PHYSIOLOGICAL, GENOMIC AND PRESERVATIVE PROPERTIES OF *PEDIOCOCCUS* ISOLATES FROM MEATS UNDER LOW TEMPERATURE STORAGE

BY

OLUBUNMI PAULINE, DUYILEMI

(124721)

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CERTIFICATION

I certify that Olubunmi Pauline DUYILEMI carried out this study under my supervision for the award of Doctor of Philosophy (Microbiology) in the Department of Microbiology, University of Ibadan

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Supervisor

Abiodun A. Onilude
B.Sc., M.Sc (Ife), Ph.D (Ibadan)
Professor of Microbiology
Department of Microbiology
University of Ibadan, Nigeria

DEDICATION

This Thesis is dedicated to my wonderful Parents Prof. B.O. and Dr (Mrs) A.N. Duyilemi

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ABSTRACT

The need for meat preservation cannot be overemphasised. However, existing methods of meat preservation including the use of artificial preservatives have toxic side effects. There is a dearth of information on the use of Lactic Acid Bacteria (LAB), which is known as good preservatives for food, in the preservation of meat. Hence, the aim of this study was to examine the use of *Pediococcus acidilactici* and low temperature in improving the quality of beef, chicken and turkey meat samples.

Samples of beef, chicken and turkey were obtained from open market retailers and stored for 28 days at 4^{o}C, 2^{o}C, -4^{o}C and -15^{o}C. From these, LAB were isolated and identified using conventional methods. Quantities of lactic acid and acetic acid were determined using high performance liquid chromatography, while diacetyl and hydrogen peroxide were determined by enzymatic methods. High production of lactic acid was used as a criterion for selecting five isolates of *Pediococcus acidilactici* and the 16S rDNA genes were amplified and sequenced. The isolates were checked for plasmid presence and tested for bacteriocin production using gel electrophoresis and agar well assay. Antimicrobials produced by the isolates were tested *in vitro* against known meat spoilage organisms: *Staphylococcus aureus, Escherichia coli, Pseudomonas faecalis, Listeria monocytogenes, Salmonella typhimurium* and *Bacillus cereus.* The isolates and their filtrates were applied to fresh meat at -4^{o}C and -15^{o}C. Microbial load, proximate and biochemical parameters of the meat samples were monitored at seven days interval for 28 days. Data were analysed using ANOVA at p = 0.05.

One hundred and ten LAB isolates from beef [*Lactobacillus* (24), *Pediococcus* (6), *Leuconostoc* (9)], chicken [*Lactobacillus* (27), *Pediococcus* (4), *Leuconostoc* (2)] and turkey [*Lactobacillus* (31), *Pediococcus* (5), *Leuconostoc* 2] were identified. Fast freezing (-15^{o}C) and freezing (-4^{o}C) gave significantly lower LAB count (4.1 ± 0.03 - 5.1 ± 0.02 logcfu/mL) than those of chilling (2^{o}C) and refrigeration (4^{o}C) (4.2 ± 0.04 - 5.4 ± 0.02 logcfu/mL) in all the meat samples. Lactic acid and acetic acid production peaked at 30.7 g/L and 32.0 mg/mL respectively while diacetyl and hydrogen peroxide production peaked at 40.8 ng/L and 16.0 µg/L respectively. Sizes of the 16S rDNA from the five strains of *P. acidilactici* ranged from

145 bp to 161 bp. Band size of plasmid DNA ranged from 861 - 20643 bp. Bacteriocin inhibition zones ranged from 1.0 to 6.5 mm. The highest zone of inhibition of antimicrobial action was 12 mm against *P. faecalis.* Proximate and biochemical analyses gave lower values compared with control samples: pH (4.8 / 5.7), thiobarbituric acid (0.2 / 0.5 mg malonaldehyde/kg), free fatty acid (0. 2 / 0.5 KOH/g lipid), total volatile nitrogen (0.6 / 1.4 mgN/100) and crude fat (3.4 / 4.5 %); but increased crude protein (22.1 / 17.7 %). Lowest microbial load (total bacteria count 2.2 logcfu/ml, coliform count 1.4 logcfu/ml, fungal count 2.0 logcfu/ml) and highest LAB count (4.9 logcfu/ml) were observed on the 28th day.

Pediococcus acidilactici with optimum physiological characteristics prolonged the keeping quality of meat under low temperature storage.

Keywords: Lactic acid bacteria, Holding temperature, Biopreservative, Physiological properties, Meat

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INTRODUCTION

1.1 General Introduction

Meat comprises of voluntary or striped muscle which together with fat and connective tissues forms flesh or butcher meat. It is a valuable part of human diet because (a) it is the most concentrated and most easily assimilable of nitrogeneous foods and is a good source of first class protein for example, it contains those amino – acids which are essential for life; (b) it is stimulating to metabolism due to its high protein content by assisting the body in the production of heat and energy (c) it is satisfying for the presence of fat in the diet, delays emptying of the stomach. Meat contains fat, and therefore remains in the stomach hours and allays hunger (d) after suitable treatment, which include processes of ripening and cooking, meat acquires a palatable flavor, acts as stimulant to gastric secretion and is readily digested (Thornton and Gracey, 1974).

Meat has long been considered a highly desirable and nutritious food, but unfortunately it is also highly perishable because it provides the nutrients needed to support growth of many types of microorganisms (Shay and Egan, 1991). Fresh meat is a highly perishable food product and unless appropriate actions are taken to preserve it, it can spoil in relatively short time (Ercolini *et al.,* 2006; Koutsoumanis *et al*., 2006). Factors affecting meat spoilage include intrinsic (for example, pH, water activity, composition, type and extent of initial contamination**)** and extrinsic parameters (like temperature and packaging atmosphere). Among these, temperature is considered the most important factor (Giannakourou *et al.,* 2001; Jay *et al*., 2003; Koutsoumanis *et al.,* 2006). Lactic acid bacteria have traditionally been used in food processing because of their characteristic flavour changes, ability to lower the pH and to produce antimicrobial agents (Lucke 2000; Barakat *et al.,* 2000; Aymerich *et al*., 2003; Leroy and De Vuyst 2005). Lactic acid bacteria (LAB) preserve foods as a result of

competitive growth and the production of inhibitory substances, such as lactic acid, acetic acid, ethanol, diacetyl, hydrogen peroxide, reuterin and bacteriocins (Mortvedt - Abildgaard *et al.*, 1994; Stiles, 1996; Cocolin e*t al.*, 2000; Andrighetto *et al.*, 2001). In addition to preservation, a number of nutritional, technological and health benefits are associated with the use of LAB which usually can be attributed to specific metabolic properties (Liao *et al.*, 1994; Koutsoumanis *et al.*, 2006).

1.1.1 Microbiology of raw meat

The characteristic microbial populations that develop in meat and meat products are the result of the effect of the prevailing environmental conditions on the growth of the type of microbes initially present in the raw material or introduced by cross-contamination (Chang *et al.*, 2003). The intrinsic and extrinsic factors governing microbial growth determine the type and number of bacteria present in meat. Intrinsic factors are predominantly chemical while extrinsic ones are concerned with storage and processing conditions. The latter, whose major parameters are temperature and oxygen availability, are often manipulated to extend the shelf life of meat products. Meat is an excellent substrate for bacterial growth, which requires restrictive methods to prevent contamination. The extrinsic factors, often controlled to extend the shelf life of meat, are mainly related to storage and processing conditions and can be considered as a set of hurdles applied for its stabilization. **(**Giannakourou *et al.*, 2001**).** Hygiene during slaughter and dressing of carcasses together with prompt and adequate cooling are of major importance for meat quality and safety. (Ghafir *et al*., 2005)

Meat is an excellent substrate for bacterial growth; hence, if preservative methods are not used, it becomes easily spoilt. The low temperatures used during carcass chilling constitute the first barrier for microbes to develop. In addition, the microbiology of meat carcasses is highly dependent on the conditions under which animals were reared, transported, slaughtered and

processed. Conditions at, and spread of contamination during slaughter and time-temperature of storage are important factors that will determine the microbiological quality of the meat (Barkocy-Gallagher *et al.,* 2003; Fegan *et al.,* 2004; Fike and Spire, 2006; Looper *et al.,* 2006).

The widespread distribution of meat products in national and international trade has reportedly brought about spread of food-borne disease through meat consumption (Chang *et al.,* 2003**).** During the 70s, the establishment of microbiological criteria for chilled and frozen meat as well as hygienic standards in slaughter houses and during sanitary inspection of live animals were of major consideration by meat processors to improve hygiene (Nottingham, 1982). More recently, intervention technologies to reduce pathogens in meat, which have received considerable attention, have proved to be effective. After carcass dressing, the microbiotas were observed to comprise of a mixture of mesophiles and psychrotrophs, which are gradually selected during meat chilling (Castillo *et al.*, 2001). Under these conditions, mesophile growth will no longer occur and a psychrotrophic microbiota will develop. Since most pathogens are mesophiles, meat obtained in good hygienic conditions would not be expected to pose sanitary risks. Carcass decontamination strategies using sanitizing solutions such as organic acids, trisodium phosphate or Ozone (Dubal *et al.,* 2004; Ransom *et al.,* 2003) have been successfully applied for pathogen reduction in beef carcasses, especially when combined with pre-chill treatments (Beach *et al.,* 2002).

1.1.2 Bioprotective cultures

The preservation of foods using their natural and controlled microbiota and/or their antimicrobial metabolites has been termed *bioprotection* or *biopreservation* to differentiate it from artificial (chemical) preservation (Stiles, 1996). Antagonistic cultures added to foods to inhibit pathogens and/or extend shelf life while changing the sensory properties as little as possible are called protective cultures (Lucke, 2000). The main purpose of biopreservation is the extension of storage life as well as the enhancement of food safety. LAB have a major potential

for use in biopreservation because they are not only safe for consumption but naturally dominate the microbiota of many foods during storage. LAB are "generally recognized as safe" (GRAS) due to their typical association with food fermentations and their long tradition as food-grade bacteria. In addition, antimicrobial peptides produced by LAB can be easily broken down by digestive proteases, so they will not produce gut microbiota disturbance. LAB can exert a bioprotective or inhibitory effect against other microorganisms as a result of the competition for nutrients and/or the production of bacteriocins or other antagonistic compounds such as organic acids, hydrogen peroxide and enzymes. A distinction can be made between starter cultures and protective cultures in which metabolic activity (acid production, protein hydrolysis) and antimicrobial action constitute the main objective respectively. Food processors face a major challenge with consumers demanding not only safe foods with a long shelf life, but also expressing their preference for minimally processed products, less severely damaged by heat and freezing and not containing chemical preservatives **(**Koutsoumanis *et al*., 2006**)**. Hence, bacteriocins appear as an attractive option to provide at least, part of the solution.

1.1.3 Shelf stability and the "hurdle effect" in fresh meat

The microbial safety and stability as well as the nutritional and sensory quality of meat and meat products are based on the application of combined preservative factors (Leistner, 2000). These factors were introduced empirically in traditional foods while for novel food products hurdles were intelligently selected and intentionally applied (Leistner, 2000). Hurdle technology refers to the deliberate combination of existing (temperature, preservatives) and novel preservation techniques (gas packaging, bacteriocins) to establish a series of more selective preservative factors (hurdles) that spoilage and pathogenic microorganisms should not be able to overcome (Leistner, 1997).

Postmortem chilling of livestock carcasses is primarily employed to ensure food safety and maximize shelf life, with less emphasis on maintaining tenderness and color in the final product. Low temperature reduces the rate of biochemical

reactions and microbial growth. Since temperature is the most important factor affecting the microbiota of meat, cooling of carcasses will be the first hurdle that spoilage bacteria have to overcome during meat conditioning. The chilling process of carcasses after slaughter has a strong impact on the quality and palatability of meat as well as on the rate of spoilage onset. The chilling rate affects more than just the meat bacterial microbiota; tenderness can be harmed by the rapid cooling of pre-rigor muscle. As the requirements of the various quality characteristics are often conflicting, optimal conditions for chilling must be a compromise (Savell *et al.,* 2005). After slaughtering, muscle glycogen is converted to lactic acid via glycolysis and, if glycogen reserves are high, a final pH of 5.4 - 5.6 is attained. At this pH, the growth of many bacteria of importance in spoilage may be partially or totally inhibited. The ultimate pH value achieved by meat during conditioning can also be considered as a hurdle for bacterial growth. The inhibitory effect of lactic acid on Gram-negative psychrotrophs in meat appears to be related to pH reduction at chilling temperatures (Koutsoumanis and Taoukis, 2005**).** However, some *Pseudomonas* species are essentially unaffected by the pH of regular meat hence other hurdles such as vacuum-packaging must be applied in order to inhibit their growth.

The prevalence of certain species will be determined by their relative initial level, affinity for the substrates, substrate availability, the relative growth rate of the competing species at different temperatures, and the production of antimicrobial metabolites **(**Koutsoumanis *et al*., 2006**).**

1.2 Statement of problem

In Nigeria, preservation of meat and meat products has become an issue of concern due to economic losses experienced by retailers and consumers. Red meat and poultry are very popular food commodity and their consumption has increased over the last decades. Meat is a highly perishable food commodity providing an almost perfect medium for microbial growth. It is thus of utmost importance for the meat industry to develop new and effective method of preservation to extend product shelf life.

A number of nutritional, technological and health benefits are associated with the use of lactic acid bacteria starters under low temperature storage. This is usually attributed to specific metabolic properties.This demand has stimulated research interest in biopreservation. Hence a safe mode of preservation under low temperature to avoid economic losses is required to enhance food safety and extend shelflife.

1.3 Justification for the Current Work

This research work aims at exploiting the potentials of lactic acid bacteria and their physiological characteristics together with low temperature as a means of meat preservation. Lactic acid bacteria originally isolated from meat and meat products are probably the best candidates for improving the microbiology safety of these foods, because they are well adapted to the conditions in meats and should therefore be more competitive than lactic acid bacteria from other sources.

The use of lactic acid bacteria has been suggested as alternative means of preserving meats.These organisms have a unique characteristic of being non-toxic, non–carcinogenic and non- immunosuppressive. Thus they are biocompatible. It is expected that the use of lactic acid bacteria, low temperature and some biotechnology approaches will improve the shelf life of meats and thus circumvent the problem of meat spoilage. These will also help in reducing economic losses by corporate bodies and families.

1.4 Objectives

The current study is aimed at achieving the following objectives:

1. Isolation and identification of species of psychrophilic lactic acid bacteria from stored beef, chicken and turkey using both conventional and molecular methods.

2. Physiological characterization of the isolates obtained including production of antimicrobials, enzyme production and properties.

3. Study of survival patterns of identified species at low temperature and in the presence of different metabolites.

4. Plasmid analysis of obtained isolates and establishment of possible linkage to survival, antagonistic activity as well as metabolite production.

5. Study of possible patterns of antagonistic activity against known food pathogens.

6. Effects of the isolates and their filterates on fresh meat stored at low temperature including nutritional and proximate properties.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Lactic acid bacteria (LAB) and Meat Preservation

In the meat industry, the use of lactic acid bacteria (LAB) as starter culture is common (Schillinger and Lucke, 1989; Bohme *et al.,* 1996; Martin *et al.*, 1997; Leroy and De Vuyst, 1998; Onilude *et al.,* 2002). The use of lactic acid bacteria for meat preservation has been reported by (Huang and Lin, 1993; Guerrero *et al.,* 1995; Papa *et al.,* 1995; Holzapfel *et al.*, 1995; Buncic *et al.,* 1997). Indeed, homofermentative and mesophilic LAB are generally used as starter cultures.

During the last decade, research on preservation strategies of microbiological origin has flourished. In particular, the use of lactic acid bacteria (LAB) and/or their metabolites (e.g. lactic acid, bacteriocins etc) for use in bio-preservation of foods has gained increasing attention (Lucke, 2000; Rodgers, 2003; Devlieghere *et al*., 2004). Successful studies have been published on the effectiveness of bacteriocin-producing strains in foods (Holzapfel *et al*., 1995; Aymerich *et al*., 2000; Rodriguez *et al*., 2002; Jacobsen *et al*., 2003). Furthermore, concerns have been raised with respect to possible resistance development (Ennahar *et al*., 2000) as a consequence of widespread use of these peptides as food preservatives, although this is not generally accepted in literature (Cleveland *et al*., 2001). Recently, some studies have demonstrated that LAB, that do not produce bacteriocins, are capable of controlling microbial growth in food products (Nilson *et al*., 1999) more specifically in refrigerated, anaerobically packaged, sliced and cooked meat products (Bredholt *et al*., 1999; Kotzekidou and Bloukas, 1998; Amezquita and Brashears 2002; Vermeiren *et al*., 2006a; Vermeiren *et al*., 2006b). In cooked meat products, protective cultures have mainly been evaluated for their potential to inhibit food pathogens such as *Listeria monocytogenes* and less is known about the possible use of protective cultures for controlling spoilage. It is important in the development

of a protective culture that the assessment of its influence on the sensory characteristics of the treated products is reviewed.

The most frequently isolated LAB from dry sausages processed with different technologies are *Lactobacillus sakei, Lactobacillus curvatus, Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Pediococcus acidilactici* and *Pediococcus pentosaceus* (Schillinger and Lucke, 1987; Hammes, 1990; Rovira *et al*., 1994).

LABS from dairy products and of intestinal origin are considered to be the main source of probiotics (Heller, 2001). However, strains of the aforementioned LAB species found in fermented sausages such as *L. brevis, L. Plantarum, L. fermentum* and *P. pentosaceus* have also been characterized as probiotic (Osullivan *et al*., 1992; Klingberg *et al*., 2005). In order to act as a probiotic in the gastrointestinal tract, bacteria must be able to survive the acidic conditions of the stomach and resist the bile acids at the beginning of the small intestine (Erkkila and Petaja, 2000; Hyronimus *et al*., 2000; Park *et al*., 2002). Other authors have included the ability to tolerate the presence of pancreatic enzymes as criterion for selecting probiotic cultures (Salminen *et al*., 1998; Ronka *et al*., 2003).

2.2 Low Temperature Storage

Even though the effects of both chilling and freezing of foods are well documented (International Institute of Refrigeration, IIR, 1986), the effects of freeze chilling on quality of foods have only recently been systematically investigated (Guldager *et al*., 1998; Boknaes *et al*., 2000; Martinsdottir and Magnusson, 2001; Emborg *et al*., 2002; Fagan *et al*., 2003; Redmond and Gormley, 2003; Redmond *et al*., 2004; Redmond *et al*., 2005). Freeze - chilling involves initial freezing of a foodstuff with frozen storage being followed by thawing and distribution of the product at chill temperatures. Freeze-chilling has already been commercially used at the retail level for chilled pre-packaged raw fish fillets which are replacing the traditional iced fish counter in

supermarkets (Fagan *et al*., 2002). Freeze chilling offers a number of advantages over both frozen and chilled products such as:

(a)Foodstuffs can be prepared in bulk, frozen and stored at deep freeze (below -4^{o}C) temperatures until required. Subsequently, a part or the whole batch of product can be thawed and further processed; (b) Freeze chilling enables chilled foods to reach distant markets in the form of frozen product which is subsequently thawed at its final destination prior to retail display as a chilled food.(c)Freeze chilling can reduce the level of product recalls enabling routine microbiological testing to be completed before the product is released from the manufacturing plant (Fagan *et al*., 2003).

Work so far on freeze chilling has been limited to its application to fish (whiting, mackerel, salmon) (Guldager *et al*, 1998; Boknaes *et al*., 2000, 2001, 2002; Emborg *et al*., 2002 Fagan *et al*., 2003;), ready to eat meals such as lasagna (Redmond *et al*., 2005), steamed broccoli and instant mashed potatoes (Redmond *et al.*, 2002) and cooked green beans and carrots (Redmond *et al*., 2004). In general, the defect caused by LAB is described as "souring", which is less offensive than the putrefaction that develops aerobically (Stiles, 1991). Microflora and spoilage pattern of chicken carcasses packaged under CO_2 conditions are similar to those observed for red meat (Gill, 1986).

Poultry meat is a very popular food commodity around the world and its consumption has increased over the last decades in many countries. Some of the reasons for its popularity are relatively low cost of production, low fat content, high nutritional value, distinct flavor and a variety of processed poultry products commercially available (Barbut, 2002). Poultry meat is a highly perishable food commodity providing an almost perfect medium for microbial growth (Jay, 1992) and it is thus of utmost importance for the poultry industry to develop new and effective methods of preservation to extend product shelf life (Chouliara *et al*., 2007, 2008).

Studies on meat spoilage have been concerned largely with the processes occurring in red meat and poultry. At low temperatures in air, meat spoilage is mainly the result of the activity of motile and non-motile Gram-negative, psychotrophic, aerobic rods dominated by *Pseudomonas spp.* Other organisms, including *Brochothrix thermosphacta,* lactic acid bacteria and cold tolerant enterobacteriaceae are also capable of multiplication but they usually account for a small proportion of the total flora (Dainty and Mackey, 1992; Garcia – Lopez *et al*., 1998).

2.3 Cold Adapted Enzymes

The fact that cold-adapted bacteria grow at chill temperatures at rates that are either equivalent to, or not much slower than mesophiles at room or body temperatures means that they must contain proteins (enzymes) that are adapted to function at low temperatures (Russel, 2000). This adaptation has evolved over many generations and is fixed in the genome. The resulting amino acid sequence of each enzyme gives a protein folded into a three - dimensional structure that remains conformationally flexible and thus catalytically active in cold (Garcia – Lopez *et al*., 1998). Different enzymes have evolved different mechanisms for achieving cold activity, but some common evolutionary adaptations have been identified: these include a reduction in the number of hydrogen bonds, salt bridges, proline and arginine contents, aromatic interactions, and hydrophobic clustering, together with increases in solvent interactions and additional surface loops (Russell, 2000). Not every kind of change is found in each enzyme, but the overall effect is to decrease the number of enthalpy-driven interactions between amino acid side chains and give a protein that is more flexible at low temperatures (Gerday *et al.*, 1997).

A corollary of the enhanced activity at low temperatures is the fact that cold-adapted enzymes are more thermolabile than their mesophilic counterparts, so that at quite moderate temperatures (typically 40-50^{o}C),

they become too flexible, loose catalytic efficiency and eventually denature. This means that psychrotrophic bacteria are usually killed by mild heat treatment, which could be an advantage in preservation regime in which mild heating is followed by refrigerated storage (Gounot and Russell, 1999). Enzymes are found either free within the cytoplasm or in the membrane, but all of the structural data on cold-active enzymes come from studies of soluble cytoplasmic ones. Nothing is known about the structure of membrane bound cold adapted enzymes compared to their mesophilic or thermophilic counterparts, but they will presumably also have x-helical sections that span the hydrophobic core of the membrane where they interact with the fatty acid chains of membrane lipids. Therefore, it is certain that they too will be specifically adapted in order to function at low temperatures and that this adaptation will depend not only on their intrinsic protein structure but also on the physical properties of the surrounding lipids (Russell, 2002).

2.4 Antimicrobial peptides produced by meatborne LAB

Lactic acid bacteria (LAB) have long been used in fermentations to preserve the nutritive qualities of various foods. The primary antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH (Daeschel, 1989). In addition, LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass (LMM) compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl- 2,3, butanedione, uncharacterized compounds, and high-molecular-mass (HMM) compounds like bacteriocins (Jay, 1986; Klaenhammer, 1988; Piard and Desmazeaud, 1991, 1992). All of these can antagonize the growth of some spoilage and pathogenic bacteria in foods.

2.4.1.1 Organic Acids and pH

Levels and types of organic acids produced during the fermentation process depend on LAB species or strains, culture composition and growth conditions (Lindgren and Dobrogosz, 1990). The antimicrobial effect of organic acids lies

in the reduction of pH, as well as the undissociated form of the molecules (Podolak *et al*., 1996). It has been proposed that the low external pH causes acidification of the cell cytoplasm, while the undissociated acid, being lipophilic, can diffuse passively across the membrane (Kashket, 1987). The undissociated acid acts by collapsing the electrochemical proton gradient, or by altering the cell membrane permeability which results in disruption of substrate transport systems (Snijders *et al*., 1985). Lactic acid is the major organic acid of LAB fermentation where it is in equilibrium with its undissociated and dissociated forms, and the extent of the dissociation depends on pH (Lindgren and Dobrogosz, 1990).

2.4.1.2 Hydrogen Peroxide

Hydrogen peroxide is produced by LAB in the presence of oxygen as a result of the action of flavoprotein oxidases of nicotinamide adenine dinucleotide (NADH) peroxides. The antimicrobial effect of H_2O_2 may result from the oxidation of sulfhydryl groups causing denaturing of number of enzymes, and from the peroxidation of membrane lipids thus increasing membrane permeability (Kong and Davison 1980). H_2O_2 may also be as a precursor for the production of bactericidal free radicals such as superoxide (O_2) and hydroxyl (OH) radicals which can damage DNA (Byczkowski and Gessner, 1988).

2.4.1.3 Carbon dioxide

Carbon dioxide is mainly produced by heterofermentative LAB. The precise mechanism of its antimicrobial action is still unknown. However, CO_2 may play a role in creating an anaerobic environment which inhibits enzymatic decarboxylations, and the accumulation of CO_2 in the membrane lipid bilayer which may cause a dysfunction in permeability (Eklund, 1984). CO_2 can effectively inhibit the growth of many food spoilage microorganisms, especially Gram-negative psycrotrophic bacteria (Farber and Peterkin, 1991). The degree of inhibition by CO_2 varies considerably between the organisms. CO_2 at 10% (v/v) could lower the total bacterial counts by 50% (v/v) (Wagner and Moberg,

1989), and at 20-50% it had a strong antifungal activity (Lindgren and Dobrogosz, 1990).

2.4.1.4 Diacetyl

Diacetyl, i.e., an aroma component, is produced by strains within all genera of LAB by citrate fermentation. It is known to inhibit the growth of Gram-negative bacteria by reacting with arginine during its utilization (Jay, 1986). Jay (1986) showed that Gram-negative bacteria were more sensitive to diacetyl than Gram-positive bacteria; the former were inhibited by diacetyl at 200 μg/ml. Diacetyl at 344 μg/ml inhibited strains of *Listeria, Salmonella, Yersinia, E. coli* and *Aeromonas.*

Among HMM compound, bacteriocins have attracted a great interest in the food industry due to their applicational potentiality in food preservation. Bacteriocins are ribosomally synthesized, extracellularly released bioactive peptides or peptide complexes (usually 30-60 amino acids) which have a bactericidal or bacteriostatic effect on other (usually closely related) species (Garneau *et al*., 2002). In all cases, the producer cell exhibits specific immunity to the action of its own bacteriocin. They are generally considered to act at the cytoplasmic membrane and dissipate the proton motive force through the formation of pores in the phospholipids bilayer (Montville *et al*., 1995). Nisin is the best defined, and the only purified bacteriocin produced by LAB that has been approved for use in food products (Hansen, 1994).

2.5 Bacteriocin production by LAB

The preservative ability of LAB in foods is attributed to the production of antimicrobial metabolites including organic acids and bacteriocins **(**Castellano *et al.,* 2004**).** Acid production as a result of carbohydrate catabolism is a common feature among LAB, although not all LAB can produce antimicrobial peptides during growth. Since numerous bacteriocins have been isolated over the past three decades, the production of these antagonistic substances seems to be a common phenotype among LAB. They vary in size from small (3kDa), heavily post-

translationally modified peptides to large heat labile proteins (Chen and Hoover, 2003).

Bacteriocins produced by LAB are a heterogeneous group of peptides and proteins. The latest revised classification scheme divides them into two main categories: the lanthionine-containing lantibiotics (class I) and the non-lanthionine-containing bacteriocins (class II) while the large; heat-labile murein hydrolases (formerly class III bacteriocins) constitute a separate group called bacteriolysins (Cotter *et al*., 2005)

Lactococcal bacteriocins are produced by several species of *Lactococcus lactis* isolated from dairy, vegetable and meat products (Twomey *et al.,* 2002; Guinane *et al.,* 2005). Under favorable conditions, nisin has a wide spectrum of inhibition against Gram-positive microorganisms. It has been extensively characterized, the precise structure of its molecule and its mechanism of action having been determined (Cotter *et al.,* 2005). Although nisin is the only commercially exploited lantibiotic to date, efforts are being made to develop applications for other lantibiotics. Lacticin 3147, a two-peptide lantibiotic produced by *L. lactis* subsp. *lactis DPC3147* isolated from Irish kefir grains, exhibits a bactericidal mode of action against food spoilage and pathogenic bacteria (Ryan *et al.,* 1996).

The high heat stability and broad pH range of lacticin 3147 make it attractive for use in the food industry. Even though most lactococcal bacteriocins were isolated from dairy and vegetable products, several nisin-producing *L. lactis* strains were isolated from fermented sausages, indicating the potential use of lactococci in meat fermentation. Nisin-producing *L. lactis* strains from Spanish fermented sausages (Rodriguez *et al.,* 1995) and from traditional Thai fermented sausage (Noonpakdee *et al.,* 2003) were effective in inhibiting closely related LAB, *L. monocytogenes, C. perfringes, Bacillus cereus* and *S. aureus.* Moreover, *Lactobacillus sakei* L45 isolated from Norwegian dry sausages and *Lb. sakei* 148 from Spanish fermented sausages secrete lactocin S, a lantibiotic whose moderate spectrum of activity comprises LAB and *Clostridium* (Aymerich *et al.,* 1998). The

abundance of LAB strains producing lantibiotic bacteriocins suggests the significance of these substances in fermented products. These bacteriocins are present in different products and geographical environments.

Class II bacteriocins include a very large group of small (<10kDa) heat-stable peptides, which unlike lantibiotics are unmodified bacteriocins. Four different groups have been recently suggested (Cotter *et al.,* 2005): class Ila includes pediocin-like bacteriocins, class l1b two-peptide bacteriocins, class He cyclic bacteriocins and class IId non-pediocin like single linear peptides (Ennahar *et al*., 2000). Class IIa bacteriocins have a narrow spectrum of activity but display a high specific activity against *L. monocytogenes.* Pediocin-like bacteriocins can be considered as the major subgroup among non-lantibiotic peptides, not only because of their large number but also because of their significant biological activities and potential applications **(**Cotter *et al.,* 2005**).** Pediocin-like peptides contain between 37 and 48 amino-acid residues, which are involved in the N-terminal region of the conserved "pedio-cin-box" motif. The C-terminal domains are less conserved and are thought to determine the non-listerial antimicrobial spectrum. According to differences in the C-terminal, class IIa bacteriocins may be grouped into three subgroups. Bacteriocins that fit into these groups include pediocin PA-1/AcH, sakacin P and enterocin A (subgroup 1); leucocin A and mesentericin Y105 (subgroup 2); and curvacin A and carnobacteriocin B2 (subgroup 3) (Fimland *et al.,* 2005).

Pediocin PA-1 is produced by *Pediococcus acidilactici* isolated from American-style sausages and *Pediococcus pentosaceus* Z102 from Spanish-style sausages. Sakacin A is produced by two different *Lb. sakei* strains (Lb706 and CTC494) and *Lactobacillus curvatus* LTH1174, isolated from fermented sausages. Enterocin A is produced by *Entrococcus faecium* CTC492 isolated from Spanish dry fermented sausages. These three bacteriocins were reported to be active against other LAB, *L. monocytogenes* and *Clostridium* (Aymerich *et al.,* 1998). In addition, *Lc. gelidum* and *Lc. mesenteroides* isolated from chill stored vacuum-packaged meat secrete leucocin A and mesentericin Y105 respectively, which are inhibitory

against LAB, *L. monocytogenes* and *Enterococcus faecalis* (Hechard and Sahl, 2002). *Lc. mesenteroides* LI24 and *Lb. curvatus* L442 isolated from dry fermented sausages also exhibit a strong anti-listerial activity (Mataragas *et al.,* 2002). Due to their high anti-listerial potential, these bacteriocin producer-LAB are of considerable interest as biopreservative cultures.

The two-peptide bacteriocins (class lIb) require the combined activity of both peptides to exert their antimicrobial activity. Each peptide displays very low activity, if any, when tested individually, most of these bacteriocins requiring a 1:1 peptide ratio for optimal bactericidal effect (Garneau *et al.,* 2002). Lactococcin G and lactacin F produced by *L. lactis* and *Lb. johnsonii*, respectively, were isolated and characterized in the early 1990s (Garneau *et al.*, 2002). These bacteriocins of dairy origin are the first reported two-peptide bacteriocins with a narrow spectrum of inhibition against other lactobacilli, *E. faecalis and Clostriduim.* The best characterized two-peptide systems are plantaricins EF/JK and plantaricin S produced by *Lb. plantarum* CM and LPCO10 respectively, of vegetable origin (Andersen, 1995). On the other hand, lactocin 705 secreted by *Lb. curvatus* CRL705 (formerly *Lb. casei),* isolated from Argentine fermented sausages, is the first two-peptide bacteriocin reported from a meat-associated strain. Lactocin 705 showed to be antagonistic toward other LAB and *B. thermosphacta* when assayed in meat systems (Castellano *et al.,* 2004; Castellano and Vignolo, 2006). Class He bacteriocins comprise a few examples of cycle peptides while class Hd includes the remaining isolated antimicrobial substances. Bacteriocins from Class I and II are among the best biochemical and genetically characterized antimicrobial peptides and the most likely to be used in food applications due to their target specificity.

2.6 Biogenic Amines

Biogenic amines (BA) are organic bases with aliphatic, aromatic or heterocyclic structures that can be found in several foods, in which they are mainly produced by microbial decarboxylation of amino acids, with the exception of physiological polyamines (Silla Santos**,** 1996**)**. Biogenic

amines are compounds commonly present in living organisms in which they are responsible for many essential functions. They can be naturally present in many foods such as fruits and vegetables, meat, fish, chocolate and milk, but can also be produced in high amounts by microorganisms through the activity of amino acid decarboxylases (Suzzi and Gardini, 2003).

BA accumulation in foods requires the availability of precursors (amino-acids), the presence of microorganisms with amino acid decarboxylases and favourable conditions for their growth and decarboxylating activity (ten Brink *et al*., 1990). Arnino acid decarboxylation can have an important energetic role in nutritionally poor environments. In fact, bacterial decarboxylation systems can generate a translocation of charge across the cytoplasmiatic membrane, influencing the membrane potential (Konings *et al.*, 1997).

2.7 Different types of Biogenic Amines

In general, histamine, putrescine, cadaverine, tyramine, 2-phenylethylamine, spermine and spermidine are the most important BA in foods (Shalaby, 1996). Amino-acid decarboxylases are enzymes present in many microorganisms of food concern. Many lactic acid bacteria belonging to the genera. *Latobacillus, Enterococcus, Carnobacterium, Pediococcus, Lactococcus* and *Leuconostoc* are able to decarboxylate amino-acids (Bover-Cid and Holzapfel, 1999; Lonvaud-Funel, 2001). Excessive consumption of these amines can be of health concern. The degrees of diseases determined by their action on nervous, gastric and intestinal systems and blood pressure. (Suzzi and Gardini, 2003).

Biogenic amines are mainly produced by the decarboxylation of certain amino-acids by microbial action. Since the ability of microorganisms to decarboxylate amino-acid is highly variable, being in most cases strain-specific, the detection of bacteria processing amino acid decarboxylase activity is important to

estimate the risk of biogenic amine food content and to prevent biogenic amine accumulation in food products. Molecular methods for the early and rapid detection of microorganisms are becoming an alternative to traditional culture methods. PCR methods offer the advantage of speed, sensitivity, simplicity and specific detection of amino-acid decarboxylase genes. Moreover, these molecular methods detect potential biogenic amine risk formation in food before the amine is produced (Landete *et al*., 2007).

Several toxicological problems resulting from the ingestion of food containing relatively high levels of biogenic amines have been reviewed (ten Brink *et al*., 1990; Vidal – Carou *et al*., 1990). Histamine and tyramine have been the most studied biogenic amine due to toxicological effects derived from their vasoactive and psychoactive properties. Histamine has been recognized as a causative agent of scombroid poisoning (histaminic intoxication), whereas tyramine has been related to food-induced migraines and hypertensive crisis in patients under anti depressive treatment with mono-amine oxidase inhibitor (MADI) drugs. Alcohol and other biogenic amines, such as the diamines, putrescine and cadaverine, may boost the toxicity of the above amines. In addition, diamines are known to be potential precursors of carcinogenic nitrosamines, especially when nitrosable agents are present in food. Apart from these toxicological aspects, Biogenic amines are of concern in relation to food hygiene. The occurrence and relatively high levels of certain biogenic amine has been reported as indicators of deterioration process and or defective elaboration (Karmas 1981; Vidal – Carou *et al*., 1990).

2.8 Microbes associated with Meat Products

Numerous reviews have suggested that pathogens/ microorganisms in meat or other fermented foods may be inhibited by some bacteriocin-producing meat LAB (Foegeding *et al*., 1992; Laukova *et al.*, 1999; Zhao *et al*., 2002; Tyopponen *et al*., 2003) focused on the application of bacteriocin-producing LAB to control the growth of pathogenic microorganisms in biofilms i.e., the real mode of microbial attachment to processing surfaces and equipment.

An increased incidence of recent outbreaks of illness as well as many challenge studies have shown that important meat-borne pathogens, such as *Escherichia coli* 0157:H7, *Salmonella* and *Listeria monocytogenes* may survive and pose a health risk in dry fermented sausages (Farber *et al*., 1993; CDC, 1995; Sauer *et al*., 1997; Cosansu and Ayhan, 2000; Bremer *et al*., 2004; Moore, 2004). The ubiquitous nature of *Listeria monocytogenes,* its hardiness and ability to grow at refrigeration temperatures and anaerobic conditions makes this well recognized pathogen a threat to the safety of public health. It is regarded as a major food safety problem because it can cause serious illnesses and death (McLauchlin *et al*., 2004). It has been detected in a variety of foods (Farber and Peterkin, 1991) particularly in meat products (Samelis and Metaxopoulos, 1999).

Consumers are drawn to natural foods with no chemical preservatives added. This demand has stimulated research interest in biopreservation that refers to the use of antagonistic microorganisms or their metabolic products to inhibit undesired microorganisms in foods to enhance food safety and extend shelf-life (Schillinger *et al*., 1996). The antibacterial proteinaceous molecules referred as bacteriocins produced by lactic acid bacteria (LAB) may be considered as promising biopreservatives (Chen and Hoover, 2003; Cotter *et al*., 2005). Among the bacteriocins active againt *Listeria,* the (antibiotic nisin has been widely studied and is currently used, in many countries, as preservatives in food products (Schillinger *et al*., 1996; Cleveland *et al*., 2001; Chen and Hoover, 2003). Another group of anti-listerial peptides forms a subclass known as the *anti-Listeria* bacteriocins, alternatively named class IIa bacteriocins (Cleveland *et al*,, 2001; Ennahar *et al*., 2000 ; Chen and Hoover, 2003) which are characterized by the consensus sequence YGNGV(X)C(X)4C (X)V(X)4A (where X represents any amino acid). Numerous reviews have suggested that some LAB were able to control the growth of some pathogenic microorganisms such as *L. monocytogenes* in food products (Callewaert and De Vuyst, 2000; Tantillo *et al*., 2002 ; Mataragas *et al*., 2003). These food-borne LAB have been described as bacteriocin producers.

Lactic acid bacteria (LAB) are the prevailing microorganisms on chill-stored fresh meat packaged under vacuum in modified atmosphere with increased CO_2 (Egan, 1983; Dainty and Mackey, 1992). Development of a lactic microflora markedly extends the storage life of meats packaged in this way, but actual extension of storage life depends on several factors, including the type(s) of LAB found on the meat (Shay and Egan, 1991; Borch and Agerhem, 1992; Dainty and Mackey, 1992). The genera of LAB most frequently encountered on vacuum or modified atmosphere packaged meat are *Lactobacillus*, *Leuconostoc* and *Carnobacterium* (Shaw and Harding, 1984; Dainty and Mackey, 1992; McMullen and Stiles, 1993). A widely used practice in meat marketing is the vacuum packaging of primal cuts for distribution and extended storage followed by removal of the meat from the package for preparation of retail cuts. The retail cuts are usually marketed in an oxygen-permeable film for display in an open, refrigerated cabinet to facilitate consumer selection. The bacteriological implications and the role of LAB in this transition from an anaerobic environment to aerobic storage have been examined for the natural microbial flora growing on pork (Greer *et al*., 1993) and after the inoculation of beef steaks with *Lactobacillus* cultures (Smith *et al*., 1980). Both studies showed that LAB may contribute to undesirable sensory changes of meat during aerobic storage.

Salmonella prevalence in beef carcasses at different stages of the beef meat production as well as trimmings was reported to be in the range of 3-12% (Barkocy-Gallagher *et al.,* 2003; Ghafir *et al.,* 2005) while a lower detection rate (0-0.2% of beef carcasses) was obtained in a recent national survey in Australia (Philips *et al.*, 2006). When the occurrence of *Salmonella* in pig slaughter-houses was investigated, the incidence of contamination showed a high variability with an average of 1.7% to 12% for pork carcasses, a higher incidence in environmental samples having been reported (Hald *et al.,* 2003; Pala and Sevilla, 2004). Some tissues (lymph nodes and tonsils) were also involved in *Salmonella* spread during pig slaughter, indicating an increased risk for pork meat contamination (Vieira-Pinto *et al.*, 2005).

Food poisoning staphylococci are widely distributed; meat contamination being generally associated with highly manual-handled foods. The most common etiological agent is *Staphylococcus aureus* and its related heat-stable enterotoxins (Balaban and Rasooly, 2000). Studies in slaughtered pigs and bovines showed that *S. aureus* was the pathogenic bacterium most frequently detected in slaughter houses and processing rooms. Although the microbiological quality of beef is highly dependent on the hygiene of slaughter-houses, dressing operations and processing lines, where workers' hands and the environment of locations associated with the evisceration process are the principal sources (Desmarchelier *et al.*,1999; Shale *et al.*, 2006). In addition, the presence of staphylococci in bioaerosols from red-meat abattoirs as well as bovine mastitis in dairy cows may also constitute a risk of foodborne pathogen contamination (Pitkala *et al.*, 2004; Shale *et al.*, 2006). Pig carcasses are often important sources of contamination with *Staphylococcus aureus* mainly due to the sequential steps of the slaughter, which involve scalding, dehairing, polishing, trimming, washing, chilling and cold chain systems for transportation and merchandising (Spescha *et al.*, 2006). Time and temperature abuse of a food product contaminated with enterotoxigenic staphylococci can result in enterotoxin formation. The occurrence of enterotoxigenic *S. aureus* in foods of animal origin was reported to be extremely variable, ranging from 6% to 90% (Blaiotta *et al.*, 2004; Holeckova *et al.*, 2004). Determination of the occurrence of staphylococcal enterotoxins by means of molecular techniques improves the possibility of tracing the enterotoxigenic strain sources and setting up preventive strategies (Blaiotta *et al.*, 2004).

Enterohemorrhagic *E. coli* shiga toxin-producing (STEC) has emerged as a foodborne pathogen more significantly than other well-known ones because of the severe consequences for humans, its low infection dose, its unusual acid tolerance and its apparent special but inexplicable association with ruminants used for food (Tarrant, 1998). This pathogen causes serious complications in humans such as hemorrhagic colitis and hemolytic uremic syndrome (Griffin and Tauxe, 1991). The increased prevalence of *E. coli* 0157:H7 in foods may be

associated with the consolidation of the beef industry into fewer but larger production and processing units. Countries such France, the United Kingdom, the United States, Canada, Japan, Australia and Argentina have tested beef carcasses and/or beef products for the presence of *E. coli* 0157, confirming that cattle are the major reservoir of STEC pathogenic for humans. Global testing of beef cattle feaces revealed wide ranges of prevalence rates for 0157 STEC (0.2 – 27.8%) with a high incidence of hemolytic uremic syndrome (Omisakin *et al*., 2003; Andral *et al*., 2004; Blanco *et al*., 2004; Gleeson *et al*., 2005; Hussein and Bollinger, 2005). In particular, young beef steers from the main beef-producing area of Argentina have been reported to be important reservoirs of STEC strains; however, its importance as agents of human diseases has still to be established (Meichtri *et al.*, 2004). Ojo et al., (2010) documented this organism in Nigeria.

L. monocytogenes has continued to raise food safety concerns for over two decades, especially with respect to ready-to-eat products. *Listeria* is a significant public health pathogen because of its clinical severity and high mortality rates, worldwide foodborne outbreaks in which meat products were implicated have been taking place during the last two decades (Okutani *et al.*, 2004; Mead *et al*., 2005; Vaillant *et al.*, 2005). Due to its ubiquitous character, *L. monocytogenes* can grow at temperatures ranging from 1 to 45^{o}C, at pH 4.6 - 9.6, in the presence of high salt concentrations, and can survive attached to processing equipment by the formation of biofilm, which could be a potential contamination source (Autio *et al.*, 1999).

Several investigators have reported higher incidences of *Listeria spp.* on ground meat than on carcasses or boneless meat cuts. Its prevalence, mainly due to cross-contamination, has increased from the farm to the manufacturing plants (Lin *et al.*, 2006; Thevenot *et al.*, 2006). The slaughter-house environment (cutting boards, knives and equipment) constitutes the primary source of *Literia*-contaminated meat products. Listerial contamination by human contact (hands and gowns of slaughterhouse workers) probably

accounts for part of the environmental contamination (Peccio *et al.,* 2003; Marzoca *et al.,* 2004). High average values for *Listeria spp.* and *L. monocytogenes* (73% and 75%, respectively) were reported in frozen beef (Hassan *et al.,* 2001). Although many risk assessment strategies have been developed to control *L. monocytogenes* in foods (Chen *et al.,* 2003; Salvat and Fravalo, 2004; ILSI Research Foundation; Risk Science Institute.

Species within the genus *Campylobacter* and *Yersinia* have also emerged as pathogens of human public health concern (CDC, 2002; Moore *et al.,* 2005). *Campylobacter* is the most common cause of human foodborne illness in the United States, this being related to its high degree of virulence and its widespread prevalence in foods of animal origin. *Campylobacter* has been isolated from beef at retail sale, indicating that beef can be a potential vehicle for the transmission of this pathogen to humans (Kramer *et al.,* 2000). *C. jejuni* can be transferred from hides to meat during slaughter and dressing of beef carcasses (Inglis *et al.,* 2005). Even after high initial carcass prevalence, chilling showed to be an efficient critical control point to eliminate *Camplylobacter* from carcass surfaces (Pearce *et al.,* 2003; Pezzotti *et al.,* 2003). A high prevalence of antimicrobial resistance was frequently observed in *Campylobacter* strains, *E. coli* being generally more, resistant than *C. jejuni* (Pezzotti *et al.,* 2003). On the other hand, *Yersinia enterocolitica* is frequently associated with pigs and pork products and can be transmitted to humans through the consumption of raw, undercooked or recontaminated processed meats. The prevalence of *Y. enterocolitica* in pig herds has been reported to range between 40% and 65%, tonsils and oral cavity being important reservoirs (Bhaduri *et al.,* 2005; Gurtler *et al.,* 2005).

2.9 Other Pathogens associated with Meat Products

Other pathogens of human health concern that may be present but remain undetected in slaughtered animals include streptococci, clostridia and corynebacteria. Among these, *Clostridium perfringes* is the leading cause of bacterial foodborne illness in countries where meat and poultry consumption is high.

Meat animals are subjected to a number of clostridial diseases and these bacteria may be present in their carcasses. Enterotoxigenic type A strains carrying a chromosomal *cpe* gene, necessary for food poisoning, have been strongly associated with food poisoning outbreaks (Wen and McClane, 2004). In a study carried out in England and Wales, the consumption of red meats was implicated in infectious intestinal outbreaks in which *C. perfringes* was the most frequently reported organism. A decrease in the number of cases linked to foods containing red meat is in agreement with the steady decline in red meat consumption (Smerdon *et al.*, 2001).

After slaughter and dressing of carcasses, bacterial growth will depend on storage conditions. During storage, environmental factors such as temperature, gaseous atmosphere and meat pH will select certain bacteria for growth.Cold storage of meat will decrease bacterial growth, only "10%" of the bacteria initially present being able to grow at refrigeration temperatures **(**Jones, 2004**)**. Oxygen restriction using vacuum or modified atmospheres will drastically reduce the presence of *Pseudomonas* while bacterial flora will be gradually selected towards CO_2-tolerant organisms. Under these conditions, the dominating microorganisms are *Brochothrix thermosphacta,* lactic acid bacteria (LAB), mainly *Lactobacillus, Leuconostoc* and *Carnobacterium* (Fontana *et al.*, 2006; Jones, 2004). The presence of *Enterococcus* in beef, poultry and pork carcasses or fresh meat, indicating fecal contamination during slaughter, has also been reported. Both beneficial and detrimental roles in foods have been ascribed to *Enterococcus.* They play an important role in the development of flavor in traditional cheeses and sausages and, as probiotics, can provide important health benefits. As detrimental organisms-they have been implicated in outbreaks of foodborne illness through their antibiotic resistance and as carriers of virulence factors. The prevalence of antibiotic resistance in streptococci and enterococci isolated from the production chain of swine commodities has been extensively reported (Houben, 2003; Rizzotti *et al.*, 2005; Sapkota, *et al.*, 2006).

According to a complete genome analysis, it was proposed that *L. sakei* can be used to control pathogens in meat because its metabolism is particularly well adapted to a meat medium (Chaillou *et al*., 2005). *Lactobacillus* species represent the LAB strains currently found in meat starter cultures (Tyopponen *et al*., 2003), Moreover, *L. sakei* and *Lactobacillus curvatus* isolates from meat often contain bacteriocinogenic strains. Up till now, several bacteriocins are known to be produced by *L. sakei* strains: sakacin A (Schillinger and Lucke, l989), sakacin M (Sobrino *et al*., 1992), SakacinP (Ttchaczek *et al*., 1994), sakacin674 (Hoick *et al*., 1994), sakacinB (Samelis *et al*., 1994), sakacin K (Hugas *et al*., 1995), Uctocin S (Skaugen *et al*., 1997), bavaricin MN (Kaiser and Montville. 1996). Sakacin T (Aymerich *et al*., 2000), sakacin G (Simon *et al*., 2001), sakacin X (Vaughan *et al*., 2003) and sakacin Q (Mathiesen *et al*., 2005). AH sakacins possess strong antilisterial activity. Also, three bacteriocins produced by *L curvatus* strains have been found: curvacin A (Tichaczek *et al*., 1993), curvaticin 13 (Sudirman *et al*., 1993), and curvaticin FS47 (Garver and Murijma, 1994). Curvacin A produced by *L. curvatus* LTH 1174 is identical to sakacin K produced by *L. sakei* CTC 494 and to sakacin A from *L. sakei* Lb 706 (Axelsson and Holck, 1995; Aymerich *et al*., 2000; Leroy and De Vuyst. 2005). Similarly, sakacin M was demonstrated to be identical to the lantibiotic lactocin S (Rodriguez *et al*,, 1995).

2.10 Recent Approaches in Meat Preservation

Recent approaches in the preservation of meat products are increasingly directed toward biocontrol using bacteriocinogenic *Lactobacillus* species as protective microflora to inhibit growth of *L monocytogenes* and other undesired microorganisms (Schillinger *et al*., 1996; Bredholt *et al*., 2001; Hugas *et al*., 2003; Castellano *et al*., 2004; Vermeiren *et al*., 2004). Moreover, numerous studies have been conducted on the environmental conditions and medium composition requirements for optimized bacteriocin production by the *Lactobacillus* species. Most of these studies indicate that the highest bacteriocin titre was obtained at pH and temperatures values lower than the optima for growth (Abildgaard *et al*., 1995; De Vuyst *et al*., 1996; Krier *et al*., 1998; Bogovic-

Matijasic *et al*., 1998; Aasen *et al*., 2000; Messens *et al*., 2002; Mataragas *et al*., 2003; Todorov and Dicks, 2005).

As lactic acid bacteria are fastidious with respect to nutrient requirements, a rich medium with yeast extract and protein hydrolysates was reported to be required for optimal growth and bacteriocin production (De Vuyst *et al*., 1996; Aasen *et al*., 2000; Todorov and Dicks, 2005). However, the use of rich, ideal media to simulate food products can lead to an overestimation of the growth of nutrient demanding microorganisms such as *Lactobacillus* species. As an example, it was shown that the MRS broth, which was used in most of these studies, contains higher levels of manganese than cooked ham which has been demonstrated to stimulate growth of *L. sakei* (Devlieghcre *et al*., 1998).

Bacteriocinogenic *L. sakei 2512* was first isolated from the screening of an industrial collection of LAB strains using a luminescent target strain (Simon *et al*., 2001). This strain was shown to produce a class IIa bacteriocin, named sakacin G, which was characterized by partial sequencing of the purified bacteriocin and subsequent gene cloning and sequencing (Simon *et al*., 2002). Two copies of the structural gene of this peptide were found located on a 35 kb plasmid. In the present study, the effect of this strain as a protective culture toward *Listeria* was evaluated using challenge tests on sliced cooked ham. A new liquid medium was designed in order to simulate meat and was used to evaluate the inhibition of *Listeria* by bacteriocinogenic *Lactobacilli* and to screen a collection of LAB strains for their antilisterial activity. Two new bacteriocinogenic strains were detected from meat isolates. One of these strains was demonstrated to produce sakacin X and probably sakacin T. Two novel bacteriocins produced by the' other strain were purified and partially characterized (Simon *et al*., 2001).

2.11 Molecular Typing methods

Various molecular typing methods such as restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), ribotyping and PCR-derived

techniques such as repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (RIC-) PCR have been used to distinguish between isolated bacterial biotypes. Moreover, RAPD analysis has been used to estimate the diversity among several genera of bacteria such as *Lactobacillus, Bacillus,* and S*taphylococcus* isolated from many sources and 16S rDNA sequence analysis has made the taxonomic identification of biotypes feasible (Morea *et al*., 1998; Rebeechi *et al*., 1998; Morea *et al*., 1999).

Randomly amplified polymorphic DNA (RAPD), partial rDNA sequence analysis, and physiological assays were used to characterize the growth and the changes of dominant microbial populations during curing. The physiological characterization of the strains contained in this typical sausage such as acid production, proteolytic and lipolytic activities and nitrate reduction was carried out to gain an insight into the role played by microbial strains belonging to different genera in meat fermentation and curing. Phenotypic methods relying on physiological or biochemical criteria have been widely used for LAB identification (Montel *et al*., 1991). In order to overcome the tediousness, ambiguousness, and time consumed by these methods, molecular methods such as rRNA hybridization probes (Nissen and Dainty, 1995), species-specific PCR (Kwon *et al*., 2004; Aymerich *et al*., 2006). PCR- denturing gel electrophoresis (Cocolin *et al*., 2004) real-time PCR (Furet *et al*., 2004) have been developed for LAB species identification. Randomly amplified polymorphic DNA (RAPD)-PCR analysis has been used to estimate the biodiversity among LAB (Aymerich *et al*., 2006).

Lactobacillus sake, L. curvatus, L. plantarum, Lactobacillus pentosus, Lactobacillus casei, Pediococcus pentosaceus and Pediococcus acidilactici are the speices most used as commercial meat LAB starter cultures (Hammes and Hertel, 1998; Hugas and Monfort, 1997). In meat fermentations, the main function of LAB is to obtain a rapid pH drop of the batter, which in turn favours (i) product safety by inactivating pathogens, (ii) product stability and shelf life by inhibiting undesirable changes caused by spoilage microorganisms or abiotic reactions, and (iii) creates the biochemical conditions to attain the new sensory properties of the ripe products

through modification of the raw materials (Lucke, 2000). Currently the use of starters as functional flora is gaining importance; designed starter cultures have properties additional to those of the more classic type, helping to optimize the sausage fermentation process and to produce tastier, safer, and healthier products.

With the increasing demand for biological preservation techniques the application of lactic acid bacteria (LAB) as starter or protective cultures is gaining interest (Holzapfel *et al.*, 1995). Some LAB show special promise as they do not pose any health risk to man and are able to prevent the outgrowth of undesirable bacteria and opportunistic pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes*.

2.12 Consumers Concern about Meat Products

Consumers may be more concerned about safety in food than in any other products, including medicines (Prendergast, 1997). Awareness of the consequences of the meat-borne pathogen *Escherichia coli* 0157:H7 has increased in the general public opinion, making this organism a household name in the 21st century (Ransom *et al*., 2003). Undoubtedly the major threat to food safety is the emergence of "new" pathogens. The recent role of *Listeria monocytogenes*, *E. coli* 0157:H7, *Campylobacter jejuni, Yersinia enterocolitica* and *Vibrio parahemolyticus* as foodborne microorganisms have been associated with the increase of outbreaks compared to traditional food pathogens (Elmi, 2004; Mead *et al*., 2005; Moore *et al*., 2005). Changes in the food chain will continue to create opportunities for the emergence of new diseases and the re-emergence of old ones. Since pathogens do not recognize national boundaries, the rapidity with which individual microorganisms can circumnavigate the globe spreading infections, makes the control of communicable diseases an enormous challenge for governments as well as for the public and primary health care systems.

The manufacture of an increasing range of novel meat products as functional foods and the inclusion of ingredients considered beneficial for health (Fernandez-Gines *et al*., 2005) may also pose additional dangers with respect to

safety. Additionally, the presence in meat products of chemical additives and residues of agrochemical and veterinary drugs is also perceived by consumers as a health risk. Even when the level of these residues seldom exceeds the regulatory limits in meat products (Tarrant, 1998), the use of antibiotics in intensive animal production poses the additional risk of bacterial resistance, which constitutes a microbiological hazard rather than a strictly chemical residue.

Consumers' concerns about the freshness of meat are continually increasing. Reliable methods for assessing the microbiological quality and/or freshness of meat would benefit both consumers and the meat industry. Traditionally, shelf-life studies of perishable meat and meat products are carried out by evaluating the microbiological and sensory quality of the product as a function of storage time. Because traditional methods (based on direct microbial analysis) are costly and time-consuming, alternative methods, involving chemical changes due to microbial growth, have also been suggested as quality indicators of meat (Dainty, 1996).

2.13 Other Benefits of LAB

Lactic acid bacteria (LAB) have a major potential for use in bio-preservation because they are safe to consume, during storage they naturally dominate the microbiota of many foods (including vacuum-packaged meat and meat products), and they are also able to inhibit undesirable microorganisms, such as *L. monocytogenes* (De Martinis *et al*., 2002; Sakala *et al*., 2002). Purified bacteriocins from LAB may also offer a promising solution to improve the safety of food products and to extend their shelf life (Nilson *et al*., 1999).

The use of protective cultures in meats is more acceptable to both producers and consumers than the addition of semi-purified bacteriocins (Buncic, 1997). Bacteriocin activity may be reduced by its binding to food components, by action of proteases and other enzymes and by an uneven distribution in the food system (Schillinger *et al*., 1996; Lucke, 2000). Bacteriocin production may also

be influenced by several factors, such as pH, temperature as well as and salt and ethanol concentrations (De Vuyst *et al*., 1996; Leroy and De Vuyst, 1999).

Sensory and microbiological analyses are most often used to evaluate the freshness or spoilage of meat and meat products. The disadvantages of sensory analysis, which is probably the most acceptable and appropriate method, is its reliance on highly trained panelists, which makes it costly and impracticable for routine analysis. On the other hand, microbiological analysis, either traditionally (total viable counts) or with the use of molecular tools (real time PCR, DGGE) are often misleading and it is more meaningful to measure the microflora fraction causing spoilage (Nychas *et al*., 2007). Unfortunately, microbiological analyses are lengthy (traditional, conventional microbiology) or costly and high-tech (molecular tools), and destructive; therefore, efforts have been made to replace both microbiological and sensory analyses with biochemical changes occurring in muscle (e.g. various microbial metabolic products, termed as Chemical Spoilage Indices – CSI), that could be used to assess meat spoilage (Huis in't Veld, 1996).

2.14 The Genus *Pediococcus*

Pediococci are Gram-positive, facultative cocci belonging to the group of lactic acid bacteria. Taxonomically, the genus is clustered within the *Lactobacillus casei* – *Pediococcus* group. The genus consists of seven species, *Pediococcus acidilactici, Pediococcus cellicola, P. clausenii, P. damnosus, P. dextrinicus, P. inopinatus, P. parvulus* and *P. pentosaceus.* (Zhang *et al.,* 2005). Two species *P. acidilactici* and *P. pentosaceus,* have been widely used for fermentation of vegetables, meats, silage and for cheese production (Simpson and Taguchi, 1995; Simpson *et al.,* 2002). Furthermore, they have been used as probiotics or biological growth promoters in animal feed. (Reuter, 1997). Other species like *P. damnosus* and *P. clausenii* are often found as beer spoilage bacteria (Barney *et al.,* 2001; Dobson *et al.,* 2002).

Pediococcus are homofermentative LAB commonly found in a variety of plant materials that are used industrially in food fermentations (Graham and Mckay, 1985; Giacomini *et al.*, 2000). A number of species of *Pediococci* including *P. pentosaceus*, have been the subject of study because of their plasmid contents and/or their ability to produce *Pediocins* (Graham and Mckay, 1985; Kim *et al.*, 1992; Giacomini *et al.*, 2000; Osmanagaoglu *et al.*, 2000; Ramesh *et al.*, 2000; Alegre *et al.*, 2009).

2.15 Plasmids

Lactobacilli generally appear to contain multiple plasmids which can vary in size from 1.2 to 169 kb (Mayo *et al.*, 1989). Generally, 1-10 plasmids have been found in *lactobacilli*, and in the case of *Lactobacillus plantarum* LPC25, 16 plasmids have been identified (Ruiz-Barba *et al.*, 1991b). *Lactobacillus* plasmids were first isolated from *Lb. casei* (Chassy *et al.*, 1976) and then from a variety of other lactobacilli while 23 have been sequenced to date, many still remain cryptic with regard to their role in cellular performance and functioning. A few plasmid-encoded functions have been discovered and applied to vector construction strain identification, detection, and modification (Wang and Lee, 1997). According to Wang and Lee (1997) *Lactobacillus* plasmid functions can be divided into four main groups:

hydrolysis of proteins, metabolism of carbohydrates, amino acids and citrate, production of bacteriocins, exopolysaccharides and pigments resistance to antibiotics, heavy metals and phages.

The first *Lactobacillus* strain to be efficiently and reproducibly transformed was *Lb. casei*, with the vector pSA3 by Chassy and Flickinger (1987). Since then, many other strains of lactobacilli have been successfully transformed with different electroporation protocols, Kullen and Klaenhammer (2000) reported that the plasmid vectors most widely used for lactobacilli are of three types:

Plasmids based on rolling circle replication (PCR) replicons; Plasmids with two origins of replication (one for *Escherichia coli* and a second for gram-positive bacteria), and *Lactobacillus* vectors with an alternative replication origin for gram-negative bacteria.

This growing interest in the characterization of *Lactobacillus* replicons themselves as potential useful vectors, has for example, led to the development of a *Lactobacillus, E. coli* shurtle vector that was designed based on the replicon of the *Lactobacillus fermentum* plasmid (Pavlova *et al.,* 2000). This vector was transformed and stably maintained in several *Lactobacillus* strains and was used to successfully express the s-layer proteins gene of *Lactobacillus acidophilus* in a heterologous *Lactobacillus* strain. In a more recent study, a derivative of pRV 500 from *Lactobacillus sakei* was constructed that carried the pRV 500 replicon (Alpert et al., 2003). This vector was also found to be maintained at a reasonable rate over 20 generations in several lactobacilli making this plasmid another potentially useful tool for different applications in lactobacilli (Alpert *et al.,* 2003), genetic engineering using these plasmid vectors has also led to the development of lactobacilli designed for therapeutic purposes, such as the delivery of antigens like the B subunit of cholera toxin, x-amylase, or an epitope from human immunodeficiency virus (HIV) at the mucosal surface (Perdigon *et al.,* 2001).

Lactobacillus paracasei NFBC338 is a human probiottic strain that was originally isolated from the GIT, and has since been used for the manufacture of Cheddar cheese (Gardiner *et al.,* 1998; Stanton *et al.,* 1998) and spray-dried powders (Desmond *et al.,* 2001, 2002; Gardiner *et al.,* 2000). Due to the commercial importance of this strain, the plasmid complement was isolated and by a combination of sequence and restriction digests analysis found to amount to approximately 88.643 bp of DNA.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

Samples of beef, chicken and turkey used in this study were obtained from Retail outlets in Ekiti, Oyo and Lagos States respectively in South Western Nigeria. The samples were collected from three different sources in each State. They were brought to the Microbial Physiology and Biochemistry Laboratory of the Department of Microbiology, University of Ibadan in sterile containers with icepacks for immediate treatment.

3.1.1 Treatment of Samples

The samples (beef, chicken and turkey) were subjected to refrigeration, chilling, freezing and fast freezing treatments. The samples were packed in sterile polyethylene bags. The treatments were carried out using a blast freezer (50 horsepower, Presco Model), Deep freezer (Ariston, Model No. PG504), refrigeration and chilling using Haier Thermocool (Model No. HR 137) for 28days.

3.2 Isolation Medium for Cultivation of Lactic Acid Bacteria

The medium used for cultivation and isolation of *Lactobacilli* strain was de Man Rogosa and Sharpe (MRS) Agar (de man *et al*., 1960). Components of the isolation medium (Appendix I) were weighed into a 500ml Erlenmeyer flask containing 500ml of distilled water. The solution in the flask was homogenized for 10 minutes to completely dissolve the components of the medium. The pH was adjusted to 5.5 for the purpose of this study. On each occasion, the medium was sterilized at 121^{o}C for 15 minutes before cooling.

3.2.1 Isolation and Culture Methods

3.2.2 Isolation from Collected Samples

Isolations were made from the samples using the pour plate method (Harrigan and McCance, 1966). Ten grams (10g) of each sample were weighed and

homogenized in 90ml sterile distilled water using a Stomacher homogenizer (Lab Blender Model 80, Seward Medical London) for 30s at 'normal' Speed. Ten milliliters of sterile pipette was used to transfer the homogenized solution into 90ml of sterile distilled water, to make a dilution of 1:100. The dilution was repeated serially up to 10 fold. Thereafter, the higher dilutions of various samples were pipetted and mixed with molten MRS medium in Petri dishes. The plating was done in duplicate. The plates were swirled round for even distribution of inoculum and setting of agar. After solidifying, the Petri dishes were incubated in anaerobic jar using H_2 and CO_2 generating kits (Mecrk Anaerocult type A).

At the end of the incubation period, the isolates were subcultured and repeated streaking was done to obtain pure cultures. The colonial morphology and cellular characteristics of the various colonies obtained were studied.

3.2.3 Maintenance of Pure Cultures

The isolated *Lactobacilli* were subcultured into maintenance medium consisting of MRS broth with 12% glycerol (v/v) and incubated at 37^{o}C until growth became visible. The stock cultures were stored at 4^{o}C pending subsequent use for a period of two to four weeks before subculturing into fresh maintenance medium.

3.3 Identification Procedures

3.3.1 Morphological Characterization

The shape, form and cell arrangement of probable isolates were elucidated under the light microscope with an oil immersion lens. Based on the observation, the acid – forming microorganisms were then eventually grouped according to cell shape, as cocci and rods.

3.3.3.1.1 Macroscopic Examination

Pure isolates of LAB were grown on MRS agar plates. Various characteristics (colour, opacity, elevation, margin, shape, size and cellular characteristics) were observed by the naked eyes in order to aid their identification.

3.3.1.2 Microscopic Examination

Microorganisms were observed under the microscope by preparing smears on a grease-free slide. The slide of specimen was placed on the microscope stage and fixed with the clips, and viewed under the oil immersion objective by turning the oil immersion lens gently into position until the cells were brought into position.

3.3.1.3 Simple Staining

This is a direct staining in which the organism is stained by immersion. The heat fixed smear of the culture on the slide was placed on the staining rack and flooded with the appropriate stains as in 3.3.1.4

3.3.1.4 Gram Staining

The method of Pelczar and Chan (1977) was used. A sterile wire loop was used to slightly touch a colony of each isolate on the subcultured plate and emulsified in a drop of water on a grease free clean slide, to make a thin smear. The smear was then air-dried, heat-fixed and stained. The smear was flooded with 2% Crystal violet stain and left for 60 seconds, after which it was drained quickly. Two drops of Gram's iodine solution was then added to act as a mordant. The solution was left for 60 seconds on the slide and washed off under tap water. 95% ethanol was used to wash the slide until it appeared free of the crystal violet stain after which it was rinsed and flooded with 4.2% Safranin for 30 seconds. The stained smear was blotted dry and examined microscopically under oil immersion objective to determine the Gram reaction and cellular characteristics.

3.3.2. Biochemical Characterization of Isolates

Various tests were carried out on the bacterial isolates for possible identification. A 24-hour old culture was used for every biochemical test carried out except otherwise stated.

3.3.2.1 Catalase Test

The method of Seelay and Van Demark (1972) was used for this test using 18 hour - old cultures. A sterile wire loop was used to touch a colony of isolate on MRS agar plate and transferred into a drop of 3% H_2O_2 (Hydrogen peroxide) on a clean side and the reaction was observed. Evolution of gas as white froth indicated a catalase positive reaction while the absence of froth indicated a negative result.

3.3.2.2 Oxidase Test

Whatman number 1 filter paper was used for this test. By means of a sterile wire loop, few drops of oxidase reagents were applied on the Whatman paper to form a purple spot. The wire loop was sterilized again and used to touch colony of the test isolates and then transferred on the reagent spot on the Whatman paper. Formation of a very deep purple colouration indicated a positive reaction, while absence of deep purple colouration indicated a negative reaction (Seeley and Van Demark, 1972).

3.3.2.3 Methyl Red Test

Glucose phosphate peptone broth was prepared as described by Harrigan and McCance (1966). Ten milliliters of the broth were dispensed into screw cap tubes and sterilized. Inoculation with test organisms was subsequently done and incubated at 35^{o}C for 2 – 5 days. After incubation a few drops of methyl red indicator was added to the culture and a resultant definite red colouration was considered positive.

3.3.2.4 Indole Production

Two percent (w/v) peptone broth was prepared and ten milliliters were dispensed into screw cap tubes and sterilized. Inoculation with test organisms were subsequently done and incubated for 72 hours at 35^{o}C after which test with Kovac's (1928) reagent was carried out. Formation of an alcoholic layer with red colouration indicated indole production, while absence of such colouration indicated negative result.

3.3.2.5 Voges-Proskauer Test

The isolates were each cultured in methyl red broth, 1ml of alpha-naphthol solution and 1ml of 10% NaOH (sodium hydroxide) was added after two days of incubation at 30^{o}C. This is to know whether the organisms after producing acid from glucose are capable of producing acetylmethyl carbinol from the acid. Appearance of a pale pink colouration within 5 minutes was recorded as positive. The solution was left for up to 1 hour to check for slow reaction (Barrit, 1936).

3.3.2.6 Oxidative/Fermentative Test

The medium of Hugh and Leifson (1953) was used. Fifteen milliliters of the medium were dispensed into screw cap tubes, were inoculated with each test organisms. One of the two screw cap tubes was covered with sterile vaspar (paraffin + wax) and the tubes were incubated for five days at 35^{o}C. Acid production was shown by change in colour from blue to yellow. Fermentative organisms will have produced in both tubes while oxidative organisms will produce acid only in the tube without vasper seal.

3.3.2.7 Homofermentative/Heterofermentative Test

The semi-solid medium of Gibson and Abdelmalek (1945) as modified by Stainer *et al*., (1964) was used. Twenty milliliters of the medium were dispensed into MacCartney bottles and sterilized. The test organisms were each inoculated in replicates and sterile agar seal poured onto the surface of each

bottle. Production of gas was indicated by gas bubbles or by forcing of the seal up the tubes. Uninoculated bottles served as control.

3.3.2.8 Oxygen Relationship of the Isolates

Yeast extract agar was distributed into screw cap tubes and sterilized in the autoclave. The tubes were inoculated with each of the isolates by the stabbing technique, using an inoculating needle. The tubes were incubated at 30^{o}C for 5 days. Areas of growth along the tubes were noted. When growth occurs only at the surface of the tube, the microbe is said to be an obligate aerobe. An organism is said to be an obligate anaerobe when it grows only at the bottom of the tube. A microorganism that grows along the length of the tube is said to be facultatively anaerobic. A microaerophilic organism is that which grows near the surface of the tube (Holding and Collec, 1971).

3.3.2.9 Sugar Fermentation Test

The fermentation patterns were determined using modified MRS medium from which meat extract and glucose had been omitted (Sharpe *et al*., 1966), but containing 0.05% (w/v) Bromocresol purple as indicator in the basal medium. Filter-sterilized solutions of the carbohydrates were added to a final concentration of 2%. Twenty milliliters of the solution were dispensed into screw cap tubes with Durham tubes inverted into each and then sterilized. The test organisms were inoculated into each of the screw cap tubes and incubated at 30^{o}C for 4 days. Tubes in which Bromocresol purple changed to yellow indicated a positive result and displacement of solution by air or gas production in the Durham tubes. Uninoculated tubes served as control.

3.3.2.10 Nitrate Reduction Test

The ability of the isolates to reduce nitrate to nitrite and ammonia (Payne, 1973) is often determined by this test. Nitrate peptone water medium was used. Five milliliters of the medium were dispensed into screw cap tubes with inverted Durham tubes and sterilized. The tubes were then inoculated with the test organisms and incubated at 35^{o}C for 4 days. The presence of nitrate was

determined by adding to each test tube 0.5ml of 1% sulphanillic acid in 5ml acetic acid followed by 0.5ml of 0.6% dimethyl-napthylamine in 5ml acetic acid. The development of red colouration gives a result and presence of gas in Durham tubes indicated the production of nitrogen.

3.3.2.11 Production of Ammonia from Arginine

The production of ammonia from arginine was tested using the method of Doring (1988). A modified MRS broth (MRS-arginine broth) without glucose and meat extract, but containing 0.3% arginine and 0.2% sodium citrate instead of ammonium citrate was used. The MRS broth without arginine was used as a control medium. 18-hour old cultures were inoculated into 10ml of each broth in a test tube and incubated at 30^{o}C for 4 days. The test samples of the culture medium after growth were placed on a spot plate to which Nesler's reagent had been applied. Cultures producing yellow or orange colour as compared to that produced by a similarly inoculated control medium indicated the production of ammonia from arginine.

3.3.2.12 Casein Hydrolysis

Skim milk agar prepared by adding 1% (w/v) skim milk to MRS agar (Harrigan and McCance, 1966) was used. The agar was sterilized by autoclaving at 110^{o}C for 10 minutes. On cooling, the medium was dispensed into sterile Petri dishes and then left to solidify. The plates were then streaked across once with the isolates and then incubated for 3 days at 35^{o}C. Uninoculated plates served as control. At the end of inocubation, a clear zone around the line of streaking indicated casein hydrolysis while the absence of a clear zone indicated a negative result.

3.3.2.13 Gelatin Hydrolysis

Nine milliliters of 10% gelatin broth (Harrigan and McCance, 1966) were dispensed into screw cap tubes and sterilized. The tubes were inoculated with the test organisms and incubated with the test organisms and incubated at 35^{o}C for 7 days. Uninoculated tubes served as control. After the incubation period,

the tubes were placed in a clean beaker containing some ice block cubes for 5 minutes, after which they were observed. Solidification of the broth indicated a negative result. This shows that gelatin was not hydrolysed by the organism. However, broth not solidified after placing in ice-cubes indicated a positive reaction meaning, gelatin was hydrolysed by the inoculated organism.

3.3.2.14 Starch Hydrolysis

Equimolar amount of soluble starch was prepared and added to MRS agar without glucose or meat extract to give 1% soluble starch agar medium. The medium was sterilized at 121^{o}C for 15 minutes before being poured to set in sterile plates, single streaks of cultures were made on the dried plates before being incubated at 30^{o}C for 48 hours. The plates were flooded with Gram's iodine after incubation. Unhydrolysed starch formed a blue colouration with iodine. Clear zones around the region of growth indicated starch hydrolysis by the culture.

3.3.2.15 Motility

The organisms were grown in MRS broth for 18 hours at 30^{o}C. After the incubation period, a few drops of broth were put on a cavity slide and examined under the X40 objective lens of the microscope. The motile cells were seen to move about randomly. This kind of movement was differentiated from Brownian or molecular movement in which case the cells move about in or to and fro manner without any change in position except as influenced by current in the fluid (Seeley and Van Demark, 1972).

3.3.2.16 Growth at Different pH

The pH values of the different MRS broth medium were adjusted to pH 4.5 and 9.6 using 0.1N HCl. Ten milliliters of the medium were distributed into tubes before autoclaving. After autoclaving, the pH values of the medium were rechecked and then the media were inoculated with the test isolates before incubation at 30^{o}C for 48 – 120 hours. Turbidity of the broth compared with the uninoculated controls was used as indicator of growth of the culture.

3.4 Molecular Procedure

Based on the results of Tests of Proteolytic activity, lactic acid production, diacetyl and hydrogen peroxide, *Pediococcus acidilactici* was chosen as the candidate organism for further study of survival patterns.This was therefore subjected to molecular identification.

3.4.1.1 Extraction of genomic DNA of LAB isolates

DNA extraction from the LAB isolates was carried out using a modified GES (5 M guanidine thiocyanate (Fisher Scientific BPE221-250), 0.1 M EDTA (Sigma E-5134), and 0.5% N-lauroyl-sarcosine sodium salt (Sigma L-5777) (w/v) DNA extraction method (Pitcher *et al*., 1989). Aliquots of 1.5 ml of overnight cultures grown in appropriate broth were centrifuged (Biofuge, Heraeus, Germany) in eppendorf tubes at 13,000g for 1 min. Pellets obtained were washed in 1 ml of ice cold lysis buffer (25 mM Tris-HCl (Sigma T-6066), 10 mM EDTA, 50 mM sucrose (BDH GPR 302997J) pH 8). The pellets were re-suspended in 100 µl of lysis buffer in addition to 50 $mgml^{-1}$ lysozyme (Sigma L-6876) and incubated at 37^{o}C for 30 min. Volumes of 0.5 ml GES solution were added and mixed well. This was incubated at room temperature for 15 min. The lysate was then cooled on ice for 2 min and 0.25 ml of 7.5 M ammonium acetate (Fisher Scientific A3440/60) (also cooled on ice) was added, vortexed and incubated on ice for 10 min.

Aliquots (0.5ml) of 24:1 chloroform: isoamylalcohol (Sigma CO549-1QT) were added, vortexed and centrifuged for 10 min at 13,000g. Aliquots of 800 µl of the upper phase were removed quantitatively and placed into a clean eppendorf tube. Cold isopropanol (Fisher Scientific P/7500/21) (0.54 vol) was added and mixed for 1 min. This was then centrifuged at 13,000g for 5 min and the supernatant removed from the pellet. The pellet was washed three times in 500 µl of 70% ethanol and dried at 37^{o}C for 15 min. Aliquots (50 µl) of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) were added and 5 µl of the DNA were checked on 1% agarose (Biogene, Kimbolton, UK) gels in 200 ml 1

X TAE (4.84 g Tris base, 1.14 g glacial acetic acid, 2 ml 0.5 M EDTA, pH 8) buffer and the DNA samples were then stored at -20^{o}C for future use.

3.4.1.2 PCR amplification of 16s rDNA gene

The modified method of Bulut *et al* (2005) was used. Amplification of 16S rDNA gene-ITS region, was performed by using the following primers:
Forward (16S ITS For), 5'-AGAGTTTGATCCTGGCTCAG-3'
Reverse (16S ITS Rev), 5'-CAAGGCATCCACCGT-3'
16s rDNA V3, Forward, 5'-CCTACGGGAGGCAGCAG-3'
16s rDNA V3, Reverse, 5'-ATTACCGCGGCTGCTGG-3'.
The V3 primer was used for ease of sequencing of the gene, using the variable region 3 (V3), for the genetic identification of the isolates.

Each of the polymerase chain reactions (PCR) was performed in a 50 µl reaction volume containing 50 ng genomic DNA as the template (equivalent to 1µl), 0.2 mM- deoxynucleoside triphospates, dNTPs, (Promega, U120A-U123A, Madison, WI, USA), 2.5 mM-$MgCl_2$, 10 pmol each (0.1 µl volume) of the DNA primers in 1 x PCR buffer (Promega, UK), and 1.25 units Taq DNA polymerase (Promega, UK). Amplification conditions were as follows: an initial denaturation step of 5 min at 94 ^{o}C; 40 amplification cycles, each consisting of 1 min denaturation at 94 ^{o}C, 1 min annealing at 42 ^{o}C, and 1 min elongation at 72 ^{o}C, steps. Reactions were terminated with a final extension step for 10 min at 72 ^{o}C. PCR amplifications were performed in a Thermocycler (Techne-Progene, Cambridge, UK).

3.4.1.3 Gel electrophoresis of 16s rDNA PCR Products

Electrophoresis of the amplified 16s rDNA PCR products was performed on the Bio-Rad Contour-Clamped Homogenous Electric Field (CHEF) DRII electrophoresis cell. This was done through 1.5% (w/v) Agarose gel (general purpose, Biogene) in 0.5 X TAE buffer at 84 volts for 1.5 - 2 hrs. A 100bp (promega G210A) and 1Kb DNA ladder (promega G571A) were used as a molecular size markers.

3.4.1.4 Sequencing and Analysis of 16s rDNA gene

3.4.1.5 Purification of Amplified PCR 16s rDNA gene

A 75 µl of the PCR 16s rDNA amplified products were resolved in 1% agarose gels with the conditions earlier described**.** The resulting bands in agarose gel were carefully excised with sterile scarpels and then purified using purification kit (Wizard PCR preps DNA purification kit, USA). The purification process involved the introduction of the excised bands into 1.5ml eppendorf tubes and suspended in water bath that was maintained at 65^{o}C. One ml of PCR preps purification resin was added and the tubes were incubated for about 5 minutes or until the agarose gel melted completely. The DNA/purification resin mix was pipetted into the syringe barrel and the syringe plunger was used to slowly push the slurry into the attached minicolumn. The minicolumn was washed with 2 ml of 80% isopropanol, to remove contaminants from the DNA. The eppendorf tubes, on which the columns were mounted, were now centrifuged at 10,000 x g for 2 minutes at 4^{o}C, to remove remaining resin and isopropanol from the purified DNA in the columns. The minicolumns were transferred into new eppendorf tubes and 40µl TE buffer (pH 7.5) was added into each of the columns, and left to stand for 1 minute. The minicolumns were centrifuged at 10,000 x g at 4^{o}C for 20 secs, to elute the DNA into the centrifuge tubes. The purified DNA was kept at 4^{o}C until use.

3.4.1.6 Qualitative and Quantitative Determination of Purified 16s rDNA gene

This was determined using software – NanoDrop version 3.1.0, Coleman Technologies Inc., USA. Determination was carried out according to the software manufacturer's instructions.

3.4.1.7 Drying of the Purified 16s rDNA genes

To a known volume of the purified DNA, 0.1 volume of Sodium acetate (3 M, pH 5.0) and 2.0 volume of 100% ethanol were added. This was then incubated at -20^{o}C for 1 hr. IT was brought out and left to stand at room temperature for 5 minutes, and then centrifuged at 13, 000 x g at 4oC for 45 minutes. The liquid

was removed, leaving only the DNA in the eppendorf tubes. The DNA was then dried at 37^0C for 30 minutes.

3.4.1.8 Sequencing of 16s rDNA gene

The dry DNA samples (obtained using the V3 primer) were sequenced using a computer analytical sequencer (MGW-Biotech, Germany) with the V3 primer Rev, acting as the basis. The generated nucleotide sequences were subjected to analysis.

3.4.1.9 Analysis of the 16s rDNA gene Sequences

The generated sequences of the 16s rDNA genes were subjected to alignment in the databases at the BLAST, Basic Local Alignment and Search Tool, website http://www.ncbi.nlm.nih.gov/blast/. The isolates were then identified based on the result of the analysis.

3.4.2 Selection of Test Organisms

Five of the *Pediococcus acidilactici* species identified were selected as test organisms based on their high proteolytic activity and lactic acid production. The test organisms were subcultured in the isolation medium before being used for all subsequent tests. The cultures were then grown at 37^oC for 24 hours.

3.5 Enzyme Studies

3.5.1 Determination of Inoculum Size

The inoculum size was determined by introducing a loopful of 24 – hour old culture into 5ml of sterile distilled water in a test tube. It was mixed for few minutes. Thereafter, 1ml of the mixture was dispensed into a sterile test tube. 1 drop of crystal violet was added to it. A sterile syringe was used to introduce the mixture into the Neubeur counting chamber and was viewed under low power objective of the microscope.

3.5.2 Production of the Enzyme

Four, 250 Erlenmeyer flasks each containing 200ml sterile MRS broth (Appendix 1) were inoculated with 0.10ml aliquot containing a load of 1.10 x 10^4 cfu/ml of *Pediococcus acidilactici*. The Erlenmeyer flasks were inoculated in a J.P. selecta s.a. 0338954 Model incubator at 35^{o}C for 96 hours. After the time interval, the enzyme (protease) was harvested by ultra centrifugation using Damon/IEC B-20A model centrifuge set at 10,000rpm for 10 minutes at 4^{o}C. The harvested crude supernatant, now served as protease and used for further work.

3.5.3 Protease Assay

This was carried out using the modified method of Kunitz (1946). Protease activity was measured using casein (BDH) as substrate, generally in 0.1M Citrate phosphate buffer (pH 5.5). 1% (w/v) casein solution was prepared in the buffer solution and was heat-denatured at 100^{o}C for 15 minutes in a water bath and allowed to cool. The reaction mixture consisted of 1ml of the substrate thoroughly mixed with 0.5ml of enzyme extract. Incubation was for one hour at 35^{o}C after which the reaction was terminated by addition of 3ml of cold (2^{o}C) 10% Trichloroacetic acid (TCA) and 1 ml of 1% casein added to the control. The reaction mixtures were then centrifuged at 10,000 rpm at 4^{o}C for 5 minutes. The optical density readings of the carefully decanted supernatant fluid were then measured with Pye Unicam SP6-250 visible spectrophotometer at 660nm wavelength against a blank containing the control. One protease unit was defined as the amount of enzyme which catalysed the release of 1µmol tyrosine from Bovine serum albumin (BSA) or Casein per minute.

3.5.4 Protein Estimation

The modified Lowry Folin-Ciocalteau's method was used for the protein assay (Lowry *et al*., 1951). To 0.1ml of the enzyme extract was added a Reagent C (Reagent A plus Reagent B). Reagent A contained a mixture of 8.4g sodium hydroxide and 4.2g Sodium carbonate and water. Reagent B contained a mixture of 0.1g Sodium tartarate and 0.05g cupric sulphate. Thereafter 3ml of

Reagent C and 0.3ml of Reagent D (Folin-Ciocalteau's) reagent were added. The mixture was thoroughly mixed and incubated for 30 minutes at 28^{o}C. The optical density reading of the resulting coloured solution was measured with Pye Unicam SPG-250 visible Spectrophotometer at 660nm wave length against a blank containing the control.

3.5.5 Optimization of Culture Conditions for the Production of Protease

The various conditions that affect the production of protease were subjected to variation to determine the ones best suited for the production.

3.5.5.1 Effect of Inoculum Concentration on Protease Production

The modified method of Olutiola and Nwaogwgwu (1982) was used. Approximately, 10ml aliquot of MRS broth was dispensed into each of several screw capped tubes and sterilized. They were inoculated with the test organisms in 4 sets. The first set was inoculated with 0.05ml, the second set was inoculated with 0.1ml, the third set was inoculated with 0.15ml and the fourth set was inoculated with 0.2ml of the broth culture. They were all incubated at 35^{o}C for enzyme production for 24 hours. At the end of the incubation period, the broth cultures were centrifuged at 10,000rpm for 30 minutes and the supernatant used as enzyme in protease assay.

3.5.5.2 Effect of Incubation Period on Protease Production

The modified method of Olutiola and Nwaogwgwu (1982) was used. The test organisms were grown in MRS broth for 24 hours, 36 hours, 48 hours and 96 hours at the optimum temperature for enzyme production of each organism. The broth cultures were centrifuged using at 10,000rpm at 5^{o}C for 30 minutes and the supernatant used as enzyme in protease assay.

3.5.5.3 Effect of Temperature on Protease Production

About 10ml of MRS broth was dispensed into screw cap tubes and sterilized. The tubes were allowed to cool, each tube was inoculated with 0.5ml of broth cultures containing the test organisms before incubation at 10^{o}C, 20^{o}C, 30^{o}C

and 35°C for 1 hour. The broth cultures were centrifuged at 10,000rpm at 5°C for 30 minutes and the supernatant used as enzyme for protease assay.

3.5.5.4 Effect of pH on Protease Production

The MRS broth used for cultivation of isolates was prepared with 0.1N HCl and adjusted to pH levels of 5.0 and 5.5. 10ml of the different MRS broth was dispensed into separate screw cap tubes and sterilized. After cooling, the tubes were all inoculated and labeled. The tubes were then incubated at 35°C for 24 hours. The broth cultures were centrifuged at 10,000rpm at 5°C for 30 minutes and the supernatant used as enzyme in protease assay.

3.5.6 Characterization of Produced Protease Enzyme

3.5.6.1 Effect of Substrate Concentration on Protease Activity of Isolates

This was determined using the method of Keay *et al*., (1970). 1%, 2%, 3%, 4%, and 5% (w/v) casein solutions were separately prepared in 0.1M citrate phosphate buffer (pH 5.5). The casein solutions were heat-denatured at 100°C for 15 minutes in a water bath. Protease assay was carried out using the different concentrations of substrate.

3.5.6.2 Effect of Temperature on Protease Activity of Isolates

This was determined using the method of Keay *et al*., (1970). 1% (w/v) casein solution was prepared in 0.1M citrate phosphate buffer (pH 5.5). The casein solution was heat-denatured at 100°C for 15 minutes in a water bath. The optimum temperature for protease activity was investigated by inoculating 0.5ml of each isolate into 1% casein solution as carried out above but cultures were incubated at different temperature (4°C, 20°C, 28°C, 30°C, 35°C) for 24 hours. Protease assay was then carried out.

3.5.6.3 Effect of pH on Protease Activity of Isolates

This was determined using the method of Keay *et al*., (1970). 1% casein solution was prepared in 0.1M citrate phosphate buffer and the pH was adjusted with dilute citric acid. pH range of 4.0, 4.5, 5.0, 5.5 and 6.0 was obtained. The

casein solutions were then heat-denatured at 100^{o}C for 15 minutes in a water bath and allowed to cool. The protease assay was carried out using the casein solutions of different test pH as the substrate.

3.5.6.4 Effect of Enzyme Concentration of Isolates on Protease Activity

This was determined using the method of Keay *et al*., (1970) 1% casein solution was prepared in 0.1M citrate phosphate buffer at (pH 5.5). The casein solution was heat-denatured at 100^{o}C for 15 minutes in a water bath. The concentration of the enzyme extracts were varied at (0.5ml, 1ml, 1.5ml, 2.0ml, and 2.5ml). Protease assay was carried out using the different concentration of enzyme extracts.

3.5.7 Purification of Protease Enzyme

3.5.7.1 Ammonium Sulphate Precipitation

The protease enzymes were partially purified using the Ammonium sulphate precipitation method (Olutiola and Cole, 1980). The clarified extracts were treated with 24.3g, 24.5g and 15.7g of solid Ammonium sulphate (Analytical grade) to 0-40%, 40-80% and 80-100% saturation respectively. The mixture for each batch of saturation was stirred continuously for 15 minutes during which the Ammonium sulphate dissolved into the medium. The mixture was kept at 4^{o}C **(**Haier Thermocool Model No HR 137 model refrigerator) for 24 hours after which it was centrifuged with Cecil CE 2021 2000 series at 10,000rpm for 5 minutes. The supernatant was then treated to the next batch of saturation until final batch. The precipitates for each batch were resuspended to the initial volume of culture filtrate with 0.1M citrate phosphate buffer (pH 5.5) and dialysed in a tubular cellulose membrane against 2 litres of the same buffer for 24 hours at 4^{o}C. The precipitates were then pooled together and used as partially purified enzyme for enzyme assay.

3.5.7.2 Gel Filtration

Dissolution of Sephadex

10 grams of each of the different grades of Sephadex G-100, G-25, C-50 were weighed out separately into clean bowls. 50ml of sterile water was added into each bowl and allowed to dissolve into a paste form. 300ml of 0.1M citrate phosphate buffer pH 5.5 was added and then the mixture was kept in the refrigerator for 3 days with constant mixing.

3.5.7.3 Soaking and Loading of the Column

The refrigerated soaked Sephadex was mixed together after the third day and dispensed into a clean chromatography column (640 x 25mm) with the use of a clean funnel. The Sephadex was allowed to compact in the column with occasional pipetting of supernatant (buffer) and refilling of the column with Sephadex to the zero point of the column. This was carried out for 48 hours which the samples were injected.

3.5.7.4 Fractionation of Sephadex G-100

The column of Sephadex G100 prepared according to the method described by Olutiola and Cole, (1980), was surrounded by a water jacket at 4^{o}C. The column was equilibrated with 0.1M citrate phosphate buffer (pH 5.5). 2ml of the enzyme concentration was applied to the column and eluted with 0.1M citrate phosphate buffer. The eluted fractions were collected in a calibrated 5ml tube. Each eluted fraction was analyzed for protease assay.

3.5.7.5 Fractionation on Sephadex G-25

Fractions which showed appreciable protease activity after passing it through Sephadex G100 were combined and applied to column of Sephadex G-25. 2ml each of the separate fractions were applied to the column and eluted with 0.1M citrate phosphate buffer. The eluted fractions were collected as above and analysed for protease assay.

3.5.7.6 Fractionation on Sephadex C-50

Fractions (39) and (50) which showed appreciable protease activity after passing it through Sephadex G-25 were applied separately to column of Sephadex C-50. 2ml each of the separate fractions was applied to the column and eluted with 0.1M citrate phosphate buffer (pH 7.0). The eluted fractions were collected as above and analysed for protease assay.

3.5.7.7 Electrophoretic Separation of Extracellular Protease

Protease samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-Page) on vertical electrophoresis kit SE 245 series, using a discontinuous gradient gel buffer systems as described by Laemmli (1970)**;** Filho and De Azevedo (1978).

The following solutions were prepared:

(a) 30% acrylamide, 0.8% w/v Bis-acrylamide. Thirty grammes of acrylamide (BDH) and 0.8g of N, N^1 methylene bis-acrylamide (BDH) were dissolved in 60ml distilled water in a 100ml standard volumetric flask and made up to mark. The solution was stored at 4^oC.

(b) Stacking gel buffer

All the components were dissolved in 500ml distilled water in a 1 litre standard volumetric flask. The pH was adjusted to 6.8 with sodium hydroxide solution. The solution was then made up to mark with distilled water and stored at 4^oC.

(c) Running gel buffer: 1.5M Tris, 8mM EDTA, 0.4% SDS pH 8.8

All the components were dissolved in 900ml of distilled water in a 1 litre standard volumetric flask. The pH was adjusted to 8.8 with hydrochloric acid. The solution was then made up to mark with distilled water and stored at 4^oC.

(d) 1% Ammonium Persulphate

One gramme of Ammonium persulphate $(NH_4)_2S_2O_8$) was dissolved in 1ml distilled water and used immediately.

(c) Electrode buffer: Glycine, SDS, Sodium Salt EDTA

All components were dissolved in four litre distilled water with constant stirring. The solution was kept at 4^{o}C.

3.5.7.8 Sample Buffer

Six milliliters of glycerol were mixed with 1.2ml of sodium phosphate buffer (pH 7.0), 1.5g of sodium dodecyl sulphate (SDS), 30mg dithiothreitol (DDT) and 10mg bromophenol blue. Distilled water was then added to make 20ml. The mixture was dispensed into test-tubes in aliquots of 1ml each and stored at -20^{o}C until needed.

3.5.7.9 Procedure

The stacking gel and running gel were prepared on the day of the experiment. The running gel solutions were poured into the gel trays to a level of about 4cm below the maximal filling level. Distilled water was layered on the gel surface using a Pasteur pipette, to ensure an even surface and also to avoid evaporation of gel while polymering gel was allowed to polymerize for 1 – 2 hours.

The water on the gel was poured off and the stacking gel was poured onto the polymerized running gel and a comb (1.l5mm thick) was gently inserted to obtain wells. Then it was allowed to polymerize for the hour before removing the comb. The gel was then clamped to the electrophoresis chamber. The upper and lower chambers were filled with electrode buffer and the bubbles formed were removed with a syringe.

The protein samples were prepared by mixing sample buffer (in a dilution of 1:1 (v/v) with enzyme samples. The mixtures were placed in a dry bath and heated for 4 minutes at 95^{o}C. Equal volumes of samples were applied to the bottom of the sample wells with a Hamilton syringe. Molecular weight standards (Bio Rad high and low ranges) were then applied into wells alongside samples.

Gel electrophoresis was conducted at 120 volts in a vertical gel electrophoresis apparatus (Mighty Small 11, Amersham Pharmacia Biotech, Cat No: 17-0446-01) for about 1.5 hour or until the bromophenyl blue dye had migrated to the bottom of the gel.

At the end of the electrophoresis, the gel was carefully removed from the glass plates and stained by soaking in the staining solution for 20 minutes with gentle agitation. Excess stain was then removed by immersing the gel for 1 hour in several changes of the destaining solution each lasting 15 – 20 minutes.

3.6 Microbiological Analysis

Meat samples (10g) were aseptically removed from storage and added to 90ml of peptone water and homogenized with a Lab Blender (Model 80, Seward Medical London). From this homogenate, a 1:10 dilution was subsequently made using peptone water followed by making a 10-fold serial dilution of beef, chicken and turkey at each of the appropriate time intervals during storage. All counts were expressed as log colony forming units.

3.6.1 Preparation of Sample/ Isolation Procedure

The meat samples were aseptically weighed and homogenized. From each sample, a 1:10 dilution was subsequently made using peptone water followed by making a 10-fold serial dilution. 0.1ml from each dilution was then subcultured in replicates in MRS Agar. The MRS agar were incubated anaerobically using the Gas Pack System (Merck, Anaerocult type A)

3.7 Proximate and biochemical Analyses of the Different Meat Samples

3.7.1 Proximate Analysis

Proximate composition of samples was determined according to A.O.A.C (2000) methods.

3.7.1.1 Moisture Content Determination

The moisture content of the samples was determined using A.O.A.C (2000) method. The sample 5 g was weighed (S) into already-weighed (W_1) clean

drying cans. The cans were placed in a well ventilated oven (Fisher Scientific Isotemp oven, by Fisher Scientific Co. USA, model 655F) maintained at 105 $\pm$ 2^0C. After 16-18 hours the drying cans were then transferred into a dessicator to cool after which the final weights were taken (W_2).

Percentage moisture content (MC) = $\frac{W_1 - W_2}{S} \times 100$

Percentage dry matter = 100 – MC

Where,

W_1 = Weight of empty moisture content can with sample before drying

W_2= Weight of moisture content can with sample after drying

S = Weight of sample

3.7.1.2 Crude Protein Determination

The crude protein in the samples were determined by the routine semi-micro Kjeldahl, procedure/technique. This consists of three techniques of analysis namely digestion, distillation and titration.

Digestion

0.5 g of each finely ground dried sample was weighed carefully into the Kjeldahl digestion tubes to ensure that all sample materials got to the bottom of the tubes. To this were added 1 Kjeldahl catalyst tablet and 10 ml of Conc. H_2SO_4. These were set in the appropriate hole of the Digestion Block Heaters in a fume cupboard. The digestion was left on for 4 hours, after which a clear colourless solution was left in the tube. The digest was cooled and carefully transferred into 100 ml volumetric flask, thoroughly rinsing the digestion tube with distilled water and the flask was made up to mark with distilled water.

Distillation

The distillation was done with Markham Distillation Apparatus which allows volatile substances such as ammonia to be steam distilled with complete collection of the distillate. The apparatus was steamed out for about ten minutes. The steam generator was then removed from the heat source to allow the developing vaccum to remove condensed water. The steam generator was

then placed on the heat source (i.e heating mantle) and each component of the apparatus was fixed up appropriately.

Determination: 5ml portion of the digest above was pipette into the body of the apparatus via the small funnel aperture. To this was added 5ml of 40% (W/V) NaOH through the same opening with the 5ml pipette.

The mixture was steam-distilled for 2 minutes into a 50 ml conical flask containing 10 ml of 20% Boric Acid plus mixed indicator solution placed at the receiving tip of the condenser. The Boric Acid plus indicator solution changes colour from red to green showing that all the ammonia liberated have been trapped.

Titration

The green colour solution obtained was then titrated against 0.01N HCL contained in a 50 ml Burette. At the end or equivalent point, the green colour turns to wine colour which indicates that all the nitrogen trapped as Ammonium Borate $[NH_4)_2BO_3]$ have been removed as Ammonium chloride (NH_4CL).

The percentage nitrogen in this analysis was calculated using the formula:

% N= Titre value x Atomic mass of Nitrogen x Normality of HCl used x 4

or % N = Titre value x Normality/Molarity of HCL used x Atomic mass of N x Volume of flask containing the digest x $\frac{100}{1}$

Weight of sample digested in milligram x vol. of digest for steam distillation. The crude protein content is determined by multiplying percentage Nitrogen by a constant factor of 6.25 i.e. % CP = % N x 6.25.

3.7.1.3 Crude Fat Determination

1g of each dried sample was weighed into fat free extraction thimble and pug lightly with cotton wool. The thimble was placed in the extractor and fitted up with reflux condenser and a 250 ml soxhlet flask which has been previously dried in the oven, cooled in the desicator and weighed. The soxhlet flask is

then filled to ¾ of its volume with petroleum ether (b.pt. 40^0-60^0C), and the soxhlet flask. Extractor plus condenser set was placed on the heater. The heater was put on for six hours with constant running water from the tap for condensation of ether vapour. The set is constantly watched for ether leaks and the heat source is adjusted over several times at least 10-12 times until it was short of siphoning. It was after this was noticed that any ether content of the extractor was carefully drained into the ether stock bottle. The thimble containing sample was then removed and dried on a clock glass on the bench top. The extractor, flask and condenser were replaced and the distillation continues until the flask is practically dry. The flask which contained the fat, its exterior cleaned and dried to a constant weight in the oven. If the initial weight of dry soxhlet flask is Wo and the final weight of oven dried flask + oil/fat is W_1, percentage fat is obtained by the formula:

$$\frac{W_1 - W_0}{\text{Wt. of sample}} \times \frac{100}{1}$$

Carbohydrate content was calculated by difference.

3.7.1.4 Peroxide Value Determination

This was determined according to the method of Pearson *et al.*, (1981) 2g of meat sample was weighed into an Erlenmeyer flask, 1g of potassium iodide and 20 ml of solvent mixture (acetic acid and chloroform 1:1) were added. The mixture was placed in a water bath for 5 min. It was transferred quickly into a flask containing 20 ml of 5% potassium iodide. It was titrated with standard 0.002 m. $Na_2S_20_3$ using starch as indicator. A colourless solution was observed. A blank was also determined

$$\text{Peroxide value} = \frac{(\text{Blank} - \text{sample}) \times \text{molarity} \times 1000}{\text{Weight of sample}}$$

3.7.1.5 Water Holding Capacity Determination

This was determined according to the method Pearson *et al.*, (1981) 2g of meat was weighed into a 250 ml Erlenmeyer flask. The flask was filled with distilled water and left for 2 day. The water was decanted and the sample in the flask

was weighed (W_2). It was thereafter dried in the oven at 105^{o}C for 2 days. The dry content was weighed (W_3).

$$\text{Water Holding Capacity (\%)} = \left(\frac{W_2 - W_3}{(W_3 - W_1)}\right) \times 100$$

3.7.2 Biochemical Analysis

3.7.2.1 Determination of Free Fatty Acid (FFA)

For total FFA determination, 5g of each of the sample were weighed into 250ml Erlenmeyer flask. 25ml of diethyl ether and 25ml of 95% alcohol were added to the samples. 1ml of phenolphthalein solution was added as indicator. The reaction mixture was neutralized with 0.1N NaOH. The mixture was shaken vigorously until a pink colour which persists for 15 seconds was obtained. Finally the mixture was titrated with 0.25N sodium hydroxide.

3.7.2.2 Determination of Thiobarbituric Acid (TBA)

TBA values were determined for the samples as described by Brewer *et al*., (1992). Ten grams of the samples were blended with 15ml of cold extracting solution containing 9% perchloric acid. The resulting slurries were transferred quantitatively to 100ml volumetric flasks and made up to 50ml each with distilled water. The slurries were filtered through Whatman No. 2 filter paper. Fifty milliliter of each of the filtrates was transferred to test tubes and 5ml of 0.02N TBA reagent was added into each and mixed thoroughly. The tubes were kept in the dark for 17 hours and absorbance read at 530nm with spectrophotometer. TBA values were calculated from the standard solution of tetrathoxypropane and expressed mg malonaldehyde/kg.

3.7.2.3 Determination of pH

pH determination in bacterial broth cultures was carried out by the method of Guererro *et al*., (1995). The bacterial isolates were inoculated into sterile MRS broths and incubated at 37^{o}C for different period of time. The pH was then measured using a calibrated pH meter, by simple insertion of the electrode and taking the reading when figure stabilizes.

3.7.2.4 Determination Lactic Acid

The lactic acid was determined in MRS broth cultures by titrating 25 ml against 0.1M NaOH in a 100ml erlenmeyer flask, to the first trace of pink colouration. This was repeated two times and the average titre value was taken.

1ml of 0.1M NaOH = 9.008mg lactic acid (Mante *et al.*, 2003)

3.7.2.5 Determination of Diacetyl

Diacetyl production was determined by transferring 25ml of broth cultures of LAB into 100ml Erlenmeyer flasks. Seven and half milliliters of 1M Hydroxylamine solution was added to a similar flask for residual titration. Both flasks were titrated against 0.1N HCl to a greenish end point using bromophenol blue as indicator. The equivalence factor of HCl to diacetyl is 21.52 mg. The concentration of diacetyl was then calculated according to the method of Food Chemicals Codex (1972)

$$Ak = \frac{(s-b) \times (100E)}{W}$$

Ak = percentage of diacetyl, b = Amount (ml) of 0.1N HCl consumed in titration sample; E = Equivalence factor; W = volume of sample; s = No of ml of 0.1N HCl consumed in titration of sample; b = No of ml of 0.1N HCl consumed in titration of blank.

3.7.2.6 Determination of Hydrogen Peroxide

Hydrogen peroxide was determined by introducing 25ml of broth cultures of test organisms into separate 100ml flasks. To each was added 25ml of dilute H_2SO_4, which was then titrated against 0.1N potassium permanganate ($KMnO_4$). Each milliliter of 0.1N $KMnO_4$ is equivalent to 1.701 mg of H_2O_2. A decolourization of the sample was regarded as the end point. (AOAC, 1990)

$$H_2O_2 = \frac{\text{ml } KMnO_4 \times \text{N } KMnO_4 \times \text{ME X100}}{\text{ml } H_2SO_4 \times \text{Vol of sample}}$$

3.6.5 Antimicrobial activities of the LAB isolates

3.6.5.1 Preparation of culture supernatant

The LAB strains were grown in MRS broth for 24 h at 30 ^{o}C or 37^{o}C. The cultures were centrifuged at 3600rpm for 15 mins. The supernatants were then collected for use in antimicrobial assay.

3.6.5.2 Antibacterial activity assay

The antimicrobial test of the LAB cultures was by the modified method of Suwanjinda *et al.* (2007). Serial dilutions of the 24 hr MRS broth cultures of respective LAB were made up to 10^{-7}. From the dilutions 10^{-5}, 10^{-6}, 10^{-7}, 0.1 ml of each was surface inoculated on sterile solid MRS agar plates, using sterile glass spreader to ensure even distribution. This was done in duplicates and then incubated at 37^{o}C for 24 hrs. Plates containing 10-50 colonies of the LAB were then selected and carefully overlaid with test indicators (70µl in 5ml of 0.7% BHI or Nutrient agar, mixed vigorously with vortex mixer). Plates were allowed to solidify and then incubated at 4^{o}C for 1 hr and subsequently at 30 or 37^{o}C for 24 hours. Plates were then examined for zones of inhibition around the LAB colonies.

The second method used for antimicrobial activity assay was the disc assay method (El-Adawy, 2001; Gurira and Buys, 2006). A sterile filter paper disc (Whatman AA, 6) was dipped into the CFNS for 30 min, and then applied on plates previously seeded with 0.7% BHI, MRS and Nutrient agar containing indicator organisms. The plates were incubated overnight at 30 or 37^{o}C for 24 hrs and the diameter of the resulting zone of inhibition was measured in mm as the distance from the edge of the paper disc to the edge of the clearing zone. Clear zones extending for 0.5mm or more were considered as positive for inhibition (Litopoulou-Tzanetaki *et al*., 1989).

3.6.5.3 Antimicrobial action due to bacteriocin

The cell-free supernatant was pH neutralized to pH 6.0–6.5 using NaOH (10M and 1 M solution). For the determination of antimicrobial activity due to

bacteriocin production, the cell free neutralized supernatant (CFNS) was treated with catalase (Sigma, Germany; 500 IU ml^{-1}, sterile) to obtain crude bacteriocin (CB) which was used in bacteriocin assay (Franz *et al*., 1996; Albano *et al*., 2007)

3.6.6 Characterization of crude bacteriocins of *Pediococcus* Strains

3.6.6.1 Effect of enzymes on activity of bacteriocin

Strains were grown in MRS broth for 18 h at 37 °C. The cells were harvested (8000 ×g, 10 min, 4 °C) and the cell-free supernatant adjusted to pH 6.0 with 1 M NaOH and catalase, 500 IU ml^{-1}.

One milliliter cell-free supernatant was incubated for 2 h in the presence of 1 mg/ml each of proteinase K, pronase, pepsin and trypsin (Boehringer, Mannheim GmbH, Germany), α-amylase (Sigma) and catalase (Boehringer, Mannheim), respectively. Antimicrobial activity was monitored by using the agar-spot test method (Van Reenen *et al*., 1998).

3.6.6.2 Effect of surfactants on activity of bacteriocin

One percent (1%) (w/v) sodium dodecyl sulphate (SDS), Tween 20, Tween 80, Urea, Triton X-114, Triton X-100, Oxbile and NaCl were added to bacteriocin-containing cell free supernatants. EDTA was added to cell-free supernatants to yield final concentration of 1mM. Untreated cell-free supernatants and detergents at these respective concentrations in water were used as controls. All samples were incubated at 37 °C in a Water Bath Model No DK- 600, Gulfex Medical and Scientific, England for 5 h and then tested for antimicrobial activity by using the agar-spot test method as earlier described.

3.6.6.3 Effect of pH on activity of bacteriocin

The effect of pH on the activity of bacteriocins was tested by adjusting cell-free supernatants from pH 2.0 to 12.0 (at increments of two pH units) with sterile 1 M NaOH or 1 M HCl. After 1 h of incubation at room temperature (25 °C), the

samples were re-adjusted to pH 6.5 with sterile 1 M NaOH or 1 M HCl and catalase and tested for antimicrobial activity by using the agar spot test method.

3.6.6.4 Effect of temperature on activity of bacteriocin

The effect of temperature on bacteriocin activity was tested by incubating cell-free supernatants, adjusted to pH 6.0 – 6.5 at -15, -4, 50, 100, and 121°C, respectively, for 60 min. *Escherichia coli* ATCC 5218 was used as indicator strain.

3.6.7 Determination of lactic acid and acetic acid concentrations produced by LAB in MRS broth by higher performance liquid chromatography (HPLC)

3.6.7.1 Preparation of Inocula

Liquid suspension of each LAB isolate was prepared from an 18hr – old colony introduced into 10ml sterile distilled water with sterile inoculating loop. Inocula of the respective LAB isolates were then prepared by introducing one millitre from the suspension into a sterile 9ml MRS broth. This was incubated for 18 hrs at 37^{o}C in a shaking incubator set at speed 200 rpm, after which serial dilutions were made and plated to determine the CFU/ml.

3.6.7.2 Standardization of Inocula

Prior to use as inocula, the respective LAB cultures were standardized by bringing culture supernatants to same approximate optical density (OD), using sterile MRS broth.

3.6.7.3 Preparation of MRS broth supernatants for lactic and acetic acid determinations

One hundred and ten microliter (100 µl) each, representing 3.1x10^{7} – 2.37x10^{8}cfu/ml, of respective inocula was inoculated into 30ml sterile MRS broth (Oxoid, UK) and incubated at 37^{o}C in a shaking incubator, set at speed of 200 rpm, for 48 hours. Samples were taken for HPLC analysis at 24hr – interval

for 120hr. MRS broth supernatants were centrifuged at 3500 rpm for 15 min, and filter sterilized through 0.2µm syringe filters and kept at -80^{o}C, pending analysis.

3.6.7.4 Sample Preparations for HPLC Injection

The organic acids in the MRS LAB broth supernatants were extracted using the modified method of Fernandez-Garcia and McGregor, (1994). The Cartridge (Strata X 33u Polymeric Reversed Phase, 30mg/ml, Phenomenex, UK) was conditioned by slowly passing 1 ml of Methanol (HPLC grade) through, followed by 1 ml of 10% methanol in HPLC mobile phase (NaH_2PO_4: Methanol, 98:2). The mixture of the mobile phase (10% Methanol in NaH_2PO_4: Methanol, 98:2) and sample supernatant (Ratio 3:1, 900µl mobile phase and 300µl supernatant) was then passed through the pre-conditioned cartridge. A few drops (200-250µl) were allowed to run off while the remaining was collected in Eppendorf tubes, for injection into HPLC.

3.6.7.5 Preparation of Standards for Standard Curve

Various concentrations of standards of lactic and acetic acids were prepared, with a view to prepare standard curves for estimation of organic acids in the sample supernatants. Concentrated solutions of 1.2kg/L and 1.05kg/L of lactic and acetic acids respectively, were used for preparing varying concentrations of standards. A one ml volume of respective acids were transferred into 100 ml volumentric flasks, and diluted to volume with deionised (DO) water, resulting in 12g/L for lactic acid and 10.5g/L for acetic acid, as stock solutions. Standard concentrations (g/L) of 0.5, 1.0, 2.5, 5.0, 7.5, 10, 12.5, 15, 17.5 and 20 of each of the acids were then prepared from the respective stock solutions, by serial dilutions with DO water. Preparations of standards for HPLC injection were made in the same way as the sample.

3.6.7.6 The HPLC System Used

The HPLC system used consisted of LC-10ADVP pump (Shimadzu, UK), equipped with injection valve of 20µl capacity; UV detector (SpectroMonitor

3000, LDC/Milton Roy, Florida, USA); Data recorder (Picolog for windows, Release 5.12.1, St Neots, UK); C18 analytical column (a reverse phase Techsphere ODS-2 5U, 250mm length, 4.6mm Internal diameter).

3.6.7.7 Chromatographic Conditions

The chromatographic conditions used for the organic acid analysis was a modified form that described by Zotou *et al.* (2004). Analysis was performed at ambient temperature with a mobile phase consisting of 0.02M NaH_2PO_4 (adjusted to pH 2.55 $\pm$ 0.02 with H_3PO_4) and methanol (98% v/v) and delivered at a flow rate of 1ml/min. The prepared samples, for HPLC injections, were detected by their UV absorbance monitoring at 220 nm with sensitivity setting of 0.002 absorbance units full scale (AUFS). The pressure of pump was 13.5$\pm$0.5 Mpa. Sample injections into HPLC were made using a 50 µl stainless steel syringe holder, with a maximum of 20 µl being injected into the injection valve, while the remaining was collected as waste through the waste outlet.

3.7.0 Quantitative estimation of hydrogen peroxide (H_2O_2) by enzymatic method

This was determined by the modified methods of some research workers (Gilliland, 1969; Villegas and Gilland, 1998; Jaroni and Brashears, 2000). A colony each from 18 hr - old plates was used to inoculate MRS broth and incubated for 18hr at 37^{o}C, in a shaking incubator set at 200 rpm. The broth cultures were then centrifuged at 10,000 rpm for 10 min and the cells were washed twice in cold sterile sodium phosphate buffer (0.2M, pH 6.5). The cells were suspended in 5ml of sterile deionised water (DO), using the vortex machine for even mixing. The suspensions were then brought to same approximate optical density (OD) at 540nm with sterile DO, using the Cecil CE 2021 (2000 series) spectrophotometer. Thereafter 2ml of each of the cell suspension s was used to inoculate 20ml of sterile Sodium phosphate buffer, with or without 55.5 mM glucose, in 30ml capacity universal bottles. Incubation was done over 48 hours, with samples taken at 24 hr intervals for H_2O_2 analysis, OD and CFU/ml determinations. For the hydrogen peroxide

determination, the culture broth samples, taken at respective time intervals, were centrifuged at 16,000 x g. The cells were removed and the supernatant was assayed for hydrogen peroxide.

3.8.0 Hydrogen peroxide (H_2O_2) assay using horseradish peroxidase

Prior to analysis of hydrogen peroxide production by the LAB isolates, analysis was carried out on standard concentrations of H_2O_2 in Sodium phosphate buffer (0.2M, pH 6.5). The data obtained was then used to construct a standard curve, from which extrapolations were made for quantification of H_2O_2 levels in the LAB samples.

Samples were assayed for hydrogen peroxide by placing 2.5 ml of cell free supernatant into test tubes containing 0.5 ml of a 0.1% aqueous solution of peroxidase (Horseradish Type VI-A; Sigma Chemical) and 0.05 mL of a 1% aqueous solution of o-dianisidine (Sigma Chemical Co., UK). A blank was prepared using 2.5 mL of sodium phosphate buffer instead of the sample supernatant fluid. Tubes were incubated for 10 min at 37^{o}C. The reaction was stopped by adding 0.1mL of 4N HCl to each test tube (Villegas and Gilland, 1998, modified). Absorbance reading (A400 nm) of each sample was determined and peroxide content was determined by comparing the A400 nm to a standard curve (Gilliland, 1969).

3.9 Statistical Analysis of Results

All analyses were carried out in triplicate. Data obtained were subjected to analysis of variance (ANOVA) using the General Linear Models procedure of Statistical Analysis System Software (SAS Institute; Inc., 2000). Differences between and within Means were established by separation using Duncans Multiple Range Test (Duncan, 1955) within 95% Confidence level.

CHAPTER FOUR

4.0 RESULTS

A total of one hundred and ten (110) strains of lactic acid bacteria (LAB) were isolated from fresh and treated beef, chicken and turkey samples. They were identified with particular reference to Bergey's Manual of Systematic Bacteriology (Sneath *et al*., 1986) and grouped into three genera: *Lactobacillus* (82); *Pediococcus* (15) and *Leuconostoc* (13). They were further identified as *Lactobacillus plantarum; Lactobacillus brevis; Lactobacillus buchneri; Leuconostoc mesenteriodes; Lactobacillus curvatus; Lactobacillus casei; L. sakei, L. bulgarius* and *Pediococcus acidilactici* (Figure 4.1).

Table 4.1 shows the viable count of LAB (log_{10}cfu) in different stored meat products subjected to refrigeration temperature for various time intervals. There was significant ($p<0.05$) increase in LAB count of beef from Day 0 to Day 28. Beef obtained from Ekiti BEK increased from (4.20±0.04 to 5.34±0.04); while Beef obtained from Lagos BLA increased from (4.11 ± 0.10 to 5.40 ± 0.03) and Beef obtained from Ibadan BIB increased from 4.20 ± 0.03 to 5.53 ± 0.07. Chicken samples stored at refrigeration temperature followed similar trend with beef samples by having non – significant ($p<0.05$) increase on day 0 – 28 day of storage. Turkey samples at refrigeration temperature recorded non-significant decrease with Turkey obtained from Ekiti TEK 4.26 ± 0.02 to 4.23± 0.08. Turkey obtained from Lagos TLA and turkey obtained from Ibadan TIB showed non - significant increase on day 0 to 28 day of storage respectively; TLA: 4.42 ± 0.01 – 5.24 ± 0.02; TIB: 4.23 ± 0.02 to 5.29 ± 0.02.

Table 4.2 shows the viable count of LAB (Log_{10}cfu) in different stored meat products subjected to chilling temperature for various time intervals. LAB count of Beef samples recorded non- significant increases in viable count on day 7 to day 28. LAB counts of beef obtained from Lagos BLA and beef obtained from Ibadan BIB increased on day 0 to 28 to a final count of 4.93 ± 0.02 and 5.04 ± 0.02 respectively. LAB count of beef obtained from Ekiti BEK recorded the highest value of 5.42 ± 0.02 on the 28^{th} day of storage. Chicken

samples showed non- significant increases from day 0 to day 28. LAB count of chicken obtained from Ekiti CEK showed a significant increase of 5.27 ± 0.01; LAB counts of chicken obtained from Lagos CLA significantly increased to 5.27 ± 0.01 and chicken obtained from Ibadan CIB increased to 4.96 ± 0.42 on 28 day respectively. Turkey samples stored at chilling temperature followed similar trend with beef and chicken samples. They recorded a final count of TEK 4.96 ± 0.02; TLA 4.92 ± 0.01 and TIB 4.90 ± 0.00.

Viable count of LAB (log_{10}cfu) in different stored meat products subjected to freezing temperature for various time intervals (Table 4.3) showed a significant($p<0.05$) increase in the LAB count of beef samples on day 0 to 28, except BEK which decreased in count from 4.20 ± 0.04 to 4.11 ± 0.03. A slight increase was observed with BLA from 4.11±0.10 to 4.12±0.00. Chicken samples displayed a non - significant increase from day 0 to day 28. Turkey samples followed similar trend of increase in LAB count. TEK and TIB showed a final LAB count of 5.02±0.08 and 5.08±0.00cfu/ml respectively. LAB count of Turkey obtained from lagos TLA recorded a non - significant decrease from day 0 to 28 of storage (4.42 ± 0.01 to 4.15 ± 0.07).

Table 4.4 shows the viable count of LAB (log_{10}cfu) in different stored meat products subjected to fast freezing temperature. LAB count of BEK recorded non - significant increase of 4.20 ± 0.04 – 4.36 ± 0.02. LAB count of BLA recorded significant increase from 4.11 ± 0.15 to 4.83 ± 0.02. LAB count of BIB showed a non - significant increase from 4.20± 0.03 to 4.20 ± 0.12. Chicken samples increased significantly from day 0 to day 28 but LAB count of CLA recorded the highest count of 45.12± 0.02 Turkey samples subjected to fast freezing temperature increased significantly from day 0 to day 28.

Table 4.1: Viable count of LAB (log_{10}cfu) in different stored meat products subjected to refrigeration temperature for various time intervals

Duration of treatment (days)	Type of meat/ Source/ Count (log_{10}cfu/ml)								
	Beef			**Chicken**			**Turkey**		
	BEK	BLA	BIB	CEK	CLA	CIB	TEK	TLA	TIB
0	**4.20± 0.04^{b}	4.11±0.10^{b}	4.20±0.03^{a}	4.11±0.01^{b}	4.10±0.03^{a}	4.32±0.06^{b}	4.26±0.02^{b}	4.42±0.01^{b}	4.23±0.02^{a}
7	4.28 ± 0.01^{b*}	4.50 ± 0.17^{b}	5.36 ± 0.02^{a}	5.09 ± 0.00^{b}	5.26 ± 0.04^{a}	5.21 ± 0.01^{a}	5.18± 0.01^{a}	5.16 ± 0.00^{a}	5.12 ± 0.00^{a}
14	5.28 ± 0.03^{a}	5.31 ± 0.02^{a}	5.36 ± 0.00^{a}	5.17 ± 0.03^{a}	5.31 ± 0.04^{a}	5.44 ± 0.03^{a}	5.22 ± 0.04^{a}	5.22 ± 0.02^{a}	5.26 ± 0.10^{a}
21	5.31 ± 0.02^{a}	5.36 ± 0.03^{a}	5.43 ± 0.00^{a}	5.21 ± 0.03^{a}	5.44 ± 0.02^{a}	5.29 ± 0.00^{a}	5.25 ± 0.02^{a}	5.30 ± 0.04^{a}	5.25 ± 0.05^{a}
28	5.34 ± 0.04^{a}	5.40 ± 0.03^{a}	5.43 ± 0.07^{a}	5.41 ± 0.03^{a}	5.41 ± 0.01^{a}	5.22 ± 0.30^{a}	4.23 ± 0.00^{b}	5.24 ± 0.02^{a}	5.29 ± 0.02^{a}

**Each value is a mean of triplicate determinations ± Standard Error
*Means followed by the same letters in the superscript are not significantly different from one another ($p< 0.05$) by Duncan's Multiple Range Test (DMRT)

BEK Beef sample Ekiti; BLA Beef sample Lagos; BIB Beef sample Oyo; CEK Chicken sample Ekiti; CLA Chicken sample Lagos; CIB Chicken sample Oyo; TEK Turkey sample Ekiti; TLA Turkey sample Lagos; TIB Turkey sample Oyo

Table 4.2: Viable count of LAB (log_{10}cfu) in different stored meat products subjected to chilling temperature for various time intervals

Duration of treatment (days)	Type of meat/source/count (log_{10}cfu)								
	Beef			**Chicken**			**Turkey**		
	BEK	BLA	BIB	CEK	CLA	CIB	TEK	TLA	TIB
0	**4.20± 0.04^{c}	4.11±0.10^{a}	4.20±0.03^{b}	4.11±0.01^{b}	4.10±0.03^{a}	4.32±0.06^{b}	4.26±0.02^{b}	4.42±0.01^{b}	4.20±0.02^{b}
7	5.13 ± 0.05^{a*}	5.28 ± 0.03^{a}	5.10 ± 0.02^{a}	5.30 ± 0.03^{a}	5.23 ± 0.04^{a}	5.10 ± 0.03^{a}	5.18 ± 0.05^{a}	5.08 ± 0.02^{a}	5.12 ± 0.00^{a}
14	5.07 ± 0.04^{b}	5.13 ± 0.00^{b}	5.06 ± 0.01^{a}	5.08 ± 0.01^{a}	5.08 ± 0.01^{b}	5.01 ± 0.01^{a}	5.11 ± 0.01^{a}	5.40 ± 0.23^{a}	5.26 ± 0.22^{a}
21	5.27 ± 0.06^{a}	5.23 ± 0.09^{a}	5.03 ± 0.11^{a}	5.03 ± 0.03^{a}	5.03 ± 0.00^{b}	4.84 ± 0.01^{b}	5.07 ± 0.01^{a}	5.53 ± 0.20^{a}	5.30 ± 0.02^{a}
28	5.43 ± 0.02^{a}	4.93 ± 0.02^{a}	5.04 ± 0.02^{a}	5.27 ± 0.01^{b}	5.27 ± 0.13^{a}	4.96 ± 0.42^{a}	4.96 ± 0.02^{b}	4.92 ± 0.01^{b}	4.90 ± 0.00^{b}

**Each value is a mean of triplicate determinations± Standard Error
*Means followed by the same letters in the superscript are not significantly different from one another ($p< 0.05$) by Duncan's Multiple Range Test (DMRT)

BEK Beef sample Ekiti; BLA Beef sample Lagos; BIB Beef sample Oyo; CEK Chicken sample Ekiti; CLA Chicken sample Lagos; CIB Chicken sample Oyo; TEK Turkey sample Ekiti; TLA Turkey sample Lagos; TIB Turkey sample Oyo

Table 4.3: Viable count of LAB (log_{10}cfu) in different stored meat products subjected to freezing temperature for various time intervals

Duration of treatment (days)	Type of meat / Source/ Count (log_{10}cfu)								
	Beef			Chicken			Turkey		
	BEK	BLA	BIB	CEK	CLA	CIB	TEK	TLA	TIB
0	**4.20± 0.04^{b}	4.11±0.10^{b}	4.20±0.03^{b}	4.11±0.01^{b}	4.11±0.03^{b}	4.32±0.06^{b}	4.26±0.02^{b}	4.42±0.01^{a}	4.20±0.02^{b}
7	4.80 ± 0.00^{a*}	4.25 ± 0.00^{a}	5.10 ± 0.00^{a}	5.12 ± 0.06^{a}	5.12 ± 0.06^{a}	5.09 ± 0.03^{a}	5.09 ± 0.03^{a}	4.22 ± 0.05^{a}	4.14 ± 0.00^{b}
14	4.29 ± 0.01^{b}	4.19 ± 0.02^{b}	5.01 ± 0.05^{a}	5.08 ± 0.02^{a}	4.27 ± 0.07^{b}	5.12± 0.01^{a}	5.03 ± 0.01^{a}	4.30 ± 0.03^{a}	4.23 ± 0.02^{b}
21	4.12 ± 0.09^{c}	4.25 ± 0.05^{a}	4.94 ± 0.23^{b}	5.12 ± 0.23^{a}	5.12 ± 0.14^{a}	5.17 ± 0.06^{a}	5.12 ± 0.50^{a}	4.20 ± 0.03^{a}	4.18 ± 0.05^{b}
28	4.11 ± 0.03^{c}	4.12 ± 0.0^{b}	4.34 ± 0.03^{b}	5.10 ± 0.06^{a}	4.28 ± 0.14^{b}	5.07 ± 0.03^{a}	5.02 ± 0.08^{a}	4.15 ± 0.07^{a}	5.08 ± 0.00^{a}

**Each value is a mean of triplicate determination ± Standard Error
*Means followed by the same letters in the superscript are not significantly different from one another ($p< 0.05$) by Duncan's Multiple Range Test (DMRT)

BEK Beef sample Ekiti; BLA Beef sample Lagos; BIB Beef sample Oyo; CEK Chicken sample Ekiti; CLA Chicken sample Lagos; CIB Chicken sample Oyo; TEK Turkey sample Ekiti; TLA Turkey sample Lagos; TIB Turkey sample Oyo

Table 4.4: Viable count of LAB (log_{10}cfu) in different stored meat products subjected to fast freezing temperature for various time intervals

Duration of treatment (days)	Type of meat/ Source/ Count (log_{10}cfu)								
	Beef			Chicken			Turkey		
	BEK	BLA	BIB	CEK	CLA	CIB	TEK	TLA	TIB
0	**4.20± 0.04^{c}	4.11±0.10^{c}	4.20±0.03^{b}	4.11±0.01^{c}	4.11±0.03^{c}	4.32±0.06^{b}	4.26±0.02^{d}	4.42±0.01^{c}	4.20±0.02^{c}
7	4.24 ± 0.12^{c*}	5.04 ± 0.15^{a}	5.08 ± 0.05^{a}	5.01 ± 0.07^{a}	4.90 ± 0.09^{b}	5.01± 0.11^{a}	5.01 ± 0.11^{a}	5.08 ± 0.09^{a}	4.97 ± 0.22^{a}
14	5.05 ± 0.09^{a}	4.89 ± 0.00^{b}	5.01 ± 0.10^{a}	4.86 ± 0.05^{b}	4.90 ± 0.04^{b}	4.94 ± 0.04^{a}	4.94 ± 0.04^{b}	4.83 ± 0.00^{b}	4.64 ± 0.03^{b}
21	4.71 ± 0.06^{b}	4.96 ± 0.22^{a}	4.11 ± 0.04^{b}	4.72 ± 0.19^{b}	4.86 ± 0.03^{b}	4.79 ± 0.02^{b}	4.79 ± 0.02^{c}	4.40 ± 0.01^{c}	4.39 ± 0.20^{b}
28	4.36 ± 0.02^{c}	4.83 ± 0.02^{b}	4.20 ± 0.12^{b}	4.20 ± 0.10^{c}	5.12 ± 0.02^{a}	4.66 ± 0.02^{b}	4.66 ± 0.02^{c}	4.68 ± 0.04^{b}	4.24 ± 0.03^{c}

**Each value is a mean of triplicate determination ± standard error
*Means followed by the same letters in the superscript are not significantly different from one another ($p< 0.05$) by Duncan's Multiple Range Test (DMRT)

BEK Beef sample Ekiti; BLA Beef sample Lagos; BIB Beef sample Oyo; CEK Chicken sample Ekiti; CLA Chicken sample Lagos; CIB Chicken sample Oyo; TEK Turkey sample Ekiti; TLA Turkey sample Lagos; TIB Turkey sample Oyo

The distribution of isolated LAB strains in the meat samples are shown in figure 4.1. *L. plantarum* had the highest percentage occurrence of 22.7% which was followed by *L. brevis* (13.6%) *Pediococcus acidilactici* was 13.64% while *L. bulgaricus* recorded the least percentage occurrence of 4.55% (Figure 4.1).

Presented in Table 4.5 is the biochemical characterization and probable identities of various LAB isolated from meat samples. The table shows that all the strains were Gram positive, catalase negative, oxidase negative and non spore forming. They all tested positive to methyl red, also negative to Voges Proskauer; gelatin hydrolysis, starch hydrolysis and casein hydrolysis. They all tested negative to nitrate reduction, H_2S production and indole test. They showed varied reactions (positive/negative) to carbohydrate utilization tests. Most of the LAB identified were homofermentative while few were heterofermentative.

Table 4.6 shows the morphological and cellular characteristics of the isolates on MRS Agar. All the isolates were creamy in colour, translucent, small and have uniform, round and smooth colonies with varying cellular characteristics.
Optimization of the growth conditions of *Pediococcus acidilactici* isolates were carried out to select strains for further studies. Production of different antimicrobials (lactic acid, diacetyl and hydrogen peroxide) by the isolates was also monitored. (Table 4.7). Lactic acid production ranged from 1.100 $\pm$ 0.22 to 2.883 $\pm$ 0.13 g/l. *P. acidilactici* BEKBLT recorded the highest lactic acid production of 2.883 g/l. This was followed by *Pediococcus acidilactici* strains TEK1U, TLA14R, CIBIU, BIB7C recording 2.702 $\pm$ 0.13, 2.162 $\pm$ 0.22, 1.802 $\pm$ 0.13 and 1.712 $\pm$ 0.22 g/l respectively. The least lactic acid production (1.100 g/l) was recorded for strain CLA7BLT. Diacetyl production ranged from 0.070 to1.962 ng/l. The highest value was recorded by *Pediococcus acidilactici* strain BIB7C; it was followed by Strain CLA7BLT with value of 1.125 $\pm$ 0.12 ng/l. The lowest diacetyl production (0.070 $\pm$ 0.00) was recorded by strain BLA7F. Considerable values of hydrogen peroxide were recorded (Table 4.7). It was in the range of 0.425-1.275 µg/ml**,** highest values were recorded by *Pediococcus*

acidilactici strains BEK7R, TLA14R and BEK14C. The lowest values were recorded by *Pediococcus* strains CLA7C, CLA7BLT, BEKBLT, BIB7C and CLA14BLT respectively.

Pediococcus acidilactici strains were grown at different pH of 3.2, 5.5, 6.5, 7.5 and 8.5 respectively (Table 4.8). pH 3.2 recorded optical density of 0.474 to 1.772. The highest pH was observed in CLA7BLT (1.772). This was followed by *Pediococcus* strains TLA14R, BEKBLT, BLA7F, TEK1U and BIB7C which recorded optical density readings of 1.478, 1.331, 1.280, 1.262 and 1.082 respectively. The lowest growth (0.474) at pH 3.2 was recorded for *Pediococcus* strain BEK7R. At pH 5.5 optical density reading ranged from 0.698 to 2.228. The highest value was recorded by *Pediococcus* strain BIB7C (2.228). This was followed by BEKBLT, BEK7R, BLA7F and TLA14R recording 2.220, 2.200, 2.125 and 2.065 respectively. Growth at pH 6.5 was in the range of 1.092 to 1.970. At pH 7.5 recorded optical density reading showed values ranging from 1.406 to 2.600. At pH 8.5, the optical density reading showed values ranging from 0.773 to 1.846. Growth at different temperatures of - 15^0C, - 4^0C, 4^0C, 28^0C and 35^0C were optimized (Table 4.9). At 4^0C, optical density reading was observed and recorded from 1.300 to 2.275. The highest value was recorded by *Pediococcus* strain BEKBLT while the least value (1.300) was recorded by BEK7R. At -4^0C growth rate ranged from 1.130 to 2.155. Optimal growth was recorded by Strain BEK14C while the lowest was observed in *Pediococcus* strain BEK7R and TLA14R respectively.

Growth at -15^0C showed values of 1.799 to 2.570. The highest value was recorded by *Pediococcus* strain isolated from stored beef at fast freezing temperature BEKBLT while the least was observed in *Pediococcus* strain CIBIU**.** At 28^0C, growth ranged was from 1.020 to 2.220. The highest (2.220) was observed in *Pediococcus* strain TLA7F while the lowest was observed in *Pediococcus* strain BLA7F. At 35^0C, the growth ranged from 1.165 to 2.345. Optimum growth was observed in *Pediococcus* strain CLA14BLT and the least was recorded in TEK1U.

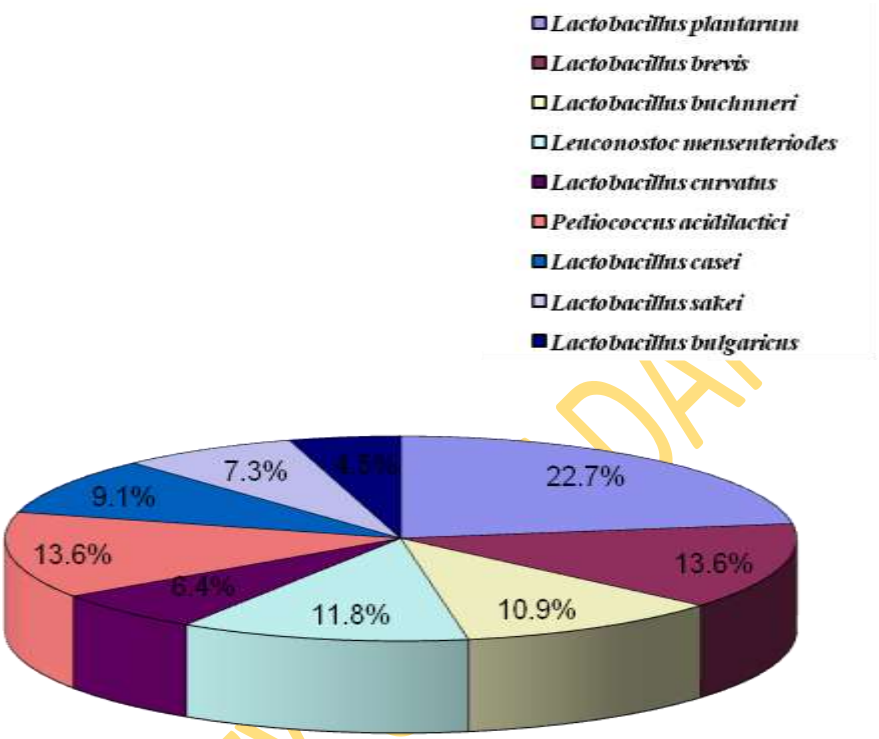

Fig. 4.1: Percentage occurence of Lactic Acid Bacteria species isolated from different meat sources under low temperature

Table 4.5: Biochemical characteristics of Lactic acid bacteria from beef, chicken and Turkey meat samples

S/N	Isolate code	Catalase test	Oxidase test	Gram's reaction	Mp	VP	Gelatin hydrolysis	Starch hydrolysis	Casein hydrolysis	Growth in 4% in NaCl	Growth At pH 4.5	Growth at pH 9.6	Growth at 45°C	Growth at -15°C	Production of NH_3 from Arginine	Nitrate reduction	H_2S production	Indole test	0/relation-ship	Oxidative fermentatio	Homo/ Hetero fermentatio	Motility	Glucose	Lactose	Fructose	Galactose	Maltose	Sucrose	Ribose	Xylose	Mannitol	Mannose	Sorbitol	Melozitose	Arabinose	Cellobiose	Rhamnose	Raffinose	Probable identity
1	CLA[1]U	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	*Lactobacillus plantarum*
2	BLA[1]U	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
3	TLA[1]U	-	-	+	+	-	-	-	-	+	St	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	-	-	-	-	-	-	+	+	-	+	-	-	*Lactobacillus bulgarius*
4	BLA[7]F	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
5	BLA[7]BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	st	+	+	+	-	+	+	-	+	-	-	+	-	-	-	*Lactobacillus cuvatus*
6	BLA[7]R	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HE	-	+	+	+	+	+	S	+	-	+	+	-	+	+	+	-	+	*Lactobacillus brevis*
7	BLA[7]C	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HM	-	+	St	+	+	+	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*
8	CLA[7]F	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
9	CLA[7]BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
10	CLA[7]C	-	-	+	+	-	-	-	-	+	St	+	+	+	-	-	-	-	FA	F	HE	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	*Leuconostoc mesenteroides*
11	CLA[7]R	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*
12	TLA[7]R	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	st	+	-	+	+	+	-	-	*Lactobacillus buchneri*
13	TLA[7]F	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
14	TLA[7]BLT	-	-	+	+	-	-	-	-	+	+	+	+	-	+	-	-	-	FA	F	HM	-	+	st	+	-	+	-	-	-	+	+	-	+	-	+	-	-	*Lactobacillus sakei*
15	TLA[7]C	-	-	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	FA	F	HM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus casei*
16	BER[U]1	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HE	-	+	+	+	+	+	+		+	-	+	+	-	+	+	-	+	*Lactobacillus brevis*
17	TEK[1]U	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
18	CEK[1]U	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	st	+	+	+	-	+	+	-	+	-	-	+	-	-	-	*Lactobacillus curvatus*
19	BIB[1]U	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*
20	CIB[1]U	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
21	TIB[1]U			+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus casei*
22	BEK[7]F	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*
23	BEK[7]C	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HE	-	+	+	+	-	+	+	+	+	+	+	+	+	-	+	-	-	*Leuconostoc mesenteoides*
24	BEK[7]BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	*Pediococcus acidilactici*
25	BEK[7]R	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-	-	+	*Pediococcus acidilactici*
26	BIB[7]F	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HE	-	+	+	+	+	+	+	S	+	-	+	+	-	+	+	-	+	*Lactobacillus brevis*

27	BIB^{7}C	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*

Table 4.5 Contd

S/N	Isolate code	Catalase test	Oxidase test	Gram's reaction	Mp	VP	Gelatin hydrolysis	Starch hydrolysis	Casein hydrolysis	Growth in 4% NaCl	Growth At pH 4.5	Growth at pH 9.6	Growth at 45°C	Growth at -15°C	Production of NH_3 from Arginine	Nitrate reduction	H_2S production	Indole test	0/F relationship	Oxidative fermentation	Homo Hetero fermentation	Motility	Glucose	Lactose	Fructose	Galactose	Maltose	Sucrose	Ribose	Xylose	Mannitol	Mannose	Sorbitol	Melozitose	Arabinose	Cellobiose	Rhamnose	Raffinose	Probable identity
28	BIB^{7}R	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*
29	BIB^{7}BLT	-	-	+	+	-	-	-	-	+	+	+	+	-	-		-	-	-	FA	F	HE	-	+	-	+	+	+	+	+	-	+	+	+	+	-	+	-	-	*Leuconostoc mesentiroides*
30	TEK^{7}C	-	-	+	+	-	-	-	-	+	+	+	+	-	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	-	-	+	+	-	+	-	-	*Lactobacillus sakei*
31	TEK^{7}R	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	-	+	*Lactobacillus brevis*
32	TEK^{7}F	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*
33	TEK^{7}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus casei*
34	CFEK^{7}C	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus casei*
35	CER^{7}R	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*
36	CEK^{7}F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	st	-	+	+	+	-	-	*Lactobacillus buchneri*
37	CEK^{7}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Leuconostoc mesenteoides*
38	CLA^{14}C	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	*Lactobacillus brevis*
39	BLA^{14}C	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	-	-	-	+	+	+	-	-	+	-	-	*Lactobacillus sakei*
40	CLA^{14}R	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus casei*
41	BLA^{14}C	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	*Lactobacillus buchneri*
42	BLA^{14}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	*Leuconostoc mesenteoides*
43	CLA^{14}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
44	BLA^{14}F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus casei*
45	BLA^{14}F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	st	-	+	+	+	-	-	*Lactobacillus buchneri*
46	TEK^{14}C	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	-	+	+	+	-	+	+	+	-	+	-	-	+	-	-	-	*Lactobacillus curvatus*
47	TEK^{14}F	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*
48	TEK^{14}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	-	+	*Lactobacillus brevis*
49	TEK^{14}R	-	-	+	+	-	-	-	-	+	+	+	+		+		-	-	-	FA	F	HE	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	*Leuconostoc mesentiroides*
50	TLA^{14}R	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*

51	TLA^{14}C	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
52	TLA^{14}R	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
53	TLA^{14}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus casei*
54	TLA^{14}F	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*

Table 4.5 Contd

S/N	Isolate code	Catalase test	Oxidase test	Gram's reaction	Mp	VP	Gelatin hydrolysis	Starch hydrolysis	Casein hydrolysis	Growth in 4% NaCl	Growth At pH 4.5	Growth at pH 9.6	Growth at 45°C	Growth at -15°C	Production of NH_3 from Arginine	Nitrate reduction	H_2S production	Indole test	O²relationship	Oxidative fermentati	Homo Hetero fermentati	Motility	Glucose	Lactose	Fructose	Galactose	Maltose	Sucrose	Ribose	Xylose	Mannitol	Mannose	Sorbitol	Melozitose	Arabinose	Cellobiose	Rhamnose	Raffinose	Probable identity
55	CLA^{14}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	-	-	-	+	+	+	+	-	+	-	-	*Lactobacillus sakei*
56	CLA^{14}R	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	*Lactobacillus brevis*
57	CLA^{14}C	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*
58	CLA^{14}F	-	-	+	+	-	-	-	-	+	St	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	-	-	-	-	-	+	+	+	+	-	-	-	*Lactobacillus bulgaricus*
59	BEK^{14}F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	st	+	-	+	+	-	-	*Lactobacillus buchneri*
60	BEK^{14}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*
61	BEK^{14}R	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	st	+	+	+	-	+	+	-	+	-	-	+	-	-	-	*Lactobacillus curvaturs*
62	BEK^{14}C	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	+	*Pediococcus acidilactici*
63	BEK^{14}F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	-	*Leuconostoc mesentiroides*
64	CEK^{14}C	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	-	+	-	-	-	+	+	-	+	-	+	-	-	*Lactobacillus sakei*
65	CEK^{14}F	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	*Lactobacillus brevis*
66	CEK^{14}R	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	*Lactobacillus buchneri*
67	CEK^{14}BLT	-	-	+	+	-	-	-	-	+	St	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-	*Lactobacillus bulgaricus*
68	TEK^{14}R	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*
69	TEK^{14}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	*Lactobacillus brevis*
70	TEK^{14}C	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	-	+	-	-	-	+	+	-	+	-	+	-	-	*Lactobacillus sakei*
71	TEK^{14F}	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*
72	BIB^{29}C	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	*Leuconostoc mesentiroides*
73	CIB^{29}F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	st	+	-	+	+	-	-	*Lactobacillus buchneri*
74	TIB^{29}R	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*
75	BIB^{21}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*

76	TIB[21]F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	-	-	*Leuconostoc mesentiroides*
77	CIB[21]BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	*Lactobacillus brevis*
78	BEK[21]R	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*
79	CEK[2]BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	-	+	+	-	+	-	-	+	-	-	-	*Lactobacillus curvaturs*
80	TEK[21]F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus casei*
81	TLA[21]R	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	+	*Lactobacillus brevis*

Table 4.5 Contd

S/N	Isolate code	Catalase test	Oxidase test	Gram's reaction	Mp	VP	Gelatin hydrolysis	Starch hydrolysis	Casein hydrolysis	Growth in 4% NaCl	Growth At pH 4.5	Growth at pH 9.6	Growth at 45°C	Growth at -15°C	Production of NH_3 from Arginine	Nitrate reduction	H_2S production	Indole test	0°relation-ship	Oxidative fermentati	Homo Hetero fermentati	Motility	Glucose	Lactose	Fructose	Galactose	Maltose	Sucrose	Ribose	Xylose	Mannitol	Mannose	Sorbitol	Melozitose	Arabinose	Cellobiose	Rhamnose	Raffinose	Probable identity
82	BLA[21]BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	-	+	+	-	+	-	-	+	-	-	-	*Lactobacillus curvatus*
83	CLA[21]C	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	*Lactobacillus buchneri*
84	TIB[21]R	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	*Lactobacillus Buchneri*
85	BLA[21]C	-	-	+	+	-	-	-	-	+	St	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	-	-	-	-	+	-	+	+	+	-	-	-	*Lactobacillus bulgaricus*
86	TLA[21]F	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	*Lactobacillus plantarum*
87	BIB[21]R	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	*Leuconostoc mesentiroides*
88	CIB[21]C	-	-	+	+	-	-	-	-	+	St	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-	-	*Lactobacillus bulgaricus*
89	TEK[21]C	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	st	+	-	+	+	-	-	*Lactobacillus buchneri*
90	BEK[21]F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	*Lactobacillus buchneri*
91	TIB[21]F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus sakei*
92	BLA[21]C	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Leuconostoc mesentiroides*
93	CLA[21]BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	*Lactobacillus brevis*
94	TEK[28]F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus casei*
95	TIB[28]F	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*
96	CIB[28]BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	*Lactobacillus casei*
97	BIB[28]R	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*
98	CLA[28]F	-	-	+	+	-	-	-	-	+	+	+	+	+	-		-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	*Lactobacillus buchneri*
99	TLA[28]BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+		-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*

100	BLA^{28}R	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Leuconostoc mesentiroides*
101	CEK^{28}C	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus curvaturs*
102	BIB^{29}F	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	*Lactobacillus brevis*
103	BIB^{28}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*
104	BEK^{29}C	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Leuconostoc mesentiroides*
105	BEK^{28}F	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	*Lactobacillus plantarum*
106	TEK^{28}R	-	-	+	+	-	-	-	-	+	St	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus bulgarius*
107	TEK^{28}C	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	*Lactobacillus brevis*
108	TIB^{28}BLT	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	*Lactobacillus sakii*
109	CIB^{28}C	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HM	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	*Lactobacillus plantarum*
110	BIB^{29}F	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	FA	F	HE	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	*Lactobacillus brevis*

Table 4.6: Morphological and Cellular Characteristics of Isolates

	Isolate code	Colour	Opacity	Elevation	Margin	Shape	Size	Cellular characteristics
1	CLA^{1}U	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
2	BLA^{1}U	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
3	TLA^{1}U	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive long rods occurring in chains
4	BLA^{7}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
5	BLA^{7}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive curved rods, occurring in pairs and short chains
6	BLA^{7}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
7	BLA^{7}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
8	CLA^{7}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
9	CLA^{7}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
10	CLA^{7}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
11	CLA^{7}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
12	TLA^{7}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains

13	TLA^{7}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
14	TLA^{7}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, with rounded ends occurring singly and in short chains
15	TLA^{7}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods with square ends, occurring in chains
16	BER^{U}1	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
17	TEK^{1}U	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
18	CEK^{1}U	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive curved rods, occurring in pairs and short chains
19	BIB^{1}U	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
20	CIB^{1}U	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
21	TIB^{1}U	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods with square ends, occurring in chains
22	BEK^{7}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
23	BEK^{7}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains

Table 4.6 Contd.

	Isolate code	**Colour**	**Opacity**	**Elevation**	**Margin**	**Shape**	**Size**	**Cellular characteristics**
24	BEK^{7}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
25	BEK^{7}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
26	BIB^{7}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
27	BIB^{7}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
28	BIB^{7}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
29	BIB^{7}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
30	TEK^{7}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, with rounded ends occurring singly and in short chains
31	TEK^{7}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
32	TEK^{7}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains

33	TEK^{7}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods with square ends, occurring in chains
34	CFEK^{7}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods with square ends, occurring in chains
35	CER^{7}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
36	CEK^{7}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains
37	CEK^{7}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
38	CLA^{14}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
39	BLA^{14}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, with rounded ends occurring singly and in short chains
40	CLA^{14}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods with square ends, occurring in chains
41	BLA^{14}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains
42	BLA^{14}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
43	CLA^{14}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
44	BLA^{14}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods with square ends, occurring in chains
45	BLA^{14}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains
46	TEK^{14}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive curved rods, occurring in pairs and short chains

Table 4.6 Contd.

	Isolate code	**Colour**	**Opacity**	**Elevation**	**Margin**	**Shape**	**Size**	**Cellular characteristics**
47	TEK^{14}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
48	TEK^{14}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
49	TEK^{14}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
50	TLA^{14}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
51	TLA^{14}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
52	TLA^{14}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
53	TLA^{14}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods with square ends, occurring in chains

54	TLA^{14}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
55	CLA^{14}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, with rounded ends occurring singly and in short chains
56	CLA^{14}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
57	CLA^{14}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
58	CLA^{14}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive long rods occurring in chains
59	BEK^{14}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains
60	BEK^{14}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
61	BEK^{14}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive curved rods, occurring in pairs and short chains
62	BEK^{14}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive cocci, occurring in tetrads and in chains
63	BEK^{14}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
64	CEK^{14}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, with rounded ends occurring singly and in short chains
65	CEK^{14}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
66	CEK^{14}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains
67	CEK^{14}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive long rods occurring in chains
68	TEK^{14}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
69	TEK^{14}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
70	TEK^{14}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, with rounded ends occurring singly and in short chains

Table 4.6 Contd

	Isolate code	**Colour**	**Opacity**	**Elevation**	**Margin**	**Shape**	**Size**	**Cellular characteristics**
71	TEK^{14F}	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
72	BIB^{29}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
73	CIB^{29}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains
74	TIB^{29}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
75	BIB^{21}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains

76	TIB^{21}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
77	CIB^{21}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
78	BEK^{21}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
79	CEK^{2}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive curved rods, occurring in pairs and short chains
80	TEK^{21}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods with square ends, occurring in chains
81	TLA^{21}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
82	BLA^{21}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive curved rods, occurring in pairs and short chains
83	CLA^{21}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains
84	TIB^{21}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains
85	BLA^{21}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive long rods occurring in chains
86	TLA^{21}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
87	BIB^{21}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
88	CIB^{21}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive long rods occurring in chains
89	TEK^{21}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains
90	BEK^{21}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains

Table 4.6 Contd

	Isolate code	Colour	Opacity	Elevation	Margin	Shape	Size	Cellular characteristics
91	TIB^{21}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, with rounded ends occurring singly and in short chains
92	BLA^{21}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
93	CLA^{21}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains

94	TEK^{28}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods with square ends, occurring in chains
95	TIB^{28}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
96	CIB^{28}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods with square ends, occurring in chains
97	BIB^{28}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
98	CLA^{28}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, occurring singly and in short chains
99	TLA^{28}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
100	BLA^{28}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
101	CEK^{28}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive curved rods, occurring in pairs and short chains
102	BIB^{28}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
103	BIB^{28}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
104	BEK^{28}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive coccoid rods occurring in chains
105	BEK^{28}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
106	TEK^{28}R	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive long rods occurring in chains
107	TEK^{28}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains
108	TIB^{28}BLT	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods, with rounded ends occurring singly and in short chains
109	CIB^{28}C	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive rods occurring singly and in short chains
110	BIB^{28}F	Creamy	Translucent	Small convex	Entire	Circular	Small	Gram positive slender rods, occurring singly and in short chains

Table 4.7: Production of different antimicrobials by *Pediococcus acidilactici* isolates from beef, chicken and turkey samples

Isolate Code	Antimicrobials / Recorded values		
	Lactic Acid (g/l)	Diacetyl (ng/l)	Hydrogen Peroxide (µg/l)
BLA^{1}U	**1.261± 0.13^{ef}	0.094 ± 0.00^{h}	0.850 ± 0.12^{b}
BLA^{7}F	1.200± 0.13^{ef*}	0.070 ± 0.00^{h}	0.850 ± 0.12^{b}
CLA^{7}C	1.340± 0.13d^{ef}	1.177 ± 0.00^{b}	0.425 ± 0.12^{e}
CLA^{7}BLT	1.100 ± 0.22^{f}	1.125± 0.12^{c}	0.425 ± 0.12^{c}
TLA^{7}F	1.441 ± 0.13^{cdef}	0.863± 0.00^{f}	0.850 ± 0.12^{b}
TEK^{1}U	2.702 ± 0.13^{a}	0.942± 0.00^{e}	0.850 ± 0.12^{b}
CIB^{1}U	1.802 ± 0.13^{bc}	0.864 ± 0.00^{f}	0.850 ± 0.12^{b}
BEK^{7}BLT	2.883 ± 0.13^{a}	0.942 ± 0.12^{e}	0.425 ± 0.00^{c}
BEK^{7}R	1.621 ± 0.22^{cde}	0.864± 0.12^{f}	1.275 ± 0.12^{a}
BIB^{7}F	1.712 ± 0.22^{cd}	1.962 ± 0.21^{a}	0.425 ± 0.22^{c}
CLA^{14}BLT	1.402 ± 0.13^{cdef}	0.550 ± 0.20^{g}	0.425 ± 0.12^{c}
TLA^{14}R	2.162 ± 0.12^{bc}	1.021 ± 0.10^{d}	1.275 ± 0.12^{a}
TLA^{14}C	1.441 ± 0.12^{cdef}	0.550 ± 0.12^{g}	0.850 ± 0.12^{b}
TLA^{14}R2	1.261 ± 0.22^{ef}	1.099 ± 0.22^{c}	0.850 ± 0.12^{b}
BEK^{14}C	1.117 ± 0.00f	0.942 ± 0.12^{e}	1.275 ± 0.11^{a}

**Each value is a mean of triplicate determination ± Standard Error
*Means followed by the same letters in the superscript are not significantly different from one another ($p< 0.05$) by Duncan's Multiple Range Test (DMRT)

BLA1U: untreated beef Lagos
BLA^{7}F: beef freezing Lagos
CLA^{7}C: chicken chilling Lagos
CLA^{7}BLT: chicken fastfreezing Lagos
TLA^{7}F: turkey freezing Lagos
TEK^{1}U: untreated turkey Ekiti
CIB^{1}U: untreated chicken Ibadan
BEK^{7}BLT: beef fastfreezing Ekiti
BEK^{7}R: beef refrigeration Ekiti
BIB^{7}F: beef freezing Ibadan,
CLA^{14}BLT: chicken fastfreezing Lagos
TLA^{14}R: turkey refrigeration Lagos
TLA^{14}C: turkey chilling Lagos
TLA^{14}R2: turkey refrigeration Lagos
BEK^{14}C: beef chilling Ekiti

Table 4.8: Growth at different pH of *Pediococcus acidilactici* isolates from meat samples under low temperature storage

	pH / Growth values $(O.D)_{540}$				
Isolate Code	**3.2**	**5.5**	**6.5**	**7.5**	**8.5**
$BLA^{1}U$	$^{**}0.793 \pm 0.12^{cd}$	1.992 ± 0.12^{b}	1.7160 ± 0.10^{abc}	1.978 ± 0.12^{bcd}	1.076 ± 0.12^{bcd}
$BLA^{7}F$	$1.280 \pm 0.12^{b*}$	2.125 ± 0.10^{a}	1.798 ± 0.12^{ab}	1.934 ± 0.12^{bcd}	1.210 ± 0.12^{bc}
$CLA^{7}C$	0.695 ± 0.12^{cd}	1.990 ± 0.12^{b}	1.578 ± 0.12^{abc}	1.650 ± 0.10^{cde}	$*0.773 \pm 0.11^{d}$
$CLA^{7}BLT$	1.772 ± 0.12^{a}	2.000 ± 0.20^{b}	1.680 ± 0.11^{abc}	1.880 ± 0.12^{bcd}	1.162 ± 0.12^{bcd}
$TLA^{7}F$	0.480 ± 0.22^{d}	1.348 ± 0.12^{c}	1.058 ± 0.12^{de}	1.926 ± 0.00^{bcd}	1.186 ± 0.10^{bcd}
$TEK^{1}U$	1.262 ± 0.12^{b}	2.120 ± 0.11^{a}	1.822 ± 0.12^{ab}	2.100 ± 0.12^{b}	1.328 ± 0.12^{b}
$CIB^{1}U$	0.982 ± 0.11^{bc}	2.160 ± 0.12^{a}	1.092 ± 0.11^{de}	1.636 ± 0.22^{de}	0.813 ± 0.22^{cd}
$BEK^{7}BLT$	1.331 ± 0.12^{b}	2.220 ± 0.22^{a}	1.790 ± 0.12^{ab}	2.100 ± 0.20^{b}	1.083 ± 0.12^{bcd}
$BEK^{7}R$	0.474 ± 0.22^{d}	2.220 ± 0.22^{a}	1.344 ± 0.22^{cd}	2.055 ± 0.22^{bc}	1.268 ± 0.22^{b}
$BIB^{7}F$	1.082 ± 0.00^{e}	2.228 ± 0.20^{a}	1.741 ± 0.10^{e}	1.674 ± 0.12^{cde}	1.052 ± 0.12^{bcd}
$CLA^{14}BLT$	0.574 ± 0.12^{d}	2.228 ± 0.12^{a}	1.970 ± 0.12^{a}	1.993 ± 0.12^{bcd}	1.076 ± 0.12^{bcd}
$TLA^{14}R2$	1.023 ± 0.00^{bc}	1.888 ± 0.00^{b}	1.514 ± 0.22^{bc}	1.840 ± 0.00^{bcd}	1.846 ± 0.22^{a}
$TLA^{14}C$	0.629 ± 0.11^{cd}	1.888 ± 0.11^{b}	1.480 ± 0.11^{bc}	2.600 ± 0.12^{a}	1.006 ± 0.20^{bcd}
$TLA^{14}R$	1.478 ± 0.12^{b}	2.065 ± 0.00^{b}	1.720 ± 0.00^{bc}	1.406 ± 0.12^{e}	1.152 ± 0.12^{bcd}
$BEK^{14}C$	0.640 ± 0.12^{cd}	1.850 ± 0.12^{a}	1.586 ± 0.00^{abc}	1.414 ± 0.12^{e}	1.150 ± 0.00^{bcd}

**Each value is a mean of triplicate determination ± Standard Error
*Means followed by the same letters in the superscript are not significantly different from one another ($p< 0.05$) by Duncan's Multiple Range Test (DMRT)

BLA1U: untreated beef Lagos
$CLA^{7}BLT$: chicken fastfreezing Lagos
$CIB^{1}U$: untreated chicken Ibadan
$BIB^{7}F$: beef freezing Ibadan,
$BLA^{7}F$: beef freezing Lagos
$TLA^{7}F$: turkey freezing Lagos
$BEK^{7}BLT$: beef fastfreezing Ekiti
$CLA^{14}BLT$: chicken fastfreezing Lagos
$CLA^{7}C$: chicken chilling Lagos
$TEK^{1}U$: untreated turkey Ekiti
$BEK^{7}R$: beef refrigeration Ekiti
$TLA^{14}R$: turkey refrigeration Lagos

TLA^{14}C: turkey chilling Lagos TLA^{14}R2: turkey refrigeration Lagos BEK^{14}C: beef chilling Ekiti

Table 4.9: Growth at different temperature of *P. acidilactici* isolates from different meat samples

	Temperature / Growth values (O.D.) $_{540}$				
Isolate Code	**4^{0}C**	**-4^{0}C**	**15^{0}C**	**28^{0}C**	**35^{0}C**
BLA^{1}U	**2.050±0.12^{abc}	1.792±0.12^{abc}	2.455±0.12^a	1.894±0.12^a	2.010±0.12^{ab}
BLA^{7}F	1.688±0.12^{c*}	1.216±0.10^{de}	2.490±0.12^a	1.020±0.12^c	1.170±0.11^c
CLA^{7}C	2.085±0.12^{abc}	2.000±0.12^{ab}	2.382±0.13^a	1.305±0.12^{bc}	1.870±0.12^b
CLA^{7}BLT	1.888±0.22^{abc}	1.678±0.12^{bc}	2.510±0.22^a	2.035±0.22^a	2.135±0.12^{ab}
TLA^{7}F	1.860±0.12^{abc}	1.766±0.12^{abc}	2.520±0.12^a	2.220±0.12^a	2.045±0.22^{ab}
TEK^{1}U	2.165±0.12^{ab}	1.852±0.12^{abc}	2.560±0.13^a	2.015±0.13^a	1.165±0.13^c
CIB^{1}U	2.030±0.12^{abc}	1.948±0.10^{bc}	1.799±0.12^c	1.368±0.12^{bc}	2.130±0.12^b
BEK^{7}BLT	2.275±0.00^a	1.972±0.12^{ab}	2.570±0.12^a	2.130±0.12^a	2.150±0.10^b
BEK^{7}R	1.300±0.22^d	1.130±0.22^e	1.910±0.22^{bc}	2.148±0.22^a	2.130±0.22^c
BIB^{7}F	1.816±0.22^{bc}	1.536±0.22^{cd}	2.520±0.12^a	1.448±0.22^b	1.482±0.22^c
CLA^{14}BLT	2.265±0.12^a	1.756±0.12^{abc}	1.810±0.10^c	2.105±0.22^a	2.345±0.12^a
TLA^{14}R	1.792±0.22^{bc}	1.130±0.22^e	2.190±0.00^{ab}	1.940±0.12^a	1.995±0.22^{ab}
TLA^{14}C	2.130±0.12^{ab}	1.824±0.12^{abc}	2.430±0.22^a	1.990±0.22^a	2.050±0.22^{ab}
TLA^{14}R2	2.090±0.13^{abc}	1.700±0.12^{bc}	2.530±0.12^a	2.080±0.00^a	2.085±0.12^{ab}
BEK^{14}C	1.826±0.12^{bc}	2.155±0.12^a	2.240±0.12^{ab}	1.848±0.12^a	1.975±0.22^{ab}

**Each value is a mean of triplicate determination ± Standard Error

*Means followed by the same letters in the superscript are not significantly different from one another ($p< 0.05$) by Duncan's Multiple Range Test (DMRT)

BLA1U: untreated beef Lagos BLA^{7}F: beef freezing Lagos CLA^{7}C: chicken chilling Lagos
CLA^{7}BLT: chicken fastfreezing Lagos TLA^{7}F: turkey freezing Lagos TEK^{1}U: untreated turkey Ekiti
CIB^{1}U: untreated chicken Ibadan BEK^{7}BLT: beef fastfreezing Ekiti BEK^{7}R: beef refrigeration Ekiti
BIB^{7}F: beef freezing Ibadan, CLA^{14}BLT: chicken fastfreezing Lagos TLA^{14}R: turkey refrigeration Lagos

TLA^{14}C: turkey chilling Lagos TLA^{14}R2: turkey refrigeration Lagos BEK^{14}C: beef chilling Ekiti

Growth at different concentration of NaCl for isolates from beef, chicken and turkey samples (Table 4.10) revealed growth values of 0.013±0.22 to 2.155 ±0.22 at 1% NaCl concentration, *P. acidilactici* CIB recorded the optimum. At 2% NaCl, rate of growth ranged from 1.678± 0.12 to 2.240 ± 0.12; *P. acidilactici* obtained from stored beef under fast freezing recorded the highest value. At 3% NaCl, 0.180 ± 0.12 to 1.930 ± 0.12 was recorded with *P. acidilactici* BEKBLT recording the highest. At 4% NaCl rate of growth rate ranged from 0.262 ± 0.22 to 1.518±0.12 with *P. acidilactici* BEKBLT also recorded the highest.

Based on the results obtained from the optimization studies above, ten strains (BLA1U, TEK1U, CIB1U, BEKBLT, BIB7C, TLA14R, CLA7C, BEK7R, CLA14BLT and TLA14C) of *Pediococcus acidilactici* were subjected to plasmid analysis and curing procedures. Plasmid DNA was isolated and samples were subjected to agarose gel electrophoresis.

Result of the plasmid DNA subjected to agarose gel electrophoresis are shown in figure 4.2 Bands were identified from top to bottom; the molecular masses of standard plasmid DNA are represented of lanes M1, and M_2. Plasmids DNA from 10 strains of *Pediococci* are represented on lanes 1 to 10. Result reveals that *Pediococci* isolate contain plasmid ranging from 861 bp to 20643 bp. No plasmid was observed in lane 1(BLA1U). Lane 2 (TEK1U) reveals plasmid presence of 861 bp molecular weight, lane 3 (CIB1U), 4 (BEKBLT) and 5 (BIB7C) had plasmids of 861 bp, 1277 bp and 20643 bp respectively. Lane 7 (TLA14R) was found to contain plasmid of 20643 bp; lane 8 (CLA7C) had a plasmid of 861 bp. Lane 9 (CLA14BLT) showed plasmids of 20643 bp and 861 bp. Lane 10 (TLA14C) showed no plasmid presence. Fig 4.3 showed results of cured plasmid of ten *P. acidilactici* strains subjected to acridine orange treatment to remove their plasmids. The result showed the absence of plasmid in all strains of *P. acidilactici*.

Table 4.10: Growth of *Pediococcus* isolates at different concentrations of NaCl isolates from beef, chicken and turkey samples

Isolate	NaCl concentration / Growth values (O.D.) $_{540}$			
	1%	2%	3%	4%
BLA^{1}U	**1.178 ± 0.11^{b}	2.170 ± 0.11^{a}	1.738 ± 0.11^{a}	1.334 ± 0.11^{b}
BLA^{7}F	0.413 ± 0.12^{c*}	2.170 ± 0.12^{ab}	0.180 ± 0.12^{d}	1.404 ± 0.12^{b}
CLA^{7}C	2.030 ± 0.11^{a}	1.844 ± 0.12^{bc}	0.228 ± 0.00^{d}	1.455 ± 0.12^{b}
CLA^{7}BLT	1.752 ± 0.12^{a}	1.964 ± 0.22^{ab}	1.848 ± 0.12^{a}	0.291 ± 0.00^{gf}
TLA^{7}F	1.758 ± 0.00^{a}	1.924 ± 0.12^{ab}	1.898 ± 0.22^{a}	1.110± 0.12^{bcd}
TEK^{1}U	2.075 ± 0.12^{a}	1.791 ± 0.11^{d}	1.627 ± 0.12^{c}	1.650± 0.12^{a}
CIB^{1}U	2.155 ± 0.22^{a}	1.932 ± 0.22^{ab}	1.334 ± 0.10^{b}	1.364± 0.22^{bc}
BEK^{7}BLT	2.150 ± 0.12^{a}	2.240 ± 0.12^{ab}	1.903 ± 0.12^{a}	1.518± 0.12^{a}
BEK^{7}R	0.013 ± 0.22^{d}	1.996 ± 0.12^{ab}	1.638 ± 0.12^{ab}	1.118± 0.22^{bcd}
BIB^{7}F	2.095 ± 0.10^{a}	1.880 ± 0.22^{ab}	1.858 ± 0.11^{a}	1.280 ± 0.10^{bc}
CLA^{14}BLT	2.015 ± 0.12^{a}	1.882 ± 0.12^{ab}	1.904 ± 0.12^{a}	0.850± 0.12^{de}
TLA^{14}R	1.912 ± 0.22^{a}	1.678 ± 0.12^{c}	1.872 ± 0.00^{a}	0.262 ± 0.22^{fg}
TLA^{14}C	2.020 ± 0.12^{a}	1.802 ± 0.12^{bc}	1.506 ± 0.22^{ab}	0.970 ± 0.12^{cde}
TLA^{14}R2	2.095 ± 0.10^{a}	1.802 ± 0.00^{bc}	1.810 ± 0.00^{a}	1.177 ± 0.22^{g}
BEK^{14}C	1.318 ± 0.12^{b}	2.045 ± 0.00 ^{ab}	1.822 ± 0.20^{a}	0.352 ± 0.12^{fg}

**Each value is a mean of triplicate determination ± Standard Error
*Means followed by the same letters in the superscript are not significantly different from one another ($p< 0.05$) by Duncan's Multiple Range Test (DMRT)

BLA1U: untreated beef Lagos
BLA^{7}F: beef freezing Lagos
CLA^{7}C: chicken chilling Lagos
CLA^{7}BLT: chicken fastfreezing Lagos
TLA^{7}F: turkey freezing Lagos
TEK^{1}U: untreated turkey Ekiti
CIB^{1}U: untreated chicken Ibadan
BEK^{7}BLT: beef fastfreezing Ekiti
BEK^{7}R: beef refrigeration Ekiti
BIB^{7}F: beef freezing Ibadan,
CLA^{14}BLT: chicken fastfreezing Lagos
TLA^{14}R: turkey refrigeration Lagos
TLA^{14}C: turkey chilling Lagos
TLA^{14}R2: turkey refrigeration Lagos
BEK^{14}C: beef chilling Ekiti

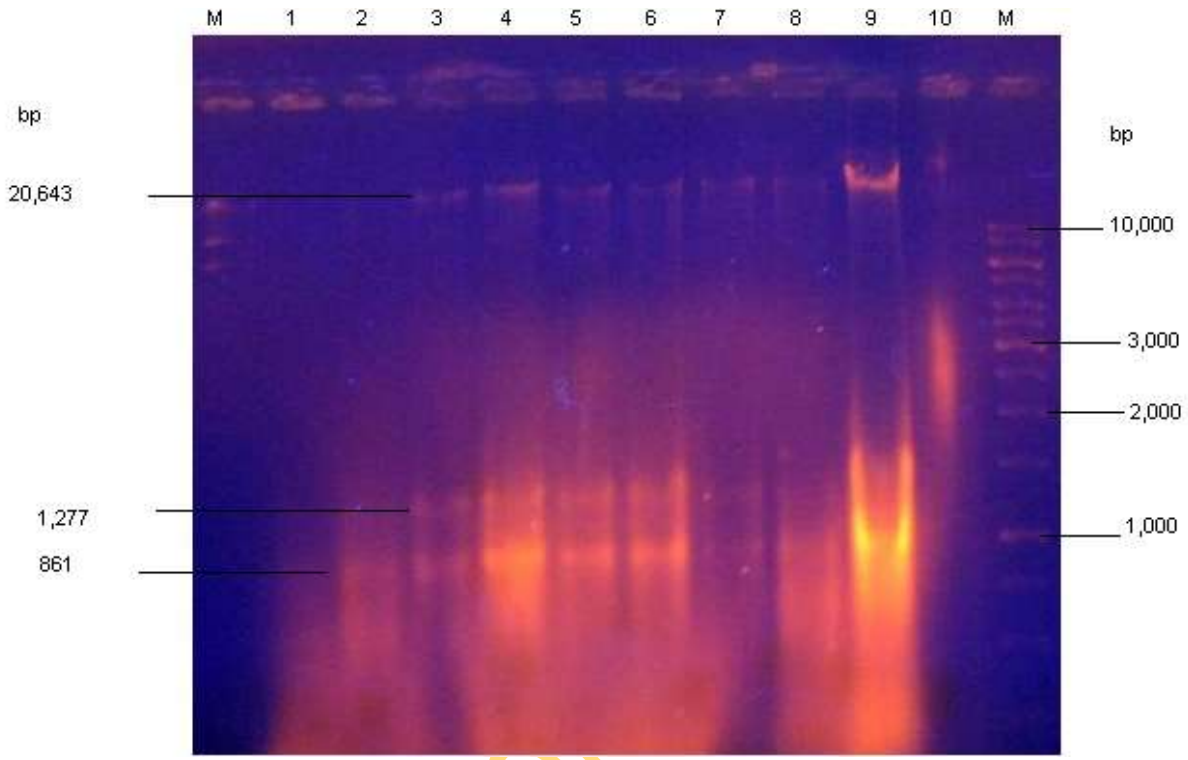

Figure 4.2: Electrophoretogam showing separation of plasmids in each of the *Pediococcus acidilactici* isolates from meats under low temperature storage

Lane M1 and M2 Markers
Lane 1 (BLA1U) no plasmid
Lane 2 (TEK1U) 861bp
Lane 3 (CIB1U)20643bp; 1277bp and 861bp
Lane 4 (BEKBLT) 20643bp, 1277bp and 861bp
Lane 5 (BIB7C) 20643bp; 1277bp and 861bp
Lane 6 (TLA14R) 20643bp, 1277bp and 861bp
Lane 7 (CLA7C) 20643bp
Lane 8 (BEK7R) 861bp
Lane 9 (CLA14BLT) 20643bp and 861bp
Lane 10(TLA14C) no plasmid

BLA1U: untreated beef Lagos
TEK^{1}U: untreated turkey Ekiti
CIB^{1}U: untreated chicken Ibadan
BEK^{7}BLT: beef fastfreezing Ekiti
BIB^{7}C: beef freezing Ibadan
TLA^{14}R: turkerefrigeration Lagos
CLA^{7}C: chicken chilling Lagos
BEK7R: beef refrigeration Ekiti
CLA^{14}BLT: chicken fastfreezing Lagos
TLA^{14}C: turkey chilling Lagos

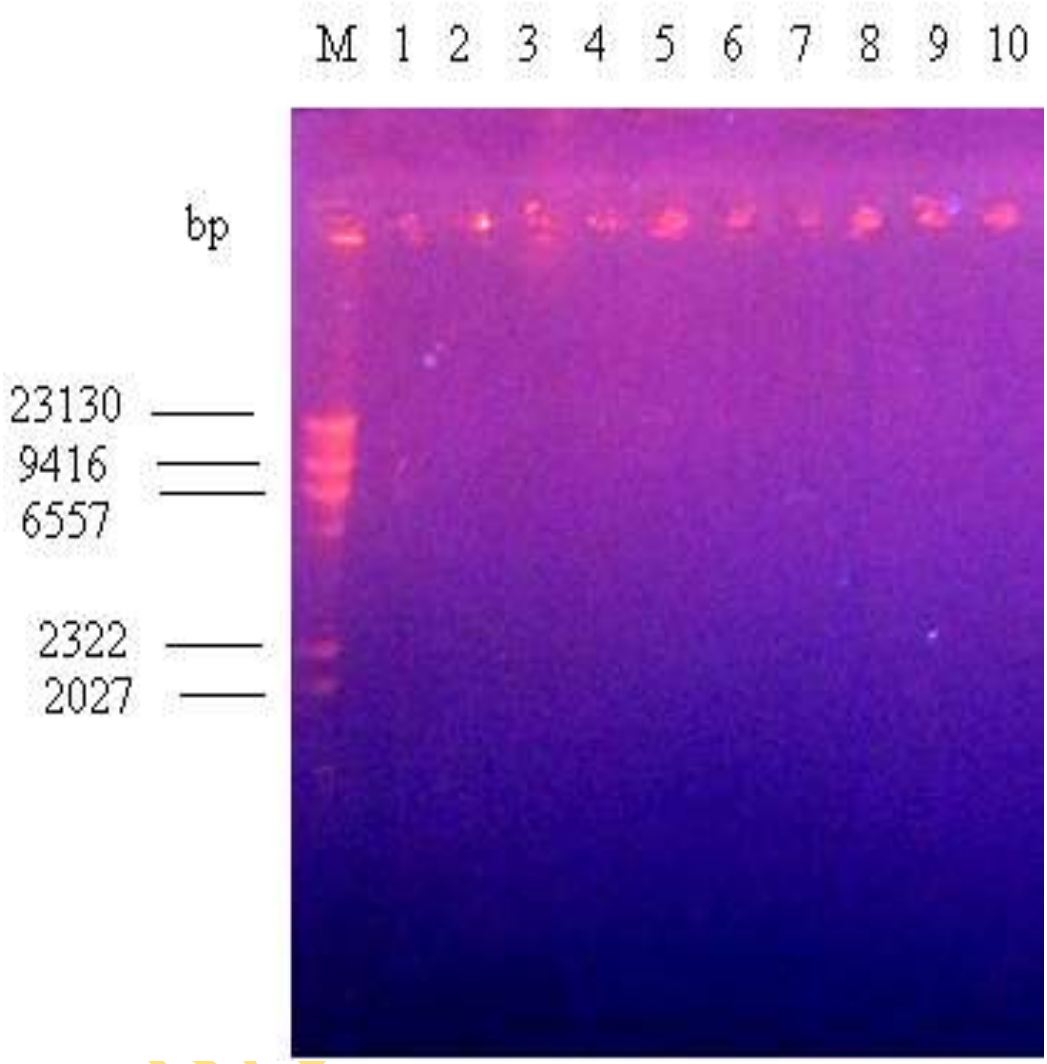

Figure 4.3 Electrophoretogam showing cured plasmids in each *Pediococcus acidilactici* isolates from meats under low temperature storage

Lane M Marker
Lane 1 (BLA1U)
Lane2 (TEK1U)
Lane 3 (CIB1U)20643bp; 1277
Lane 4 (BEKBLT)
Lane 5 (BIB7C)

Lane 1- 10 *Pediococcus* isolate
Lane 6 (TLA14R)
Lane 7 (CLA7C)
Lane 8 (BEK7R)
Lane 9 (CLA14BLT)
Lane 10(TLA14C)

BLA1U: untreated beef Lagos
$TEK^{1}U$: untreated turkey Ekiti
$CIB^{1}U$: untreated chicken Ibadan
$BEK^{7}BLT$: beef fastfreezing Ekiti
$BIB^{7}C$: beef freezing Ibadan

$TLA^{14}R$: turkerefrigeration Lagos
$CLA^{7}C$: chicken chilling Lagos
BEK7R: beef refrigeration Ekiti
$CLA^{14}BLT$: chicken fastfreezing Lagos
$TLA^{14}C$: turkey chilling Lagos

Antagonistic activities of *Pediococcus acidilactici* strains were tested against eight indicator organisms (Table 4.11) *P. acidilactici* TLA14R showed the highest zone of inhibition (6.5 mm) against *Staphylococcus aureus* ATCC 25923 while strain TEK1U and BIB7C showed the least inhibition zone of 1.5 mm against the same indicator strain. All strains of *Pediococcus acidilactici* tested showed higher zone of inhibition against *Escherichia coli* ATCC 5218 ranging from 6.5–10 mm respectively. *P. acidilactici* strains BEKBLT and BIB7C showed the highest inhibition zone of 10 mm while strain CIB1U recorded the lowest zone of inhibition of 6.5 mm against *Escherichia coli* ATCC 5218. For *Pseudomonas aeruginosa* ATCC 27853, no activity was detected with strain BIB7C. *P. acidilactici* BEKBLT displayed the highest zone of inhibition against *Enterococcus faecalis* ATCC 29212 (8.0 mm); *Salmonella typhimurium* wild type (8.0 mm); *Bacillus cereus* wild type (8.00 mm) and *Listeria monocytogens* wild type (6.00 mm). No activity was recorded against *Bacillus subtilis* wild type and control samples.

Table 4.12 shows the comparison between parental and cured strains subjected to antimicrobial activity test. Non of the cured isolates was susceptible to the eight meat spoilage microorganisms tested, except two isolates of *Pedioccoccus acidilactici* BEKBLT and TLA14R that recorded zone of inhibition of 1.5 mm against *Escherichia coli* ATCC 5218. Physiological and sugar fermentation by parental and cured strains of *P. acicilactici* showed similar results in the tests carried out **(**Table 4.13)

Their qualities and quantities after purification of the LAB isolates are shown in Table 4.14. The result showed that TLA14R had the highest concentration of 18.23 ng/µl while the lowest (6.62 ng/µl) was recorded for BIB7C. The A260/280 measurement fell between the range of 1.66 and 2.0 (Schurman et al., 2004)

The result of the nucleotide sequencing of the 16S rDNA genes of selected five of the isolates are shown in Table 4.15. The length of nucleotide sequences (in

bases) obtained for the isolates are 151, 161, 145, 152 and 151 for BEKBLT, BIB7C, CIB1U, TEK1U and TLA14R respectively with BIB7C having the highest length.

Computer generated chromatograms of the nucleotide sequences of the 16S rDNA gene of the LAB isolate obtained from stored beef under fast freezing. Colours **black**, **blue**, **green** and **red** represent nucleotide bases guanine (**G**), cytosine (**C**), adenine (**A**) and thymine (**T**) respectively. The relative height of peaks depicts the level of quality as well as quantity of the DNA in the test samples. The nucleotide sequences starts from A through B and C and terminates at D (Appendix 2).

Presented in Table 4.16 is the result of the ten topmost sequences producing significant alignments when the nucleotide sequences subjected to Basic Local Alignment Search Tool (BLAST) in the gene bank Database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), for strain BEKBLT. Over 100 species of organisms were shown to have significant alignments in the gene database.

All the ten topmost species shown to produce significant alignment with *Pediococcus acidilactici* isolate have expected value (E value) of between 3e-69 and 4e-68 and maximum identification (Max identity of 98%). Six of the strains were all *Pediococcus acidilactici,* another two with accession numbers EU169995.1 and AB362985.1 were *Pediococcus* sp., the third was *Pediococcus pentosaceus* with accession number EU667384.1, while the fourth was uncultured bacterium with accession number AM277654.1, whose species was not specified by the author that made the submission of the 16S rDNA gene nucleotide sequences into the database. (Table 4.16)

Table 4.11: Antagonistic activity of *Pediococcus acidilactici* isolates against selected indicator organisms at 27±2°C

Indicator code	Zones of Inhibition (mm)				
	TEK1U	**CIB1U**	**BEKBLT**	**BIB7C**	**TLA14R**
Staphylococcus aureus ATCC 25923	1.5	6.0	5.0	1.5	6.5
Escherichia coli ATCC 5218	8.0	6.5	10.0	10.0	8.0
Pseudomonas aeruginosa ATCC 27853	8.0	6.5	12.0	ND	5.0
Enterococcus feacalis ATCC 29212	6.0	6.0	6.0	ND	4.0
Salmonella typhimurium wild type	5.0	6.0	8.0	6.5	4.0
Bacillus cereus wild type	6.5	5.0	8.0	ND	5.0
Listeria monocytogenes wild type	5.0	ND	6.0	6.0	6.0
Bacillus subtilis wild type	5.0	6.0	ND	4.0	4.0
Control	ND	ND	ND	ND	ND

ND – Not Detected

TEK1U: Untreated turkey Ekiti
CIB1U: Untreated chicken Ibadan
BEKBLT: Beef fast freezing Ekiti
BIB7C: Beef chilling Ibadan
TLA14R: Turkey refrigeration Lagos

Table 4.12: Effect of curing on the antimicrobial activity of *Pediococcus acidilactici* strains against indicator organisms

Indicator	Zones of Inhibition (mm)									
	Parental strain					Cured strain				
	TEK1U	CIB1U	BEKBLT	BIB7C	TLA14R	TEK1U	CIB1U	BEKBLT	BIB7C	TLA14R
Staphylococcus aureus ATCC 25923	1.5	6.0	6.5	5.0	6.5	ND	ND	ND	ND	ND
Escherichia coli ATCC 5218	8.0	6.5	10	10.0	8.0	ND	ND	1.5	ND	1.5
Pseudomonas aeruginosa ATCC 27853	5.0	6.5	12	ND	5.0	ND	ND	ND	ND	ND
Enterococcus feacalis ATCC 29212	6.0	6.0	6.0	ND	4.0	ND	ND	ND	ND	ND
Salmonella typhimurium wild type	5.0	6.0	8.0	6.5	4.0	ND	ND	ND	ND	ND
Bacillus cereus wild type	6.5	5.0	8.0	ND	5.0	ND	ND	ND	ND	ND
Listeria monocytogenes wild type	5.0	ND	6.0	6.0	6.0	ND	ND	ND	ND	ND
Bacillus subtilis wild type	5.0	6.0	ND	4.0	4.0	ND	ND	ND	ND	ND
Control	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND – Not Detected

TEK1U : Untreated turkey Ekiti
CIB1U: Untreated chicken Ibadan
BEKBLT: Beef fast freezing Ekiti
BIB7C: Beef chilling Ibadan
TLA14R: Turkey refrigeration Lagos

Table 4.13: Physiological and sugar fermentation by parental and cured *Pediococcus acidilactici* strains

	Extent of fermentation									
	Parental strain					**Cured strain**				
Physiological / Biochemical Test	**TEKU**	**CIBU**	**BEKBLT**	**BIB7C**	**TLA14R**	**TEK1U**	**CIB1U**	**BEKBLT**	**BIB7C**	**TLA14R**
Catalase test	-	-	-	-	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-	-
Growth in 4% NaCl	+	+	+	+	+	+	+	+	+	+
Growth at -15^{0}C	+	+	+	+	+	+	+	+	+	+
Growth at 45^{0}C	+	+	+	+	+	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	-	+	St	-	+
Fructose	+	+	+	+	+	+	St	-	+	-
Arabinose	+	+	+	+	+	St	-	+	-	St
Galatose	+	+	+	+	+	+	+	-	+	+
Raffinose	+	+	+	+	+	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-

+ Positive; - Negative; St - slightly positive

TEK1U : Untreated turkey Ekiti CIB1U: Untreated chicken Ibadan

BEKBLT: Beef fast freezing Ekiti BIB7C: Beef chilling Ibadan TLA14R: Turkey refrigeration Lagos

Table 4.14: Qualities and quantities of the 16s rDNA genes of the *Pediococcus acidilactici* isolates obtained by PCR using V3 primer after purification.

S/N	Sample ID	16S rDNA conc (ng/uL)	A260nm	A260/280	A260/230
1	BEKBLT	15.35	0.504	1.75	0.04
2	BIB7C	6.62	0.265	1.66	0.03
3	CIB1U	12.67	0.338	1.50	0.02
4	TEK1U	9.42	0.223	2.0	0.03
5	TLA14R	18.23	0.548	1.57	0.06

TEK1U: Untreated turkey Ekiti
CIB1U: Untreated chicken Ibadan
BEKBLT: Beef fast freezing Ekiti
BIB7C: Beef chilling Ibadan
TLA14R: Turkey refrigeration Lagos

Table 4.15: Nucleotide Base Sequences of the 16S rDNA genes of the *Pediococci* Cultures

Isolate	Nucleotide Base sequences	N0
BEKBLT	TCTGGTTATACCGTCACTGGGTGAACAGTTACTCTCACCC ACGTTCTTCTTTAACAACAGAGCTTTACGAGCCGAAACCC TTCTTCACTCACGCGGCGTTGCTCCATCAGACTTGCGTCCA TTGTGGAAGATTCCCTACTGCTGCCACCCG	**151**
BIB7C	TGGTATACCGTCACTGGGTAAACAGTTACTCTTACCCACGT TCTTCTTTAACAACAGAGCTTTACGAGCCGAAACCCTTCTTC ACTCACGCGGCGTTGCTCCATCAGACTTGCGTCCATTGTGGA AGATTCCCTACTGCTGCCACCCGTAGGAATGTTCTT	**161**
CIB1U	GGTTATACCGTCCTGGGTAAACAGTTACTCTTACCCACGT TCTTCTTTAACAACAGAGCTTTACGAGCCGAAACCCTTCT TCACTCACGCGGCGTTGCTCCATCAGACTTGCGTCCATTG TGGAAGATTCCCTACTGCTGCCACC	**148**
TEK1U	TCTGGTTATACCGTCACTGGGTAAACAGTTACTCTTACCCAC GTTCTTCTTTAACAACAGAGCTTTACGAGCCGAAACCCTTCT TCACTCACGCGGCGTTGCTCCATCAGACTTGCGTCCATTGTG GAAGATTCCCTACTGCTGCCACCCGT	**152**
TLA14R	TCTGGTTATACCGTCACTGGGTGAACAGTTACTCTCACCC ACGTTCTTCTTTAACAACAGAGCTTTACGAGCCGAAACCC TTCTTCACTCACGCGGCGTTGCTCCATCAGACTTGCGTCCA TTGTGGAAGATTCCCTACTGCTGCCACCCG	**151**

TEK1U : Untreated turkey Ekiti
CIB1U: Untreated chicken Ibadan
BEKBLT: Beef fast freezing Ekiti
BIB7C: Beef chilling Ibadan
TLA14R: Turkey refrigeration Lagos

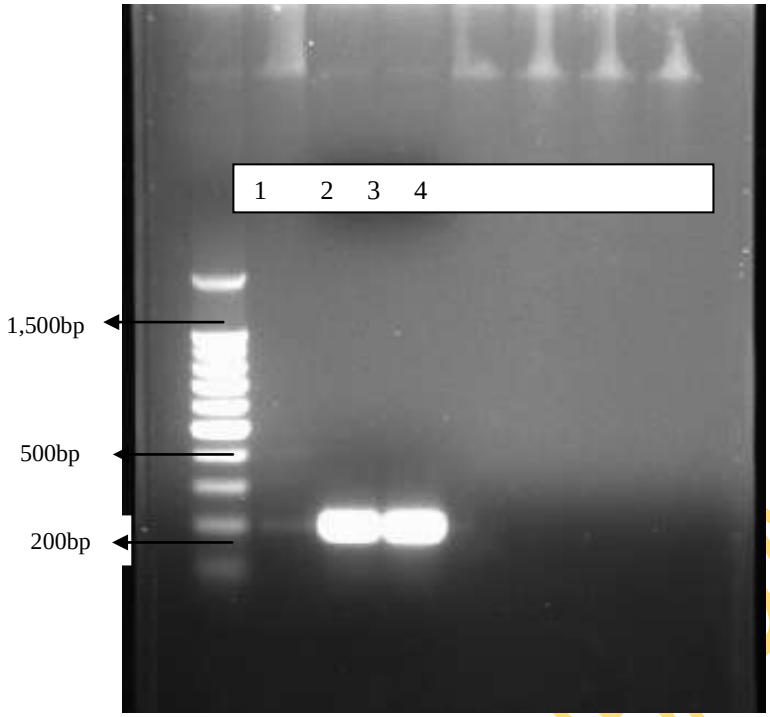

Fig. 4.4: Gel image of the 16SrDNA `PCR products

Lane1 - 100bp DNA ladder

Lane 2 - Control (no PCR product)

Lane 3 - PCR product of 16S rDNA, V3 region (*P. acidilactici* from stored beef under fast freezing temperature**)**

Lane 4 - PCR product of 16S rDNA, V3 region (*P. acidilactici* from stored beef under chilling temperature BIB7C*)*

Table 4.16. Basic Local Alignmet Search Tool result of stored beef under fast freezing temperature showing sequences producing significant alignments in the gene bank Database

	Accession no	Description	Max score	Total score	Query coverage	E value	Max ident
1	EU667384.1	Pediococcus pentosaceus isolate INT 02 16S ribosomal RNA gene, partial sequence	268	268	100%	3e-69	98%
2	AM277654.1	Uncultured bacterium partial 16S rRNA gene,	268	268	100%	3e-69	98%
3	EU082179.1	Pediococcus acidilactici strain. F328 16S ribosomal RNA gene, partial sequence	265	265	100%	4e-69	98%
4	EU180608.1	Pediococcus acidilactici strain NS96 16S rRNA, partial sequence	265	265	100%	4e-68	98%
5	EU263132.1	Pediococcus acidilactici strain CFR2193 16S ribosomal RNA gene, partial sequence	265	265	100%	4e-68	98%
6	EU169995.1	Pediococcus sp. SD2 16S ribosomal RNA gene, partial sequence	265	265	100%	4e-63	98%
7	AB362985.1	Pediococcus sp NGRI 0510 16S ribosomal RNA gene, partial sequence	265	265	100%	4e-68	98%
8	EU147316.1	Pediococcus acidilactici strain BFE 8262 16S ribosomal RNA gene, partial sequence	265	265	100%	4e-68	98%
9	EU147314.1	Pediococcus acidilactici strain BFE 8390 16S ribosomal RNA gene, partial sequence	265	265	100%	4e-68	98%
10	EU147314.1	Pediococcus acidilactici strain BFE 8390 16S ribosomal RNA gene, partial sequence	265	265	100%	4e-68	98%

Figure 4.5, 4.6a and 4.6b to show the results of alignment of the 16S rDNA gene nucleotide sequences of the *P. acidilactici* BEKBLT isolate with five organisms, producing significant alignment in their sequences with this strain in the gene database. Organism with (accession number EU1667384.1) showed that there was mismatch at two positions of the aligned nucleotide sequences thus leading to 98% maximum identities Fig 4.5

The result of alignment of the 16S rDNA gene nucleotide sequences of the *P. acidilactici* obtained from stored beef under fast freezing showed a gap and mismatch in the nucleotide sequence of an uncultured bacterium with accession number AM277654.1. (Fig 4.5). Two gaps were noted for the other three strains of *Pediococcus acidilactci* with accession numbers EU082179.1, EU180608.1 and EU263132.1 respectively. There were gaps in Positions 8 and 9 of their nucleotide sequence alignment. Also a mismatch was noted at the 149th positions of the aligned nucleotide sequences of strains, thus resulting to 98% maximum identities.(Fig 4.6a and b)

Lactic acid and Acetic acid production by the five selected *Pediococcus acidilactici* strains (TEK1U, CIB1U, BEKBLT, BIB7C and TLA14R) were determined using the high performance liquid chromatography (HPLC). Diacetyl and hydrogen peroxide were determined using the enzymatic methods. Various concentrations of lactic and acetic acids were used to prepare standards. The concentrations are 0.5, 2.5, 5.0, 7.5, 10.0, 15.0 and 20 mg/ml respectively. Chromatograms of standard concentration of 2.5mg/l of lactic and acetic acids are shown in Appendix 3. Acetic acid had higher peak and retention time than lactic acid of the same concentration. The retention time is on the x-axis while absorbance unit (AU) is on y-axis. The rentention time (RT) for lactic acid and acetic acid were 7.91 and 8.04 mins respectively. Chromatograms generated by HPLC for other concentrations and respective standards curves are shown in Appendix 4 to 7. Standard concentration of Diacetyl is shown in Appendix 10.

|EU667384.1|*Pediococcus pentosaceus* INT 02 16S ribosomal RNA gene, partial sequence. Length=152 Score = 268 bits (145), Expect = 3e-69 Identities = 149/151 (98%), Gaps = 0/151 (0%) Strand=Plus/Minus

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TCTGGTTATTACCGTCACTGGGTGAACAGTTACTCTCACCCACGTTCTTCTTTAACAACAG

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject
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||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query       ATTGTGGAAGATTCCCTACTGCTGCCACCG
            |||||||||||||||||||||||||||||
Sbjct       ATTGTGGAAGATTCCCTACTGCTGCCACCG
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|EU147314.1|*Pediococcus acidilactici* Strain BFE 8260 16S ribosomal RNA gene, partial sequence. Length=1437 Score = 265 bits (143), Expect = 4e-68 Identities = 150/153 (98%), Gaps = 2/153 (1%) Strand=Plus/Minus

```
Query
```

2 GAPS

```
TCTGGTT--ATACCGTCACTGGGTGAACAGTTACTCTCACCCACGTTCTTCTTTAACAAC

|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject
TCTGGTTAAATACCGTCACTGGGTGAACAGTTACTCTCACCCACGTTCTTCTTTAACAAC

Query
AGAGCTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTGCGT

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject
AGAGCTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTGCGT

Query       CCATTGTGGAAGATTCCCTACTGCTGCCACCCG
            |||||||||||||||||||||||||||||||||
Subject     CCATTGTGGAAGATTCCCTACTGCTGCCACCCG
```

Figure 4.5: Alignment of 16s rDNA nucleotide sequences of *Pediococcus pentosaceus* (accession no EU667384.1) and Pediococcus *acidilactici* (accession no EU147314.1) in genebank database.

|EU082179.1| ***Pediococcus acidilactici*** **strain F328 16S rRNA, partial Length=535, Score = 265 bits (143), Expect = 4e-68, Identities = 150/153 (98%), Gaps = 2/153 (1%)**

Strand=Plus/Minus

Query **2 GAPS**

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```

|EU180608.1| *Pediococcus acidilactici* strain NS96 16S rRNA,

partial Length=1439, Score = 265 bits (143),

Expect = 4e-68, Identities = 150/153 (98%), Gaps = 2/153 (1%)

Strand=Plus/Minus

Query **2 GAPS**

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AGAGCTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTGCGT

        Query       CCATTGTGGAAGATTCCCTACTGCTGCCACCCG
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        Subjcet     CCATTGTGGAAGATTCCCTACTGCTGCCACCCG
```

Figure 4.6a: Alignment of 16s rDNA nucleotide sequences of isolate from stored beef under fast freezing against *Pediococcus acidilactici* (accession no |EU082179.1) and *Pediococcus acidilactici* (accession no EU180608.1) in genebank database.

|EU263132.1| *Pediococcus acidilactici* strain CFR2193 16S rRNA, partial Length=535, Score = 265 bits (143),
Expect = 4e-68, Identities = 150/153 (98%), Gaps = 2/153 (1%)

Strand=Plus/Minus

Query **2 GAPS**

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TCTGGTT--ATACCGTCACTGGGTGAACAGTTACTCTCACCCACGTTCTTCTTTAACAAC

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
        Subject
TCTGGTTAAATACCGTCACTGGGTGAACAGTTACTCTCACCCACGTTCTTCTTTAACAAC

        Query
AGAGCTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTGCGT

||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
        Subject
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        Query       CCATTGTGGAAGATTCCCTACTGCTGCCACCCG
                    |||||||||||||||||||||||||||||||
        Subject     CCATTGTGGAAGATTCCCTACTGCTGCCACCCG
```

Figure 4.6b: Alignment of 16S rDNA nucleotide sequences of isolate from stored beef under fast freezing against against *Pediococcus acidilactici* (accession number EU263132.1) in the gene bank database.

Figure 4.7a shows the graphical representation of lactic and acetic acids produced by *Pediococcus acidilactici* obtained from untreated turkey TEK1U in MRS broth. There was decrease in lactic acid production at 24 hr to 72 hr (5.84 – 3.06 g/l). Peak production of lactic acid and acetic acid by strain TEK1U was reached at 96 hr, which recorded 24.37 g/l and 28.76 mg/ml respectively. At 121 hr lactic acid reduced to 20.19 g/l and no acetic acid was recorded at the same period. Figure 4.7b shows the pH production and optical density by *Pediococcus acidilactici* TEK1U reveals a slight decrease from 4.56 – 4.54 at 24 hr and 48 hr respectively. It further increased with peak value of (4.84) recorded at 96 hr. The optical density at 540 nm wavelength increased at 24 hr to 121 hr. (1.904 – 2074). Hydrogen peroxide production with and without glucose in phosphate buffer was examined (Fig. 4.8a). Better growths were recorded at each interval with the addition 50 mM glucose. The peak values of hydrogen peroxide with or without glucose were recorded at 72 hrs as 13.76 µg/ml and10.80 µg/ml respectively. Diacetyl production by *P. acidilactici* TEK1U in MRS broth showed a decreased in value from 26.69 – 18.84 ng/l. The peak diacetyl production by TEK1U was 40.80 ng//l at 96 hr (Fig. 4.8b**).**

Figure 4.9a shows the result of lactic and acetic acids produced by *P. acidilactici* obtained from untreated chicken CIB1U. Lactic acid increased from an initial value of 4.21 g/l at 24h to 13. 77 g/l at 121 h. The peak value of (17.76 g/l) was recorded at 72 hr. Acetic acid production followed similar trend with lactic acid, but its peak 18.62 mg/ml was reached at 96 hr. The pH value recorded a slight decrease from 4.55 – 4.51 at 121h. Optical density (OD) reading decreased in value from 2.62 to 1.87 at 121 hr. (Fig. 4b). Hydrogen peroxide production with glucose recorded higher yield than isolates without glucose except at 72-96 hr (Fig 4.10a). Diacetyl production in *P. acidilactici* CIB1U decreased initially and later remained constant at 96 - 121 h, value recorded was 25.12 ng/l (Fig. 4.10b).

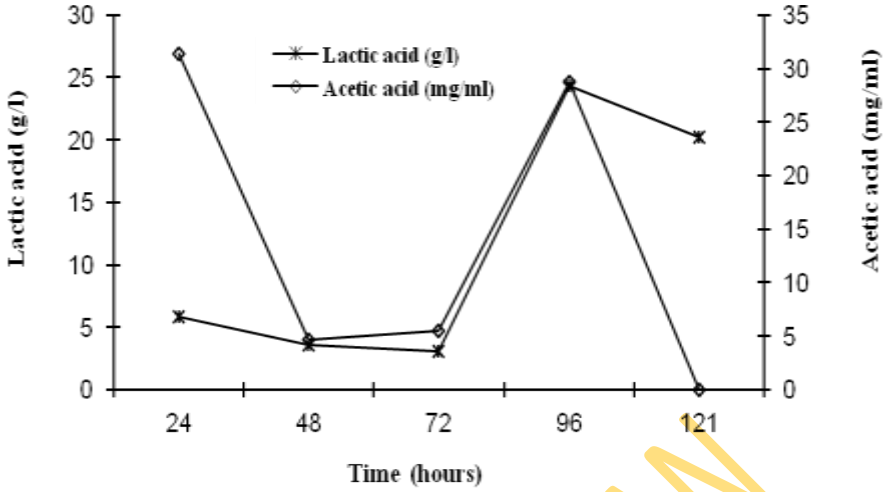


Fig. 4.7a: Time Course of lactic acid and acetic acid production by *Pediococcus acidilactici* from untreated turkey in Man Rogosa and Sharpe broth

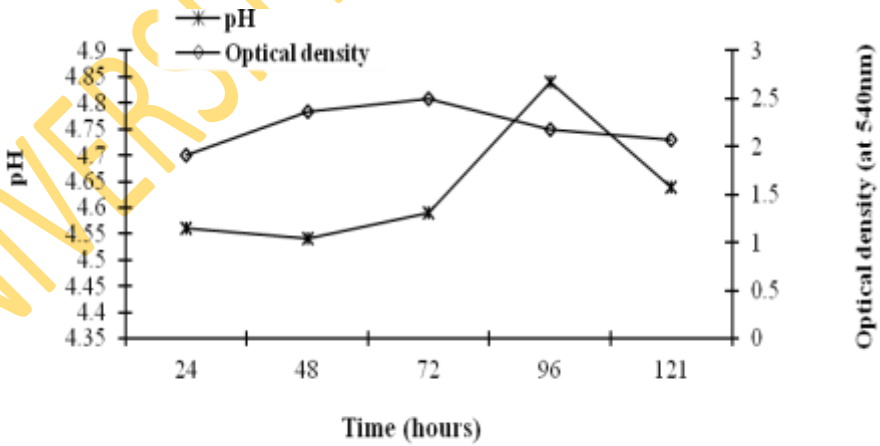


Fig. 4.7b: pH and growth measurements for *Pediococcus acidilactici* from untreated turkey in Man Rogosa and Sharpe broth at different time intervals

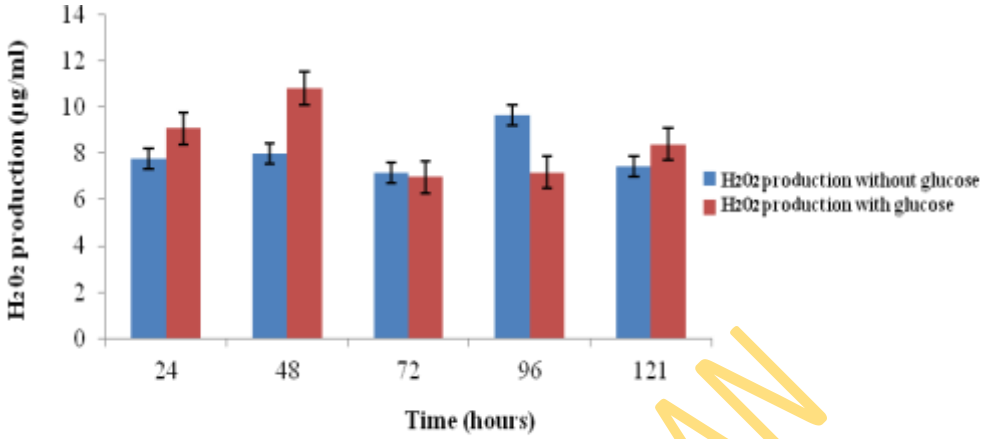


Fig. 4.8a: Hydrogen peroxide production by *Pediococcus acidilacti* from untreated turkey in the presence and absence of glucose in growth medium

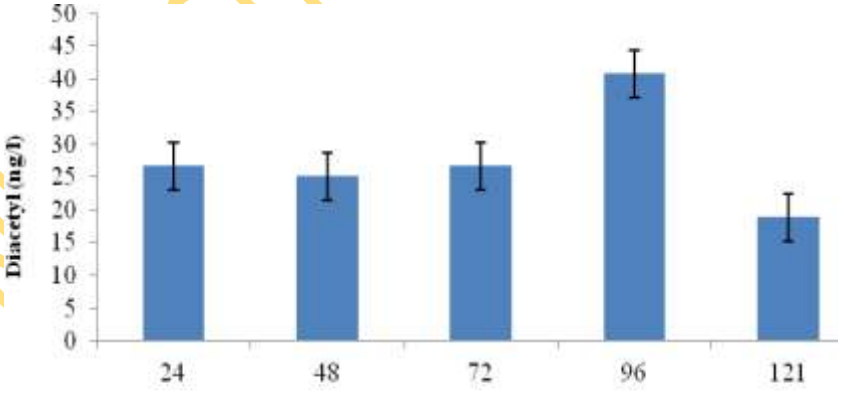


Fig. 4.8b: Diacetyl production by Pediococcus acidilactici from untreated turkey in Man Rogosa and Sharpe broth

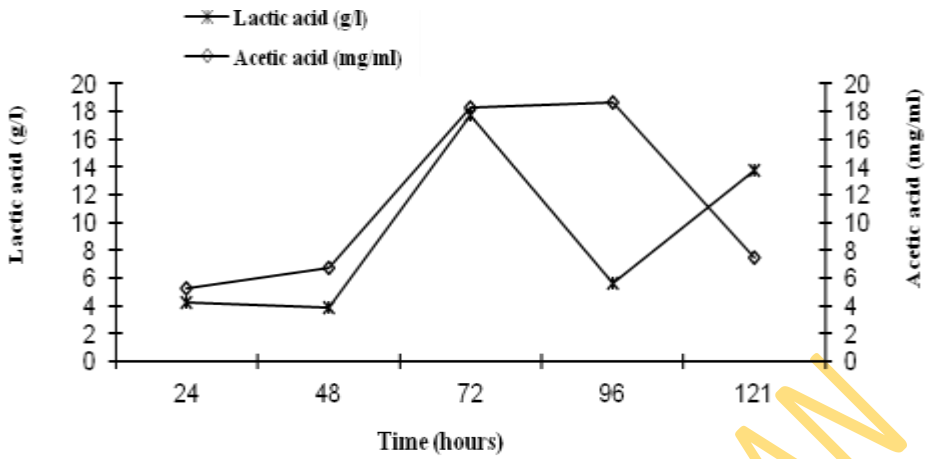


Fig. 4.9a: Time course of lactic acid and acetic acid production by *Pediococcus acidilactici* from untreated chicken in Man Rogosa and Sharpe broth

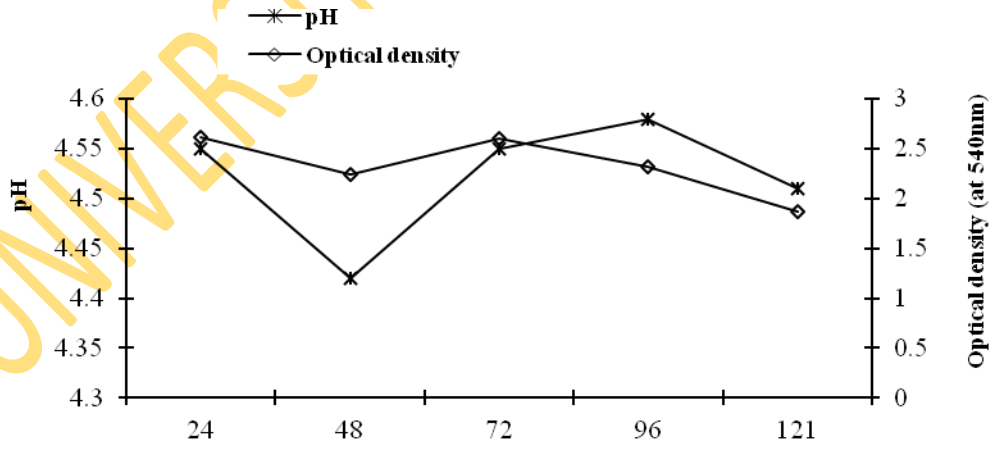


Fig. 4.9b: pH and growth measurements for *Pediococcus acidilactici* from untreated chicken in Man Rogosa and Sharpe broth at different time intervals

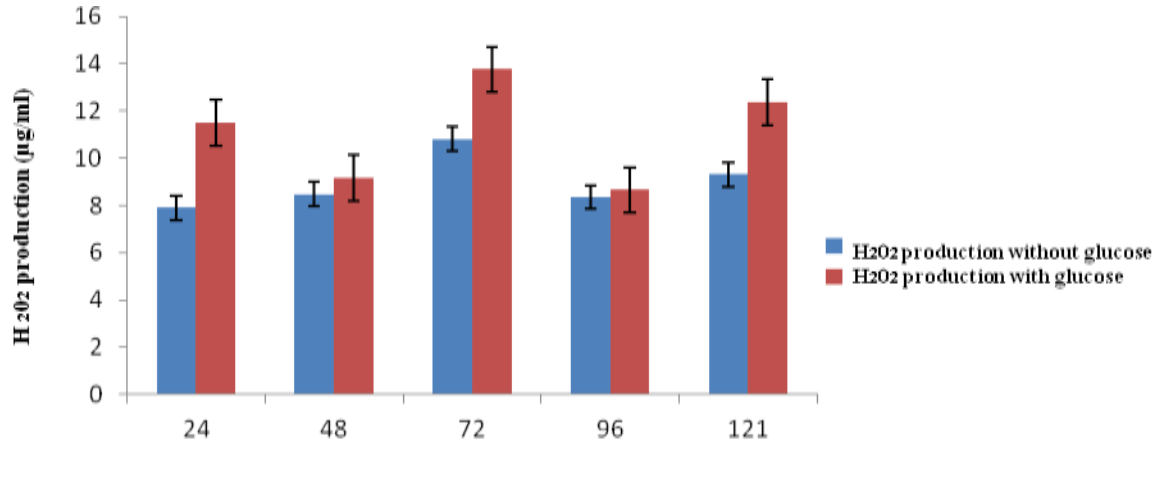


Fig. 4.10a: Hydrogen peroxide production by *Pediococcus acidilactici* from untreated chicken in the presence and absence of glucose in growth

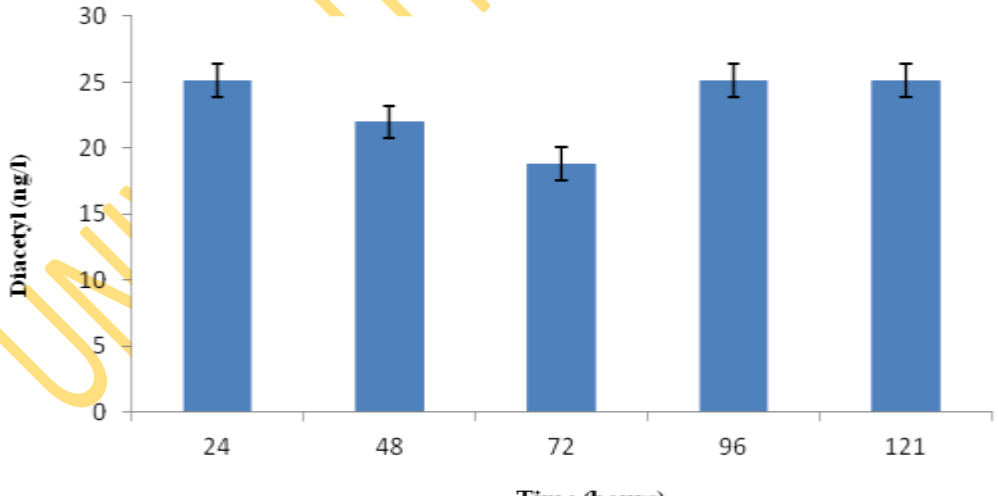


Fig. 4.10b: Diacetyl production by *Pediococcus acidilactici* from untreated chicken in Man Rogosa and Sharpe broth

Pediococcus acidilactici isolated from stored beef under fast freezing temperature BEKBLT produced highest lactic acid (30.74 g/l) at 72 hr while the least was recorded as 4.46 g/l at 24 hr. Acetic acid production followed similar trend (Fig. 4.11a). The pH decreased from 4.50 to 4.38 at 121 hr. OD reading decreased from 2.495 to 1.689 at 121 hr (Fig. 4.11b). Hydrogen peroxide productions with 55.5 mM glucose were higher than production without glucose. Peak values with and without glucose (16.00 µg/ml and13.06 µg/ml) were recorded at 121 hr. The least values (10.29 µg/ml and 9.73 µg/ml) were recorded at 96 hr (Fig. 4.12a). Diacetyl production by *P.acidilactici* BEKBLT recorded the highest and lowest values of 21.98 ng/l and 10.99 ng/l at 72 h and 96h respectively (Fig 4.12b).

Fig 4.13a shows the graphical representation of lactic and acetic acids produced *P. acidilactici* isolated from stored beef under chilling temperature BIB7C in MRS broth. Both acids increased from 24 h and reached their peaks (22.41 g/l and 26.43 mg/ml) at 72 h respectively. The pH and optical density of *P. acidilactici* BIB7C decreased from (4.56 - 4.38) and (2.069 – 1.890) respectively (Fig 4.13b). Hydrogen peroxide production by *Pediococcus acidilactici* BIB7C with and without 55.5 mM glucose followed the same trend with other strains except at 72 and 121 hr. (Fig. 4.14a). Diacetyl production increased from 10.99 ng/l – 18.84 ng/l at 121 hr. The peak diacetyl production (23.55 ng/l) was at 96 hr. (Fig. 4.14b). *Pediococcus acidilactici* isolated from stored turkey under refrigeration TLA14R recorded highest lactic and acetic acid production of 16.3 g/l and 19.34 mg/ml at 96 h and 121 h respectively (Fig 4.15a). The least values were recorded at 48 h. pH and optical density reading of TLA14R decreased to 4.38 and 1.838 respectively(Fig. 4.15b). Hydrogen peroxide production with and without 55.5 mM glucose followed the same trend with other strains. The highest value of (12.49 µg/ml) was produced with glucose at 121 h while the least value recorded was 8.39 µg/ml at 24 h. (Fig. 4.16a). Diacetyl production by *P. acidilactici* TLA14R recorded an increase from (18.84 ng/l to 20.41 ng/l) at 121 h. The least (4.13 ng/l) diacetyl was produced at 48 h (Fig 4.16b).

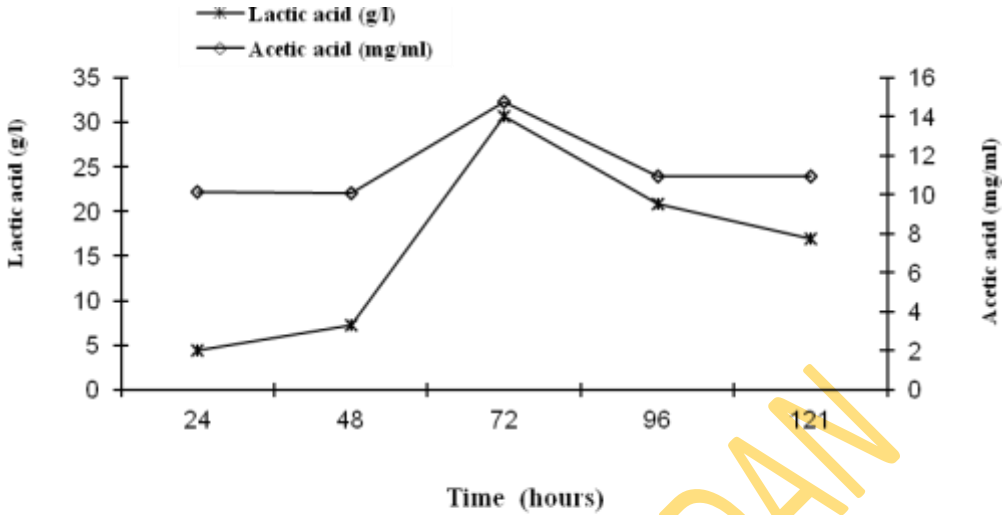


Fig. 4.11a: Time course of lactic acid and acetic acid production by *Pediococcus acidilactici* from stored beef under fast freezing in Man Rogosa and Sharpe broth

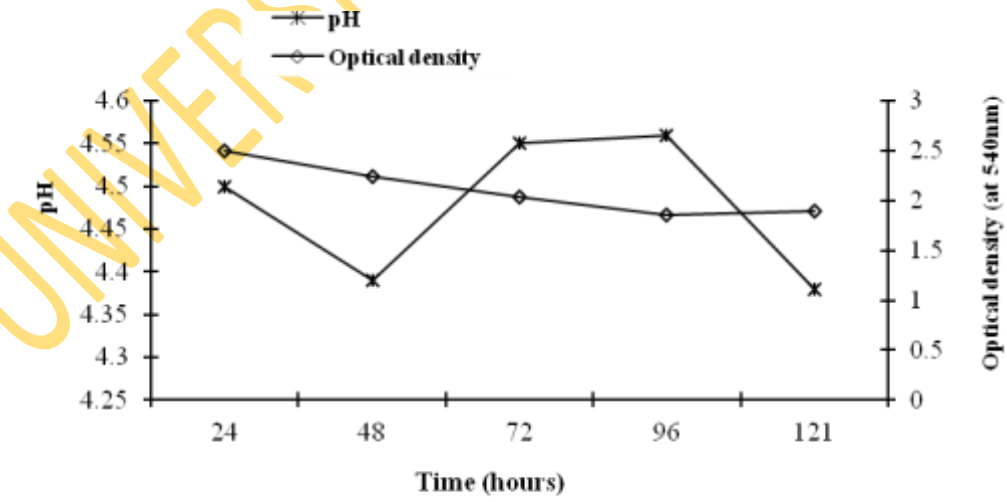


Fig. 4.11b: pH and growth measurements for *Pediococcus acidilactici* from stored beef under fast freezing in Man Rogosa and Sharpe broth at different time intervals

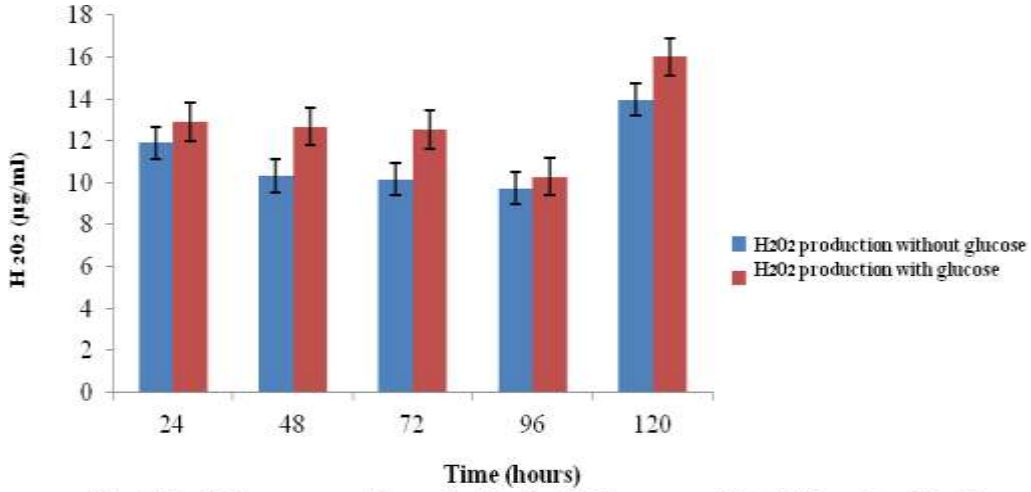


Fig. 4.12a: Hydrogen peroxide production by *Pediococcus acidilactici* from stored beef under fast freezing in the presence and absence of glucose in growth medium

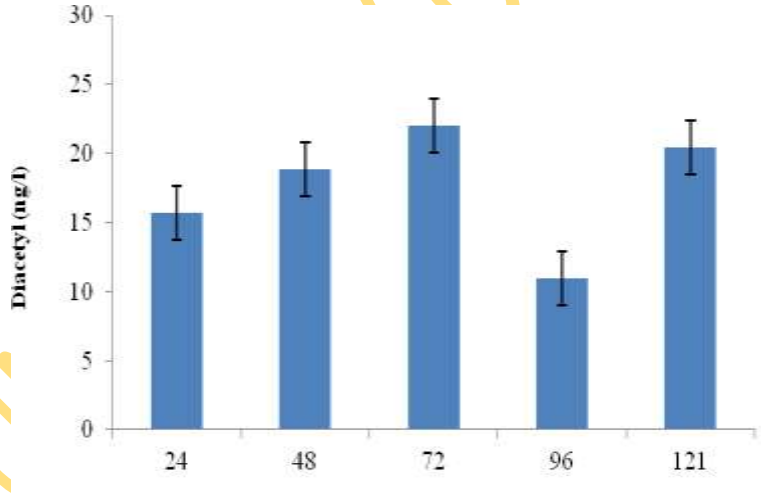


Fig. 4.12b: Diacetyl production by Pediococcus acidilactici from stored beef under fast freezing in Man Rogosa and Sharpe broth

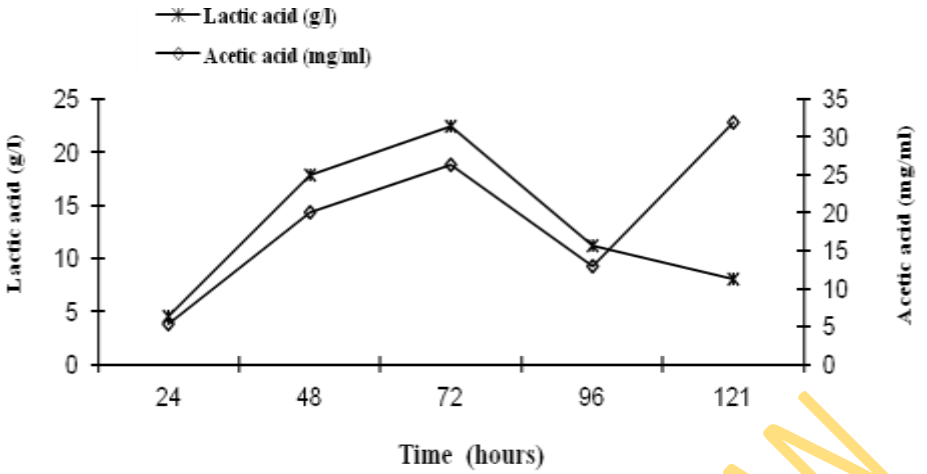


Fig. 4.13a: Time course of lactic acid and acetic acid production by *Pediococcus acidilactici* from stored beef under chilling in Man Rogosa and Sharpe broth

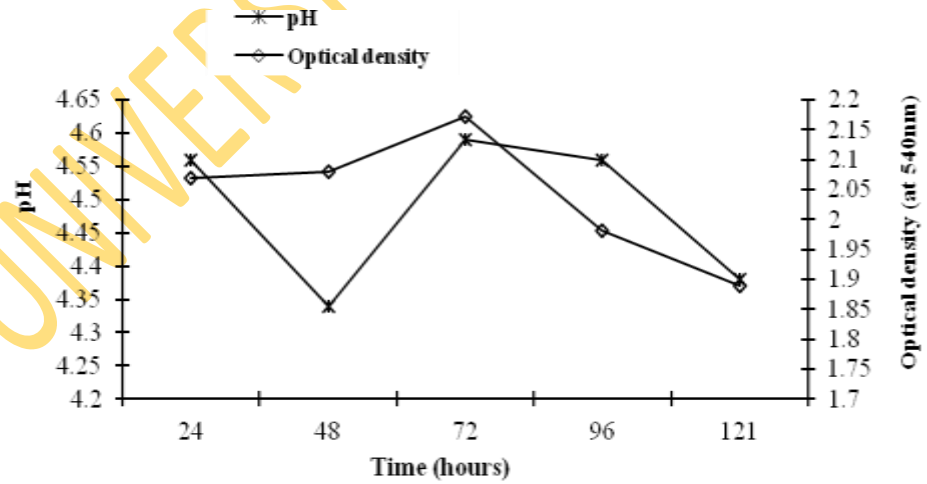


Fig. 4.13b: pH and growth measurements for *Pediococcus acidilactici* from stored beef under chilling in Man Rogosa and Sharpe broth at different time intervals

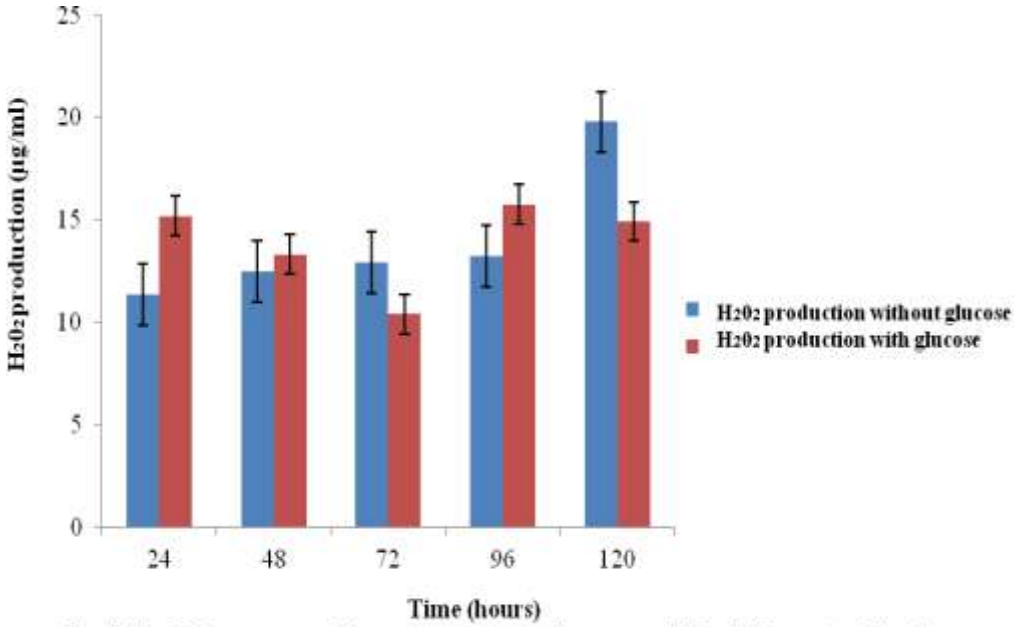


Fig. 4.14a: Hydrogen peroxide production by *Pediococcus acidilactici* from stored beef under chilling in the presence and absence of glucose in growth medium

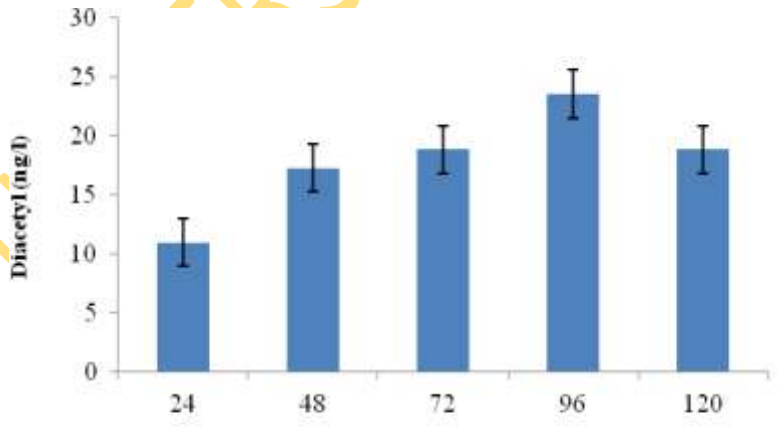


Fig. 4.14b: Diacetyl production by Pediococcus acidilactici from stored beef under chilling in Man Rogosa and Sharpe broth

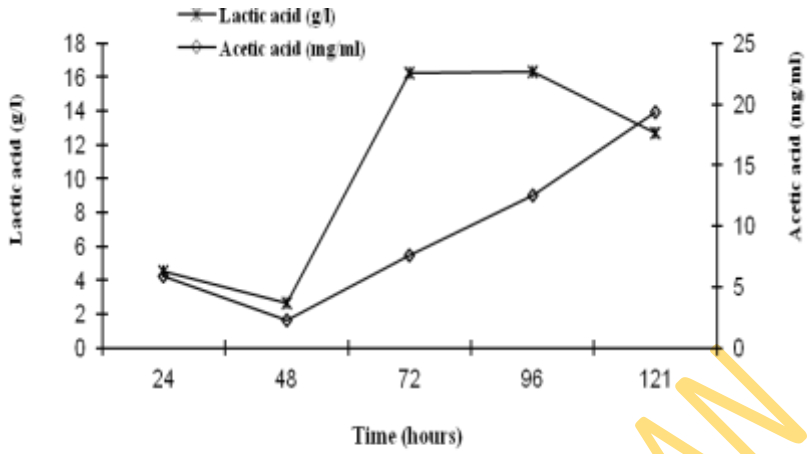


Fig. 4.15a: Time course of lactic acid and acetic acid production by *Pediococcus acidilactici* from stored turkey under refrigeration in Man Rogosa and Sharpe broth

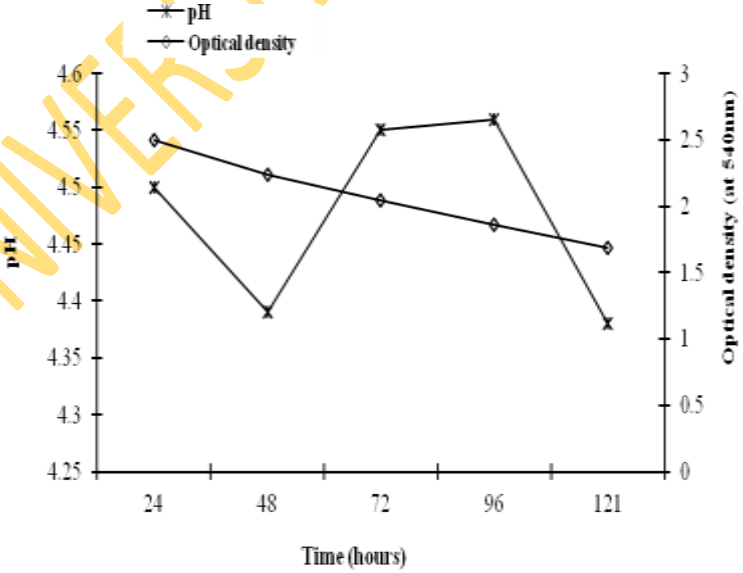


Fig. 4.15b: pH and Growth measurements for Pediococcus acidilactici from stored turkey under refrigeration in Man Rogosa and Sharpe broth

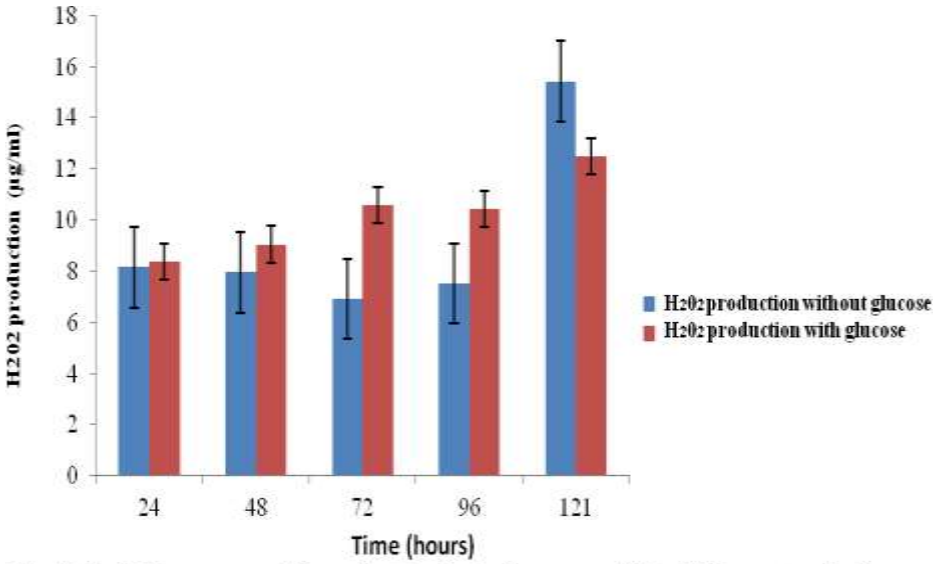


Fig. 4.16a: Hydrogen peroxide production by *Pediococcus acidilactici* from stored turkey under refrigeration in the presence or absence of glucose in growth medium

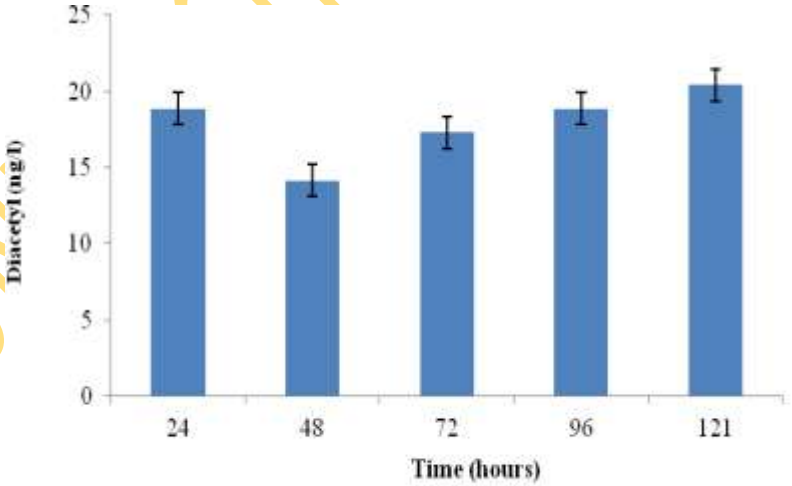


Fig. 4.16b: Diacetyl production by Pediococcus acidilactici from stored turkey under refrigeration in Man Rogosa and Sharpe broth

Based on the results obtained in (Table 4.11), *Escherichia coli* ATCC 5218 was chosen for optimization and characterization of bacteriocin. Bacteriocin production was detected and optimized in five *P. acidilactici* strains namely (TEK1U, CIB1U, BEKBLT, BIB7C and TLA14R) respectively. The effect of pH on the activity of bacteriocin production in *P. acidilactici* strains are shown in (Table 4.17), pH range of 2 to 10 were optimized for bacteriocin production. pH values of (2 – 6) recorded higher zones of inhibition (4.0 – 6.5mm) than Basic pH (8 – 10) recording 2.0 – 4.0 mm. pH 12 did not show any zone of inhibition in all strains against *Escherichia coli* ATCC 5218. At pH 2, 4, 6 and 8, *P. acidilactici* isolated from stored beef under fast freezing temperature BEKBLT recorded the highest zones of inhibition against *Escherichia coli* ATCC 5218.

Effect of incubation temperature on bacteriocin activity was varied (Table 4.18). *P. acidilactici* BEKBLT displayed the highest zone (6.0 mm) of inhibition at -15^{o}C while isolate from untreated chicken CIB1U showed the least zone of inhibition 3.5mm. *P. acidilactici* TLA14R recorded no activity against *Esherichia coli* ATCC 5218 at -15^{o}C. At -4^{0}C and 37^{o}C all the strains recorded higher zone of inhibition against indicator organism ranging from 5.0 – 6.5 mm. At 50^{o}C, *P. acidilactici* BEKBLT, TLA14R displayed zone of inhibition of 5.0 mm against indicator organism, while no activity was shown by strains CIB1U and BIB7C. At 100^{o}C no activity was detected in test strains except strain BEKBLT that recorded 6.0 mm against indicator organism. At 121^{o}C no activity was recorded in all the test strains.

Table 4.17: Effect of pH on the activity of bacteriocin production by *Pediococcus acidilactici* strains against typed *Escherichia coli* strain ATCC 5218

	Zones of inhibition (mm)				
pH	**TEK1U**	**CIB1U**	**BEKBLT**	**BIB7C**	**TLA14R**
2	4.0	6.0	6.0	6.0	2.5
4	4.5	4.0	6.5	6.0	6.0
6	4.0	4.5	5.0	5.0	5.0
8	4.0	3.0	5.0	4.0	2.0
10	4.0	3.0	6.0	2.0	4.0
12	ND	ND	ND	ND	ND
Control	ND	ND	ND	ND	ND

ND – Not Detected
TEK1U – Untreated turkey sample Ekiti ;
CIB1U- Untreated chicken sample Oyo ;
BEKBLT- Beef sample fast freezing Ekiti ;
BIB7C- Beef sample chilling Oyo ;
TLA14R- Turkey sample refrigerated Lagos

Table 4.18: Effect of different temperatures on the activity of bacteriocin production by *Pediococcus acidilactici* strains against Typed *Escherichia coli* strain ATCC 5218

	Zones of inhibition (mm)				
Temperature	**TEK1U**	**CIB1U**	**BEKBLT**	**BIB7C**	**TLA14R**
-15^{0}C	ND	3.5	6.0	5.0	ND
-4^{0}C	6.0	6.0	5.0	6.5	6.0
37^{0}C	6.0	5.0	5.5	6.0	6.5
50^{0}C	4.0	ND	5.0	ND	5.0
100^{0}C	ND	ND	6.0	ND	5.0
121^{0}C	ND	ND	ND	ND	ND
Control	ND	ND	ND	ND	ND

ND – Not Detected
TEK1U – Untreated turkey sample Ekiti
CIB1U- Untreated chicken sample Oyo
BEKBLT- Beef sample fast freezing Ekiti
BIB7C- Beef sample chilling Oyo
TLA14R- Turkey sample refrigerated Lagos

The influence of different enzymes on bacteriocin activity of the five strains is shown in (Table 4.19). Alpha Amylase recorded highest zone of inhibition (6.5 mm) with strain TLA14R. It was followed by TEK1U recording 6.0 mm. CIBIU, BEKBLT and BIB7C displayed zone of inhibition of 5 mm respectively against test strain. Proteinase k, trypsin, pepsin, α-chymotrypsin and pepsin inhibited bacteriocin production in all strains. *P. acidilactici* BEKBLT displayed the highest zone of inhibition against indicator organism with the addition of catalase (6.0 mm); Lysozyme (6.0 mm) and Mitomycin (6.0mm) respectively.

Effect of surfactants on bacteriocin activity was considered by the test organisms (Table 4.20). Tween 20 and Tween 80 recorded zones of inhibition of (2.0 – 6.0 mm) against *Escherichia coli* in all strains. *P acidilactici* BEKBLT recorded highest zone of 6.0 mm with Tween 20 while TEK1U and TLA14R recorded highest zone of 6.0 mm with the addition of Tween 20. Strain CIBIU recorded the least zones of inhibition with Tween 20 and Tween 80. With the addition of EDTA, SDS, and Urea *P. acidilactici* BEKBLT recorded highest zones of inhibition against indicator organism. Triton X – 100 inhibited bacteriocin production in all strains.

Various organic solvents were tested for bacteriocin production (Table 4.21). Chloroform inhibited bacteriocin production in all strains, Diethyl inhibited bacteriocin production in all strains except strain BEKBLT and TLA14R that recorded 5.0mm and 4.0mm zone of clearing respectively**.** Petroleum ether recorded zones of inhibition of 5mm with strains CIBIU and BIB7C while no activity was recorded by other strains. Amyl alcohol exhibited zones of inhibition of (4.0mm – 6.0mm) with all strains; BEKBLT recorded the highest zone of 6.0 against indicator organism. Hexane exhibited zones of inhibition of 5.0 mm and 5.5 mm against strains BEKBLT and TLA14R respectively. Other strains inhibited bacteriocin production in the presence of Hexane. No activity was detected in control samples.

The antimicrobial activities ($AUmL^{-1}$) of the bacteriocin produced by different strains of *P. acidilactici* were optimized and monitored at different pH, temperature, enzyme, surfactant and organic solvents. The values represent the reciprocal of the highest dilution showing incubation of the indicator lawn. Variations in pH showed an indicator lawn of 1800 – 7200 AU mL^{-1} **(**Table 4.22); temperature recorded 900 – 7200 AU mL^{-1} (Table 4.23), effects of different enzymes recorded 900 – 3600 AU mL^{-1} (Table 4.24) different surfactants recorded 900 – 7200 AU mL^{-1} (Table 4.25) and organic solvents recorded 900 – 7200 AU mL^{-1} (Table 4.26) respectively.

The mean value of proximate analysis of fresh beef, chicken and turkey are presented in Table 4.27. There was significant difference between the pH of samples. Turkey had the highest pH of 6.39 $\pm$ 0.17 while beef had the lowest value of 5.79 $\pm$ 0.04. Turkey had higher moisture content of 76.02 $\pm$ 0.22% than beef and chicken. A slight difference was observed between the moisture content of beef and chicken 75.31± 0.23 and 75.43± 0.37% respectively. Low content of ash was recorded in the three samples 0.99 – 1.04%. There was no crude fibre in beef and chicken samples, while 0.03% was recorded for turkey. There was significant difference in the protein content of the samples. Beef recorded the highest protein content of 20.17 $\pm$ 0.08% while turkey recorded the least value of 17.33 $\pm$ 0.58%. Significant differences in the crude fat content of samples were observed. Chicken recorded the highest crude fat content of 5.57 $\pm$ 0.03% while beef had the least value of 3.87 $\pm$ 0.09%. There was significant difference between the ash content of turkey and other samples. Turkey recorded a value of 1.04 $\pm$ 0.03% while beef and chicken recorded values of 0.99 $\pm$ 0.03% and 1.00 $\pm$ 0.00% respectively.

Table 4.19: Effect of different enzymes on the activity of bacteriocin production by *Pediococcus acidilactici* strains against Typed *Escherichia coli* strain ATCC 5218

Enzyme	Zones of inhibition (mm)				
	TEK1U	CIB1U	BEKBLT	BIB7C	TLA14R
α –Amylase	6.0	5.0	5.0	5.0	6.5
Proteinase k	ND	ND	ND	ND	ND
Catalase	4.0	5.0	6.0	5.0	5.0
Trypsin	ND	ND	ND	ND	ND
Lysozyme	2.0	5.0	6.0	5.0	6.0
α- Chymotrypsin	ND	ND	ND	ND	ND
Pepsin	ND	ND	ND	ND	ND
Pronase E	ND	ND	ND	ND	ND
Mictomycin C	3.0	5.0	6.0	4.0	6.0
Control	ND	ND	ND	ND	ND

ND – Not Detected
TEK1U – Untreated turkey sample Ekiti
CIB1U- Untreated chicken sample Oyo
BEKBLT- Beef sample fast freezing Ekiti
BIB7C- Beef sample chilling Oyo
TLA14R- Turkey sample refrigerated Lagos

Table 4.20: Effect of different surfactants on the activity of bacteriocin production by *Pediococcus acidilactici* strains against typed *Escherichia coli* strain ATCC 5218

Surfactant	Zones of inhibition (mm)				
	TEK1U	**CIB1U**	**BEKBLT**	**BIB7C**	**TLA14R**
Tween 20	*5.0	4.0	6.0	5.0	4.0
Tween 80	6.0	2.0	5.0	5.0	6.0
EDTA	5.0	5.0	6.5	4.0	5.0
SDS	4.0	1.0	6.5	5.0	4.0
Urea	4.5	4.0	6.0	5.0	4.0
Triton X-100	ND	ND	ND	ND	ND
Control	ND	ND	ND	ND	ND

ND – Not Detected
TEK1U – Untreated turkey sample Ekiti
CIB1U- Untreated chicken sample Oyo
BEKBLT- Beef sample fast freezing Ekiti
BIB7C- Beef sample chilling Oyo
TLA14R- Turkey sample refrigerated Lagos

Table 4.21: Effect of different organic solvents on the activity of bacteriocin production by *Pediococcus acidilactici* strains against typed *Escherichia coli* strain ATCC 5218

Organic solvents	Zones of inhibition (mm)				
	TEK1U	CIB1U	BEKBLT	BIB7C	TLA14R
Chloroform	ND	ND	ND	ND	ND
Diethylether	ND	ND	5.0	ND	4.0
Petroleum ether	ND	5.0	ND	5.0	ND
Amyl alcohol	4.0	5.0	6.0	5.0	4.0
Hexane	ND	ND	5.0	ND	5.5
Control	ND	ND	ND	ND	ND

ND – Not Detected
TEK1U – Untreated turkey sample Ekiti
CIB1U- Untreated chicken sample Oyo
BEKBLT- Beef sample fast freezing Ekiti
BIB7C- Beef sample chilling Oyo
TLA14R- Turkey sample refrigerated Lagos

Table 4.22: Effect of pH on the antimicrobial activity of bacteriocin produced by different *Pediococcus acidilactici* strains

	Antimicrobial activity ($AUmL^{1}$)				
pH	**TEK1U**	**CIB1U**	**BEKBLT**	**BIB7C**	**TLA14R**
2	*7200	3600	7200	7200	7200
4	7200	7200	7200	7200	7200
6	3600	7200	3600	7200	7200
8	3600	7200	3600	3600	1800
10	3600	1800	1800	3600	3600
12	-			-	-
Control	-	-	-	-	-

*Each value is a reciprocal of the highest dilution showing inhibition of the indicator lawn
- Nil
TEK1U – Untreated turkey sample Ekiti
CIB1U- Untreated chicken sample Oyo
BEKBLT- Beef sample fast freezing Ekiti
BIB7C- Beef sample chilling Oyo
TLA14R- Turkey sample refrigerated Lagos

Table 4.23: Effect of temperature on the antimicrobial activity of bacteriocin produced by different *Pediococcus acidilactici* strains

	Antimicrobial activity ($AUmL^{-1}$)				
Temperature	**TEK1U**	**CIB1U**	**BEKBLT**	**BIB7C**	**TLA14R**
-15^0C	-	7200	7200	7200	-
-4^0C	*7200	3600	7200	7200	3600
37^0C	3600	7200	7200	7200	7200
50^0C	900	-	1800	-	3600
100^0C	-	-	900	-	3600
121^0C		-	-	900	-
Control	-	-	-	-	-

*Each value is a reciprocal of the highest dilution showing inhibition of the indicator lawn
- Nil
TEK1U – Untreated turkey sample Ekiti
CIB1U- Untreated chicken sample Oyo
BEKBLT- Beef sample fast freezing Ekiti
BIB7C- Beef sample chilling Oyo
TLA14R- Turkey sample refrigerated Lagos

Table 4.24: Effect of different enzymes on the antimicrobial activity of bacteriocin produced by different *Pediococcus acidilactici* strains

	Antimicrobial activity ($AUmL^{-1}$)				
Enzyme	**TEK1U**	**CIB1U**	**BEKBLT**	**BIB7C**	**TLA14R**
α –Amylase	*7200	3600	3600	7200	7200
Proteinase k	-	-	-	-	-
Catalase	7200	7200	7200	7200	7200
Trypsin	-	-	-	-	-
Lyzozyme	3600	7200	7200	7200	7200
α- Chymotrypsin	-	-	-	-	-
Pepsin	-	-	-	-	-
Pronase E	-	-	-	-	-
Mictomycin C	1800	900	3600	3600	3600
Control	-	-	-	-	-

*Each value is a reciprocal of the highest dilution showing inhibition of the indicator lawn
- Nil
TEK1U – Untreated turkey sample Ekiti
CIB1U- Untreated chicken sample Oyo
BEKBLT- Beef sample fast freezing Ekiti
BIB7C- Beef sample chilling Oyo
TLA14R- Turkey sample refrigerated Lagos

Table 4.25: Effect of different surfactants on the antimicrobial activity of bacteriocin produced by different *Pediococcus acidilactici* strains

	Antimicrobial activity ($AUmL^{-1}$)				
Surfactant	**TEK1U**	**CIB1U**	**BEKBLT**	**BIB7C**	**TLA14R**
Tween 20	*7200	7200	7200	7200	3600
Tween 80	7200	3600	3600	7200	7200
EDTA	3600	1800	3600	3600	1800
SDS	7200	7200	1800	3600	7200
Urea	1800	3600	1800	900	3600
Triton X-100	-	-	-	-	-
Control	-	-	-	-	-

*Each value is a reciprocal of the highest dilution showing inhibition of the indicator lawn
ND – Not Detected - Nil
TEK1U – Untreated turkey sample Ekiti
CIB1U- Untreated chicken sample Oyo
BEKBLT- Beef sample fast freezing Ekiti
BIB7C- Beef sample chilling Oyo
TLA14R- Turkey sample refrigerated Lagos

Table 4.26: Effect of different organic solvents on the antimicrobial activity of bacteriocin produced by different *Pediococcus acidilactici* strains

	***Pediococcus acicilactici* Strains /Antimicrobial activity ($AUmL^{-1}$)**				
Organic solvent	**TEK1U**	**CIB1U**	**BEKBLT**	**BIB7C**	**TLA14R**
Chloroform	-	-	-	-	-
Diethylether	-	-	-	7200	7200
Petroleum ether	-	3600	-	1800	3600
Amy alcohol	3600	7200	3600	900	7200
Hexane	-	-	3600	-	1800
Control	-	-	-	-	-

*Each value is a reciprocal of the highest dilution showing inhibition of the indicator lawn
- Nil
TEK1U – Untreated turkey sample Ekiti
CIB1U- Untreated chicken sample Oyo
BEKBLT- Beef sample fast freezing Ekiti
BIB7C- Beef sample chilling Oyo
TLA14R- Turkey sample refrigerated Lagos

Table 4.27: Proximate Composition of Fresh Beef, Chicken and Turkey Samples Obtained from Abattoirs in Oyo State, Nigeria

	Beef	Chicken	Turkey
pH (Unit)	**5.79 ±0.04[a]	6.28±0.11[b]	6.39±0.17[c]
Moisture	75.31±0.23[a*]	75.43±0.37[ab]	76.02±0.22b
Ash	0.99±0.03[a]	1.00±0.00[a]	1.04±0.03[b]
Crude fibre	0.00±0.00[a]	0.00±0.00[a]	0.03±0.03a
Protein	20.17±0.08[c]	18.00±0.00[b]	17.33±0.58[a]
Crude fat	3.87 ±0.09[a]	5.57±0.03[c]	5.10 ±0.58[b]
Carbohydrate	1.11 ±0.03[b]	0.86 ±0.03[a]	1.09 ±0.09[b]
Energy level	104.67 ±0.33[c]	120.00 ±0.00[a]	103.67 ±0.33[b]
FFA	0.37 ±0.13[b]	0.43 ±0.06[c]	0.31 ±0.06[a]
TBA	0.27 ±0.02[b]	0.35 ±0.06[c]	0.20 ±0.11[a]
PV	0.49 ±0.07[a]	0.53 ±0.33[a]	1.27 ±0.06[b]
TVB- N	0.22 ±0.11[a]	0.60 ±0.00[a]	0.73 ±0.33[b]
WHC	56.62 ±0.97[a]	54.39 ±0.89[a]	50.60 ±0.03[a]

**Each value is a mean of triplicate determination ± Standard Error
*Means followed by the same letters in the superscript are not significantly different from one another ($p < 0.05$) by Duncan's Multiple Range Test (DMRT)

Units Moisture (%); Ash (%); Crude fibre (%); Protein (%); Crude fat (%); carbohydrate (%); Energy level (Kcal)

FFA – Free Fatty Acid (KOH/g lipid)
TBA – Thiobabituric acid (mg malonaldehyde / kg)
PV – Peroxide value (milli equivalent fat / kg of fat)
TVB-N – Total Volatile Base Nitrogen (mgN/100g)
WHC – Water Holding Capacity (%)

Table 4.27 shows that carbohydrate content was not significantly different in beef and turkey samples 1.11 ± 0.03 and 1.09 ± 0.09% while chicken recorded the least value of 0.86 ± 0.03%. Significant difference was observed in the energy level of samples with slight difference in values recorded 102.00 ± 0.00 – 104.67 ± 0.33. There was significant difference in Thiobarbituric acid (TBA) and free fatty (FFA) analyses values recorded for the three samples, considerable values were recorded for TBA; 0.20 ± 0.11 – 0.35 ± 0.06; FFA: 0.31±0.06 – 0.43±0.06**.** Peroxide value recorded for turkey was highest (1.27 ± 0.06) while those of beef and turkey were 0.49 ± 0.07 and 0.53 ± 0.33 respectively. There was no significant difference in total volatile base nitrogen of turkey and other samples. Turkey had the highest value of 0.73 ± 0.33, while beef and turkey samples recorded 0.22 ± 0.11 and 0.60 ± 0.00 respectivly. No significant difference was noted in the water holding capacity of the samples. Beef gave the highest value of 56.62 ± 0.97 while turkey recorded the lowest value of 50.60 ± 0.03 (Table 4.27).

Due to results obtained in antimicrobial analysis, characterization of bacteriocin of the *P. acidilactici* strains, *P. acidilactici* BEKBLT was chosen to inoculate meat samples.

Physico-chemical analysis and changes in microbial load of beef, chicken and turkey samples inoculated with *P. acidilactici* BEKBLT under fast freezing temperature were examined (Table 4.28– 4.31). They were monitored at 7 -day interval for 28days against control samples. pH of treated beef sample subjected to fast freezing decreased significantly from 5.79 ± 0.04 to 4.82 ± 0.00 while a non significant increase was observed in control sample on day 28.

Free fatty acid (FFA) analysis showed significant decrease and increase in inoculated and control sample from (0.35 ± 0.01 to 0.13 ± 0.01 and 0.35 ± 0.01 to 0.41 ± 0.01) respectively (Table 4.28).

Thiobabituric acid (TBA) value of treated beef sample slightly increased significantly from (0.27 ± 0.02 to 0.29 ± 0.02) against control sample (0.43 ±

0.00) on day 28. Peroxidase value (PV) increased significantly on day 0 to day 28. For treated sample (0.49 ± 0.01 to 0.60 ± 0.00) while higher value was recorded in control sample, although non significant value of 2.13 ± 0.07). Water holding capacity (WHC) significantly decreased in treated sample from 56.62 ± 0.01 to 42.40 ± 0.30 against control sample which showed a non significant increase to (59.31 ± 0.01). Inoculated beef sample recorded a non significant increase in Crude protein (CP) content from 20.17 ± 0.09 to 22.10 ± 0.01% against control sample, which showed a significant decrease to 17.78 ± 0.22%. Crude fat (CF) content showed a non significant decrease from 3.87 ± 0.09% to 3.47 ± 0.00% for treated sample, while control sample showed a significant increase to 4.57 ± 0.07%. on day 28 Total volatile base nitrogen (TVB-N) increased significantly in treated and control samples from 0.22 ± 0.01 to 0.75 ± 0.00; and 1.73 ± 0.03 respectively. (Table 4.28).

Table 4.29 shows the physioco-chemical analyses of chicken samples under fast freezing temperature. The pH value of inoculated chicken sample stored under fast freezing temperature, recorded a non significant decrease from 6.28 ± 0.01 to 6.25 ± 0.02. A non significant increase was recorded in control sample to 6.57 ± 0.02 on day 28. FFA content significantly decreased in treated and control samples. The initial value of FFA content was 0.43 ± 0.01 which later recorded 0.27 ± 0.01 and 0.29 ± 0.02 for treated and control samples. For TBA value of chicken, a non significant decrease was observed in treated chicken sample from 0.35 ± 0.01 to 0.20 ± 0.02 against control sample that recorded 0.53 ± 0.01 on the 28th day.

PV of chicken significantly increased in both treated and inoculated samples. Highest value was recorded by control sample as 1.37 ± 0.03 while treated sample gave 1.00 ± 0.02 respectively on day 28. WHC of treated chicken decreased significantly from 56.33 ± 0.04 to 40.73 ± 0.37**;** while that of control showed a non significant increase to 57.20 ± 0.12 on day 28. CP content of chicken sample subjected to fast freezing showed a significant increase in treated sample from 18.03 ± 0.03 to 21.83 ± 0.20, while the control showed a

non significant increase to 20.20 ± 0.01. CF content increased in treated sample from 5.57 ± 0.03 to 5.63 ± 0.0 while control showed a significant increase to 7.40 ± 0.12 on the 28th day. The total volatile base nitrogen of inoculated chicken recorded a non significant increase in treated sample from 0.60 ± 0.00 to 0.70 ± 0.06**.** Control recorded a significant increase of 1.33 ± 0.33(Table 4.29).

Turkey samples subjected to fast freezing temperature for 28 days (Table 4.30) showed significant decrease in pH of treated sample from 6.39 ± 0.01 to 5.79 ± 0.02, control recorded a significant increase of 6.61 ± 0.01 on the 28th day. FFA content increased significantly in treated and control samples. Control gave the highest value of 0.43 ± 0.01. TBA value increased significantly in inoculated and control sample from 0.20± 0.01 to 0.32 ±0.00; 0.42 ±0.03 respectively. PV values of inoculated sample increased during storage, but later remain constant on day 28. PV of control increased from 1.27 ± 0.07 to 1.57 ± 0.03**.**

WHC increased and decreased significantly in treated turkey and control samples to 52.60 ± 0.31 and 40.24 ± 0.12 respectively. Crude protein content increased significantly in treated turkey sample from 17.33 ± 0.03 to 17.73 ± 0.18%, while control recorded a non-significant decrease of 17.17 ± 0.03. CF and TVB –N increased in control samples to 5.20 ± 0.17 and 1.77 ± 0.09 respectively. Crude fat content of treated turkey sample recorded a non-significant increase of 5.17 ± 0.17 while total volatile base nitrogen remained the same at the end up 28 days (Table 4.30).

Table 4.28: Physico-Chemical Analysis of Beef Samples Under Fast Freezing Temperature For 28 Days

STORAGE PERIOD		Parameter / Obtained Values							
		pH	FFA	TBA	PER VALUE	WHC	CP	CRUDE FAT	TVB-N
O	S	**5.79 ± 0.04^{a}	0.35 ± 0.01^{c}	0.27 ± 0.02^{b}	0.49 ± 0.01^{b}	56.62 ± 0. 01^{a}	20.17± 0.09^{b}	3.87 ± 0.09^{b}	0.22 ± 0.01^{c}
	C	5.79 ± 0.04^{a*}	0.35 ± 0.01^{c}	0.27 ± 0.02^{b}	0.49 ± 0.01^{b}	56.62 ± 0. 07^{a}	20.17 ± 0.09^{b}	3.87 ± 0.09^{b}	0.22 ± 0.01^{c}
7	S	4.83 ± 0.02^{b}	0.53 ± 0.02^{a}	0.25 ± 0.01^{b}	0.63 ± 0.03^{a}	51.61 ± 0.01^{b}	21.70 ± 0.06^{a}	3.48 ± 0.03^{c}	0.40 ± 0.00^{b}
	C	6.13 ± 0.13^{a}	0.42 ± 0.01^{a}	0.33 ± 0.01^{a}	1.50 ± 0.06^{c}	50.41 ± 0.01^{b}	21.17 ± 0.12^{a}	4.57 ± 0.07^{a}	0.80 ± 0.00^{a}
14	S	4.89 ± 0.00^{b}	0.54 ± 0.02^{c}	0.21 ± 0.01^{b}	0.67 ± 0.03^{a}	50.03 ± 0.03^{b}	19.70 ± 0.06^{a}	4.20 ± 0.00^{a}	0.37 ± 0.03^{b}
	C	6.10 ± 0.029	0.39 ± 0.00^{b}	0.29 ± 0.01^{a}	2.40 ± 0.00^{a}	58.27 ± 0.07^{a}	19.48 ± 0.03^{e}	3.70 ± 0.06^{a}	1.77 ± 0.03^{c}
21	S	4.86 ± 0.01^{b}	0.34 ± 0.00^{c}	0.20 ± 0.00^{b}	0.63 ± 0.3^{a}	49.80 ± 0.20^{b}	20.17 ± 0.03^{b}	3.93 ± 0.03^{b}	0.77 ± 0.03^{a}
	C	6.07 ± 0.04^{a}	0.11 ± 0.00^{d}	0.35 ± 0.03^{a}	2.07 ± 0.03^{a}	59.53 ± 0.01^{a}	18.40 ± 0.00^{d}	4.37 ± 0.08^{a}	1.50 ± 0.00^{b}
28	S	4.82 ± 0.04^{b}	0.13 ± 0.01^{d}	0.29 ± 0.02^{a}	0.60 ± 0.00^{a}	42.40 ± 0.30^{c}	22.10 ± 0.01^{b}	3.47 ± 0.00^{b}	0.75 ± 0.00^{a}
	C	5.77 ± 0.00^{a}	0.41 ± 0.01^{b}	0.43 ± 0.00^{a}	2.13 ± 0.07^{b}	59.31 ± 0.01^{a}	17.78 ± 0.22^{d}	4.57 ± 0.07^{b}	1.73 ± 0.03^{a}

**Each value is a mean of triplicate determination ± Standard Error

*Means followed by the same letters in the superscript are not significantly different from one another ($p < 0.05$) by Duncan's Multiple Range Test (DMRT)

pH; FFA –free fatty acid (%); TBA - thiobabituric acid analysis (mg malonaldehyde/kg); Peroxide value - (mg/kg fat); WHC water holding capacity - (%); CP - crude protein (%); CF - crude fat (%) ; TVB-N total volatile nitrogen (mg N/100g)

Table 4.29: Physico-Chemical Analysis of Chicken Samples Under Fast Freezing Temperature For 28 Days

STORAGE PERIOD		Parameter / Obtained Values							
		pH	FFA	TBA	PER VALUE	WHC	CP	CRUDE FAT	TOTAL V.NITROGEN
O	S	**6.28 ± 0.01^{b}	0.43 ± 0.01^{a}	0.35 ± 0.01^{a}	0.63 ± 0.09^{c}	56.33 ± 0.04^{b}	18.03± 0.03^{b}	5.57 ± 0.03^{c}	0.60 ± 0.00^{a}
	C	6.28 ± 0.01^{b*}	0.43 ± 0.01^{a}	0.35 ± 0.01^{a}	0.63 ± 0.09^{c}	56.33 ± 0.04^{b}	18.03± 0.03^{b}	5.57 ± 0.03^{c}	0.60 ± 0.00^{a}
7	S	6.31 ± 0.02^{a}	0.26 ± 0.01^{b}	0.33 ± 0.01^{a}	1.20 ± 0.00^{b}	57.60 ± 0.40^{a}	19.33 ± 0.03^{c}	4.60 ± 0.10^{c}	1.43 ± 0.09^{a}
	C	6.67 ± 0.06^{a}	0.49 ± 0.01^{b}	0.35 ± 0.01^{a}	0.60 ± 0.00^{c}	52.13 ± 0.13^{c}	18.47 ± 0.17^{c}	6.07 ± 0.03^{b}	0.67 ± 0.03^{bc}
14	S	6.25 ± 0.02^{b}	0.22 ± 0.00^{c}	0.28 ± 0.01^{b}	1.40 ± 0.00^{a}	50.40 ± 0.12^{c}	20.77 ± 0.33^{b}	6.03 ± 0.03^{a}	1.40 ± 0.00^{a}
	C	6.58 ± 0.01^{b}	0.56 ± 0.00^{a}	0.33 ± 0.02^{a}	0.67 ± 0.03^{c}	58.87 ± 0.07^{a}	21.07 ± 0.03^{a}	6.20 ± 0.06^{b}	0.67 ± 0.03^{bc}
21	S	6.31 ± 0.01^{a}	0.25 ± 0.00^{b}	0.28 ± 0.01^{b}	1.37 ± 0.03^{ab}	49.13 ± 0.13^{d}	21.67 ± 0.03^{a}	5.73 ± 0.07^{b}	1.10 ± 0.00^{b}
	C	6.62 ± 0.01^{a}	0.22 ± 0.00^{e}	0.41 ± 0.02^{b}	1.40 ± 0.00^{a}	55.20 ± 0.12^{d}	17.87 ± 0.03^{d}	5.43 ± 0.03^{c}	1.41 ± 0.01^{a}
28	S	6.25 ± 0.02^{b}	0.27 ± 0.01^{b}	0.20 ± 0.02^{a}	1.00 ± 0.02^{ab}	40.73 ± 0.37^{c}	21.83 ± 0.20^{a}	5.63 ± 0.09^{b}	0.70 ± 0.06^{bc}
	C	6.57 ± 0.02^{b}	0.29 ± 0.02^{d}	0.53 ± 0.01^{a}	1.37 ± 0.02^{ab}	57.20 ± 0.12^{b}	20.20 ± 0.01^{b}	7.40 ± 0.12^{a}	1.33 ± 0.33^{a}

**Each value is a mean of triplicate determination ± Standard Error

*Means followed by the same letters in the superscript are not significantly different from one another ($p< 0.05$) by Duncan's Multiple Range Test (DMRT)

pH; FFA –free fatty acid (%); TBA - thiobabituric acid analysis (mg malonaldehyde/kg); Peroxide value - (mg/kg fat); WHC water holding capacity - (%); CP - crude protein (%); CF - crude fat (%) ; TVB-N total volatile nitrogen (mg N/100g).

Table 4.30: Physico-Chemical Analysis of Turkey Samples Under Fast Freezing Temperature For 28 Days

		Parameter / Obtained Values							
STORAGE PERIOD		pH	FFA	TBA	PER VALUE	WHC	CP	CRUDE FAT	T VB-N
O	S	$^{**}6.39 \pm 0.01^{a}$	0.31 ± 0.01^{c}	0.20 ± 0.01^{c}	1.27 ± 0.07^{b}	50.60 ± 0.00^{d}	17.33 ± 0.03^{d}	5.10 ± 0.06^{b}	0.73 ± 0.03^{b}
	C	$6.39 \pm 0.02^{a*}$	0.31 ± 0.01^{c}	0.20 ± 0.01^{c}	1.27 ± 0.07^{b}	50.60 ± 0.00^{d}	17.33 ± 0.03^{d}	5.10 ± 0.06^{b}	0.73 ± 0.03^{b}
7	S	5.79 ± 0.02^{b}	0.26 ± 0.02^{d}	0.22 ± 0.00^{c}	1.53 ± 0.07^{a}	55.73 ± 0.13^{a}	20.67 ± 0.03^{a}	5.50 ± 0.00^{a}	0.90 ± 0.06^{a}
	C	6.71 ± 0.01^{a}	0.86 ± 0.00^{a}	0.21 ± 0.01^{b}	1.47 ± 0.3^{ab}	40.47 ± 0.27^{bc}	17.80 ± 0.00^{a}	5.77 ± 0.03^{a}	0.87 ± 0.03^{a}
14	S	5.79 ± 0.02^{b}	0.44 ± 0.01^{a}	0.29 ± 0.01^{b}	1.10 ± 0.00^{c}	54.10 ± 0.06^{b}	18.03 ± 0.03^{b}	5.17 ± 0.03^{b}	0.37 ± 0.03^{c}
	C	6.63 ± 0.00^{b}	0.82 ± 0.01^{a}	0.21 ± 0.01^{b}	1.00 ± 0.00^{d}	40.83 ± 0.03^{b}	17.53 ± 0.03^{b}	4.90 ± 0.06^{c}	1.37 ± 0.03^{b}
21	S	5.80 ± 0.00^{b}	$0.33 \pm 0.00b^{c}$	0.36 ± 0.05^{a}	1.40 ± 0.00^{ab}	53.30 ± 0.05^{bc}	17.67 ± 0.03^{c}	4.47 ± 0.03^{c}	0.77 ± 0.06^{ab}
	C	6.64 ± 0.03^{b}	0.41 ± 0.01^{b}	0.35 ± 0.03^{a}	1.40 ± 0.00^{b}	40.10 ± 0.00^{c}	17.10 ± 0.00^{d}	4.50 ± 0.00^{d}	1.77 ± 0.03^{a}
28	S	5.79 ± 0.02^{b}	0.36 ± 0.01^{b}	0.32 ± 0.00^{ab}	1.27 ± 0.03^{b}	52.60 ± 0.31^{c}	17.73 ± 0.18^{c}	5.17 ± 0.17^{b}	0.73 ± 0.70^{b}
	C	6.61 ± 0.01^{b}	0.43 ± 0.03^{b}	0.42 ± 0.03^{a}	1.57 ± 0.03^{a}	40.24 ± 0.12^{bc}	17.17 ± 0.03^{d}	5.20 ± 0.15^{b}	1.77 ± 0.09^{a}

*Each value is a mean of triplicate determination ± Standard Error

*Means followed by the same letters in the superscript are not significantly different from one another ($p < 0.05$) by Duncan's Multiple Range Test (DMRT)

pH; FFA –free fatty acid (%); TBA - thiobabituric acid analysis (mg malonaldehyde/kg); Peroxide value - (mg/kg fat); WHC water holding capacity - (%); CP - crude protein (%); CF - crude fat (%) ; TVB-N total volatile nitrogen (mg N/100g)

The microbial load profile in beef, chicken and turkey samples subjected to fast freezing temperature (Table 4.31) shows different microbial groups of treated and control samples. Total bacteria count (TBC) of beef, chicken and turkey decreased from 2.66 ± 0.03 to 2.16 ± 0.12; 3.88 ± 0.49 to 3.25 ± 0.13 and 3.07 ± 0.23 to 2.18 ± 0.02 log_{10}cfu/ml) respectively. TBC of control recorded a significant increase in beef, chicken and turkey on the 28 day to 4.74 ± 0.21, 4.95 ± 0.26 and 5.06 ±0.08 log_{10}cfu/ml respectively. *Enterococcal* and fungi counts decreased in treated samples but increased counts were recorded in control samples. Chicken had the highest *enterococcal* count of 7.44 ± 0.44 log_{10}cfu/ml; while beef recorded the lowest count of 5.94 ± 0.22 in control samples. Fungi count in control samples for beef was highest 4.97 ± 0.29 while chicken recorded the least fungi count 4.17 ± 0.36 for control samples. *Lactobacilli* count increased in all treated samples to 3.71 log_{10}cfu/ml for beef; 4.87 log_{10}cfu/ml for chicken and 3.59 log_{10}cfu/ml for turkey. In contrast, control samples recorded lower *lactobacilli* counts (Table 4.31).

Physico-chemical analysis and changes in microbial load of beef, chicken and turkey samples inoculated with *P. acidilactici* BEKBLT under freezing temperature (Table 4.32 – 4.35). They were monitored at 7 -day interval for 28days against control samples. Significant increase was noticed in pH of treated sample from 5.79 $\pm$ 0.04 to 6.21$\pm$ 0.02, against control sample that showed a non significant increase of 6.56 $\pm$ 0.00. FFA decreased significantly in treated sample from 0.35 $\pm$ 0.01 to 0.31 $\pm$ 0.02, while control sample recorded a significant increase of 0.51 $\pm$ 0.01. TBA values increased significantly in treated and control samples. Control recorded the highest TBA value of 0.70 $\pm$ 0.03 while treated sample showed 0.45 $\pm$ 0.06. PV increased significantly in both samples. Treated beef sample increased from 0.49 $\pm$ 0.01 to 0.78 $\pm$ 0.00 while control increased to 2.19 $\pm$ 0.01(Table 4.32). WHC of both samples (inoculated and control) decreased at the end of 28th day. Treated beef significantly decreased from 56.62 $\pm$ 0.01 to 54 .61 $\pm$ 0.07 while control recorded a non-significant decrease of 50.47 $\pm$ 0.04. CP content under freezing temperature showed a significant increase in treated beef samples. From 20.17

± 0.09 to 20.21 ± 0.01while control samples recorded a significant decrease of 16.22 ± 0.01. Crude fat content showed a non significant decrease in treated beef from 3.87±0.09 to 3.81±0.07 against control sample that increased significantly to 4.62 ± 0.00**.** TVB-N increased significantly in both treated and control samples. Control recorded the highest value of 1.86 ± 0.08 against 0.78 ± 0.01 for treated samples. (Table 4.32)

Chicken samples subjected to freezing for 28 days (Table 4.33) showed a non significant decrease in pH from 6.28±0.01 to 5.63±0.18. Control also recorded a significant decrease to 6.20 ± 0.00. FFA decreased from 0.43±0.01 to 0.30 ± 0.21 against control that recorded 0.55±0.06. PV increased in both samples. Control recorded the highest value of 1.65 ± 0.17 against 0.81 ± 0.02 for treated chicken sample. WHC increased significantly in both samples. Crude protein content increased significantly in treated sample from 18.03 ± 0.03 to 20.05 ± 0.08 but decreased significantly to 17.25 ± 0.14 in control. CF content in chicken sample under freezing temperature (Table 4.33) increased in treated and control samples, control recorded the highest value of 7.80 ± 0.14% against treated sample (6.09 ± 0.13%). TVB-N increased significantly with control recording the highest value of 1.38 ± 0.11 on day 28 (Table 4.33).

Turkey samples subjected to freezing temperature (-4^{o}C) for 28 days (Table 4.34) recorded a non significant decrease in pH of treated sample from 6.39 ± 0.01 to 5.74 ± 0.09. Control sample showed a non significant increase of 6.40 ± 0.22 on day 28. FFA, TBA and PV increased in a treated and control samples, control samples recorded highest values of 0.61 ± 0.13; 0.53 ± 0.14 and 1.62 ± 0.12 against values of treated samples 0.32 ± 0.02, 0.40 ± 0.17 and 1.36 ± 0.03 respectively.

Table 4.31: Microbial load profile in different meat samples subjected to fast freezing treatment for 28 days

	Meat type / Storage Time (days) / Group Count (log cfu ml^{-1})														
Microbial groups	BF	CK	TK	BF	CK	TK	BF	CK	TK	BF	CK	TK	BF	CK	TK
	0			7			14			21			28		
Total bact. Count	**2.66 ± 0.03^{a}	3.88± 0.49^{a}	3.07± 0.23^{a}	2.68± 0.33^{a}	3.84 ± 0.28^{a}	3.07± 0.63^{b}	3.25± 0.13^{a}	3.81± 0.02^{a}	2.49± 0.23^{a}	3.20± 0.36^{a}	3.38± 0.06^{a}	2.18±0.06^{a}	2.16±0.12^{a}	3.25± 0.13	2.18± 0.02^{a}
Enteriococcal count	2.16 ±0.01^{c*}	2.63 ±0.06^{d}	2.28 ±0.14^{c}	2.16 ±0.02^{e}	2.62 ±0.11^{d}	1.82 ±0.11^{b}	1.53 ±0.02^{b}	1.72 ±0.02^{c}	1.82 ±0.01^{b}	1.38 ±0.05^{a}	1.63 ±0.17^{b}	1.72 ±0.08^{a}	1.38 ±0.22^{d}	1.55 ±0.06^{a}	1.70 ±0.14^{a}
LAB count	2.27 ±0.01^{a}	2.66 ±0.18^{ab}	2.91 ±0.35^{a}	2.87 ±0.01^{ab}	2.63 ±0.02^{a}	3.21 ±0.04^{b}	2.49 ±0.04^{b}	2.77 ±0.06^{b}	3.35 ±0.24^{c}	3.30 ±0.03^{c}	3.14 ±0.12^{c}	3.50 ±0.24^{d}	3.71 ±0.15^{d}	4.87 ±0.33^{d}	3.59 ±0.05^{d}
Fungi count	3.06 ±0.01^{b}	2.60 ±0.03^{d}	3.73 ±0.02^{c}	2.85 ±0.02^{b}	2.52 ±0.00^{c}	3.73 ±0.10^{c}	2.40 ±0.01^{a}	2.40 ±0.01^{a}	3.71±0.01^{c}	2.40 ±0.04^{a}	2.25 ±0.01^{b}	2.86 ±0.00^{b}	2.11 ±0.30^{a}	2.03 ±0.04^{a}	1.99 ±0.13^{a}
CONTROL															
Total bact. Count	2.66 ± 0.03^{a}	3.88± 0.49^{c}	3.07± 0.23^{a}	2.84± 0.02^{b}	3.63 ± 0.24^{a}	3.62± 0.12^{b}	3.76± 0.02^{c}	3.77± 0.15^{b}	3.85± 0.17^{c}	4.26± 0.01^{d}	3.94± 0.20^{c}	4.06±0.08^{d}	4.74±0.21^{e}	4.95± 0.26^{d}	5.06± 0.08^{e}
Enterococcal count	2.16 ±0.26^{a}	2.63 ±0.06^{d}	2.28 ±0.14^{c}	2.96±0.02^{b}	3.77±0.32^{b}	2.25±0.16^{b}	4.11±0.01^{c}	5.23±0.23^{c}	5.06±0.18^{c}	5.56±0.03^{d}	6.14±0.23^{d}	6.03±0.14^{d}	5.94±0.22^{e}	7.44±0.44^{e}	7.33±0.24^{e}
LAB count	2.27 ±0.01^{a}	2.66 ±0.18^{ab}	2.91 ±0.35^{a}	2.26±0.08^{b}	2.71±0.13^{c}	2.25±0.14^{d}	2.30±0.08^{c}	2.54±0.14^{a}	2.79±0.17^{a}	2.26±0.14^{d}	2.57±0.14^{a}	2.15±0.17^{c}	2.26±0.15^{d}	2.28±0.14^{d}	2.03±0.24^{e}
Fungi count	3.06 ±0.01^{b}	2.60 ±0.03^{d}	3.73 ±0.02^{c}	2.73±0.24^{b}	4.28±0.28^{b}	4.28±0.03^{b}	4.59±0.40^{c}	4.74±0.20^{b}	3.09±0.14^{c}	3.25±0.11^{e}	3.42±0.11^{c}	3.64±0.02^{d}	4.97±0.29^{d}	4.17±0.36^{d}	4.39±0.04^{e}

**Each value is a mean of triplicate determination ± Standard Error

*Means followed by the same letters in the superscript are not significantly different from one another ($p< 0.05$) by Duncan's Multiple Range Test (DMRT)

BF - Beef sample; CK - Chicken sample; TK - Turkey sample

Table 4.32: Physico-Chemical Analysis of Beef Samples Under Freezing Temperature For 28 Days

STORAGE PERIOD		Parameter / Obtained Values							
		pH	FFA	TBA	PER VALUE	WHC	CP	CRUDE FAT	TOTAL V.NITROGEN
O	S	**5.79 ± 0.04[a]	0.35 ± 0.01[c]	0.27 ± 0.02[b]	0.49 ± 0.01[b]	56.62 ± 0.01[a]	20.17± 0.09[b]	3.87 ± 0.09[b]	0.22 ± 0.01[c]
	C	5.79 ± 0.04[a*]	0.35 ± 0.01[c]	0.27 ± 0.02[c]	0.49 ± 0.01[b]	56.62 ± 0.01[a]	20.17± 0.09[b]	3.87 ± 0.09[b]	0.22 ± 0.01[c]
7	S	5.81±0.01[b]	0.42±0.02[a]	0.36±0.01[c]	0.54±0.00[a]	56.59±0.06[d]	20.02±0.07[a]	3.80±0.12[b]	0.21±0.06[a]
	C	6.34±0.18[a]	0.33±0.02[ab]	0.38±0.14[a]	0.79±0.13[a]	54.15±0.07[c]	20.20±0.02[d]	4.23±0.18[a]	0.61±0.17[a]
14	S	5.78±0.00[b]	0.48±0.00[b]	0.31±0.01[a]	0.62±0.02[b]	54.14±0.12[b]	18.56±0.52	3.81±0.06[b]	0.31±0.12[b]
	C	6.59±0.01[a]	0.38±0.14[b]	0.43±0.17[b]	1.20±0.05[b]	54.15±0.07[c]	17.73±0.06[b]	4.24±0.01[a]	0.63±0.03[b]
21	S	5.16±0.01[a]	0.51±0.01[c]	0.40±0.02[a]	0.61±0.03[b]	52.21±0.10[a]	19.32±0.03[c]	3.24±0.00[a]	0.50±0.14[c]
	C	6.63±0.2[a]	0.31±0.27[a]	0.43±0.23[b]	2.21±0.13[c]	52.37±0.01[b]	17.80±0.00[c]	4.45±0.03[b]	1.19±0.01[c]
28	S	6.21±0.02[c]	0.31±0.02[a]	0.45±0.06[b]	0.78±0.00[c]	54.61±0.07[c]	20.21±0.01[c]	3.81±0.07[b]	0.78±0.01[d]
	C	6.56±0.00[a]	0.51±0.01[c]	0.70±0.03[c]	2.19±0.01[c]	50.47±0.04[a]	16.22±0.01[a]	4.62±0.00[c]	1.86±0.08[d]

**Each value is a mean of triplicate determination ± Standard Error

*Means followed by the same letters in the superscript are not significantly different from one another ($p < 0.05$) by Duncan's Multiple Range Test (DMRT)

pH; FFA –free fatty acid (%); TBA - thiobabituric acid analysis (mg malonaldehyde/kg); Peroxide value - (mg/kg fat); WHC water holding capacity - (%); CP - crude protein (%); CF - crude fat (%) ; TVB-N total volatile nitrogen (mg N/100g)

Table 4.33: Physico-Chemical Analysis of Chicken Samples Under Freezing Temperature For 28 Days

STORAGE PERIOD		Parameter / Obtained Values							
		pH	FFA	TBA	PER. VALUE	WHC	CP	CRUDE FAT	T VB-N
O	S	$^{**}6.28 \pm 0.01^{b}$	0.43 ± 0.01^{a}	0.35 ± 0.01^{a}	0.63 ± 0.09^{c}	56.33 ± 0.04^{d}	18.03 ± 0.03^{b}	5.57 ± 0.03^{c}	0.60 ± 0.00^{a}
	C	6.28 ± 0.01^{b}*	0.43 ± 0.01^{a}	0.35 ± 0.01^{a}	0.63 ± 0.09^{c}	56.33 ± 0.04^{d}	18.03 ± 0.03^{b}	5.57 ± 0.03^{c}	0.60 ± 0.00^{a}
7	S	6.11 ± 0.13^{c}	0.56 ± 0.06^{b}	0.40 ± 0.00^{b}	0.96 ± 0.13^{a}	58.16 ± 0.03^{d}	18.06 ± 0.02^{a}	5.51 ± 0.13^{a}	0.88 ± 0.11^{a}
	C	6.21 ± 0.01^{a}	0.50 ± 0.08^{c}	0.36 ± 0.01^{a}	0.95 ± 0.00^{d}	54.41 ± 0.01^{a}	17.31 ± 0.06^{a}	5.88 ± 0.11^{a}	1.37 ± 0.06^{c}
14	S	5.74 ± 0.18^{b}	0.48 ± 0.12^{a}	0.42 ± 0.00^{b}	1.17 ± 0.01^{c}	56.75 ± 0.29^{c}	19.39 ± 0.01^{b}	5.63 ± 0.17^{b}	0.90 ± 0.03^{b}
	C	6.26 ± 0.03^{a}	0.57 ± 0.12^{d}	0.41 ± 0.13^{b}	1.26 ± 0.23^{b}	53.87 ± 0.76^{a}	17.23 ± 0.11^{a}	6.03 ± 0.35^{b}	0.98 ± 0.00^{a}
21	S	5.53 ± 0.24^{a}	0.53 ± 0.17^{b}	0.40 ± 0.02^{b}	0.98 ± 0.00^{b}	52.82 ± 0.12^{a}	19.58 ± 0.24^{b}	6.20 ± 0.12^{d}	0.92 ± 0.01^{b}
	C	6.21 ± 0.01^{a}	0.22 ± 0.00^{a}	0.49 ± 0.01^{c}	1.55 ± 0.29^{c}	57.96 ± 0.12^{b}	17.42 ± 0.12^{a}	6.44 ± 0.23^{c}	0.96 ± 0.01^{a}
28	S	5.63 ± 0.18^{ab}	0.30 ± 0.21^{b}	0.36 ± 0.01^{a}	0.81 ± 0.02^{a}	56.42 ± 0.01^{b}	20.05 ± 0.08^{c}	6.09 ± 0.13^{c}	0.92 ± 0.04^{c}
	C	6.20 ± 0.00^{a}	0.55 ± 0.06^{b}	0.58 ± 0.11^{d}	1.65 ± 0.17^{d}	56.80 ± 0.11^{b}	17.25 ± 0.14^{a}	7.80 ± 0.14^{b}	1.38 ± 0.11^{c}

*Each value is a mean of triplicate determination ± Standard Error

*Means followed by the same letters in the superscript are not significantly different from one another ($p < 0.05$) by Duncan's Multiple Range Test (DMRT)

pH; FFA –free fatty acid (%); TBA - thiobabituric acid analysis (mg malonaldehyde/kg); Peroxide value - (mg/kg fat); WHC water holding capacity - (%); CP - crude protein (%); CF - crude fat (%) ; TVB-N total volatile nitrogen (mg N/100g)

Table 4.34: Physico-Chemical Analysis of Turkey Samples under Freezing Temperature for 28 Days

		Parameter / Obtained Values							
STORAGE PERIOD		pH	FFA	TBA	PER VALUE	WHC	CP	CRUDE FAT	TOTAL V.NITROGEN
O	S	**6.39 ± 0.01[a]	0.31 ± 0.01[c]	0.20 ± 0.01[c]	1.27 ± 0.07[b]	50.60 ± 0.00[d]	17.33± 0.03[d]	5.10 ± 0.06[b]	0.73 ± 0.03[b]
	C	6.39 ± 0.02[a*]	0.31 ± 0.01[c]	0.20 ± 0.01[c]	1.27 ± 0.07[b]	50.60 ± 0.00[d]	17.33± 0.03[d]	5.10 ± 0.06[b]	0.73 ± 0.03[b]
7	S	6.30±0.00[b]	0.35±0.17[a]	0.26±0.00[a]	1.29±0.01[b]	52.35±0.29[a]	17.43±0.01[b]	5.17±0.01[b]	0.91±0.30[a]
	C	6.46±0.02[a]	0.54±0.00[a]	0.29±0.13[a]	1.35±0.17[a]	50.60±0.34[a]	17.22±0.01[a]	5.28±0.14[a]	0.98±0.21[c]
14	S	6.25±0.02[b]	0.43±0.16[b]	0.31±0.17[a]	1.20±0.00[a]	52.47±0.17[b]	16.99±0.05[b]	5.24±0.04[b]	0.77±0.41[a]
	C	6.20±0.31[a]	0.66±0.00[c]	0.33±0.04[a]	1.46±0.23[b]	54.63±0.07[a]	17.04±0.21[a]	5.34±0.34[a]	1.37±0.00[a]
21	S	5.82±0.11[a]	0.48±0.01[c]	0.36±0.02[b]	1.20±0.00[a]	55.18±0.04[b]	16.29±0.02[a]	5.21±0.01[b]	0.81±0.24[a]
	C	6.61±0.03[a]	0.66±0.00[c]	0.43±0.01[b]	1.46±0.11[b]	51.74±0.31[a]	17.10±0.08[a]	5.32±0.11[a]	1.77±0.00[b]
28	S	5.74±0.09[a]	0.32±0.02[a]	0.40±0.17[b]	1.36±0.03[c]	55.20±0.18[b]	17.34±0.07[b]	5.11±0.06[a]	0.72±0.02[a]
	C	6.40±0.22[a]	0.61±0.13[b]	0.53±0.14[c]	1.62±0.12[c]	50.72±0.74[a]	17.26±0.00[a]	5.40±0.12[b]	1.77±0.00[b]

**Each value is a mean of triplicate determinations ± Standard Error

*Means followed by the same letters in the superscript are not significantly different from one another ($p < 0.05$) by Duncan's Multiple Range Test (DMRT)

pH; FFA –free fatty acid (%); TBA - thiobabituric acid analysis (mg malonaldehyde/kg); Peroxide value - (mg/kg fat); WHC water holding capacity - (%); CP - crude protein (%); CF - crude fat (%) ; TVB-N total volatile nitrogen (mg N/100g)

WHC of turkey samples showed lower value (50.72 ± 0.74) in control sample than 55.20 ± 0.18 recorded for treated sample on the 28th day. CP content increased slightly in treated sample from 17.33 ± 0.03 to 17.34 ± 0.07 while it decreased to 17.26 ± 0.00 in control. CF content of turkey increased in both samples tested with control recording the highest. TVB-N decreased slightly in treated sample from 0.73 ± 0.03 to 0.72 ± 0.02, while control sample increased to 1.77 ± 0.00 at the end of treatment (Table 4.34).

Microbial load profile of beef, chicken and turkey under freezing temperature for 28 days (Table 4.35) showed slight decrease in (TBC) of chicken and turkey from (3.88 ± 0.49 to 3.81 ± 0.00 log_{10}cfu/ml and 3.07 ± 0.23 to 3.01 ± 0.17 log_{10}cfu/ml) respectively. TBC count of control samples increased in chicken and turkey to 5.23±0.17 log_{10}cfu/ml and 5.26 ± 0.02 log_{10}cfu/ml respectively. Total bacteria count of beef increased in treated sample from 2.66 ± 0.03 to 3.46 ± 0.17 log_{10}cfu/ml against 4.47 ± 0.17 log_{10}cfu/ml recorded by control on day 28. *Enterococcal* count of treated beef and turkey significantly increased from 2.16 ± 0.01 to 2.68 ± 0.04 log_{10}cfu/ml and 2.28 ± 0.14 – 3.72 ± 0.01 log_{10}cfu/ml respectively. Control samples of beef and turkey recorded significant increase in *Enterococcal* count to a final count of 5.72 ± 0.02 log_{10}cfu/ml and 8.33 ± 0.04 log_{10}cfu/ml respectively. Chicken samples subjected to freezing temperature recorded a significant decrease in *Enterococcal* count from 2.63 ± 0.06 log_{10}cfu/ml to 2.50 ± 0.17 log_{10}cfu/ml while control samples recorded a count of 8.01 ± 0.15 log_{10}cfu/ml at the end of 28 days.

LAB count significantly increased in all samples subjected to freezing temperature with beef showing the highest count of 3.39 ± 0.18 log_{10}cfu/ml while treated chicken and turkey samples recorded 2.82 ± 0.02 log_{10}cfu/ml and 3.37 ± 0.01 log_{10}cfu/ml respectively. Control samples of beef, chicken and turkey recorded significant decrease in LAB count as 2.41 ± 0.13 log_{10}cfu/ml; 2.48 ± 0.34 log_{10}cfu/ml and 2.34 ± 0.02 log_{10}cfu/ml) respectively. Fungi counts increased significantly in beef and chicken treated samples from 3.06 ± 0.01 to

3.22 ± 0.16 log_{10}cfu/ml; and 2.60 ± 0.03 to 3.35 ± 0.02 log_{10}cfu/ml. Control samples of beef and chicken recorded increase fungi counts of 5.01 ± 0.24 log_{10}cfu/ml and 5.19 ± 0.17 log_{10}cfu/ml on day 28. Significant decrease in fungal count was observed in treated turkey sample from 3.73 ± 0.02 to 3.14 ± 0.17 log_{10}cfu/ml. Control recorded a significant increase of 5.48 ± 0.00 log_{10}cfu/ml. (Table 4.35).

Proteolytic activity of *P. acidilactici* isolated from stored beef under fast freezing temperature was cultivated in MRS broth at pH 5.0 and varied at -10^{o}C , 20^{o}C, 30^{o}C and 35^{o}C respectively. Values obtaned ranged from 12.37 to 30.91 units/ml. The optimum result was obtained at the temperature of 30^{0}C, incubation period of 72 hrs and inoculum concentration in 0.05 ml. At other temperatures, protease activity decreased with increase in inoculum size (Figures 4.17a- d).

Figures 4.18a-d shows proteolytic activity of *P. acidilactici* isolated from stored beef under fast freezing temperature was cultivated in MRS broth at pH 5.5. The range of proteolytic activity was 24.73 to 49.46 units/ml. Highest activity was recorded at -10^{0}C and 20^{0}C respectively. Both were observed at 96 hr and 0.1 ml inoculum size. The least activity was recorded at 30^{0}C, 48 h and 0.2 ml inoculums size. Proteolytic activity increased with increase in inoculums size of 0.1 ml but later declined.

Growth of *P. acidilactici* was observed at pH 5.0 **(**Fig 4.19a-d **)** and pH 5.5 (Fig 4.20a-d). At 5.0, growth in MRS broth ranged from 0.09 to 0.18 while pH 5.5 recorded higher growth of 0.15 to 0.39. The highest growth at pH 5.5 was recorded at -10^{0}C, 72 hr and 0.1 inoculum concentration also at 35^{0}C; 96 hr and 0.2 inoculum concentration respectively.

Extracelular Protein content determination in MRS broth at pH 5.0 gave a result of 0.02 – 0.80 mg/ml (Fig 4.21 a-d) while protein content determination in

MRS broth at pH 5.5 ranged from 0.04 to 4.00 mg/ml. The highest protein contents were recorded at -10, 72hr and 0.15 respectively (figure 4.22 a-d).

Effect of substrate concentration on proteolytic production in *P. acidilactici* isolated from stored beef under fast freezing temperature was optimized from 1% to 5%. It was observed that protease activity decreased with increase in concentration. 1% recorded the highest activity of 32.0 units/ml while the least activity 20.0 units/ml was recorded at 5% casein concentration Fig. 4.23

Lineweaver-Burke plot for the hydrolysis of different concentration of casein by the partially purified protease of ***P. acidilactici*** isolated from stored beef under fast freezing temperature revealed that V_{max} and K_m of substrate to the enzyme is 100 and 5.0 respectively. (Figure 4.24)

Table 4.35: Microbial load profile in different meat samples subjected to freezing treatment for 28 days

Microbial Group	Meat type / Storage Time (days) / Group Count (log cfu ml^{-1})														
	BF	CK	TK	BF	CK	TK	BF	CK	TK	BF	CK	TK	BF	CK	TK
		0			7			14			21			28	
Total Bact Count	$^{**}2.66 \pm 0.03^{a}$	3.88 ± 0.49^{a}	3.07 ± 0.23^{a}	2.77 ± 0.01^{a}	3.60 ± 0.19^{a}	3.18 ± 0.15^{b}	3.38 ± 0.15^{b}	4.02 ± 0.02^{bc}	3.55 ± 0.01^{c}	3.47 ± 0.00^{c}	4.22 ± 0.01^{c}	3.53 ± 0.17^{c}	3.46 ± 0.05^{c}	3.81 ± 0.0^{ab}	3.01 ± 0.17^{a}
Enterococcal Count	$2.16 \pm 0.01^{c*}$	2.63 ± 0.06^{d}	2.28 ± 0.14^{c}	2.40 ± 0.01^{a}	2.16 ± 0.03^{a}	2.28 ± 0.34^{a}	2.51 ± 0.01^{b}	2.50 ± 0.23^{b}	3.66 ± 0.00^{b}	2.63 ± 0.28^{c}	2.49 ± 0.13^{b}	3.79 ± 0.02^{c}	2.68 ± 0.04^{c}	2.50 ± 0.17^{b}	3.72 ± 0.01^{b}
LAB count	2.27 ± 0.01^{a}	2.66 ± 0.18^{ab}	2.91 ± 0.35^{a}	2.85 ± 0.17^{a}	2.57 ± 0.04^{a}	3.31 ± 0.13^{b}	3.20 ± 0.00^{c}	2.66 ± 0.01^{b}	3.12 ± 0.00^{a}	2.90 ± 0.12^{b}	2.71 ± 0.01^{b}	3.14 ± 0.02^{a}	3.39 ± 0.18^{d}	2.82 ± 0.02^{c}	3.37 ± 0.01^{c}
Fungi count	3.06 ± 0.01^{b}	2.60 ± 0.03^{d}	3.73 ± 0.02^{c}	3.60 ± 0.19^{b}	3.31 ± 0.33^{ab}	3.82 ± 0.12^{b}	3.07 ± 0.02^{a}	3.36 ± 0.03^{c}	3.84 ± 0.00^{b}	3.08 ± 0.1^{a}	3.44 ± 0.08^{c}	3.16 ± 0.01^{a}	3.22 ± 0.16^{a}	3.35 ± 0.02^{a}	3.14 ± 0.17^{a}
CONTROL															
Total bact. Count	2.66 ± 0.03^{a}	3.88 ± 0.49^{c}	3.07 ± 0.23^{a}	3.11 ± 0.12^{a}	3.03 ± 0.02^{a}	3.66 ± 0.10^{a}	3.87 ± 0.04^{b}	3.63 ± 0.14^{b}	4.63 ± 0.17^{b}	4.38 ± 0.04^{c}	3.86 ± 0.04^{c}	4.93 ± 0.17^{c}	4.47 ± 0.14^{c}	5.23 ± 0.17^{d}	5.26 ± 0.02^{d}
Enterococcal Count	2.16 ± 0.26^{a}	2.63 ± 0.06^{d}	2.28 ± 0.14^{c}	2.68 ± 0.00	3.99 ± 0.17^{a}	4.65 ± 0.03^{a}	4.77 ± 0.06^{a}	5.22 ± 0.00^{b}	6.01 ± 0.06^{b}	5.65 ± 0.04^{b}	6.31 ± 0.01^{c}	7.17 ± 0.05^{c}	5.72 ± 0.02^{c}	8.01 ± 0.15^{d}	8.33 ± 0.04^{d}
LAB count	2.27 ± 0.01^{a}	2.66 ± 0.18^{ab}	2.91 ± 0.35^{a}	2.44 ± 0.00	2.76 ± 0.03^{b}	2.31 ± 0.01^{a}	2.44 ± 0.02^{a}	2.46 ± 0.02^{a}	2.47 ± 0.04^{b}	2.44 ± 0.00^{a}	2.46 ± 0.02^{a}	2.47 ± 0.07^{b}	2.41 ± 0.13^{b}	2.48 ± 0.34^{a}	2.34 ± 0.02^{a}
Fungi count	3.06 ± 0.01^{b}	2.60 ± 0.03^{d}	3.73 ± 0.02^{c}	3.06 ± 0.00^{a}	4.36 ± 0.02^{a}	4.99 ± 0.13^{a}	3.51 ± 0.13^{a}	5.65 ± 0.24^{b}	5.02 ± 0.00^{a}	4.48 ± 0.23	5.67 ± 0.06^{b}	5.01 ± 0.17^{b}	5.01 ± 0.24	5.19 ± 0.17^{c}	5.48 ± 0.00^{c}

**Each value is a mean of triplicate determination s± Standard Error

*Means followed by the same letters in the superscript are not significantly different from one another ($p < 0.05$) by Duncan's Multiple Range Test (DMRT)

BF - Beef sample; CK - Chicken sample; TK - Turkey sample

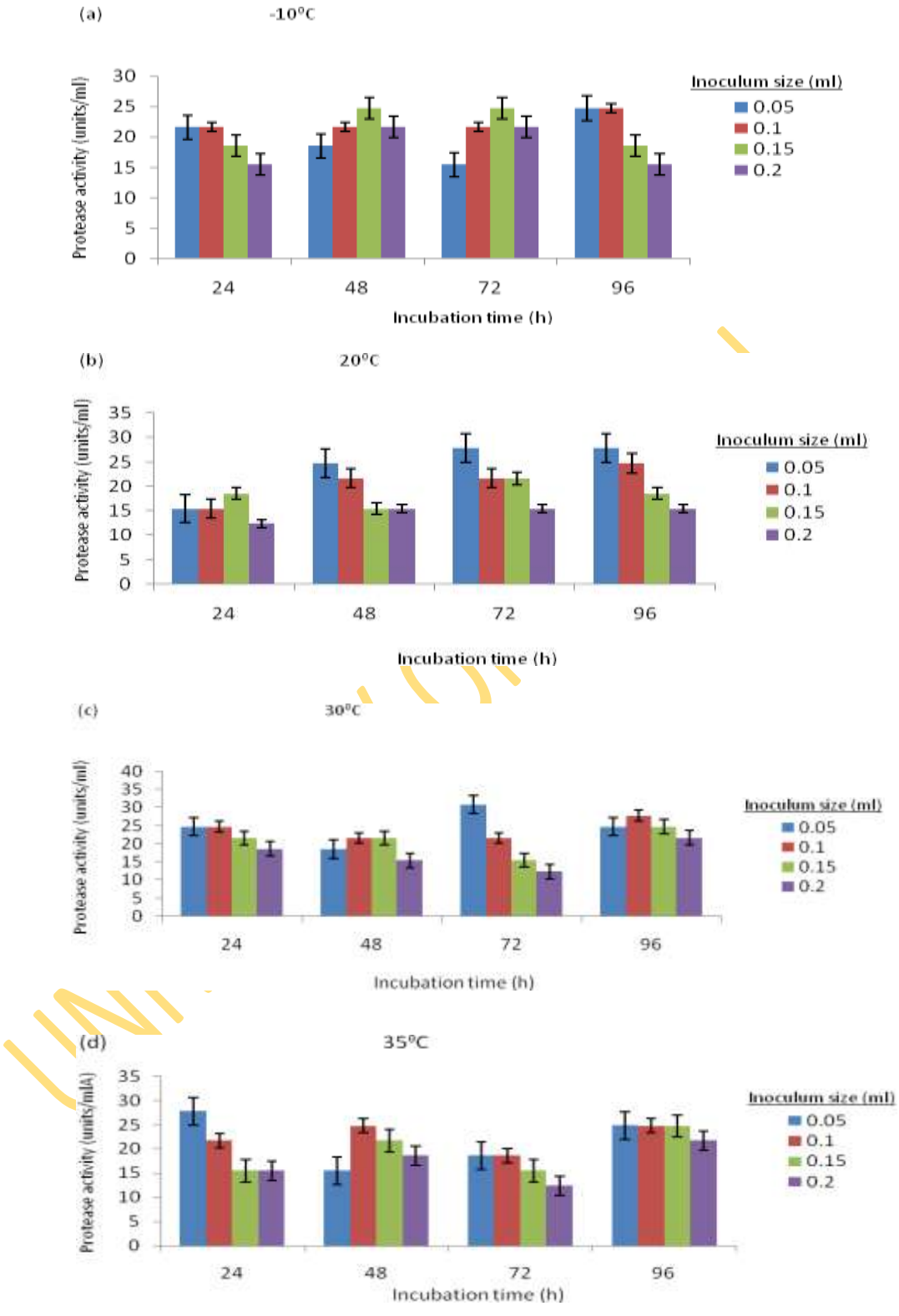


Fig. 4.17 a-d: Proteolytic activity of *P. acidilactici* from stored beef under fast freezing at different temperatures at pH 5.0

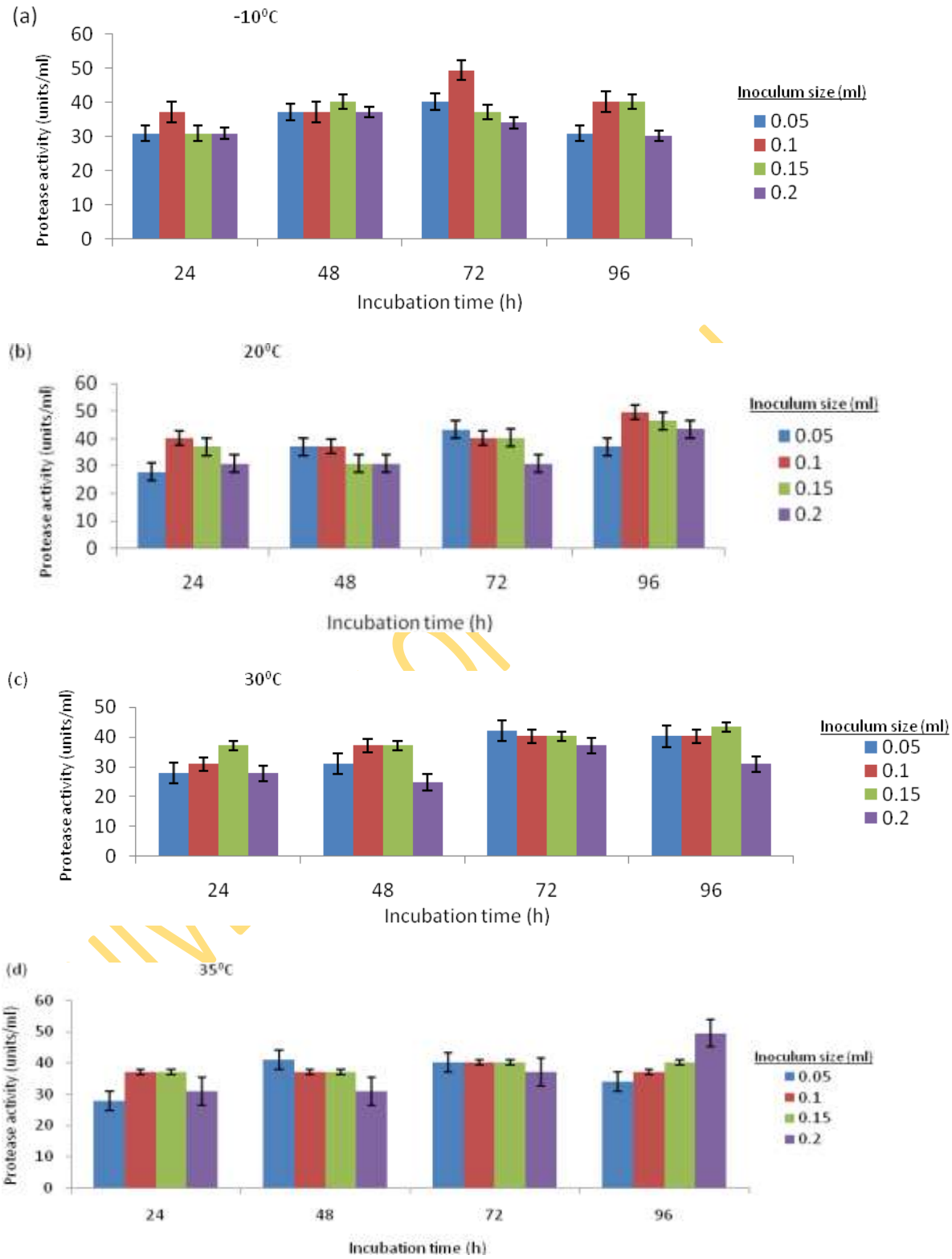


Figure 4.18 a-d: Proteolytic activity of *P. acidilactici* from stored beef under fast freezing at different temperatures at pH 5.5

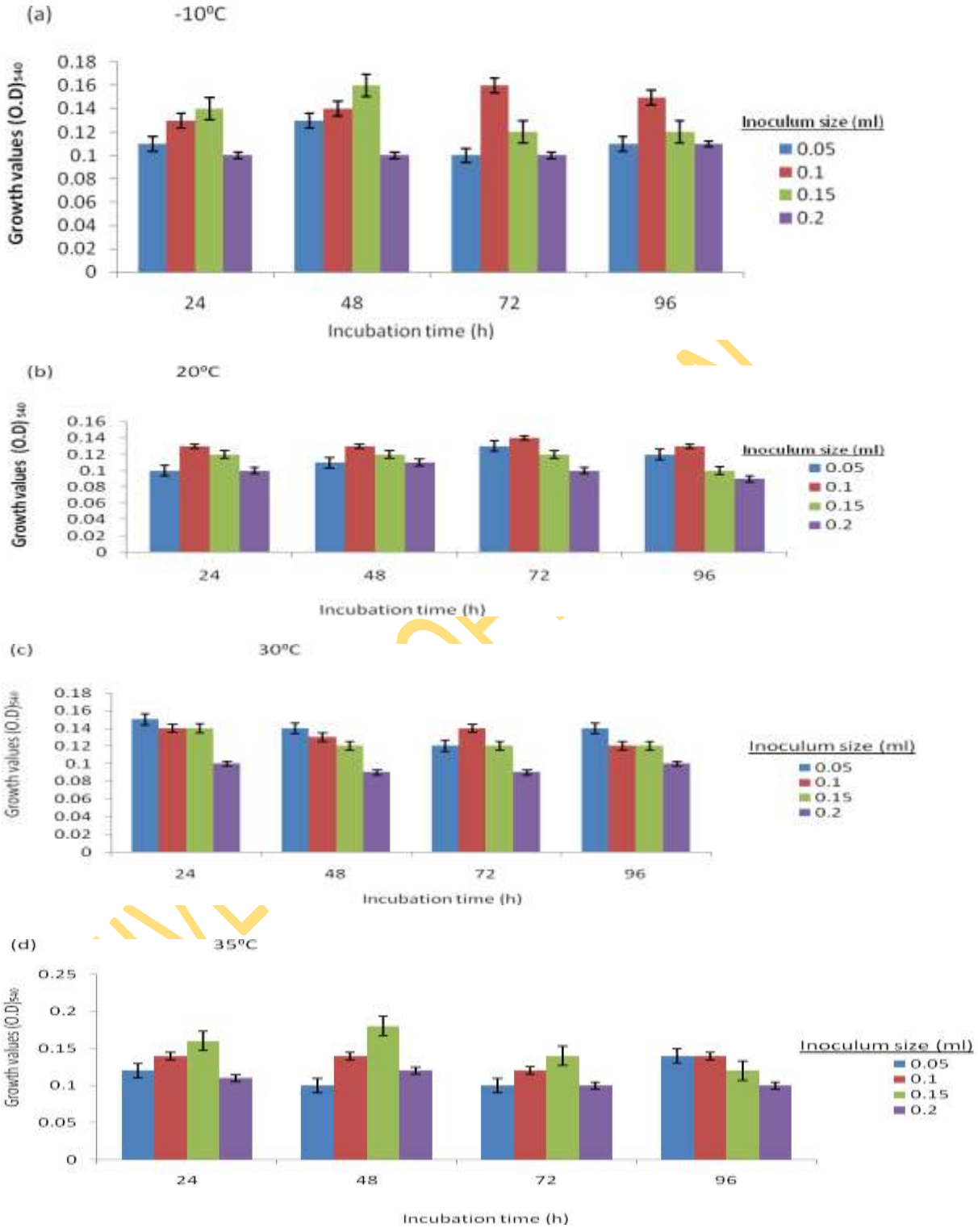


Fig. 4.19 a-d: Growth of *P. acidilactici* from stored beef under fast freezing in Man Rogosa and Sharpe broth at pH 5.0

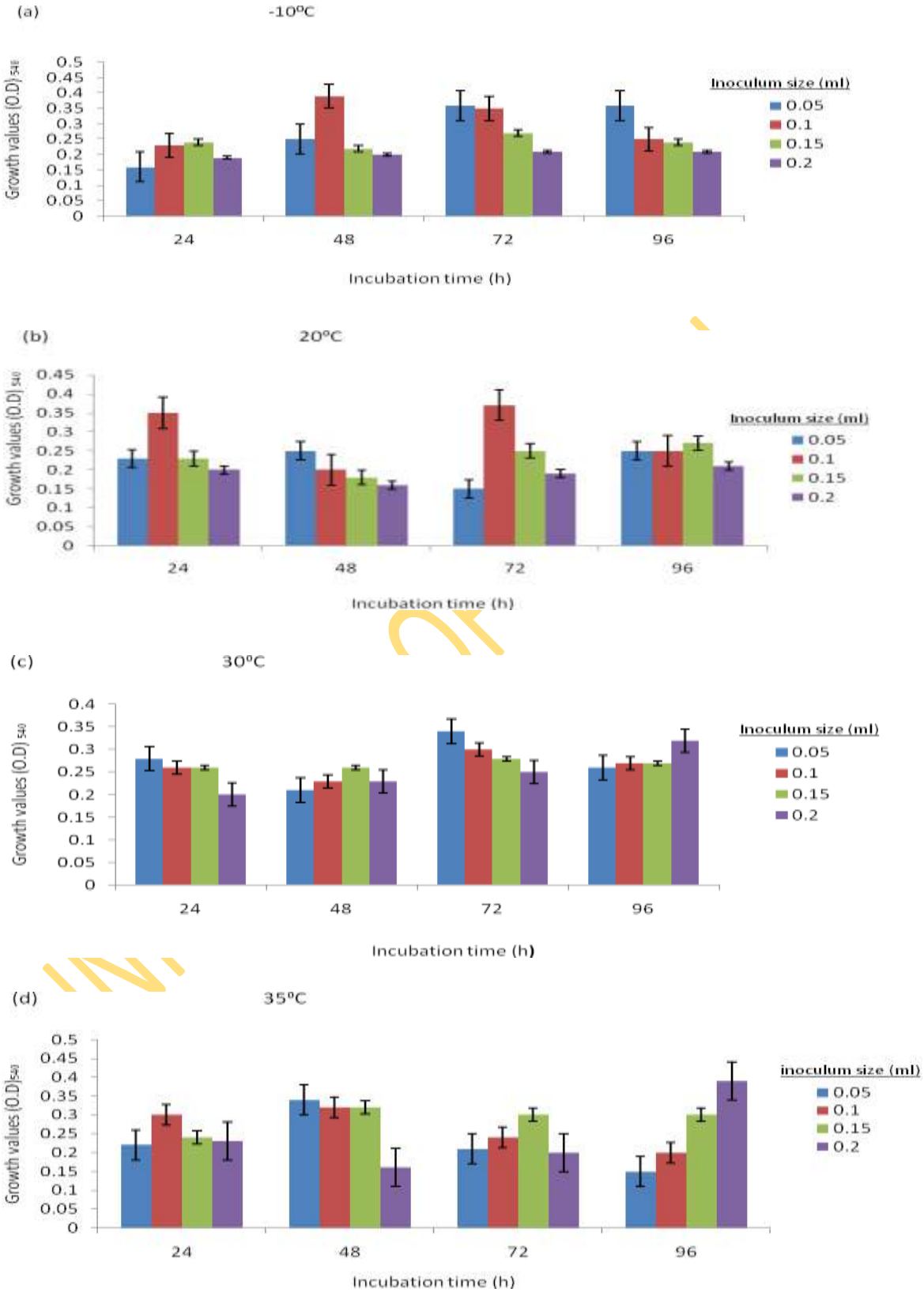


Fig. 4.20 a-d: Growth of *P. acidilactici* from stored beef under fast freezing in Man Rogosa and Sharpe broth at pH 5.5

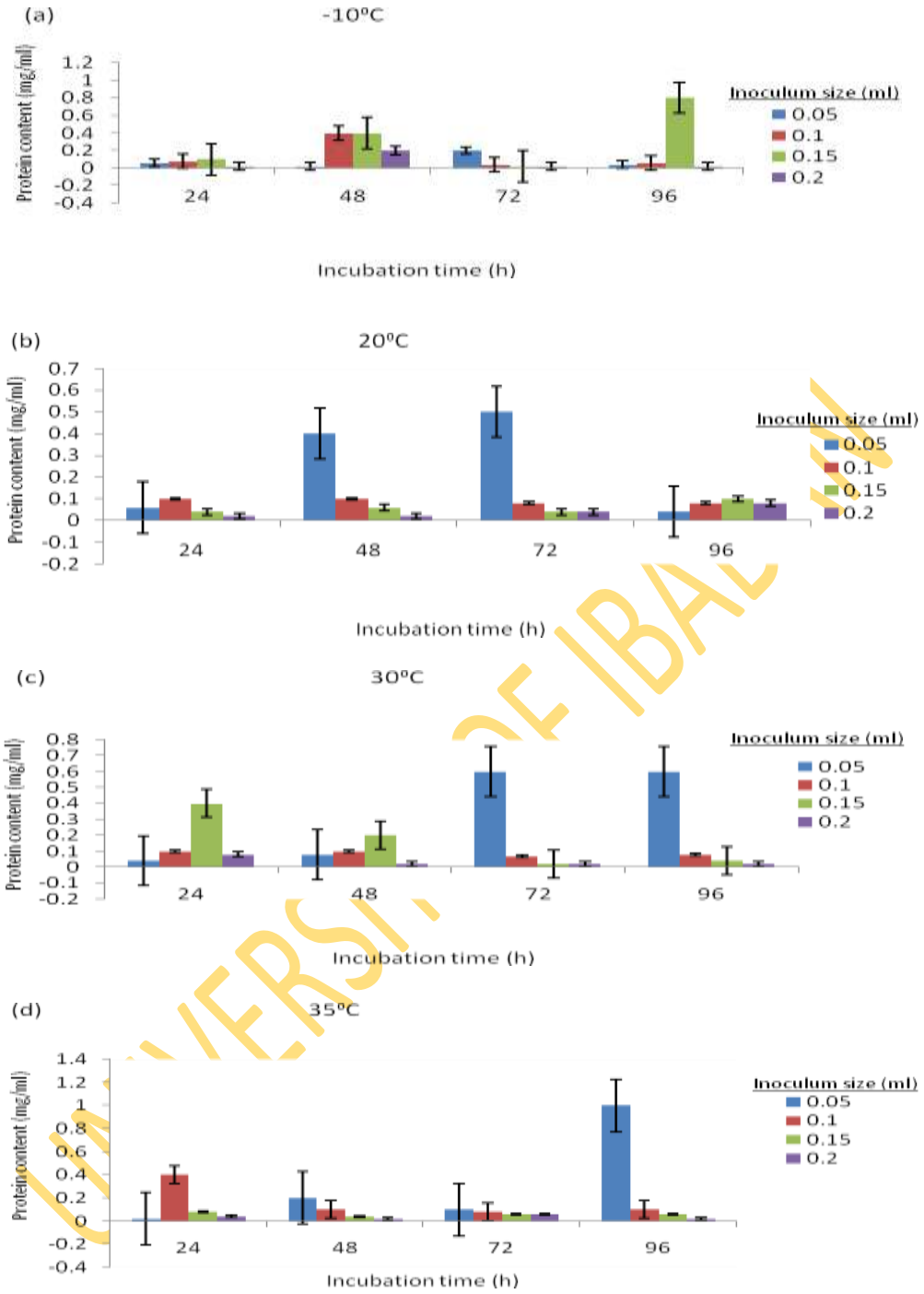


Fig. 4.21 a-d: Extracellular Protein content (mg/ml) of *P. acidilactici* from stored beef under fast freezing cultivated in Man Rogosa and Sharpe broth at pH 5.0

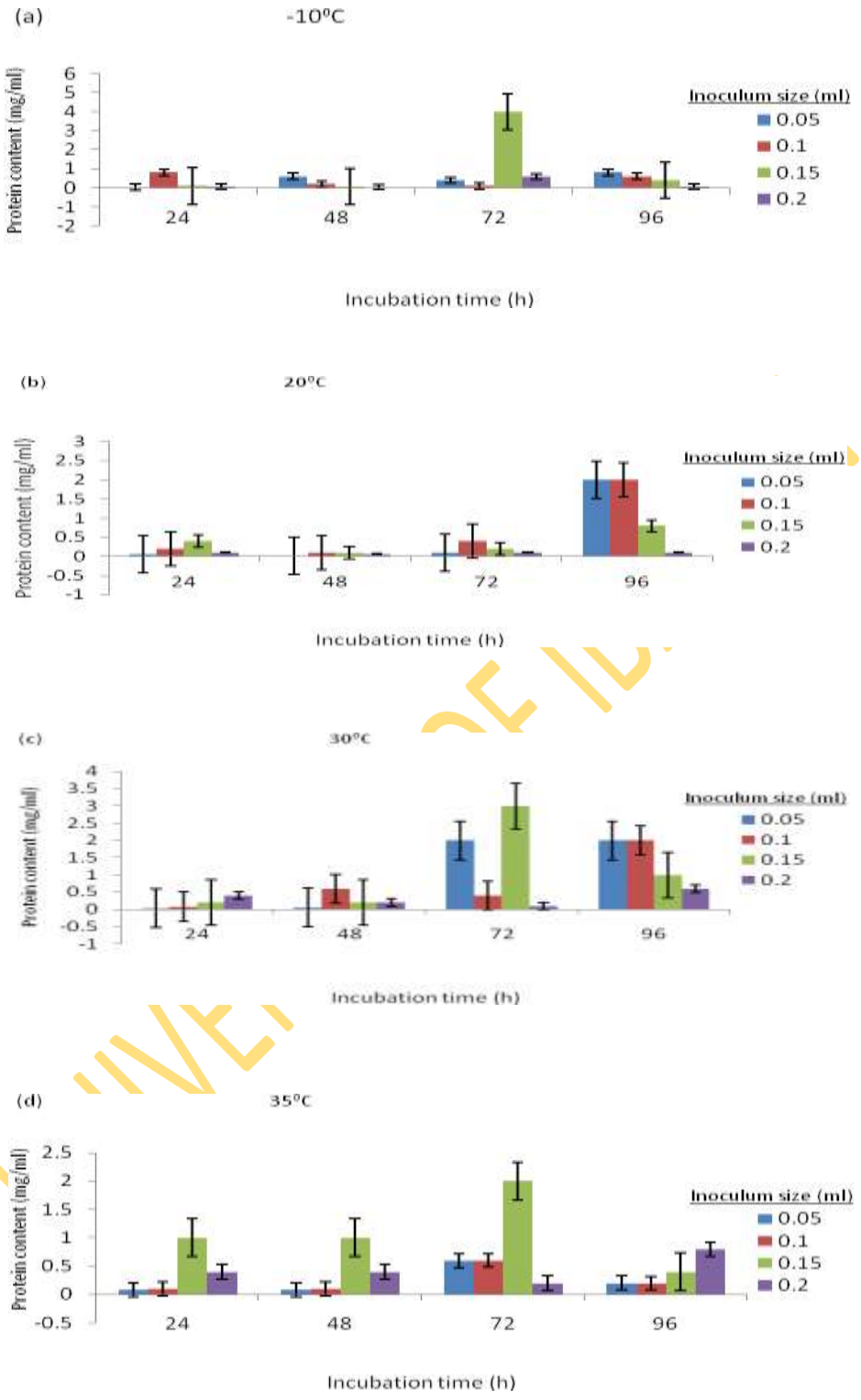
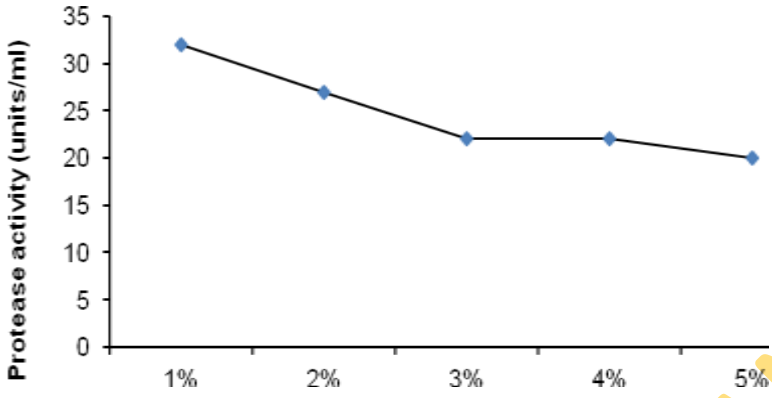


Fig. 4.22 a-d: Exracellular Protein content (mg/ml) of *P. acidilactici* from stored beef under fast freezing cultivated in Man Rogosa and Sharpe broth at pH 5.5



. **Casein**

Fig. 4.23: Effect of substrate concentration on protease activity of *Pediococcus acidilactici* from stored beef under fast freezing temperature

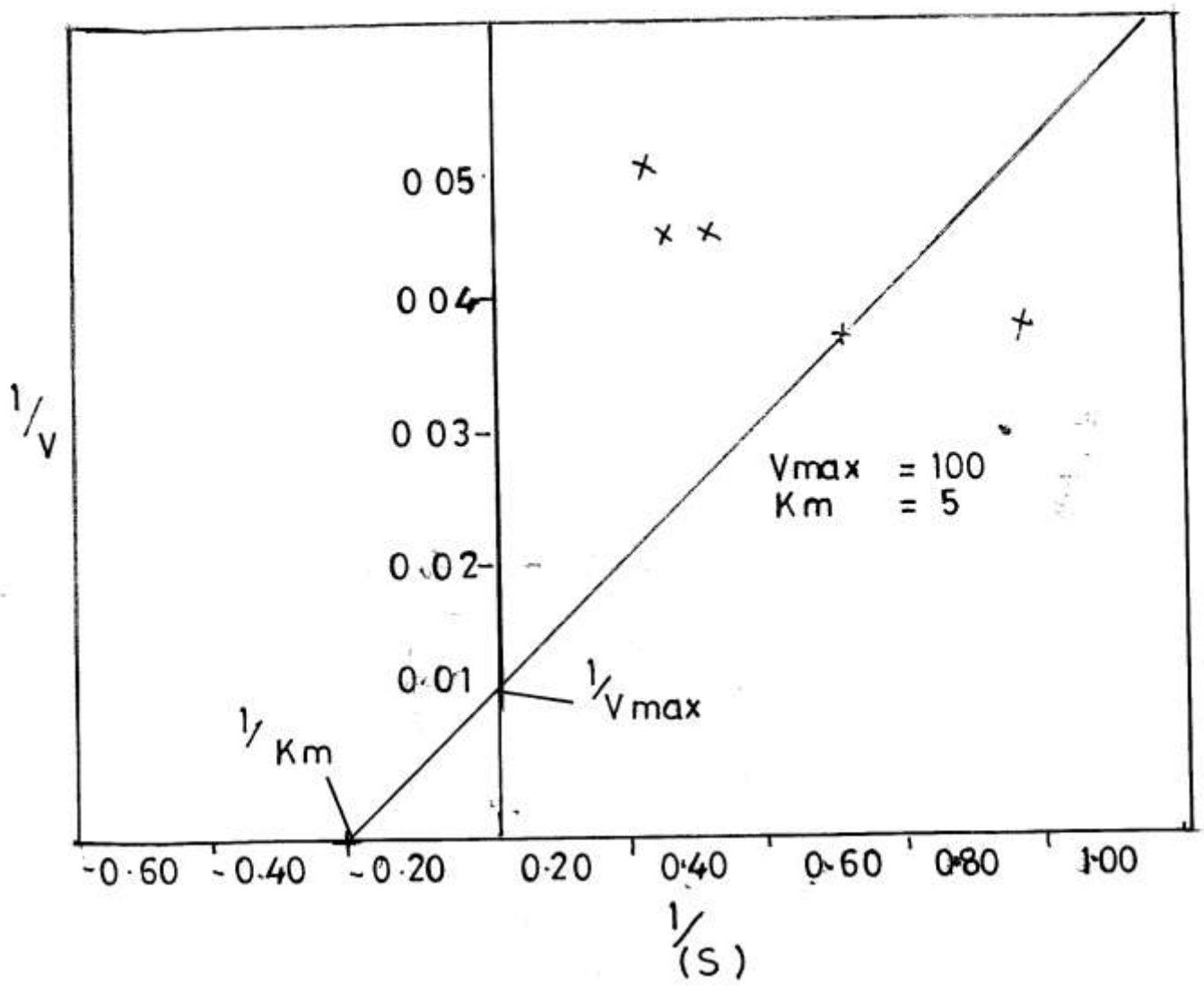


Fig. 4.24 Lineweaver-Burke plot of the hydrolysis of different concentrations of casein by the partially purified protease from *Pediococcus acidilactici* from stored beef under fast frozen temperature

Effect of varying temperature on proteolytic activity of *P. acidilactici* isolated from stored beef under fast freezing temperature BEKBLT showed the highest protease activity was observed at -4^{o}C and -15^{o}C, it decreased in activity as the temperature increased from 20^{o}C to 40^{o}C Figure 4.25a. Effect of pH on protease activity increased from pH 4 to pH 5.5 and declined at pH 6. pH 5.5 recorded the highest activity of 42.03 units/ml. The lowest value was recorded at pH 6.0 as 7.0 units/ml Fig 4.25b.

Effect of enzyme concentration on proteolytic activity of *P. acidilactici* BEKBLT Fig 4.26 followed similar trend with pH, it showed increase in protease activity from 14.0 to 22.0 units/ml and further dropped to 14.0 units/ml

Lineweaver-burke plot of enzyme concentration against protease activity of *P. acidilactici* BEKBLT shows that the Vmax and km of protease to substrate is 50 and 2.5 respectively Fig.4.27.

Effect of cations on proteolytic activity of *P. acidilactici* BEKBLT revealed the effect of different concentration (0.1 M to 0.5 M) of Na^{+}, K^{+}, Ca^{2+}, Mg^{2+} and Mn^{2+} respectively. 0.1M of Mn^{2+} gave the highest protease activity of 49.04units/ml. In most of the results lower concentrations gave higher protease activity than higher concentrations Fig.4.28a

Effect of anions on proteolytic activity of *P. acidilactici* BEKBLT shows the effect of Cl^{-}, $N0_3^{-}$, $S0_4^{2-}$, Cl_2^{-} and $N0_3^{-}$ varied at concentrations of (0.1- 0.5 M). 0.1 M of $N0_3^{-}$ gave the highest proteolytic activity of 37.36Units/ml. Cl_2, $S0_4^{2-}$ and $N0_3^{-}$ decreased with increase in concentration. The least proteolytic activity of 4.67 units/ml was recorded by Cl_2 at 0.5 M concentration fig. 4.28b.

Enzyme extracted from *P. acidilactici* BEKBLT was subjected to characterization studies. It was partially purified by ammonium sulphate precipitation, dialysed, passed through sephadex grades of G100, C50 and G25 and subjected to SDS-PAGE for molecular weight determination**.** Separation by

ion exchange chromatography of major proteins and enzyme activity of fractions of *P. acidilactici* BEKBLT towards casein showed two absorption peaks of AA and BB on sephadex G 100. Protease activity and protein concentration of peaks AA and BB recorded (27.0 units/ml / 0.06 mg/ml; 28.0 Units /ml / 0.06 mg/ml) respectively Fig. 4.29.

Separation by ion exchange chromatography of high molecular weight protein and enzyme activity of fractions of *P. acidilactici* BEKBLT towards casein revealed three absorption peaks of AAa, AAb and AAc on sephadex C50. Enzyme activity and protein concentration of (30.0 units /ml / 0.06 mg/ml; 29.0 units/ml/0.07 mg/ml and 25.0 units/ml 0.06 mg/ml were recorded for three absorption peaks respectively fig. 4.30. Separation by ion exchange chromatography of low molecular weight protein and enzymic activity of fraction of *P. acidilactici* BEKBLT towards casein showed two absorption peaks of BBa and BBb. On Sephadex G 25. Proteolytic activity and protein concentration recorded for both peaks were (23.0 units ml^{-1} /10 mg/ml; 20.0 $unitsml^{-1}$/0.06 mg/ml) respectively. (Fig. 4.31)

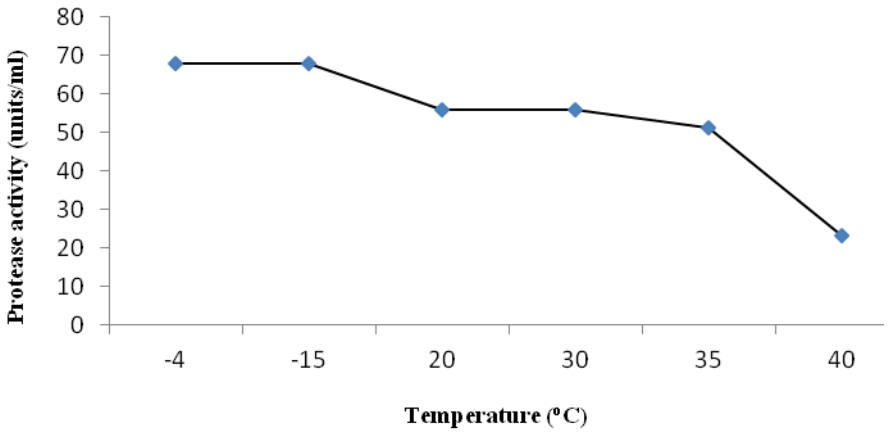


Fig. 4.25a: Effect of temeprature on protease activity of *Pediococcus acidilactici* from stored beef under fast freezing temperature

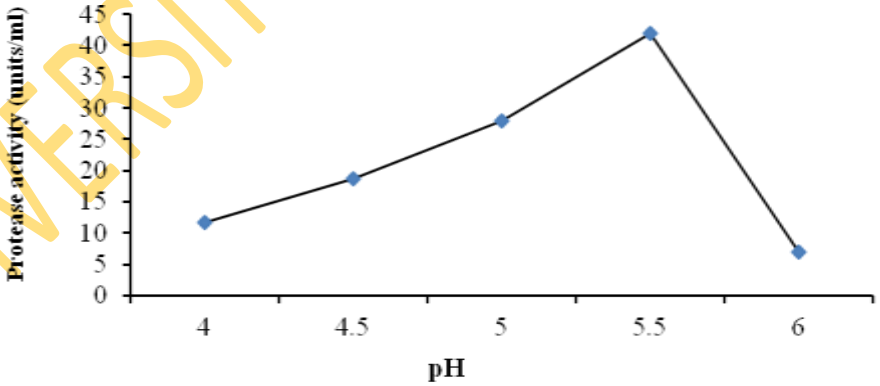


Fig. 4.25b: Effect of pH on protease activity of *Pediococcus acidilactici* from stored beef under fast freezing temperature

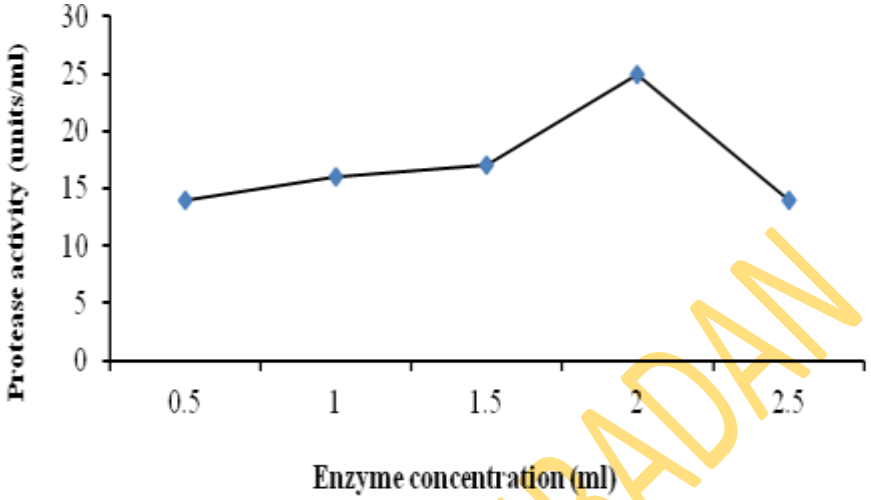


Fig. 4.26: Effect of Enzyme concentration on protease activity of *Pediococcus acidilactici* from stored beef under fast freezing temperature

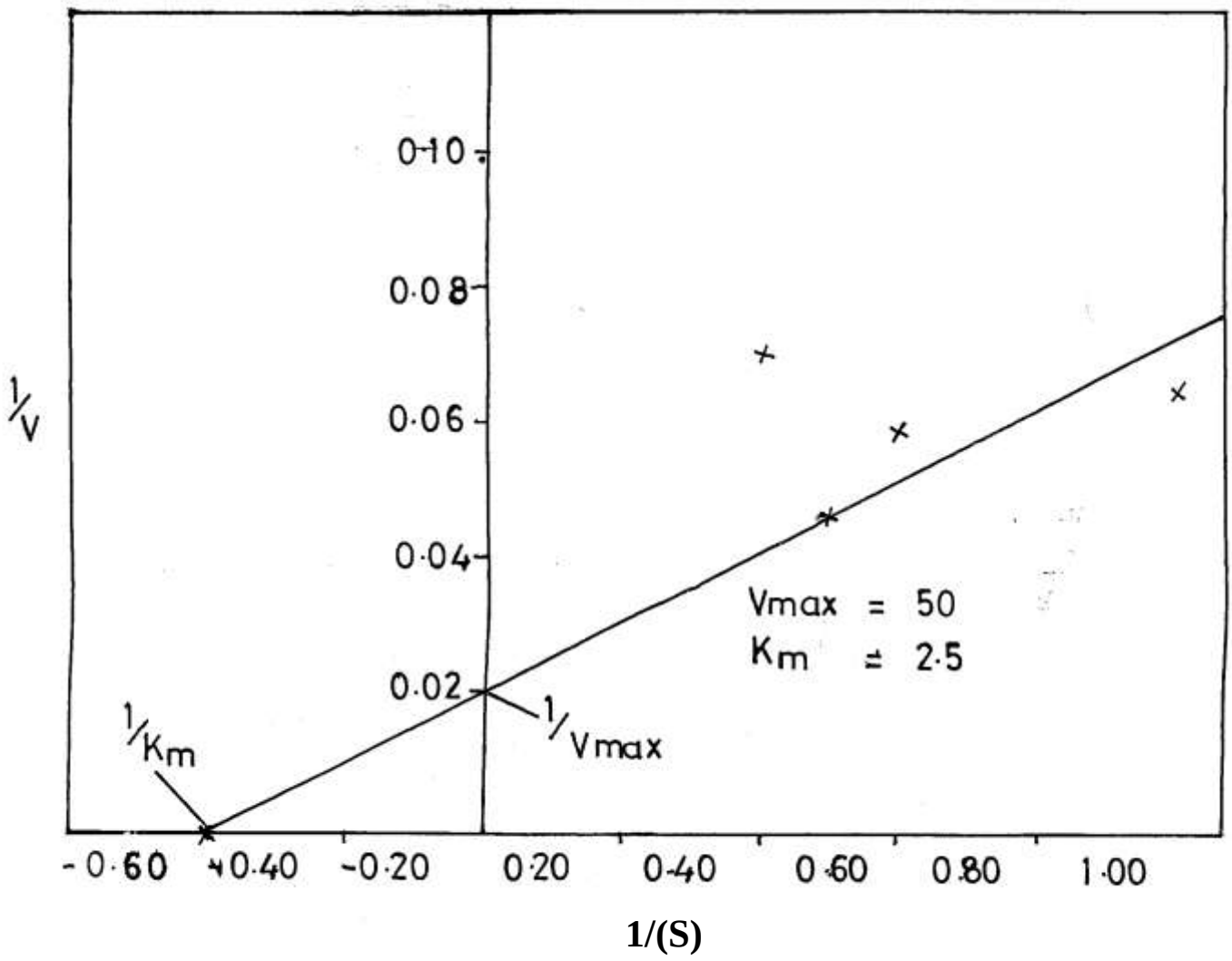


Fig. 4.27 Lineweaver –Burke plot for the hydrolysis of casein by different concentration of partially purified protease from *Pediococcus acidilactici* from stored beef under fast frozen temperature

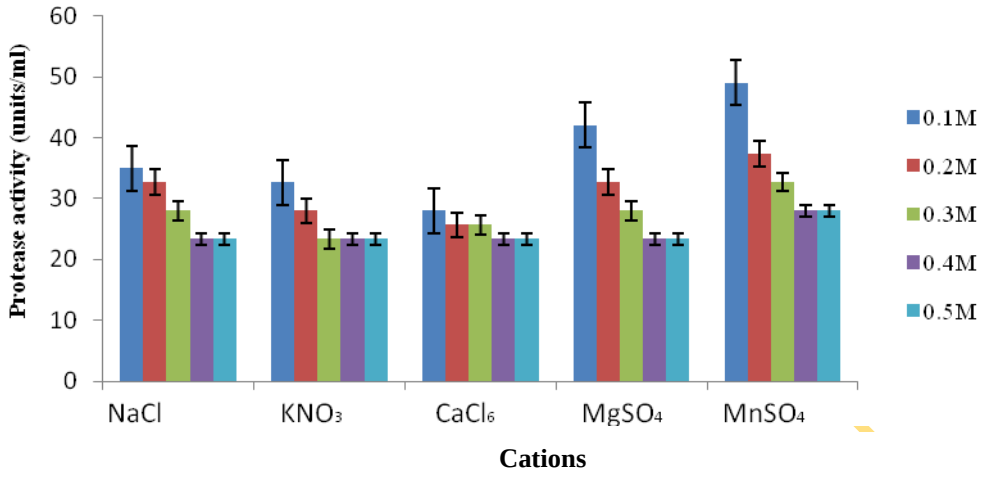


Fig. 4.28a: Effect of Cations at different concentrations on protease activity of *Pediococcus acidilactici* from stored beef under low temperature storage

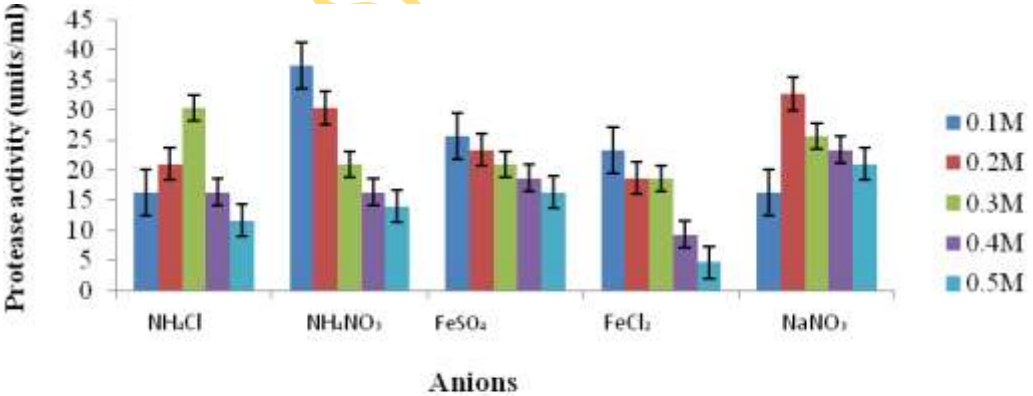


Fig. 4.28b: Effect of anions at different concentrations on protease activity of *Pediococcus acidilactici* from stored beef under fast freezing

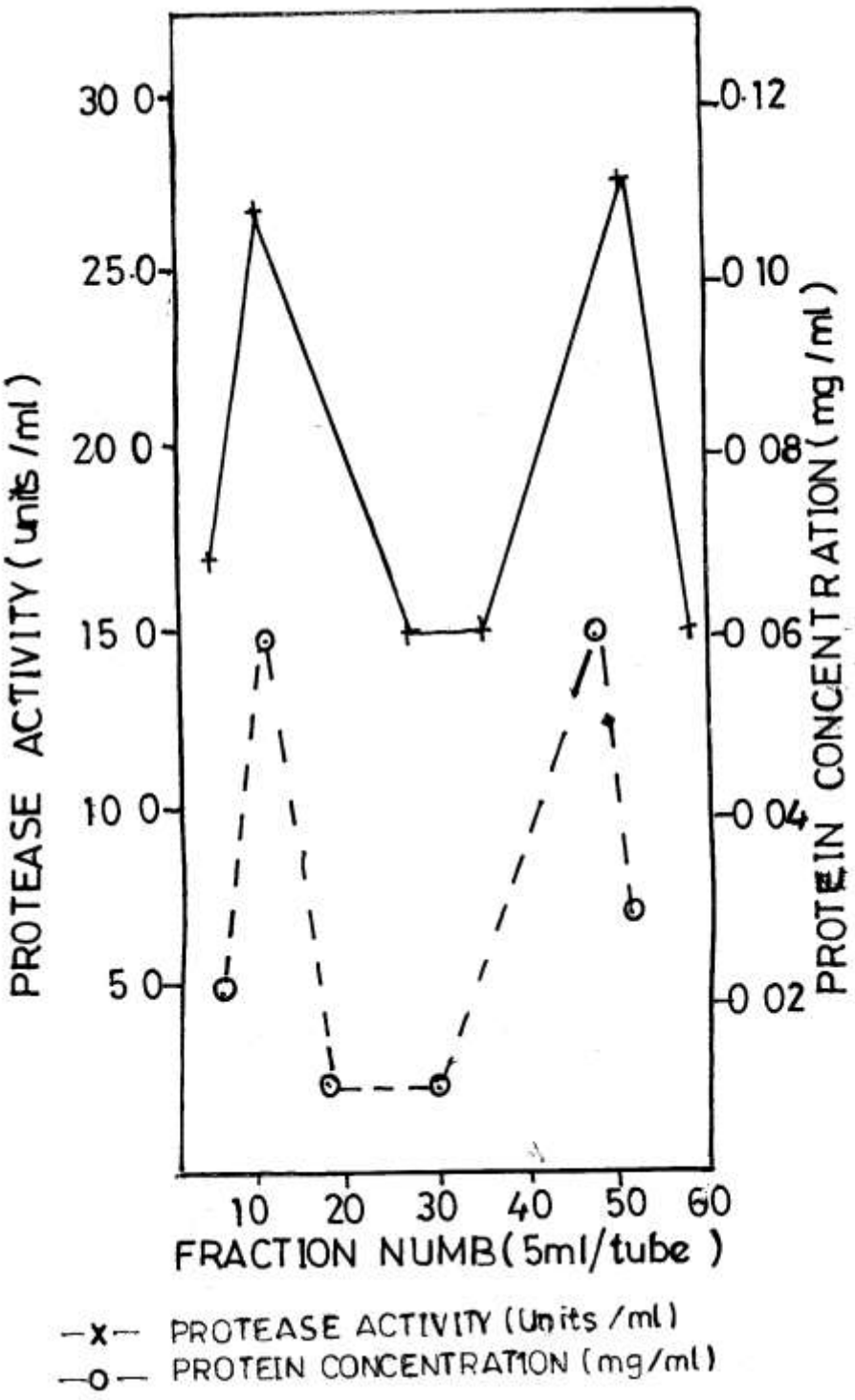


Fig. 4.29 Seperation by ion exchange chromatography of major proteins and enzymic activity of fractions from *Pediococcus acidilactici* from stored beef under fast frozen temperature towards casein

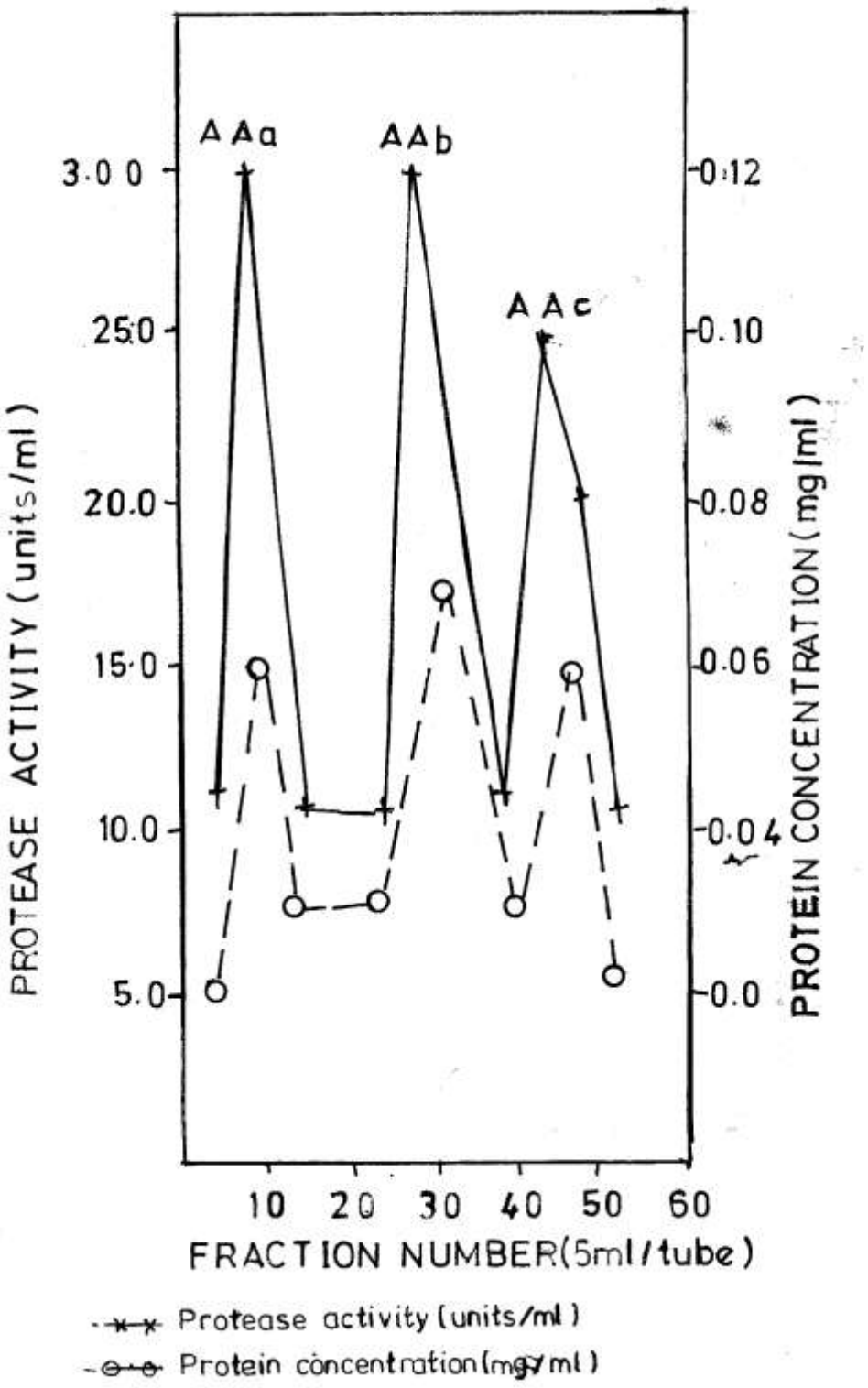


Fig. 4.30 Seperation by ion exchange chromatography of high molecular weight proteins and enzymic activity of fractions from *Pediococcus acidilactici* from stored beef under fast frozen temperature towards casein

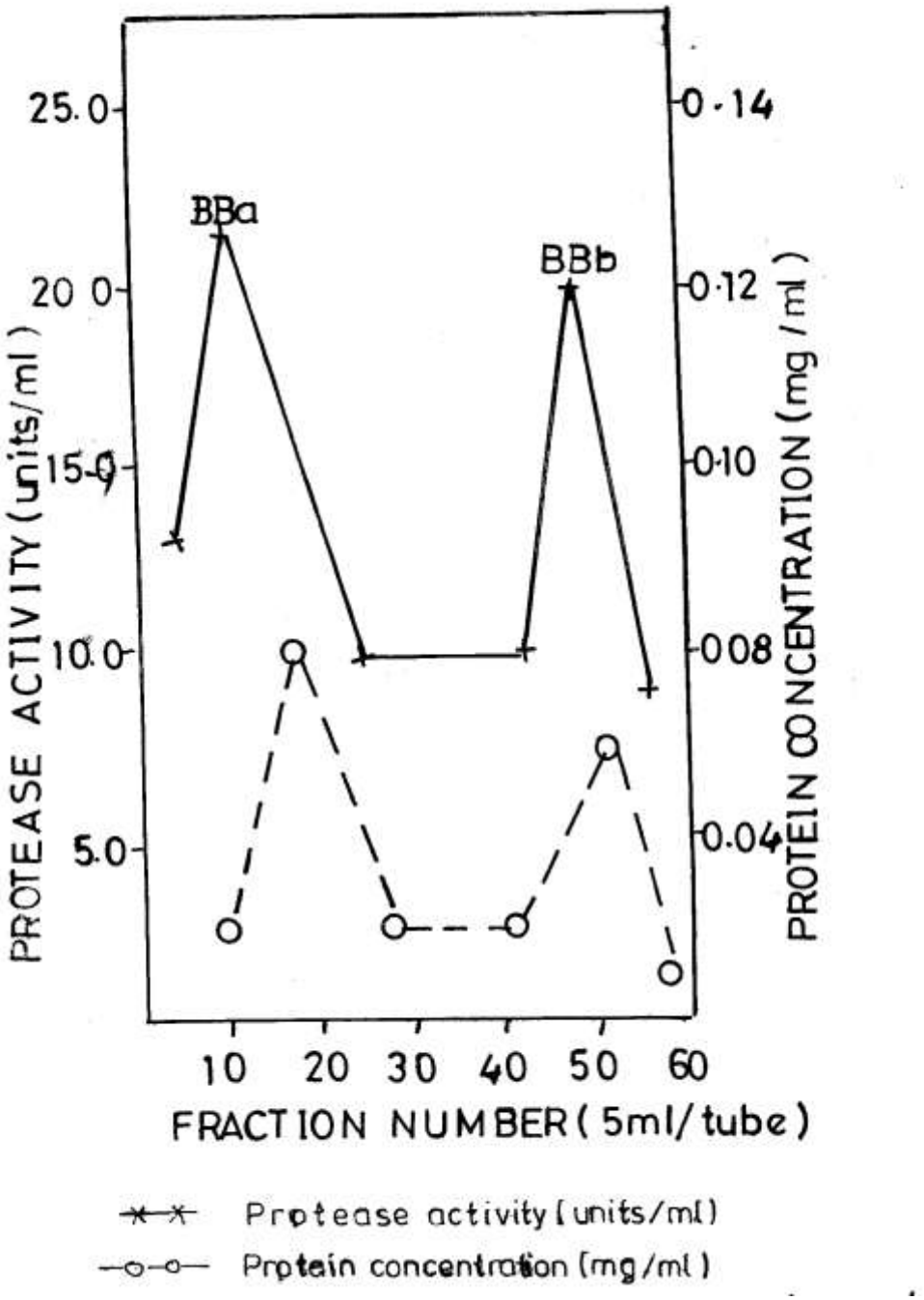


Fig. 4.31 Seperation by ion exchange chromatography of high molecular weight proteins and enzymic activity of fractions from *Pediococcus acidilactici* from stored beef under fast frozen temperature towards casein

CHAPTER FIVE
DISCUSSION

A total of one hundred and ten strains of lactic acid bacteria (LAB) were isolated from fresh and treated samples of beef, chicken and turkey. They were phenotypically characterized and identified with reference to Bergey's Manual of Systematic Bacteriology (Sneath *et al*., 1986). The species identified include *Lactobacillus plantarum; Lactobacillus brevis; Lactobacillus buchneri; Leuconostoc mensenteroides; Pediococcus acidilactici; Lactobacillus curvatus; Lactobacillus casei; Lactobacillus sakei* and *Lactobacillus bulgaricus.* In this study, LAB were isolated, screened and characterized to search for potential starter culture that could be used in the preservation of meat under low temperature. In meat, lactic acid bacteria (LAB) constitute a part of the initial microflora (Rountsiou and Cocolin, 2006) which develop after meat processing. In general, conditions that favour their growth result in an extension of the storage life and enhance safety of chilled meats. Recent approaches in the preservation of meat products increased the use of LAB as protective microbiota to inhibit spoilage and pathogenic bacteria (Kotzekidou and Bloukas, 1996; Bredholt *et al*., 1999; 2001; Pidcock *et al.*, 2002).

The isolation and identification of similar LAB isolates from meat have also been reported by various workers. Stiles (1991) reported the identification of aciduric *Lactobacillus sp* including *Lactobacillus sakei; Lactobacillus curvatus* and *Lactobacillus plantarum* from fresh meat samples. Hamasaki *et al.,* 2003 have reported the isolation of *Lactobacillus plantarum* and *Pediococcus acidilactici* from cooked meat stored at 10^0C. Nychas and Tassou (1996), Borch *et al* (1996), Korkeala *et al.* (1992) and Rato *et al.* (2002) are research workers that have isolated and identified lactic acid from meat products. Other researchers that have reported the isolation and identification of lactic acid bacteria from meat samples are Ahn and Stiles (1990), Guerrero *et al.* (1995) Leisner *et al.* (1995) Aymerich *et al.* (1998, 2003), Lucke (2000) Bromberg *et al.* (2004), Leroy and De Vuyst (2005) and Jones *et al.* (2008). Marceau *et al.* (2003) reported that *Lactobacillus sakei* is commonly found on fresh meat and

is a predominant flora observed in vacuum packaged meat. In Iberian dry fermented sausages, *P. pentosaceus*, *P. acidilactici*, *Lactococcus lactis* and *L. brevis* have been isolated as the predominant LAB, together with isolates of *L. plantarum* and *L. curvatus* (Benito *et al.*, 2007). LAB such as *Pediococcus pentasaceous* and *Pediococcus acidilactici* were used to prevent the growth of fish borne bacteria in mackerel fish chunks (Kannappan and Manja, 2004)

The Lactic acid bacteria (LAB) occupies a central role, and has a long and safe history of application and consumption in fermented foods and beverages (Papamanoli *et al.*, 2003). LAB are widely distributed in nature, and are commonly found in vegetables, grains, milk and fresh meat. In several food fermentations, LAB are oftentimes the dominating microorganisms, resulting in acidification and eventually inhibiting spoilage and pathogenic bacteria (De Vuyst and Vandamme, 1994; Stiles, 1996; Papamanoli *et al.*, 2003; Cotter *et al.*, 2005). LAB are considered as food grade organisms that are safe to consume and have long history of use in food (Bredholt *et al*., 2001). Reasons for suitability of LAB include their natural origin and their contribution to food safety and/or offer of one or more organoleptic, technological, nutritional and health advantages (Papamanoli *et al.*, 2003).

In this work, fast freezing and freezing gave significantly lower LAB count than that of chilling and refrigeration in this work. Temperature seems to be the most important factor that influences the spoilage as well as the safety of meat (Koutsoumanis and Taoukis, 2005). Indeed modern lifestyle and evolution of consumer requirements over the past decade have led to significant increase in the demand of fresh raw meat (Hugas, 1998).

Consumers are drawn to natural foods with little or no chemical preservative contents (Hequet *et al.*, 2009). This perception has stimulated research interest in bio-preservation that depends on the use of antagonistic micro-organisms or their antimicrobial products, to inhibit undesired microorganisms in order to enhance safety and extend shelf life of fresh meat products (Holzapfel *et al.*,

1995). Bio-preservation has been proven to be a promising natural preservation technique (Vermeiren *et al.,* 2005). Therefore the use of LAB as bio-preservative to extend the shelf-life of fresh meat can improve microbial stability and safety in commercial meat preservation (Castellano *et al.,* 2008). LAB are advantageous from a technological and hygienic point of view in meat preservation. Hence research on improving the quality and shelf life of meat is vital to maximize the potentials from the valuable materials.

Biopreservation has gained increased attention as a means of naturally controlling the shelf life and safety of meat and meat products. Some LAB, among those commonly associated with meats, demonstrate antagonism toward pathogenic and spoilage organisms. (Holzapfel *et al.*, 1995; Rodriguez *et al.*, 2002; Castellano *et al.*, 2008). Their antagonism refers to inhibition through competition for nutrients and/or production of one or more antimicrobially active metabolites such as lactic and acetic acids; hydrogen peroxide, antimicrobial enzymes, bacteriocins and reuterin (Holzapfel *et al.,* 1995; Stiles 1996 and Lucke, 2000). Although it is generally assumed that this antimicrobial activity is due to organic acids, diacetyl and hydrogen peroxide, the inhibition could be attributed to a family of antimicrobial peptides collectively known as bacteriocins (Cotter *et al*., 2005). Those peptides are ribosomally synthesized, normally contain 30 to 60 residues and usually have bactericidal and bacteriostatic effect toward closely related species (Mortvedt *et al.,* 1991). Nowadays, only the bacteriocin nisin has been widely used in the food industry. Nonetheless, the application of nisin to meats may be limited because of its low solubility in meat pH, the inability of the producer organism to grow in meats, its efficiency to inhibit all spoilage and pathogenic microorganisms associated with meats, and its inactivation by the enzyme glutathione S-transferase of raw beef (De Martins *et al.*, 2002).

In this study, LAB isolates were screened and subjected to lactic acid, acetic acid and diacetyl production. The isolates were also grown at different pH, temperature and varying concentration of salt. *Pediococcus acidilactici* of

stored beef under fat freezing temperature exhibited better results in terms of the above parameters. Results revealed the ability of some isolates to be salt tolerant at varying concentration. LAB that are salt tolerant, psychrotrophic and adapted to meat have a good potential to be used for biopreservation of meat and meat products (Stiles, 1996). Lactic acid bacteria produce organic acids which lowers the pH of products or bacteriocins which inhibit pathogens without altering the product (Montville and Chikindas, 2007). Lactic acid has been tested as a chemical meat-sanitizing agent by several authors (Van Nethen *et al.*, 1994; Borpuzari and Borpuzari, 1995). However, extention of meat shelf life by the addition of chemical preservatives like lactic acid and nitrate is not accepted by consumers who demand little or no chemical preservatives. (Cudjoe and Kapperud, 1991; Stiles, 1996; Hugas, 1998).

Biopreservation has been proven to be a promising natural preservation technique (Vermeiren *et al.,* 2005). Lactic acid bacteria originally isolated from traditional sausages are probably the best candidates for improving the microbiological safety of these foods, because they are well adapted to the conditions in meat and should therefore be more competitive than LAB from other sources (Ammor *et al.,* 2006). The use of homofermentative lactic acid bacteria (LAB) whose main metabolism is lactic acid as bioprotector is an alternative to meat chemical preservation, because of their ability to inhibit the growth of pathogens and spoilage microorganisms (Signorini *et al.,* 2007). *Pediococcus pentasaceus* has been utilized as a starter culture in the industrial fermentation of meats (Erkkila *et al.,* 2001) and as a potential biopreservative (Rodgers *et al.*, 2003). The culture of *Pediococcus pentosaceus* has been used among other forms, as frozen concentrate and traditional starter culture in the manufacture of fermented dry and semi - dry sausages (Raccach, 1987).

Lactobacillus sakei, Lactobacillus curvatus; Lactobacillus plantarum; Lactobacillus pentosus; Lactobacillus casei; Pediococcus pentosaceus and *Pediococcus acidilactici* are species most used as commercial meat LAB starter cultures (Hugas and Monfort, 1997; Hammes and Hertel, 1998). Inoculation of

sausage batter with homofermentative lactobacilli and/or *Pediococci* improved the quality and safety of the final product and standardized the production process (Campbell-Platt and Cook, 1995; Hugas and Monfort, 1997; Lucke, 2000).

Pediococcus acidilactici isolates were shown to contain plasmid DNA. After determining that plasmid DNA was present in *Pediococcus acidilactici* strains, studies to determine whether among the resident plasmids might encode for a detectable phenotypic character such as carbohydrate utilization and antimicrobial activity was carried out using curing studies. Curing studies suggested that utilization of carbohydrate was not plasmid linked in the five isolates examined. This is because parental strain and their respective cured strains exhibited similar reactions.

The present study revealed that *P. acidilactici* isolates exhibited substantial amount of inhibitory activity against foodborne pathogens and food spoilage organisms including certain Gram negative bacteria. *Pediococcus* isolates displayed wide zones of inhibition against *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium* and *Listeria monocytogenes* among other selected indicator organisms tested**.** Lactic acid bacteria are commonly isolated from meats (Berry *et al.*, 1990; Schillinger and Lucke, 1987; Schillinger and Lucke, 1989). These organisms can inhibit the natural microflora of meat, which include spoilage bacteria and, if present, pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* (Bacus, 1984). Ammor *et al.*, 2006 reported that strains of *L. sakei* were able to inhibit *L. innocua* and other *Lactobacilli* while strains of *Escherichia. coli, Pseudomonas aeruginosa, Serratia marcescens* were resistant. Tantillo *et al.* (2002) reported that *Staphylococcus aureus* was much more resistant than *L. innocua*. The ability of *L. sakei* species to display antibacterial activity against *Listeria* species was shown in several reports (Liserre *et al.*, 2002; Mataragas *et al.*, 2003; Nieto-Lozano *et al.*, 2002).

Several studies have shown considerable interest in *L. sakei* for the bioprotection of meat products (Vold *et al.,* 2000; Bredholt *et al.,* 2001; Katikou *et al.,* 2005). For example, Katikou *et al.* (2005) have shown that *L. sakei* CECT 4808 was more efficient than *L. curvatus* CECT 904 to reduce the development of *Enterobacteriaceae* in refrigerated vacuum – packed sliced beef. According to the complete genome analysis, it was proposed that *L. sakei* could be used to control pathogens in meat because its metabolism is particularly well adapted to meat environment (Chaillous *et al.,* 2005) and the environmental conditions that prevail during manufacturing process (Marceau *et al.*, 2003, 2004). The ability of *L. sakei* and *L. plantarum* species to display antibacterial activity against *Listeria* species was shown in several reports (Mataragas *et al.,* 2003; Dicks *et al*., 2004).

In this study, the nucleotide sequencing of the genes of Pediococcus *acidilactici* strains were examined. The PCR of the variable regions (V) of the 16S rDNA gene were targeted using specific primers, because of the small fragments of the DNA usually associated with the V region, thus making PCR fractions easy to handle, sequence and analyze

Presently, the nucleotide sequencing of 16S rDNA genes is one of the ultimate and most popular aspect of tools for molecular identification of organisms, effort was made towards targeting the V region rather than the ITS internally transcribed spacer (Klijn *et al.,* 1991; Ercolini *et al*., 2001; Ercolini *et al*., 2003; Schuurman *et al.,* 2004; Clarridge, 2004; Fujita, 2007).

Sequencing of the nucleotides of the 16S rDNA genes of the respective LAB isolates were finally carried out using automated sequencing facilities. The nucleotide sequences of the LAB isolates revealed comparable result with the PCR 16S rDNA V3 regions, when resolved in 1% agarose gel on electrophoresis. The number of nucleotide bases for the LAB strains were less than 200 base pairs (bp), indicating loss of part of the PCR products during purification or rather contaminants. This was expected, as some of the

contaminants of the PCR products including proteins, RNA, residual salts, excess PCR primers, dNTPs, enzyme and buffer components (from PCR amplification) may all contribute to the size of the PCR products, and such are usually removed during the purification process, prior to sequencing (Marugg *et al.,* 1992*;* Yepyhardi, 2007). Also the V3 region of the 16S rDNA genes of most LAB are usually less than 200 bp (Ercolini *et al.*, 2001, 2003).

The computer generated chromatograms of the nucleotide sequences has been achieved by recent advances in the technology of nucleic acid sequencing, engaging the abilities of radioactively or fluorescently labeled dideoxyribonucleotide tri phosphates (ddNTPs) to absorb light at different wavelengths (Russel, 2000). The various ddNTPs comprising of ddGTP, ddCTP, ddTTP and ddATP are usually labelled with the nucleotide bases tagged with colours of **black**, blue, red and green respectively and each colour absorb light at varying wavelengths. The ddNTPs are typical of the usual deoxyribonucleotide tri phosphates (dNTPs) except that the former had one oxygen atom less at the carbon 3 of the deoxyribose sugar and they are very useful in bringing about an enforcing termination whenever they are added to growing DNA chain during the amplification process of the sequencing reaction (Metzenberg, 2003). The relative height of the peaks of chromatograms depicts the quantity of the respective base nucleotides present in the DNA sample being sequenced. Also the presence of only four lines, especially at the beginning of the nucleotide sequences is an indication of good quality of the purified 16S rDNA genes, which otherwise would be an impure sample of DNA (Russel, 2000). In such a case, the result of sequencing of the DNA would usually return as fail, the instance which was obtained during initial trials in the course of this research study.

The nucleotide sequences obtained from the respective LAB isolates were subjected to basic local alignment search tool (BLAST) analysis at the geneBank database (http://www.ncbi.nlm.nih.gov/) in the quest for the identities of the strains. The primary purpose of the BLAST was to compare the

nucleotide sequences of the organism being investigated to those that have been obtained by other researchers in their studies and that have been deposited in the database for others to access. In this way ambiguities and confusion normally associated with the phenotypic method of identification are eliminated. In instances where no matches are obtained with those in the database, the organism in question could be a novel type whose genomes have not been exploited by other researchers.

The nucleotide sequence of the 16S rDNA gene of Pediococcus acidilactici isolated from stored beef under fast freezing temperature was able to yield a significant number of hits during the BLAST query. Over one hundred strains of organisms in the database compared favourably with the LAB strain in their nucleotide alignment with the test organisms. Among the first ten organisms in the geneBank database, that showed significant alignment with the LAB strain, six of strains were *P. acidilactici*, two were *Pediococcus* sp., without a species name attached; while the last was *Pediococcus pentosaceus*. This implies that the quest for the identification of the strain BEKBLT was finally achieved and this LAB strain shall hence forth be referred to as *P. acidilactici* BEKBLT, referring to *Pediococcus acidilactici* isolated from beef under fast freezing temperature. The entire ten topmost organisms had required e-values to accept the identity of the test isolate (BLAST, 2008). When performing the nucleotide alignments of the test organism with the topmost five organisms in the database, showing significant alignment, two gaps were observed in three of the isolates, thus giving 98% maximum identities. This implying a good comparison of the base pairs of the nucleotide sequences. This serves to buttress the confirmation of the identity of the *P. acidilactici.*

The use of PFGE, 16S rDNA PCR-RFLP and analysis of nucleotide sequences have been widely reported by many research workers as useful molecular tools being engaged in the characterization and identification of lactic acid bacteria (Mora *et al.*, 2000; Jang *et al*., 2003; Deveau and Moineau, 2003; Botina *et al*., 2006). According to many of the researchers such tools have helped in

combating and erasing the problems known to be associated with the biochemical or phenotypic methods of characterization of microorganisms especially lactic acid bacteria. Some of such problems that could have significant adverse effect on research findings have to do with many microorganisms that have been falsely identified by the latter method. In enhancing the quality of research findings efforts should be geared towards imbibing molecular tools in the identification procedures of microorganisms. Although it may seem to be financially expensive and demanding at the early stage, but it will be very worthwhile on the long run (Ammor, 2007).

Determination of bacteriocin activity was carried out on five selected strains of *P. acidilactici* (TEK1U, CIB1U, BEKBLT, BIB7C and TLA14R). Characterization of bacteriocin at different pH, temperature, enzyme, organic solvents and surfactants showed varying zones of inhibition and antimicrobial activity against *Escherichia coli* ATCC 25923. *P*ediococcus *acidilactici* isolated from stored beef under fast freezing temperature exhibited notable zones of inhibition and antimicrobial activities. The use of bacteriocins, the organism which produced them or both is attractive to the food industry, because consumers demand for natural products and increasing concern about foodborne diseases (Montville and Winkowski, 1997). Current research on LAB bacteriocins are conducted aiming to broaden their application as natural food preservation (D'Angelis *et al.,* 2009). Several lactic acid bacteria (LAB) associated with meat products are important natural bacteriocin producers (Castellano *et al.,* 2008). Several publications have reported that bacteriocinogenic LAB especially *Pediococci* strains, could be used as bioprotective cultures for food manufacturing processes in attempts to control L. *monocytogenes* (Dicks *et al.,* 2004; Nieto-Lozan *et al.,* 2006). Amezquita and Brashears (2002) found positive results for the bacteriocin producing *Pediococcus acidilactici* on frankfurters after 5 - 6 days at 5^0C.

The present study revealed that the bacteriocins from *P. acidilactici* isolates exhibited substantial amount of inhibitory activity against typed *Escherichia*

coli strain ATCC 5218. This can be used as food preservative replacing the chemical preservatives in order to safeguard human health (Pal *et al*., 2010). Bacteriocin-producing lactic acid bacteria could be used to lower the level of pathogens in meat products as compared to non bacteriocin producing LAB (Hugas *et al.,* 1995). According to the literature, the frequency of isolation of bacteriocin producing strains varies. This variation can be attributed to sample intrinsic differences in experimental set-up of the antibacterial activity test and diversity of indicator microorganisms used for initial screening (De Martinis *et al.,* 2002).

Lactic acid bacteria are known to have an antagonistic activity towards a variety of microorganisms. Bacteriocin production is one of the properties responsible for the antibacterial activity against closely related species and possible Gram positive food spoilers and pathogens (Klaenhammer, 1993; De Vuyst and Vandamme, 1994). Bacteriocins produced by lactic acid bacteria are either small thermostable peptides or large thermoliable proteins. Large numbers of bacteriocin producers have been found among different general of the lactic acid bacteria (Piard and Desmazeaud, 1992; Hoover and Steenson, 1993; De Vuyst and Vandamme, 1994; Dodd and Gasson, 1994). Bacteriocins are of interest for potential application in the food industry because of their antimicrobial activity and their technologically favourable properties (Ray and Daeschel, 1992; De Vuyst and Vandamme, 1994). Several LAB associated with meat products are important natural bacteriocin producers (Castellano *et al.,* 2008).

Numerous reviews have suggested that some LAB were able to control the growth of some pathogenic microorganisms such as *L. monocytogenes* in food products (Callewaert and De Vuyst , 2000; Mataragas *et al.,* 2002; Tantillo *et al.,* 2002). These food-borne LAB have been described as bacteriocin producers. De Martinis *et al*. (2002) isolated and identified the LAB strain *L. sakei*, from fresh pork sausage. The microorganism was capable to inhibit the multiplication of *L. monocytogenes* in culture media by bacteriocin production.

Bacteriocinogenic *L. sakei* was tested in a model food system by Alves *et al.* (2003) and it was more effective to inhibit *L. monocytogenes* than a non bacteriocinogenic strain of the same species.

Meat-borne LAB produce a range of bacteriocins that are generally active towards other LAB (contributing to the competitiveness of the producing strain) and food borne Gram-positive pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus, Clostridium perfringes* and *Bacillus cereus* (Aymerich *et al.*, 2000; Enan *et al.*, 1996; Messi *et al.*, 2001; Noonpakodee *et al.*, 2003). Antimicrobial peptides produced by LAB can easily be broken down by digestive proteases, so they will not produce gut microbiota disturbance Castellano *et al.*, (2008). Based on literature, it can be concluded that antifungal and antibacterial compound of LAB do exist and have the potential for being effective in combating food borne bacteria, yeasts and molds (Rodriguez *et al.*, 2002 and Mataragas *et al.*, 2003). Castellano *et al.*, 2008 observed that *Lactobacillus curvatus* CRL 705 used as protective culture in fresh beef was effective in inhibiting *Listeria innocua* and *Brochothrix thermosphacta* as well as the indigenous contaminant LAB, by retaining its inhibiting effect at low temperature and having a negligible effect on meat pH. The antibacterial activity of bacteriocin of *Enterococcus faecium* MMZ17 was tested against various gram-positive and gram-negative bacteria. Some gram-positive bacteria including lactic acid bacteria and several pathogenic strains such as *Listeria monocytogenes* CECT 4032, *Enterococcus faecalis* ATCC 19433, *Enterococcus faecalis* JH 2-2, *P. acidilactici, Lactococcus lactis* spp. Cremoris ATCC 11603, *L. casei* DSM 20011; *L. sakei* ATCC 19433 and *Leuconostoc mesenteroides* were sensitive, while variety of gram-negative bacteria were not inhibited.

Proteinase K, trypsin, pepsin, α-chymotrypsin, pepsin and chloroform inhibited bacteriocin production by *Pediococcus acidlactici* strains in this work. De Martinis *et al.*, 2002 showed the bacteriocin presented bactericidal effect against *L. monocytogenes* and it was inactivated by pepsin, heat stable (60^{o}C/60

min; 100°C/20 min) and did not loose activity during storage at 10^{0}C for 24 h. Belgacem *et al.,* 2008 reported that antibacterial activities of four *Enterococcus faecium* were stable after a heat treatment at 60^{0}C for 30 min, and 100^{0}C for 15 min respectively. Inhibitory activity of these extracts was lost after treatment with proteinase k, trypsin and α-chymotrypsin whereas it was not affected by lysozyme and catalase (Belgacem *et al.,* 2008). This inactivation suggests that inhibition was due to a proteinaceous agent and not to hydrogen peroxide or acidity (Supernatant adjusted at pH 6.5) Montville and Kaiser, (1993).

Effect of pH on the activity of bacteriocin by five strains of *Pediococcus acidilactici* against typed *Escherichia coli* strain ATCC 5218 was in the pH range of 2 - 10 while no activity was detected in all strains at pH 12. The pH stability of the antibacterial substances was not changed in a wide pH range 3-9, with a maximum of activity at pH 6 and 7. It was shown that certain bacteriocin-like producing LAB may repress the growth of some undesirable microorganisms. PA-1 is a bacteriocin produced by *Pediococcus acidilactici* with antimicrobial activity against *L. monocytogenes* and several strains of *L. inoccua (*Albano *et al.,* 2007). This bacteriocin is stable over a wide range of temperature and pH conditions, and sensitive to a number of digestive proteases, suggesting that it might be a promising alternative to chemical preservatives in some applications (Albano *et al.,* 2007)

The recovery of active lactocin 705 was strongly dependent upon the pH of the growth medium, and was highest in the 6.5 - 7.5 range. The optimum pH for nisin production was found to occur in the 5.5 – 6.1 range (Hurst, 1981). The optimum pH for *Pediocin* ACH was close to 4.0, while no bacteriocin activity was detectable at pH 5.5 or higher (Biswas *et al.,* 1991). On the whole, the variability in the pH optima seems to reflect the growth of the organisms as well as the activity (Vignolo *et al.,* 1995). Lactic acid bacteriocins are generally stable at acid or neutral pH, indicating that the substances are well adapted to the environment of the producer bacteria.

Titres of 900- 7200 $AUmL^{-1}$ were recorded during optimization and monitoring of bacteriocins by varying different pH, temperature, enzyme, surfactant and organic solvent in this work. Inhibitory activity was detected when the producer strain *L. casei* CRL 705 was incubated at 15, 20, 25 and 30^{o}C. Titres of inhibitory activity as high as 1066 $AUmL^{-1}$ were obtained and $cfuml^{-1}$ were maximal at 20^{0}C. When different initial pH values in MRS broth were assayed, bacteriocin activity showed a maximum between pH 6.5 and 7.5, with a titre of 2133 $AUmL^{-1}$ after 24 h.

Proximate and biochemical analyses of the beef, chicken and turkey samples with changes in microbial load were monitored at 7 -day interval for 28days. During storage, the pH of meat samples tend to drop in inoculated meat samples, while control samples recorded increase in pH under freezing and fast freezing temperature storage. This is associated with an increase in the LAB population. This is consistent with the view that pH decreased under low temperature progressively, it selects for the development of aciduric subpopulations. Low pH is associated with acidifying LAB growth, favoured by microaerophilic conditions (Mendonca *et al.,* 1989). The results obtained in this work were in agreement with those reported by Guerrero *et al.,* (1995) regarding the significant reduction of pH in beef treated with homofermentative LAB. The increase in meat pH could be due to glucose exhaustion that reduces LAB growth (Gill and Newton, 1980). Increase in pH as storage progressed could be due to the bacterial activity that resulted in the production of ammonia, amines and other basic substances (Strange *et al.,* 1977; Wing *et al.,* 1983; Nychas *et al.,* 1998 and Greer, 1989). pH is an important determinant of microbial growth. The production of organic acids is undoubtedly the determining factor on which the shelf life and safety of the final product depends (Ammor and Mayo, 2007).

Decreasing pH pattern with extending storage period was observed in inoculated samples of beef, chicken and turkey under freezing and fast freezing temperature. Similar reports were recorded by Brewer *et al.*, 1992; Lin and Lin,

2002 on fresh pork sausages, low fat Chinese-style sausages and other sausages. Increase in pH was recorded in control samples. Increase pH is attributed to increase in the total negative charges leading to rise in pH of meat (Kim and Slavik, 1996). Consequently increasing the ionic strength (IS). Statistically, non significant pH increase in beef and turkey control samples on day 7 may have been caused by conversion of lactic acid to $C0_2$ and water (Jezek and Buchtova, 2007). Balamatsia *et al.* (2007) reported that the pH of fresh chicken muscle was 6.32.

Water holding capacity (WHC) under fast freezing temperature was observed to decrease with prolonged storage while control samples increased with increase in storage period. At freezing temperature similar trend was observed with fast freezing but higher values were recorded for samples inoculated with *Pediococcus acidilactici*. Devatkal and Mendiratta (2004) concluded that water holding capacity of different tissues depend on the percentage of low ionic soluble, high ionic soluble and insoluble protein fractions and is a function of total soluble proteins. Freezing produce some changes in the tissue, which reduces the water holding capacity after thawing (Sanguinetti *et al.,* 1985; Devatkal and Mendiratta, 2004). Loss of water holding capacity in prolonged storage of meats may be due to the rate in postmortem pH falls; ice crystal fermentation, high ionic strength, protein denaturation, drip loss and above all, the bulk of meat storage and the capacity of the chilling / freezer facilities (Lawrie, 1998; Kandeepan and Biswasi, 2007). High pH combined with connective tissue proteins might be responsible for higher water holding capacity (Venegas *et al*., 1988). Lower water holding capacity was attributed to higher content of sarcoplasmic and stroma proteins than myofibrillar proteins (Swift and Berman 1959; Venegas *et al.,* 1988; Ramirez *et al.,* 1995; Devatkal *et al.,* 2004).

Limited information is available on production of Total Volatile Base Nitrogen (TVB-N) in beef, chicken and turkey under low temperature. TVB-N could be used as a quality indicator for meat (Ageena, 2001) and in association with the

amino acid decarboxylase activity of microorganism during storage (Ageena, 2001). TVB-N levels were monitored as the main parameter of freshness. TVB-N are produced by decomposition of proteins into simpler substances (ammonia, trimethylamine, creatine, purine bases, free amino acids (Scherer *et al.,* 2006; Jezek and Buchtova, 2007). TVB-N is a product of bacterial spoilage and often used as a chemical index to assess quality and shelf life of seafood products (Connell, 1990). Because ammonia production increases due to the deamination of amino acids during spoilage. TVB-N has proposed an index of fresh meat quality and maximum acceptability limit values between 20 and 30 mgN/100 g have been suggested for beef and pork respectively (Singhal *et al.*, 1997; Byun *et al*., 2003; Balamsia et al., 2006).

In this study, both inoculated and control samples (beef, chicken and turkey) did not exceed TVB-N acceptability limit. They increased gradually in TVB-N in meat over storage period, which could be due to changes and proteolysis in myofibrillar protein during storage; it could be due to increased accumulation of free nitrogen groups that might lead to higher TVB-N value (Ageena, 2001). TVB-N is primarily a consequence of the enzyme decarboxylation of specific amino acids due to microbial enzyme activity (Halasz *et al.*, 1994; Bardocz, 1995; Ruiz-Capillas and Jimenez-Colmenero, 2004).

It is important to investigate the effect of freezing and fast freezing because free fatty acids (FFA) are major contributor to various aspects of meat quality and are central to the nutritional value of meat (Wood *et al.,* 2008). Free fatty acid values were observed to increase in control samples under freezing temperature. Increase in FFA could be due to citrate metabolism by some species of lactic acid bacteria, thus enhancing FFA production (Brewer *et al*., 1992). Increase in FFA in this study agrees with the work of (Dominguez Fernandez and Zumalacegui- Rodriguez 1991; Montel *et al.*, 1993; Onilude *et al*., 2002). Increase could be due to the action of lipolytic enzymes (Lipase and phospholipase) on lipid leading to increased FFA which contribute positively to the generation of undesirable aroma as well as flavor. The course of hydrolytic

processes in muscle/meat was monitored on the basis of FFA levels determination (Jezek and Buchtova, 2007). Low values of free fatty acid were recorded by beef and chicken inoculated samples in this work, while increase in FFA values was observed in control samples under fast freezing temperature**.** **S**imilar results were reported by Muller *et al.,* 1991 and Kim *et al.,* 1998, on frozen stored beef and structured beef steak.

Thiobabituric acid analysis (TBA) is a measure of malonaldehyde, one of the degradation products of lipid hydro peroxides formed through oxidation of unsaturated fatty acids (Nawar, 1996). There was a trend towards decrease in TBA value of inoculated samples against increase in TBA values of control samples. TBA value is often used as an index of lipid oxidation in meat products during storage. Tarladgis *et al.,* 1960 found that the TBA number at which rancid odour was first perceived was between 0.5 and 1.0. This threshold has served as a guide for interpreting TBA test results for 28 days of storage. In this study, both control and treated samples showed TBA values less than 0.5mg malonaldehyde/kg meat. Brewer *et al.,* 1992 and Celik, 1995 showed that TBA value in raw beef was 0.22mg malonaldehyde/kg. Meat increased to 0.91mg malonaldehyde/kg after 15days of low temperature storage. It was probably due to lipid oxidation resulting from action of lipase or phospholipase (Raharjo *et al*., 1992). There is an important parameter that influences the quality and acceptability of meat (Cheath and Ledward, 1996; Ma *et al*., 2007). TBA value is an indication of rancidity (Brewer *et al*., 1992) that is, the lesser the amount, the less rancidity. This is in agreement with research findings of Onilude *et al*.,(2002) who reported increase in the TBA values of Tsire samples. White *et al*., 1970; Waldman *et al*., 1974; Brewer et al., 1992 observed a gradual increase in the TBA values of beef sausages at 24h intervals during storage.

Most work performed on the changes of microbial associations in meat during storage have focused on relationship between viable counts of spoilage-related microbial groups (Ercolini *et al.,* 2006). However, this study further

investigated microbial groups and physiochemical changes during storage of meat under freezing and fast freezing temperature at 7 -day interval for 28days; to evaluate the microbiological acceptability and effectiveness of preservation methods, total viable count, enterococcal count and fungal count of samples. For all inoculated samples, the counts were well below 6logcfu/g, which is considered as the critical for meat spoilage (Mbugua and Karuri, 1994). The value of 7logcfu/g is considered as upper acceptability limit for fresh poultry meat as defined by ICMSF (1986). Ismail *et al*. (2000) reported mean TVC count of 3.32 – 5.77 logcfu/g for various raw and processed chicken products. The correlation between microbial growth and chemical changes has been recognized as a means of revealing indicators that may be useful for quantifying as well as the degree of spoilage of animal and muscle foods (Jay, 1986; Koutsoumanis and Nychas, 1999; Ellis and Goodcare, 2001; Nychas and Skandamis, 2006; Olafsdottir *et al*., 2006). TVC count of 7-8 log cfu/g has been used to mark the end of microbiological shelf-life of fresh poultry (Senter *et al.*, 2000) upper acceptability limit.

Few studies Nychas *et al.*, (1998); Byun *et al.,* (2003) Rokka *et al.,* (2004) and Balamatsia *et al.,* (2006) have been published on the use of chemical indicators for predicting the microbial quality of meat (including fresh poultry and products) in contrast to wealth of information that is available for various fish species (Connell, 1990). LAB counts determined were increasing progressively with storage time attaining highest and final count of 4.87cfu/g and 3.39 cfu/g at fast freezing and freezing respectively. LAB Strains with fast rates at low temperature have potential as protective cultures; this indicates greater competiveness for nutrients and gives the LAB a selective advantage over slower growing competitors (Bredholt *et al.,* 1999). However, fast growth rates of LAB are most often accompanied by fast lactic acid production, which could result in a rapid and large pH decrease. The behavior exhibited by LAB was fully anticipated given the fact that LAB are facultative anaerobic and are able to grow both in the presence and absence of oxygen (Patsias *et al.,* 2008). In this study, meat samples inoculated with *P. acidilactici* isolated from stored

beef under fast freezing temperature reduced spoilage indices. Since LAB has been used to prevent / inhibit spoilage bacteria in meat (Ammor *et al.,* 2006).

Efforts have been made to replace both microbiological and sensory analyses with biochemical changes occurring in muscle that could be used to assess meat spoilage (Huis in't Veld, 1996; Mataragas *et al.,* 2007). Most of the works performed on the changes of microbial associations in meat during storage have focused on comparisons between viable counts of microorganisms. However, microbial analysis alone as a spoilage index may not be exhaustive enough to understand the actual shifts in microbial ecology of raw meat in response to different storage conditions (Ercolini et al., 2006). Sensory and microbiological analyses are most often used to evaluate the freshness or spoilage of meat and meat products. The disadvantages of sensory analysis, which is probably the most acceptable and appropriate method, is its reliance on highly trained panelists, which makes it costly and impracticable for routine analysis (Mataragas *et al.,* 2007; Nychas *et al*., 2007.

The use of chemical preservatives has fallen into disfavor with consumers, who are requesting fresh, natural food products that are mild and light, with less acid, sugar or fat (Gould, 1996). Microbiological and physico-chemical analyses are recognized as a means of circumventing meat spoilage (Dainty, 1996). It is noteworthy that, population of fungi and bacteria in fresh and processed poultry products have been determined only in few studies. Knowledge of the presence and numbers of fungi in poultry and poultry products would be useful when developing technologies to retard spoilage of poultry. In this study, low numbers of enteriobacteriaceae (<3.0 logcfu/ml) were observed in inoculated beef, chicken and turkey samples stored under fast freezing temperature throughout storage period. Control samples recorded higher values especially at freezing temperature.

CONCLUSION

The aim of this work was to screen and evaluate the efficacy of Lactic acid bacteria in combination with low temperature as well as physiological and microbiological analyses as suitable parameters of assessment of beef, chicken and turkey qualities.

The antimicrobially active metabolites produced by the isolates are stable over a wide range of temperature and pH conditions, and sensitive to a number of digestive proteases, suggesting that it might be a promising alternative to chemical preservatives in some applications. Bacteriocin – like compounds produced by *Pediococcus acidilactici* as well as their bactericidal mode of action suggests they may have application as additives within meat products from the inhibition of food borne strains / pathogens thus contributing to a more stable and safer end product.

This study has provided evidences that inoculating meat samples with *Pediococcus acidilactici* isolated from stored beef under fast freezing temperaturecould help in improving meat quality by inhibiting meatborne bacteria and producing a wide range of inhibitory compounds. *Pediococcus acidilactici* isolated from stored beef under fast freezing temperature produced many extracellular compounds which facilitate improved biochemical quality of meat, reduced microbiota and safety of frozen beef, chicken and turkey samples. Careful screening of meat LAB should take into consideration physicochemical and microbial screening of strains. This would contribute towards improving the quality and safety of meat.

In conclusion, method used for strain selection shows that *Pediococcus acidilactici* isolated from stored beef under fast freezing temperature is a potential candidate for biopreservation of meats. It could be an efficient strain to prevent meat contamination by spoilage and pathogenic bacteria. The findings of this work contribute to the knowledge of screening and identification of candidate isolate with wide range of antimicrobial abilities.

Based on the result obtained, correlation between microbial growth and chemical changes has been recognized as a means of revealing indicators that may be useful for quantifying as well as the degree of spoilage of meat samples.

RECOMMENDATION

Pediococcus acidilactici isolated from stored beef under fast freezing temperature showed fast rate of growth and greater competiveness for nutrients. It could serve as potential protective cultures, thus it gives the LAB a selective advantage over slower growing competitors. However, fast growth rates of LAB are most often accompanied by fast lactic acid production, which could result in a rapid and large pH decrease. *Pediococcus acidilactici* isolated from stored beef under fast freezing temperature has served as basis for enabling meat preservation under freezing and fast freezing storage, thus large scale and optimization of its application in meat systems is recommended.

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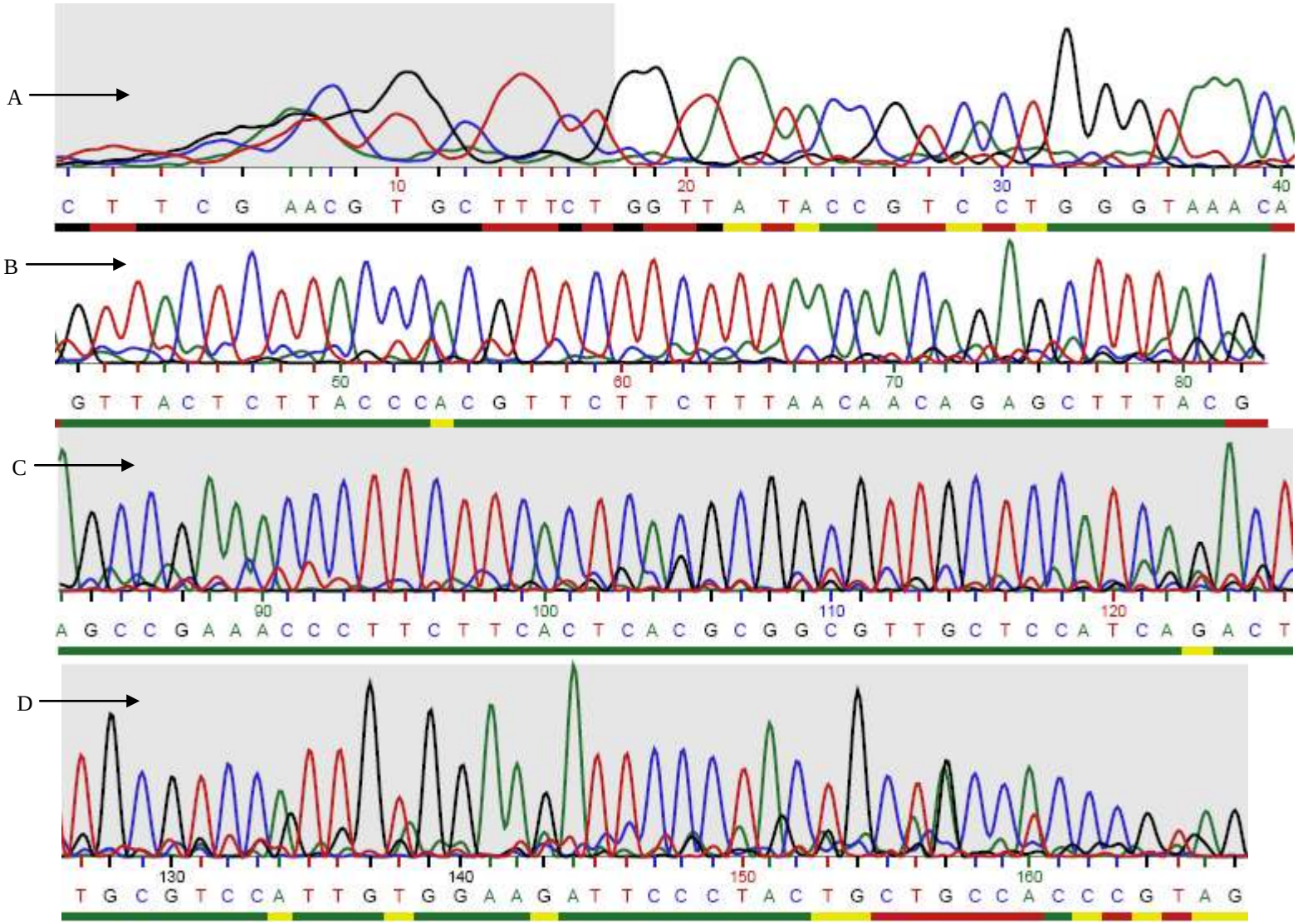
APPENDICES

Appendix 1

Composition of MRS medium (Man, Rogosa and Sharpe)

Peptone	1.0g
Yeast extract	0.5g
Lab- lemco	1.0g
Dipotassium hydrogen phosphate	0.2g
Sodium acetate	0.5g
Magnesium sulphate	0.2g
Manganese sulphate	5.0mg
Tween 80	0.1ml
Triammonium citrate	0.2g
Agar	1.5g
Distilled water	100ml

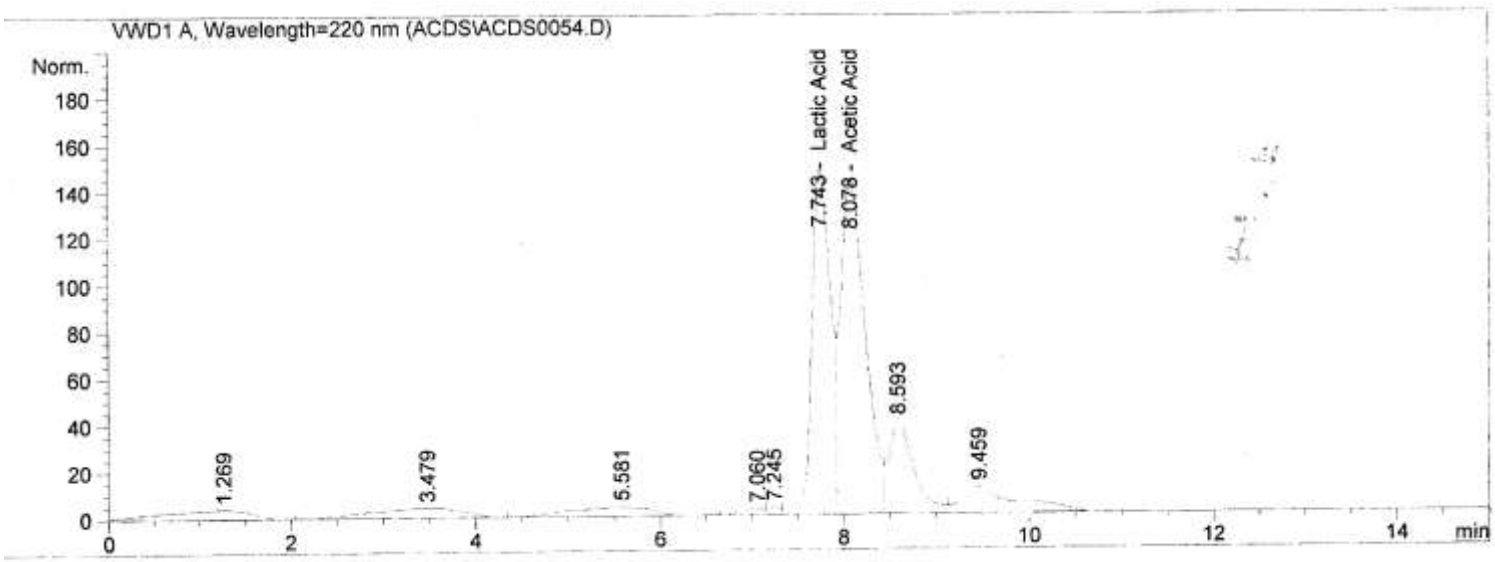


Appendix 2: Computer generated chromatograms of the nucleotide sequences of 16S rDNA gene of LAB isolate of stored beef under fast

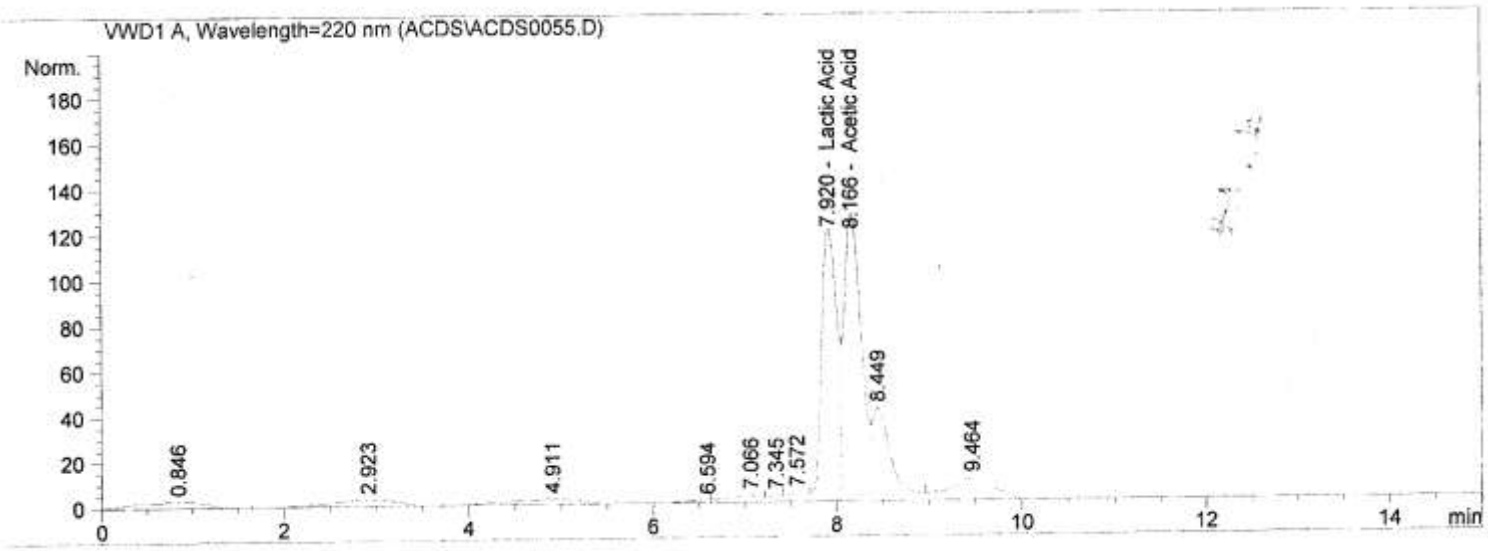
freezing temperature.

Appendix 3: Chromatogram of standard concentration of 2.5mg/l of lactic acid and acetic acid

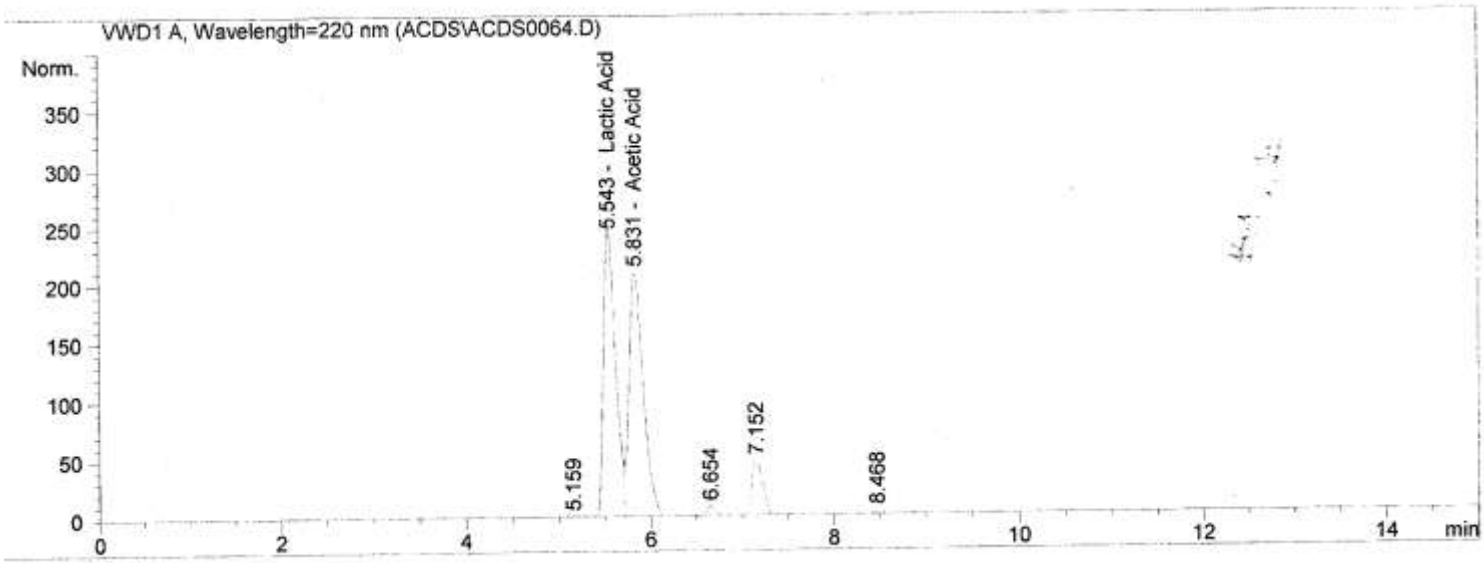
Appendix 4: Chromatogram of standard concentration of 5.0mg/l of lactic acid and acetic acid



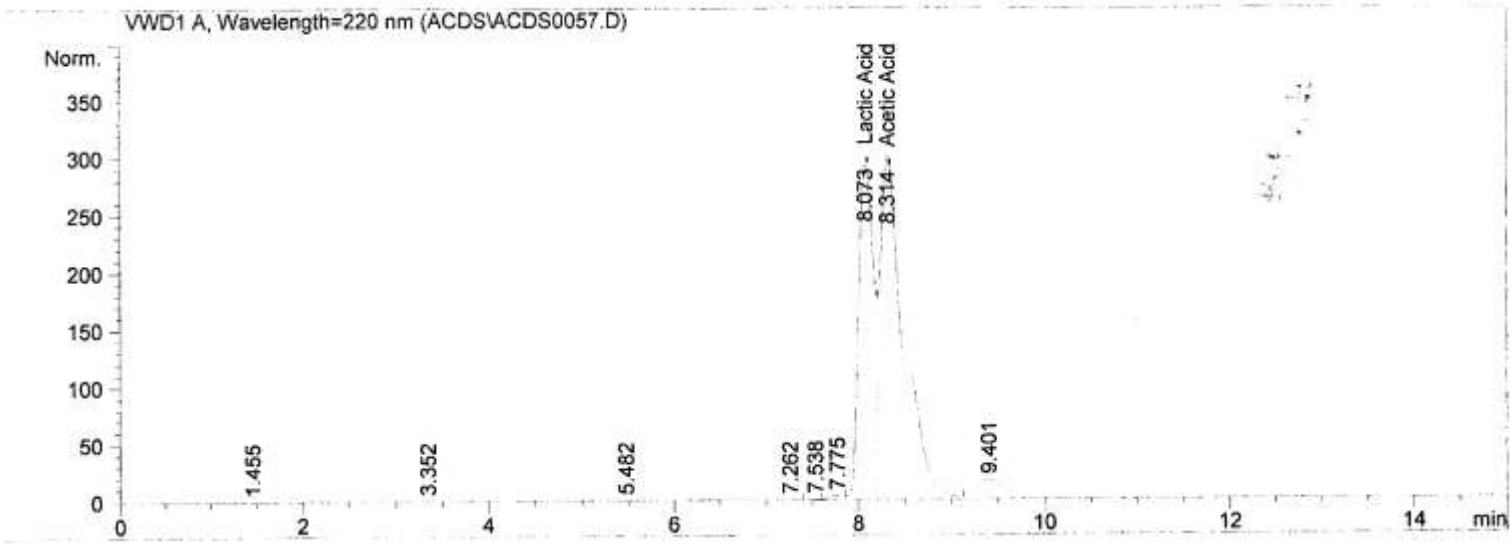
Appendix 5: Chromatogram of standard concentration of 7.5mg/l of lactic acid and acetic acid



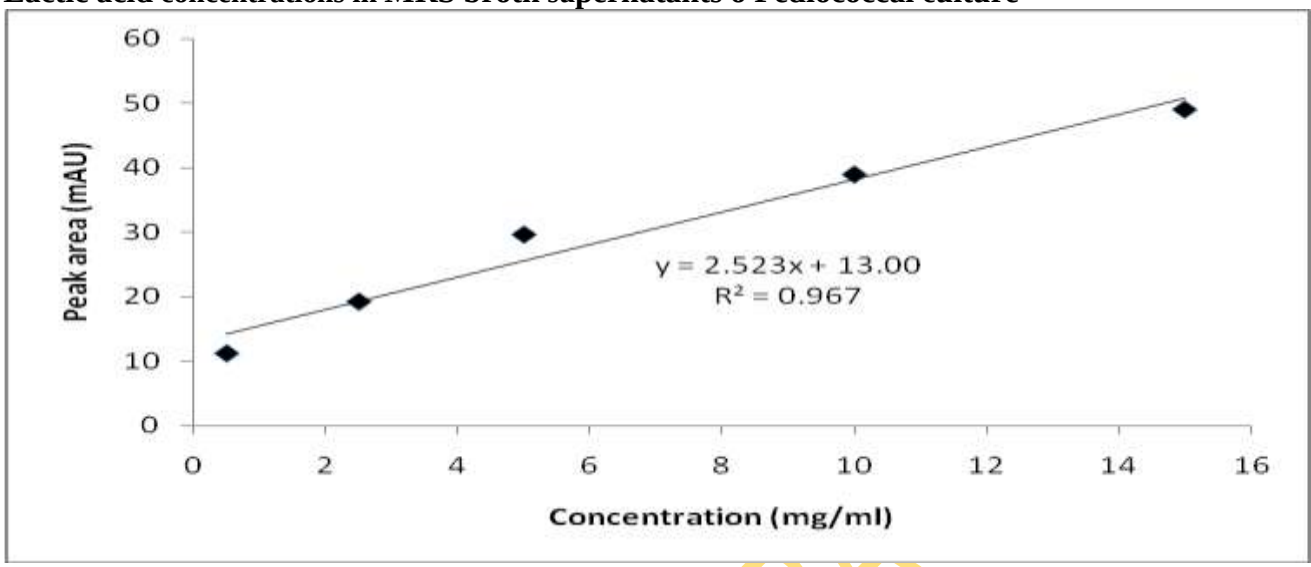
Appendix 6: Chromatogram of standard concentration of 10.0mg/l of lactic acid and acetic acid



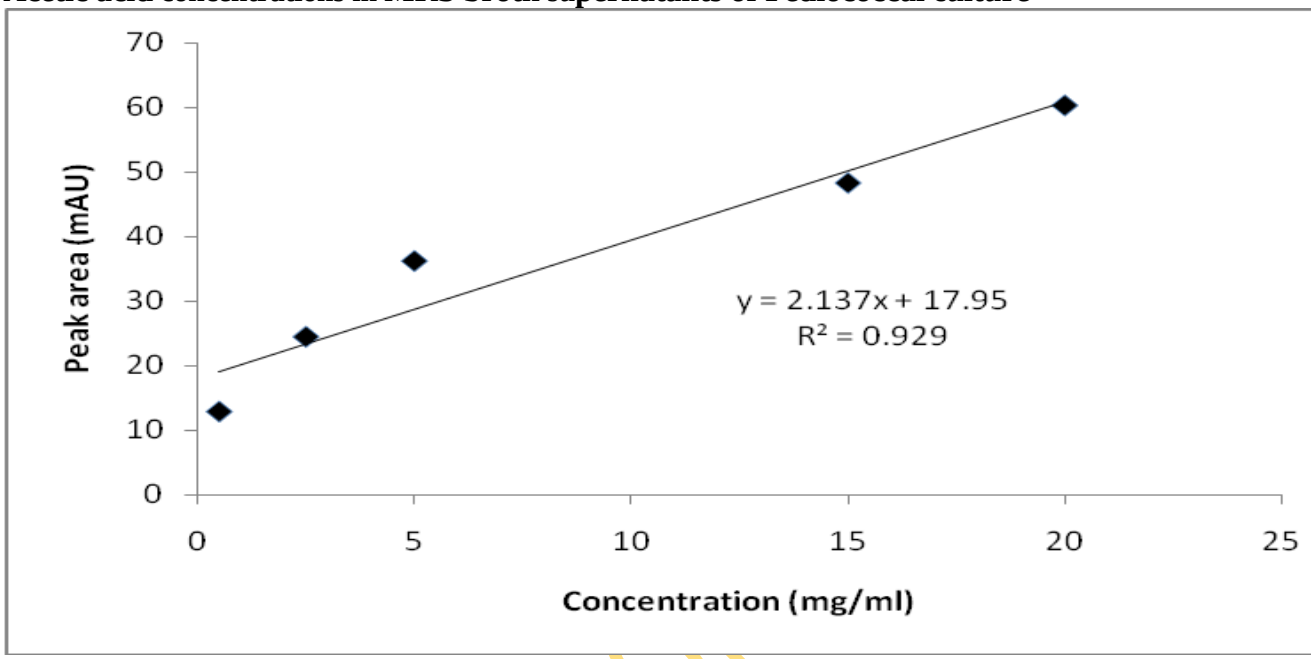
Appendix 7: Chromatogram of standard concentration of 15.0mg/l of lactic acid and acetic acid



Appendix 8: Standard Curve of Lactic acid, as obtained by HPLC, for use in estimation of Lactic acid concentrations in MRS broth supernatants o Pediococcal culture



Appendix 9: Standard Curve of Acetic acid, as obtained by HPLC, for use in estimation of Acetic acid concentrations in MRS broth supernatants of Pediococcal culture



Appendix 10: Diacetyl standard concentration