BIOPRESERVATION OF MILK USING BACTERIOCINSPRODUCING LACTIC ACID BACTERIA ISOLATED FROM *KUNUN ZAKI* IN ZARIA, KADUNA STATE, NIGERIA

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BY

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DEDICATION

I dedicate this research work to my beloved, caring, encouraging and wonderful parents (Alhaji Adamu Aliyu Muhammad and Hajiya Aisha Muhammad Tako), my adoring, patient husband (Malam Muhammad Yahaya) and to my beloved daughters (Amina and Aisha) who with their joint effort made this work possible.

DECLARATION

I declare that the work in this dissertation entitled "Biopreservation of milk using *bacteriocin*producing Lactic acid bacteria isolated from *kunun Zaki* in Zaria, has been carried out by me in the Department of Microbiology.The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Amina GogoADAMU _____ _____

Signature Date

CERTIFICATION

This dissertation entitled BIOPRESERVATION OF MILK USING *BACTERIOCIN* PRODUCING LACTIC ACID BACTERIA ISOLATED FROM *KUNUN ZAKI* IN ZARIA, Kaduna State, Nigeria BY Amina Gogo ADAMU meets the regulations governing the award of M.Sc. Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Prof. E.D. Jatau	_____	_____
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ABSTRACT

Bacteriocins from Lactic acid bacteria are used in preservation of foods due to their beenGenerally Regarded as Safe (GRAS) status.Consumers awareness over chemical free foods has led to the search for alternative method of food preservation using Lactic acid bacteria. Lactic acid bacteria were isolated from *kunun zaki* in Zaria metropolis, Kaduna State, Nigeria. Sampling technique used was a non probability type, convenience method. The pH of *Kunun Zaki* sample ranged 5.68 ± 0.09 - 5.80±0.09, mean count of Lactic acid bacteria ranged $1.14 \times 10^6 \pm 2.4 \times 10^4$ - $1.8 \times 10^6 \pm 2.3 \times 10^4$,Analytical Profile Index (API 50 CH KIT) was used to identify the organisms to Species level as *Lactococcus lactis, Lactobacillus plantarum, Lactobacillus acidophilus, Pediococcus pentosaceous, Lactobacillus fermentum* and *Lactobacillus paracasei*. *Lactococcus lactis, Lactobacillus plantarum, Lactobacillus acidophilus* and *Pediococcus pentosaceous* had the potential for bacteriocin activity following screening by agar well diffusion assay, zones of inhibition were measured as *Staphylococcus aureus* (3.10 ± 0.03 -8.10 ± 0.07mm), *Escherichia coli* (1.20 ± 0.34mm), *Pseudomonas aeruginosa* (0.64±0.07 -2.11±0.42mm), *Listeria monocytogenes*(3.10 ± 0.03-9.25 ± 0.40mm),*Salmonella* Typhimurium(1.28 ±0.92-1.36±0.62mm) and *Clostridium botulinum* (2.34 ± 0.08-7.43 ± 0.53mm). Production study reveal that pH 5 and temperature 37^0C were optimum for bacteriocin activity. Assessment of the Biopreservative potential of bacteriocin on milk showed that the shelf life of milk was extended at 4^0C for 6 days on addition of Nisin and 5 days when Plantaricin, Acidophilin and Pediocin were inoculated. At 8^0C, the shelf life of milk was extended for 4 days after inoculation of Nisin, Plantaricin, Acidophilin and Pediocin while the initial shelf life of milk without bacteriocin (control) was 4 days, at ambient temperature, shelf life of milk was extended for 2 days (after inoculation of Nisin, Plantaricin, Acidophilin and Pediocin) while the initial shelf life of milk without bacteriocin (control) was 1 day. pH of milk ranged from 5.4±0.1-4.0±0.1 while total titratable acidity ranged from 0.0217±0.001 -0.120±0.003. Findings from the work showed that the milk sample inoculated with bacteriocin was acceptable. Proximate composition of milk sample such as Moisture content (64.00±1.62 - 66.67±2.67) of bacteriocin inoculated milk showed a significant difference ($P \leq 0.05$) compared with milk without bacteriocin (control), Ash content (4.30±0.2 - 4.60±0.6), Fat content (3.10±0.1 - 3.37±0.1), Protein content (20.00 ±0.06-21.07±0.54) and carbohydrate content (3.10±0.61 - 6.77±0.43) by difference also showed that bacteriocins from Lactic acid bacteria isolated from *kunun Zaki* could serve as a food additives for biopreservation. The use of bacteriocins from Lactic acid bacteria as a natural preservative in milk has shown to be effective against foodborne pathogens and may be used as an alternative to the commonly used preservatives in response to consumers demand for natural products.

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CHAPTER ONE

1.0 INTRODUCTION

The use of microorganisms in food fermentation is one of the oldest method for producing and preserving food.Much of the world depends upon various fermented food that are staples in the diet (Mohammed *et al.*,2013). Biopreservation refers to extended storage life and enhanced safety of foods using the natural microflora and their antimicrobial products for example bacteriocins (Mohammed *et al*.,2013). Lactic acid bacteria (LAB) have a major potential for use in biopreservation because they are safe to consume and during storage they dominate the microflora of many foods.In milk,brined vegetables,many cereal products and meat with added carbohydrate,the growth of LAB produce a new plant product(Mohammed *et al*.,2013)

Lactic acid bacteria (LAB) are Gram positive, fastidious, acid tolerant, generally non-sporulating, catalase negative, devoid of cytochrome and non-respiring rod or cocci that produce lactic acid as a major or sole product of fermentative metabolism (Holzapfel *et al*., 2001).

Lactic acid bacteria comprise these major genera: *Lactobacillus, Lactococcus, Enterococus, Pediococcus, Streptococcus, Lactospaera, Melissoccus, Oenocuccus, Tetragenococcus, Bifidobacterium, Proprionibacterium and Weisella (*Carr *et al*., 2002). Some of the most common specie include*L acidophilus, L. plantarum, L. casei*, *L. fermentum, L. lactis, L. delbruekii bulgaricus*, *B. bifidum*, *B. infantis*, *E. faecum* and *E. faecalis* (Parada *et al*., 2007).

These micro-organisms are found in milk, meat, fermented products and beverages, inhibiting the growth of pathogenic and deteriorating micro-organisms maintaining the nutritive quality and improving the shelf life of food (Carr*et al*., 2002). Lactic acid

bacteria (LAB) are generally recognized as safe (GRAS), and have an important role in the preservation of foods and fermented products that can be used as natural competitive microbiota or as specific starter cultures under controlled conditions (Cintas *et al*., 2001).

Lactic acid bacteria can produce various antimicrobial compounds which can be classified as low molecular carbon mass compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), and high molecular mass compounds such as bacteriocins (Nawful,2014).

Bacteriocins are ribosomally synthesized antimicrobial peptides that are active against other bacteria, either of the same species (narrow spectrum), or across genera (broad spectrum) (Cotter *et al*., 2005). Bacteriocins may be produced by other bacteria, those produced by LAB are of particular interest in the food industry since these bacteria have generally been recognized as safe (GRAS) (Altuntas *et al*.,2010). Bacteriocins have been known for approximately 90 years and nisin, the widely studied bacteriocin was discovered in 1928 and it is the first bacteriocin used in the food system as biopreservative (Gurakan, 2007).Nisin produced by numerous *Lactococcus lactis*strainis commercially available as biopreservative and has been approved in over 50 countries for many applications in a large range of food products (Javed, 2009). More than 300 different bacteriocins have been described for the genera *Lactobacillus, Lactococcus, Leuconostoc,Pediococcus* and *Enterococcus* (Murua *et al*., 2003). Among the various antimicrobial metabolites produced by LAB, bacteriocins are often the most inhibitors of bacteria (Deegan *et al*., 2006). Generally, the compounds are named according to the genus or species of the bacterial strains that produce them for example plantaricin is produced by *Lactobacillus plantarum* (Joerger and Hoover, 2000). Lactic acid bacteria, through their fermentative activities also exert a positive effect, imparting desirable

flavours and inhibiting a variety of food spoilage and pathogenic organisms (Buddle *et al*., 2003).Milk is an important source of all basic nutrients required for mammals including human beings.Milk from various animals, mammals such as cow, buffalo, goat, sheep, camel is used for different nutritional purposes which includesfeeding to young ones and preparation of some nutritional products such as milk cream,butter,yoghurt, and sour milk (Mohammad,2005).

In Nigeria, *Kunun zaki* and Ogi are the two of the most common Lactic acid fermented products popular with consumers. Consumption of these fermented foods have many advantages including enhanced nutritional value, digestibility, therapeutic benefits and safety against pathogens (Oranusi *et al*.,2003). The traditional methods of preparation of *Kunun zaki* have been described and there is a general agreement on the dominance and beneficial effects of lactic Acid Bacteria(LAB) in the fermentation processes of these foods (Omemu *et al*.,2007).

1.1 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 pore a major threat to consumers and there is increasing trend towards less processed food (Saranj, *et. al.,* 2013).

The contamination of milk and its products by *Listeria monocytogenes, Salmonella* Typhimurium, *Escherichia coli, Staphylococcus aureus and Clostridium botulinum* have been the major source of several foodborne outbreaks (CDC,1999).

The resistance of *Listeria monocytogenes, Salmonella* Typhimurium, *Escherichia coli, Staphylococcus aureus and Clostridium botulinum* to most commonly used preservatives have created problems for the food industry (WHO,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).

1.2 Justification of the Study

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,the 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 nanomolar range and have no toxicity(Deraz.,*et al*.,2005).

1.3 Aim

To determine the biopreservative potential of bacteriocins on milk using bacteriocins producing Lactic acid bacteria isolated from *Kunun Zaki*.

1.4 Objectives

1. To isolate and identify Lactic acid bacteria from *Kunun Zaki*.
2. To screen for Lactic acid bacteria and to partially purify the bacteriocins produced by the Lactic acid bacteria.

3. To determine the effect of Temperature and pH on the bacteriocins produced by lactic acid bacteria
4. To assess the biopreservative potential of bacteriocins of Lactic acid bacteria on milk .
5. To determine the proximate composition and sensory attributes of the milk to which bacteriocin is added.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 History of food preservation.

The single most important development permitting the formation of civilization was the ability to produce and store large quantities of food. Hunter-gatherer societies lived from day to day either starving or gorging themselves based upon the amount of food they could find in a day. When it became possible for one person to produce more food than they needed, time from gathering food could be apportioned to culture and science. Following this trend, it became beneficial to be able to store as much food as possible in order to minimize the amount of time spent gathering that food (Soomro *et al*., 2002). Food storage has always been at odds with food spoilage. Some of the earliest evidence of food preservation comes from the post-glacial era, from 15,000 to 10,000 BC. The first use of biological methods was from 6000 to 1000 BC when fermentation was used to produce beer, bread, wine, vinegar, yoghurt, cheese and butter. In 1864, Louis Pasteur proved that microorganisms in foods were the cause of food spoilage, that heat treatment of food killed these microbes and that sealed containers helped to preserve food by preventing recontamination from atmospheric air(Soomro *et al*., 2002). A major development in the distribution and storage of foods came in 1940 with the availability of low cost home refrigerators and freezers. Other developments included the artificial drying, vacuum packaging, ionizing radiations and chemical preservation. Now-a-days consumers are concerned about the synthetic chemicals used as preservatives in food, and there is increasing trend towards less processed food (Saranj *et al*., 2013). These untreated foods can harbour dangerous pathogens which can multiply under refrigeration and without oxygen. A solution to this dilemma is the use of antimicrobial metabolites of fermentative microorganisms. Many antimicrobial chemicals have been

in use for a long time without any known adverse effects. Many of the organic compounds which have stirred interest are antimicrobial metabolites of bacteria used to produce, or associated with fermented foods (Soomro *et al*.,2002).

In fermentation, the raw materials are converted by microorganisms (bacteria, yeast and molds) to products that have acceptable qualities of food. In common fermented products such as yogurt, lactic acid is produced by the starter culture bacteria to prevent the growth of undesirable microorganisms (Ray and Daeschel, 1992). Food fermentations have a great economic value and it has been accepted that these products contribute in improving human health. Lactic acid bacteria (LAB) have contributed in the increased volume of fermented foods worldwide especially in foods containing probiotics or health promoting bacteria.

2.2 Lactic Acid Bacteria

The Lactic acid bacteria (LAB) comprise a clade of Gram positive, acid tolerant, non-sporulating, non-respiring rod or cocci that are associated by their common metabolic and physiological characteristics. These bacteria are usually found in decomposing plants and Lactic acid products, produce lactic acid as the major metabolic end product of carbohydrate fermentation (Saranranj *et al*.,2013). This trait has historically linked LAB with food fermentation as acidification inhibits the growth of spoilage agents. The LAB group comprises the genera *Lactobacillus, Streptococcus, Lactococus, Leuconostoc, Pediococcus, Aerococcus, Alloicoccus, Dolosigranulum, Enterococcus, Globicatella, Lactospaera, Oenococcus, Carnobacterium, Tetragenococcus, Vagoccus and Weissella*. Historically, the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* form the core of the LAB group (Mahsa *et al*.,2013).

Lactic acid bacteria were first discovered by Scheele (Saranranj *et al*., 2013) from sour milk. Pasteur discovered in 1857, that the souring of milk was caused by the

microorganisms. Lactic acid was first produced commercially by Clinton processing company, USA. Lactic acid bacteria had been widely used for the fermentation of many fermented product such as cheese, sourdough, buttermilk, brined vegetables, yoghurt and sauerkraut (Salim *et al*.,2006).

Lactic acid bacteria are widespread in nature and predominant microflora of milk and its products. Lactic acid bacteria are one of the important groups of microorganisms in food fermentation. A wide variety of strains are routinely used as starter cultures to manufacture dairy products such as curd, cheese, whey and yoghurt (Ayad, 2004). These bacteria produce organic acids, hydrogen peroxide and several enzymes during fermentation (Frederic and De Vuyst, 2004). These compounds not only contribute to desirable effect on food flavor and texture, but also inhibit undesirable microflora and extending shelf life of products (Van *et al*., 2002). Growth of spoilage and pathogenic bacteria in the fermented foods were inhibited due to the production of antimicrobial substances by lactic acid bacteria as their competition for nutrients (Amezquite and Brashars, 2002).

2.2.1 Genus *Lactobacillus*

Lactobacillus: The genus *Lactobacillus* is a diverse group of microorganisms consisting of a number of different species. They are non-spore formers, Gram positive rods ranging from 0.5-1.2 x 1-10 um in size and produce lactic acid as a fermentation end product (Aasen *et al*.,2000).The characteristics of Lactobacillus are rods, usually long and slender, that forms chains in most species. They are microaerophilic, but some are strict anaerobe, catalase-negative and Gram positive, and they ferment sugars to yield lactic acid as the main product. They ferment sugars chiefly to Lactic acid if they are homofermentative, with small amount of acetic acid, carbon-di-oxide and trace products, if they are heterofermentative, they produce appreciable amounts of volatile

products, including alcohol, in addition to lactic acid. Most species of this non spore forming bacterium ferment glucose into lactate hence the name *Lactobacillus.Lactobacillus* is used in industries for the production of fermented food products (Saranraj *et al*.,2013).

2.2.2 *Lactobacillus plantarum*:

Lactobacillus plantarum is an important species in the fermentation of various plant and some animals products and is known to produce antimicrobial substance, example Plantaricin that are active against certain bacteria and pathogens(Cebeci and Gurakan,2003).*Lactobacillus plantarum* has the ability to adapt to many different condition(De Vries *et al*.,2006).*Lactobacillus plantarum* has the coding capacity for the uptake and utilization of many different sugars, uptake of peptides, and formation of most amino acids (Geogleva *et al*.,2009).

2.2.3 *Pediococci*:

The characteristics of cocci occur in single, in pairs or in short chains or in tetrads and are Gram positive, catalase - negative and microaerophilic. They are homofermentative, fermenting sugars to yield 0.5 to 0.9 percent acid, mostly lactic and they grow fairly well in salt brines up to 5.5 percent and poorly in concentrations of salt up to about ten percent. They grow at the temperature 45°C but best at 32°C. Pediococcus have been found growing, during the fermentation of brined vegetables (Piva and Headon,1994).Outside Nisin,it is well known that several strains of *Pediococcus acidilactici* and *P.pentosaceous* ,significant member of lactic acid bacteria were found to produce pediocins (Anastasidou, 2009).Pediocins-like bacteriocins(Class ii LAB bacteriocins)are known as small(<10 kDa),heat stable,non-lanthionine containing membrane active peptides(Xiraphi *et al*.,2008).Pediocin AcH or PA-1 was the first and most thoroughly characterised bacteriocin among the Class 11a bacteriocins,and it is

produced by several *P.acidilactici* strains.One of the most important characteristics of Pediocin –like bacteriocins is their high antimicrobial activity against *Listeria monocytogene* (Altuntas *et al*.,2010).*Listeria monocytogenes* is a Gram –positive bacterium that is capable of growth at low temperatures and is known for its ability to adapt to various environmental conditions such as acidity and salt. Because of this as well as its wide distribution in the environment and food, it is of great concern for the food industry.

More than 20 members of the pediocin-like bacteriocins have been identified to date.A major concern for the food industry is the psychrotrophic pathogenic microorganism,ubiquitous in nature.

2.2.4 *Lactobacillus fermentum*

Lactobacillus fermentum is a gram positive species of the bacterium in the genus *Lactobacillus*.It is commonly found in fermenting animal and plant material(medical dictionary).A few strains are considered probiotic or "friendly"bacteria in animal. Species in this genus are used for a wide variety of application.These applications include food and feed fermentations.It has been found that some strains for *Lactobacillus fermentum* have natural resistance to humans(Klein,2011).One of the ways in which *Lactobacillus fermentum* have been seen as a probiotic is its ability to reduce cholesterol levels. One of the mechanisms by which *L.fermentum* may remove cholesterol through invivo is by the adsorption of cholesterol, which as a result accelerates cholesterol metabolism(Pan *et al*.,2011).

2.2.5 *Lactobacillus paracasei*:

Lactobacillus paracasei is a gram positive nonspore forming homofermentative rod that is a common inhabitant of the intestinal tract (Mitsuoka,1996).*L.paracasei* strains are also found naturally in fermented vegetables, cereal, milk and meat. Strains of this

species are used in many food products, including traditionally fermented milks and cheese. Selected strains of this species are also used in probiotic foods and dietary supplements. *L.paracasei* is listed in the inventory of microorganisms with documented history of use in human food (Mogensen,2002).

2.2.6 *Lactococcus lactis*

Lactococcus lactis was the first bacterium that was isolated in pure culture in 1873 by Joseph Lister. *Lactococcus lactis* is a homofermentative bacterium. Its primary function is rapid lactic acid production from lactose.The characteristics of *Lactococcus* include carbon metabolism, extracellular and intracellular proteolytic system.This species have two subspecies and a biovar:*Lactococcus lactis* subsp *lactis*;*Lactococcus lactis* subsp cremoris; *Lactococcus lactis* subsp.*lactis* biovar diacetylactis (Sungathi *et al*.,2014). *Lactococcus lactis* is a safe microorganism that has been consumed over thousands of years.

2.3Application of lactic acid bacterial bacteriocins in food preservation:

Bacteriocins have several characteristics that make them ideal food preservatives. Many bacteriocins are capable of resisting inactivation at the relatively high temperatures used in food processing and can remain functional over a broad pH range. Bacteriocins are usually inactivated by one or more of the proteolytic enzymes present in the digestive tract of humans and would be digested just like any other protein in the diet. Bacteriocins are nontoxic, odourless, colourless and tasteless.The efficacy of using bacteriocins as food preservatives will need to be determined for each food system.Solubility, stability, sensory impact, heat and pH tolerance, and types and number of organisms inhibited will need to be evaluated for each bacteriocin in each food category under a variety of storage conditions.

2.3.1. Application of bacteriocins in the preservation of diary products:

Several researchers have demonstrated the effectiveness of Nisin and /or Nisin-producing strains against pathogenic bacteria such as *Clostrdium butulinum* in cheese and against *L.monocytogenes* in cheeses such as Camembert,Manchego and Ricotta(Nunez *et al*.,1997).Other bacteriocins have been tested in milk and Dairy products,such as Pediocin AcH in milk and cheddar and Munster cheeses against *L.monocytogenes, S.aureus,* and *E.coli* O157:H7(Rodriguez *et al*.,2005),Lacticin 3147 against undesirable LAB ,*L.monocytogenes* and *B.cereus* in Cheddar,Cottage Cheese and Yoghurt(Morgan *et al*.,2001), ingredients used in the manufacture of these products are raw cheese, butter, skimmed milk powder, often various added flavours, phosphate or citrate emulsifying salt and added water. Spores of anaerobic clostridial species are often present in some of these ingredients, particularly the cheese. The composition of the processed cheese in terms of the relatively high pH and moisture content combined with low redox potential (anaerobic conditions) can favour the outgrowth of spores which may cause subsequent spoilage due to the production of gas, off-odours and liquefaction of the cheese. The potential for the growth of *C.botulinum* in processed cheese products particularly spreads, is of considerable significance.

2.3.2 Application of bacteriocins in the preservation of meat products:

Concern on high level of nitrite in cured meat has led various workers to consider alternative preservation systems, which include a reduction in nitrite levels and these have included nisin (Rayan *et al*.,1998).The principal concern with the use of nitrite for curing of meat is the eventual formation of carcinogenic N-nitrosamines. Recently attempts have been made to use nisin A as an alternative to nitrite. Nisin A is not apparently the bacteriocin of choice for meat preservation in contrast to its effectiveness in dairy products. Bacteriocins produced by LAB associated with meat and meat

fermentations such as *Pediococcus,Leuconostoc,Carnobacterium* and *Lactobacillus* spp are likely to have much potential as meat preservatives(Shahidi,1991).*Listeria monocytogenes* is a food-borne pathogen which is ubiquitous in the environment and can be isolated from foods of different origin, including meat and meat products.Nisin and butylated hydroxyl anosole (BHA)were added to study the shelf life of meat sausage(Shureshkumar *et al*.,2010).Recently, some biopreservation techniques have been applied to meat products and these involved the introduction of a competitive microflora of LAB as protective culture for chill-stored ready- to- eat meat products, including bacteriocin producing LAB, and the use of purified anti-listerial bacteriocins added directly as natural preservatives. Yousef *et al*.(1991)investigated the growth of *L.monocytogenes* in packed wienwe sausage,a fully cooked cured meat product which is susceptible to contamination by *L.monocytogene* before packaging.These researchers provided evidence that *Pediococcus* inoculants or purified pediocin can function as biopreservatives to eliminate gram-positive pathogenic bacteria in cooked meats during extended refrigerated storage.

2.3.3 Application of bacteriocins in the preservation of fish:

The application of nisin in the preservation of fish products has been studied by Taylor *et al*.(1990) who showed that nisin treatment of cod, herring, and smoked mackerel fillets inoculated with *Clostridium botulinum* spores brought about a delay in toxin production of 5 days at 10^0C ,but only a half a day at 26^0C.

2.3.4 Application of bacteriocins in preservation of vegetables:

vegetables that are packaged and ready –to-use as a convenience product generally have a refrigerated storage life of one week and support the growth of a microbial population that is contaminated by *Pseudomonas* and *Enterobacteriaceae* (Huxoll and Bolin 1989).The possibility of preserving ready -to –use vegetables with bacteriocin

producing LAB has been investigated(Vescova *et al*.,1995).Under the conditions of this study it was shown that inoculation of the salads with the strains of *Lactobacillus casei* or *Pediococcus pentosaceous* resulted in the domination of the vegetables with these bacteria and a dramatic decrease in *Enterobacteriaceae* that dominated the uninoculated control samples. During the 8-day storage period at 8^0C the inoculated LAB grew at the pH of the salads decreased from about 5.2 to 4.8. The study indicated that the inoculation of ready to use vegetables with Lactic acid bacteria is effective.

2.3.5 Application of bacteriocins in preservation of Alcohol Beverages:

Research in Europe has demonstrated the potential of nisin in controlling spoilage bylactic acid bacteria in beer(Ogden,1985) and wine(Radler,1990).Applications identified in the brewing industry by adding to fermenters for controlling and preventing contamination, reducing pasteurization process and increasing the shelf life of unpasteurised or bottle conditioned beers. Similar applicationalso occur in wine industry. Nisin is also used in distilled alcohol production, both for beverages and industrial production.When added to fermentation mashes that is naturally contaminated with Lactic acid bacteria, the latter's activity can be controlled and cause increased alcohol yield by allowing the yeast less competitive for substrate(Henning *et al*.,1986).

2.4 Classification of Lactic acid bacteria bacteriocins

There is a wide number of bacteriocins produced by different LAB, and they can be classified according to their biochemical and genetic characteristics(Rajaram *et al*.,2010).

2.4.1 Class 1: thelantibiotics

This is a class of peptide substances that are small(<5 kDa) heat –stable peptides acting on membrane structures.The lantibiotics bacteriocinswere initially divided into two subclasses based on structural similarities.Subclass Ia included relatively elongated,

flexible and positively charged peptides; they generally act by forming pores in the cytoplasmic membranes of sensitive target species. Subclass Ib peptides are characteristically globular, more rigid in structure and are either negatively charged or have no net charge.They exert their action by interfering with essential enzymatic reactions of sensitive bacteria.The best example in this group is Nisin produced by *Lactococcus lactis.*subsp,*Lactis.* All forms of Nisin are antimicrobially active against Gram positive bacteria, such as *Listeria* sp,*Micrococcus* sp and sporeforming bacteria like *Bacillus* sp and *Clostridium* sp.

2.4.2 Class II: Non-Lantibiotics

Class II bacteriocins are also small (<10 kDa) relatively heat stable, non-lanthionine containing membrane active peptides.This group was divided into three subgroups:

Class IIa : peptides active against Listeria, examples include Pediocin PA-1 and Sakacin P.

ClassIIb: they are formed by a complex of two distinct peptides. In this group are Lactoccocin G and Plantaricins.

Class IIc: small peptides,heat stable.In this subclass are found only the bacteriocins divergicins and acidocin B.

2.4.3 Class III: Big peptides

This group consists of heat labile proteins which are in general of large molecular weight(>30kDa).In this class are Helveticins J and V,Acidophilin A and Lacticins A and B.

2.5. Improvement of Food Safety using Bacteriocins

Bacteriocins have often been mooted as potential valuable biological tools to improve the food safety and reduce the prevalence of foodborne illnesses. There already exist many control measures within the food industry to prevent or minimise bacterial

contamination, including good manufacturing practices effective sanitation and hygiene measures with respect to raw materials, the food plant, the food products, the food processing personnel (Moberg, 1989) and other basic fundamentals of an effective Hazard Analysis Critical Control Point (HACCP) programme. These measures facilitate the identification, evaluation and control of food safety hazards (National Advisory Committee on Microbiological Criteria for Foods (NACMCF, 1998). However, despite these precautions, foodborne outbreaks do occur alarmingly frequently. *L. monocytogenes* is of particular concern to the food industry and susceptible consumers that include pregnant women, infants, immunocompromised individuals and the elderly. *L. monocytogenes* is ubiquitous in the environment and is extremely resilient, surviving refrigeration temperatures and high salt concentrations. Statistics for Wales and England showed 194 hospital cases of *Listeriosis* in 2000, of which 68 resulted in death (Adak *et al*., 2002).Within the United States, the microorganism is estimated to be responsible for 2500 cases, resulting in 500 deaths annually. A policy of zero tolerance in ready to eat foods has been implemented in the United States for *L. monocytogenes*, but outbreaks continue to occur. A recent outbreak associated with contamination of hot dogs resulted in 101 cases and 21 deaths and the recall of 500, 000 lbs. of contaminated hot dogs and meats (Donnelly, 2001). This highlights the considerable burden this food pathogen can place on consumers and the food industry. It would seem particularly important to provide an additional hurdle in food to prevent such outbreaks, and bacteriocins would be an economically feasible option. *L. monocytogenes* is not the only concern, there exists a substantial list of food pathogens that result in foodborne illness every year, including many Gram-negative pathogens such as *Escherichia coli* VTEC 0157, *Campylobacter and Salmonella* among others (Adak *et al*., 2002). Thus, bacteriocins may be best applied when providing an extra obstacle to prevent the growth of

pathogenic and spoilage bacteria, especially in situations where contamination could occur post-production.There are at least three ways in which bacteriocins can be incorporated into a food to improve its safety i.e., using a purified/semi-purified bacteriocin preparation as an ingredient in food, by incorporating an ingredient previously fermented with a bacteriocin-producing strain, or by using a bacteriocin-producing culture to replace all or part of a starter culture in fermented foods to produce the bacteriocin in situ. The use of purified bacteriocins is not always attractive to the food industry, as in this form they may have to be labelled as additives and require regulatory approval. Nisin is utilised as an additive and was assigned the number E234 (EEC, 1983 EEC commission directive 83/463/EEC). The two other alternatives (fermented ingredient/starter culture) do not require regulatory approval or preservative label declarations. These options are frequently regarded as more attractive routes through which bacteriocins can be incorporated into a food.

2.6 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 ,T.J and Bruno,M.E.C.(1994). The PMF, which is composed of a chemical component (the pH gradient; vpH) 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(McAuliffe *et al*.,2001). Collapse of the PMF, induced by bacteriocin action, leads to cell death through cessation of energy-requiring reactions.

2.7 Lactic acid bacteria used as probiotics:

The concept of probiotics was in use in the early 1900s,however the term was coined in 1965 by Lilly and Stillwell. Probiotic is a preparation of live organisms which when applied to man or animal,beneficiary affects the host by improving the properties of the indigenous microbiota (Havenaar and Huis Veld,1992). Probiotics are viable organisms and supportive substances that improve intestinal microbial balance,such as *Lactobacillus acidophilus* and bioactive proteins . Antimicrobial compounds produced by LAB have provided these organisms with a competitive advantage over other microorganisms.Probiotics act through suppression of viable count by production of antibacterial compounds,competition for nutrients and adhesion sites,alteration of microbial metabolites and stimulation of immunity (Mishra and Lambert 1996). *Lactobacillus* spp and *Bifidobacterium*spp are prominent members of the commensal intestine flora and are the commonly studied probiotic bacteria.They cause reduced lactose intolerance, alleviation of some diarrhoeas, lowered bood cholesterol, increased immune responses and prevention of cancer (Salminen *et al*.,1998).

The selection criteria for probiotic LAB include: human origin, safety, viability/activity in delivery vehicles,resistance to acid and bile,adherence to gut epithelial tissue, ability to colonise the G.I.T, production of antimicrobial substance,ability to stimulate a host immune response and the ability to influence metabolic activities such as vitamin production,cholesterol assimilation and lactose activity (Salminen *et al*.,1996). Microorganisms used as probiotics; *Lactobacillus acidophilus , L. plantarum,L. casei, L. fermentum, Bifidobacterium bifidum Enterococcus faecalis, Lactococcus lactis*

subspecie lactis.B infantis, B.adolescents, B.longum, S.thermophilus, L.casei (Conway, 1996).

2.8 Exopolysaccharides (EPSs) from Lactic Acid Bacteria (LAB)

Exopolysaccharides have found their most valuable application in the improvement of the rheology, texture and 'mouthfeel' of fermented milk products, such as yoghurt. There is a high consumer demand for smooth and creamy yoghurt products, which is typically met by increasing the content of fat, sugars, proteins or stabilizers (e.g. pectin, starch, alginate or gelatin). Consumer demand for products with low fat or sugar content and low levels of additives, as well as cost factors, make Exopolysaccharides a viable alternative (Jolly *et al*., 2002). Although having no taste of their own, Exopolysaccharides from Lactic acid bacteria increase the time the milk product spends in the mouth, and hence impart an enhanced perception of taste (Duboc and Mollet 2001). An additional hypothesized physiological benefit is that Exopolysaccharides will remain for longer in the gastrointestinal tract, thus enhancing colonization by probiotic bacteria (German,1999). In addition, Lactic acid bacteria Exopolysaccharides have been claimed to have antitumor effects (Kitazawa, 1998) immunostimulatory activity (Chabot *et al*., 2001) and to lower blood cholesterol (Nakajima,1992). Exopolysaccharides are long-chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives. These sugar units are mainly glucose, galactose and rhamnose, in different ratios (De Vuyst ,2001). They are secreted into their surroundings during growth and are not attached permanently to the surface of the microbial cell (Laws, 2001). This distinguishes them from the structurally similar capsular polysaccharides (CPSs), which do remain permanently attached to the surface of the cell. Exopolysaccharides from microbial sources can be classified into two groups – homopolysaccharides (e.g. cellulose, dextran,mutan, alternan, pullulan, levan and curdlan) and

heteropolysaccharides e.g. gellan and xanthan ((Laws, 2001). Homopolysaccharides consist of repeating units of only one type of monosaccharide (D-glucose or D-fructose) and can be divided into two major groups: glucans and fructans. By contrast, heteropolysaccharides from LAB have repeating units that demonstrate littlemstructural similarity to one another ((De Vuyst, 2001).. The heteropolysaccharides are constructed from multiple copies of oligosaccharides (Laws, 2001) which contain between three and eight residues two or more different monosaccharides are usually present in each repeating unit and show different linkage patterns.

2.9.1 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

iii. regarded as safe”(GRAS) in certain applications

iv. Realization that bacteriocinogenicity is not a rare occurrence within the lactic acid bacteria

v. Consumer awareness and resistance to traditional chemical preservatives

vi. Justifiable concerns over the safety of existing food preservatives such as sulphites and nitrites.

vii. Possibility of use of bacteriocins production and immunity as selectable genetic markers in starter culture bacteria.

viii. 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.

ix. Willingness of federal funding agencies,food commodity groups,and food processing corporations to find both basic and applied researches

2.9.2 Effectiveness of bacteriocins in food systems

The application of bacteriocins particularly nisin,in food systems has been extensively reviewed(Ross *et al*.,2002).It is now known that the production and activity of bacteriocins in foods can be influenced by many factors:

factors negatively affecting production (Cleveland *et al*.,2001) include:inadequate physical conditions and chemical composition of food (pH ,temperature, nutrients,e.t.c.) spontaneous loss in production capacity;inactivation by phage or producing strain;and antagonistism effect of other microorganisms in food. Nisin for example, is 228 times more soluble at pH 2 than at pH 8.

The effectiveness of bacterioin activity in food is negatively affected by:resistance development of pathogens to the bacteriocins, inadequate environmental condition for the biological activity;higher retension of bacteriocin molecules by food system components (e.g fat); inactivation by other additives;slower diffusion and solubility and/irregular distribution of bacteriocin molecules in the meat matrix

2.9.3 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 loses 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.

2.9.4 Compound and Antimicrobial Spectrum Produced by Lactic Acid Bacteria

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).

2.9.4.1Hydrogen 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 lactoperoxidase,oxidase endogenous thiocyanate.The oxidized intermediary products are toxic to different bacteria(Leroy et al., 2006).

2.9.4.2 Diacetyl, Acetaldehyde and Acetoin

Heterofermentative LAB produce active acetaldehyde by decarboxylation of pyruvate.This product then condences 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 diary 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 diary 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.

2.9.4.3 Carbondioxide

The influence of carbondioxide on product preservation is two fold, except for its own antimicrobial activity,it creates an anaerobic environment by replacing the existent molecular oxygen. The antifungal activity of CO2 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).

2.9.4.6 ***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).

2.9.4.5 Other Low Molecular Mass Antimicrobials

Other low molecular mass compounds with antimicrobials activity against Gram positive and Gram negative bacteria, moulds and yeast have been described,including antifungal cyclic dipeptides,phenyllactivc acid,4-hydroxyphenyllactic acid and 3-hydroxy fatty acid(Valeriop *et al*.,2004).

2.9.4.6 Traditional Production of .Kunun zaki (cereal based beverage):

Kunun zaki is a cereal based beverage in Nigeria.The cereal utilized in its production are millet,sorghum and maize in decreasing order of preference(Gaffa *et al*.,2002a).The traditional production process involves steeping the grains in local household utensils such as buckets, drum, calabashes or earthen vessels. The steeping duration depends on the cereal used but vary between twelve and seven two hours for millet, sorghum and maize .(Gaffa,2000).Grinding of the steeped grains mixed with spices(ginger cloves, red or black pepper)is done with local milling machines and part of the slurry(3/4 volume) is gelatinized with boiling hot water (Gaffa *et al*., 2002b).The remaining part of the slurry(1/4 volume ungelatinised)containing liquefying agents(sweet potato tuber paste, malted rice, extract of cadaba farinos stem)is mixed with the gelatinized portion when the temperature is about 60-70^{o}C.The mixture is altogether left overnight at room temperature for chance fermentation and filtered using local sieve the next morning.The filterate *Kunun Zaki* is consumed as a beverage with or without sugar as a sweetener. The nutrient content and microbiological quality of this product has been reported (Gaffa *et al*.,2002b).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Study Area:

The study area was Zaria metropolis, this included Zaria and Sabon Gari Local Government Areas of Kaduna State, Nigeria.

3.2 Sample Collection

Traditionally prepared samples of fermented *kunun-zaki*were obtained from different hawkers on the streets and markets in Zaria metropolis, North-west Nigeria. The samples were packaged in 50 ml sterile ice packed bottles and immediately transferred to the laboratory in the Department of Microbiology, ABU,Zaria for analysis.

3.3 pH Determination

The pH of the *kunun zaki*samples weredetermined by dispensing 10ml of the sample homogenised into a conical flask and pH was taken using a pH meter model number, Crison MicropH 2000.The pH meter was first calibrated using buffer 7 and then pH of the samples were measured.

3.4 Isolation of Lactic Acid Bacteria

Twenty five millileters (25ml)of *kunun zaki* was mixed with two hundred and twenty five millileters (225ml) of peptone water to obtain a stock homogenate. One millileter from the stock homogenate was transferred into sterile test tubes containing 9ml of sterile peptone water, it was serially diluted to 10^{-6}, Iml from the diluted sample was pour plated on Mann Rogosa and Sharp agar (MRS)plates in triplicate then inverted and

incubated anaerobically at 37^0C for 72 hours and the developed colonies counted using a colony counter (Omemu *et al*.,2011).

3.5 Characterisation of Lactic Acid Bacteria

The Lactic acid Bacteria(LAB),were characterized based on Colonial morphology, cell morphology(Mohammed *et al*.,2013) and biochemical tests(Oyeleke and Manga ,2008).

3.6Biochemical Tests

3.7.1 Microscopic Examination of the Isolates

Discrete colonies of Lactic acid bacteria which were streaked on the Mann Rogosa and Sharpe agar slant were picked after incubation for 24hours and smeared on a slide. The dried smear was fixed and covered with crystal violet stain for 60 seconds. Afterwhich it was rapidly washed off with clean water.All the water were tipped off and the smear was covered with Lugol's iodine for 60 seconds. The Lugol's iodine was then washed off with clean water and decolourised rapidly(few seconds) with acetone alcohol which was washed immediately with clean water and then the smear was covered with safranin stain for 30 seconds which was also washed with clean water after which the back of the slide was wiped clean and placed in a draining rack for the smear to air-dry and Finally, the smear was examined microscopically with the oil immersion objective. A dark purple colour indicated Gram positive bacteria.

3.7.2 Catalase Test

The test was carried out by pouring 2ml of Hydrogen peroxide solution into a test tube and with the aid of a sterile Wire loop several colonies of the Lactic acid bacteria isolates were removed and immersed in the Hydrogen peroxide solution. Active bubbling was an indicative of a positive reaction.

3.7.3 Endospore Test

A smear of the Lactic acid bacteria isolate was prepared on a grease-free slide and fixed, the slide was placed over boiling water(in a beaker) resting it on the Rim with the bacterial film uppermost, after 40seconds, large droplets of water condensed underneath the slide. Then the smear was flooded with 5% aqueous solution of malachite green and allowed to stand for 2minutes while the water continues to boil. The slide was removed from the boiling water and washed with running water and then air-dried. Using an oil immersion lens, it was observed under the microscope. Endospores stained green while vegetative cells stained red.

3.7.4 Motility Test

Motility slants were prepared, using an inoculating needle, a growth of the Lactic acid bacteria isolates was picked and stabbed on the agar slant at right angles to the bottom of the test tube. It was incubated at 37^0C for 24 hours and pattern of growth of the organism was observed. Motile organisms grew away from the line of stab while non-motile organisms grew along the line of stab.

3.7.5 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**.**

3.8 Identification of Lactic Acid Bacteria using Analytical Profile Index (API) 50 CH Kit (Biomeruex)

Lactic acid bacteria were subcultured on a Mann Rogosa and sharpe agar plates. Portions of growth of each Lactic acid bacteria isolates were aseptically transferred from a freshly inoculated stock culture using a swab to an ampule of Analytical Profile Index (API 50 CH KIT) basal medium and emulsified to give a final turbidity equivalent to McFarland standard 0.5 (10^8cells). Each tube of the API 50 CH strip was inoculated with the bacterial suspension using a sterile pippete. The tray with the strips in it was covered loosely with a lid,and incubated at 37^0C for 72hours.Reactions were visually examined after 24 and 48 hours which was determined to be positive or negative based on colour change in the tube. The result which form the biochemical profiles were identified using an apiweb software version. These profiles were then compared to those listed in the API 50 CHL analytical profile index.

3.9 Screening for Lactic Acid Bacteria with Potential Activity

Collection of pathogenic bacteria:

Pathogenic bacteria (*Staphylococcus aureus, Clostridium botulinum ,Escherichia coli O157:H7, Pseudomonas aeruginosa, Listeria monocytogenes and Salmonella* Typhimurium were collected at the Teaching Laboratory, Department of Microbiology , ABU, Zaria. They were identified and subcultured on their respective selective media. The cell free supernatant from the samples were introduced into the wells bored with cork borer. The plates were allowed to set for 1hr to allow the antibacterial to diffuse. The plates were incubated at 37^0C for 48hours after which the plates were examined for clear zones of inhibition (Babatunde *et al*.,2014).

3.10 Extraction and Partial Purifiction of Bacteriocins:

3.10.1 Extraction of Bacteriocins

A cell free solution was obtained by centrifugation at 10000g for 20minutes at 4°C. The culture was adjusted to pH 7.0 by the addition of sodium hydroxide to get the required pH. The cell free solution obtained was the crude extract.

3.10.2 Partial Purification of Bacteriocins

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 4^0C. 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 4^0C for further use(Aly *et al*.,2003).

3.11 Effect of pH on Bacteriocin Activity

The activity of the bacteriocin was quantified as described by Mohammed*et al*., (2013) while the production studies was determined (Okpara *et al*.,2014). Cell free supernatants of the Lactic acid bacteria isolates were diluted using normal physiological saline, 5ml from each dilution was heated at different pH values (2.0- 7.0). The pH of samples were adjusted to pH 2, 3, 4, 5 and 6 with 0.1M hydrochloric acid (HCl) and pH 8, 9 and 10 with 1M Sodium hydroxide (NaOH) and subsequently incubated at ambient temperature for 1hour, the activity of the sample against test organisms was monitored by agar well diffusion method by streaking the pathogenic organisms with Mueller Hinton agar, 2ml of the cell free supernatants were poured into the holes bored with cork borer. It was allowed to set for the antibacterial diffuse and then incubated at 37^0C for 48hrs after which the plates were examined for clear zones of inhibition. The activity of the sample at pH 7.0 was used as control.The antibacterial activity of the

bacteriocin produced was defined as the reciprocal of the highest dilution showing inhibition of microorganisms multiplied by 100 and expressed as activity units per ml (Au/mL).

3.12 Effect of Temperature on Bacteriocin Activity

The activity of the bacteriocin was quantified (Mohammed *et al*.,2013) while the production studies was also determined (Okpara *et al*.,2014). Cell free supernatants of the isolates were diluted using normal physiological saline, 5ml from each dilutionwere poured into a sterile test tubes were heated at different temperatures ranging from 10^0C - 60^0C in a hot air oven for 30minutes,following heat treatment and then cooled to ambient temperatures. Afterwards, the activity of the heat treated samples on pathogenic organisms were monitored by agar well diffusion method by streaking the pathogenic organisms on Mueller Hinton agar, and 2ml of the the cell free supernatants were poured into the holes bored with a cork borer. It was allowed to set for the antibacterial diffuse and subsequently incubated at 37^0C for 48hrs after which the plates were examined for clear zones of inhibition.The antibacterial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition of microorganisms multiplied by 100 and expressed as activity units per ml (Au/mL).

3.13 Biopreservation Study of Milk (without bacteriocin) and Milk (with bacteriocin)

3.13.1 pH Determination

The pH of the milk samples weredetermined by dispensing 10mls of the milk sample into a conical flask and pH was taken using a pH meter model number Crison MicropH, 2000.The pH meter was first calibrated using buffer 7.0 and the pH of the samples were measured.

3.13.2 Enumeration of Lactic Acid Bacteria from Milk

Twenty five milliliters (25ml) of the milk sample was mixed with two hundred and twenty five milliliters (225ml) of peptone water in a sterile beaker to obtain a stock homogenate. One milliliter from the stock homogenate was transferred into sterile test tubes containing 9ml of peptone water, it was serially diluted to 10^{-6}, Iml from the diluted 10^{-6} sample was pour plated and incubated anaerobically at 37^0C for 48 hours and the developed colonies were counted using a colony counter and expressed as:

Cfu/ml =Number of colonies x dilution factor /inoculum volume (Omemu *etal.*, 2011).

3.13.3 Screening for Lactic Acid Bacteria from Milk Sample with Potential Bacteriocin Activity

The pathogenic bacteria (*Staphylococcus aureus, Clostridium botulinum,Escherichia coli O157:H7,Pseudomonas aeruginosa , Listeria monocytogenes and Salmonella* Typhimurium) were collected at the Teaching Laboratory, Department of Microbiology ,ABU, Zaria. They were identified and were subcultured on their respective selective media.These pathogenic bacteria were streaked on Mueller Hinton agar poured in petridishes and were left to set, the Lactic acid bacteria isolates which were subcultured on Mann Rogosa and Sharp agar plates, were grown on Mann Rogosa and Sharp broth in sterile centrifuge tubes were incubated at 37^0C for 48hours. A cell free solution was obtained by centrifugation (10000g for 20minutes at 4°C). The cell free supernatant

(2ml) from the milk samples were introduced into the wells bored with cork borer and were allowed to set for antibacterial to diffuse, then the plates were incubated at 37^0C for 48hours and examined for clear zones of inhibition ((Babatunde *et al*.,2014).

3.13.4 Biopreservative Potential of Bacteriocins from Lactic Acid Bacteria on Milk Sample

Shelf life extension of milk was determined based on Total viable count and subjective appearance of the milk sample before and after every twenty four hours of inoculation of bacteriocins from Lactic acid bacteria on milk. The milk samples inoculated with bacteriocin were then stored at two refrigeration temperature of 4^0C and 8^0C and at ambient temperature and milk sample without bacteriocin served as a control (Mohammed *et al*.,2013a).

3.13.5 Total Viable Count (TVC) of Milk Sample

Milk sample (25ml) was mixed with 225ml of peptone water in a sterile beaker to obtain a stock homogenate. One milliliter of the sample from the stock culture was aseptically transferred into test tubes containing 9ml of peptone water, it was serially diluted to 10^6, 1ml from the dilution was pour plated on Plate Count Agar (PCA) on sterile petridishes in triplicate and inverted then incubated aerobically at 37^0C for 24 hours while for anaerobic counts, plates were incubated anaerobically at $37^0$for 48hours using a candle jar. Colonies that developed were counted using a colony counter and expressed as Cfu/ml ==Number of colonies x dilution factor /inoculum volume

3.13.6 Potential of BLAB to extend the shelf life of milk sample

Five millileter (5ml) of bacteriocin was added to ninety five millileter (95ml) of the milk sample and were refrigerated at two different temperatures (4, and 8^0C) and at ambient temperature (25^0C) in sterile conical flasks covered with cotton wool and alluminium foil for 13days. Samples were removed at twenty four hours interval and checked for total viable count by pourplating on plate count agar, colour and aroma of the milk samples were also considered to determine the shelf-lifeof the milk that is control (milk without Bacteriocin) and bacteriocin inoculated milk ((Mohammed *et al*.,2013).

3.14 Physico-chemical,Proximate Composition and Sensory Properties of Milk Inoculated with Bacteriocin

Physico-chemical Analysis of Milk was carried out according to the method described by AOAC (2005).

3.14.1 pH Determination

The pH of the milk sample was determined by dispensing 10ml of the milk sample into a conical flask and pH was taken using a pH metermodel number Crison MicropH, 2000.The pH meter was first calibrated using buffer 7.0 and then pH of the samples were measured..

3.14.2 Determination of Total Titratable Acidity(TTA)

The total titratable acidity of milk was measured by adding 1ml of phenolphthalein indicator to 200ml of boiled distilled water and neutralized to pink by titrating with standard 0.1M Sodium hydroxide. Five millileter (5ml) of the milk sample was transferred into the solution separately using different sterile volumetric pipette. This was titrated to the same end point with standardized 0.1M NaoH. Total acidity was calculated as:

% Lactic acid = Titre value x normality of alkali x 9 / volume of sample.

Proximate analysis

Moisture, Ash, Protein,Fat, and Carbohydrate content

3.14.3 Determination of Moisture Content

Metallic dishes were dried in an oven at 80^0C for 20 minutes, and allowed to cool in a desiccator and weighed. Five millileter of milk was placed in the dishes and weighed. The dishes with the samples were then dried in an oven at 80^0C 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 as :

W2- W3 X 100 / W2- W1

3.14.4

Determination of Ash Content

Crucibles were cleansed and dried in the oven, after drying they were cooled in the dessicator and weighed (W1). Two millileter of the milk sample was placed in the crucibles and weighed (W2).They were transferred into the muffle furnace at 550^0C for 2 hours then removed and cooled in the dessicator and weighed (W3).

The % ash was calculated as :W3- W1 X 100 / W2 – W1

3.1.4.5 Determination of Protein Content

This comprises of three stages as follows:

Digestion:

Two millileter of the milk sample was weighed into a Kjeldahl flask afterwhich Copper catalyst and 15ml 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, afterwhich it was transferred with several washings into 100ml with distilled water.

Distillation:

Ten millileter of boric indicator was placed under the condenser afterwhich ten millileter 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 millileter of 40% NaOH solution afterwhich it was steam through for five minutes to collect ammonium sulphate (40ml).

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:Final reading - initial reading – blank (0.2) x standard number of nitrogen (1.4) / initial weight x standard number of protein (6.25).

3.14.6 Determination of Fat Contents

An empty filter paper was weighed and labeled W1, afterwhich 2ml of milk 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 afterwhich it was removed and transferred to an oven and dried and was weighed as W3

The % Fat was calculated as: W2 –W3 X 100 / W2 – W1

3.14.7 Determination ofTotal Carbohydrate Content

The percentage of Carbohydrate content of milk was determined by subtracting from 100,the sum of the percentage moisture,ash,protein and fat.The remainder value gives the carbohydrate content of the sample.

The % CHO was calculated as :100 – (% moisture + % ash + % protein + % fat)

3.14.8 Sensory Property of Milk

The method ofRanganna (2008) was employed. Sensory quality attributes such as colour, aroma, texture, taste and general acceptability of the bacteriocin incorporated milk was evaluated using a five-point Hedonic scale. The milk 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 analysed using ANOVA Analysis of variance (ANOVA).

3.15 Data Analysis

Results were presented in tables and charts.Analysis of variance(ANOVA), and Duncan's Multiple range test were used to analyse the results**.**

CHAPTER FOUR

4.0 RESULTS

Table 4.1 is the pH of the *Kunun Zaki* samples in various location of Zaria metropolis,Sabongari had the highest mean pH (5.80± 0.089), while Zaria cityhad the least mean pH(5.68±0.09).

The fermented *kunun Zaki* analyzed had the highest mean counts from Sabongari($1.83x10^6 \pm 2.31x10^4$ cfu/ml) with Tudun wada($1.14x10^6 \pm 2.4x10^4$cfu/m) showing the least mean counts(Table 4.2).

The value and percent of occurrence of Lactic acid bacteria in *kunun zaki* samples is as presented in Table 4.3. *Lactobacillus plantarum* had the highest number of occurrence(6) 40%,while *Lactobacillus lactis* (1) 7% and *Pediococcus pentosaceous*(1) 7% had the lowest number of occurrence.*Lactococcus lactis* inhibited all the pathogenic bacteria tested with different clear zones of inhibition with *Staphylococcus aureus* (8.10 ± 0.07mm), showing the largest zone of inhibition while *Escherichia coli* (1.10 ± 0.34mm) showed the smallest zone of inhibition. *Lactobacillus plantarum* on the other hand inhibited the growth of *Listeria monocytogenes* with9.25 ± 0.40mm clear zone of inhibition and no zone of inhibition for *Salmonella* Typhimuriumand *Escherichia.coli*. *Lactobacillus acidophilus* inhibited the growth of *Staphylococcus aureus* showing 3.10 ± 0.03mm zone of inhibition,and no zone of inhibition was detected for *Escherichia.coli* and *Salmonella* Typhimirium.*Pediococcus pentosaceous* alsoinhibited the growth of *Staphylococcus aureus* showing highest zone of inhibition on *Listeria monocytogenes* (10.26 ± 1.10mm)and No zone of inhibition was detected for *Escherichia.coli* (Table 4.4).

Table 4.1 Mean pH of *Kunun Zaki* Samples in Zaria Metropolis

Location	pH
Sabongari	5.80 ± 0.08
Samaru	5.68 ±0.98
Tudunwada	5.70±0.89
Zaria city	5.68±0.09

SEM= Standard error of mean Values are Mean ± SEM

Table 4.2: Lactic Acid Bacteria Counts(cfu/ml) of *Kunun Zaki* samples from Zaria Metropolis

Location	Lactic acid bacteria Count (Cfu/ml)
Sabon Gari	$1.83x10^6 \pm 2.31x10^4$
Samaru	$1.16x10^6 \pm 2.08x10^4$
Tudun wada	$1.14x10^6 \pm 2.4x10^4$
Zaria city	$1.16x10^6 \pm 1.1x10^4$

Values are mean ± SEM **CFU – Colony Forming Unit**

Table 4.3 A Value and Percentage of Lactic Acid Bacteria in *Kunun Zaki*from Zaria Metropolis

Organisms	Frequency	Percent
L lactis	1	7
L. plantarum	6	40
L. acidophilus	3	20
L. fermentum	2	13
L. paracasei	2	13
P. pentosaceous	1	7
Total	15	100

Table 4.4: Zones of inhibition(mm)of LAB on foodborne pathogens

Organism	*L.lactis*	*L.plantarum*	*L.acidophilus*	*P.pentosaceous*
S.aureus	8.10 ± 0.07	5.24 ± 0.067	3.10 ± 0.03	5.24 ± 0.04
E. coli	1.20 ± 0.34	0	0	0
L.monocytogenes	3.53 ± 0.65	9.25 ± 0.40	3.10 ± 0.03	10.26 ± 1.10
P. aeruginosa	2.11 ± 0.42	0.83 ± 0.94	0.64 ± 0.07	1.04 ± 0.03
C.botulinum	7.43 ± 0.53	3.36 ± 0.56	2.34 ± 0.08	3.46 ± 0.56
*S.*Typhimurium	1.36 ± 0.62	0	0	1.28 ± 0.92

SEM= Standard error of mean Values are mean ± SEM

Figure 4.1 is the Effect of pH on bacteriocin activity on *Staphylococcus aureus,*,at pH5 optimum activity was noted,*Lactobacillus plantarum* showed the highest activity (5200Au/ml) while *Lactobacillus acidophilus*(2300Au/ml) showed the least activity.

Effect of pH on bacteriocin activity on *Escherichia coli*showed that only*Lactococcus lactis* showed activity on *E.coli*, optimum activity was noted at pH 5(950Au/ml),least activity was noted at pH 7 (200Au/ml).(Figure 4.2).

Effect of pH on bacteriocin activity on *Listeria monocytogenes* showed that optimumpH was also noted at pH5 (5300Au/ml)by *Pediococcus pentosaceous* while*Lactobacillus acidophilus*atpH 5(2500Au/ml) showed the least activity.(Figure 4.3).

Effect of pH on *Pseudomonas aeruginosa*on bacteriocin activity showed that optimum pH was noted at pH 5 (1300Au/ml) by *Lactococcus lactis* while *Lactobacillus acidophilus*atpH 5(300Au/ml) showed the least activity. (Figure 4.4).

At pH 5,optimum activity was attained by*Clostridium botulinum*on*Lactococcus lactis* (3800Au/ml) while *Lactobacillus acidophilus* pH 5(2400Au/ml) showed the least activity.(Figure 4.5).

Figure 4.6 is the effect of pH on bacteriocin activity on*Salmonella* Typhimurium, optimumpH was also noted at pH5 (1000Au/ml)by *Lactococcus lactis* while *Pediococcus pentosaceous* pH 5(900Au/ml) showed the least activity.

Effect of temperature on bacteriocin activity on *Staphylococcus aureus* showed that optimumtemperaturewas also noted at 37°C (3500Au/ml)by*Lactobacillus plantarum* while*Lactobacillus acidophilus*at 37°C(1500Au/ml) showed the least activity.(Figure 4.7) On the otherhand, *Lactococcus lactis* (900Au/ml) showed the optimum activity at optimumtemperature37°C on *Escherichia coli.*(Figure4.8).

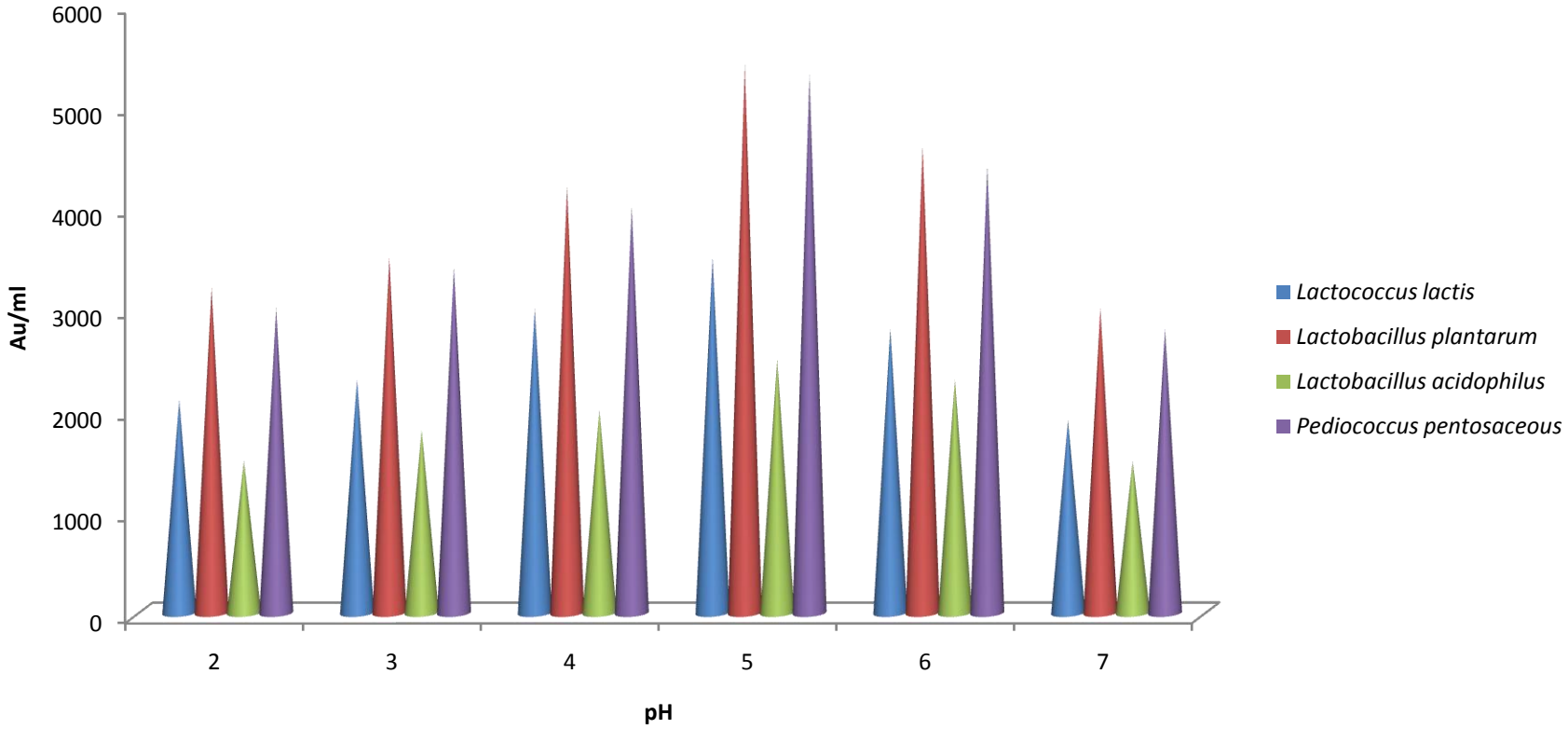

Figure 4.1: Effect of pH on bacteriocin activity on *Staphylococcus aureus*

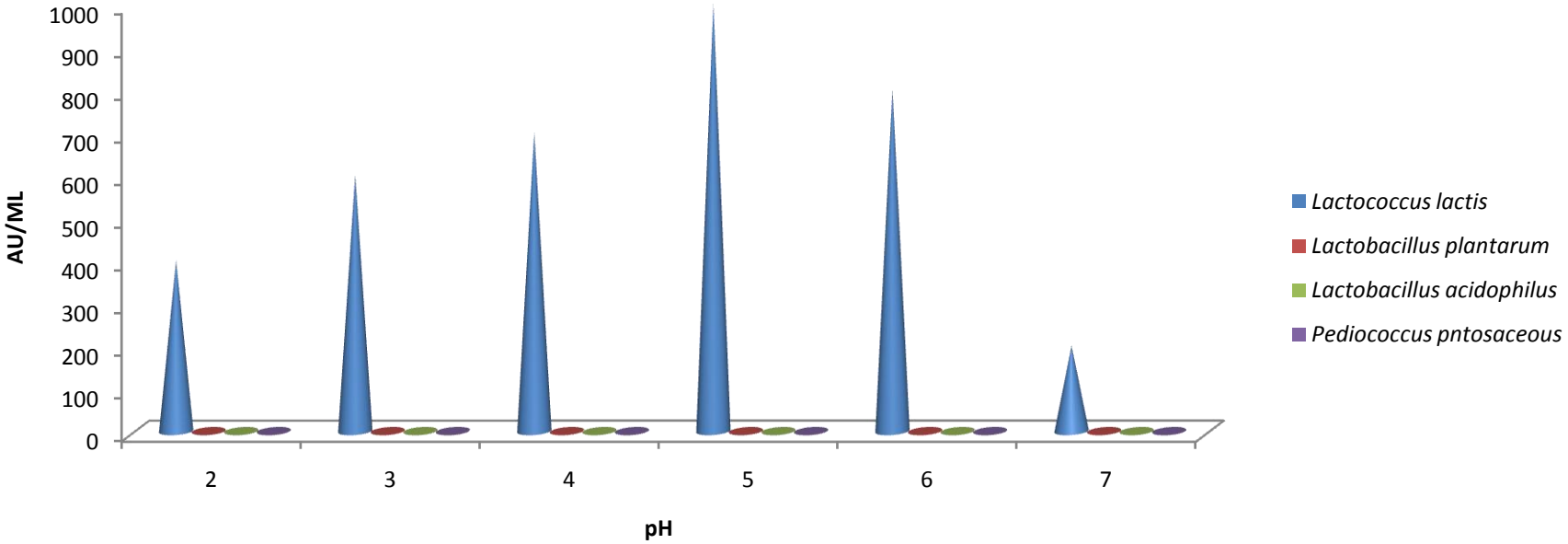

Figure 4.2: Effect of pH on bacteriocin activity on *Escherichia coli*

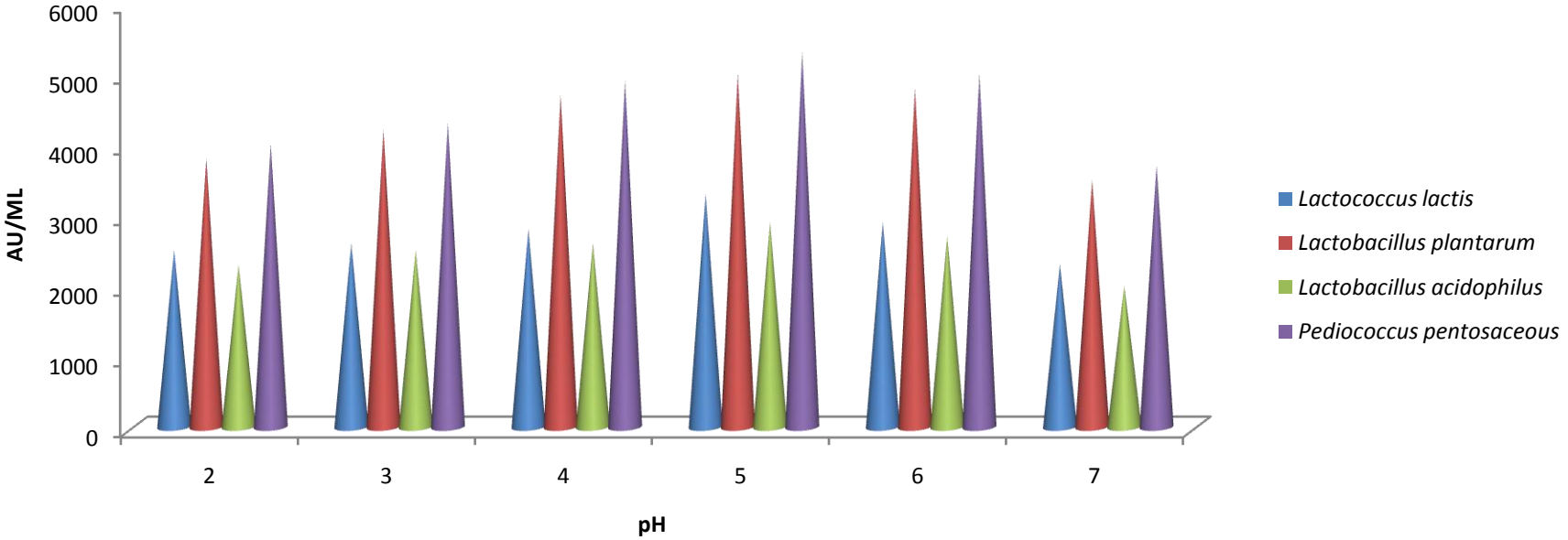

Figure 4.3: Effect of pH on bacteriocin activity on *Listeria monocytogenes*

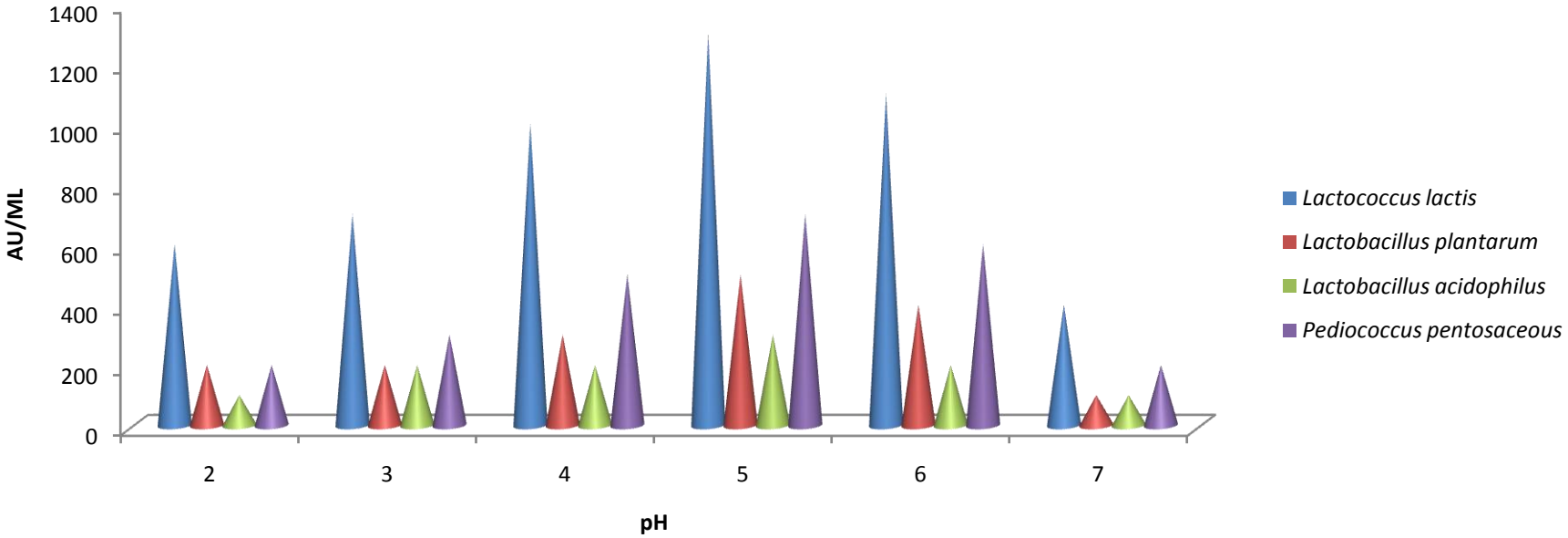

Figure 4.4: Effect of pH on bacteriocin activity on *Pseudomonas aeruginosa*

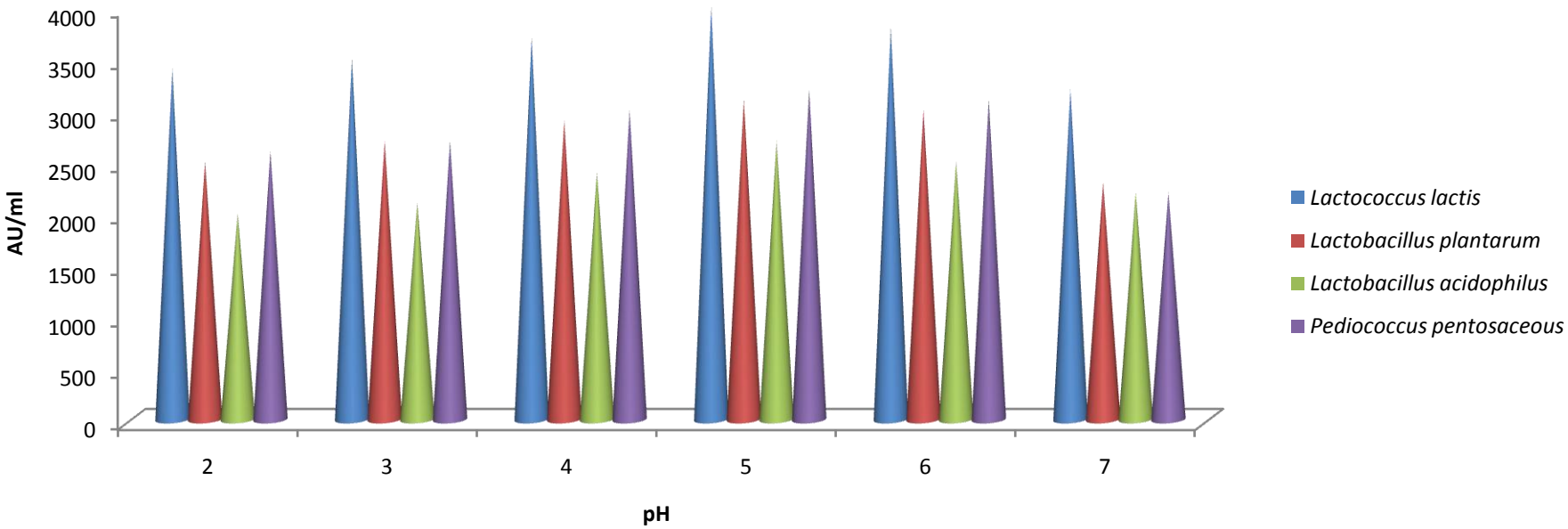

Figure 4.5: Effect of pH on bacteriocin activity on *Clostridium botulinum*

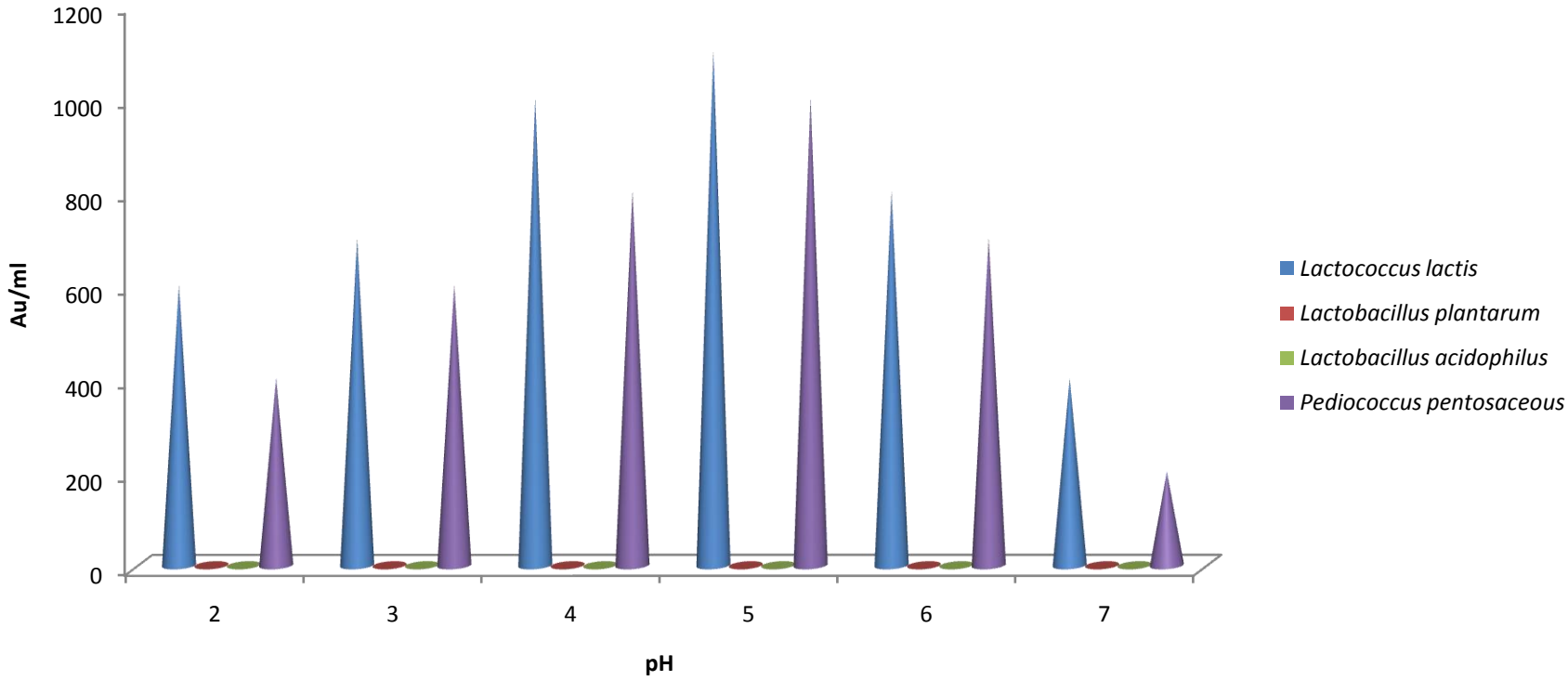

Figure 4.6: Effect of pH on bacteriocin activity on *Salmonella* Typhymurium

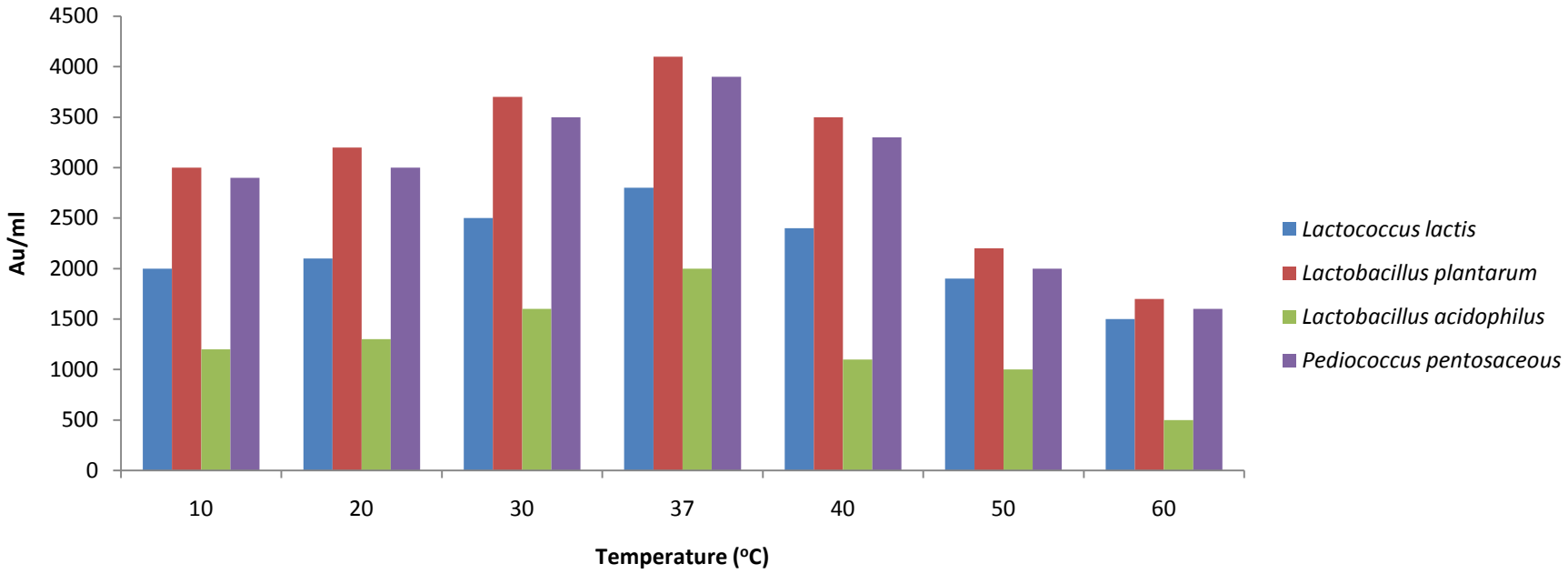

Figure 4.7: Effect of temperature on bacteriocin activityactivity on*Staphylococcus aureus*

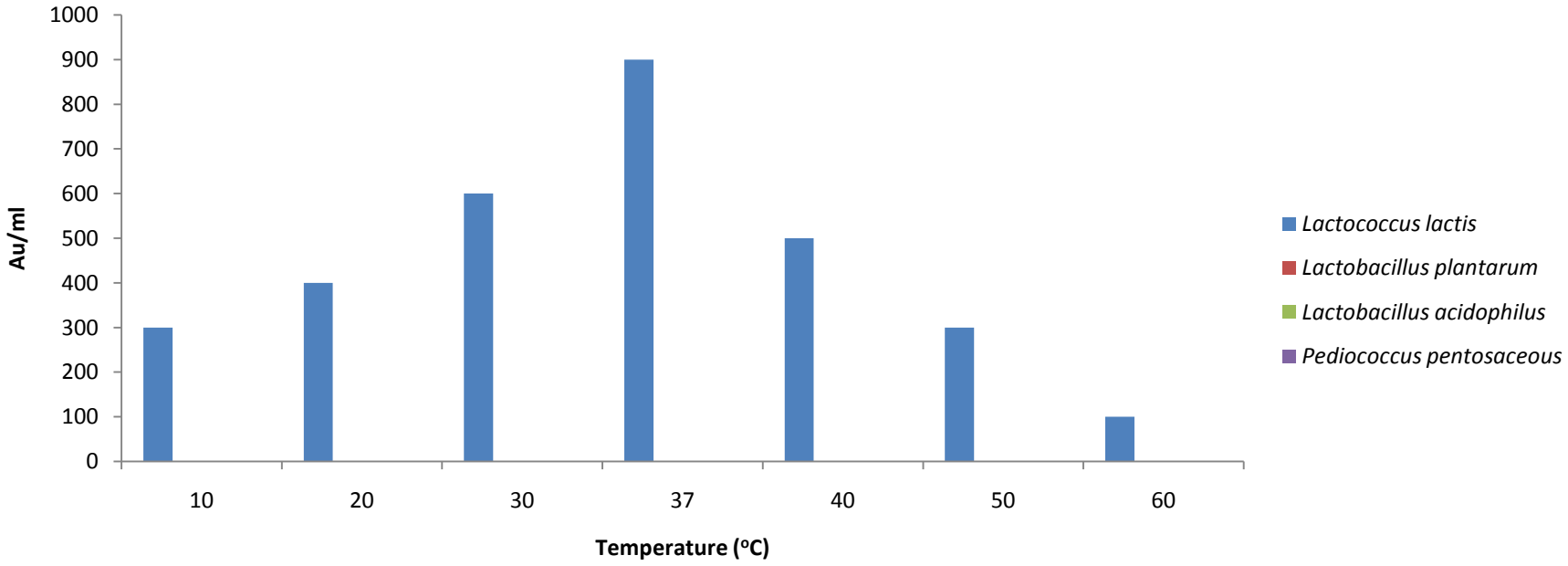

Figure 4.8: Effect of temperature on bacteriocin activity on *Escherichia coli*

Figure 4.9 is the effect of temperature on bacteriocin activity on *Listeria monocytogenes*, optimum temperature was attained at 37°C (5000Au/ml) by *Pediococcus pentosaceous* while least activity was shown by*Lactobacillus acidophilus*at 37°C(2300Au/ml). *Lactococcus lactis*showed optimum temperature at 37°C (1000Au/ml) on*Pseudomonas aeruginosa* while least activity was shown by *Lactobacillus acidophilus*at 37°C(200Au/ml).Figure 4.10.

Figure 4.11 showed the effect of temperature on bacteriocin activity on *Clostridium botulinum*, optimum temperature was attained at 37°C (5000Au/ml) by *Lactococcus lactis* while least activity was shown by *Lactobacillus acidophilus*at 37°C(2300Au/ml). On the otherhand, *Lactococcus lactis*showed optimum temperature at 37°C (9000Au/ml) by *Salmonella* Typhimuriumwhile least activity was shown by*Pediococcus pentosaceous* at 37°C(600Au/ml). (Figure 4.12).

Figure 4.13 is the bacterial load of Nisin inoculated milk sample. At 4^{o}C, initial shelf life of milk (control, milk without Nisin) was six days ,while the shelf life of the milk sample inoculated with Nisin was extended for six days, there was a decrease in bacterial load from 1.6 $x10^{6}\pm$ 1.0 x $10^{5}$to 8.3 $x10^{5}$ ± 1.0 x 10^{4} cfu/ml while spoilage occurred on the thirteenth day (1.7 x $10^{6}\pm$ 1.1 x 10^{6}cfu /ml) on the other hand, at 8^{0}C refrigeration storage temperature, total number of days of shelf life of the milk was eight days ,initial shelf life of milk (control, milk without Nisin) was four days, while the shelf life of the milk sample inoculated with Nisin was extended for four days, a decrease in bacterial load from 1.6 $x10^{6}\pm$ 1.0 x $10^{5}$to 8.9 $x10^{5}$ ± 1.0 x 10^{4} cfu/mlwas observed while spoilage occurred on the nineth day (1.7 x $10^{6}\pm$ 1.0 x 10^{5}cfu /ml). However, total number of days of shelf life of milk was three days at ambient temperature, initial shelf life of milk (control, milk without Nisin) was one day, while

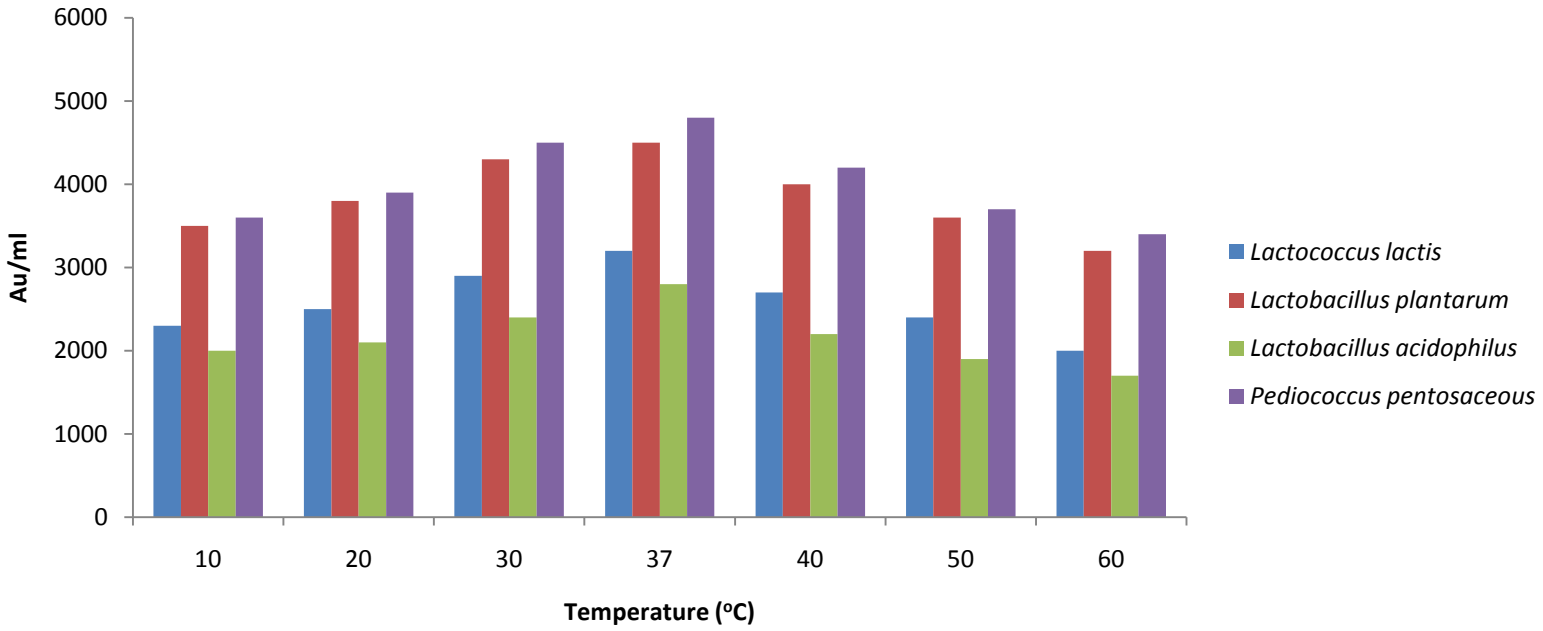

Figure 4.9: Effect of temperature on bacteriocin activity on *Listeria monocytogenes*

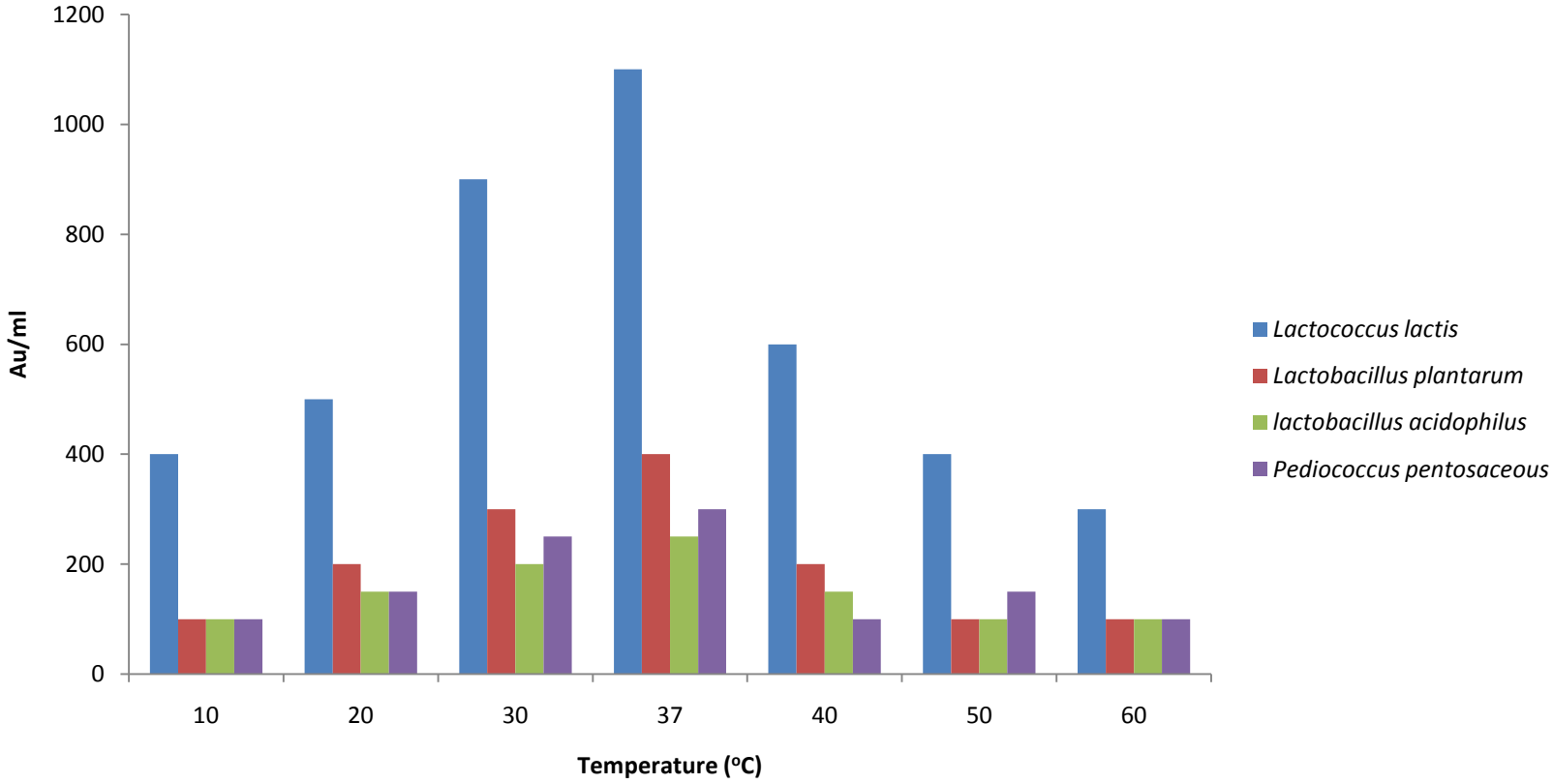

Fig.4.10: Effect of temperature on bacteriocin activity on *Pseudomonas aeruginosa*

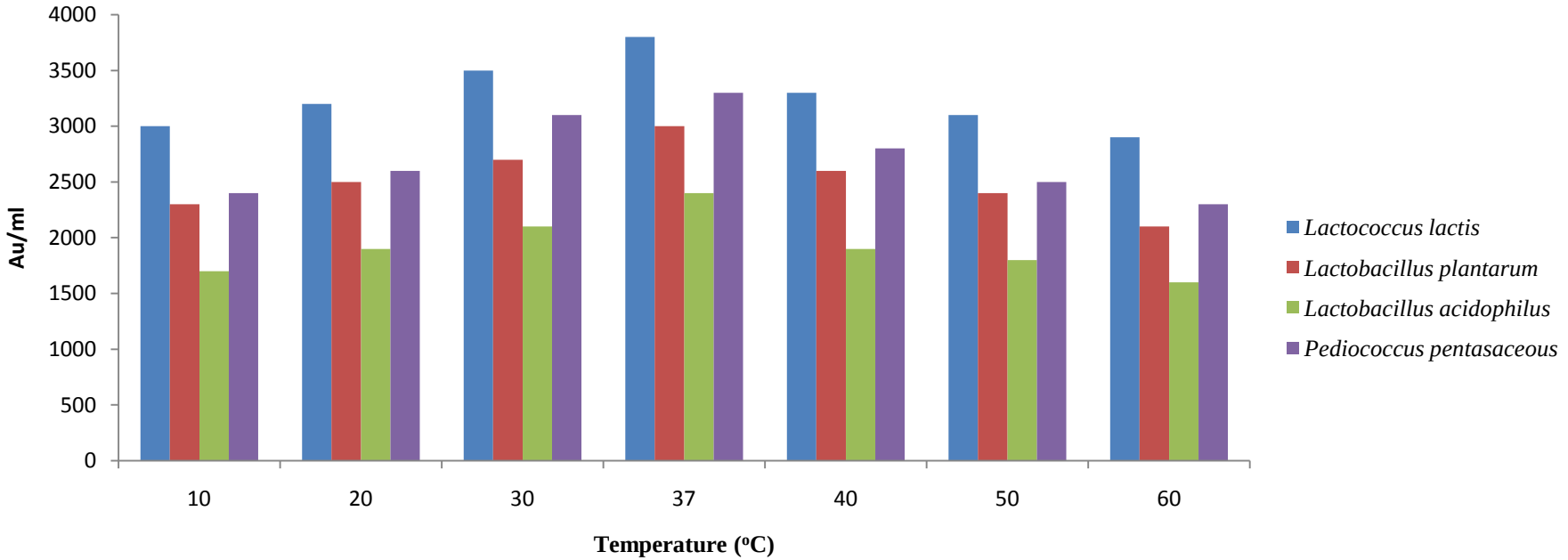

Figure 4.11: Effect of temperature on bacteriocin activity on*Clostridium botulinum*

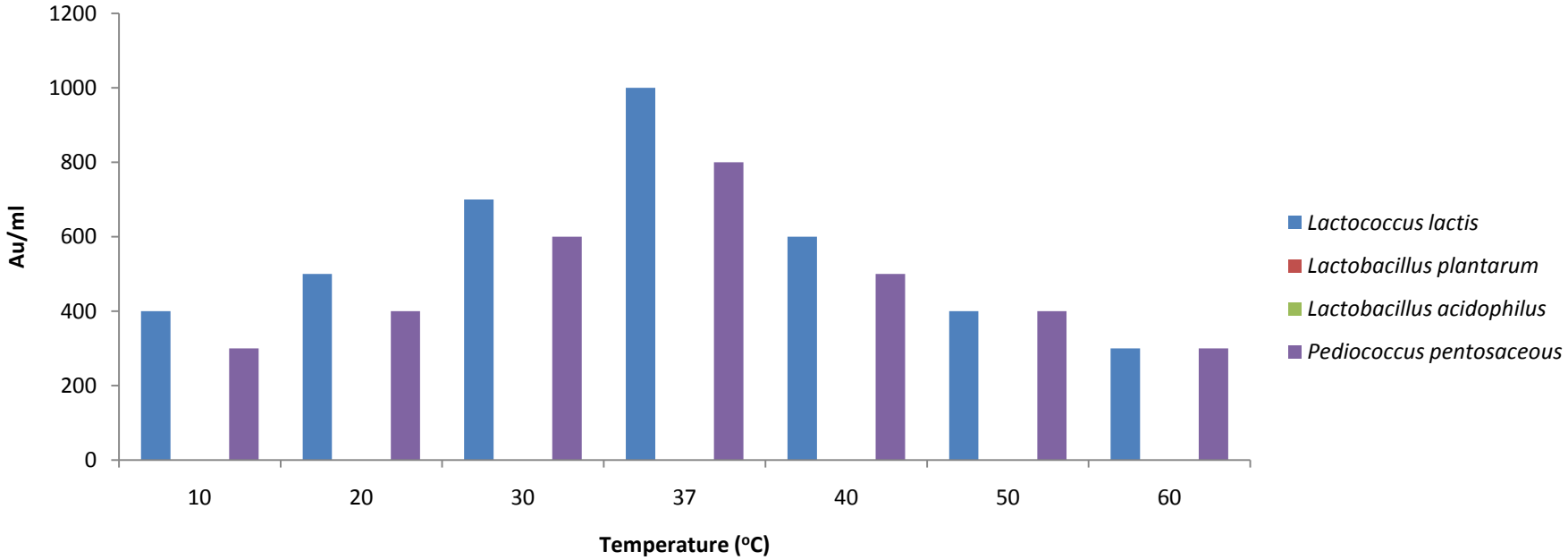

Figure 4.12: Effect of temperature on bacteriocin activity on*Salmonella* Typhimurium

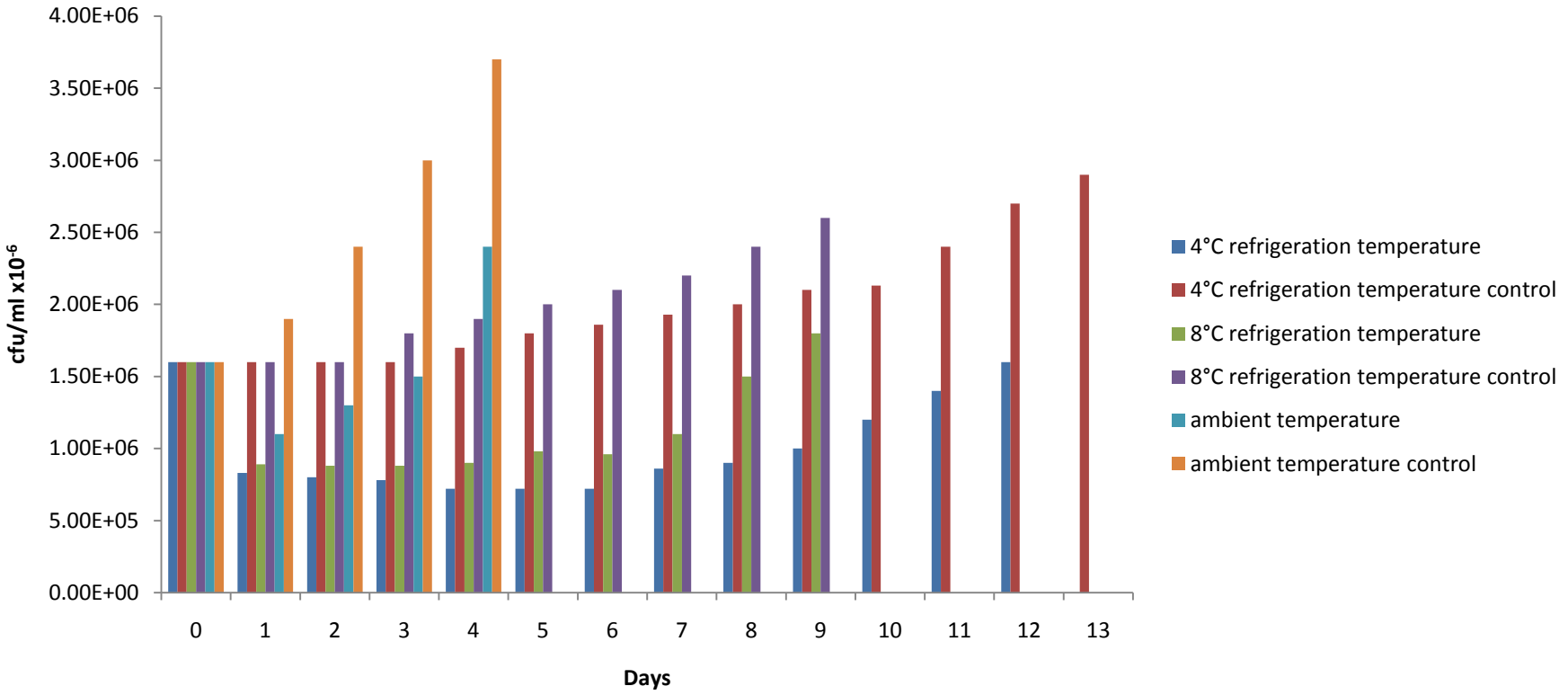

Figure 4.13:Bacterial load of Nisin inoculated milk sample kept at different refrigeration temperature

the shelf life of the milk sample inoculated with Nisin was extended for two days, there was a decrease in bacterial load from 1.6 $x10^6 \pm$ 1.0 x $10^5$to 1.2 $x10^5 \pm$ 1.0 x 10^4 cfu/ml after inoculation with Nisin while spoilage occurred on the fourth day (2.6 x $10^6 \pm$ 1.0 x 10^5cfu /ml). Figure 4.14 is the milk sample inoculated with Plantaricin stored at refrigeration temperature 4^0C, total number of days of shelf life of milk was eleven days at 4^oC, initial shelf life of milk (control, milk without Plantaricin) was six days ,while the shelf life of the milk sample inoculated with Plantaricin was extended for five days, there was a decrease in bacterial load from 1.6 $x10^6 \pm$ 1.0 x $10^5$to 8.4 $x10^5 \pm$ 1.0 x 10^4 cfu/mlwhile spoilage occurred on the twelveth day (1.8 $x10^6 \pm$ 1.0 x 10^5 cfu/ml). At 8^oC, initial shelf life of milk (control, milk without Plantaricin) was four days ,while the shelf life of the milk sample inoculated with Plantaricin was extended for four days, there was a decrease in bacterial load from 1.6 $x10^6 \pm$ 1.0 x $10^5$to 9.0 $x10^5 \pm$ 1.0 x 10^4 cfu/ml while spoilage occurred on the nineth day (1.8 $x10^6 \pm$ 1.5 x 10^5 cfu/ml).

Inoculation of Acidophilin into milk at 4^0C refrigeration temperature caused a reduction of total viable count from 1.6 $x10^6 \pm$ 1.0 x $10^5$to 8.3 $x10^5 \pm$ 1.5 x 10^4 cfu/ml after twenty four hours while spoilage occurred on the twelveth day (1.7 $x10^6 \pm$ 1.0 x 10^5cfu/ml). On the otherhand 8^0C refrigeration caused a reduction of total viable count from 1.6 $x10^6 \pm$ 1.0 x $10^5$to 9.1 $x10^5 \pm$ 1.5 x 10^4 cfu/ml after inoculation with Acidophilin while spoilage occurred on the nineth day (1.9 $x10^6 \pm$ 5.7 x 10^5cfu/ml). Further more, the bacterial load at ambient temperature after twenty four hours of inoculation of Acidophilin decreased from 1.6 x $10^6 \pm$ 1.0 x $10^5$to 1.2 x $10^6 \pm$ 1.5 x 10^5 while spoilage occurred on the fourth day (2.6 x $10^6 \pm$ 2.0 x 10^5cfu/ml). (figure 4.15).

Inoculation of Pediocin into milk at 4^0C refrigeration temperature caused a reduction of total viable count from 1.6 $x10^6 \pm$ 1.0 x $10^5$to 8.3 $x10^5 \pm$ 5.7 x 10^4 cfu/ml after twenty four hours while spoilage occurred on the twelveth day (1.7 $x10^6 \pm$ 1.5 x 10^5cfu/ml)

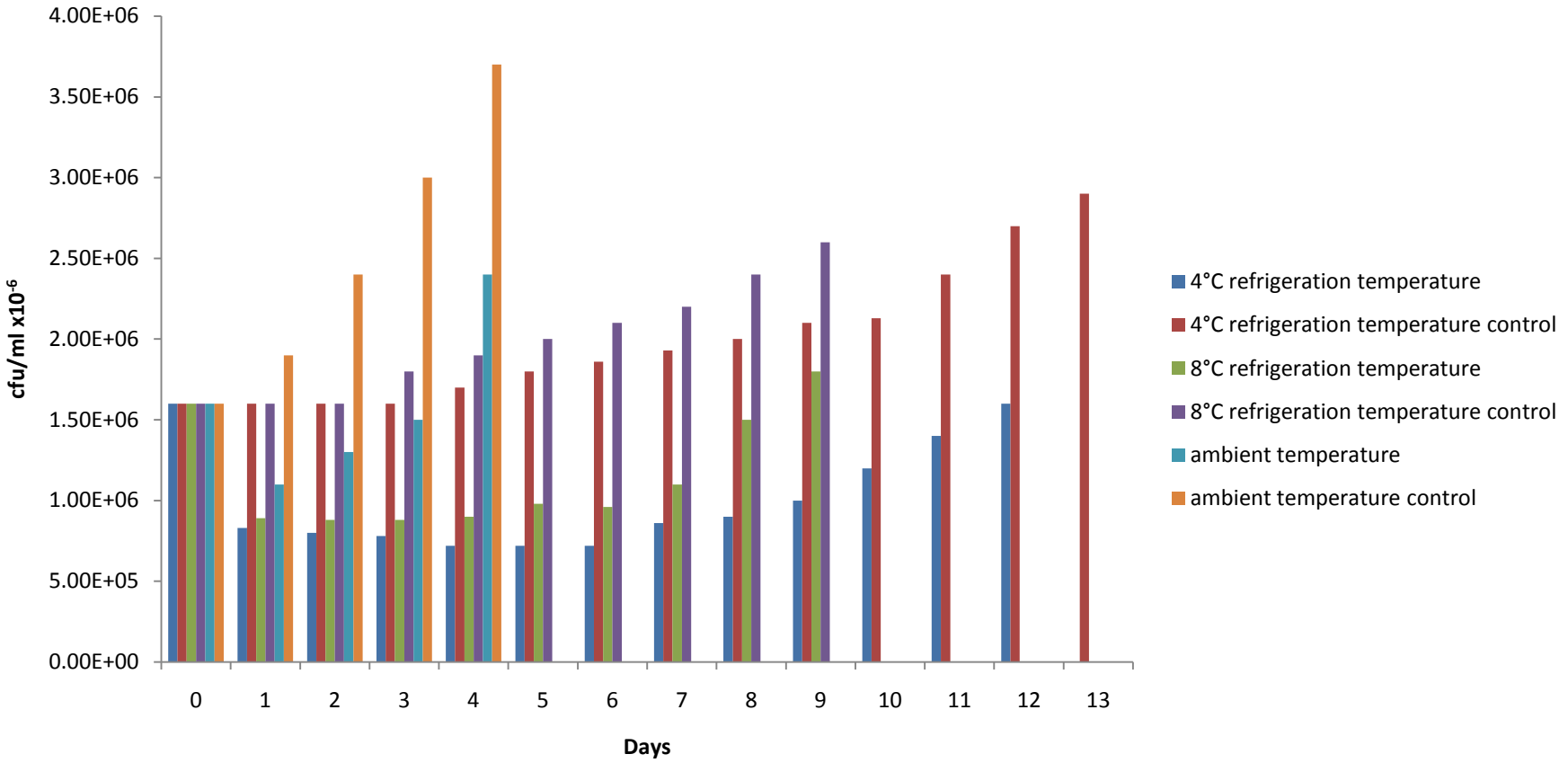

Figure 4.14: Bacterial load of Plantaricin inoculated milk sample kept at different refrigeration temperature

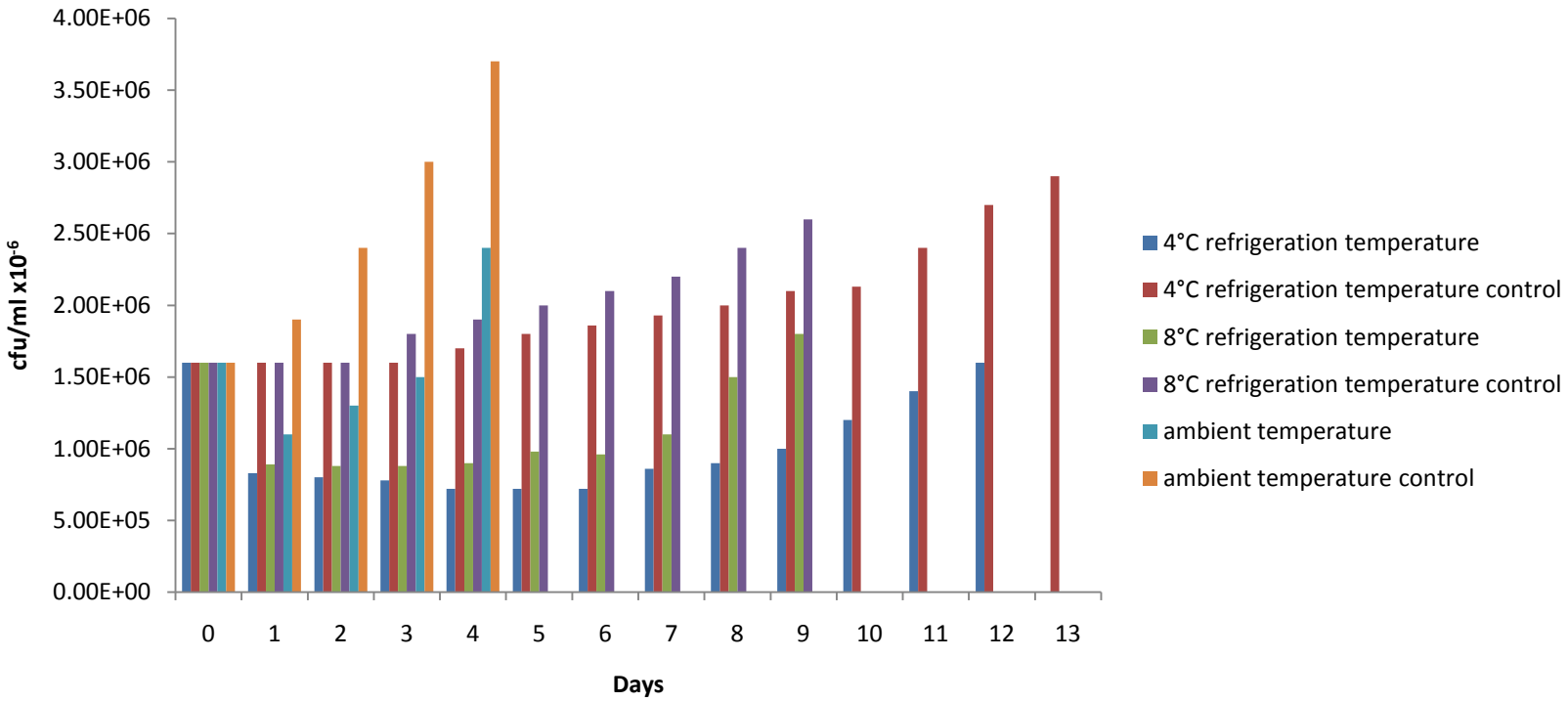

Figure 4.15 :Bacterial load ofAcidophilin inoculated milk sample kept at different refrigeration temperature

8^0C refrigeration caused a reduction of total viable count from 1.6 x10^6± 1.0 x $10^5$to 8.9 x10^5 ± 1.5 x 10^4 cfu/ml after inoculation with Pediocin while spoilage occurred on the nineth day (1.8 x 10^6± 5.7 x 10^5). However, the bacterial load at ambient temperature after twenty four hours of inoculation of Pediocin decreased from 1.6 x 10^6± 1.0 x $10^5$to 1.1 x 10^6 ± 5.7 x 10^5 cfu/ml while spoilage occurred on the fourth day (2.4 x 10^6± 2.0 x 10^5cfu/ml). (figure 4.16).

Table 4.5 showed the result of the Sensory properties of the milk sample inoculated with bacteriocins compared to the control (milk sample without bacteriocins),the result showed that there was a significant difference (P-value < 0.05)between the bacteriocin inoculated milk sample compared with the control without bacteriocins.The bacteriocin inoculated milk samples were better acceptable, in terms of aroma, texture, appearance, and taste. Proximate composition of bacteriocin inoculated milk sample showed that there was a statistical significance difference between the moisture content of the bacteriocin inoculated milk sample and the control (milk without bacteriocin).(Table 4.6).

Figure 4.17 showed the pH of bacteriocin inoculated milk sample. A gradual decrease in pH was observed throughout the period of storage. pH decreases from initial 5.4 ± 0.1 to 3.9 ± 0.1 while that of the control (milk without bacteriocin) decreases from 5.93 ± 0.11 to 5.0 ± 0.1.

On the otherhand, the Total Titratable Acidity of bacteriocin inoculated milk sample increased gradually throughout the period of storage. Total Titratable Acidity increased

from 0.0217 ± 0.001 to 0.148 ± 0.001 while that of the control (milk without bacteriocin) increased from 0.0217 ± 0.001 to 0.1200 ± 0.003.(Figure 4.18).

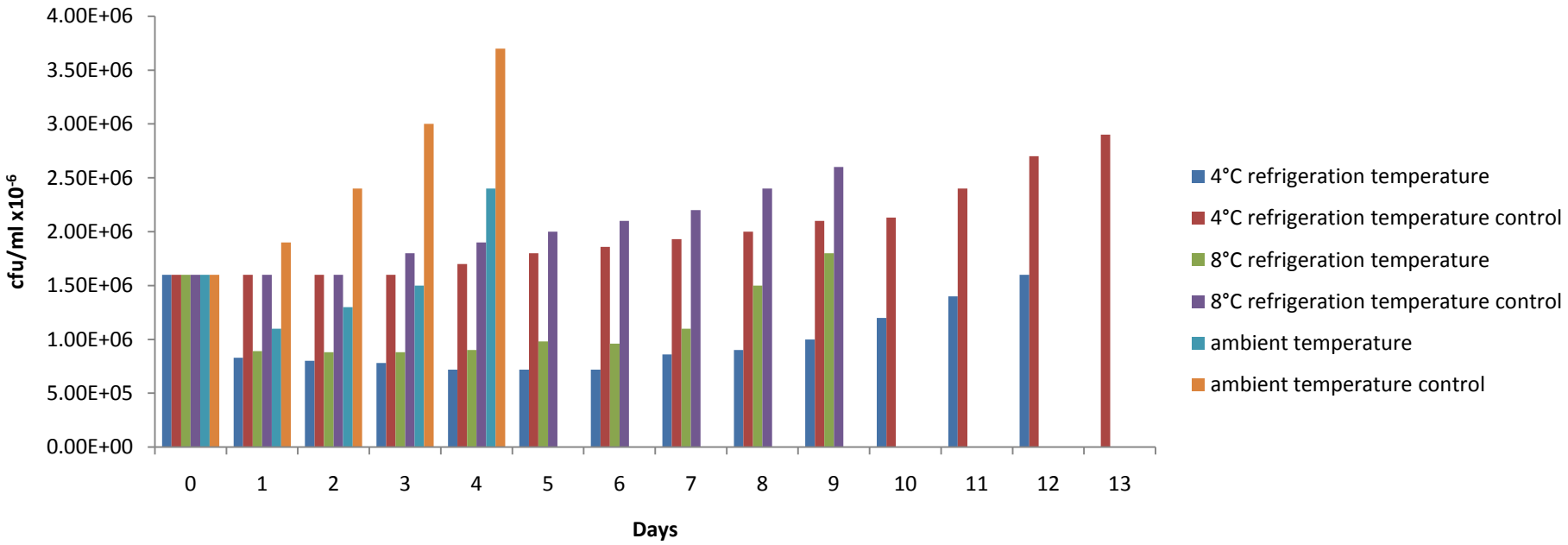

Figure 4.16: Bacterial load of Pediocin inoculated milk sample kept at different refrigeration temperatures

Table 4.5 Sensory properties of milk inoculated with bacteriocins

Sample	Appearance	Aroma	Texture	Taste	Acceptability
Nisin	5.00±0.33[b]	5.50±0.37[b]	5.00±0.26[b]	5.10±0.37[b]	5.50±0.37[b]
Plantaricin	5.20± 0.29[b]	5.20±0.29[b]	4.80±0.37[b]	4.90±0.38 [b]	5.00±0.33[b]
Acidophilin	4.90±0.35[b]	5.10±0.28[b]	5.00±0.28[b]	4.80±0.40[b]	2.70±0.36[a]
Pediocin	5.10±0.31[b]	5.00±0.33[b]	5.00±0.33[b]	4.80±0.40[b]	5.10±0.31[b]
Control	2.70±0.36[a]	2.70±0.37[a]	2.90±0.37[a]	3.10±0.23[a]	2.80±0.29[a]

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

Table 4.6: Proximate Composition of Milk Samples Inoculated with Bacteriocins

Bacteriocin	Moisture	Ash	Protein	Fat	Total Carbonhydrate
Nisin	64.00±1.16[a]	4.60±0.07[a]	21.07±0.54[a]	3.13±0.07[a]	6.10±0.61[a]
Plantaricin	65.67±1.20[a]	4.57±0.06[a]	20.33±0.88[a]	3.10±0.06[a]	6.57±0.35[a]
Acidophilin	65.67±1.45[a]	4.30±0.08[a]	20.00±0.58[a]	3.13±0.08[a]	6.77±0.43[a]
Pediocin	65.67±2.67[a]	4.30±0.08[a]	20.03±0.68[a]	3.37±0.08[a]	6.03±0.27[a]
Control	70.00±0.51[b]	4.30±0.04[b]	19.33±0.58[b]	3.32±0.04[b]	3.03±0.27[b]

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

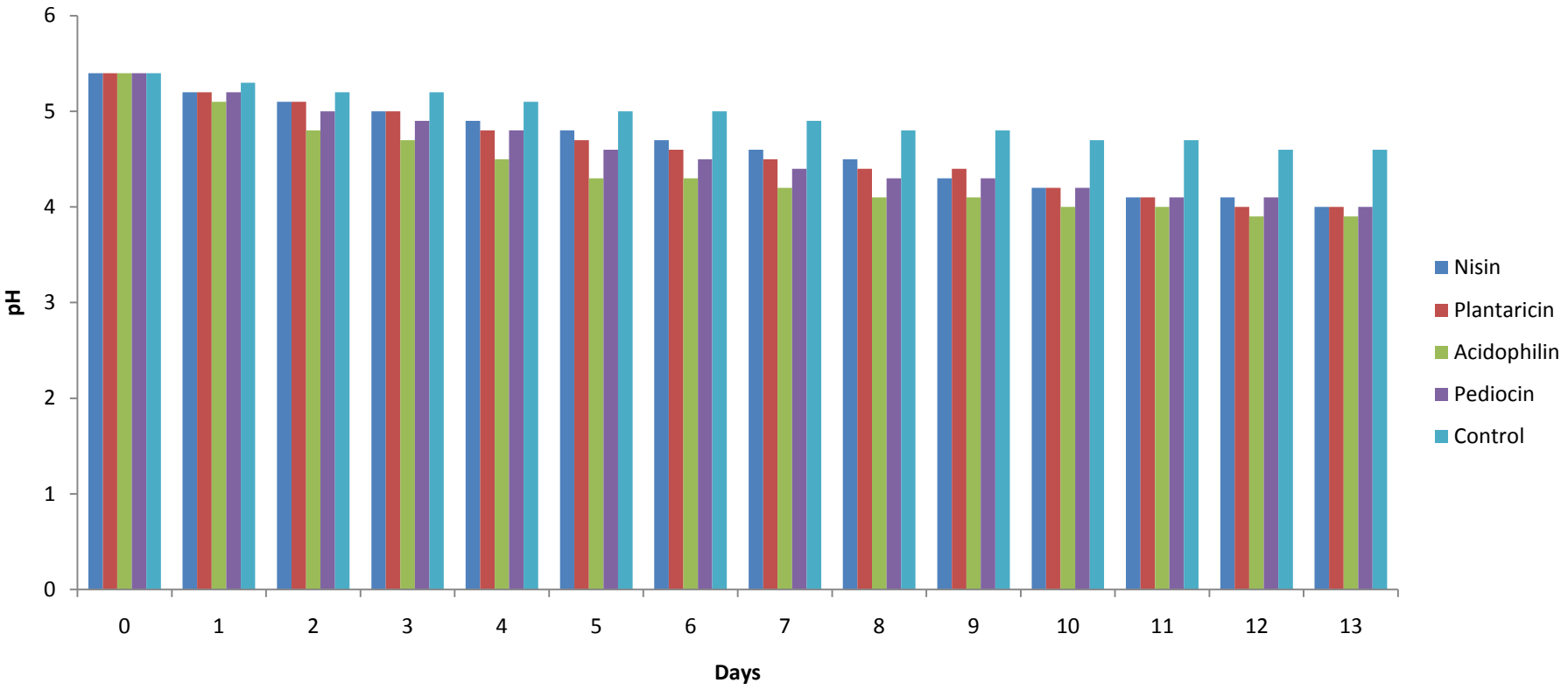

Figure 4.17 pH of bacteriocin inoculated milk samples

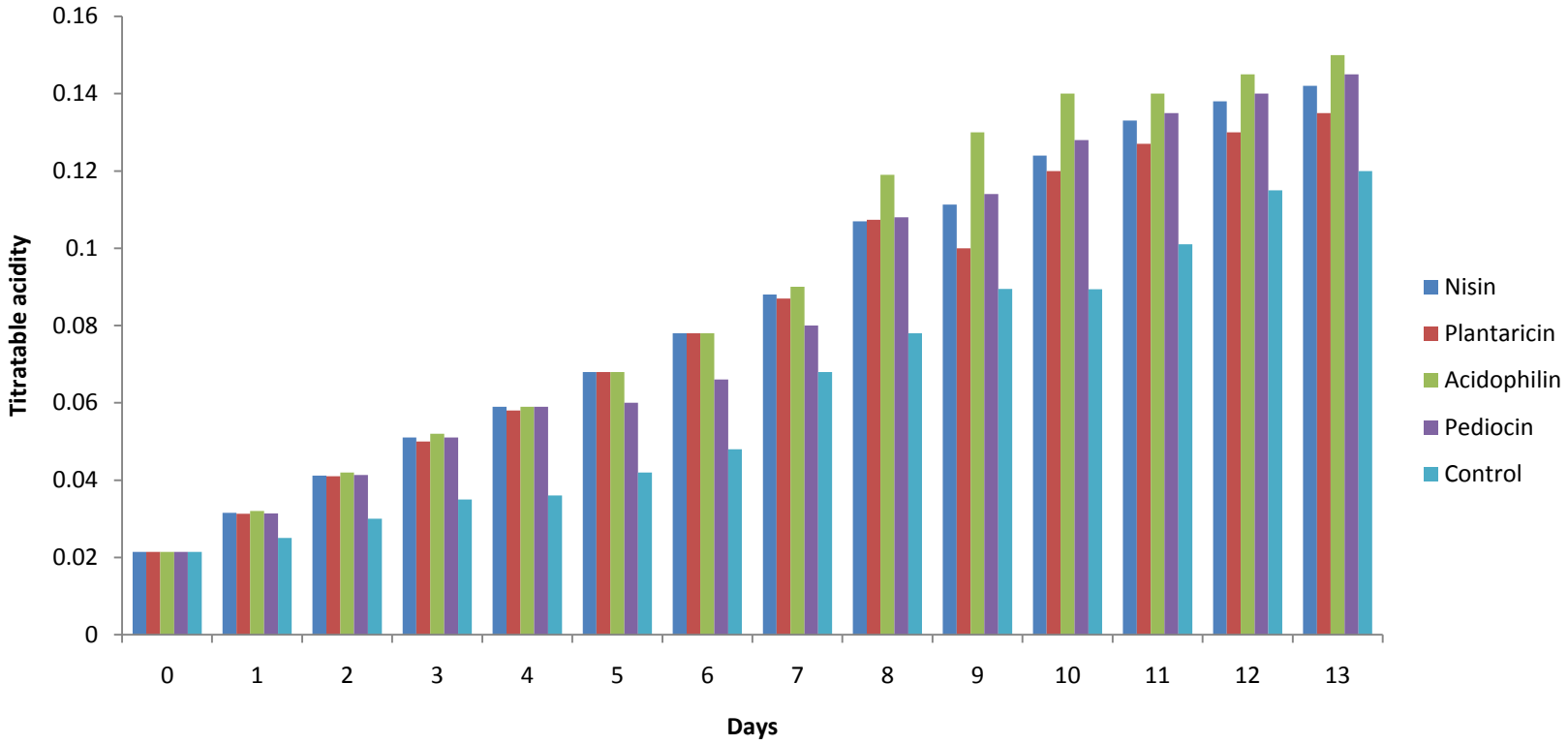

Figure 4.18 Titratable acidiy of the bacteriocin inoculated milk samples

CHAPTERFIVE

5.0 DISCUSSION

The pH observed indicates acidity as a result of presence of Lactic acid bacteria in the sample, which lowers the pH Values, and can also inhibit the growth of other competing pathogens of foods.Lactic acid bacteria are naturally present in all fermented foods like cereal,milk,vegetables,meat and fish (Okpara *et al*.,(2014). However, Okpara *et al*., (2014),observed a pH of kunun zaki as 3.5-5.5 which was different from that obtained in this study. The isolation of lactic acid bacteria in this beverage implies that they may contain potentially bacteriocin producing Lactic acid bacteria which may probably inhibit or antagonise some food pathogens thereby making the beverage suitable for consumption. Similar result was reported by Babatunde *et al*., (2014).

Lactic acid bacteria have a long history of use in a variety of cereal fermentation with predominance of *Lactobacillus plantarum* whichhad 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). 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. 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.

However, low zone of inhibition produced by the lactic acid bacteria against the Gram negative bacteria might be due to the nature of the cytoplasmic membrane of the Gram negative barrier because of the protective barrier provided by the Lipopolysaccharide (LPs) of the outer membrane of the Gram negative bacteria, which are generally active

against Gram positive bacteria, this agrees with the work of Okpara *et al*., (2014) and that bacteriocins usually have a narrow spectrum of activity inhibiting most Gram positive bacteria especially those closely related to the producer organism.

Bacteriocin producing *Lactococcus lactics* and *Pediococcus pentosaceous* in "*kunun zaki*" is an indication of potential of growth of this strain in variable ecological complex environment and has been selected for application in food biopreservation. Strongest activity was observed on *L.monocytogene* by *P.pentosaceous*.This might be due to the antilisterial activity it possesses. *Pediococcus* has been approved as a food additive to control *L.monocytogene* in meat and dairy products.However, Ogunbanwo *et al*., (2004) reported strongest activity of the inhibitory compounds against*S.aureus* ATCC12600.

Similarly, broad inhibitory spectrum by *Lactococus lactics* (Nisin) was observed on all the foodborne pathogens, this might be attributed to its low pH, or probably due to its dual mechanism of action. Nisin form pores and disrupt the proton motive force and the pH equilibrium causing leakage of ions and hydrolysis of ATP resulting in cell death (Ross *et al*.,2006).However, Babatunde *et al*. (2014),reported a resistance of *E.coli* on*Lactoocus lactis*.

*Clostridium botulium*exhibited high susceptibility on *Lactococcus lactics* and *Listeria monocytogenes* this might be due to its low pHor antibotulinum activity or partly due to the presence of receptor sites in the cell wall of the organism. Nisin is produced by *Lactococcus lactics* and belongs to the class 1 lantibiotics, Soomro *et al.,* (2002) reported that Nisin is active against many Gram positive bacteria including *Listeria* spp. It prevents the growth of germinating *Bacillus* and *Clostridial* spores of processed and natural cheeses, inhibits the growth of some psychotrophic bacteria in Cottage, cheese, extends the shelf life of milk in warm countries, prevents the growth of spoilage lactobacilli in beer and wine fermentations and provides additional protection against

Bacillus and *Clostridial* spores in canned foods. Many bacteriocins of LAB are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in various foods. Nisin is permitted as a food additives in more than 50 countries including the US and Europe under the trade name Nisaplin (soomro *et al*, 2002). Inhibition of the growth of other Gram negative bacteria including *E.coli*, *Salmonella typhi* and*Pseudomonas aeruginosa*, implies a broad spectrum of inhibition by Nisin, this might be the reason why it has been approved by FDA for use as food preservative in cheese, vegetables, fish, alcohol and dairy products. Several researchers have demonstrated the effectiveness of nisin and / or nisin-producing strains against pathogenic bacteria such as *Clostridium botulinum* in cheese and against *L.monocytogenes* in cheeses such as Camembert (Nunez*et al*.,1997), and Manchego other bacteriocins have been tested in milk and dairy products, such as pediocin AcH in milk and cheddar and munster cheeses against *L.monocytogenes, S.aureus, and E.coli* 0157: H7 (Rodriguez *et al*.,2005).

Lactobacillus plantarum showed a broad inhibitory effect on food spoilage organisms except *E,coli*. This might be due to the class of bacteriocin it belongs or probably due to the activity of Plantaricin synthesized by the organism. *Lactobacillus plantarum* belongs to the class IIb Non lantibiotics,they are relatively heat stable, non-lanthionine containing membrane active peptides. Many reports have shown that the antimicrobial substances produced by *Lactobacillus* sp. could fight against *Salmonella* and other harmful pathogens affecting animal health and the agent of food poisoning. Similarly, Mollendorf *et al*.,(2006) observed that bacterial metabolites of*L.plantarum* displayed a wide inhibitory activity on various species of pathogens such as *Salmonella typhymurium, Escherichia coli and Clostridium perferinges* and other Gram-positive bacteria such as *Bacillus cereus, Staphylococcus aureus, Streptococcus pneumoniae*and

Enterococcus faecum Moghadam *et al*., (2010) also reported that metabolites of *L. plantarum* had broad inhibitory spectrum against *E. coli, Listeria monocytogenes, S.* Typhymurium *and Vancomycin resistant enterococci* (VRE) due to the presence of 2 classes of bacteriocins, plantaricin Ef and plantaricin W.

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 *L. plantarum* on *P. aeruginosa*, 2.5mm on *E. coli*, 2mm on *S. aureus* .

Least activity displayed by *Lactobacillus acidophilus* in the inhibition of pathogens especially Gram negative bacteria might be due to the genetic make up of the organism or probably due to a weak receptor sites in the cell wall of the organism. Bacteriocin produced by *L.acidophilus* has been successfully used as safe and effective natural inhibitors of pathogenic and food spoilage organisms.This finding is in contrast with that reported by Saranj *et al*., (2013) who observed an antibacterial activity against a wide range of Gram negative and Gram positive pathogens. *P.pentosaceous* showed susceptibility on *Listeria monocytogenes* might be due to the antilisteria activity it possesses and synthesized by *L.monocytogene* or probably due to the class II (Non lantibiotics) it belongs, they are relatively heat stable and can withstand high temperature and pH. The inhibitory effect produced by *Pediococcus pentosaceus* is in agreement with that observed by Cosansu *et al*., (2007) who reported that the bacteriocins produced by *Pediococcus* sp have high antimicrobial activity especially against *L.monocytogens*.

Low, moderate and high temperature tolerance by the LAB isolates indicates that foods that harbour Lactic acid bacteria can be used refrigerated or frozen foods or high temperature or even at room temperature in order to prevent the growth of pathogenic and spoilage psychrotophic organisms. Temperature 37^0C was observed to be the

optimum for bacteriocin activity. However, Biswas *et al*., (1991) observed that the optimum temperature for bacteriocin activity was 25^0C.

Stability of Lactic acid bacteria at low pH ranges justify its use in fermented foods like kunun zaki, yoghurt, 'Eko'and locust bean. Most of the fermented foods are acidic, and acidity inhibits the growth of pathogenic organism thereby enhancing safety to fermented foods. Optimum pH of 5 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.

Addition of Nisin into milk sample resulted in a decrease in Total viable count at 4^0C for six days (Nisin)and five days (Plantaricin, Acidophilin and Pediocin) indicates a bacteriostatic potential for LAB bacteriocins. Lactic acid bacteria are reported to be inhibitory against many pathogenic organisms, the decrease in the total viable count of milk after inoculation with Nisin might be due to the exracellular compound that might have been released and attacked the cytoplasm of the foodborne pathogen that might be present in the milk sample. This finding is contrary to the work of Pinto *et al*.(2011) who extended the shelf life of Ricotta-type cheese up to 8weeks in the presence of Nisin. The natural and GRAS antimicrobial Nisin produced by LAB have been the subject of intense study because of their potential use as biopreservatives in the food industry. (O'sullivan *et al*.,2002). Nisin has been approved by FDA for application as food additives for biopreservation (Galvez *et al*., 2007).

At 8^0C,the shelf life of milk sample inoculated with Nisin was extended for 4days while the shelf life of milk sample inoculated with Nisin and stored at ambient temperature was extended for 2days.The decrease in total viable count of milk during the shelf life extension of milk and the difference observed in the shelf life extension days of milk

sample might be due to the presence of bacteriocin, and or probably due to the storage condition the milk samples were kept, because 4^0C is lower than 8^0C,and ambient temperature is higher than 8^0C.Low temperature slow down chemical changes and the growth of many pathogenic microorganisms. Similarly, Gallo *et al*.(2007) reported that the reduction of L – innocua in liquid cheese whey with a combination of low pH and nisin (PH=5.5,3001U/ml of nisn) was more at 2^0C than at 7^0C.

A decrease in total viable count and shelf life extension of milk sample inoculated with Plantaricin might be as a result of bacteriocin that was inoculated, and the storage temperature of the milk sample, cooling reduces the bacterial growth but does not eliminate the microorganism already present in the milk. High temperature supports bacterial growth than low temperature. This finding is inconsistent with that of FSAI.(2005) who reported that the shelf life of many food products depends on storage temperatures 4^0C and 10^0C.

The effect of acidophilin to extend the shelf life of milk could be attributed to low pH, faster diffusion rate of acidophilin molecules in the food matrix, or probably due to regular distribution of the acidophilin in the milk sample. Ostile *et al*.(2003),reported that *Lactobacillus acidophilus* is a slow growing organism and survives well in milk and dairy products,very stable and has a high resistance towards acids in fermented dairy products. Total viable count of milk sample was reduced after 24hours inoculation with acidophilins.The result obtained is greater than that obtained by Mohammed *et al*,(2013),who extended the shelf life of milk sample inoculated with acidophilin at 4^0C(3days),8^0C(2days) and ambient temperature (1day). Extension of shelf life of milk by Pediocin might be attributed to the incorporation of pediocin which has a bacteriacidal effect on pathogenic organisms in the milk sample,hence,extending the shelf life of milk. Several reports are available on the preservation of milk and milk

based foods with added antimicrobial compounds, such as nisin and pediocin from LAB in the last few years (Pinto *et al.*, 2011).

Nisin and Other antimicrobial like pediocin from LAB have been shown to reduce or inhibit *L.monocytogenes* in diary and meats, particularly in pasteurised cheese.

Mean value obtained for Proximate composition showed that Moisture content of the the milk samples inoculated with bacteriocins ranged from 64.00±1.16to 70.00±0.51. Moisture content is a measure of the water content in a food sample. The moisture content accounts for the textual property of the food sample. High moisture content supports microbial growth thereby reducing the shelf life of milk while low moisture content causes reduction in microbial growth due to low water activity and hence increased the shelf life of milk.. The moisture content observed is similar to 63.6-68.4% that was reported by Lawal and Adedeji (2013).

A decrease in pH with a corresponding increase in total titratable acidity was observed in the bacteriocin inoculated milk samples. This attribute enhances acidity to the fermenting milk as well as the buffering capacity of the milk. Acidification is detrimental to other pathogens in the same environment.This plays an important role in the shelf life extension of milk. pH also plays an important role in the shelf life of milk, during fermentation, the pH is lowered as a result of presence of lactic acid bacteria. At low pH pathogenic organisms cannot withstand and survive hence making the environment unfavourable for their growth. This finding is similar to that reported by (Uaboi *et al*., 2010) who observed a value of pH decrease of 5.8 - 3.2 and an increase of Total Titrable Acidity of 0.049-0.137%. Sensory attributes such as colour, acceptability, aroma, texture and taste indicated that the milk inoculated with bacteriocin was significantly acceptable. The significant difference between the fermented milk samples and the control might be due to the inoculation of bacteriocins which inhibits a variety

of food spoilageand pathogenic organisms, which might have imparted flavor, texture,aroma and taste to the fermented milk samples which might have also resulted in preservation of the milk sample and hence better acceptability of the milk sample. However, Wirjantoro *et al*. (2001) reported that milk sample with or no bacteriocin was acceptable although milk sample with bacteriocin was superior in flavour with no off odour.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Lactic acid bacteria were isolated and identified using the Analytical Profile Index (API 50 CH KIT).

L.lactis,L.plantarum,L.acidophilus and P.pentosaceous had the potential for bacteriocin activity.

The bacteriocins (Nisin,Plantaricin,Acidophilin and Pediocin) from Lactic acid bacteria proved to be an effective antibacterial agent on foodborne pathogens in milk sample.

Temperature, pH tolerance, inhibitory activity, stability during storage played an important role in the shelf life extension of milk and may be used as an alternative to the most commonly used chemical preservative(Nitrite and Nacl).

Sensory property and physicochemical parameters showed evidence that the bacteriocin has potential application as biopreservatives in food items.

6.2 Recommendations

Based on the outcome of this study the following recommendations are made :

i. Bacteriocins could be produced via fermentation by native or genetically engineered organisms purified and added to foods as pure chemicals.
ii. The combination of two or more bacteriocins will also provide promising results, particularly if the bacteriocins belong to different cellular components.
iii. Continued study of the physical and chemical properties, mode of action, structure and function, relationships of bacteriocin is necessary if their potential in food preservation is to be exploited.

iv. Studies should aim at screening for local indigenous foods widely consumed in our localities for bacteriocin producing LAB and to examine their potential to inhibit the growth of spoilage and food borne pathogens.

v. Large scale production of bacteriocins in Industries should be encouraged in response to consumers demand for natural products.

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APPENDICES

APPENDIX I:Conventional Biochemical Test of the LAB Isolates

isolates code	Physical appearance	Colonial morphology	Cell morphology	Gram reaction	Catalae test	Motility test	Endospore test	Oxidase test	Inference
KN1	Whitish	Oval, round protruding	Cocci, arranged in chain	Gram positive	-	-	-	-	LAB
KN2	Cream	Little sticks,smooth round colonies	Long rods arranged in pairs	Gram positive	-	-	-	-	LAB
KN3	Cream	Beige,smooth round colonies	Thick short rods arranged in pairs	Gram positive	-	-	-	-	LAB
KN4	White	Round with jagged ends	Cocci in clusters	Gram positive	-	-	-	-	LAB
KN5	Cream	Small round colonies	Short rods arranged singly	Gram positive	-	-	-	-	LAB
KN6	White	Small oval colonies	Rod, arranged in chains	Gram positive	-	-	-	-	LAB

Key:KNI-KN6=ISOLATE CODES

Appendix II: Carbohydrate fermentation profiles of Lactic acid bacterial isolates using API 50 CH Kit .

	0	GLY	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG	Inference
KN1	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	-	+	+	+	
KN2	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	-		-	-	
KN3	-	-	-	+	-	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	-	-	+	-	-	-	-	-		-	-	+	-	
KN4	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	+	-	+	-	-	
KN5	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	-	-	+	+	+	
KN6	-	+	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	+	-	+	-	-	
KN7	-	-	-	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	
KN8	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	-	-	-	-	+	-	+	-	-	
KN9	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	
KN10	-	-	-	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+	+	+	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	+	-	
KN11	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	
KN12	-	-	-	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	
KN13	-	-	-	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+	-	+	+	+	-	+	+	-	-	-	+	-	-	-	-	-	-	-	+	-	+	
KN14	-	-	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	-	-	+	-	+	+	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	+	-	+	+	-	
KN15																																																			

Key:= KN1-KN15=Isolate codes

Inference:KN1=*L.lactis*,KN2=*P.pentosaceous*,KN3=*L.acidophilus*,KN4=*L.paracasei*,KN5=*L.fermentum*,KN5=*L.fermentum*,KN6=*L.acidophilus*,KN7=*L.plantarum*,KN8=*L.plantarum*,KN9=*L.acidophilus*,KN10=*L.plantarum*,KN11=*L.plantarum*,KN12=*L.paracasei*,KN13=*L.fermentum*,KN14=*L.plantarum*,KN15=*L.plantarum*

2GLY=Glycerol,ERY=Erythritol,DARA=D-Arabinose,LARA=L-Arabinose,RIB=D-Ribose,DXYL=D-Xylose,LXYL=L-Xylose,ADO=D-Adonitol,MDX=Metyl-βD-Xylopyranoside,GAL=D-Galactose,GLU=Glucose,FRU=Fructose,MNE=D-Mannose,SBE=L-Sorbose,RHA=L-Rhamnose,DUL,=Dulcitol,INO=Inositol,MAN=D-Mannitol,SOR=D-Sorbitol,MDM=Metyl-αD-Mannopyranoside,MDG=Metyl-α D-Glucopryanoside,NAG=N-Acetylglucosamine,AMY=Amygdaline,ARB=Arbutine.ESC=Esculine,SAL=Salicine,CEL=D-Celibiose,MAL=D-Maltose,LAC=D-lactose,MEL=D-Melibiose,SAC=D-Saccharose,TRE=D-Trehalose,INU=Inuline,MLZ=D-Melezitose,RAF=D-Raffinose,AMD=Amidon,GLYG=Glycogene,XLT=Xylitol,GEN=Gentiobiose,TUR=D-Turanose,LYX=D-Lyxose,TAG=D-Tagatose,DFUC=D-Fucose,LFUC=L-Fucose,DARL=D-Arabitol,LARL=L-Arabitol,GNT=Potassiumgluconate,2KG=Potassium2-cetogluconate,5KG=Potassium5-cetogluconate.

Appendix III: Physical examination of Bacteriocin inoculated milk sample kept at $4^{O}C$ refrigeration temperature

Days	Nisin	Plantaricin	Acidophilin	Pediocin	control
Day0	cream,good flavour	cream, good flavour	cream, good flavour	cream, good flavour	cream, good flavour
Day1	white,very good flavour	White, very good flavour	White, very good flavour	White, very good flavour	cream, good flavour
Day2	white,very good flavour	White, very good flavour	White, very good flavour	White, very good flavour	cream, good flavour
Day3	white,very good flavour	White, very good flavour	White, very good flavour	White, very good flavour	cream, good flavour
Day4	white,very good flavour	White, very good flavour	White, very good flavour	White, very good flavour	cream, good flavour
Day5	white,very good flavour	White, very good flavour	White, very good flavour	White, very good flavour	cream, good flavour
Day6	white,very good flavour	White, very good flavour	White, very good flavour	White, very good flavour	Cream,good flavour
Day7	white,very good flavour	White, good flavour	White, good flavour	White, good flavour	Off cream,off flavour
Day8	white,very good flavour	ite, good flavour	White, good flavour	White, good flavour	Off cream,off flavour
Day9	White,good flavour	White, good flavour	White, good flavour	White, good flavour	Off cream,off flavour
Day10	White, good flavour	White, good flavour	White, good flavour	White, good flavour	Off cream,off flavour
Day11	White, good flavour	White, good flavour	White, good flavour	White, good flavour	Off cream,off flavour
Day12	White, good flavour	Off white,off flavour	Off white,off flavour	Off white,off flavour	Off white,off flavour
Day13	Off white,off flavour				

Appendix IV: Physical examination of the bacteriocin inoculated milk kept at $8^{o}C$ refrigeration temperature

Days	Nisin	Plantaricin	Acidophilin	Pediocin	Control
1	cream,good flavour	cream, good flavour	cream, good flavour	cream, good flavour	cream, good flavour
1	white,very good flavour	white, very good flavour	white, very good flavour	white, very good flavour	cream, good flavour
2	white,very good flavour	white, very good flavour	white, very good flavour	white, very good flavour	cream, good flavour
3	white,very good flavour	white, very good flavour	white, very good flavour	white, very good flavour	cream, good flavour
4	white,very good flavour	white, very good flavour	white, very good flavour	white, very good flavour	cream, good flavour
5	white, good flavour	white, good flavour	white, good flavour	white, good flavour	off cream,off flavour
6	white,good flavour	white, good flavour	white, good flavour	white, good flavour	off cream,off flavour
7	white, good flavour	white, good flavour	white, good flavour	white, good flavour	off cream,off flavour
8	white, good flavour	white, good flavour	white, good flavour	white, good flavour	off cream,off flavour
Day9	off cream,off flavour	off cream,off flavour	off cream,off flavour	off cream,off flavour	off cream,off flavour

APPENDICE V: Physical examination of the bacteriocin inoculated milk sample kept at ambient temperature

Days	Nisin	Plantaricin	Acidophilin	Pediocin	Control
0	cream,good flavour	cream, good flavour	cream, good flavour	cream, good flavour	cream, good flavour
1	white, good flavour	White, good flavour	White, good flavour	White, good flavour	cream, good flavour
2	white, good flavour	White, good flavour	White, good flavour	White, good flavour	off cream,off flavour
3	white, good flavour	White, good flavour	White, good flavour	White, good flavour	Off odour,curdling
4	Off odour, curdling	Off odour, curdling	Off odour, curdling	Off odour, curdling	Off odour, curdling