Properties 8

2.2.4 Detoxification 9

2.2.5 Antibiotic Activities 9

2.3 Lactic Acid Bacteria 10

2.3.1 Sources of LAB 11

2.3.2 Role of Lactic Acid Bacteria for Food Industry 12

2.3.3 LAB as Starter-Cultures in Cheese Industry 12

2.3.4 Importance of the Metabolites of LAB 13

2.3.5 Compound and Antimicrobial Spectrum Produced by Lactic Acid Bacteria 14

2.4 Food Fermented Lactic Acid Bacteria Used as Probiotic 15

2.5 Bacteriocins 16

2.5.1 Brief History on Bacteriocin 17

2.5.2 Classification of bacteriocins 18

2.5.2.1 Class I bacteriocins 18

2.5.2.2 Class II bacteriocins 18

2.5.2.3 Class III bacteriocins 18

2.5.2.4 Class IV bacteriocins 19

2.5.3 Characteristics of bacteriocins 19

2.5.4 Mechanism of Action of Bacteriocins 19

2.5.5 Use of Bacteriocins in Food Systems 20

2.5.6 Bacteriocins Produced by LAB 21

2.5.7 Microbial Origin of Bacteriocin 21

2.5.7.1 Bacteriocins of Archaea 22

2.5.7.2 Bacteriocins of Gram-negative Bacteria 22

2.5.7.3 Bacteriocins of Gram-positive Bacteria 23

2.5.8 Factors that contribute to the increasing number of applied Investigations on bacteriocins of Lactic acid bacteria 23

2.5.9 Application of Purified Bacteriocins to Dairy Products 24

2.5.10 Bacteriocins and Food Applications 25

2.5.11 Requirements and regulatory status for bacteriocins 26

2.6 Millet dough ball (*Fura)* 28

2.6.1 Preparation of millet dough ball (*Fura)* 28

CHAPTER THREE

3.0 **MATERIALS AND METHODS** 31

3.1 Study area 31

3.2 Sample Collection and processing 32

3.3 Culture Media Used 32

3.4 Isolation of Lactic Acid Bacteria (LAB) 32

3.5 Characterization and Identification of Lactic Acid Bacterial 32

3.6 Molecular Identification and Characterization of the isolates 37

3.6.1 DNA extraction 37

3.6.2 Polymerase chain reaction (PCR) 38

3.6.3 Integrity of the amplification 38

3.6.4 Purification of Amplified Product 39

3.6.5 Sequencing 40

3.7 Screening for Lactic Acid Bacteria with Potential Activity 40

3.7.1 Collection of pathogenic bacteria (indicator organism) and inhibitory activity of LAB 40

3.7.2 Inhibitory activity of LAB 40

3.8 Bacteriocin production 41

3.9 Extraction of Bacteriocin 41

3.10 Partial Purification of Bacteriocin 41

3.11 Determination of bacteriocin titre 42

3.12 Characterization of Bacteriocin 42

3.12.1 Heat Resistance 42

3.12.2 pH Sensitivity 42

3.12.3 Stability of bacteriocin during storage 43

3.13 Preparation of millet dough ball (*fura)* 43

3.14 Potential of BLAB to extend the shelf life of millet dough ball (*Fura)* sample (Shelf life study of *fura)* 44

3.14.1 Physical Appearance of Fermented *Fura* Product produced 44

3.14.2 Flavor Determination 45

3.15 Proximate composition of millet dough ball (*Fura)* 45

3.15.1 Determination of Moisture Content 45

3.15.2 Determination of Ash Content 45

3.15.3 Determination of crude fiber content 46

3.15.4 Determination of Protein Content 46

3.15.5 Determination of Fat 47

3.15.6 Determination of Total Carbohydrate Content 47

3.15.7 Sensory Property of millet dough ball (*Fura)* 47

3.16 Data Analysis 48

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION 49

4.1 Results 49

4.1.1 Total viable counts of lactic acid bacteria in fermented cheese 49

4.1.2 Morphology, Cultural and Physiological Characteristic of Lactic acid Bacteria in Fermented *Fura* 49

4.1.3 Potential of Lactic Acid Bacteria for Bacteriocin Production 52

4.1.4 Molecular Characterization of Lactic Acid Bacteria producing Bacteriocin (LAB A and LAB B) 53

4.1.5 Effect of lactic acid bacteria on the indicator organisms 54

4.1.6 Purification of Bacteriocin by Lactic Acid Bacteria 54

4.1.7 Effect of Temperature on Bacteriocin Activity (Au/ml) produced by *Lactobacillus plantarum* and *Lactobacillus bulgaricus* against indicator Organisms (*staphylococcus aureus and salmonella* sp) 57

4.1.8 Effect of Storage Stability Temperature on Bacteriocin Activity (Au/ml) produced by of *Lactobacillus plantarum* and *Lactobacillus bulgaricus against indicator organism* (*staphylococcus aureus and salmonella* sp) 58

4.1.9 Effect of pH on Bacteriocin Activity (Au/ml) produced by strain of *Lactobacillus plantarum* and *Lactobacillus bulgaricus* against indicator organisms (*Staphylococcus aureus and Salmonella* specie) 59

4.1.10 Bacterial load of Plantaricin and Bulgarican inoculated millet dough ball at 4°C (refrigeration) temperature 60

4.1.11 Physicochemical and microbial qualities of fermented millet dough ball (f*ura*) enriched with bacteriocin producing LAB 62

4.1.12 Sensory properties of millet dough ball (f*ura*) inoculated and uninoculated 64

4.1.13 Proximate Composition of millet dough ball (f*ura*) Samples Inoculated with Bacteriocins 65

4.2 Discussion 66

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS 74

5.1 Conclusion 74

5.2 Recommendations 74

REFERENCES 75

APPENDICES 86

LIST OF TABLES

Tables **Pages**

4.1 Total viable counts of lactic acid bacteria in fermented cheese 49

4.2 Morphology, Cultural and Physiological Characteristic of Lactic acid Bacteria in Fermented *Fura* 51

4.3 Bacteriocin producing ability of lactic acid bacteria (LAB) 52

4.4 Inhibition of Indicator Microorganisms by Lactic Acid Bacteria 54

4.5 Purification of Bacteriocin Produced by *Lactobacillus plantarum* AAAbd and *Lactobacillus bulgaricus* MN945906 56

4.6 Bacterial load of Plantaricin and Bulgarican inoculated millet dough ball (f*ura*) sample kept at 4°C (refrigeration) temperature 62

4.7 Physicochemical and microbial qualities of fermented millet dough ball (f*ura*) enriched with bacteriocin producing LAB 63

4.8 Sensory properties of millet dough ball (f*ura*) inoculated with Bacteriocins 64

4.9 Proximate Composition of millet dough ball (f*ura*) Samples Inoculated with Bacteriocins 65

LIST OF FIGURES

Figures		Page
2.1	Process Flow Chart for millet dough ball (*Fura)* Production	30
3.1	Map of Niger State showing Bosso local government Effect of Temperature on Bacteriocin Activity (Au/ml) produced by	31
4.1	Strain of *Lactobacillus plantarum (plantaricin)* and *Lactobacillus bulgaricus* (bulgarican) against indicator organisms (*staphylococcus aureus and salmonella* sp)	58
4.2	Effect of storage stabilityTemperature on Bacteriocin Activity (Au/ml) produced by strain of *Lactobacillus plantarum (plantaricin)* and *Lactobacillus bulgaricus* (bulgarican) against indicator organisms (*staphylococcus aureus* and *salmonella* sp)	59
4.3	Effect of storage stabilityTemperature on Bacteriocin Activity (Au/ml) produced by strain of *Lactobacillus plantarum (plantaricin)* and *Lactobacillus bulgaricus* (bulgarican) against indicator organisms (*staphylococcus aureus* and *salmonella* sp)	60

LIST OF PLATES

Plates **Page**

3.1 Preparation of millet dough ball (*fura)* 44

4.1 Amplified image of *Lactobacillus bulgaricus* strain MN945906 and *Lactobacillus plantarum* strain AAAbd 53

LIST OF APPENDICES

Appendix		Page
A	Composition and preparation of media	87
B	Nucleotide sequence of *Lactobacillus plantarum* strain AAAbd and *Lactobacillus bulgaricus* strain MN945906	89
C	Activity plates	92
D	Shelf life study of millet dough ball (fura) inoculated with Bacteriocin	94
E	Sensory evaluation questionnaire	95

LIST OF ABBREVIATION

Abbreviation	Meaning
ANOVA	Analysis of viariance
AOAC	Association of Official Analytical Chemists
AU/ ml	activity units per ml
BLAB	bacteriocin producing lactic acid bacteria
CDC	Centre for diseases control
CFU	colony forming unit
DNA	Deoxyribonucleic acid
HCl	hydrochloric acid
LAB	Lactic acid bacteria
NaOH	sodium hydroxide
mRNA	messenger RNA
MRS	De Man, Rogosa and Sharpe agar
PCR	Polymerase chain reaction
RNA	Ribo nucleic acid
TC	Trichloroacetic acid
WHO	World health organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Food products such as fermented foods are among the main constituent of human diet and have been manufactured and consumed since the development of human civilizations (Marco *et al.*, 2017). The method whereby microorganisms and their enzymes bring about these desirable changes in food materials is known as fermentation. Food fermentation methods can be categorized by the metabolites and microorganisms involved (Admassie, 2018). Fermentation can also be described based on the food substrates, which include meats and fish, dairy, vegetables, soy beans and other legumes, cereals, starchy roots and grapes and other fruits. Raw materials that contain high concentration of monosaccharide and disaccharide or in some cases starch are fermented by lactic acid bacteria (Marco *et al.*, 2017).

Lactic acid bacteria (LAB) are group of Gram positive, non-sporulating, anaerobic or facultative aerobic cocci or rods, fastidious on artificial media, however they grow readily in most food substrates and lower the pH rapidly to a point where competing organisms are no longer able to grow and it is one of the main fermentation products of the metabolism of carbohydrates (Hayek *et al.*, 2013). Lactic acid bacteria for thousands of years have been exploited in the production of fermented foods because of their ability to produce desirable changes in the taste, flavor, texture and to inhibit pathogenic and spoilage microorganisms. Lactic acid bacteria are generally regarded as safe (GRAS) which plays an essential role in food fermentation, preservation and a wide variety of strains were routinely employed as starter cultures in the manufacture of dairy, meat, vegetable and bakery products. They contribute to the enhancement of the

sensory, quality and safety features of these fermented foods (Holzapfel and Wood, 2014). Ashrethalatha *et al*, (2016)., Bennani *et al*., (2017)**.,** Colombo *et al* (2018) All conducted research on the isolation and characterization of lactic acid bacteria from different sources and having anti-microbial activity against food spoilage organism.

Their antimicrobial activity has been attributed to fermentation products such as organic acids, carbon dioxide, hydrogen peroxide, diacetyl e.t.c, which can inhibit pathogenic and spoilage microorganisms, extending the shelf life and enhancing the safety of food products. Several research has been conducted on the antimicrobial activity of lactic acid bacteria (Abdelhadi *et al.,* 2016).

Cheese is a diary product derived from milk produced in a wide range of flavors, textures and forms by coagulation of the milk protein casein, it is also an excellent source of protein, fats and minerals such as calcium, iron and phosphorus, vitamins and essential amino acids, thus making it an important food in the diet of both old and young, usually from the milk of cows, buffalo, goats or sheep ,it is an important food component in the healthy diet of human's highly nutritious rich food and source of protein, peptides, amino acids, vitamins, salt, and essential minerals including calcium. Cheese however, has been shown to have antimicrobial properties that prevent disease, it has been used as drugs for certain infections when common antimicrobial agents failed it had also been found that soft cheese has growth inhibitory activity against common bacteria that caused diarrhea in South West Nigeria (Oladipo and Jadesimi, 2013 ; Ajayi *et al*., 2018). During production, the milk is usually acidified and adding the enzymes rennet caused coagulation (Sangoyomi *et al*., 2010). The solids are separated and pressed into final form. It is mostly produced in some parts of Southern Nigeria and predominately in the Northern parts.

Millet dough ball (*Fura)* is an indigenous fermented cereal based foods majorly consumed in the Northern part of Nigeria. It is a thick ball snack that is produced mainly from millet or sorghum and spices such as pepper, cloves and ginger (Ogodo *et al*., 2018). It is a semi solid dumpling meal made from millet or sorghum and is used traditionally as stable food in most West African countries as well as Nigeria and Ghana. Though, millet dough ball (f*ura*) has a short shelf life of 3 to 4 days at the temperature of about 5°C and 1-2 days at room temperature of 25°C while at 35°C it can only last for 18hours with unacceptable quality, after which they can be Deteriorate by microorganisms whose presence poses health risk as they can be source of infection when consumed (Achi and Ukwuru, 2015).

Bacteriocins are generally defined as peptides or proteins ribosomal synthesized by bacteria that inhibit or kill other related or unrelated microorganisms (Leroy and De Vuyst, 2004; Cotter *et al*., 2005). Bacteriocins may have a narrow spectrum, by inhibiting bacteria taxonomically close, or a broad spectrum, by inhibiting a wide variety of bacteria (Cotter *et al.,* 2005; Mills *et al*., 2011). Bacteriocins have attracted considerable interest for their use as safe food preservatives, as they are easily digested by the human gastrointestinal tract (Mills *et al*., 2011). The use of bacteriocins as natural food preservatives fulfills consumer demands for high quality and safe foods without the use of chemical preservatives. However, the application of bacteriocins as food additives can be limited for various reasons, such as effectiveness of pathogen elimination or its high price (Chen and Hoover, 2003). Research interest in bacteriocins has continued over the past years, as investigators continuing to search for new and more effective bacteriocins to address both biologic and economic concerns.

The application of bacteriocins for biopreservation of foods usually includes the following approaches, inoculation of food with the bacteriocin-producer strain, addition

of purified or semi-purified bacteriocin as food additive and use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing (Chen and Hoover, 2003). An increasingly number of bacteriocins have been isolated and identified from Gram-positive and Gram-negative microorganisms.

Three major Classes of bacteriocin has been propose Class I – small post-translationally modified peptides; Class II – unmodified bacteriocins; and Class III – larger peptides (Célia *et al.,* 2018).

Many studies showed the potential of applying bacteriocins or bacteriocin-producing strains into foods, such as meat, dairy products, fish, alcoholic beverages, salads, and fermented vegetables (O'Sullivan *et al.,* 2003; Ramu *et al.,* 2015). Bacteriocins produced by LAB have attracted special interest as potential alternative safe commercial food preservatives. LAB have been used as food and feed preservatives for centuries, and bacteriocin-producing LAB could replace chemical preservatives for the prevention of bacterial spoilage and the outgrowth of pathogenic bacteria in food products (Ramu *et al.,* 2015).

1.2 Statement of the Research Problem

Modern day consumers prefer preservatives of natural origin to those of chemical origin due to side effects like cancer, cardiovascular diseases and aging which pose a major threat to consumers and there is increasing trend towards less processed food (Saranj *et. al.,* 2013). The contamination of *fura* and its products by *Salmonella* typhimurium, *Escherichia coli, Staphylococcus aureus and klebseilla* specie have been the major source of several foodborne outbreaks (Center for disease control, 2015). The resistance of *Salmonella typhimurium, Escherichia coli, a*nd *Staphylococcus aureus* to most commonly used preservatives have created problems for the food industry (World Health Organization, 2002). In recent years bacterial antibiotics resistance has been

considered a problem due to the extensive use of classical antibiotics in the treatment of human and animal diseases and has been restricted for use in foods (Jack *et al*., 1995).

1.3 Justification for the Research

Fermentation, low or high temperature, acidity, use of stabilizers, chemical additives, thermal pasteurization, sterilization and freezing has been used to control food spoilage but the excessive use of these technique change the flavour, taste and texture of food and can be toxic to the consumers (Aqeela, 2015).

Lactic acid bacteria (LAB) has also made it possible for human to increase the shelf life of food and food products by using antimicrobial activity of LAB without damaging food contents (Soomro *et al.,* 2002). Different compounds such as diacetyl, organic acids, hydrogen peroxide and proteins (bacteriocins) are produced during lactic fermentation and used against food spoiling microorganisms.

Since consumers are concerned about the safety of chemical preservatives, the potential applications of bacteriocins from lactic acid bacteria in foods have received increasing attention in recent years (Papagianni and Anastasiadou, 2009). The preservation of foods by natural and microbiological methods may be satisfactory approach to reduce the incidence of food borne illnesses (Altuntas *et al*., 2010). Due to their resistance to temperature and low pH, bacteriocins are digested by human peptidases, thus avoiding resistance and problems associated to the presence of residues in food (Javed, 2009). In order to control the abusive use of antibiotics in food, one plausible alternative is the application of bacteriocins produced by Lactic acid bacteria, since they are active in Nano-molar range and have no toxicity (Deraz *et al*., 2005).

1.4 Aim and Objectives of the Study

The aim of the study was production of bacteriocin by lactic acid bacteria isolated from cheese to enhance the shelf life of millet dough ball (*fura*)

The objectives of the study were to:

i. isolate, characterize and identify lactic acid bacteria from cheese
ii. produce, extract and partially purify bacteriocins produced by the Lactic acid bacteria
iii. determine the effect of temperature, storage and pH on the bacteriocins produced by lactic acid bacteria
iv. evaluate the effect of the addition of purify bacteriocin producing LAB on millet dough ball (*Fura)* on the shelf life (inoculated and control).
v. Determine the proximate composition and sensory attributes of the millet dough ball (*Fura)* to which bacteriocin is added.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Fermentation

Fermentation are chemical changes in foods brought about by enzymes from living microorganisms such as bacteria and yeasts, that use the nutrients in the food as an energy source which result in a transformation of the original food into one with organic acids and other compounds beneficial for health. Fermented foods have a unique flavor that is tangy, pungent, and aromatic. There are dozens of fermented foods ranging from drinks to side dishes (Foroutan, 2012).

Fermented foods are found rich in nutritional values (vitamin K, trace minerals, B-vitamins and probiotics) with their easy preparation and available as more economical diet as health point view (Srivastava, 2018). Beneficial effects of fermented foods on health are reported and fermentation is used by many people due to its capacity to preserve foods, enhance shelf life, and improve flavour. Fermented foods have important part in most of world people diet due to have many cultures which is associated with many health benefits (Srivastava, 2018).

Microorganism's contributions in fermentation process are associated with many health benefits and in this regards, lactic acid bacteria (LAB) are most studied microorganisms which synthesize vitamins and minerals as well as also produce biologically active peptides with enzymes (proteinase and peptidase) with removal some non-nutrients (Themelis *et al*., 2017). Fermented foods and beverages are reported as first processed food products consumed by most of humans being at worldwide and these are mainly yogurt and cultured milk, wine and beer, sauerkraut, kimchi and fermented sausage and these products has shown their initial values due to having their improved shelf life, safety and organoleptic properties. Fermented foods have shown their important due to

enhanced nutritional and functional properties via transformation of substrates and formation of bioactive or bioavailable end products which are recent interest about their food choice. Many fermented foods have living microorganisms with genetically similar to microbial strains used as probiotics.

2.2 The Role of Fermentation to Food

2.2.1 Flavor enhancement

Fermentation makes the food palatable by enhancing its aroma and flavor. These organoleptic properties make fermented food more popular than the unfermented one in terms of consumer acceptance (Blandino *et al*., 2003)

2.2.2 Nutritional quality

A number of foods especially cereals are poor in nutritional value, and they constitute the main staple diet of the low-income populations. However, LAB fermentation has been shown to improve the nutritional value and digestibility of these foods. The acidic nature of the fermentation products enhances the activity of microbial enzymes at a temperature range of 22-25 °C (Mokoena *et al.,* 2005). The enzymes, which include amylases, proteases, phytases and lipases, modify the primary food products through hydrolysis of polysaccharides, proteins, phytates and lipids respectively. Thus, in addition to enhancing the activity of enzymes, LAB fermentation also reduces the levels of anti-nutrients such as phytic acid and tannins in food leading to increased bioavailability of minerals such as iron, protein and simple sugars. The number of vitamins is also increased in the ferment (Santos *et al*., 2008).

2.2.3 Preservative properties

The preservative activity of LAB has been observed in some fermented products such as cereals, and yogurt. Lowering the pH below 4 through acid production, inhibits the growth of pathogenic microorganisms which can cause food spoilage, food poisoning

and disease (Ananou *et al*., 2007). For example, LAB has antifungal activities (Schnurer *et al*., 2005). By doing this, the shelf life of fermented food is prolonged.

2.2.4 Detoxification

Detoxification of mycotoxins in food through LAB fermentation has been demonstrated over the years (Chelule *et al*, 2010). Using LAB fermentation for detoxification is more advantageous in that it is a milder method which preserves the nutritive value and flavor of decontaminated food. In addition to this, LAB fermentation irreversibly degrades mycotoxins without leaving any toxic residues. The detoxifying effect is believed to be through toxin binding effect (Chelule *et al.*, 2010).

2.2.5 Antibiotic activities

Fermentation has been demonstrated to be more effective in the removal of gram negative than the gram-positive bacteria, which are more resistant to fermentation processing (Guandalini *et al*., 2006). As such, fermented food can control diarrhea diseases in children (Guandalini *et al*., 2006). Moreover, LAB is also known to produce protein antimicrobial agents such as bacteriocins. Bacteriocins are peptides that elicit antimicrobial activity against food spoilage organisms and food borne pathogens but do not affect the producing organisms. LAB also synthesizes other anti-microbial compounds such as, hydrogen peroxide, reuterin, and reutericyclin (Sauer *et al*., 2008). Other applications of LAB include their use as probiotics that restore the gut flora in patients suffering from diarrhea, following usage of antibiotics that destroy the normal flora (Ananou *et al.,* 2007).

2.3 Lactic Acid Bacteria

Lactic acid bacteria (LAB) species are indigenous to food-related habitats, including plant (fruits, vegetables, and cereal grains) and milk environments. In addition, LABs are naturally commensal in small intestine, colon and vagina of human and animals (Goodarzi, 2018). They are characterized as Gram-positive cocci or rods, non-aerobic but aerotolerant, able to ferment carbohydrates for energy, lactic acid production, nonmotile and non-sporeforming. Physiologically LAB possess unique properties like resistance to bacteriophages, proteolytic activity, lactose and citrate fermentation, production of polysaccharides, high resistance to freezing and lyophilization, capacity for adhesion and colonization of the digestive mucosa, and production of antimicrobial substances. In the first case two molecules of lactate are generated (as in *Streptococcus* and *Lactococcus),* and in the second, lactate, ethanol and carbon dioxide are produced, as in *Leuconostoc* and some lactobacilli. Lactic acid bacteria are also able to produce small organic substances that contribute with aroma and give specific organoleptic attributes to the products (Caplice and Fitzgerald, 1999). These microorganisms are found in milk, meat and fermented products, as well as in fermented vegetables and beverages inhibiting the growth of pathogenic and deteriorating microorganisms, maintaining the nutritive quality and improving the shelf life of foods. They have also been used as flavor and texture producers. Lactic acid bacteria include various major genera: *Lactobacillus, Lactococcus, Carnobacterium, Enterococcus, Lactosphaera, Leuconostoc*, *Melissococcus, Oenococcus*, *Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella*. Other genera are: *Aerococcus, Microbacterium*, *Propionibacterium* and *Bifidobacterium* (Carr *et al.,* 2002). *Lactobacillus acidophilus, L. plantarum, L. casei, L. casei rhamnosus, L. delbrueckii*

bulgaricus, L. fermentum, L. reuteri, Lactococcus lactis lactis, Lactococcus lactis cremoris, Bifidobacterium bifidum, B. infantis, B. adolecentis, B. longum, B. breve, Enterococcus faecalis, Enterococcus faecium, are some of the most common species (Garrity, 1984) and some strains are recognized as probiotics (Fuller, 1989; Parada *et al.*, 2003). Sugar fermentation followed by a reduction in pH due to the production of lactic and other organic acids is an important factor for the inhibition of growth of undesired microorganisms. The low pH makes organic acids lipossoluble, allowing them to break through the cell membrane and reach the cytoplasm of pathogens (Haller *et al*, 2001). The competition for essential nutrients, accumulation of D-amino-acids and diminution of the oxirredutive potential also contribute to their inhibitory effect (Parada *et al.,* 2007).

The basic function of LAB is in food fermentation and recognized as GRAS (generally recognized as safe). LAB can be utilized in production of various fermented products of dairy, meat and vegetable. It helps in protection of nutritional qualities of raw material, enhances the shelf life of food and protects food against spoilage and pathogenic organisms. Major compounds produced in LAB fermentation are organic acids, hydrogen peroxide and bacteriocins. Additionally LAB can be utilized in production of probiotics. Some of these bacteria produce antagonistic substances, called bacteriocins, which in small amounts are very active against pathogens (Klaenhammer *et al.,* 1994; Moreno *et al.*, 2006).

2.3.1 Sources of LAB

Some LAB is associated with the mouth flora, intestine and vagina of mammals, while others are present in fermented seafood, such as *Lactobacillus plantarum* and *Lactobacillus reuteri*, which are reported to be associated with plaa-som fermented Thai fish (Saithong *et al*., 2010) LAB are the most important bacteria used in the

fermentation industry of dairy products, such as yogurt, cheese, sour milk and butter, and in combination with yeast are commonly used to ferment cereal products such as dough (Muhialdin *et al*., 2011)

2.3.2 Role of lactic acid bacteria for food industry

Fermentation is one of the oldest ways of food preservation. This is made possible by the use of Lactic Acid Bacteria or LAB that plays the major role in this process. Even with the absence of oxygen, it drives the reactions of converting the carbohydrate to lactic acid with addition of carbon dioxide and other organic compounds such as mannitol and dextran. It also contributes to smell, taste, texture and color of the foods. Due to its many uses in the food industry, a study by (Capozzi, 2012) highlighted the impact of the potential biotechnological applications LAB in food-making process. Genetically engineered tactics with better fermentation efficiency better shelf life, nutritional and sensory properties for the product. When food products are made, undesirable contaminants can lead to poor flavor, low yield and food poisoning. LAB can be genetically engineered to grow faster than contaminants, as well as inhibit and destroy the growth of contaminants including pathogens by producing antimicrobial agents (Matunga, 2003). Because LAB has many uses, cultures are being kept. In order to preserve LAB for future uses, freezing is frequently used. Nonetheless, its analytical way of preservation decreases the acidifying and viability ability when thawed. Genetically modified (GM) LAB is being created in order to increase the performance of the said bacteria even subjected to such condition.

2.3.3 LAB as starter-cultures in cheese industry

Milk is a highly perishable food raw material, therefore, its transformation in cheese or other form of fermented dairy product cheese-making is based on application of LAB in the form of defined or undefined starter cultures that are expected to cause a rapid

acidification of milk through the production of lactic acid, with the consequent decrease in pH, thus affecting a number of aspects of the cheese manufacturing process and ultimately cheese composition and quality (Briggiler-Marco *et al.,* 2007) The starter-culture applied in this natural fermentation, is usually a poorly known micro flora mix that although having a predominance of LAB, may also contain non-LAB microorganisms, and its microbial diversity and load is usually variable over time. In fact, studies directed to characterize traditional cheeses show that those made from raw milk harbor a diversity of LAB (Bernardeau *et al.,* 2008) depending on geographical region, where a few may show particular interesting technological features that upon optimization may have industrial applications. For example, because wild strains need to withstand the competition of other microorganisms to survive in their hostile natural environment, they often produce antimicrobials substances called bacteriocins, which are natural antibacterial proteins that can be incorporated directly into fermented foods or indirectly as starter culture (Bernardeau *et al.,* 2008).

2.3.4 Importance of the metabolites of LAB

LAB is well known for their antifungal activity, which is related to the production of a variety of compounds including acids, alcohols, carbon dioxide, diacetyl, hydrogen peroxide, phenyl acetic acid, bacteriocins and cycle peptides (Gerez *et al*., 2009). These compounds were added to several foods in order to conserve them from food-borne and spoilage microorganisms. The main acids produced by LAB are lactic acid and acetic acid, besides certain other acids depending upon the strain of LAB. These acids will be diffused through the membrane of the target organisms in their hydrophobic un-dissociated form then used to reduce the cytoplasmic pH and stop metabolic activities. Other factors that contribute to the preservative action of the acids are the sole effect of pH, the extent of the dissociation of the acid and the specific effect of the molecule itself

on the microorganisms. Bacteriocins exhibit good potential for use in the food industry and as bio-preservation agents. Bacteriocins are small, ribosomally synthesized, antimicrobial peptides or proteins that display inhibition activity toward related species, with no reports about fungal inhibition. The notable property of LAB supernatant is the heat stability of the antifungal compounds present in it. This will promote the use of LAB supernatant and/or antifungal compounds in heat-treated foods. The supernatant of certain LAB observed to be active within a wide range of pH, starting from as low as three and up to nine depending upon the strain (Muhialdin *et al*., 2011).

2.3.5 Compound and antimicrobial spectrum produced by lactic acid bacteria

2.3.5.1 Organic acids: the amount and type of acids produced during fermentation influence the subsequent microbial activity in the fermented materials. Acetic acid, for example is more antagonistic against yeasts compared to lactic acid. Some oxidative yeasts are able to utilize organic acids as a carbon and energy source and consequently cause spoilage through deacidification in fermented especially plant materials where they are naturally present (Niku *et al*., 1999). The inhibitory effect of organic acids is mainly caused by undissociated form of the molecule, which diffuses across the cell membrane towards the more alkaline cytosol and interferes with essential metabolic functions. The toxic effects of lactic and acetic acid include the reduction of the intracellular pH and dissipation of the membrane potential (Valerio *et al*., 2004).

2.3.5.2 Hydrogen Peroxide: Antimicrobial activity of hydrogen peroxide is attributed to its strong oxidizing effect on the bacterial cell and to the destruction of basic molecular structures of cell proteins (Leroy *et al*., 2006)).In raw milk, hydrogen peroxide produced by LAB can after after being catalysed by lacto peroxidase, oxidase endogenous thiocyanate. The oxidized intermediary products are toxic to different bacteria (Leroy *et al*., 2006).

2.3.5.3 Diacetyl, Acetaldehyde and Acetoin: Heterofermentative LAB produce active acetaldehyde by decarboxylation of pyruvate. This product then condenses with pyruvate, forming d-acetolactate and it is converted by alpha acetolactate synthesis to diacetyl. The product of decarboxylation of alpha acetolactate and reduction of diacetyl is acetoin (Collicins *et al*., 2009). Diacetyl (2,3-butanedione) is best known for the buttery aroma that it imparts to fermented dairy products, but this property as well as high concentration needed to provide preservation of food limit the use of diacetyl as food preservative. Similarly an acetaldehyde usually present in fermented dairy products in concentration smaller than necessary for inhibition of undesired microorganisms also play a role in controlling the growth of contaminants together with other antimicrobial metabolites of LAB.

2.3.5.4 Carbondioxide: The influence of carbondioxide on product preservation is twofold, except for its own antimicrobial activity, it creates an anaerobic environment by replacing the existent molecular oxygen. The antifungal activity of CO_2 is due to the inhibition of enzymatic decarboxylation and to its accumulation in the membrane lipid bilayer resulting in dysfunction in permeability (Strom *et al*., 2002).

2.3.5.5 Reuterin and Reutericyclin: Selected isolates of *Lactobacillus reuteri* produce two compounds reuterin and reutericyclin, both active towards Gram positive bacteria, Reutericyclin is a tetramic acid derivative and reuterin is a mixture of monomeric hydrated monomeric and cyclic dimeric forms of Bhydroxypropionaldehyde with a broader spectrum of inhibitory activity, including Gram negative bacteria, fungi and protozoa (De Vuyst *et al*., 2006).

2.4 Food Fermented Lactic Acid Bacteria Used as Probiotic

Live microbes associated with food fermentations can provide beneficial functions in the gastro intestinal tract is consistent with the emerging view that core health benefits

of probiotic cultures can be assigned to a species, rather than to specific strains of a species (Salminen *et al*., 2014). At least this is the case for some species of LAB for which certain strains have long been applied as probiotics. Most human trials have found that the LAB strains tested may exert anti-carcinogenic effects by decreasing the activity of an enzyme called β-glucuronidase which can generate carcinogens (Heterocyclic amines) in the digestive system. Lactobacilli de- conjugate the bile salts in the intestine to form bile acids and thereby inhibit micelle formation. This leads to decreased absorption of cholesterol (Prasad *et al.,* 2011). Several small clinical trials have indicated that consumption of milk fermented with various strains of LAB may result in modest reductions in blood pressure. It is thought that this is due to the ACE (Angiotensin-converting enzyme) inhibitor-like peptides produced during fermentation. Increasing or improving phagocytosis as well as increasing the proportion of T-lymphocytes and natural killer cells. Probiotics are also known to reduce dental carries in children as well as aid in the treatment of Helicobacter pylori infection (Narwal, 2011). Therefore, a reasonable argument could be made that these foods should be considered to have similar health benefits as those conferred by probiotic lactobacilli of the same species.

2.5 Bacteriocins

Bacteriocins are bacterial ribosomally synthesized peptides or proteins with antimicrobial activity. Initially bacteriocins are mainly described to the colicins which are relatively large proteins of up to 80 kDa and were primarily obtained from E. coli. They were capable to kill very closely related bacteria upon binding to the inner membrane or other cytosolic targets (García-Bayona *et al.,* 2017).

Bacteriocins produced by LAB which are capable to kill closely related micro-organisms. Bacteriocins produced by LAB are thermostable cationic molecules contains

up to 60 amino acid residues and hydrophobic patches. The affectivity of LAB bacteriocin is mainly due to electrostatic interactions with negatively charged phosphate groups on target cell membranes through initial binding, forming pores and killing the cells leads to lethal damage and autolysin activation to digest the cellular wall. But by nature these bacteriocins are inactivated by proteases in the gastrointestinal tract. However, LAB bacteriocins are considered good bio-preservative agents due to non-toxic, non-immunogenic, thermo-resistance characteristics and broad bactericidal activity. These bacteriocins are most effective against Gram-positive bacteria and some, damaged, Gram-negative bacteria including various pathogens such as *L. monocytogenes, Bacillus cereus, S. aureus, Salmonella* etc.

However, today world is recognizing the bacteriocins as small, heat-stable cationic peptides synthesised by Gram positive bacteria called LAB and possess wider spectrum of inhibition (Mokoena, 2017). Traditionally LAB is regarded as safe for use in food so the food bio-preservation is mainly rely on the use of LAB bacteriocins.

2.5.1 Brief history on bacteriocin

Bacteriocins were first discovered by A. Gratia in 1925. He was involved in the process of searching for ways to kill bacteria, which also resulted in the development of antibiotics (Narasimhulu *et al*., 2010) and the discovery of bacteriophage (Bell *et al*., 2011) all within a span of a few years. He called his first discovery a colicine because it killed *E. coli.* Escherichia coli (*E. coli*) belong to a group of pathogenic bacteria called gram-negative enterobacteria that also includes Salmonella and Pseudomonas (Wasi and Ahmad, 2010, St-Onge *et al*., 2010). *E. coli* and Salmonella found in contaminated foods can cause diarrhea, and even death in severe cases (Wasi and Ahmad, 2010, St-Onge *et al*., 2010). Bacteriocins are categorized in several ways, including producing strain, common resistance mechanisms, and mechanism of killing. There are several

large categories of bacteriocin which are only phenomenologically related. These include the bacteriocins from gram positive bacteria- the colicins, microcins, bacteriocins from Archaea. Besides the production of bacteriocins, some LAB are able to synthesize other antimicrobial peptides that may also contribute to food preservation and safety (Kristiansen *et al*., 2011)

2.5.2 Classification of bacteriocins

According to Klaen hammer, bacteriocins can be classified into four groups on the basis of their molecular mass, thermo-stability, enzymatic sensitivity, presence of post translationally modified amino acids, and mode of action. (Singh *et al.,* 2013).

2.5.2.1 Class I bacteriocins: The class I bacteriocins are small peptide inhibitors and include nisin and other lantibiotics. They are further divided into two subgroups on the basis of structure and charge of the compound:

Group Ia, which consists of screw-shaped, amphipathic, small cationic peptides that produce voltage-dependent pores by unspecific interaction with the membrane of the target cell.

Group Ib, which consists of anionic or neutral peptides having a globular shape.

2.5.2.2 Class II bacteriocins: This group comprises heat-stable peptides with molecular masses smaller than 10 kDa and with no modified amino acids. Members of this class can be further sub-classified into four groups:

i. Group IIa: Consists of anti-listerial peptides showing the consensus sequence YGNGV at their N-terminal sequence.
ii. Class IIa: bacteriocins have a large potential for use in food preservation as well medical applications, due to their strong antilisterial activity, and broad range of activity.

iii. Class IIb: bacteriocins (two-peptide bacteriocins) require two different peptides for activity. Other bacteriocins can be grouped together as

iv. Class IIc (circular bacteriocins). These have a wide range of effects on membrane permeability, cell wall formation and pheromone actions of target cells.

2.5.2.3 Class III bacteriocins: This group consists of peptidic antibiotics that are heat-labile proteins with a molecular mass larger than 30 kDa.

2.5.2.4 Class IV bacteriocins: This group consists of either glycoproteins or lipoproteins that require non-protein moieties for their activity (Singh *et al*., 2013)

2.5.3 Characteristics of bacteriocins

i. Bacteriocins must have two requirements to become lethal, to be cationic and highly hydrophobic. Most of the class I and II bacteriocins are cationic at pH 7.0. Their high isoelectric point allows them to interact at physiological pH values with the anionic surface of the bacterial membrane, which leads to insertion of the hydrophobic moiety into the bacterial membrane and build up the trans membrane pore which cause gradient dissipation and cellular death

ii. Heat-stability is another property of bacteriocins (low molecular weight). Complex pattern of mono sulfide and disulfide intra molecular bonds helps in the stabilization of secondary structures by reducing the number of possible unfolded structures (entropic effect) (Cintas *et al*., 1995) observed that most of the supernatants of bacteriocins producing strains are resistant to autoclaving conditions and heat treatment (100 and 121 ^{0}C). However, some bacteriocins produced by *Lactobacillus* strains (helveticin J) were inactivated by 10 to 15min treatments of 60-100 ^{0}C.

iii. It has high solubility, antimicrobial activity, and thermostability at pH 2.0. It is inactivated at pH 7.0. Sensitivity of nisin to digestive enzymes made it a product of choice as food preservative.

2.5.4 Mechanism of action of bacteriocins

In general, the action of bacteriocins produced by Gram-positive bacteria is directed primarily against other Gram-positive species. It has been established that the primary target for many of these small, cationic peptides is the cytoplasmic membrane of sensitive cells (Ross *et al*., 2001), where they act to dissipate the proton motive force (PMF) through the formation of discrete pores in the cytoplasmic membrane, and thus deprive cells of an essential energy source (Montville and Bruno, 1994). The PMF, which is composed of a chemical component (the pH gradient; pH) and an electrical component (the membrane potential; vi), drives ATP synthesis and the accumulation of ions and other metabolites through PMF-driven transport systems in the Membrane. Collapse of the PMF, induced by bacteriocin action, leads to cell death through cessation of energy-requiring reactions.

2.5.5 Use of bacteriocins in food systems

The bacteriocins in general have broad spectrum activity against number of micro-organisms. The application of bacteriocins, particularly nisin, in food systems is influenced by inadequate physical conditions and chemical composition of food including pH, temperature, nutrients, etc. It is also affected by spontaneous loss in production capacity, inactivation by phage of producing strain and antagonism effect of other microorganisms in foods. The negative factors in its effectively include resistance against certain pathogens, biological activity interference by certain environmental conditions, and higher retention of bacteriocin molecules like fat by food system components, inactivation by other additives, slower diffusion and solubility and/or

irregular distribution of bacteriocin molecules in the meat matrix. The pre-requisites for use of bacteriocins in food are its production from GRAS micro-organisms, high specific activity of bacteriocins, thermo stability, capability to enhance quality and flavour. Bacteriocins must be safe to human health and bio-preservation must fulfill the GRAS regulations. The common bacteriocin named nisin was first time used in 50´s to inhibit the outgrowth of Clostridium tyrobutyricum (Singh *et al*., 2018)

2.5.6 Bacteriocins produced by LAB

Although there are several microorganisms that produce bacteriocins, those produced by the lactic acid bacteria (LAB) are of particular interest to the dairy industry (Egan *et al*., 2016). LAB have long been used in a variety of food fermentations by converting lactose to lactic acid, as well as producing additional antimicrobial molecules such as other organic acids, diacetyl, acetoin, hydrogen peroxide, antifungal peptides, and bacteriocins (Egan *et al*., 2016). As a result of their extensive use in traditional fermented products, most of the LAB are Generally Regarded as Safe (GRAS), granted by the American Food and Drug Agency (FDA). The European Food Safety Authority (EFSA) also granted the Qualified Presumption of Safety (QPS) status to most of the LAB genera, such as *Lactococcus, Lactobacillus*, *Leuconostoc, Pediococcus,* and some *Streptococcus* (EFSA, 2007). Nevertheless, species of the genus Enterococcus and some Streptococcus are pathogenic, thus, they do not have GRAS status and were not proposed for QPS status (EFSA, 2007). Lactic acid bacteria bacteriocins are often active across a range of pH values, resistant to high temperatures and active against a range of food pathogenic and spoilage bacteria (Ahmad *et al*., 2017). In addition, LAB bacteriocins are sensitive to digestive proteases such as pancreatin complex, trypsin and

chymotrypsin, and thus do not impact negatively on the gut microbiota (Egan *et al*., 2016).

2.5.7 Microbial origin of bacteriocin

As mentioned before, bacteriocins are referred as ribosomally synthesized antimicrobial peptides from microorganisms and there is a great variation in their producers fundamentally divided in three groups: Archaea, Gram-negative and Gram-positive bacteria.

2.5.7.1 Bacteriocins of archaea

The Archaea synthesize their own distinct family of bacteriocin-like antimicrobial peptides named as archaeocins. The halocin S8 from halobacteria, a short hydrophobic peptide with 36 amino acids, is the first discovered member of the archaeocin family. These molecules are produced as the cells enter stationary phase. When resources are limited by microorganisms, the producer strain lyses the target cells by secretion of archaeocins and reduces the competition in the local environment (Riley *et al.,* 2002).

2.5.7.2 Bacteriocins of Gram-negative bacteria

As mentioned before, bacteriocins are initially isolated from Gram-negative bacteria. A colicin from *E. coli*, identified as an antimicrobial protein by Gratia in 1925, was the first described one for the bacteriocin family and dominated many of the related studies up to the recent past. Then, following researches pointed out that bacteriocin producer strains are not only restricted by *E. coli* but also the fact that many species of Gram-negative bacteria have production ability for colicin-like proteins. Klebicins of *Klebsiella pneumonia*, marcescins of *Serratia marcescens*, alveicins of

Hafnia alvei, cloacins of *Enterobacter cloacae* and pyocins of *Pseudomonads* are important representative examples for bacteriocins of other Gram-negative bacteria. Most bacteriocins of this group are relatively large and consequently heat-labile peptides (Mills *et al.,* 2011). An exception, microcins such as microcin V of *E. coli* breaks this rule. It characteristically contains only a few peptides and shows heat-stable property. The narrow antimicrobial activity spectrum is the main disadvantage for the bacteriocins of Gram-negative bacteria that limits their industrial-scale uses. This property calls attentions towards to the more suitable types of bacteriocins produced by Gram-positive bacteria (Riley *et al.,* 2002; Nes *et al.,* 2007).

2.5.7.3 Bacteriocins of Gram-positive bacteria

Gram-positive bacteria also produce a wide variety of bacteriocins. Their non-toxic property on eukaryotic cells and much broader inhibitory spectra make Gram-positive bacteriocins a unique useful tool for many industrial and medicinal applications. In this respect, lactic acid bacteria (LAB), a group of phylogenetically diverse Gram-positive bacteria characterized by some common morphological, metabolic and physiological properties, have attracted much interest due to their GRAS (generally regarded as safe) potential for human consumption (Balciunas *et al*., 2013). LAB are characterized by production of lactic acid in their fermentation pathway, thereby earning the name "lactic acid bacteria". In this process, a member of LAB converts at least 50 % of the carbon from sugars into two isomers of lactic acid. This group of bacteria shows a great variety depending on many physiological and morphological properties (Mills *et al.,* 2011). Members of LAB can be cocci, bacilli or coccobacilli shaped Gram-positive bacterial strains with various physiological characteristics. Due to their safe nature and valuable metabolic products (such as organic acids, diacetyl, acetoin, hydrogen peroxide,

reuterin, reutericyclin, antifungal peptides, and bacteriocins), these have a great importance in medicinal and food applications. Especially various types of bacteriocins have attracted much interest (Gálvez *et al*., 2008).

2.5.8 Factors that contribute to the increasing number of applied investigations on bacteriocins of lactic acid bacteria

i. Acceptance of nisin as safe and efficacious in the past years
ii. Approval of nisin by the food and drug administration (FDA) as a "generally regarded as safe" (GRAS) in certain applications
iii. Realization that bacteriocinogenicity is not a rare occurrence within the lactic acid bacteria
iv. Consumer awareness and resistance to traditional chemical preservatives
v. Justifiable concerns over the safety of existing food preservatives such as sulphites and nitrites.
vi. Possibility of use of bacteriocins production and immunity as selectable genetic markers in starter culture bacteria.
vii. Improvement in molecular techniques and availability of molecular biology tools to transfer, clone and sequence the genetic determinants and to engineer genetic variants of bacteriocins.
viii. Willingness of federal funding agencies, food commodity groups, and food processing corporations to find both basic and applied researches.

2.5.9 Application of purified bacteriocins to dairy products

Bacteriocins have been used in the biopreservation of various foods, either alone or in combination with other methods of preservation, known as hurdle technology (De Vuyst and Leroy, 2007; Perez *et al.,* 2014). The application of bacteriocins into foods must be tested to confirm their effectiveness. The screening of bacteriocins to be applied to

foods requires the fulfillment of some important criteria. Producing strains should be food grade (GRAS or QPS), exhibit a broad spectrum of inhibition, present high specific activity, have no associated health risks, present beneficial effects (e.g., improve safety, quality, and flavor of foods), display heat and pH stability, and optimal solubility and stability for a particular food (Cotter *et al.,* 2005; Leroy and De Vuyst, 2010). Various authors have reported that inactivation of several foodborne pathogens by bacteriocins may differ greatly depending on the food matrix used (Muñoz *et al.*, 2007). Therefore, the effectiveness of different bacteriocins to foodborne pathogens must be tested in all food systems. Recent applications of bacteriocins into dairy foods to control food-borne pathogens included the inoculation of food with LAB that produce bacteriocins or the addition of purified or semi-purified bacteriocins directly to food. Applying bacteriocin-producing LAB strains as antibacterial starter cultures and protective cultures may confer an advantage over the use of semi-purified/purified bacteriocins. In most cases, bacteriocins are adsorbed into food matrices and are easily degraded, which results in a loss of antibacterial activity. However, an alternative method is the incorporation of bacteriocins into food packaging films/coatings, which improve their activity and stability in complex food systems (Salgado *et al.,* 2015).

2.5.10 Bacteriocins and food applications

Although modern advances in technology have been developing day by day, the preservation of food is still a debated issue, resulting in economic losses due to food spoilage and undesirable effects on human health. In this respect, many chemical preservatives have been identified and successfully applied in various food processing applications (Balciunas *et al.*, 2013). However, the growing awareness of consumers and the health concerns regarding chemical food additives make natural antimicrobials more attractive. Bacteriocins, especially produced by LAB, have a great potential to

meet this request in the food industries (Mills *et al.*, 2011). In food preservation, the bacteriocins produced by LAB: (i) are generally recognized as safe substances (GRAS property), (ii) are not active and non-toxic on eukaryotic cells, (iii) become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) are usually pH and heat-tolerant, (v) have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (vi) show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (Gálvez *et al.*, 2007).The related studies indicates that the applications of bacteriocins with these features in food industries can extend shelf life of foods, provide extra protection during temperature abuse conditions, decrease the risk for transmission of food-borne pathogens through the food chain, ameliorate the economic losses due to food spoilage, reduce the application of chemical preservatives, permit the application of less severe heat treatments without compromising food safety: better preservation of food nutrients and vitamins, as well as organoleptic properties of foods, permit the marketing of novel types of foods (Gálvez *et al.*, 2007). Bacteriocins are added into food processing applications as *ex situ* produced preparations, or by inoculation with the bacteriocinogenic strains. Then these antimicrobial agents can be ready to show their specific activity in the food matrix. However, the matrix, the processing steps and the natural microbiota have a fairly complex and non-stable nature in many cases.

2.5.11 Requirements and regulatory status for bacteriocins

In general, the following features should be considered when selecting bacteriocin-producing strain for food applications:

i. The producing strain preferably should have GRAS status

ii. Depending on the application, the bacteriocin should have a broad spectrum of inhibition that includes pathogens or else high specific activity.
iii. Thermostability
iv. Beneficial effects and improved safety.
v. No adverse effects on quality and flavour.
vi. The bacteriocins produced by LAB offer several desirable properties that makes them suitable for food preservation:
vii. Are generally recognised as safe substances,
viii. Are not active and non-toxic on eukaryotic cells,
ix. Become inactivated by digestive proteases, having little influence on the gut microbiota
x. Are usually pH and heat tolerant
xi. They have a relatively broad antimicrobial spectrum, against many food –borne pathogenic and spoilage bacteria
xii. They show a bacteriocidal mode of action, usually acting on the bacterial cytoplasmic membrane, no cross resistance with antibiotics.
xiii. Their genetic determinants are usually plasmid encoded, facilitating genetic manipulation.

The accumulation of studies carried out in recent years clearly indicate that the application of bacteriocins in food preservation can offer several benefits (Thomas *et al.*, 2000).

i. An extended shelf life of foods
ii. Provide extra protection during temperature abuse conditions

iii. Decrease the risk for transmission of food borne pathogens through the food chain.
iv. Ameliorate the economic losses due to food spoilage
v. Reduce the application of chemical preservatives
vi. Permit the application of less severe heat treatments without compromising food safety; better preservation of food nutrients and vitamins, as well as organoleptic property of foods.
vii. Permit the marketing of novel "foods (less acidic, with a lower salt content, and with a higher water content
viii. They may serve to satisfy consumers and industrial demand.

2.6 Millet Dough Ball (*Fura)*

Millet dough ball popularly known as *Fura* is a semi-solid dumpling meal made from millet or sorghum and is used traditionally as stable food in most West African countries including Nigeria and Ghana (Achi and Ukwuru, 2015). In Nigeria it is mostly consumes by the Hausa-Fulani's and some other tribes in Northern Nigeria are also fast adopting Fura as a beverage. It can now be found in non-Hausa towns such as Zuru, Abuja and Minna. Millet dough ball is also available in some Southern towns such as Lagos though mainly among the Hausa residents. The popular ingredient for producing Fura is millet (Jideani *et al.,* 2001). All varieties of millet are suitable for Fura production. It is a thick ball snack that is produced mainly from millet or sorghum and spices such as ginger, pepper, black pepper and gloves.

2.6.1 Preparation of millet dough ball (*Fura)*

Millet dough ball is produced by malting of grains, which is a process of soaking grains in water to induce seed germination and starch hydrolysis. Millet flour blended with spices and compressed into balls and boiled for 30 min. While still hot, the cooked dough is worked in a mortar with the pestle (with the addition of hot water) until a smooth, slightly elastic and cohesive lump (fura) is formed. The fura dough is rolled into a 25–30 g ball by hand and dusted with flour (Jideani *et al.,* 2001). The millet dough ball is made into porridge by crumbling the fura balls into fermented whole milk (kindrimo) or fermented skim milk (nono). Sugar may be added to taste.

Fura can be considered to be functional natural food since the raw material (millet) has been reported to have protein content up to 11% protein by weight and are rich in B vitamins such as niacin, B6 and folic acid, iron, potassium, zinc, magnesium, and calcium with no gluten content (Abdulkadir and Mugadi, 2012). They are also rich in phytochemicals, including phytic acid which is believed to lower cholesterol and reduce the risk of cancer. Moreover, cereals regarded as functional foods since they provide dietary fiber, energy, protein, minerals, vitamins and anti-oxidants required for human health (Saikia and Deka, 2011). However, millet dough ball (*Fura)* has a short shelf life of 3 to 4 days at the temperature of about 5°C and 1 to 2 days at room temperature of 25°C while at 35°C it can only last for 18 hours with unacceptable quality, after which they can be deteriorated by microorganisms whose presence poses health risk as they can be source of infection when consumed (Filli *et al*., 2010., Achi and Ukwuru, 2015). Moreover, poor handling of millet dough ball (*Fura)* during processing, storage and marketing can predispose it to microbial contamination as they are molded into balls by hand during preparation, and storage may be in an unhygienic containers and environment (Adebesin *et al*., 2001) Also, improper handling and post-fermentation

processing such as pounding in mortar, molding and the point of sale can expose the *fura* product to microbial contamination (Owusu-Kwarteng *et al*., 2010).

Preparation of Millet Dough Ball (*Fura)*

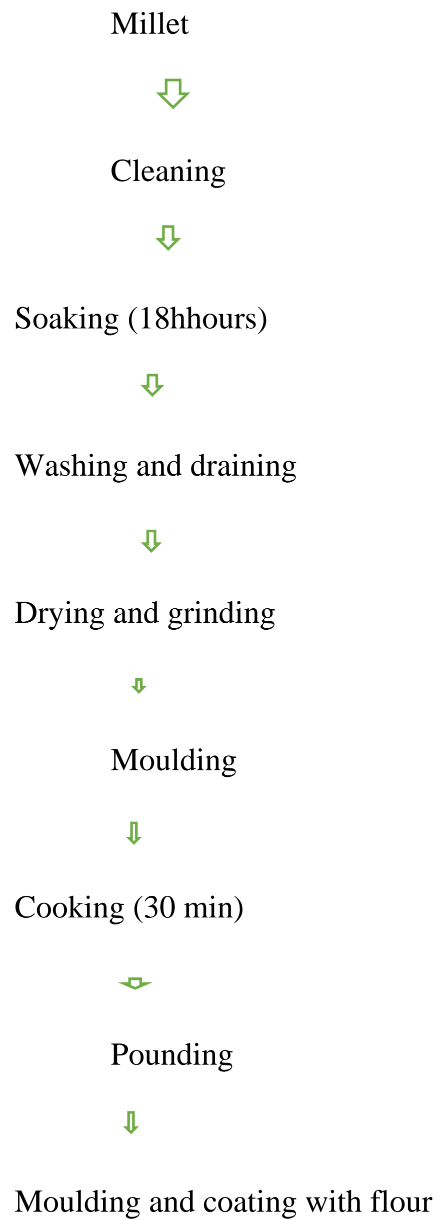


Figure 2.1: Process Flow Chart for millet dough ball (*Fura)* Production

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Niger state Nigeria. Minna is located in the north central part of Nigeria between longitudes 6.55´N and 11.3´N and latitude 9.62´E.

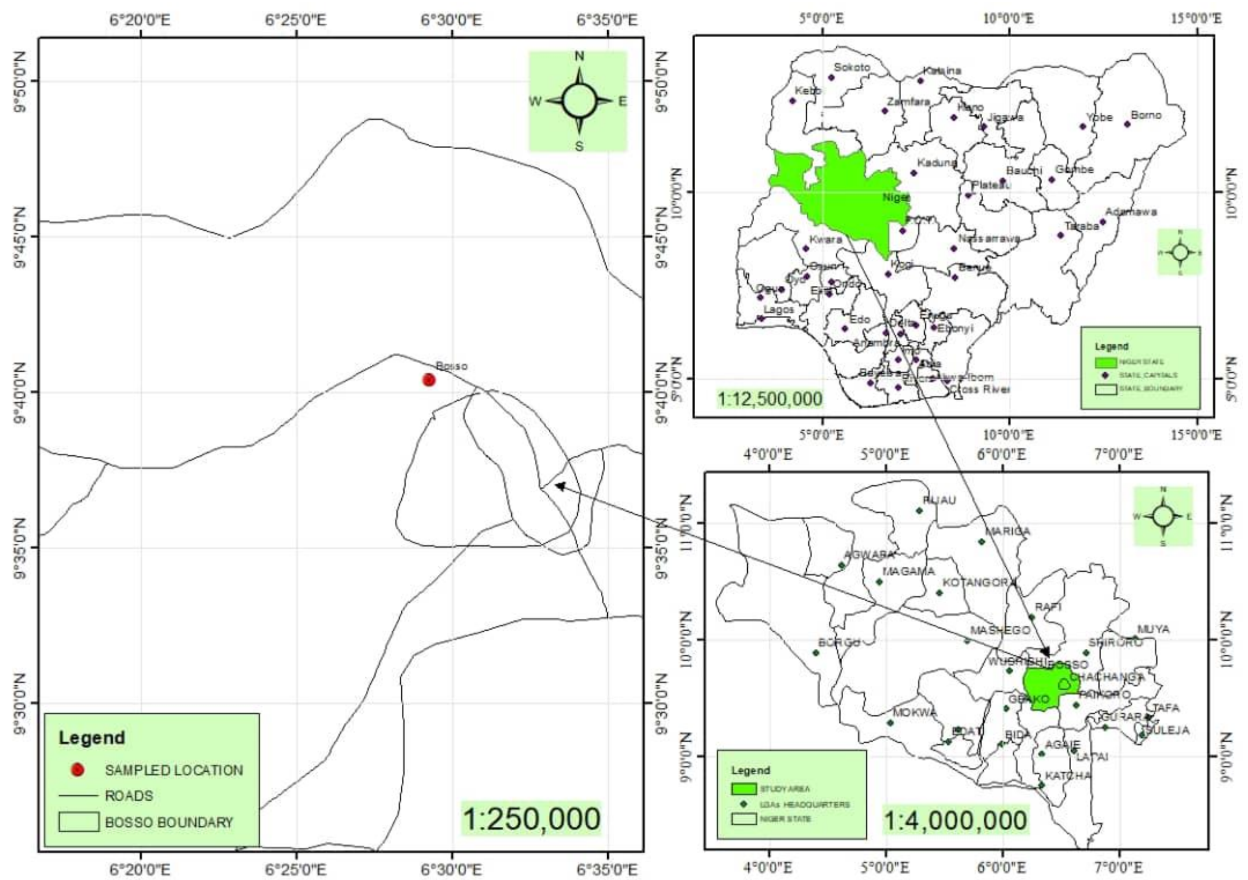


Figure 3.1: Map of Niger State Showing Bosso local government

Source: Kemiki *et al*. (2018)

3.2 Sample Collection and Processing

The cheese samples were brought from Fulani hawkers in Bosso market and put in a sterile polyethylene bag and transported immediately to the Microbiology Laboratory Federal University of Technology for analysis. The LAB isolation was carried out in the Microbiology Laboratory, Federal University of Technology Minna, Niger state.

3.3 Culture Media Used

Media used (De Man, Rogosa and Sharpe agar, Mannitol salt agar, salmonella shigella agar and nutrient agar) for this research were of analytical grade and they were prepared according to the manufacturer's instruction.

3.4 Isolation of Lactic Acid Bacteria (LAB)

Lactic acid bacterial was be isolated using the method by Graciela *et al*. (1995)**.** Ten (10g) grams of cheese sample was mixed with 9 mL sterile peptone water and homogenized for 30 second in Lab- Blender. The samples were serially diluted and 1 mL of suitable dilutions was pour in petri dish (using the pour plate method) and De Man, Rogosa and Sharpe (MRS) agar was added to the plate. The plates were incubated for 3 days at 37 °C under anaerobic conditions using the anaerobic jar. Bacterial colonies grown on the plates were individually picked up and streaked on fresh MRS agar plates to have pure single colonies. Then pure strains were maintained on MRS agar slants media at 4°C for further examination.

3.5 Characterization and Identification of Lactic Acid Bacterial

The pure LAB isolates were identify base on their reaction to Gram stain and their biochemical tests. Identification of the isolates were done by comparing their

characteristics with those of known taxa using Bergey's Manual of determinative of bacteriology and the Genera of Lactic Acid Bacteria (George *et al.,* 2014).

i. Gram Staining

Wire loop was sterilized until it becomes red hot by flaming in an open fire and then allowed to cool. The sterile wire loop was used to pick out culture from a discrete colony and a smear was made on a clean grease free slide. The smear was air dried and fixed to the slide by passing it gently swiftly over the open flame (Beccerra *et al.,* 2016). The fixed smear was be flooded with crystal violet and was allowed to stand for 60/seconds. The stain was drained and the slide was washed under a running tap. Then, the smear was cover with Lugol's iodine and allowed to stand for 60/seconds and rinsed with water. The rinsed slide was again covered with alcohol to decolorize for 30/seconds and then rinsed with water (Tshkhudo *et al*., 2013). Counter staining of smear was done by flooding slide with safranine and allowed to stain for another 30seconds, then slide was washed again with water and left to dry. Each slide was observed under oil immersion objective lens of the microscope (Beccerra *et al*., 2016). Gram positive bacteria appeared as purple/blue carrying the colour of the primary dye, while gram negative carried the red/pink colour of the secondary dye (Beccerra *et al*., 2016).

ii. Catalase Test

A clean grease free slide was labeled A and B on each end, and then two drops of 3% hydrogen peroxide (H_2O_2) was placed on the rare side of each end (at point A and B). A clean glass rod was sterilized over an open flame, cooled and used to transfer the test organism to the drop at point A on the slide. The mixture was observed instantly for the appearance of gas bubbles (effervescence), drop B served as control. Result showing

effervescence were recorded as positive, while those that showed no bubbles were recorded as negative (George *et al*., 2004).

iii. Indole tests

The isolated organisms was grown in a test tube containing 5mL of 1% peptone water and incubated at 37°C for 24hours. Kovac's reagent was then formulated by liquidating 5g pdimethylamino-benzaladehde into a mixture of 25ml concentrated HCl and 75ml amyl alcohol. Then, 0.5ml of the prepared Kovac's reagent was added into the tube with the test organism and shaken gently; a control was prepared without the inoculation of the test organism. A positive result was indicated by the appearance of a red colour ring at the reagent layer, while negative result showed no colour ring (George *et al.,* 2004).

iv. Citrate test

One hundred milliliters (100mL) of distilled water was used to dissolved 24.28g of Simmons citrate agar by heating, it was dispensed into test tubes. The tubes were sterilized by autoclaving at 121°C for 15minutes at 15 pound pressure (15 psi), and then placed in an angular position to gel into slant. The isolates was streaked on the surface of the citrate agar slants and incubated with test organism (the control) for 4 days. A positive reaction is indicated by the appearance of a colour change and retained the green colouration indicating a negative result (George *et al.,* 2004).

v. Oxidase test

Two drops of a diluted solution of the oxidase reagent (tetramethyl-p-phenalene-diamine dihydrochloride) was used to wet a piece of filter paper, and a growth of lactic acid bacteria was smeared on the wet piece of filter paper. The development of an

intense purple colour by the cells in the smear within 30 seconds indicated a positive test. Failure of the development of an intense purple colour within 30 seconds indicated a negative test (Oyeleke and Manga, 2008).

vi. Triple sugar iron agar (TSI) (hydrogen sulphide production)

The TSI agar slant was prepared in test tube and autoclaved at 121°C for 15 minutes at psi pressure. An inoculating wire loop was sterilized and used to inoculate the bacterial isolate on the TSI slant by streaking the surface of the slant and stabbing the agar to the bottom. The control was also prepared without the inoculated test organism. Agar slants and the control were incubated at 37°C for three days, with daily examination of the formation of sugar (sucrose , lactose and glucose), while the presence of cracks at the butt of the butt of the tube, pushing from the bottom indicated that gas was formed (Ralph, 2007).

vii. Carbohydrate utilization test (acid and gas production from carbohydrates)

The sugar tested were D-glucose, fructose, sucrose, lactose, D-mannitol, sorbitol, arabinose and D-mannose. One hundred millitres (100mL) of peptone water was prepared and then two grams (2g) of the test sugars and 0,08g of phenol red were added as indicator. Five millitres (5 mL) of the mixture was measured and dispensed into test tubes and inverted Durham's tube was inserted into the medium, the medium was sterilized by autoclaving at 121 °C for 15 minutes at 15 pound pressure (15psi) and allow to cool. The sterile medium was inoculated with the test organism. The test tube was incubated at 37 °C for 24 hours. The medium was observed for a colour change from red to orange /yellow which indicated that the sugar present was fermented , the

production of acid and gas was indicated by the displacement of test solution at the Durham;s tube (cheesbrough, 2003).

viii. Motility test using stab culture techniques

Inoculum was picked with sterilized straight wire loop and stab into sterile semi solid (motility medium) in test tubes, the stab was stopped about the center of the medium in the test tube. This was done in duplicates for the test bacterial isolates. The test tubes was incubated at 37 °C for 24hours, thereafter, the tubes was examined. The motile bacteria (positive growth) grew along the line of stab causing turbidity and rendering the medium opaque, while non-motile bacteria (negative growth) grew only along the line of stab (cheesbrough, 2003; Oyeleke and Manga, 2008).

ix. Urease production

The preparation of urea agar slants will be done in bijou bottles and was inoculated with the bacterial isolates, a control was prepared but without the inoculation of the test organism. Incubated of inoculated slants and control at 37 °C for 24hours, and colour changes from pale yellow to a bright pink/red colour, while medium that maintained the pale yellow color were recorded as a negative reaction (Cheesbrough, 2003).

x. Methyl red (MR) and Voges Proskauer (VP TEST)

Two milliliters of glucose phosphate peptone water was sterilized in two test tubes labelled A and B, both tubes was inoculated with the bacterial isolates and incubated at 37 °C for 48hours. Into test tube A, a Pasteur’s pipette was used to add four drops of methyl red reagent and was mixed by shaking gently. The mixture was observed for immediate colour change. A positive MR deduction was indicated by the appearance of bright red rings on the surface of the medium, while yellow coloured medium with no

red ring observed indicates a negative reaction. To tube B one milliters of 40 % potassium hydroxide (KOH) and 3ml of 5 % alcoholic alpha-naphtol was added and shaken properly. The mixture was allowed to stand for three minutes and then observed for a colour change from black to pick which indicated a positive V-P reaction while a negative reaction is recorded when there is no colour change from back. (Oyeleke and Manga, 2008).

xi. Mannitol activity

The isolate was inoculated into mannitol broth and incubated at 37 °C for 24hours and observed for colour change. Change in colour from pink to yellow indicate mannitol positive while no change in colour indicate mannitol negative (Oyeleke and Manga, 2008).

xii. Coagulase test

A drop of normal saline was placed on a clean slide and a small portion of the isolate was emulsified onto the saline until homogenous suspension was obtained. A drop of human plasma was added to each of the suspension and stirred for 5 seconds. Coagulase positive result indicate clumping, which was emulsify. Negative result showed no clumping (Fawole and Oso, 1998; Oyeleke and Manga, 2008).

xiii. Endospore Test

A smear of the LAB isolates was made on slide and the slide was fully covered with malachite green. The stained slides was placed over boiling water and allowed to steam for three minutes. It was then be rinsed gently with tap water. Slides was covered with safranine solution and was left for 30 seconds, after which it was washed and allowed to

air dry and viewed under the oil immersion objective. A positive result show that the spores present were stained green while the remaining cells was light red.

3.6 Molecular Identification and Characterization of the Isolates

3.6.1 DNA extraction

Deoxyribonucleic acid (DNA) was extracted using the protocol stated by Saitou and Nei, (1987), Briefly, Single colonies grown on medium were transferred to 1.5 mL of liquid medium and cultures were grown on a shaker for 48 h at 28 ºC. After this period, cultures were centrifuged at 4600g for 5 min. The resulting pellets were resuspended in 520 µL of TE buffer (10 mMTris-HCl, 1mM EDTA, pH 8.0). Fifteen microliters of 20 % SDS and 3 µL of Proteinase K (20 mg/ml) were then added. The mixture was incubated for 1 hour at 37 ºC, then 100 µL of 5 M NaCl and 80 µL of a 10 % CTAB solution in 0.7 M NaCl were added and votexed. The suspension was incubated for 10 min at 65 ºC and kept on ice for 15 min. An equal volume of chloroform: isoamyl alcohol 24:1 was added, followed by incubation on ice for 5 min and centrifugation at 7200g for 20 min. The aqueous phase was then transferred to a new tube and isopropanol (1: 0.6) was added and DNA precipitated at –20 ºC for 16 h. Deoxyribonucleic acid was centrifuge at 13000g for 10 min, washed with 500 µL of 70 % ethanol, air-dried at room temperature for approximately three hours and finally dissolved in 50 µL of TE buffer.

3.6.2 Polymerase chain reaction (PCR)

PCR sequencing preparation cocktail consisted of 10 µL of 5x GoTaq colourless reaction, 3 µL of 25mM MgCl2, 1 µL of 10 mM of dNTPs mix, 1 µL of 10 pmol each 27F 5’- AGA GTT TGA TCM TGG CTC AG-3’ and - 1525R, 5′-AAGGAGGTGATCCAGCC-3′ primers and 0.3units of Taq DNA polymerase

(Promega, USA) made up to 42 µL with sterile distilled water 8µL DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a Pcr profile consisting of an initial denaturation at 94 °C for 5 min; followed by a 30 cycles consisting of 94°C for 30 s, 50 °C for 60s and 72 °C for 1 minute 30 seconds and a final termination at 72 °C for 10 mins. And chill at 4°C in a GEL (Felsenstein, J. 1985; Tamura *et al.*, 2004)

3.6.3 Integrity of the amplification

The integrity of the amplified about (1.5Mb) gene fragment was checked on a 1 % Agarose gel ran to confirm amplification. The buffer (1XTAE buffer) was prepared and subsequently used to prepare 1.5 % agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60 °C and stained with 3µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (Felsenstein, J. 1985; Saitou, 1987) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µl of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel (Tamura *et al.*, 2004).

3.6.4 Purification of amplified product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µL of Na acetate 3M and 240 µL of 95 % ethanol were added to each about 40µl PCR amplified product in a new sterile 1.5 µL tube eppendorf, mix thoroughly by vortexing and keep at -20 °C for at least 30 min. Centrifugation for 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet were washed by adding 150 µL of 70 % ethanol and mix then centrifuge for 15 min at 7500 g and 4 °C. Again remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 10-15 min. then re-suspend with 20 µL of sterile distilled water and kept in -20 °C prior to sequencing. The purified fragment was checked on a 1.5 % Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nano drop of model 2000 from thermo scientific.

3.6.5 Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis

3.7 Screening for Lactic Acid Bacteria with Potential Activity

3.7.1 Collection of pathogenic bacteria (indicator organism)

Pathogenic bacteria (*Staphylococcus aureus and Salmonella* specie) were collected from the Microbiology Laboratory, Department of Microbiology, FUT, Minna. They were identified and sub cultured on their respective selective media. *Staphylococcus aureus* was subculture on mannitol salt agar (MSA) while *Salmonella* specie on salmonella

shigella agar (SSA) and incubated at 37 °C for 24hours. They were stock on agar slant for further use.

3.7.2 Inhibitory activity of LAB

The indicator organisms cell free supernatant from the samples were introduced into the wells bored with cork borer. The plates were allowed to set for 1h to allow the antibacterial to diffuse. The plates were incubated at 37 °C for 48hours after which the plates were examined for clear zones of inhibition (Babatunde *et al*., 2014).

3.8 Bacteriocin Production

Lactobacillus species were propagated in 1000 ml MRS broth (pH 7.0, glucose, 0.25 % w/v, peptone, 0.5 % w/v) for 72 h at 37 °C anaerobically in triplicate. To determine bacteriocin activity, dilutions of the bacterial culture supernatant was tested for antimicrobial activity against indicator organism by the agar well diffusion method (Ogunbanwo *et al.,* 2003).

3.9 Extraction of Bacteriocin

The cell free solution obtained was the crude extract. A cell free solution was obtained by centrifugation at 10000g for 20 minutes at 4 °C. The culture was adjusted to pH 7.0 by the addition of sodium hydroxide to get the required pH followed by filtration of the supernatant through 0.2µm pore-size cellulose acetate filter. The cell free solution obtained was the crude extract (Daba *et al.,* 1991).

3.10 Partial Purification of Bacteriocin

i. **Ammonium Sulphate Precipitation**: The crude extract was precipitated with ammonium sulphate (40 % saturation). The mixture was stirred for 2hours using a magnetic stirrer and then centrifuged at 20,000g for 1hour at 4 °C. The precipitate was resuspended in 25ml of 0.05M potassium phosphate buffer (pH 7.0). Catalase (5mg) was added and the new precipitate was filtered and stored at 4°C for further use (Aly *et al*., 2003).

ii. **Trichloroacetic acid (TC) precipitation:** Five percent (5%) equivalent of TC was added to 25 ml of Fraction 1 to precipitate the protein (bacteriocin). The mixture was centrifuged at 13,000 rpm for 10 minutes after which the supernatant was decanted. The resulting pellet was dissolved in 2 ml of potassium phosphate buffer (Fraction 2).

iii. **Ultrafiltration studies*:*** The Fraction 2 (Trichloroacetic acid precipitation) bacteriocin sample was resuspended to 1/30 volume in potassium phosphate buffer (50mM, pH 7.0). Several aliquots was filtered. Bacteriocin activity was determined by agar well diffusion (Jimenez-Diaz *et al*., 1993) and protein concentration of the fractions was determined by the Bradford method (Bradford, 1976).

3.11 Determination of bacteriocin titre

The titres of bacteriocin produced were quantified by two fold serial dilutions of bacteriocin in saline solution and 1ml from each dilution were placed in wells in plates seeded with the bioassay strain. These plates were incubated anaerobically at 37 °C for 24 hours and examined for the presence of 2 mm or larger clear zones of inhibition around the wells. The antimicrobial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn multiplied by 100 and was expressed in (AU/ ml) (Graciela *et al*., 1995)**.**

3.12 Characterization of Bacteriocin

The purified bacteriocin samples was characterized with respect to thermal, pH and stability during storage (Aqeela *et al.,* 2015).

3.12.1 Heat resistance*:* Purified bacteriocin was exposed to various heat treatments: 40, 60, 80, 100 and 121 ºC. Aliquot volumes of each Fraction will be assayed for bacteriocin (ten Brink *et al*., 1994)

3.12.2 pH sensitivity*:* Purified bacteriocin was adjusted to pH 2, 4, 6, 8, 10, and 12 with hydrochloric acid (HCl) and sodium hydroxide (NaOH), incubated for 1hour at 37 ºC. The bacteriocin was assay against indicator organisms.

3.12.3 Stability of bacteriocin during storage*:* Purified bacteriocin was stored at 4, 10 and 37 ºC for 24 hours, samples was taken from the stored material (Brinkten *et al*., 1994) to determine bacteriocin activity.

3.13: Preparation of millet dough ball (*fura*): millet dough ball was prepared as follows

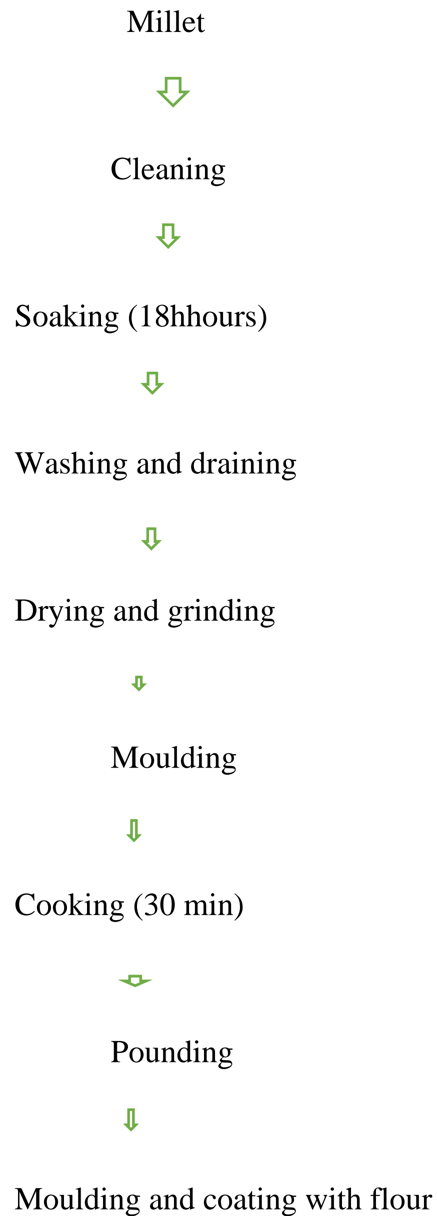


Plate 3.1: Millet dough ball (*fura)*

3.14 Potential of BLAB to extend the shelf life of millet dough ball (*Fura)* sample (Shelf life study of *fura)*

A volume of 10 mL of the bacteriocin obtained was added to 20 g of millet dough ball (*Fura)* and the two different combinations (products) were stored under refrigerated conditions (4°C) for the determination of the shelf life in comparison with the experimental control (fura without bacteriocin). The f*ura* samples were then observed daily to determine when spoilage would start. The total microbial load was determined at the beginning of spoilage (the growth of microorganisms on the food samples showed sign of spoilage). Initial plate count of samples was serially diluted at 10^6 and the plates were incubated at 37 °C for 24 h. The colony count was recorded and compared with the control (without bacteriocin) (Narayanapillai *et al*., 2012). The following parameters of the fermented millet dough ball (*Fura)* was monitor.

3.14.1 Physical appearance of fermented *Fura* product produced

The physical appearance of the fermented millet dough ball (*Fura)* will be examined before inoculating it with the bacteriocin producing lactic acid bacteria. The result was

based on the colour change of the millet dough ball product under examination (after shelf life) and the results recorded according to the colour change.

3.14.2 Flavor determination

The flavor of the fermented millet dough ball (*Fura)* was perceived before and after inoculating the products with bacteriocin producing lactic acid bacteria. The results obtained will be recorded as either pleasant or unpleasant. .

3.15 Proximate composition of millet dough ball (*Fura)*

Proximate analysis Moisture, Ash, Protein, Fat, fiber and Carbohydrate content. The proximate composition was carried out in triplicate for each sample using AOAC (2000) and the mean result was recorded.

3.15.1 Determination of moisture content: Metallic dishes were dried in an oven at 800 °C for 20 minutes, and allowed to cool in a desiccator and weighed. Five gramme of millet dough ball (*Fura)* was placed in the dishes and weighed. The dishes with the samples were then dried in an oven at 800 °C for 24 hours to achieve a constant weight and quickly transferred to a desiccator to cool. After cooling, it was weighed immediately with minimum exposure to the atmosphere. The loss in weight of the milk during drying is the Moisture content. The % moisture was calculated using equation (1)

$$W2- W3 X 100 / W2- W1$$

3.15.2 Determination of ash content: Crucibles were cleansed and dried in the oven, after drying they were cooled in the dessicator and weighed (W1). Two gramme of the millet dough ball (*Fura)* sample was placed in the crucibles and weighed (W2).They were transferred into the muffle furnace at 5500 °C for 2 hours then removed and cooled in the desiccator and weighed (W3).

The % ash was calculated using equation (2):

W3- W1 X 100 / W2 – W1

3.15.3 Determination of crude fiber content

Each sample (5 g) was transferred into 600 ml beaker and 700 ml of 1.25 % sulphuric acid added. Each beaker was heated for 30 min while being rotated periodically to prevent adherence of solids to the side of the beakers. Thereafter, each solution was filtered and rinsed with 50 ml boiling water. This was repeated with three 50 ml portions of water and subsequently sucked dry. The entire residue was reduced and remained in the beaker with boiling 1.25 % NaOH added and boiled with each residue after which the content of each beaker was removed and filtered as described above. This was then washed with 25 ml of 1% sulphuric acid, three portions of 50 ml water and 25 ml ethanol. The residue was later transferred into ashing dish and dried at 103 °C. This was followed by cooling in desiccator and weighing. The residue was thereafter ignited at 600 °C for 30 min in muffle furnace, cooled in the desiccator and reweighed (AOAC, 2000). The percentage crude fiber was calculated as:

$$\frac{\text{\% total ash} - \text{weight of ash}}{\text{Weight of sample}} \times 100$$

3.15.4 Determination of Protein Content This comprises of three stages as follows:

3.15.4.1 Digestion: 2g of the millet dough ball (*Fura)* sample was weighed into a Kjeldahl flask after which Copper catalyst and 15 mL of sulphuric acid were added, the solution was heated in a fume cupboard until it assumed a green colour. The digest was allowed to cool, after which it was transferred with several washings into 100mL with distilled water.

3.15.4.2 Distillation: Ten milliliter of boric indicator was placed under the condenser after which ten milliliter of the digest was pippete into the body of the apparatus via the small funnel aperture, it was then washed down with distilled water followed by ten

milliliter of 40 % NaOH solution after which it was steam through for five minutes to collect ammonium sulphate (40 mL).

3.15.4.3 Titration: The solution was titrated in the receiving flask using N/100 (0.01 N), hydrochloric acid and the nitrogen content hence the protein content of the sample was calculated as:

$$\frac{\text{Final reading - initial reading – blank (0.2) x standard number of nitrogen (1.4)}}{\text{Initial weight x standard number of protein.}}$$

3.15.5 Determination of fat contents

An empty filter paper was weighed and labeled W1, after which 2g of millet dough ball (*Fura)* sample was weighed into labeled thimbles (filter paper) and labelled W2.The boiling flask was then filled with petroleum spirit. The soxhlet apparatus was assembled and allowed to reflux for 8hrs after which it was removed and transferred to an oven and dried and was weighed as W3. The % Fat was calculated using equation (3):

$$\frac{W2 - W3 \text{ X } 100}{W2 - W1}$$

3.15.6 Determination of total carbohydrate content

The percentage of Carbohydrate content of milk was determined by subtracting from 100, the sum of the percentage moisture, ash, protein, fiber and fat. The remainder value gives the carbohydrate content of the sample. The % CHO was calculated using equation (4):

$$100 - (\%\text{ moisture} + \%\text{ ash} + \%\text{ protein} + \%\text{fiber} + \%\text{ fat})$$

3.15.7 Sensory property of millet dough ball (*Fura)*

The method of Ranganna (2008) was employed. Sensory quality attributes such as colour, aroma, texture and general acceptability of the bacteriocin incorporated fura was evaluated using a five-point Hedonic scale. The millet dough ball (*Fura)* sample were

served to ten panelists for rating on five-point scale ranging from score1 (dislike much), 2 (dislike),3 (like),4(like much),5(like very much) Compared with the control without bacteriocin. Sensory scores generated were statistically analyzed using ANOVA Analysis of variance (ANOVA).

3.16 Data Analysis

Results were expressed as the mean values ±standard error (S.E.) by measuring three independent replicates. Analysis variance using one-way ANOVA was done and Duncan's test was performed to test the significance difference between means values obtained among the treatments at 5% level of significance using SPSS software (version 21, IBM SPSS). Differences were considered significant at $p<0.05$.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Total viable counts of lactic acid bacteria in fermented cheese

The morphologically different colonies on each plates were observed, counted and the result recorded appropriately is shown in Table 4.1 shows that sample A had the highest counts of ($2.3 \times 10^7 \pm 2 \times 10^6$cfu/g) while sample B had the least count of ($1.6 \times 10^7 \pm 1 \times 10^6$cuf/g).

Table 4.1: Total viable count of lactic acid bacteria in fermented cheese

Cheese	Bacterial counts (Cfu/mL)
Sample A	$2.3 \times 10^7 \pm 2 \times 10^6$
Sample B	$1.6 \times 10^7 \pm 1 \times 10^6$

Keys:*CFU – Colony Forming Unit

Sample A: hawker 1

Sample B: hawker 2

4.1.2 Morphology, cultural and physiological characteristic of lactic acid bacteria in fermented *Fura*

The biochemical tests for the identification of the bacteria lactic acid bacteria isolated from cheese shown in **T**able 4.2. The cheese sample was analyzed, observed and result recorded. Four isolates were identified and were found to differ in response to sugar fermentation and gas production. The four samples were isolated as lactic acid bacteria

on morphological and microbiological bases. These lactic acid bacteria were rod/cocci in shaped. Characteristically colonies of Lactic acid bacteria were circular and slightly convex, white to creamy in colour and smooth in texture. Biochemical characteristics of these isolated colonies were Gram positive, Indole negative, acid fast negative, motility negative, oxidase negative and catalase negative, mannitol negative but positive for isolate C, activity from arginine was negative for isolate A and C. For sugar utilization tests, there was production of gas from glucose, lactose and sucrose in isolate B,C and D and acid production for all the sugar fermentation test for isolate A and B. isolate C produces gas for glucose and sucrose and acid production for lactose, fructose and sucrose.

Table 4.2: **Morphology, cultural and physiological characteristic of lactic acid bacteria in fermented *Fura***

Isolate	cell morphology	Colony morphology	Grams reaction	Catalase	Activity from arginine	Oxidase	Mannitol activity	Lactose	Glucose	Fructose	Sucrose	Motility	Probable identity
A	Rod	Creamy, smooth	G+	-	-	-	-	A	A	A	A	-	*Lactobacillus plantarum*
B	Cocci	Circular,convex	G+	-	+	-	-	AG	AG	A	A	-	*Lactococcus lactic*
C	Rod	Creamy.smooth	G+	-	+	-	+	A	G	A	AG	-	*Lactobacillus helveticus*
D	Rod	Creamy, smooth	G+	-	+	-	-	AG	G	A	-ve	-	*Lactobacillus bulgaricus*

KEY: + = Positive, - = Negative, A= Acid production, G = Gas production, AG = Acid and gas production, G+ = Gram positive

4.1.3 Potential of lactic acid bacteria for bacteriocin production

The lactic acid bacteria were selected after vigorous screening based on their ability to grow in De Man Rogosa sharpe broth, also through calorimetric analysis at 580nm wavelength, pH and inhibition against indicator organism. Isolate A and D had the highest wavelength of 0.85 nm and 0.89 nm respectively while isolate B had the least wavelength 0.25 nm. The medium of isolate A had a pH of 4.01 while the least was exhibited by isolate C. Isolate B had no zone of inhibition against indicator organism while isolate C had a low zone of inhibition with isolate A having the highest inhibition rate.

Table 4.3 Bacteriocin producing ability of lactic acid bacteria (LAB)

Isolate	**Growth of LAB (580nm)**	**pH of medium**	**Zone of inhibition**
A	0.85	4.01	+(10mm)*
B	0.25	2.08	No inhibition
C	0.38	1.28	+(2mm)
D	0.89	3.80	+(6mm)*

*: potential bacteriocin producers

A-*Lactobacillus plantarum*

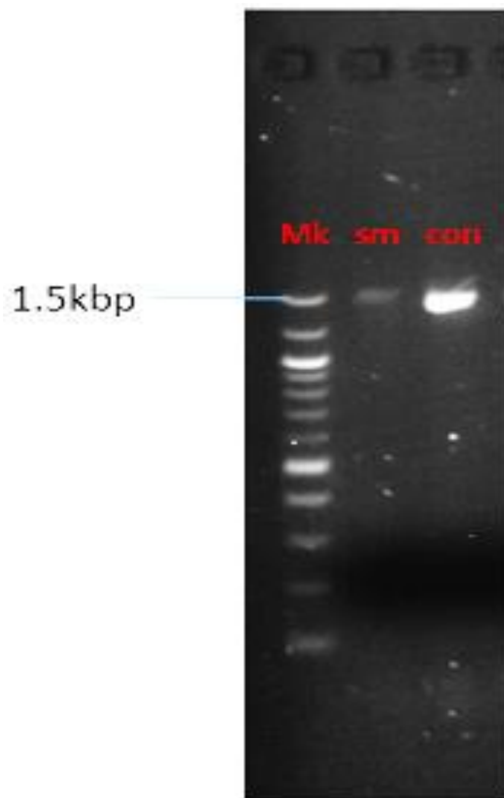
B-*Lactococcus lactic*

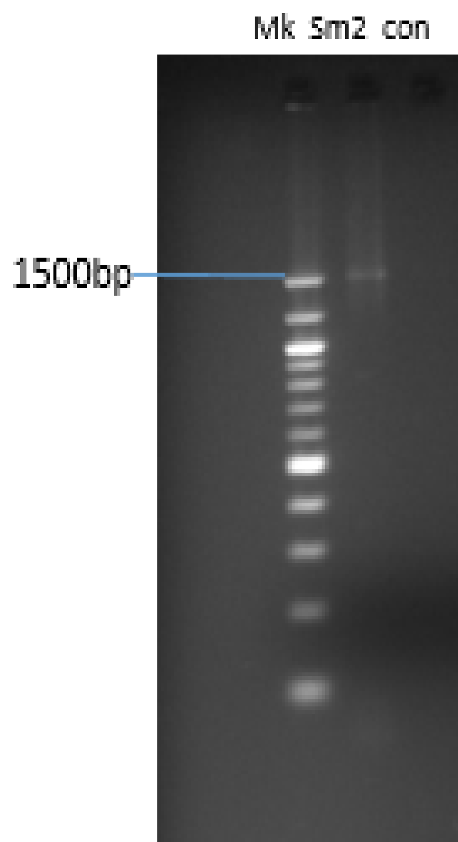
C-*Lactobacillus helveticus*

D-*Lactobacillus bulgaricus*

4.1.4 Molecular characterization of lactic acid bacteria producing bacteriocin (LAB A and LAB B)

Figure 4.1 Shows the result of sequenced amplicons of Lactobacillus specie isolated from cheese (sample) as the resulting alignment of the joined nucleotide (from 5'-3 and 3-5 with known sequence previously blasted on international institute of tropical agriculture (IITA), the gel documented images of the isolated bacterial DNA extracted after electrophoresis shows lane mk which represent molecular maker (ladder), lane SM represent DNA extracted from sample A (Appendix B).





Lactobacillus bulgaricus **strain MN945906**

Lactobacillus plantarum **strain AAAbd**

Plate 4.1: Amplified image of *Lactobacillus bulgaricus* strain MN945906 and *Lactobacillus plantarum* strain AAAbd

4.1.5 Effect of lactic acid bacteria on the indicator organisms

The result (Table 4.4) revealed lactic acid bacteria isolated from cheese inhibit all the indicator organisms tested. Similarly *Lactobacillus plantarum* has the highest zone of inhibition (12±2.00mm) against *Staphylococcus aureus* and also has the least inhibition (6±0.00mm) against *Salmonella* sp.

Table 4.4: Inhibition of indicator microorganisms by lactic acid bacteria

	Zone of inhibition diameter (mm)	
Indicator Microorganism	***Lactobacillus plantarum***	***Lactobacillus bulgaricus***
Staphylococcus aureus	12±2.00	8±0.00
***Salmonella* species**	6±0.00	10±1.00

4.1.6 Purification of bacteriocin by lactic acid bacteria

The least bacteriocin activity (400 Au/ml) was exhibited by *Lactobacillus bulgaricus* while *lactobacillus plantarum* had bacteriocin activity ranging from 800(Au/ml) to 6400(Au/ml) Similarly, the recovery of bacteriocin produced ranged from 100 % to 14.70% the highest recovery was at the culture supernatant stage with 100 % recovery for the two LAB organisms while the least recovery was 14.70 % by *Lactobacillus bulgaricus* at the ultra-filtration. The specific activity and protein content of bacteriocin also varied with organisms (Table 4.5). It was observed that there was variations in the bacteriocin activities and total activities after bacteriocins from each LAB were subjected to ammonium sulphate precipitation, trichloroacetic acid precipitation and

ultra-filtration. It was also observed that the amount of protein, specific activity and recovery throughout the three stages of purification, *Lactobacillus plantarum* showed the highest activity (6400 Au/ml) followed of *Lactobacillus plantarum* with the activity of (1600 Au/ml) at the ultrafiltration stage and trichloroacetic acid precipitation while at the ammonium sulphate precipitation stage also have the same value and while *Lactobacillus bulgaricus* had the least bacteriocin activity (400 Au/ml) at the culture supernatant stage (Table 4.5). The protein concentration reduces throughout the purification stages, *Lactobacillus plantarum* has the highest protein concentration of (19.58 µg/mL) at the culture supernatant stage while the least concentration of *Lactobacillus bulgaricus* with contration of (2.50 µg/mL). The total activity vary across the different stages, the highest activity was exhibited by *Lactobacillus plantarum* 400000 Au/ml while *Lactobacillus bulgaricus* had 200000 Au/mL all at the culture supernatant stage followed by decrease arcoss ammonium sulphate and trichloroacetic acid precipitation, followed by an increase at the ultrafiltration stage of 6400 Au/mL and 3200 Au/mL in *Lactobacillus plantarum* and *Lactobacillus bulgaricus* respectively.

Table 4.5: Purification of bacteriocin produced by *Lactobacillus plantarum* AAAbd and *Lactobacillus bulgaricus* MN945906

LAB	Purification stages	Volume of culture supernatant	Activity of bacteriocin (Au/ml)	Total activity	Protein (µg/ml)	Specific activity	Recovery (%)	Bacteriocin produced
Lactobacillus plantarum	Culture supernatant	500	800	400000	19.58	40.85	100	Plantaricin
	Ammonium sulphate precipitation	25	1600	40000	16	100	81.71	
	Trichloroacetic acid precipitation	15	1600	24000	7.5	213.33	38.3	
	Ultra filtration	10	6400	64000	4.4	1454.5	22.47	
Lactobacillus bulgaricus	Culture supernatant	500	400	200000	17	23.53	100	Bulgarican
	Ammonium sulphate precipitation	25	800	20000	10.6	75.47	62.35	
	Trichloroacetic acid precipitation	15	1600	24000	5	320	29.41	
	Ultra filtration	10	3200	32000	2.5	1280	14.7	

[i]Total activity was determined by the multiplication of volume by activity

[ii]protein concentration was determined by the Bradford method

[iii]specific activity is the activity units divided by the protein concentration((µg/mL)

[iv]recovery percentage is the remaining protein concentration as a percentage of the initial protein concentration

4.1.7 Effect of Temperature on Bacteriocin Activity (Au/ml) produced by *Lactobacillus plantarum* and *Lactobacillus bulgaricus* against indicator organisms (*staphylococcus aureus and salmonella* sp)

The bacteriocin produced by *Lactobacillus plantarum* and *Lactobacillus bulgaricus* were heat stable after heat treatment at 40, 60, 80 100 °C and 121 °C for 15 min. Figure 4.1 shows that *Lactobacillus plantarum* against *staphylococcus aureus (*indicator organism*)* has the highest bacteriocin activity of 3200(Au/ml) at 40 °C and 60 °C while at 80 °C and 100 °C the activity decreases to 800(Au/ml) and 400(Au/ml) respectively, at autoclaving temperature 121 °C it had the lowest value of 200(Au/ml). *Lactobacillus bulgaricus* against *staphylococcus aureus* has activity of 1600(Au/ml) at 40 °C and 60 °C, at 80 °C the activity decreases to 400 (Au/ml) while the lowest activity was at 100 °C and 121 °C with the bacteriocin activity of 200(Au/ml). *Lactobacillus plantarum* against salmonella specie has activity of 1600(Au/ml) at 40 °C and 60 °C, at 80 °C and 100 °C activity was 400 (Au/ml) while a lower activity of 200(Au/ml) at 121 °C. *Lactobacillus bulgaricus* against salmonella specie has activity of 1600(Au/ml) at 40 °C. While at 60 °C and 80 °C with activity of 800(Au/ml) and 200(Au/ml) at 100 °C and 121 °C.

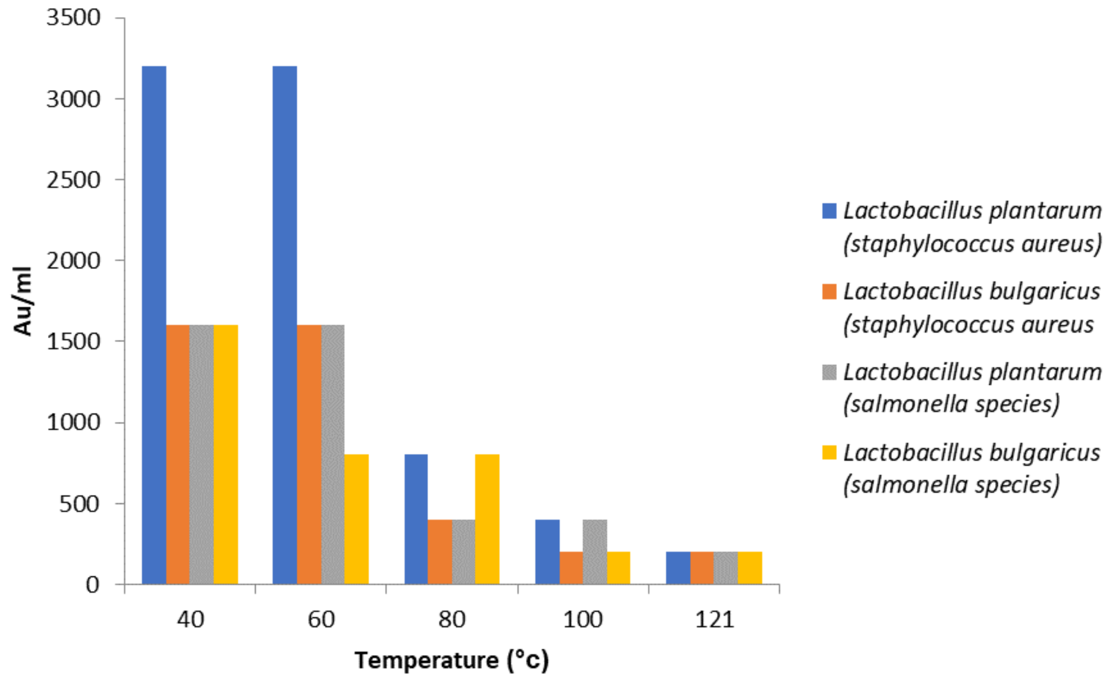


Figure 4.1: Effect of Temperature on Bacteriocin Activity (Au/ml) produced by strain of *Lactobacillus plantarum (plantaricin)* and *Lactobacillus bulgaricus* (bulgarican) against indicator organisms (*staphylococcus aureus and salmonella* sp)

4.1.8 Effect of Storage Stability Temperature on Bacteriocin Activity (Au/ml) produced by *Lactobacillus plantarum* and *Lactobacillus bulgaricus against indicator organism* (*staphylococcus aureus and salmonella* sp)

The storage temperature of *Lactobacillus plantarum* and *Lactobacillus bulgaricus* against indicator organism were stable at different storage temperature (figure 4.2) shows that *Lactobacillus plantarum* against *staphylococcus aureus* has an activity of 3200(Au/ml) at 4 °C and 10 °C storage temperature, the stability decreases to 1600(Au/ml) at 37 °C. *Lactobacillus bulgaricus* against *staphylococcus aureus* has an activity of 3200(Au/ml), while at 10 °C and 37 °C has an activity of 1600(Au/ml). *Lactobacillus plantarum* against *salmonella specie* has an activity of 1600(Au/ml) at 4 °C and 10 °C while at 37 °C the activity was decreases to 800(Au/ml). *Lactobacillus bulgaricus* against *salmonella specie*

has activity of 1600(Au/ml), at 10 °C the acitivity came down to 800(Au/ml) while 400(Au/ml) at 37 °C.

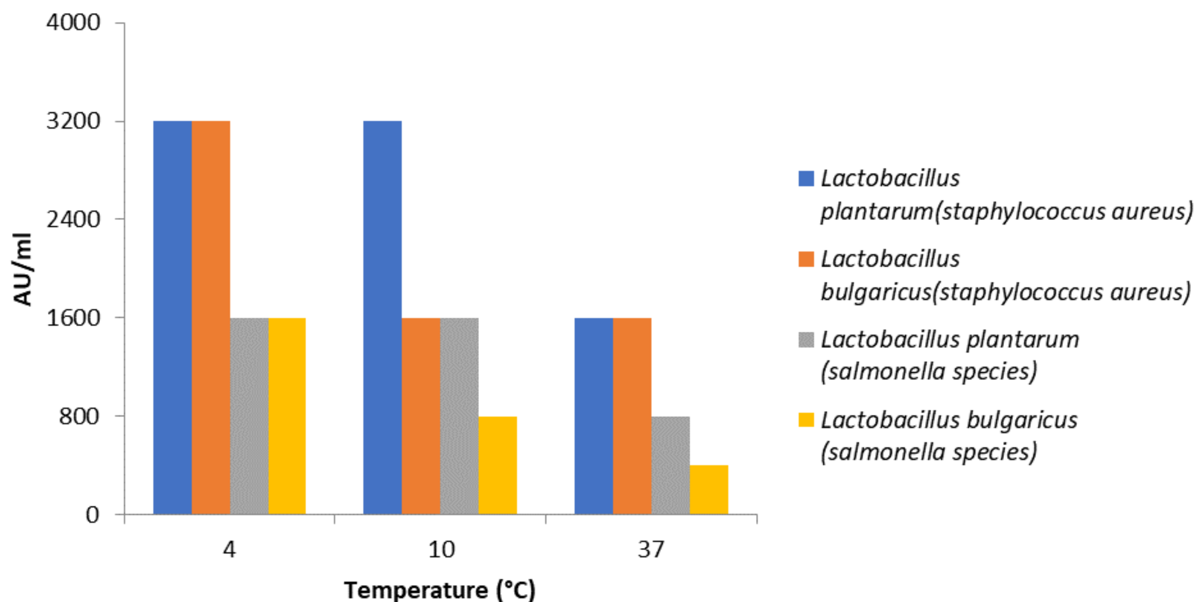


Figure 4.2: Effect of Storage Temperature on Bacteriocin Activity (Au/ml) produced by strain of *Lactobacillus plantarum* (plantaricin) and *Lactobacillus bulgaricus* (bulgarican) against indicator organisms (*staphylococcus aureus and salmonella* sp)

4.1.9 Effect of pH on Bacteriocin Activity (Au/ml) produced by strain of *Lactobacillus plantarum* and *Lactobacillus bulgaricus* against indicator organisms (*Staphylococcus aureus and Salmonella* specie)

Effect of pH on activity of bacteriocin was carried out (Figure 4.3) shows Bacteriocin produced by *Lactobacillus plantarum* has activity against *staphylococcus aureus* of 6400(Au/ml) at pH 2 and 4, there was a decline in the activity to 3200(Au/ml) at pH 6 to 8, then followed by a decrease of activity to 1600(Au/ml) at pH 10 to 12. *Lactobacillus bulgaricus* has a bacteriocin activity against *staphylococcus aureus of* 3200 (Au/ml) at pH

2 to 4. There was a decline in the activity to 1600(Au/ml) at pH 6 to 8 while pH 10 to 12 was a further to 800(Au/ml)

Lactobacillus plantarum against *salmonella specie* has pH activity of 6400 (Au/ml) at pH 2 and 8, at pH 10 to 12 the activity decreases to 800 (Au/ml). *Lactobacillus bulgaricus* against *salmonella specie* has activity of 3200 (Au/ml) at pH 2, while activity of 1600 (Au/ml) at pH 4 to 6, a further decrease to 800 (Au/ml) at pH 8 to 10 and 400 (Au/ml) at pH 12

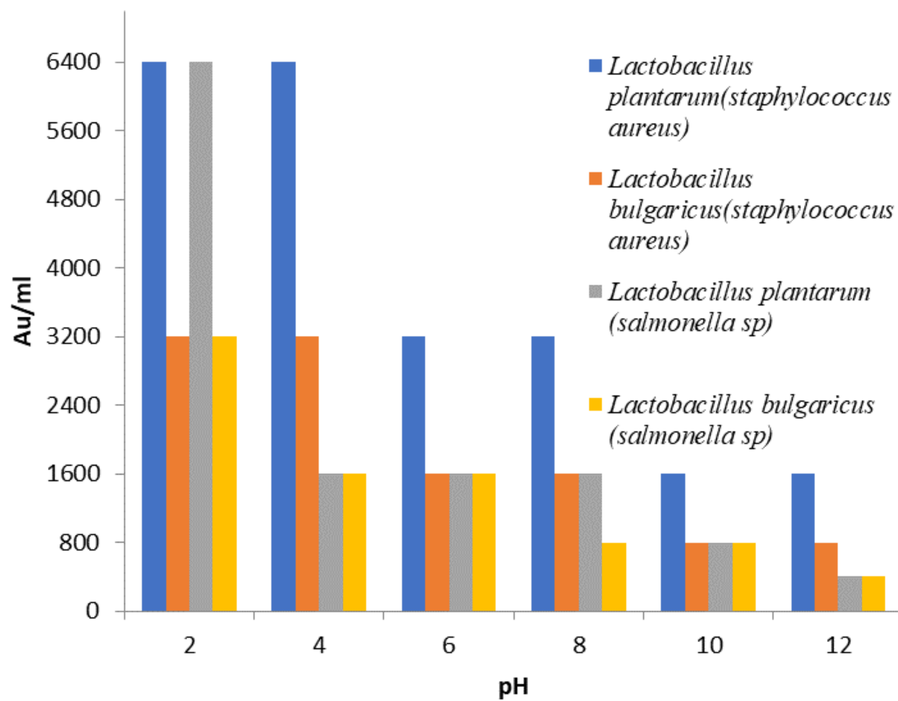


Figure 4.3 Effect of pH on Bacteriocin Activity (Au/ml) produced by strain of *Lactobacillus plantarum (plantaricin)* and *Lactobacillus bulgaricus (*bulgarican) against indicator organisms (*Staphylococcus aureus and Salmonella* specie)

4.1.10 Bacterial load of Plantaricin and Bulgarican inoculated millet dough ball (*fura*) sample kept at 4 °C (refrigeration) temperature

The bacterial counts of the samples were presented in Table 4.6 the result of the shelf life study carried out on inoculated *fura* and uninoculated *fura* (as control) which were monitored and compared throughout the 13days period of study. In the case of the samples inoculated with plantaricin at day zero, the microbial growth at day zero of both the inoculated and uninoculated samples were $9 x 10^5 \pm 1 x 10^5$ CFU/g and 1.0 $x 10^6 \pm 0.00$ CFU/g respectively. After 24hours there was a decrease in microbial count of inoculated *fura* to 7 $x 10^5 \pm 2 x 10^5$ while control had a total microbial count of 1.2 $x 10^6 \pm 3 x 10^5$ CFU/g. At day 3 the inoculated sample had a further decrease in microbial count of 8 $x 10^5 \pm 0.0$ CFU/g while the control had 1.8 $x 10^6 \pm 2 x 10^5$ CFU/g. The uninoculated had 2.3 $x 10^6 \pm 4 x 10^5$ CFU/g at day 5 while the inoculated sample had 8×10^{-3} CFU/g, this shows an increase in the microbial load of millet dough ball (f*ura*)**.** During storage of *fura*, physical examination showed signs of spoilage after 13days of storage which caused a sharp increase in the microbial count, 2.2 $x 10^6 \pm 0.00$ CFU/g for the inoculated millet dough ball (f*ura*) sample*,* while control had signs of spoilage at 5days 2.3 $x 10^6 \pm 4 x 10^5$ CFU/g.

The effect of *bulgarican* on the shelf life of *fura* is presented in Table 4.6. There was microbial growth on the inoculated sample of 9 $x 10^5 \pm 0.00$ CFU/g while the control had 1.0 $x 10^6 \pm 0.00$ CFU/g. The total microbial count in millet dough ball (control) was 1.2 $x 10^6 \pm 3 x 10^5$ CFU/g as compared to 4 $x 10^5 \pm 0.00$ CFU/g inoculated sample within 24hours. At day 3 the inoculated sample had a lower microbial counts of 8 $x 10^5 \pm 2 x 10^5$ CFU/g while the control had 1.8 $x 10^6 \pm 2 x 10^5$ CFU/g. at the 5th day the control show physical sign of spoilage which appear grey white and also with a microbial count of 28×10^{-5} CFU/g while the inoculated sample had 1.0 $x 10^6 \pm 0.00$ CFU/g. the inoculated sample of fura treated with bacteriocin from *Lactobacillus bulgaricus* during storage showed signs of spoilage after

11days of storage which caused a sharp increase in the microbial count of $2.6 \times 10^6 \pm 4 \times 10^5$ CFU/g.

Table 4.6: Bacterial load of Plantaricin and Bulgarican inoculated millet dough ball (f*ura*) sample kept at 4 °C (refrigeration) temperature

DAY	*Plantaricin*	*Bulgarican*	CONTROL
DAY0	$9x10^5±1x10^5$	$9 x10^5±2x10^5$	$1.0 x10^6±2x10^5$
DAY1	$7 x10^5±2x10^5$	$4 x10^5±3 x10^5$	$1.2 x10^6±3x10^5$
DAY3	$5 x10^5±1x10^5$	$8 x10^5±2x10^5$	$1.8 x10^6±2x10^5$
DAY5	$8 x10^5±1.5x10^5$	$1.0 x10^6±1x10^5$	$2.3 x10^6±4x10^5$
DAY7	$1.0 x10^6±1x10^5$	$1.2 x10^6±2x10^5$	TNTC
DAY9	$1.3 x10^6±5x10^5$	$1.8x10^6±1x10^5$	TNTC
DAY11	$1.6 x10^6±1x10^5$	$2.6 x10^6±4x10^5$	TNTC
DAY13	$2.2 x10^6±1x10^5$	TNTC	TNTC

Values are mean ± standard error of mean of triplicate determination. Means values with the same superscript in the same column are not significantly different at (P<0.05)

Key: TNTC= Too numerous to count

4.1.11 Physicochemical and microbial qualities of fermented millet dough ball (f*ura*) enriched with bacteriocin producing LAB

The initial physical colour of the millet dough ball (fu*ra*) was grey and the flavor after preparation was pleasant before inoculation with bacteriocin. The pH before inoculation

was at acidic pH range of 4.28 to 4. 25. The physical appearance after spoilage was grey white while the flavor was not pleasant for all the samples after spoilage.

Table 4.7: Physicochemical and microbial qualities of fermented millet dough ball (f*ura*) enriched with bacteriocin producing LAB

Parameters	*Lactobacillus plantarum*(plantaricin)	*Lactobacillus bulgaricus*(bulgarican)	Control (uninoculated)
Physical appearance before inoculation	Grey	Grey	Grey
Flavor before inoculation with bacteriocin	Pleasant	Pleasant	Pleasant
pH before inoculation with bacteriocin	4.25	4.28	4.25
Microbial count after 24hrs inoculation with bacteriocin	7 $x10^5$±2$x10^5$	4 $x10^5$±0.00	1.2$x10^6$±3$x10^5$
Physical appearance after inoculation with bacteriocin(after shelf life)	Grey white	Grey white	Grey white
Flavor after inoculation with bacteriocin after shelf life	Unpleasant	Unpleasant	unpleasant

Microbial count after shelf life	$2.2 \times 10^6 \pm 0.00$	$2.6 \times 10^6 \pm 4 \times 10^5$	$2.3 \times 10^6 \pm 4 \times 10^5$
Extended period (days) of shelf life	13	11	5

4.1.12 Sensory properties of millet dough ball (f*ura*) inoculated and uninoculated

The sensory properties of millet dough ball (f*ura*) inoculated and uninoculated showed the result of the Sensory properties of the millet dough ball (f*ura*) sample inoculated with bacteriocins compared to the control (fura sample without bacteriocins), the result in Table 4.8 showed that there was a significant difference (P-value < 0.05) between the bacteriocin inoculated fura samples compared with the control without bacteriocins. The bacteriocin inoculated millet dough ball (f*ura*) samples were better acceptable, in terms of aroma, texture, appearance, and taste.

Table 4.8: Sensory properties of millet dough ball (f*ura*) inoculated with bacteriocins

Sample	Appearance	Aroma	Texture	Acceptability
A	5.00 ± 0.00^{b}	5.00 ± 0.33^{b}	4.90 ± 0.10^{b}	5.00 ± 0.00^{b}
B	5.00 ± 1.20^{b}	4.80 ± 0.34^{b}	4.50 ± 0.33^{b}	5.00 ± 0.33^{b}
C	4.10 ± 0.33^{a}	3.70 ± 0.33^{a}	3.70 ± 0.30^{a}	3.80 ± 0.33^{a}

Values are Mean ± SEM. Values with different Superscript down the Column are significantly different ($P < 0.05$)

KEYS: A=Fura inoculated with plantaricin

B= Fura inoculated with Bulgarican

C= control (uninoculated fura)

4.1.13 Proximate Composition of millet dough ball (f*ura*) inoculated and uninoculated

Proximate composition of bacteriocin inoculated millet dough ball (f*ura*) sample showed that there was a statistical significance difference between the moisture content of the bacteriocin inoculated sample and the control (fura without bacteriocin).(Table 4.9).

Table 4.9: Proximate Composition of millet dough ball (f*ura*) Samples Inoculated with Bacteriocins (%)

Treatment	%Moisture	%Ash	%Crude protein	%Crude fiber	%Fats	%Total carbohydrate `
Plantaricin	54.36±0.09^{b}	1.12±0.01^{a}	13.72±0.12^{a}	2.53±0.01^{b}	3.14±0.00^{b}	25.125±0.19^{a}
Bulgarican	53.21±0.02^{a}	1.18±0.00^{a}	15.26±0.06^{a}	2.26±0.02^{a}	3.035±0.02^{a}	25.05±0.09^{a}
Control	59.785±0.03^{c}	1.125±0.02^{a}	6.47±6.47^{a}	2.26±0.02^{a}	3.105±0.00^{b}	24.76±0.01^{a}

Values are mean ± standard error of mean of triplicate determination. Means values with the same superscript in the same column are not significantly different at ($P<0.05$)

4.2 Discussion

Four (4) species of lactobacillus species were isolated (table 4.1) *Lactobacillus* species is a well-known food fermenter worldwide. The isolation and screening of microorganisms from natural sources has always been proven to be a successful way for obtaining industrially important strains with valuable industrial and medical applications (Yang *et al*., 2012). Previous studies on microbial community in fermented foods from different sources (meat, cheese, fish, dairy products, fruits, vegetables, cereals) have demonstrated the dominance of most of the genera of LAB in such fermented foods (Abdelgadir *et al*., 2001; Hwanhlem *et al*., 2011; Pringsulaka *et al*., 2012; Sulieman *et al*., 2006; Yang *et al*., 2012).

Lactobacillus bacteria ferment different monosaccharide and disaccharide (Pyar and Peh, 2014). The results of sugar utilization test were shown in table 4.2 shows all the isolates fermented glucose, sucrose, lactose and mannitol with evolution of gas except for isolate B on sucrose test. The presence of LAB in locally fermented food has been reported by other researchers (Admassie, 2018; Bennani *et al*., 2017; Ismail *et al*., (2019).

The isolation of lactic acid bacteria in cheese implies that they may contain potentially bacteriocin producing Lactic acid bacteria which may probably inhibit or antagonize some food pathogens thereby making the snack suitable for consumption. Similar result was reported by Sangoyomi *et al.*, (2010) who also isolated lactic acid bacteria from cheese.

The sequenced amplicons of the two lactic acid bacteria from cheese plate 4.1 shows that the molecular characterization using 16s rRNA confirms the isolates of A and B as *Lactobacillus plantarum* strain and *Lactobacillus bulgaricus* respectively. Similarly, the

occurrence of LAB in locally fermented foods were also reported by Oyeleke *et al.,* (2006) who reported frequent isolation of *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* products. This is in conformity with the report of Mohammed and Ijah (2013) who isolated and characterized LAB from fermented milk (nono), cheese (wara) and yoghurt. Also Bennani *et al*., (2017) characterized and Identify lactic acid bacteria isolated from Moroccan raw cow's milk. Lactic acid bacteria have a long history of use in a variety of cheese fermentation with predominance of *Lactobacillus plantarum* which had earlier been reported by Merih *et al*., (2011) in Boza a cereal based fermented foods and Nigerian Ogia spontaneous fermented maize product (Oranusi *et al*., 2003).

Table 4.3 showed that Lab with growth activity of 0.85nm to 0.89nm and pH of 4.01 to 3.80 and zone of inhibition of 10mm to 6mm were better bacteriocin producers than those LAB with growth ability from 0.25nm to 0.89nm, this is in accordance with the result of Mohammed *et al* ., (2012)

The antimicrobial properties of lactic acid bacteria on two pathogenic strains are shown in table 4.4 the results revealed that LAB exhibited strong inhibition on the growth *of Staphylococcus aureus* and (inhibition zone of 8±0mm, 12±2mm) and *salmonella sp*ecie (6±0 mm and 10±1mm respectively). This is in line with the work reported by Ogunshe *et al*., (2007) who recorded a good inhibition against *S. aureus* and *Salmonella dysentriae* with inhibition zones of 8 and 7 mm, respectively. However, the zone of inhibition observed in this work is greater than that observed by linan *et al*., (2014), who observed a 2mm zone of inhibition of *Lactobacillus. plantarum* on *P. aeruginosa*, 2.5mm on *E. coli*, and 2mm on *S. aureus.* Formation of different clear zones of inhibition around the wells by agar well diffusion assay may be due to the production of several antimicrobial compounds like bacteriocins (Ogunshe *et al*., 2007). This was in

agreement with earlier report by Labiou *et al*., (2005) that the inhibitory effect demonstrated against the pathogens in the wells by agar well diffusion assay is an indication of possession of antibacterial activity like bacteriocins.

During the purification procedures, each step resulted in a considerable loss of protein concentration while specific activity increases. The optimal bacteriocin recovery was achieved by including ammonium sulphate precipitation and Trichloroacetic acid precipitation. This agreed with the findings of Ivanova *et al*., (2000)

Lactobacilli are important in food fermentation as well as the production of bacteriocins which are responsible for inhibition of pathogenic and spoilage organism in food (Adams and Moss, 1995). These fact are supported by the report of Ogunbawo *et al*., (2003) that *Lactobacillus plantarun* and *Lactobacillus brevis* isolated from Nigeria fermented food products produced bacteriocins that had broad spectrum of inhibition against both pathogenic and food spoilage organism.

The heat stability of bacteriocin by bulgarican and plantaracin were heat stable after heat treatment at 40, 60, 80, 100 °C and 121 °C for 15 min. Figure 4.1 shows that at 40 and 60 °C, has the highest activity of both bacteriocin produced with activity of 3200 Au/ml, against *staphylococcus aureus*, while at autoclaving temperature 121°C, they had the lowest inhibition activity of 200 Au/ml for the two bacteriocin produced against all the indicator organisms. Similar results were recorded for a number of bacteriocins produced by *Lactobacillus* strains which was resistant at 100 °C for 15 min (Joshi *et al*., 2006). The phenomenon of heat stability of LAB bacteriocins have been reported earlier in literatures (Moigani and Amirinia, 2007; Ogunbanwo *et al*., 2004). This present research is also in agreement with the above mentioned reports as it was observed that the bacteriocin used in this study still retained its antimicrobial activity after heating at 121 °C for 15 min which means it could be placed within the heat stable low molecular

weight group of bacteriocins. This quality of the bacteriocin makes it superior in processed foodstuffs where high heat is applied. Thermostability is a very useful characteristic in case of using bacteriocin as food preservative, because many food processing procedures involve a heating step (Panesar and Bera, 2011). Moigani and Amirinia (2007) also stated that it is a good property of bacteriocin that it remains effective even at 121 °C for 15 min. Due to this property, it remains effective during many food safety processes like pasteurization.

The storage stability of bacteriocin by *Lactobacillus plantarum and Lactobacillus bulgaricus* was stable at 4 and 10 °C, slightly stable at 37 °C. Figure 4.2 shows that the maximum zone of inhibition was at 4 and 10 °C with activity of 3200 Au/mL against *Staphylococcus aureus* with the least against *salmonella* specie with the activity of 1600 – 400 Au/mL**.** The percentage of effectiveness reduced more at 37 °C as compared to 4 and 10 °C storage temperature. These implies that the bacteriocin can be stored at 10 and 4 °C, indicating that cold temperature may be the most appropriate preservation technique for storing bacteriocins. Similar results was reported by Panesar and Bera (2011) that the high stability of bacteriocin during prolong storage makes them superior and can have a positive impact on their use as bio-preservative with a view to improving the hygiene and safety of food products, especially processed foods.

The pH stability of bacteriocin obtained from *Lactobacillus plantarum* and *lactobacillus bulgaricus* in this study was observed to be active over a pH range of 2 to 6, but its activity reduced along alkaline pH range of 8 to 12 (Figure 4.3). Stability of Lactic acid bacteria at low pH ranges justify its use in fermented foods like cheese and yoghurt. Most of the fermented foods are acidic, and acidity inhibits the growth of pathogenic organism thereby enhancing safety to fermented foods. Optimum pH of 2-6 was observed to favour bacteriocin activity. This implies its use in fermented foods since

most fermented foods have pH range of 4.5-6.5.This finding is similar with that of Dhewa, (2012), who reported that pH 4.5-6.5 favoured bacteriocin activity.

This also implies that bacteriocin obtained from *Lactobacillus plantarum and Lactobacillus bulgaricus* will be effective against Gram negative bacteria such as *Salmonnela species* at acidic pH ranges and not at alkaline pH range, Also, against *Staphylococcus aureus*. Similar results were reported by Adebayo and Famurewa (2002) who opined that the bacteriocin of *Lactobacillus* were active over a wide range of pH 2 to 6 and is the optimum pH range for good inhibitory activity of bacteriocin from *Lactobacillus* strains against a wide range of various pathogenic organisms for example *S.aureus.*, while inactivation occurred mostly at pH 12. These observations are in agreement with those reported by Tatsadjieu *et al.,* (2009) in their work with LAB bacteriocins with antimicrobial activities against Chicken *Salmonella enteric and E. coli*. The findings of the present study are also in agreement with those reported by Holzapfel *et al*. (2010) who showed that *Lactobacillus. plantarum* excreted other compounds such as bacteriocins that inhibited the growth of pathogens.

Bacteriocin is pH, heat and storage temperature dependent. So, due to these qualities, bacteriocins produced by LAB constitute the best option as bio-preservative for the preservation of food at commercial level.

The effectiveness of bacteriocin isolated from *Lactobacillus plantarum* to act as bio-preservative and its role in increasing shelf life of *fura* was checked in the presence of bacteriocin as compared to control. Table 4.6 presents the result of the shelf life study carried out on inoculated millet dough ball (f*ura*) and uninoculated millet dough ball (as control) which were monitored and compared throughout the 13days period of study. In the case of the control sample at day zero, the total microbial count in millet dough ball (f*ura*) was 1.0 $x10^6$±2 $x10^5$CFU/ml, while inoculated *fura* at day zero was $9x10^5$±$1x10^5$.

CFU/mL, while a decrease after 24hours of 7 x10^5± 2 x10^5 this could be due to bactericidal effect of bacteriocin on the microorganisms initially present in the sample (Samelis *et al*., 2005). During storage of millet dough ball (f*ura*) physical examination showed signs of spoilage after 13days of storage which caused a sharp increase in the microbial count, 2.2 x10^6±1 x10^5 CFU/ml which could be due to the initiation of spoilage of millet dough ball (f*ura*) at 4 °C, while inoculated *fura* after 5 days of storage was 2.3 x10^6±4x10^5 CFU/mL. The result depicted that the microbial load of bacteriocin treated samples and the control samples after day 13 were not comparable. The result showed that there was a decrease in the microbial count in the inoculated *fura* throughout the period of study. After day zero and day 1 the microbial analysis was made after every 2 days interval. The results of this investigation have shown that the uninoculated *fura* had a shelf life of 13days before spoilage occurred. With bacteriocin, the shelf life of *fura* was increased up to 13days, this is in line with the work of Ohenhen and Ikenebomeh (2007) who monitored and compared inoculated fermented *ogi* slurry and uninoculated *ogi* slurry throughout a 60 days period of study in which the uninoculated *ogi* slurry had a mouldy flavour by 40 days of study and by the end of 60 days period of study, it was no longer acceptable nor edible in terms of colour and flavour. This observation correlated with the observation of Mensah *et al.,* (2002) that the method of preparation, handling and environmental factors were probably responsible for the early sign of spoilage observed during the study.

Bacteriocin in millet dough ball (f*ura*) inoculated with of bulgarican on the shelf life of millet dough ball (f*ura*) is presented in Table 4.6 The total microbial count in *fura* (control) was 1.0 x 10^6±2x10^5 CFU/mL as compared to 9 x 10^5±1x10^5 CFU/mL in inoculated sample at day zero. The result of this findings was not in agreement with the work of (Intarapichet and Gosaarak, 2008), who recorded a zero microbial count in

meat ball preparation at day zero because of treatment of heat during the meat-ball preparation inactivates vegetative cells, which was indicated by zero microbial count in control and treated samples at day zero (Intarapichet and Gosaarak, 2008). There was a reduction in the microbial load of the inoculated sample at day 1 to $4x10^5 \pm 1x10^5$ which could aslo be due to the action of bulgarican in the sample.

The results of this investigation have shown that the shelf life of inoculated millet dough ball (f*ura*) was over 11 days; this indicates that the product can be kept well beyond this period, whereas the uninoculated millet dough ball (f*ura*) had a shelf life of 5 days before spoilage started. Previous studies (Intarapichet and Gosaarak, 2008) also reported that crude bacteriocin from *Lactococcus lactis* TISTR 1401 prevented the growth of total aerobic bacteria up to day 6 in treated meatball batter as compared to control.

In the present study, the pH of all the millet dough ball (f*ura*) samples studied were within the acidic range of 4.25 to 4.28. This acidic property can be traced to the addition of species such as ginger, dry pepper, cloves and alligator pepper or to the presence of some lactic acid producing bacteria during overnight fermentation process (Ofudje *et al*., 2016). The pH values of millet dough ball (f*ura*) in this study agree with the report of pH values ranging from 4.10 to 5.00 in *fura* samples in Ghana (Owusu-Kwarteng *et al*., 2010). This is also similar to the pH values of cereal-based fermented beverages, which reported the pH of kunun-zaki (a non-alcoholic cereal-based fermented beverage) samples in the range of 4.00 to 4.30 in Ogun state Nigeria (Owusu-Kwarteng *et al*., 2010). The low pH values are desirable since report indicates that it inhibits the growth and survival of spoilage organisms and give fermenting organisms an advantage (Ogodo *et al.,* 2018). This low pH values can also be attributed to the presence of lactic acid bacteria which produced acid during fermentation which lower the pH.

Table 4.9 shows the proximate composition of fura (inoculated and uninoculated) the result revealed that Mean value obtained for Proximate composition showed that Moisture content of the the fura samples inoculated with bacteriocins ranged from 53.21±0.02 % to 59.785±0.03 %. Moisture content is a measure of the water content in a food sample. The moisture content accounts for the textural property of the food sample. High moisture content supports microbial growth thereby reducing the shelf life of millet dumpling ball (*fura*) while low moisture content causes reduction in microbial growth due to low water activity and hence increased the shelf life of millet dough ball (f*ura*). The moisture content observed is similar to 47.49±0.01 % to 50.66±0.24 % that was reported by Jideani and Danladi (2005).

Sensory attributes such as colour, acceptability, aroma and texture indicated that the fura inoculated with bacteriocin was significantly acceptable. The significant difference between the *fura* samples and the control might be due to the inoculation of bacteriocins which inhibits a variety of food spoilage and pathogenic organisms, which might have imparted flavor, texture and aroma to the samples which might have also resulted in preservation of the millet dough ball (f*ura*) sample and hence better acceptability.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Lactobacillus species isolated from cheese were identified as *Lactobacillus plantarum* strain AAAbd and *Lactobacillus bulgaricus* strain Mn95906. During extraction, production, purification and characterization of bacteriocins, the bacteriocins (Plantaricin, and bulgarican) from Lactic acid bacteria produced an appreciable quantity of bacteriocin that has proved to be an effective antibacterial agent on the indicator organism in millet dough ball *(fura).* Temperature, pH tolerance, inhibitory activity, stability during storage played an important role in the shelf life extension of millet dough ball (*fura)* by increasing the shelf life to 8 and 6 days. Sensory property and proximate parameters showed evidence that the bacteriocin has potential application in extending the shelf life millet dough ball *(fura)*.

5.2 Recommendations

Bacteriocins produced by *Lactobacillus plantarum* strain AAAbd and *Lactobacillus bulgaricus* strain Mn95906 were able to extend the shelf life of millet dough ball (*fura)* for 8 and 6 days respectively as compare to the control which was 5days.

Therefore food processing companies should embark on the use of bacteriocin for bio-preservation of foods which may reduce the risk of using chemicals as food additive.

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APPENDICES

Appendix A: Composition and preparation of media

Nutrient of agar medium: seven grams of nutrient agar powder was weighed and dissolved in 250mL and of distilled water. It was stirred vigorously and dissolved using hot plate after which was sterilized in autoclave for 15minutes at 121°C. It was then allowed to cool after which it was dispensed in petri dishes and allow to solidify (cheesebrough, 2000).

Nutrient broth: this was done using 1.3g of nutrient broth medium dissolved in 100mL of distilled water. It was stirred and dissolved then sterilized in an autoclave for 15minutes at 121°C and it was allowed to cool after which was dispensed into test tubes (cheesebrough, 2000).

De Man Rogosa Sharpe (MRS) Agar:

Composition: agar 12g/L, diammonium hydrogen citrate 2g/L, dipotassium hydrogen phosphate 2g/L,D(+)-glucose, 20g/L manganous sulfate, 0,05g/L meat extract, 5g/L sodium acetate, 5g/L universal peptone, 10g/L yeast extract 5g/L.

This was done using 61.15g of De Man Rogosa Sharpe (MRS) agar dissolved in 1000mL of distilled water. It was stirred and dissolved then sterilized in an autoclave for 15minutes at 121°C and it was allowed to cool after which was dispensed into petric plates.

Mannitol salt agar:

Composition: proteose peptone 10g/L, sodium chloride 75.0g/L, D-mannitol 10g/L, beef extract 1.0g/L, phenol red 09.025g/L, Agar 15.0g/L.

This was done using 111g of mannitol salt agar dissolved in 1000mL of distilled water. It was stirred and dissolved then sterilized in an autoclave for 15minutes at 121°C and it was allowed to cool after which was dispensed into petri plates,

Salmonella shigella agar (SSA):

Composition: beef extract 5.00g/L, enzymatic Digest of Casein 2.50g/L, enzymatic digest of animal tissue 2.50g/L, lactose 10.00g/L, bile salts 8.50g/L, sodium thiosulphate 8.50g/L, ferric cutrate, 1.00g/L, brilliant green 0.00033g/L, neutral red 0,025g/L, agar 13.50g/L

This was done using 60.0g of salmonela shigella agar dissolved in 1000mL of distilled water. It was stirred and dissolved then heated to boiling to dissolved the medium completely and it was allowed to cool after which was dispensed into petri plates.

Appendix B: Nucleotide sequence of *Lactobacillus plantarum* strain AAAbd and *Lactobacillus bulgaricus* strain MN945906

***Lactobacillus plantarum* strain AAAbd**

CAACGGCGTGCCTAATACATGCAGTCGAACGAACTCTGGTATTGATTGGTGC
TTGCATCATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGG
GAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCG
CATAACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCA
CTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACC
ATGGCAATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACT
GAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAA
TGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGC
TCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGT
ATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAG
CGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAA
GTGCATCGGAAACTGGGAAACTTGAGTGCAGAGAGGACAGTGGAACTCCAT
GTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCG
GCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACA
GGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTGTTG
GAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGG
GGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAG
GTCTTGACATACTATGCAAATCTAAGAGATTAGACGTTCCCTTCGGGGACAT
GGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGCT

AAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAAGTTGGG

CACTCTGGTGAGACTGCCGGTGACAAACCGGAGGACGGTGGGGATGACGTC

AAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGT

ACAACGAGTTGCGAACTCGCGAGAGTAAGCTAATCTCTTAAAGCCATTCTC

AGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTA

ATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACC

GCCCGTCACACCATGAGAGTTTGTAACACCCAAAGTCGGTGGGGTAACCTTT

TAGGAACCAGCCGCCTAAGGTGGACAGATGAT

Lactobacillus bulgaricus MN945906

GCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAGCT

GAATTCAAAGATTCCTTCGGGATGATTTGTTGGACGCTAGCGGCGGATGGGT

GAGTAACACGTGGGCAATCTGCCCTAAAGACTGGGATACCACTTGGAAACA

GGTGCTAATACCGGATAACAACATGAATCGCATGATTCAAGTTTGAAAGGC

GGCGTAAGCTGTCACTTTAGGATGAGCC

CGCGGCGCATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCAATGATGC

GTAGCCGAGTTGAGAGACTGATCGGCCACATTGGGACTGAGACACGGCCCA

AACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCT

GATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAGCTCTG

TTGTTGGTGAAGAAGGATAGAGGCAGTAACTGGTCTTTATTTGACGGTAATC

AACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG

TGGAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAATGA

TAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAACTGCATCGGAAACT

GTCATTCTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGTA

ATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTG

CAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCC

TGGTAGTCCATGCCGTAAACGATGAGCGCTAGGTGTTGGGGACTTTCCGGTC

CTCAGTGCCGCAGCAAACGCATTAAGCGCTCCGCCTGGGGAGTACGACCGC

AGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT

GTGGTTTAATTCGAAGCAAGCGAAGAACCTTACCAGGTCTTGACATCCTGTG

CTACACCTAGAGATAGGTGGTTCCCTTCGGGGACGCAGAGACAGGTGGTGC

ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG

CGCAACCCTTGTCTTTAGTTGCCATCATTAAGTTGGGCACTCTAAAGAGACT

GCCGGTGACACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTT

ATGACCTGGGCTACACACGTGCTACATGGGCAGTACAACGAGAAGCGAACC

CGCGAGGGTAAGCGGATCTCTTAAAGCTGTTCTCAGTTCGGACTCAGGCTGA

ACTCGCCTGCACGAAGCTGGAATCGCTAGTAATCGCGGATCAGCACGCCGC

GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGTC

TGCAATGCCCAAAGTCGGTGGGATAACCTTTATAGAGTCAGCCGCCTAAGG

CAGGGCAGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGCAT

Appendix C: Inhibition of indicator organism due to bacteriocins produced by lactic acid bacteria

Plate 1: zone of inhibition of *Staphylococcus aureus* from bacteriocin produced by *Lactobacillus plantarum* AAAbd

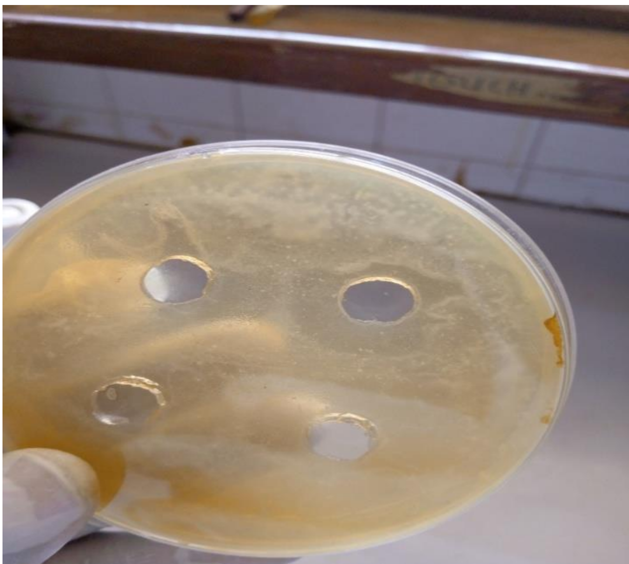

Plate 2: Zone of inhibition of *Salmonella specie* from bacteriocin produced by *Lactobacillus plantarum* AAAbd

Plate 3: zone of inhibition of *Staphylococcus aureus* from bacteriocin produced by *Lactobacillus bulgaricus* Mn95906

Plate 4: Zone of inhibition of *Salmonella* sp from bacteriocin produced by *Lactobacillus bulgaricus* Mn95906

Appendix D: Shelf life study of millet dough ball (*fura*) inoculated with bacteriocin

Shelf life study of millet dough ball (*fura*) inoculated with bacteriocin and the control

Appendix E: SENSORY EVALUATION QUESTIONNAIRE

On the Study titled:

ENHANCEMENT OF SHELF LIFE MILLET DOUGH BALLS (*FURA*) USING BACTERIOCIN PRODUCING LACTIC ACID ISOLATED FROM CHEESE

INSTRUCTIONS: On the **hedonic** scale of five-point scale ranging from score1 (dislike much), 2 (dislike), 3 (like), 4 (like much), 5(like very much) below tick the grade for the following sensorial qualities of the millet dough balls (*fura)* samples presented

		Hedonic **scale**				
Parameters		**1**	**2**	**3**	**4**	**5**
Colour	**A**					
	B					
	C					
Aroma	**A**					
	B					
	C					
Texture	**A**					
	B					
	C					
Acceptability	**A**					
	B					

	C					

Keys: A= millet dough ball inoculated with bacteriocin produced by *Lactobacillus plantarum* AAAbd (plantaricin)

B= millet dough ball inoculated with bacteriocin produced by *Lactobacillus bulgaricus* MN945906 (Bulgarican)

C= control (uninoculated fura)

Panelist Code__________________ Signature____________________

CONSENT FORM

My name is **Abdullahi, Aisha Aliyu** (MTech, Food and Industrial Microbiology), and an M.Tech student with **Federal University of Technology Minna**, Nigeria working on the study titled: **Enhancement of shelf life millet dough balls (*Fura*) using bacteriocin producing lactic acid isolated from cheese**

In the study millet dough ball (*fura*) was produced, bacteriocin was added which was produce by *Lactobacillus plantarum* AAAbd (plantaricin) and *Lactobacillus bulgaricus* MN945906 (Bulgarican) and then compare the control.

Volunteers are required to assess the overall quality of the produced *fura* through graded sensory evaluation procedure,

Sensory Evaluation Procedure

This procedure will involve issuing questionnaire to trained consuming panelist, to evaluate the sensorial quality of the millet dough ball (*Fura*) produced experimentally with bacteriocin producing lactic acid bacteria and compare with millet dough ball (Fura) without bacteriocin producing lactic acid bacteria using a hedonic scale of 5

points (from score1: dislike much to score 5: like very much) for easy comparison (the questionnaire is attached to this consent form)

Potential Harm, Risks or Discomfort (Non-maleficence)

It is not likely that there will be any harm or discomfort associated with this procedure

Right to Refuse or Withdrawal (Autonomy)

Your participation in this study is voluntary; should you change your mind, you can stop at any time, even after signing the consent form. There will be no consequences if you decline to participate.

Confidentiality

Your response to this questionnaire will be kept confidential and used solely for this research purpose, your identity shall be concealed as your name will not appear anywhere on the coded information forms. Whatever it is in our finding that could identify you will not be published except with your consent.