# CHAPTER ONE

# INTRODUCTION

In the Sudan, food processing, specifically meat was started only two to three decades ago. Because Sudanese people traditionally dislike eating out-doors and readymade meals. Recently social changes took place in this country. Radical changes in life patterns, economical stress, cultural changes, migration from rural areas, desertification together with general increase of population had radically changed our food consumption pattern .Consequently created high demand for processed food ,to cope with these, food industries were establish in the Sudan where large commercial quantities are usually produced. Spoilage of such quantities might be very costly to food producers.

Beef burger processing in Sudan differs in factories. Burgers after being processed are stored under low temperature (-20$^{o}$C) then will be taken by retailers. This temperature causes shock to the bacteria.

The economic impact of several outbreaks involving food service establishment had been determined. .In addition food producers have a fundamental responsibility to deliver safe, whole some and nutritious product to their customers in mostpossible economical manner. Accordingly, proper microbialcontrol of the ingredients and the products is necessary to determine the sanitary quality, to ensure safety to the consumers and to reduce spoilage of the products. However, in developed countries where very large food industries are found and the products are produced nationally and internationally, food producers are more aware of food –spoilage and food- poisoning bacteria. As a result microbial control in such large industries is usually achieved by subjecting the product to one or more types of preservation treatment such as freezing, irradiation, drying or addition of chemicals such as curing salts or

antibiotics( Mohammed,1990).While in the Sudan preservation of uncooked processed food, specifically processed meat is totally dependent on freezing.

Modern attitudes on bacteriological safety of frozen foods are more rational, recognizing that food poisoning organisms can gain access to foodvia the raw material or during processing and handling and that constant attention must be given to factors controlling the possible survival and transmission of these organisms in such foods(Harris, 1963).Experimental studies have provided information which is of great importance to frozen food industries. It had been found that proportion of any contamination might be destroyed by freezing and cold storage but this process can not entirely eliminated dangersfrom pathogens which gain to food before freezing. In 1900 Macfadyenreported that, after freezing, at -190$^{o}$Ffor 29h, bacterial cultures notonly survived but also retained their biochemical and other characteristics. *Staphylococcus aureus* retained itspigment producing property and pathogens such as typhoid and also diphtheria bacilli retained their pathogenicity. Although this fact was documented by many workers, still pathogens, indicators and food spoilage bacteria usually detected in frozen food are found to be viable but physiologically injured (Busta, 1976).Bacterial injury was defined by Ray (1984) as increased sensitivity of surviving cells to deleterious agents i.e. injured cells due to their developed sensitivity will not be detected in selective media.

Thereby, for effective microbiological evaluation of these products samples should be analyzed by methods which include an initial repair phase .Ifimproved recovery method including repair phase are available, high levels of microbial indictors can be detected .Ifgreater numbers of indicators are present than had originally been thought, then greater risk also exists, that viable but injured food-borne pathogens are also present.

The count of bacterial load is not always true on burger. As *Salmonella sp* counted the bacterial load it will be affected by sudden freeze. This is why this research work was carried out using certain media for growth.

Objectives:

1-To find the true bacterial load *Salmonella sp* on burger from processed fresh beef and frozen burger

2-To use the normal selective medium for counting the *Salmonella sp* before and after shock

3-To use sodium pyruvate, vit$B_6$, andB12in recovery media for counting *Salmonella sp* before and after shock

# CHAPTER TWO

# LITERATURE REVIEW

Meat is the most important source of food proteins to the people of the Sudan. Because of its high consumption rate every effort should be made to provide clean and safe meat to the consumer. Fresh and processed beef is consumed by most Sudanese, and it is subjected to contamination at various stages of production.

Quality control include the suitability of facility, control of suppliers, safety and maintenance of production equipment including cleaning and sanitation of equipment and facilities, personal hygiene of employees, control of chemicals, pest control ,and the like (Jay,2000)

Food safety is important for consumer, food producers and inspection authorities for numerous reasons, including consumer protection, producers, risk and international trade (Van Gerwen*et al.*, 2000)

Meat and meat products (minced meat, sausage, and burger, kofta…) are highly perishable and easily spoiled and contaminated by bacteria from different sources of contamination before, during and after processing, and soon become unfit and possibly dangerous to health. Inspite of increasing consumption of these productsthere are no enough studies to assess the level of contamination with some important pathogenic bacteria which cause food poisoning.

## **2**.1. **History of burger**

The origin of hamburger is a bit hazy and unclear. This is because there is no proper documentation to give us an idea about how the fast food came into being. Still, many people have claimed that the hamburger 'patty' was first

noticed in the medieval times. Tartars (a band of Mongolian and Turkish warriors) used to place pieces of beef under their saddles. Under the weight of the rider and the saddle, the pieces used to turn tender enough to be eaten raw. Thus was born the initial beef 'patty'. A food item resembling the present-day burger, to some extent, reached America around the 19th century. The dish, called Hamburg style beef, was brought to Hamburg (Germany) from Russia in the 14th century and when the German immigrants arrived in America, they brought it along with them. With time, the raw, chopped piece of beef evolved into the 'patty sandwiched in a bun'. Thus, it can be said that America had a major role in giving the world the hamburger as we know(history-of burgerhttp://lifestyle.iloveindia.com) Beef burgerconsist of more finely minced beef (70-80%), spices, seasonings, sodium chloride, and fat (20%).Thoroughly mixed to form dough texture and pressed in small rounded metal objects to form ringlike structure of a bout (10 cm in diameter and 1 cm thick)(history-of burgerhttp://lifestyle.iloveindia.com).

## 2.2 Meat contamination

Raw materials, as well as final meat produced are exposed to high risk of microbial contamination at the time of their production, processing, storage and distribution .Chemical composition of food, properties of the outside environment and specific growth requirements determine the type of microorganisms and the course of physical and chemical reaction in the contaminated food (Laciakova*etal.*, 2004)

## 2.3 Storing uncooked meat

Meat should be stored at a low temperature to help preserve its quality and prevent the growth of pathogenic bacteria. Chilling meat to below $40^{o}$F is recommended. Meat can be chilled or frozen. In order to freeze meat, you must drop the temperature below $28^{o}$F.

### 2.3.1 Refrigerating meat

Refrigeration is a good way to store meat for short periods of time. Refrigerated meat should be stored below $35^{o}$ F and wrapped to prevent dehydration, odor absorption, and contamination of the meat and other foods in your refrigerator. You may extend the shelf life of meat by removing the retail packaging and rewrapping the meat in foil. In general, you should consume refrigerated meat within four days of purchase

Cured meat products have a longer, more stable life in the refrigerator. They can be safely stored in the refrigerator for up to 60 days(http:/ag-ansc.purdue.ed/meat-gaulity/contact. Ml.).

### 2.3.2 Early work on freezing process

During the last hundred years, many workers investigated different effects of freezing on bacteria. This included death of bacteria due to freezing, effect of repeated freezing and thawing and effect of cold –shock on bacterial cells. It also included the effect of freezing on pathogenicity, and the mechanism of freeze- injury. The role of suspending media as well as the effect of cryoprotectants on frozen cells were also studied. However, many studied the effect of cold diluents on bacterial count. They also investigated the growth of injury bacteria on selective and non –selective media as influencedbynumerous factorssuch as nutrient composition, hydrogen-ion concentration, incubation temperature and presence of selective agents or other inhibitors. Observations on the effect of freezing on bacteria were initiated in 1887, when Prudden reported that, very low temperatures were more destructive to bacteria than high freezing temperatures. In addition he observed gradual death of bacteria due to frozen storage and high mortality on repeated freezing. He also reported low resistance of old cultures *of Staphylococcusaureus to* freezing and concluded that initial killing following freezing was due to immediate killing of

more viable bacteria. In 1900 Macfadyen stated that ″pathogenic bacteria probably do not lose their virulence by freezing ″ this finding was later documented by Sorrels *et al.* (1970). In 1901, Park reported the death of *Salmonella typhi* when suspended in water and stored at-50°C for short period ranging from 3 to 130 minutes. The belief that ″ice crushing″due to formation of ice crystals as the principle cause of damage to bacterial cells. In 1902,Sedgwick and Winslow, presented an observation opposing the mechanical damage theory. These authors stated that ″death rates in chilled suspension at low temperature ,were essentially the same .″from this they concluded that, there was no mechanical crushing due to ice crystals and that destruction below the freezing point was continuous as a function of time and temperature. Such observation generated the interest among researchers to investigate the mechanism of freeze-injury of bacteria.In 1905 Smith and Swingle reported that ,the critical point at which immediate destruction took place was somewhere near ,and that repeated freezing for ten times was found to destroy *Pseudomonas sp.*completely .In 1913 Keith investigated the role of different suspending media in the protection of frozen cells. He regarded the higher mortality in water resulted from complete crystallization. Hislland and Davis (1918),and Sherman and Albus(1923),were convinced that, the crushing of extracellular ice was the major factor responsible for the death of bacteria by freezing .They stated that ,″there is no critical temperature below freezing where the germicidal effect is greatly accelerated ″.In accordance , Haines (1937-38) found that no differences in mortality occurred when bacterial suspension were rapidly frozen and thawed at temperatures of -70°Cand50°C. He believed that, his result was in opposition to the mechanical destruction theory.

### 2.3.2.1 Freezing meat

Freezing is an excellent way to store meat for longer periods of time. It allows the meat to maintain most of its physical properties, taste, texture, smell, and nutritive properties. Small amounts of nutrients (salts, proteins, peptides, amino acids, and water-soluble vitamins) will be lost as drip when the meat is thawed. Frozen meat will have a different color than fresh meat. You should learn to recognize the "normal" frozen meat color so that you can identify frozen meat which may have an unusual color and may have spoiled.

Frozen storage time is extended by proper packaging, which is sealed to prevent air and moisture exchange and protects against freezer burn (areas of severe dehydration on the surface of the meat). The table below shows that the maximum recommended length of storage for different meat products at $0^{o}F$ for the preservation of optimum quality(http:/ag-ansc.purdue.ed/meat-gaulity/contact. Ml.).

**Type of meat:** Beef Ground beef

**Freezer Storage Times:** 6 months 6 months

### 2.3.2.2 Freezing of cooked meat

Precooked meat and poultry will have a "warmed over" flavor when cooked the second time. This loss or "fresh-cooked" flavor can be minimized by vegetable or seed flour based sauces or gravies. This is because the natural anti-oxidants found in many vegetables and seeds prevent the lipid oxidation which contributes to the "warmed over" taste.

Meat is available in various forms such as fresh meats, minced meats, meat balls, sausages and hamburger. Because of differences in production and processing of these products different microbial problems are encountered in them. Any additional handling such as the preparation of individual cuts may

increase the bacterial load due to contact with contaminant equipment and utensils (Hassan, 1999)**.** Beef burgerconsisted of minced meat, salt onion, garlic, spices mixture was pressed lightly on a metal object disc of 8-10cm in diameter and 0-6cm in thickness.

**2.3.2.3 Effect of freezing on bacteria**

The effects of different methods of freezing on bacteria were studied by many workers. These studies included the effect of repeated freezing and thawing, frozen storage for various period of time ,the types of freezing (slow or fast),and the rate of death of bacteria due to freezing (rapid or slow).Freezing and thawing of *Escherichia col*i has two types of lethal effect,(I) immediate ,i.e. that occurring during freezing at -20$^{o}$C or -196$^{o}$C and immediate thawing ,and (ii) delayed ,i.e. that occurring during frozen -20$^{o}$Cstorage .On this basis , Alur and Grecz(1975) concluded that cell death of*E. coli* by freezing ,cold storage ,and thawing is due to DNA degradation and loss of its vital integrity.

The acquired freeze – freeze thaw tolerance was investigated for*LactococcusLactis.*Pretreatment of microorganism at less severe temperature to initiate cold tolerance gave *L.lactis*improved cell viability after successive freezing and thawing. The ability of cell to survive freeze –thaw was dependent on factors experienced prior to freezing. Factors affecting lactic acid bacteria survival during freeze –thaw cycles were found to be different diluents, growth phase, and different cold temperature (Lee *et al*., 1999)

Also metabolic injury was noticed by Moss and Speck (1966) when *Escherichia coli* stored -20$^{o}$C and resulted in non lethal or ″metabolic ″ injury to proportion of the surviving population. The injury was manifested as an increased nutritional requirement after freezing.Injury could not grow on minimal agar medium, but could not develop on Trypticase soy agar.The

percentage of injury survivors varied among strain, but was little affected by altering freezing.

Freezing product and its effect whether the cells are frozen on the growing media or stored in freeze and dried -state .On the other hand keeping the freeze –dried cells at-20$^{o}$C resulted in marked percentage of injury cells and this may lead to false judgment when enumeration of the actual number of the cells is attempted in a selective media .This fact ought to be given some consideration when dried food is to be subjected for bacteriological examinations (Mohammed, 1987).

Amixed inoculums of *Salmonella derby* or *Salmonella typhimurium* and *Escherichia coli* was injected into the intestinal region pacific of oysters which were then frozen by four methods. Both species of *Salmonella* proved to be highly sensitive to freezing , regardless of the freezing method, and showed a survival of 1% or less after 48 hr.*E.coli* proved less sensitive , showing a wide capricious variability of survival during the first week of storage ,with survival ranging from 10% to 30 % (Digirolamo*et al*.,2006).

Bacteria isolated from processed meat that subjected to freezing at-20$^{o}$C to study the effect of freezing on bacteria cells, consequently, freeze- injured cells manifested a) an extended lag phase b) an increased sensitivity to selective agents incorporated in selective media c) in addition, and *Staph.aureus* lost their salt tolerance (Mohammed, 1990).

Mohammed,(1987) reported that the freezing of *Staphylococcusaureus, E.coli, Clostridiumperfringens* and *Pseudomonas aeruginosa* in meat was less harmful when compared with freezing done in the laboratory media at the same temperature (-20$^{o}$C) and the same period of time (24hr).

## 2.4 Cold shocked bacteria

A bacterial cell has to react immediately when exposed to unfavorable external condition, through such responses as entering the stationary or sporulation phase. Cold shock occurs when bacteria are exposed to sudden down shifts in temperature, and is characterized by the expression of a well-defined set of proteins which include helices, nucleases and a plethora of nucleic acid binding proteins which are so called cold shock proteins (CSPs) (Wilson and Nierhaus, 2004)

The study of cold shock response is now linkedwith commercial and health implications. This study is useful in avoiding potentially disastrous situations in various industries. Understanding cold-shock response of food –borne pathogens such as *Listeria* is imperative as refrigeration is a commonly used method of food storage. Cells which are cold-shock prior to freezing, exhibit better cry tolerance,therefore, food-spoilage bacteria

### 2.4.1. Effect of cold shock on bacteria

Cold stress stabilizes the secondary conformation of nucleic acids, which inhibits DNA replication, gene transcription and translation. The activity of enzymes and the rate of metabolic process, as well as membrane fluidity of compounds. The intracellular formation of ice crystals damage sub cellular causing death (Golovlev, 2003**).**

### 2.4.2. Cold shock response

In responses to low temperature, microorganisms synthesize cold shock proteins (CSPs), which are involved in the process of protein synthesis and in RNA folding (Golovlev, 2003).

The responses of prokaryotes to low temperature stress have been extensively studied in *E.coli* and are characterized by the accumulation of cold shock

proteins (CSPs). Bacteria CSPs are small proteins that consist of single nucleic acid-binding domain, which is termed the cold shock domain (CSD). The CSD is proposed to be an ancient molecule that was present before the origin of single cell life and the most evolutionary conserved nucleic acid-binding domain within prokaryotes and eukaryote.

In *Escherichia coli* ,a family of cold shock proteins (CSPs) function as transcription and antiterminors or translation enhancers at low temperature by destabilizing RNA secondary structure (Nakaminami*et al.*,2006).

### 2.4.3 Cold shock (Phoenix Phenomenon)

The effect on cold diluents of viability was first reported by Sherman and Albus (1923) who reported that95% of cells were killed within 1hrwhen the temperature of a 4 hrculture of *E.coli* was reduced suddenly from 45$^{o}$C to 10$^{o}$C, while no similar loss in viability was observed in 12 day old cultures.

A loss in viability also observed by Sherman and Cameron (1934) upon transferring *E.coli* in the lag phase from a medium at 45$^{o}$C to medium at 10$^{o}$C .*Pseudomonasaeruginosa* was shown to be sensitive to cold shock in the log phase (GorriL and McNeil, (1960). In contrast, Mackelvie*etal.* (1968) found that stationary phase cultures of *Pseudomonas aeruginosa* were susceptible to cold shock during harvesting but lag-phase cells were not. The same phenomenon i.e. cold- shock of lag phase cells was observed in *Aerobacteraerogenes* (Strange and Dark, 1962;Strange and Ness,1963) butnotin*Staphylococcusaureus* (GorriLandMcNile,1960) or in *Streptococcus hydrogenous*(Ring , 1956).Williams (1956) observed a similar fall in viable count with lag phase cells of thermoduric bacterium (Mcrococcus 9, dairy Group III B) in glucose broth at 37.5$^{o}$C,but since it never occurred when cultures were incubated at 15$^{o}$C or 25$^{o}$C, he concluded that the effect was due solely to the temperature of incubation. The more probable reason for failure to

observe the phenomenon was the small difference between the incubation temperatures of the diluents, assuming the later was at room temperature. The term (cold-shock) or the effect of sudden freezing on bacterial cells was first used by Elsanousi (1975) instead of (phoenix phenomenon) and other investigators such as Shoemaker and Pierson( 1976) also used this term.

Despite the fact that, Gram- negative organisms are more susceptible to freezing than Gram- positives, Elsanousi, (1975) reported that cold –shock phenomenon which results inlossof viability in cultures of Gram-negative bacteria, also occurred in cultures of *Clostridiumsperfringens*. Cells at different stages of growth were subjected to cold diluents for 1hr.Cells at all stages of growthwere inactivated by cold diluents. Those from the early logarithmic phase were most sensitive, viability falling by 60% over 1hr. most of which (90%) occurred in the first 15 minutes. Also the "phoenix phenomenon" was observed in *Clostridium perfringens* Hobbs, serological type 9 (HT9) in a cooked meat medium at 81.7$^{o}$C by a decrease in plate count (phase I), followed by an increase in count to the initial level (phase II) and a continued increase above the initial count (phase III).

Thisphenomenonwas reproduced in experiments with population-negative mutants derived from HT9 inoculated at various cell ages, and different assay media (sulfite-iron agar, tryptose-soytone –yeast extract agar, prereduced peptone-yeast extract agar, prereduced veal agar, and veal agar). When strict anaerobic condition were employed, it was necessary to increase the heating temperature to 52.3$^{o}$C to observe the phenomenon. The phenomenon was eliminated at 52.3$^{o}$C when a combination of strict anaerobic conditions, prereduced medial and prereduced veal diluents was employed.

The addition of nalidixic acid at the minimum point of the growth curve (end of phase I) has no effect on the appearance of phase II; however, phase III was

completely inhibited. This indicated that phase I and II were an injury-recovery process (Shoemaker and Pierson, 1976).

Several observations have been made in regard to cold shock lethality of *Clostridiumperfringens* : (i) loss of viability was not consequence of exposure of the cells to air; (ii) stationary-phase cells were much more resistant to cold shock at $4^oC$ than exponential-phase cells;(iii) at $4^oC$ 96% of an initial population of exponential-phase cells were killed upon cold shock and 95% of the remaining population was killed within 90 mins of continued exposure at $4^oC$ (iv) the minimal temperature differential for detectable cold shock lethality was between $17^oC$ and $23^oC$, and the maximum beyond which lethality was not appreciably increased was between $28^oC$ and $33^oC$ . Up to 75% of viable cold – shock cells were injury, as demonstrated by cold shocking late exponential-phase cells at $10^oC$ and using differential plating procedure for recovery.

Repair of injury was temperature dependent, and occurred in a complex medium and 0.1% peptone but not water. Nalidixic acid, chloramphenicol, and revamping did not inhibit repair of injury (Traci and Duncan, 1974).

*Campylobacter jejuni* is fastidious in terms of their temperature requirements, being unable to grow below $31^oC$, but have been found to be physiologically active at lower temperatures and to tolerate exposure to low temperatures in a strain-dependent manner. Chan *et a*l., (2001) studied nineteen field isolates of *C.jejuni* (10 of clinical and 9 of poultry origin) for their ability to prolonged exposure to low temperature ($4o^oC$). Although substantial variability was found among different strains, clinical isolates tended to be significantly more liked to remain viable following cold exposure than poultry –derived strains.

In contrast, the relative degree of tolerance of the bacteria to freezing at-$20^oC$and freeze-thawing was strain specific but independent of strain source (poultry versus clinical) and degree of cold ($4^oC$) tolerance.

Also Klein *et al*. (1999) noted that *Bacillus subtilis* has developed sophisticated mechanisms to withstand fluctuation in temperatures. Membrane fatty acids are the major determinants for a sufficiently fluid membrane state to ensure the membrans, function at all temperatures. The fatty acid profile of *B. subtilis* is characterized by a high content of branched fatty acids irrespective of the growth medium.

When Oliver (1981) conducted a study on the survival of *Vibrio vulnificus*, an estuarine human pathogen, in oyster homogenates held at $4^{o}$C. He reported a rapid and dramatic decrease in viability not attributable to either cold shock or the oyster homogenate alone but to a combination of the two. Such a decline was not observed with *Vibrio parahaemolyticus.*

Also the results showed that chilled*V.vulnificus* cells were unable to repair themselves in brain heart infusion broth at $37^{o}$C. *V.vulnificus* cells incubated on whole raw oysters at $0.5^{0}$C also exhibited a decline in viability, but of a lesser degree.

When *Campylobacter jejuni* culture that had been grown in broth at $39^{o}$C were subcultured into fresh medium at $30^{o}$C, there was a transient period of growth followed by a decline in viable-cells numbers before growth resumed once more (Vivian,2008).

When lactic acid bacterial culture were frozen at $-20^{o}$C for 24 hr, the cells viability decreased drastically, but when they were shocked at $10^{o}$ C for 2 hr prior to freezing viability improved significantly for the *Lactococcus Lactic* subsp. Lactic strains (25-37%) and *Pediococcuspentosaceus* $PO_2$ (18%), but not for the *Lactococcuslactis* subsp. *cremories* strains tested or for one strain of *lactobacillushelvetius* LBI and *Streptococcus thermophilus* TS2. (Kim and Dunn, 1997).

### 2.4.4. Cold shock genes

Various species and genera of bacteria show the same responses to low temperatures. Cold induces cold shock genes, by causing significant changes in the regulation of protein synthesis. The synthesis of major proteins in the microbial cells suppressed. However, there is synthesis of a great deal of new proteins, the so-called cold shock ones. The chief proteins in this family are*E. Coli*CspA that activates the translation of other cold shock genes and negatively regulates the expression of its own gene. *E.coli*CspA homologies were identified in many bacteria. They *can*alsobe identified in other microorganisms, including the pathogens of infectious diseases. This can be attributable to the presence of common antigens in different bacteria (Basnakak'ia, 2001).

Responses of bacteria cells to low-temperature stress are basically of two types. One type involves the cold-incused desideration of fatty acids in membrane lipids such that the membranes become more fluid to compensate for the decrease in membrane fluidity that would otherwise occur at the temperature. The other type involves the low-temperature-induced synthesis of enzymes that enhance the efficiency of these processes at low temperature. Both types of responses serve to protect the cyan bacteria cells from the detrimental effects of low-temperature stress (Los *et al*., 2000).

Also Yamanaka and Inouye (2001) found the synthesis of CspA, the major cold-shock protein of *Escherichia coli*, is dramatically induced of CspA under no stress condition, and it is thus claimed that CspA as the cold- shock proteins is a misnomer. They examined and confirmed that CspA is induced upon culture dilution at 37º C. However, its indication level is one-sixth of the cold-shock-induced level, clearly indicating that the major stress that induces CspA is cold shock.

It was fatherly found that CspA induction can be achieved not only by culture dilution but also by the simple addition of nutrients, and that it was almost completely abolished in the presence of rifampicin or nalidixic acid. Nutritional up shift causes the induction of only CspA but not other cold shock -inducible CspA homologues. The amount of CspA mRNA rabidly and transiently increased by culture dilution, but its stability was not significantly changed. These results suggest that CspA is a nutritional-up shift stress protein as well as a cold –shock stress protein, and that CspA induction following nutritional up shift may be due to transcriptional activation(Yamanaka and Inouye ,2001).

In *Escherichia coli*, the CspA family includes nine homologous proteins, CspA to CspI. Four of these proteins are transiently and significantly induced upon temperature downshift from 37$^{o}$C to 15$^{o}$C (hence the name Csp, for cold shock protein).

None of the CspA homologues appears to be singularly responsible for cold-shock adaptation, since individual Csp genes are dispensable at both normal and low growth temperatures. However, while all single, double or triple deletion mutants of Csp genes grow at low temperature, a quadruple deletion strain (CspA,CspB ,CspG ,CspE) is cold-sensitive for growth, indicating that the presence of at least one of these CspA family is required to support growth at low temperature. Overproduction of any one of the nine CspA family genes, with the exception strain, indication that the functions of these proteins redundant (Phadtare and Severinov, 2005).

The food borne pathogen *Listeria monocytogenes* has many physiological adaptations that enable survival under a wide range of environmental conditions. The microbes overcome various types of stress, including the cold stress associated with low temperature in food production and storage environments. Cold stress adaptation mechanisms are therefore important

attribute of *L.monocytogenes*, enabling these food pathogens to survive and proliferate to reach minimal infectious levels on refrigerated foods.

This phenomenon is a function of many molecular adaptation mechanisms. Therefore, an improved understanding of how cold stress is sensed and adaptation measures implemented by *L. monocytogenes* may facilitate the development of better ways of controlling these photogenes in food and related environments. Research over the past few years has highlighted some of the molecular aspect of cellular mechanisms behind cold stress adaptation in *L.monocytogenes*.*Listeriamonocytogenes* possesses three small, height homologous protein members of the cold shock protein (Csp) family. Stephan and Tasara (2006) used genes expression analysis and a set of mutants with single, double, and triple deletions of the Csp genes to evaluate the roles of CspA, CspB, and CspD in the cold and osmotic (NaCl) stress adaptation responses of *L.monocytogenes* .All these Csps are dispensable for growth at optimal temperature ($37^{o}$C). The hierarchies of their functional importance differ, depending on the environmental stress conditions: CspD, CspA/CspD in response to NaCl salt osmotic stress. The fact that Csps are promoting *L.monocytogenes* adaptation against both cold and NaCl stress has significant implications or sequential exposure of *L.monocytogenes* cells to these two stresses in food environments might inadvertently endues cross-protection responses (Stephan and Tasara 2006).

**2.5 Definition of stress on bacteria**

The term stress has been used to describe the effect of sub lethal treatments, However,Hurst( 1984) considers injury to be the preferred term because, by analogy with higher organisms, its description evokes an image of “temporary and repairable physical damage. By similar analogy, the term stress carries a more subtle meaning not necessarily causing physically damage but altering

organism behavior.Current literature pertaining to microbial injury typically does not maintain this distinction, and the terms are often used interchangeably. The term stress, however, is universally used in reference to the agents or treatment causing injury. Although there is tendency to perceive food matrices as metabolically supportive environment ,food is frequently bacteriostatic or bactericidal due to intrinsic factor such as water activity (a), pH, oxidation-reaction potential,comptive exclusion by protective culture ,and other environmental and processing stresses (Arecher,1996).

Other types of stress encountered in food environments may include exposure to acids bases, bioactive antimicrobial peptide,oxidants,osmotic pressure

Differences, starvation, heating, freezing, thawing and the presence of other innate and supplemented antimicrobial compound (Miller*et al*., 2000)some emerging technologies (e.g. High hydrostat static pressure)cause sub lethal injury, although some have argued that other technology (e.g. pulsed electric filed)do not induce injury (Wuytack*et al* .,2003;Yousef and Courentey,2003).Bacterial stresses, whichgnarly fit into three categories physical,chemical,or nutritional can occur throughout the farm –to-fork continuum and to different type of bacterial cell damage.

### 2.5.1Stress reaction, stress in survival of bacteria

The concept of stress was advanced by Hans (2004), who wrote that "stress is life is stress".Human frequently encounter stressful situation, this being the reason for the interest in stress and the nature of this phenomenon. Stress can be defined as a change in the genome, or the environment producing decrease in the growth rate or survival. Stress responses are of particular importance to microorganisms, because their habitats are subject to continual change of parameters such as temperature, osmotic pressure, and substrate viability. Abreaction to stress in bacteria is remarkably similar to those observed in

higher eukaryotes. Stressor or stress may have a chemical, physical or biological nature. The organism itself may bacteria be the of stress factor. For example, many bacteria form $H_2O_2$,super oxide radicals($O_2$) and other reactive oxygen species (ROSs).Oxidative stress induces breaks in nucleic acid chains, cleaves protein molecules (after fragment may be cross-linked),and blocks the active sites of enzyme ,energy depletion is its consequence at the organism level. The anti oxidativedefence of cells includes superoxide dismutase (SOD), Catalase, peroxides which perform the reaction.

### 2.6Definition of injury

Bacterial injury may be defined simply as the effect of one or more sub lethal treatment on microorganism (Hurst, 1984). By extension, sub lethal injury is a consequence of exposure to a chemical or physical process that damages but does not kill microorganism (Russell, 1984).Yousef and Caurtneg (2003) included damages to cellular components in their description of injury, andGilbert (1984) wrote," sub lethal injury of microorganism implies damage to structures within the cells, the expression of which entails some loss of cell function that may be transient or permanent. Most intervention strategies used for the control of pathogenic and spoilage microorganisms frequently produce a continuum of sub lethal effects, and considerable proportion of microorganisms in foods likely incur some degree of sub lethal injury duringprocessing(Zhao and Dolye,2001)

### 2.6.1Injury to microorganisms

Disinfection or sterilization treatment by heating, irradiation, or chemicals can cause injury to microorganisms at sub lethal levels. Microbial injury is the inability to grow under conditions suitable for the uninjured microorganisms. This inability of injured microorganismstogrow is explained in terms of more complex or different nutritional requirements or in termsofincreased.

Sensitivity to environmental conditions such as incubation conditions (time or temperature) or to chemical agents such as halogen compounds. Injured microorganisms can be distinguished from those that are dead or mutated by their ability to regain normal physiological activity when placed in appropriate conditions for cultivation.

The return to normal physiological function has been termed repair. The extent and severity of sub lethal injury, the mechanisms of injury, and the mechanisms and degree of recovery vary with the sterilization procedures, the species, the strains, the condition of the microorganism, and the methods of repair. Injury to spore formers has been detected at different stages of the spore cycle. The sites of injury include damage to enzymes, membrane disruption, and/or damage to DNA or RNA. Information on the sub lethal injury and recovery of microorganisms is very important in evaluating sterilization/disinfection procedures. (Zhao and Dolye, 2001)

### 2.6.2. Cause of injury

There are so many cause of injury including sub lethal heat. Freezing, freeze-drying, irradiation,high hydrostatic pressure,aerosolization,dyes, sodium azide ,salts ,heavy metals, antibiotics essential oils ,sanitizing compounds ,and other chemicals or natural antimicrobial compounds (Vivian,2008).

### 2.6.3 Repair of injured cells

Hideharu (2006) defined repair as the ability of injured cells to return to normal physiological function. Janssen and Busta (1973) reported that fast freezing and slow thawing of *Salmonella* anatomy cells in nonfat milk solids resulted in about 20% death and 50% injury of the cells surviving the treatment. Death was defined as the inability to form colonies on a non-selective plating medium Xylose-lysine-peptone agar (XLB) after freezing and thawing. Injury was defined as the inability to form colonies on selective plating medium (XLP

with 0.2% sodium deoxcholate added). The injured cells repaired rabidly and within 2 hr at 25$^{o}$C, in the presence of 0.1% milk solids, all the injured cells regained the ability to form colonies on the selective medium. The treated cells showed a 1-hrextended lag phase of growth as compared to the unfrozen cells. Milk solids concentration in the freezing and repair menstrual influenced injury, repair of injury and death. The same above investigator concluded that, the repair process was affected by the pH and temperature of environment in which the injured cells were incubated. Maximum repair occurred at pH values between 6.0 and 7.4 and temperatures from 25$^{o}$C to 42$^{o}$C. The data suggested repair did not require the synthesis of protein, ribonucleic acid, or cell-wall mucopeptide but did require energy synthesis.

After *Escherichia coli* was injured by freezing, the repair process was studied during incubation of the cells for 2 hour at 25$^{o}$C in 0.5% K2HPO4 at pH 7.0 in the presence of specific metabolic inhibitors. Data indicated that the cells synthesized energy in the form of ATP also facilitated the repair of injury. The freeze-injured cells showed extreme susceptibility to surface-active agents and lyzoyme. The repair cells, like the uninjured cells, became relatively resistant to these compounds. (Ray and Speck, 1972)

**2.7 The Pathogencityof injured cells**

In (1990) McFadden stated that "pathogenic bacteria probably do not lose their virulence by freezing". Freezing at 75$^{o}$C and storage at-20$^{o}$C of a cell suspension of *Salmonellagallinarum* resulted in a heterogeneous population of dead, metabolically injured and unharmed cells. Injured cells constituted as much as 40% of those surviving freezing and storage for one day. Replica plating of frozen and thawed cells indicated metabolic injury was repairable and not stable penicillin was used to increase the ratio of injured to uninjured cells from a frozen and thawed cell suspension. Pathogen city was evaluated by

observing percent mortality after injecting injured or uninjured cells into separate sets of chicks.

Mortality differences between wholly uninjured and predominantly injured population were small and consistent (5% level) with a hypothesis of no difference (Sorrels *et al*., 1970).

As in many bacteria, including such pathogens as *Salmonella enteritidis*, entertoxic*Escherichia coli, Vibrio vulnificus*, *Vibriocholerae*, the occurrence of available but non-cultural (VBNC) state has been described for *Campylobacter jejuni*. This bacterium has been recognized as a leading foodborne pathogen. VBNC cells from three*Campylobacterjejuni* human isolates were suspended in microcosm water at 4ºC and entered the VBNC state.

The metabolic activity of these VBNC cells was monitored by CTC reduction. Once in VBNC state, the strains were inoculated per so into newborn mice and 1-day-old chicks so as to evaluate and compare these animal models of VBNC cells recovery.

The three strains used were revived using the muring model, whereas only two strains were revived with the chick model.

The results showed that the marines' model permits better recovery than the 1-day chick model. All three strains revived exhibited an associative index very similar to that measured in the culturable state. This study indicates that the VBNC state should be considered as role in the epidemiology of *Campylobacter* infection (Cappelier*et al*.,1999).

### 2.7. 1*Escherichia coli*

Since1885, when it was first isolated from children's feces and described by the German bacteriologist Theodor Escherichia, scientific attention has been lavished on *Escherichia coli* to such an extent that it is today probably the best

understood free-living organisms. Strains of *E.coli* were first recognized as a cause of gastroenteritis by workers in England investigating summer diarrhea in infants in the early 1940s. Until 1982, strains producing diarrhea were classified to three types based on their virulence properties, enter pathogenic*E.coli* (EPEC), and enteor- invasive*E.coli* (EIEC), enterotoxogenicE.*coli* (ETEC). They are not very common causes of food borne illness in developed countries, but an important cause of childhood diarrhea in less developed countries. ETEC is also frequently associated with so called traveler's diarrhea. However since 1982 enterohemorrhagic*E.Coli* (EHEC) particularly associated with serotypes 0157:H7 has been recognized as the cause of a number of out breaks of hemorrhagic colitis and hemolytic uremic syndrome, particularly in North America where food such as under-cooked ground meat, raw milk and fresh product have been implicated. (Adams and Moss, 2008).

### 2.7.2. *Salmonella sp.*

Adams and Moss (2008) also recorded that most Salmonellae are regarded as human pathogens, though they differ in the characteristic and the severity of the illness they cause. Typhoid fever is the most severe and consequently was the earliest infection to be reliably described as a zoonotic infection since the major source of human illness is infected animals. Transmission is by the fecal-oral route, where by intestinal contents from an infected animal are ingested with food or water. A period of temperature abuse which allows the *Salmonella* to grow in the food and an adequate or absent final heat treatments is common factors contributing to outbreaks. Meats, milk, poultry and eggs are primary vehicles, they may be undercooked, allowing the salmonellas to survive or they may cross-contaminated other food that are consumed without further cooking. Human carriers are, generally, less important in the transmission of salmonellosis.

### 2.7. 3. *Staphylococcus aureus*

They were first described by the Scottish surgeon, Sir Alexander as cause of a number phylogenic (pus forming) infections in human. The presence of small number of staph. aureus in food are not uncommon. It will occur naturally in poultry and other raw meats as frequent competent of the skin flora, There, however, been out breaks caused by milk products such as dried milk and chocolate milk where growth and enterotoxin productions occurred in the raw milk and the entertoxin, but not the organism, survive subsequent pasteuralization. Contamination by food handlers is also probably a frequent occurrence in view of high rate of human carriage (Adams andMoss, 2008).

### 2.8 The effect of selective media on recovery of injured bacterial cells

When Moriachi and Irie (2004) investigated conditions facilitating recovery of sub lethally injured cells present in frozen or freeze-dried preparation of bacteria, they noted that some cells of *Streptococcus faecalis* injured by freezing required RNA resynthesis for recovery, whereas the rest did not. Addition of 6% NaCl to control recovery medium was found to inhibit RNA synthesis completely in frozen-injured cells of *Streptococcus faecalis*, but not in actones. This seems to be a principle reason why 6% NaCl inhibits thegrow of the injured cells.

Thesame investigators observed that the recovery of viable cells of freeze-dried and stored *Streptococcus thermophilus* was greatly increased by the addition of cytosine to the plating medium. The favorable effect of cytosine was mainly due to its reducing activity. On the contrary, the addition to the plating medium of certain types of peptone, such as lactalbuminhydrolyzate, resulted in a considerable decrease of viability in frozen preparations of *Vibrio metschnikovii*.

Ray and Speck (1972) recorded that freezing an aqueous suspension of *Escherichia coli* NCSM at-78º C for 10 min, followed by thawing in water at 80ºC for 30 min, resulted in the death of approximately 50% of the cells, as determinate by their inability to form colonies on trypticase soy agar containing 0.3%yeast extracts (TSYA). Among the survivors, more than 90% of the cells were injured, as they failed to form colonies on TSYA containing 0.1% deoxcholateXLD.

They also concluded that injury was reversible as the injured cells repaired in many suitable media. The rate of repair was rapid and maximum in a complex nutrient medium such as trypticase soy broth supplemented with yeast extract.

### 2.8.1 Pyridoxine

A new factor which could cure dermatitis in rats was reported in 1935 and was named vitamin B6. The active material was isolated in 1939 and pyridoxine was subsequently synthesized. First quantitative requirements for fish were described in 1944. Pyridoxine deficiency has since been reported in troutin salmon.

### 2.8.2 Vitamin B12

The ant pernicious anemia factor found to be contained in liver was isolated and crystallized in the mid forties. This substance, named vitamin B12 by its discoverers, was later to be recognized as essential for growth of chicken fed diets entirely of plant origin and was designated as animal protein factor (APF). When anemic salmon were injected with crystalline B12 in combination with folic acid and xanthopterin positive haemopoiesisoccurred within a few days, and the salmon showed rapid recovery from the anemia.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Materials

Fifty samples were collected from the final product of frozen burger from different factories in Khartoum State (Khartoum, Omdurman, Khartoum North).The samples were then analyzed within twelve hours.

### 3.1.1 Recovery media

The following media were used for growth and recovery of injured cells.

XLD Agar consisted of different concentrations (0.5% 1%and 1.5%) of sodium pyruvate.

XLD Agar consisted of different concentrations (1ml/ 2ml/ 3ml/L) of vitamin $B_{12}$

XLD Agar consisted of different concentrations (1 ml / 2ml / 3ml/L) ofvitamin$B_6$

## 3.2*Salmonella* method

Twenty-five grams of the burger, were added to225ml buffer peptone water and mixedwith stomacher bag for 2 min, then incubated at 37$^{o}$C for24hr. Ten ml of culture were transferred to tubecontaining 90 ml modified semi-solid Rappaport-Vassiliadis medium (MSRV), and incubated at41.5$^{o}$C for24hr. The cultures were then plated on Xylose lysine desoxycholate (XLD) agar and incubated at 37$^{o}$C for24hr to obtain isolated colonies. Typical colonies of *Salmonella* grown on XLD agar have a black centre and light prefri. The organism was isolated according to ISO 6579 (2007).

### 3.2.1 Biochemical tests:

The selected colonies were streaked on to the surface of pre-dried nutrient agar plates and incubated at 37$^{o}$C for 24h.Pure cultures were used for confirmationISO7218 (2007)

#### 3.2.1.1TSI Agar

The agar slant surface was streaked with sterile loop and the butt was stab. Incubated at 37$^{o}$Cfor 24h. Interpret the changes in the medium as follows: Slant (red), butt (yellow) with gas formation of hydrogen sulfide (black of the agar) indicates positive result

#### 3.2.1.2 Urea agar

The urea agar slant surface was streaked by the organism and incubated at 37$^{o}$Cfor24hr and examined at intervals. Ifthereactionis positive, ureas liberateammonia, changing the colorphenol red to rose- pink. Thereactions often appear after 2hr-4h.

### 3.2.2 Recovery method

Two steps were followed for recovery media:

#### 3.2.2.1 Before shock

Different concentrations (0.5%, 1% and 1.5%) of sodium pyruvate, were added to XLD agar medium sterilized and cooled at 45°C – 50°C.Then poured in sterile Petri dishes (about 15-25 ml) and allowed to solidity.

Full loop of –*Salmonella sp* suspension was prepared in test tube containing peptone water, and incubated at 37°C for 24h. 1 ml of the suspension was added to 9 ml normal saline and serial dilution was prepared $10^{-1}$, $10^{-2}$, $10^{-3}$etc….0.1 ml of each serial dilution was inoculated in XLD agar prepared with different concentrations of additives before the shock and incubated at 37$^{o}$C for 24 hrs.

### 3.2. 2.2 After shock

Serial dilutions were cold shocked for I hr. at -20$^{o}$C. 0.1 ml from each dilution was inoculated as before in XLD agar prepared with different concentration of additives ($B_6$, $B_{12}$, and sodium pyruvate) after the shock at -30$^{o}$C and -40$^{o}$C and incubated at 37$^{o}$C for 24 hrs.

# CHAPTER FOUR

# RESULTS AND DISCUSSION

I n this study 3 types of growth promoters, $vitB_6$,$vitB_{12}$ and sodium pyruvate were added to XLD selective medium to encourage the growth of *Salmonella* isolated from burger and cold shocked at -20$^{o}$C,-30$^{o}$C and -40$^{o}$C

## 4.1 Effect of vitB6 on growth of cold shocked *Salmonella*

The recovery *of Salmonella* with addition of $vitB_6$ on growth of cold shocked *Salmonella* with addition of $vitB_6$ in the medium at -20$^{o}$C,-30$^{o}$Cand -40$^{o}$Care shown in table 1, 2and3, respectively.

In Table 1, the effect of freezing on the loss of *Salmonella* at -20$^{o}$Cin XLD medium without the addition of $vitB_6$ was 4.8% and the recovery was 95.2%.With the addition of $vitB_6$ (concentration 1 ml) loss was 1.3% and recovery was 98.7%. In 2 ml, loss was 2.8% and recovery was 97.3%. In 3 ml, loss was 7.0% and recovery was 93.0%

In Table 2 at-30$^{o}$Cin XLD medium without the addition of $vitB_{6,}$ loss was 9.7% and the recovery was 90.3%. With the addition of $vitB_6$ (concentration 1 ml) loss was 4.3% and recovery was 95.8%. In 2 ml loss was 10.0% and recovery was 90.0%. In 3 ml loss was 11.01% and recovery was 89.0%.

In Table 3 at -40$^{o}$C in XLD medium without the addition of $vitB_{6,}$ loss was 26.3% and the recovery was 73.7%. With the addition of vitB6 (concentration 1 ml) loss was 28.5 % and recovery was 71.5%. In 2 ml, loss was 36.3% and recovery was 63.7% .In 3, ml loss was 22.8% and recovery was 77.2%.

**Table (1):** The effect of addition of vit$B_6$ (ml/L) on growth of *Salmonella*, before and after cold shock at -20°C

| Condition | log *Salmonella* count(cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with $B_6$concentration | | |
| | | 1ml | 2ml | 3ml |
| (A)Before Shock | 8.32 | 7.51 | 8.27 | 8.96 |
| (B)After shock | 7.92 | 7.41 | 8.04 | 8.36 |
| Loss | 4.8% | 1.3.% | 2.8% | 7.0 % |
| Recovery | 95.2% | 98.7% | 97.3% | 93.0% |

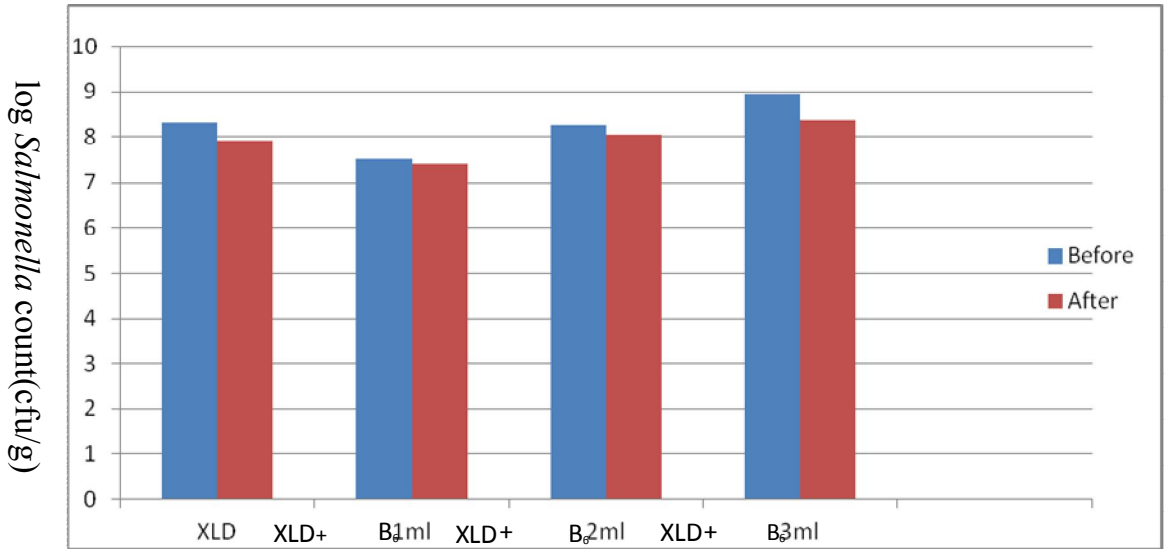

**Table (2):** The effect of addition of vit$B_6$ (ml/L) on growth of *Salmonella*, before and after cold shock at -30°C

| Condition | log *Salmonella* count(cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with $B_6$concentration | | |
| | | 1ml | 2ml | 3ml |
| (A)Before Shock | 7.94 | 7.72 | 7.83 | 7.99 |
| (B)After shock | 7.17 | 7.39 | 7.04 | 77.1 |
| Loss | 9.7% | 4.3% | 10.0% | 11.0% |
| Recovery | 90.3% | 95.8% | 90.0% | 89.0% |

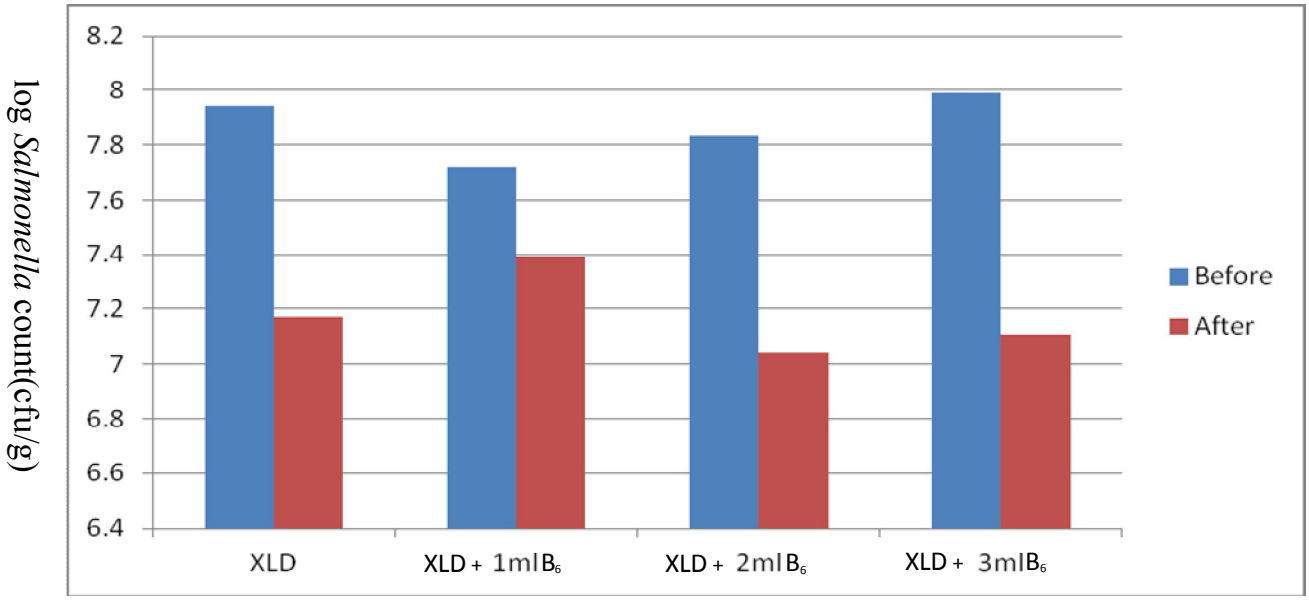


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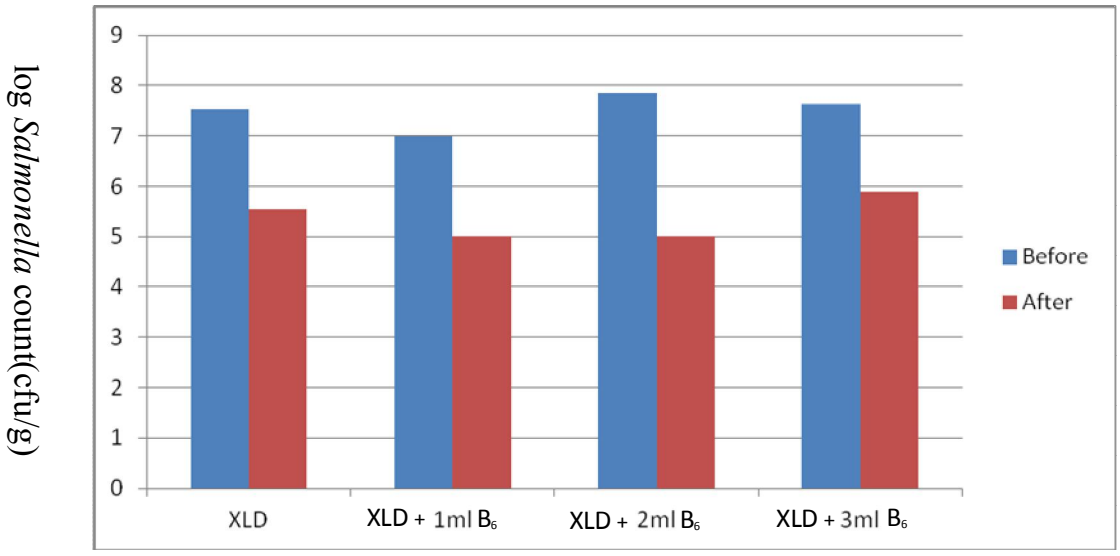
**Table (3):** The effect of addition of vit$B_6$ (ml/L) on growthof *Salmonella*, before and after cold shock at -40°C

| Condition | log *Salmonella* count(cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with $B_6$concentration | | |
| | | 1ml | 2ml | 3ml |
| (A)Before Shock | 7.51 | 7.0 | 7.86 | 7.65 |
| (B)After shock | 5.54 | 5.0 | 5.0 | 5.90 |
| Loss | 26.3% | 28.5% | 36.3 % | 22.8% |
| Recovery | 73.4% | 71.5% | 63.7% | 77.2% |



### 4.2 Effect of vit$B_{12}$ on growth of the cold shocked *Salmonella*

The recovery of *Salmonella* after addition of vit$B_{12}$ in the XLD medium at -20$^{o}$Cand 40$^{o}$Care shown in table 4, 5 and6, respectively.

In Table 4, the effect of freezing on the loss of *Salmonella* at -20$^{o}$C in XLD medium without addition of vit$B_{12}$was 0.98% and the recovery was 99.0 %. With addition of $B_{12}$ (concentration 1 ml) loss was 4.6% and recovery was 95.4%. In 2 ml, loss was 0.4% and recovery was 99.6%, In 3 ml, loss was 2.0% and recovery was 98.0%.

In Table 5 at -30$^{o}$C in XLD medium without addition of $B_{12}$ the loss was 2.5% and the recovery was 97.5 %.The loss after addition of vit$B_{12}$ (concentration 1 ml) was 3.2% and recovery was 96.8%. In 2 ml, loss was 4.8% and recovery was 95.2%. In 3 ml, loss was 5.2% and recovery was 94.8%.

In Table 6 at -40$^{o}$C in XLD medium without addition of $B_{12}$ the loss was 14.8% and the recovery was 85.2%. Loss with addition of vitB12 (concentration 1 ml) was 2.9 % and recovery was 97.1%. In 2 ml, loss was 14.6% and recovery was 85.4% .In 3ml loss was12.5% and recovery was 87.5%.

**Table (4):** The effect of addition of vit$B_{12}$ (ml/L) on growth of *Salmonella*, before and after cold shock at -20°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with $B_{12}$concentration | | |
| | | 1ml | 2ml | ml |
| (A)Before Shock | 7.14 | 7.41 | 7.14 | 7.14 |
| (B)After shock | 7.07 | 7.07 | 7.11 | 7.0 |
| Loss | 0.98% | 4.6% | 0.4% | 7.0 % |
| Recovery | 99.0% | 95.4% | 99.6% | 98.0% |

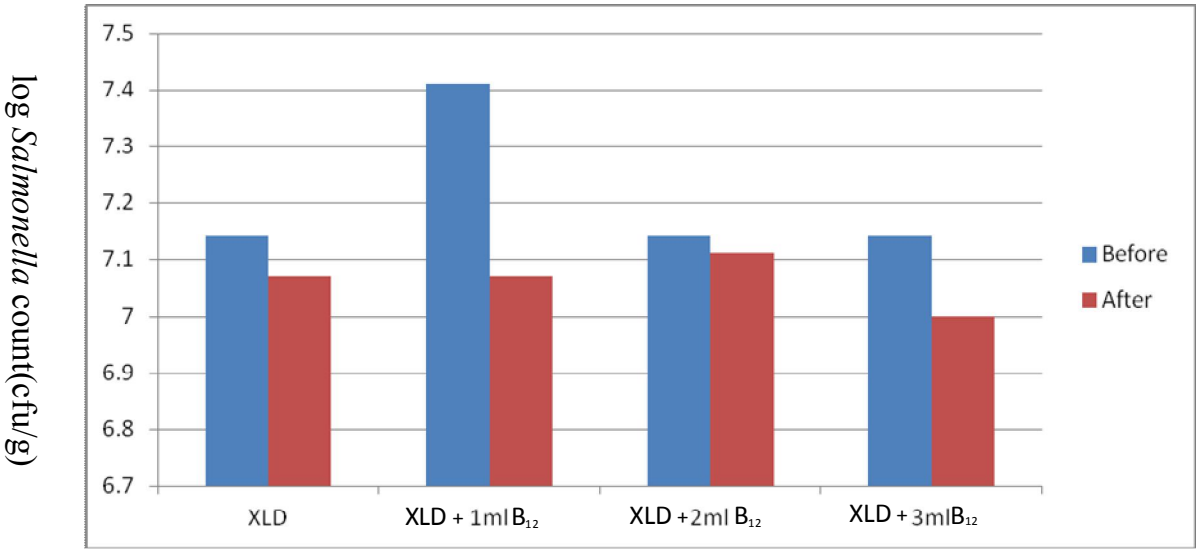


**Table (5):** The effect of addition of vit$B_{12}$ (ml/L) on growth of *Salmonella*, before and after cold shock at -30°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with $B_{12}$concentration | | |
| | | 1ml | 2ml | 3ml |
| A)Before Shock | 7.53 | 7.49 | 7.53 | 6.0 |
| (B)After shock | 7.34 | 7.25 | 7.17 | 5.69 |
| Loss | 2.5% | 3.2% | 8 .4% | 5.2% |
| Recovery | 97.5% | 96.8% | 95.2% | 94.8% |

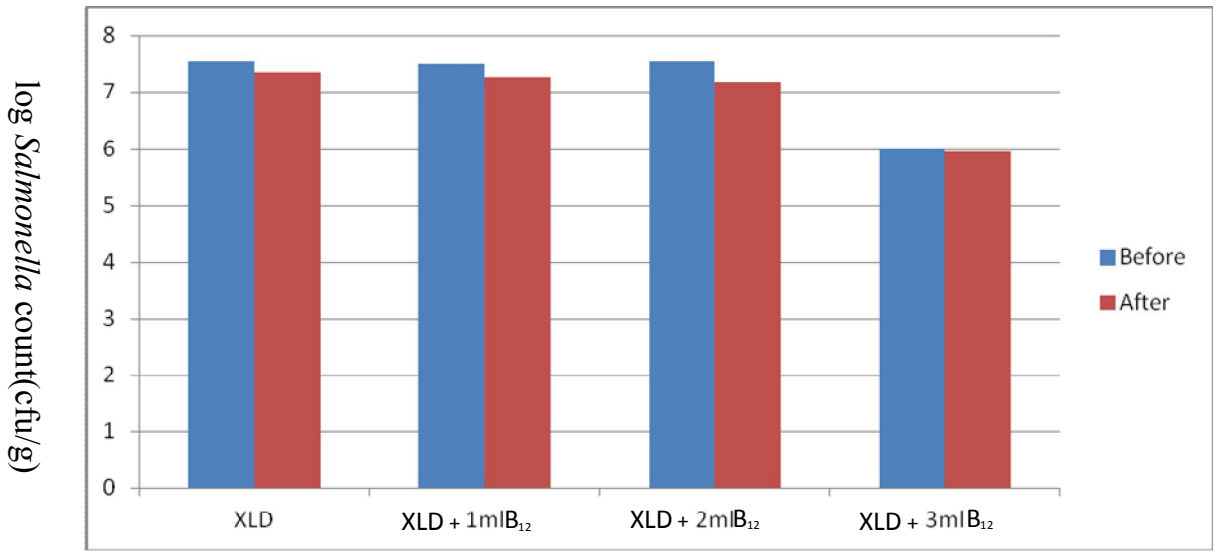
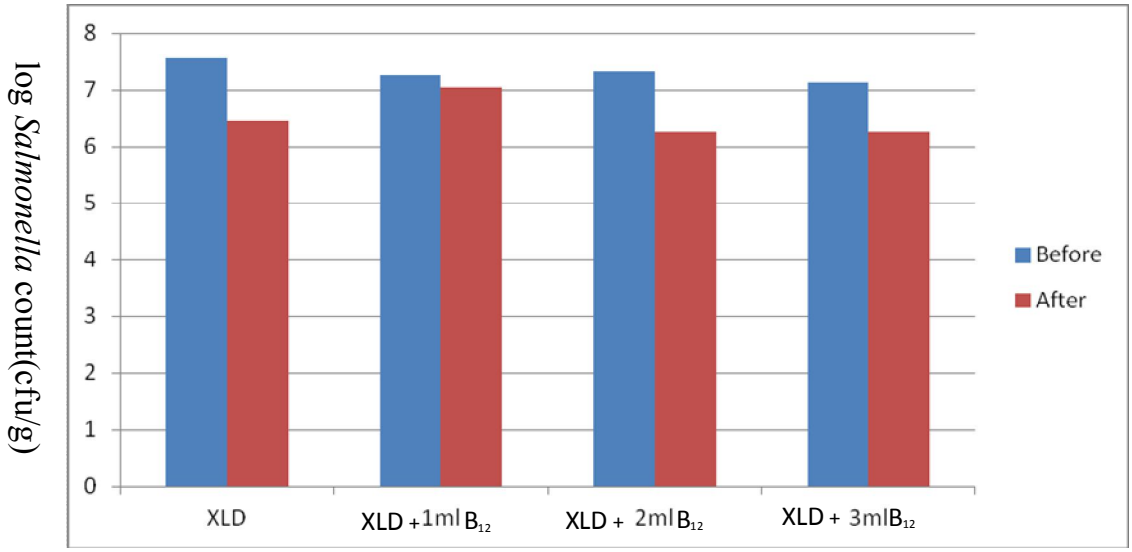


**Table (6):** The effect of addition of vit$B_{12}$ (ml/L) on growth of *Salmonellasp* inXLD medium, before and after cold shock at -40°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with $B_{12}$concentration | | |
| | | 1ml | 2ml | ml |
| (A)Before Shock | 7.56 | 7.25 | 7.32 | 7.14 |
| (B)After shock | 6.44 | 7.04 | 6.25 | 6.25 |
| Loss | 14.8% | 2.9% | 14.6% | 12.5% |
| Recovery | 85.2% | 97.1% | 85.4% | 87.5% |



### 4.3Effect of sodium pyruvate on growth of cold- shocked *Salmonella sp*

The recovery of *Salmonella sp*by addition ofsodium pyruvate (g %) in the XLD medium at -20$^{o}$C,-3$^{o}$C and -40$^{o}$Care shown in table 7, 8and 9, respectively

In Table 7 the effect of freezing on the loss of *Salmonella* at -20$^{o}$C in XLD medium without addition of sodium pyruvate was 2.0% and the recovery was 98.0%. Loss and recovery with addition of sodium pyruvate, (concentration 0.5 %) loss was 6.2% and recovery was 93.8%. In 1.0% ml loss was 6.0% and recovery was 94.0%, in 1.5% ml loss was7.0%and recovery was 92.9%.

In Table 8 at-30$^{o}$Cin XLD medium without addition of sodium pyruvate was 25.2% and the recovery was 97.8%. Loss and recovery with addition of sodium pyruvate (concentration 0.5%) loss was18.5% and recovery was 81.5%. In 1.0% ml loss was17.1% and recovery was 82.9%. In 1.5% loss was 22.5%and recovery was 77.5%.

In Table 9 at -40$^{o}$C in XLD medium without addition of sodium pyruvate was 26.77% and the recovery was73.2%. Loss and recovery with addition of sodium pyruvate (concentration 0.5 %ml) loss was 24.9% and recovery was 75.0% in 1.0% ml loss was 22.3% and recovery was 77.7%. In 1.5% ml loss was 4.5% and recovery was 95.5%.

**Table (7):** The effect of addition of sodium pyruvate (gm %) on growth of *Salmonella*, before and after cold shock at -20°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with sodium pyruvate concentration | | |
| | | 0.5% | 1.0% | 1.5% |
| (A)Before Shock | 7.91 | 7.85 | 7.94 | 8.04 |
| (B)After shock | 7.75 | 7.36 | 7.46 | 7.47 |
| Loss | 2.0% | 6.2% | 6.0% | 7.1% |
| Recovery | 98.0% | 93.8% | 94.0% | 92.9% |

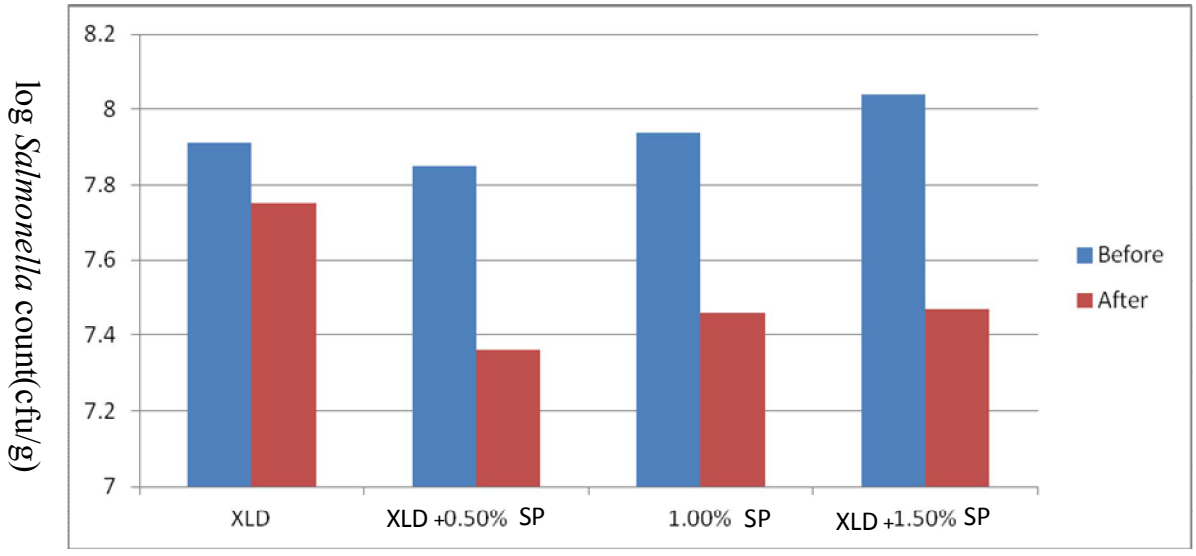


**Table (8):** The effect of addition of sodium pyruvate (gm %) on growth of *Salmonella*, before and after cold shock at -30°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with sodium pyruvate concentration | | |
| | | 0.5% | 1.0% | 1.5% |
| (A)Before Shock | 6.55 | 6.44 | 6.49 | 6.54 |
| (B)After shock | 4.90 | 5.25 | 5.38 | 5.07 |
| Loss | 25.2% | 18.5% | 17.1% | 22.5% |
| Recovery | 97.8% | 81.5% | 82.9% | 77.5% |

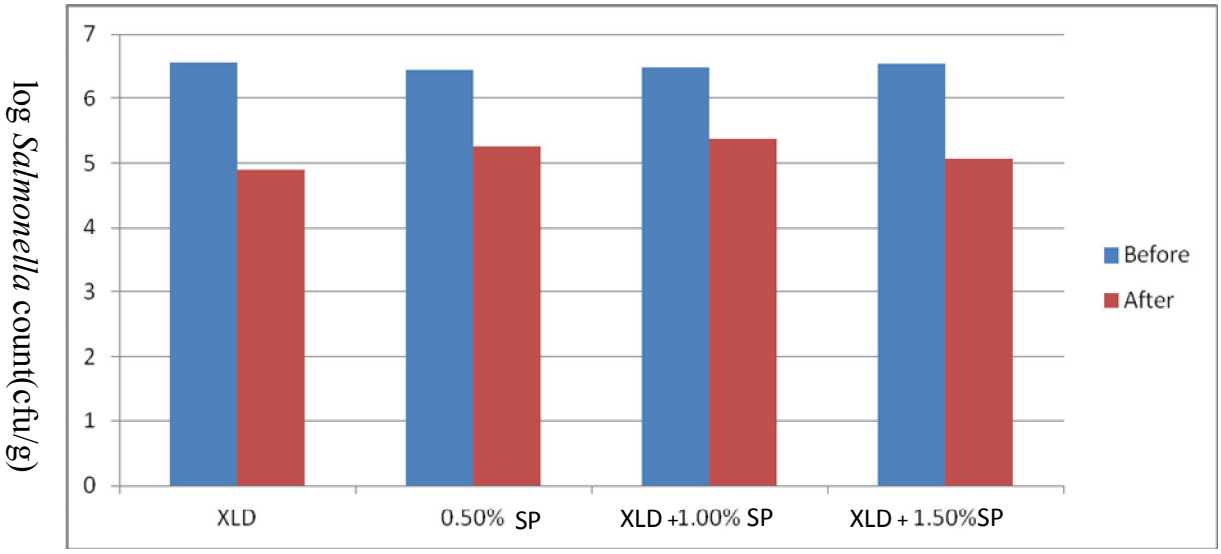
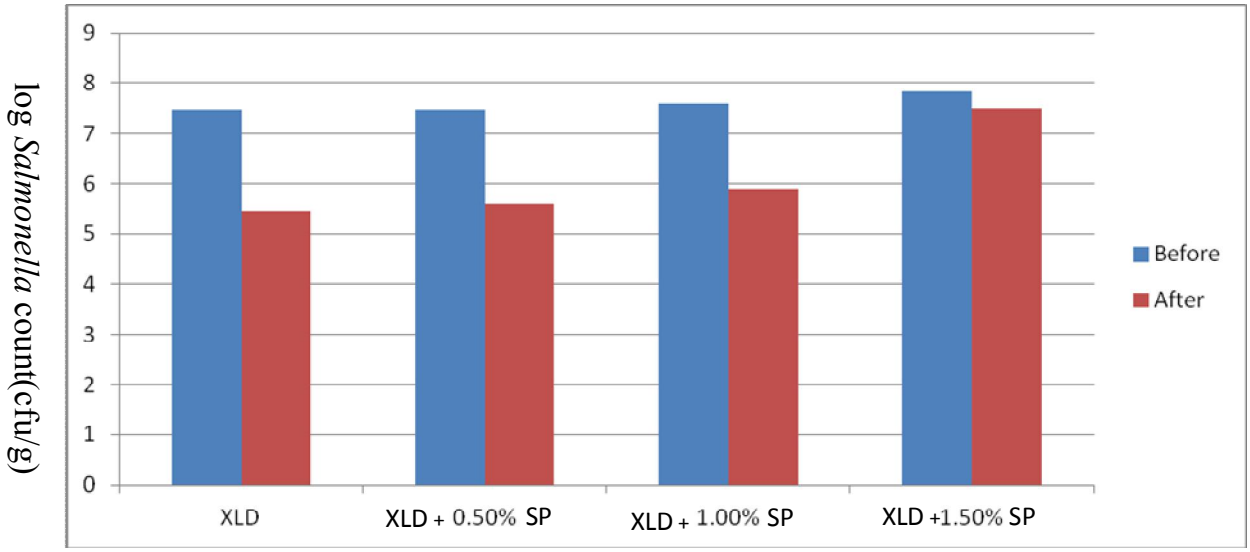


**Table (9):** The effect of addition of sodium pyruvate (gm %) on growth of *Salmonella*, before and after cold shock at -40°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with sodium pyruvate concentration | | |
| | | 0.5% | 1.0% | 1.5% |
| (A) before Shock | 7.47 | 7.46 | 7.59 | 7.84 |
| (B)after shock | 5.47 | 5.60 | 5.90 | 7.49 |
| Loss | 26.8% | 24.9% | 22.3% | 4.5% |
| Recovery | 73.2% | 75.1% | 77.7% | 95.5% |



### 4.4 Effect of sodium pyruvate and vitB$_6$ on growth of cold-shocked *Salmonella*

The recovery of *Salmonella sp* of addition of vitB$_6$ with sodium pyruvate (ml/L +gm %) in the XLD medium at -20$^o$C,-30$^o$C and -40$^o$C are shown in table 10, 11 and 12, respectively.

In Table 10 the effect of freezing on the loss of *Salmonella* at -20$^o$Cin XLD medium without addition of vitB$_6$ with sodium pyruvate was 2.1% and the recovery was 97.9%, Loss and recovery with addition of vitB6 with sodium pyruvate, concentration (1ml+0.5%) loss was 2.8%and recovery was 97.2%. In (2ml+1.0%ml) loss was 3.5%and recovery was 96.5%, in (3ml+1.5%) loss was 3.4% and recovery was 96.6%.

In Table 11 at -30$^o$C in XLD medium without addition of vitB$_6$ with sodium pyruvate was 8.0% and the recovery was 92.0%. Loss and recovery with addition of vitB6 with sodium pyruvate concentration (1ml+0.5%) loss was 4.0% and recovery was 96.0%. In (2ml+1.0%ml) loss was 4.8% and recovery was 95.2%. In (3ml+1.5%) % ml loss was 4.2 and recovery was 95.8 %.

In Table 12 at -40$^o$C in XLD medium without addition of vitB$_6$ with sodium pyruvate was 14.5% and the recovery was 85.5%. Loss and recovery with addition of vitB$_6$ with sodium pyruvate (concentration 1ml+0.5%) loss was 5.4%and recovery was 94.7%. In (2ml+1.0%ml) loss was15.1% and recovery was 84.9%. In (3ml+1.5%) % ml loss was 21.0% and recovery was79.0%.

**Table (10)**: The effect of addition of vit$B_6$ and sodium pyruvate (ml/L +gm. %) on growth of *Salmonella*, before and after cold shock at -20°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with Volume of($B_6$+SP) concentration | | |
| | | 1ml+0.5% | 2ml+1.0% | 3ml+1.5% |
| (A)Before Shock | 7.65 | 7.57 | 7.68 | 7.60 |
| (B)after shock | 7.49 | 7.36 | 7.41 | 7.34 |
| Loss | 2.9% | 2.8% | 3.5% | 3.4% |
| Recovery | 97.9% | 97.2% | 96.5% | 96.6% |

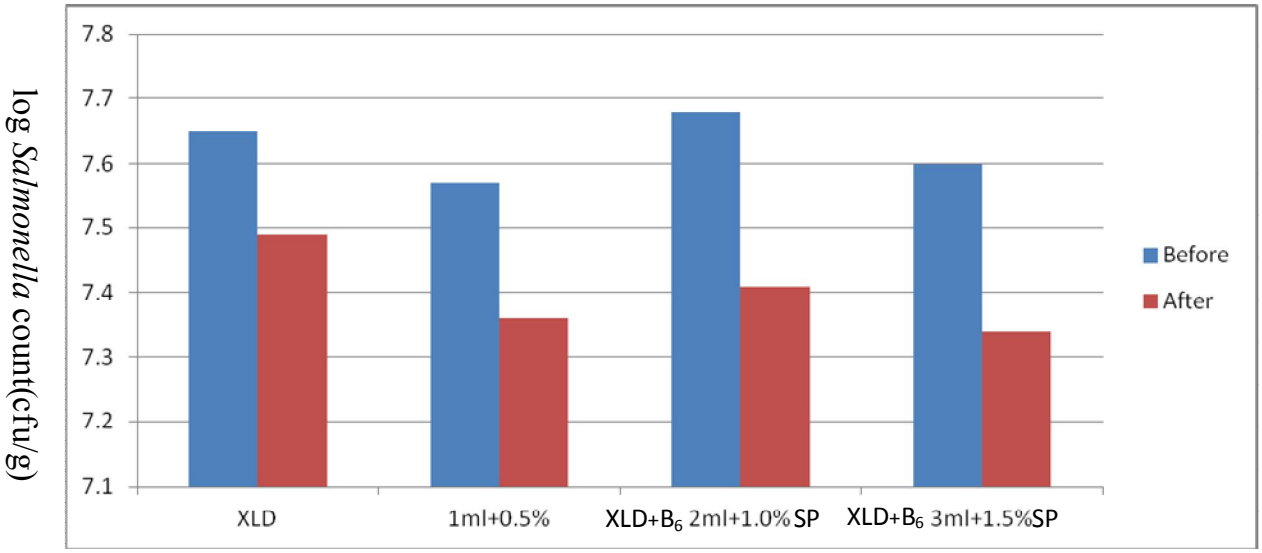


**Table (11):** The effect of addition of vit$B_6$ and sodium pyruvate (ml/L+ gm. %) on growth of *Salmonella*, before and after cold shock at -30°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | Bacterial count(cfu/g) on XLD medium | XLD with Volume of($B_6$+SP) concentration | | |
| | | 1ml+0.5% | 2ml+1.0% | 3ml+1.5% |
| (A)Before Shock | 7.90 | 7.80 | 7.89 | 7.83 |
| (B)After shock | 7.27 | 7.49 | 7.51 | 7.50 |
| Loss | 8.0% | 4.0% | 4.8% | 4.2% |
| Recovery | 92.0% | 96.0% | 95.2% | 95.8% |

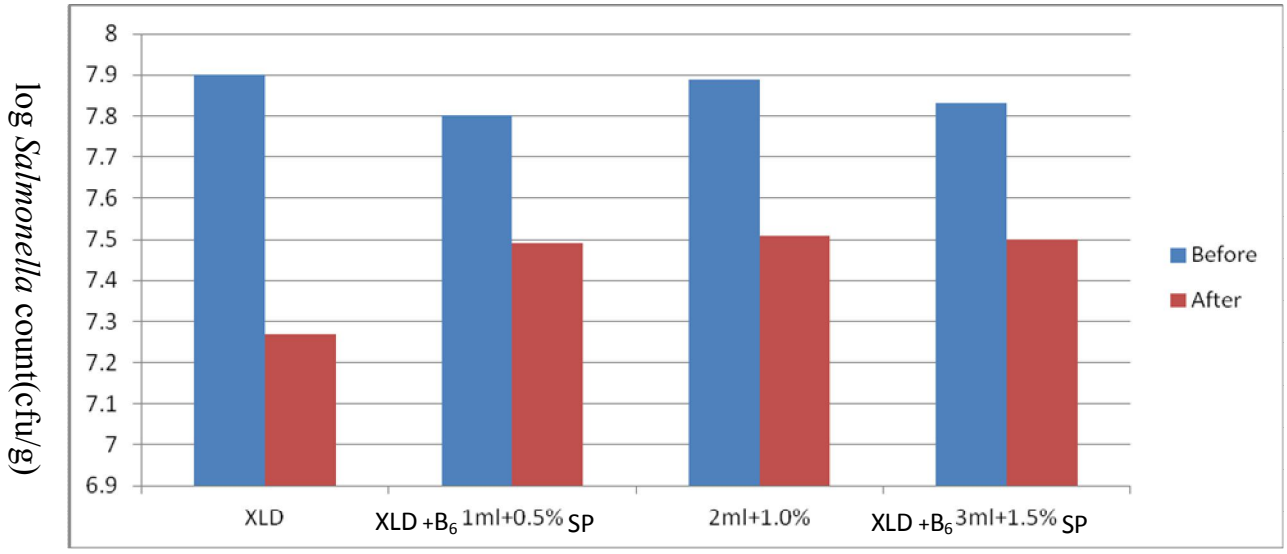
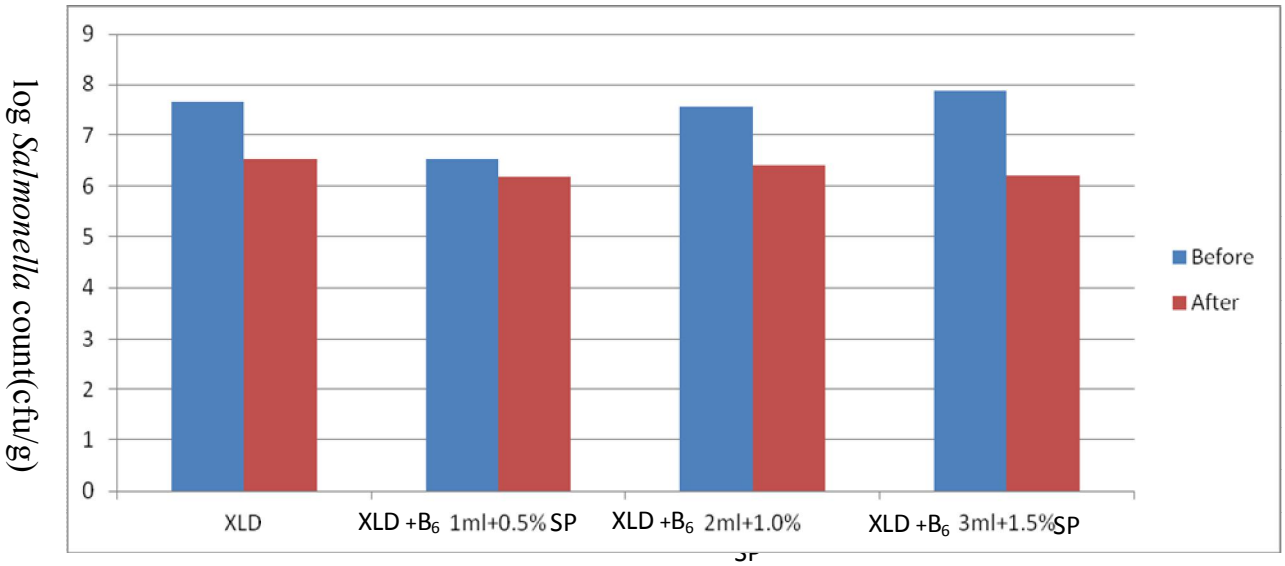


**Table (12):** The effect of addition of vit$B_6$ and sodium pyruvate (ml/L+ gm. %) on growth of *Salmonella*, before and after cold shock at -40°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with Volume of($B_6$+SP) concentration | | |
| | | 1ml+0.5% | 2ml+1.0% | 3ml+1.5% |
| (A)Before Shock | 7.66 | 6.54 | 7.55 | 7.86 |
| (B)After shock | 6.55 | 6.19 | 6.41 | 6.21 |
| Loss | 14.5% | 5.4% | 15.1% | 21.0% |
| Recovery | 85.5% | 94.7% | 84.9% | 79.0% |



## 4.5 Effect of sodium pyruvate and vitB$_{12}$ on growth of cold-shocked *Salmonella*

The recovery of *Salmonella sp*by addition of vitB$_{12}$ with sodium pyruvate (ml/L +gm. %) in the XLD medium at -20$^{o}$C,-30$^{o}$Cα -40$^{o}$Care shown in table 13, 14and15, respectively

In Table 13, the effect of freezing on the loss of *Salmonella* at -40$^{o}$Cin XLD medium without addition of vitB$_{12}$ with sodium pyruvate was 4.0% and the recovery was 96.0%.With addition of vitB$_{12}$with sodium pyruvate, concentration (1ml+0.5%) loss was 0.4% and recovery was 100.0%. In (2ml+1.0%ml) loss was 3.5%and recovery was 96.5%, in (3ml+1.5%) loss was 3.6% and recovery was 96.4%.

In Table 14 at -30$^{o}$Cin XLD medium without addition of vitB$_{12}$ with sodium pyruvate was 5.3% and the recovery was 94.7%. With addition of vitB$_{12}$ with sodium pyruvate concentration (1ml+0.5 %) loss was 7.7% and recovery was 92.3%. In (2ml+1.0%ml) loss was 7.1% and recovery was 92.9%in (3ml+1.5%) % ml loss was 4.1%and recovery was 95.9%.

In Table15 at -40$^{o}$C in XLD medium without addition of vitB$_{12}$ with sodium pyruvate was 22.7%and the recovery was 77.3%. With addition of vitB$_{12}$ with sodium pyruvate (concentration1ml+0.5%) loss was 30.0% and recovery was 70.0%. In (2ml+1.0%ml) loss was 21.4% and recovery was 78.6%. In (3ml+1.5%) ml loss was 16.8%and recovery was 83.2%.

**Table (13):** The effect of addition of vit$B_{12}$ and sodium pyruvate (ml/L +gm %) on growth of *Salmonella*, before and after cold shock at -20°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with Volume of($B_{12}$+SP) concentration | | |
| | | 1ml+0.5% | 2ml+1.0% | 3ml+1.5% |
| (A)Before Shock | 7.53 | 7.49 | 7.46 | 7.41 |
| (B)After shock | 7.23 | 7.46 | 7.20 | 7.14 |
| Loss | 4.0% | 0.4% | 3.5% | 3.6% |
| Recovery | 96.0% | 99.6% | 96.5% | 96.4% |

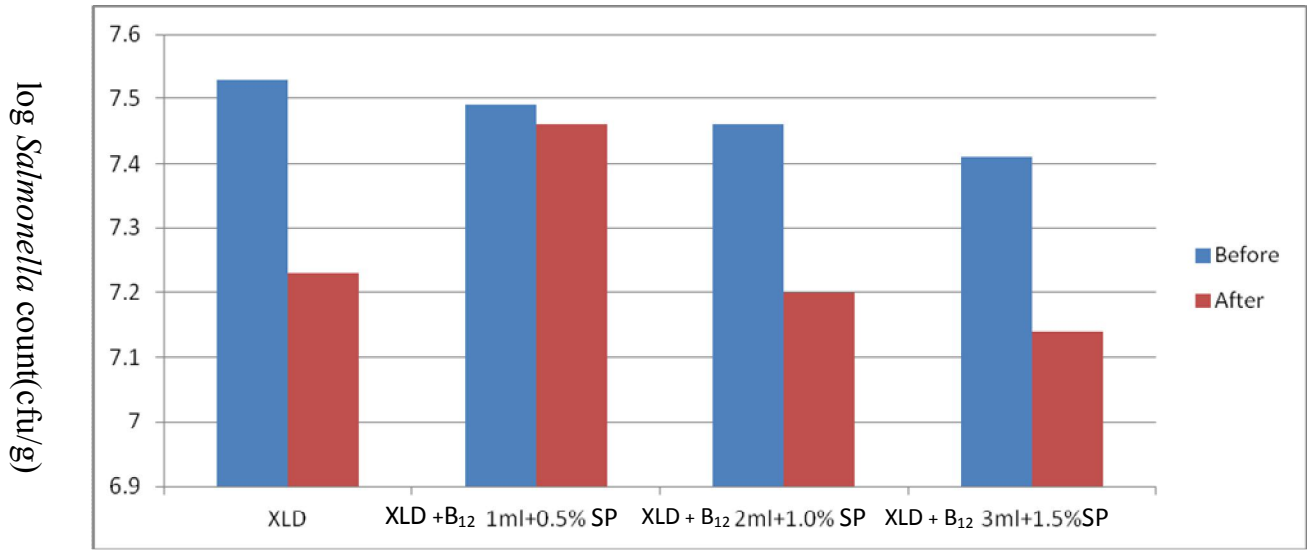


**Table (14):** The effect of addition of vit$B_{12}$ and sodium pyruvate (ml/L+ mg %) on growth of *Salmonella*, before and after cold shock at -30°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with Volume of($B_{12}$+SP) concentration | | |
| | | 1ml+0.5% | 2ml+1.0% | 3ml+1.5% |
| A)Before Shock (cfu/g) | 7.38 | 7.50 | 7.43 | 7.74 |
| (B)After shock | 6.99 | 6.92 | 6.90 | 7.42 |
| Loss | 5.3% | 7.7% | 7.1% | 4.1% |
| Recovery | 94.7% | 92.3% | 92.9% | 95.9% |

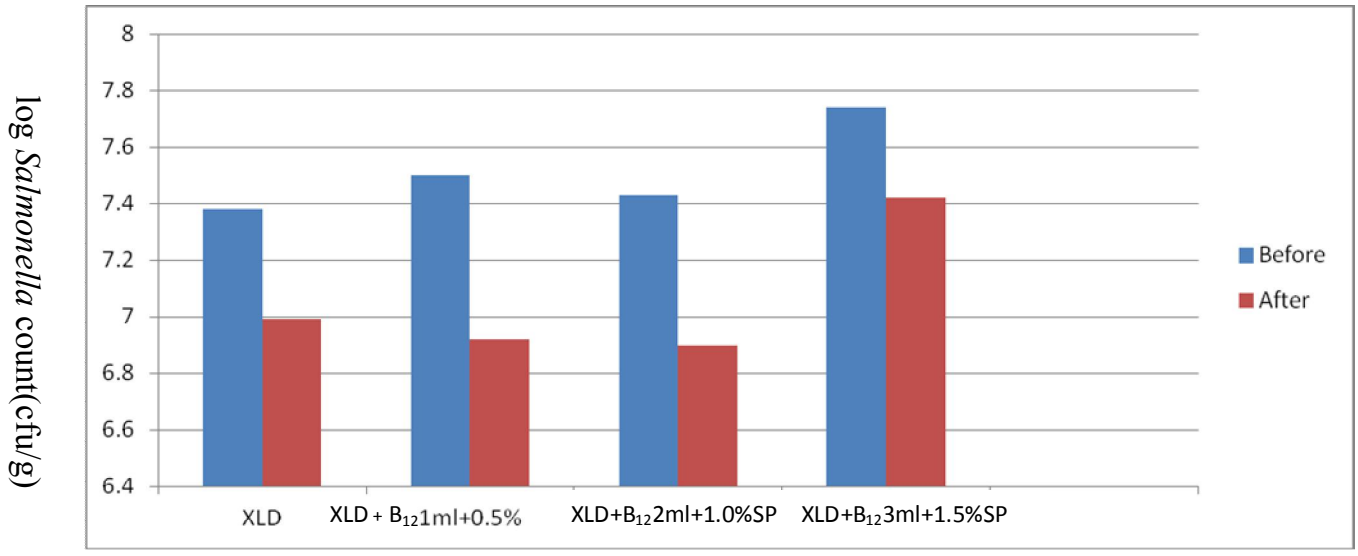
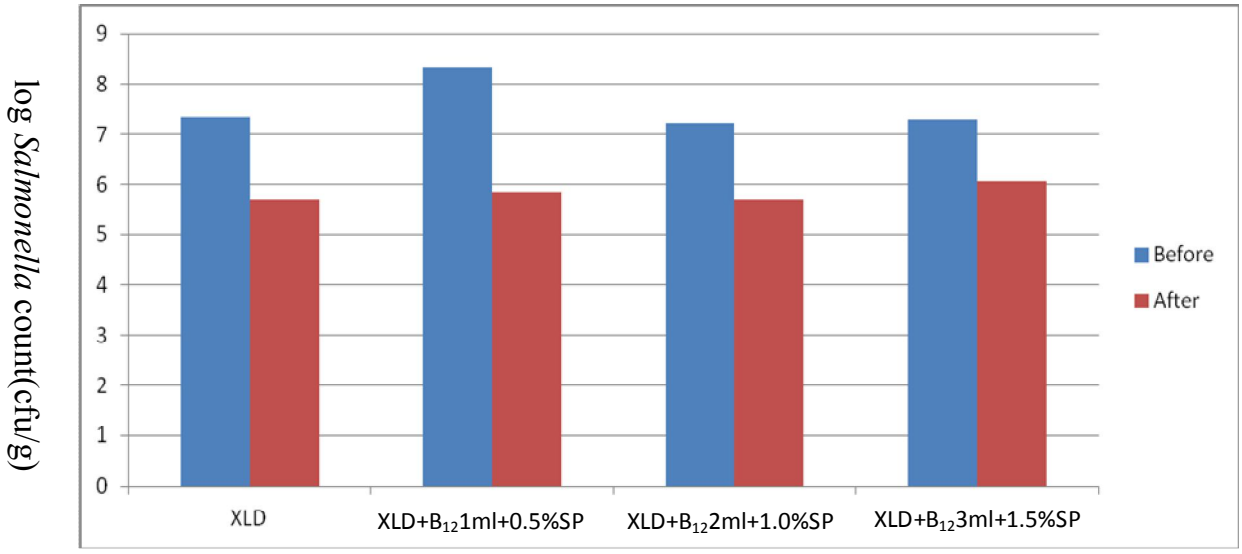


**Table (15):** The effect of addition of vit$B_{12}$ and sodium pyruvate (ml/L +mg %) on growth of, before and after cold shock at(-40°C

| Condition | Log *Salmonella* count (cfu/g) | | | |
|---|---|---|---|---|
| | XLD medium | XLD with Volume of($B_{12}$+SP) concentration | | |
| | | 1ml+0.5% | 2ml+1.0% | 3ml+1.5% |
| (A)Before Shock | 7.36 | 8.34 | 7.24 | 7.30 |
| (B)After shock | 5.69 | 5.84 | 5.69 | 6.07 |
| Loss | 23.0% | 30.0% | 21.4% | 16.8% |
| Recovery | 77.3% | 70.0% | 70.0% | 83.2% |



In this study, the evaluation of the effect of freezing and thawing on *Salmonella sp*. showed that they are susceptible to freezing and this is in line with previous findings (Moss and Speck, 1966; Mohammed, 1987 and Mubarak, 2010).

The responses of the organism tested agree with Mubarak, (2010).who recorded that responses of the organisms to freezing varies considerably, some are killed and some survive and may remain viable to different degrees during frozen storage and after swing. The colonies of *Salmonella* that developed on non –selective medium represent both injured and uninjured cells whereas only the uninjured cells developed on the selective media.

Also these results are in agreement with Mohammed (1987) who declared that at -20$^{o}$C in the cells of *E.coli* was the most susceptible cells to injury. Also at this temperature (-20$^{o}$C) the cells showed even more sensitivity. This result confirms that obtained by Mackey *et al*., (1980) who found more rapid increase in the injury of *E. coli* at higher subzero-temperature. But they did not work in the recovery. Noting that this work was done on *Salmonella sp*. Both organisms are included in the family Enterobacteriaceae which are Gram negative bacteria.

There is some work of cold shock carried out for gram positive bacteria, like the bacteria *Staph. aureus*. Cells of *Staph. aureus* were almost resistant when subjected to freezing at -20$^{o}$C .These results are inagreement with those of Minor and Marth (1976) and Egziabber*et al.* (1982). That means cold shock affects both gram negative and gram positive bacteria.

Mohammed (1987), Sinkey and Silverman (1970) observed that the cells of *E.coli* when frozen at -40$^{o}$C, no noticeable damage death occurred. In this research work the results obtained with *Salmonella sp* most cells died and the count is very low which do not agree with their findings.

The effect of freezing on the organism isolated from sterilized meat (beef) showed that freezing of these organisms in meat was less harmful when compared with freezing done in the laboratory media to the same temperature (-20$^{o}$ C).

The result obtained with *Salmonella* are in agreement with that of Mohammed (1987) andSinckey and Silverman (1970).Those out hors observed that the cells of *E.coli* when frozen at -40$^{o}$C, no noticeable damage death occurred.

Addition of sodium pyruvate to XLD medium improved the recovery of injured bacterial cell which agrees with McDonald *et al*. (1983).There are earlier reports to indicate that most often sodium pyruvate can improve the detection of microbes in stress(Krieg and Hoffman,1986 and Chang *et al*. (1993).

# CHAPTER FIVE

# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 **Conclusions**

• Injured bacteria failed to from colonies on selective media but some can form colonies on-non selective improved media.

• The effect of freezing on*Salmonella sp* varied in different temperatures and additive concentrations, some are very sensitive while others are less.

• Theinjured cells whichresulted were repairable by adding some additives, e.g. $B_6$, $B_{12}$ andsodium pyruvate.

•The effect of $B_6$ at -20$^{o}$C with concentration 1ml (1000g)gave loss 1.3% whilerecoverywas 98.7%,in2ml loss was2.8% while recovery was 97.3%,in 3ml loss was7.0% while recovery93.0%, $B_6$ at -30$^{o}$Cthe loss was 4.3% while recoverywas95.8%, no improvement was noticed at -40$^{o}$C.

•The effect of $B_{12}$ at-20$^{o}$C with concentration 1ml (1oomg) loss was 4.6%, whilerecovery was 95.4%, 2ml loss was0.4%while recovery was 99.6%, 3ml loss was 2.0 %, whilerecovery was 98.0%,and improvement was noticed at -20$^{o}$Cwith concentration of 2ml. No improvement was noticedat -30$^{o}$C,

• Sodium pyruvate at -20$^{o}$C and -30$^{o}$C showedno improvement in growth while recovery was 95.5% at -40$^{o}$C

•No effect of $B_6$+ sodium pyruvate at-20$^{o}$C, whileimprovement was noticed at -30$^{o}$C and -40$^{o}$C with concentration of 1ml+0.5%.

•The effect of $B_{12}$+ sodium pyruvate at-20$^{o}$C withconcentrationof 1ml+0.5loss was0.4% while recovery was 99.6%.

## 5.2 Recommendations

• More studies should be done on the nature of cellular damage at molecular level in injured bacteria and mechanisms of injury.

•Improvement of the most effective methods to allow enumeration and isolation of injured bacteria from frozen food samples because they are not effectively detected either by selective or non-selective media due to irreversible injury.

•The physiological effect on bacterial structure on injured cells should be studied.

•Freezer should remain at a constant temperature that is below0$^{o}$C (-10$^{o}$F) is better. If you must remove your meat from the freezer for short periods of time, it should be maintained at a temperature below 28$^{o}$F.

•Sodium pyruvate with vit$B_{12}$ (0.5gm+1ml) can be used for recovery mediaat-20$^{o}$C.

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