ISOLATION , IDENTIFICATION AND ENUMERATION OF STAPHYLOCOCCOUS AUREUS FROM FROZEN FOOD

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CERTIFICATION

I thereby certify that this project was carried out by **Omamuwagbemi Irene ASAMA** with Matriculation number **LSC1705462**in the Department of Microbiology, Faculty of life sciences, University of Benin under my supervision.

Mrs. F. O. Omorotionmwan	**DATE**
(Supervisor)	

APPROVAL

I certify that this work has been accepted in partial fulfilment of the requirement for the award of Bachelor of Sciences (B.Sc.) in the Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City.

Prof. (Mrs.) F. I. Akinibosun **DATE**

(Head of Department)

DEDICATION

This work is dedicated to the Almighty God, my strength and help, for his grace throughout the course of this pursuit.

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My deepest gratitude goes to God Almighty for his sufficient grace towards the successful completion of this program and project work.

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ABSTRACT

The main objective of food preservation has been on controlling microbial populations, with a specific emphasis on pathogenic microorganisms. Food preservation implies inhibiting the growth of microorganisms. Hostile environments for microorganisms are an adequate food preservation strategy The application of heat treatments, reduction of storage temperatures, application of good manufacturing practices and the addition of additives define the food shelf-life and safety. The aim of this study was to evaluate the presence of *Staphylococcus aureus* in frozen food such as chicken turkey and fish and to enumerate *Staphylococcus aureus* in the frozen foods' samples. The samples were gotten from various locations in Benin City. The eight (8) samples were carried to the laboratory and analyzed following standard operations and procedures. The results of the microbial assessment of the frozen foods to isolate, identify and enumerate *Staphylococcus aureus*. The total heterotrophic bacterial counts revealed that the population density of the microorganisms varied from one sample to another. The mean total heterotrophic bacterial count ranged from 7.0×10^3 to 4.2×10^4 cfu/g for the samples. The results revealed the isolates as *Serratia marcescens, Proteus mirabilis* and *Staphylococcus aureus*. which indicated a diversity of the microbial species found in the frozen food samples. The results obtained in this study revealed that the bacterial isolates present in the frozen food samples were found to harbor certain enzymes and factors, which contributes to their virulence factors. The microbial assessment of the frozen foods revealed that *Staphylococcus aureus* had the highest prevalence amongst the isolates found in the frozen food samples with a percentage occurrence of 57.14%. From the study it was ascertained that freezing as a means of preservation can reduce bacteria contamination. Due to contamination of frozen food with the bacteria isolates found in this study to be of public health significance thus the usefulness of proper freezing and preservation cannot be overemphasized.

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

The main objective of food preservation has been on controlling microbial populations, with a specific emphasis on pathogenic microorganisms. Food preservation implies inhibiting the growth of microorganisms (Alum *et al*., 2016). Hostile environments for microorganisms are an adequate food preservation strategy The application of heat treatments, reduction of storage temperatures, application of good manufacturing practices and the addition of additives define the food shelf-life and safety.

Asepsis is defined as freedom from pathogenic microorganisms' insufficient dose to cause an infection. It is a way to prevent microorganisms from reaching the food. Applied mainly to raw foods. The type of microorganism (if it is pathogenic) and the number (size of the danger and the loading treatment) must be identified to know the potential risk (Alum *et al*., 2016). Preservation processes can be used to achieve asepsis in food items such as vegetable products and animal products (Anil, 2019). There are various methods of preservation, and it may be physical (low temperatures: refrigeration, freezing, high temperatures: pasteurization, sterilization, elimination of water: concentration, drying, freeze drying, removal of air: vacuum packaging, packaging with CO2 or N2) or chemical like the use of food additives.

Food poisoning syndrome results from ingestion of water and wide variety of food contaminated with pathogenic microorganisms (bacteria, viruses, protozoa, fungi), their toxins and chemicals (Bintsis, 2018). Food poisoning must be suspected when an acute illness with gastrointestinal or neurological manifestation affects two or more persons, who have shared a meal during the previous 72 hours. The term as generally used encompasses both food-related infection and food-related intoxication. Some microbiologists consider microbial food poisoning to be different from food-borne infections. In microbial food

poisoning, the microbes multiply readily in the food prior to consumption, whereas in food-borne infection, food is merely the vector for microbes that do not grow on their transient substrate (Kumar,2019). Others consider food poisoning as intoxication of food by chemicals or toxins from bacteria or fungi. Some microorganisms can use our food as a source of nutrients for their own growth. By growing in the food, metabolizing them, and producing by-products, they not only render the food inedible but also pose health problems upon consumption. Many of our foods will support the growth of pathogenic microorganisms or at least serve as a vector for their transmission (Li and Farid, 2016). Food can get contaminated from plant surfaces, animals, water, sewage, air, soil, or from food handlers during handling and processing.

S. aureus is a Gram-positive, non-spore forming spherical bacterium that belongs to the *Staphylococcus* genus. The *Staphylococcus* genus is subdivided into 32 species and subspecies. S. aureus produces staphylococcal enterotoxin (SE) and is responsible for almost all staphylococcal food poisoning (Talarico *et al*., 1997). *Staphylococcus aureus* is a bacterium that causes staphylococcal food poisoning, a form of gastroenteritis with rapid onset of symptoms. *S. aureus* is commonly found in the environment (soil, water, and air) and is also found in the nose and on the skin of humans (Seoks and Bohach, 2007). Staphylococcal food poisoning symptoms generally have a rapid onset, appearing around 3 hours after ingestion (range 1–6 hours). Common symptoms include nausea, vomiting, abdominal cramps, and diarrhoea. *S. aureus* can cause various non-food related health issues such as skin inflammations (e.g., boils and styes), mastitis, respiratory infections, wound sepsis, and toxic shock syndrome (TSS).

1.2 AIMS AND OBJECTIVES

The aim of this study was to evaluate the presence of *Staphylococcus aureus* in frozen food such as chicken turkey and fish. The specific objectives of this microbiological assessment of the frozen foods were to:

1. to isolate and identify the microorganisms present in the frozen foods' samples
2. to enumerate *Staphylococcus aureus* in the frozen foods' samples.

CHAPTER TWO

LITERATURE REVIEW

2.1 FOOD PRESERVATION

Virtually all foods are derived from living cells from animals and plant origin and in some cases from some microorganisms by biotechnology methods. Thus, foods are for the most part composed of "edible biochemical". The main objective of food preservation has been on controlling microbial populations, with a specific emphasis on pathogenic microorganisms (Alum *et al*., 2016). Food preservation implies inhibiting the growth of microorganisms. Hostile environments for microorganisms are an adequate food preservation strategy The application of heat treatments, reduction of storage temperatures, application of good manufacturing practices and the addition of additives define the food shelf-life and safety. The most important hurdles are water activity (aw), temperature (high or low), acidity (pH), dehydration, preservatives, and non-thermal technologies**.**

Cereals, sugar, fungi (grown as food), fruits, and vegetables are susceptible to microbial and physical-chemical deterioration after harvest (Tsironi *et al*., 2020). This generates the need to use preservation techniques. The postharvest preservation foods as of fruits and vegetables is a challenge owing to their highly perishable nature which leads to spoilage if not properly preserved. Foods like meat and meat products, birds, and eggs, fish (and other marine foods), and milk and derivatives are easily altered by microorganisms unless they undergo some conservation treatment. Most of these foods must be refrigerated or even frozen immediately after harvesting, to inhibit microbial growth and loss of quality which will make it unfit for human consumption.

There are different principles of food preservation. One of which is the keeping of food without germs (asepsis), the elimination existing germs (filtration and sterilisation), then

placing an obstacle to microbial growth (low temperatures, dehydration, anaerobic conditions, chemical preservatives) and the destroying microorganisms are important forms to prevent spoilage in food caused by bacteria (Liu *et al*., 2020). Another, which is, destroying or inactivating their enzymes (scalding) and preventing or delaying chemical reactions (avoiding oxidation with the use of antioxidants) are some ways to prevent spoilage of food. Another principle is protecting food against damage by microorganisms. This is possible by ensuring that the smallest possible number of microorganisms reach the food the smaller the number of microorganisms, the greater the latency phase which is the resting phase of the microorganisms in their growth cycle. Also, contamination by germs of no active growth (in logarithmic phase), present in containers, machinery, and utensils should be avoided. Finally, unfavourable environmental conditions for food germs (humidity, temperature, WA, pH, or redox potential), or the presence of microbial inhibitors must be ensured in food (preservatives). The higher the number of unfavourable conditions, the longer it will take to start growth of the microorganisms (Sohail *et al*., 2018).

The preservation, processing and storage of the food are vital for the continuous supply of foods during seasons and off-seasons to reduce risk of hunger and make the food fit for human consumption. One very important consideration that differentiates the agricultural from all other industrial processes is their seasonal nature. The main reasons for food processing and preservation are to overcome seasonal production in agriculture; to produce value-added products; and to provide different variety in diets (Saohail *et al*., 2018). People like to eat wide varieties of foods, having different tastes, flavours, nutritional, dietetic, and other characteristics. Unfortunately, it has been estimated that as many as 2 billion people do not have enough to eat and that perhaps as many as 40 000 die every day from diseases related to inadequate diets, including the lack of sufficient food, protein, or specific nutrients. Inadequate nutrition in extreme cases can produce in

children an advanced state of protein deficiency known as kwashiorkor or the more widespread protein. Major processes of food deterioration are caused by environmental factors such as temperature, humidity, oxygen, and light which can be reason for several reaction mechanisms that may lead to food deterioration to such an extent that they are either rejected by or harmful to the consumer (Liu *et al*., 2020).

2.2 METHODS OF FOOD PRESERVATION

There are various methods of food preservation, but they are majorly grouped into two classes. These classes are the traditional methods and the modern methods of food preservation (Anilkumar, 2019). The traditional methods of preservation include salting, smoking, and drying.

While the modern methods of food preservation include, irradiation of food, use of carbon (iv) oxide, sulphur dioxide and nitrates.

Salting is the preservation of food with dry edible salt. It is related to pickling in general and more specifically to brining also known as fermenting (preparing food with brine, that is, salty water) and is one form of curing (Tsironi *et al*., 2020). It is one of the oldest methods of preserving food, and two historically significant salt-cured foods are salted fish (usually dried and salted cod or salted herring) and salt-cured meat (such as bacon). Vegetables such as runner beans and cabbage are also often preserved in this manner (Sohail *et al*., 2018).

Smoking is the process of flavoring, browning, cooking, or preserving food by exposing it to smoke from burning or smoldering material, most often wood. Meat and fish are often preserved using this method. Smoking can be done in four ways: cold smoking, warm smoking, hot smoking, and through the employment of a smoke flavoring, such as liquid smoke. However, these methods of imparting smoke only affect the food surface, and are

unable to preserve food, thus, smoking is paired with other microbial hurdles, such as chilling and packaging, to extend food shelf-life.

The use of irradiation involves use of ionising agents on the food products (Qiu *et al*., 2019). Ionisation irradiation affects bacteria, yeast, and moulds by causing lesions in the genetic material of the cell. Factors that affect the susceptibility of microorganisms to irradiation are dose level, temperature, atmosphere composition, medium including foods and type of organism. In general, the higher the dose applied the lower number of survivors.

2.3 FREEZING

Freezing, in food processing, method of preserving food by lowering the temperature to inhibit microorganism growth (Ratti, 2001). The method has been used for centuries in cold regions, and a patent was issued in Britain as early as 1842 for freezing food by immersion in an ice and salt brine. It was not, however, until the advent of mechanical refrigeration that the process became widely applicable commercially. In 1880 a cargo of meat shipped from Australia to Britain under refrigeration accidentally froze, with such good results that the process was at once adopted for long-distance shipments and other storage. In the 20th century quick, or flash, freezing was found to be especially effective with certain types of food.

Except for beef and venison, which benefit from an aging process, meat is frozen as promptly as possible after slaughter, with best results at temperatures of 0 °F (−18 °C) or lower. Fruits are frozen in a syrup or dry sugar pack to exclude air and prevent both oxidation and desiccation (Ratti, 2019).

Most commercial freezing is done either in cold air kept in motion by fans (blast freezing) or by placing the foodstuffs in packages or metal trays on refrigerated surfaces (contact freezing)

Freezing preserves food for extended periods by stopping the growth and multiplication of microorganisms that cause both food spoilage and foodborne illness and by halting the foods own enzyme activity that would otherwise cause the food to rot. Most pathogens don't multiply at freezer temperature and many of them perish because their enzymes don't work properly to maintain normal cell activity. Also, pathogens need water to grow and freezing turns the available water into solid ice crystals (Fellows, 2017). Freezing is a common practice in the meat, fish, and other animal protein-based industry, because it preserved the quality for an extended time and offers several advantages such as insignificant alterations in the product dimensions, and minimum deterioration in products colour, flavour, and texture (Prosapio, 2017).

2.4 BACTERIAL FOOD POISONING

According to various studies conducted till date, food poisoning is a very common illness. For most people it is usually mild, but food poisoning can be severe and even deadly for some individuals. Most cases of food poisoning occur when people eat food or drink water containing bacteria, bacterial toxins (substances produced by bacteria), parasites, or viruses. Food poisoning can also occur when non-infectious poisons (such as poisonous mushrooms) or heavy metals (such as lead or mercury) find their way into people's stomachs (Lynch *et* al., 2009). It is estimated that about 4 million Canadians experience food poisoning each year. People at greatest risk for food poisoning are seniors, pregnant women, young children and babies, and people with chronic medical conditions (e.g., Diabetes, AIDS, liver diseases).

Food poisoning occurs when contaminated food or water is ingested into the body of the individual. Contamination can occur anywhere along the process of obtaining and eating food – it can occur during growing, harvesting, processing, storing, or preparation stages. In most cases, bacteria, viruses, or parasites are transferred to food from other sources, making these organisms the most common causes of food poisoning (Eisenberg *et al*., 1975). However, in some fewer common types of food poisoning, the poison or toxin is naturally part of the food (e.g., poisonous mushrooms or fish). Other less common causes include shellfish and insecticides.

Food poisoning can be caused by various microorganisms such as viruses, bacteria, fungi and parasites. Viruses such as norovirus can cause food poisoning, most commonly through contaminated raw or uncooked produce and shellfish from contaminated water. Parasites such *Giardia lamblia* can also cause food poisoning through contaminated produce and water. Many bacteria can cause food poisoning, either directly or by the toxins they produce. Some of the most common include *Salmonella, E. coli, Shigella, Staphylococcus*, *Campylobacter*, and *Clostridium perfringens*. Many bacterial causes of food poisoning can be found in undercooked meats, poultry, eggs, dairy, processed meats, fish, custards, cream pies, and contaminated water (Rooney *et al*., 2004). Many other things can cause food poisoning. These include mushrooms, wild nuts, leaves, flowers, and berries, under ripe tubers, botulism, cadmium from containers, lead or arsenic from fertilizers, and acids and lead from pottery.

The symptoms and complications of almost all forms of food poisoning produce nausea, vomiting, abdominal cramps, and diarrhoea. The bacterial causes of food poisoning tend to cause these symptoms as well as fever and headache. Symptoms can start within hours to days after eating the contaminated food and last from a day to a week (Al-Bahry *et al*., 2014). Many non-infectious (not caused by bacteria and their toxins, viruses, etc.) food poisoning affects the central nervous system and cause symptoms typical of nerve poisons.

Eating shellfish contaminated with saxitoxin, for example, will produce weakness or paralysis around the mouth in a few minutes, which slowly spreads to the rest of the body. Signs of ciguatera poisoning include face pain, headache, itching, and odd sensations of alternating hot and cold. Mushroom poisoning also attacks the nervous system. Shrunken eye pupils, tears, salivation or frothing at the mouth, sweating, vertigo, confusion, coma, and sometimes seizures appear within 2 hours of eating a poisonous mushroom. Insecticides based on organophosphates cause very similar symptoms. They're likely to be milder, since it is extremely rare for large doses of insecticide to be eaten accidentally. The most common complication of food poisoning is dehydration, when your body loses too much water and electrolytes (e.g., sodium, potassium). Food poisoning caused by the bacteria *Listeria* can cause problems for unborn babies, and *E. coli* infection can cause problems with the kidneys. Other complications can include arthritis and bleeding problems. Non-infectious food poisoning can occasionally lead to permanent nervous system problems and even death.

2.5 PREVENTION OF BACTERIAL FOOD POISONING

The treatment of food poisoning depends on the cause and on its severity. For most people, food poisoning resolves quickly without treatment. For people with mild diarrhoea lasting less than 24 hours, treatment should consist of drinking clear fluids such as oral replacement solutions (Al-Bahry *et al*., 2014). These solutions contain the right balance of water, salts, and sugar needed to prevent or treat mild dehydration. Use commercially available solutions, when possible, but, if necessary, a solution can be made by mixing 1/2 teaspoonful of salt, 6teaspoonful of sugar with 1 litre of water. It may be best to stay away from solid food during diarrhoea and vomiting. Once you can take fluids, gradually start eating plain foods as tolerated. Avoid alcohol and caffeine while you are sick. People with severe symptoms or severe dehydration may need to be

admitted to the hospital so they can receive rehydration solutions intravenously (into a vein).

Most bacterial food poisonings do not need antibiotics, but some types of infections may need antibiotic treatment (Lynch *et* al., 2009). For food poisonings that cause nervous system effects, there may be other medications or antidotes that can be used. For example, in mushroom (muscarine) and insecticide poisoning, a medication called atropine can be used to counterattack toxic effects. If poisoning is very severe, a patient may require a ventilator (artificial breathing

machine), kidney dialysis, and or admission to a hospital intensive care unit.

Proper frozen storage of foods helps prevent food poisoning (Prosapio *et al*., 2017).. Prevention is often the role of government, by setting strict rules for health and public services for veterinary surveys of animal products in the food chain, from the agricultural field to manufacturing and delivering products to supermarkets and restaurants.

2.6 *Staphylococcus aureus*

Staphylococcus aureus is a bacterium that causes staphylococcal food poisoning, a form of gastroenteritis with rapid onset of symptoms. S. aureus is commonly found in the environment (soil, water, and air) and is also found in the nose and on the skin of humans. *S. aureus* is a Gram-positive, non-spore forming spherical bacterium that belongs to the Staphylococcus genus. The Staphylococcus genus is subdivided into 32 species and subspecies. *S. aureus* produces staphylococcal enterotoxin (SE) and is responsible for almost all staphylococcal food poisoning (Montville and Matthews 2008; FDA 2012). *S. intermedius*, a Staphylococcus species which is commonly associated with dogs and other animals, can also produce SE and has been rarely associated with staphylococcal food poisoning (Talan et al. 1989; Khambaty et al. 1994; Le Loir et al. 2003).

The growth and survival of *S. aureus* is dependent on several environmental factors such as temperature, water activity (aw), pH, the presence of oxygen and composition of the food. These physical growth parameters vary for different *S. aureus* strains (Stewart 2003). The temperature range for growth of *S. aureus* is 7–48°C, with an optimum of 37°C. *S. aureus* is resistant to freezing and survives well in food stored below -20°C; however, viability is reduced at temperatures of -10 to 0°C. S. aureus is readily killed during pasteurisation or cooking. Growth of *S. aureus* occurs over the pH range of 4.0–10.0, with an optimum of 6–7 (Stewart 2003). *S. aureus* is uniquely resistant to adverse conditions such as low aw, high salt content and osmotic stress. In response to low aw, several compounds accumulate in the bacterial cell, which lowers the intracellular aw to match the external aw (Montville and Matthews 2008). As such, most *S. aureus* strains can grow over a aw range of 0.83 to >0.99 (FDA 2012). *S. aureus* is a poor competitor, but its ability to grow under osmotic and pH stress means that it can thrive in a wide variety of foods, including cured meats that do not support the growth of other foodborne pathogens (Montville and Matthews 2008). *S. aureus* is a facultative anaerobe so can grow under both aerobic and anaerobic conditions. However, growth occurs at a much slower rate under anaerobic conditions (Stewart 2003).

For a non-sporing mesophilic bacterium, *S. aureus* has a relatively high heat resistance (Stewart 2003). The observed average decimal reduction value (D-value, the value at which the initial concentration of bacterial cells would be reduced by 1 log10 unit) was 4.8–6.6 min at 60°C when heated in broth (Kennedy et al. 2005). The bacteria have a higher heat resistance when it is encapsulated in oil, with a D-value at 60°C of 20.5 min for *S. aureus* in fish and oil (Gaze 1985). An extremely heat resistant strain of *S. aureus* (D-value at 60°C of >15 min in broth) has been recovered from a foodborne outbreak in India (Nema et al. 2007).

2.7 STAPHYLOCOCCAL FOOD POISONING

Staphylococcal food poisoning is an intoxication that is caused by the ingestion of food containing pre-formed SE (Argudin et al. 2010). There are several different types of SE; enterotoxin A is most associated with staphylococcal food poisoning. Enterotoxins D, E and H, and to a lesser extent B, G and I, have also been associated with staphylococcal food poisoning (Seo and Bohach 2007). LSEs are produced during the exponential phase of S. aureus growth, with the quantity being strain dependent. Typically, doses of SE that cause illness result when at least 105 – 108cfu/g of S. aureus are present (Seo and Bohach 2007; Montville and Matthews 2008). Most genes for SEs are located on mobile elements, such as plasmids or prophages. As such, transfer between strains can occur, modifying the ability of S. aureus strains to cause disease and contributing to pathogen evolution (Argudin *et al*., 2010). S. aureus produces SEs within the temperature range of 10–48°C, with an optimum of 40–45°C. As the temperature decreases, the level of SE production also decreases. However, SEs remain stable under frozen storage. SEs are extremely resistant to heating and can survive the process used to sterilise low acid canned foods. SE production can occur in a pH range of 4.5–9.6, with an optimum of 7–8. Production of SE can occur in both anaerobic and aerobic environments; however, toxin production is optimum in aerobic conditions (Stewart 2003).

SEs are resistant to the heat and low pH conditions that easily destroy S. aureus bacteria. The SEs are also resistant to proteolytic enzymes; hence SEs retain their activity in the gastrointestinal tract after ingestion. SEs range in size from 22–28 kDa and contain a highly flexible disulphide loop at the top of the N-terminal domain that is required for stable conformation and is associated with the ability of the SE to induce vomiting (Argudin et al. 2010). It has been suggested that SEs stimulate neuroreceptors in the intestinal tract which transmit stimuli to the vomiting centre of the brain via the vagus nerve (Montville and Matthews 2008; Argudin *et al*. 2010). In addition, SEs can penetrate the lining of the gut and stimulate the host immune response. The release of inflammatory

mediators, such as histamine, causes vomiting. The host immune response also appears to be responsible for the damage to the gastrointestinal tract associated with SE ingestion, with lesions occurring in the stomach and upper part of the small intestine. Diarrhoea that can be associated with staphylococcal food poisoning may be due to the inhibition of water and electrolyte reabsorption in the small intestine (Argudin *et al*., 2010).

Staphylococcal food poisoning occurs when food is consumed that contains SE produced by *S. Aureus*. Food handlers carrying enterotoxin-producing *S. aureus* in their noses or on their hands are regarded as the main source of food contamination via direct contact or through respiratory secretions (Argudin *et al*., 2010). Foods associated with outbreaks of staphylococcal food poisoning include meat and meat products, poultry and egg products, milk and dairy products, salads, cream-filled bakery products and sandwich fillings. Foods that require extensive handling during preparation and are kept above refrigeration temperature (4°C) for extended periods after preparation are often involved in staphylococcal food poisoning (Argudin *et al.,* 2010). Foods high in starch and protein are believed to favour SE production (Stewart, 2003).

Despite *S. aureus* colonising a wide range of animals, people are the main reservoir of food contamination (Montville and Matthews, 2008). Prevalence of enterotoxigenic *S. aureus* in food handlers is variable between industries and countries. The udders and teats of cows are known sources of enterotoxigenic *S. aureus*, and the occurrence of *S. aureus* in unpasteurised milk and cheese is common. The tonsils and skin of pigs, chickens and turkeys often harbour *S. aureus*, and are also potential sources of *S. aureus* contamination (Stewart, 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.1 SAMPLE COLLECTION:

A total of 5 samples which included 2 frozen chicken, 2 frozen fish and 1 frozen turkey respectively were collected from different market in Benin city. The samples of the frozen food were taken randomly from market of different sanitary status by using sterile collection bottles and transported to the laboratory.

3.1.1Transport Media

In the current research, sterilized collection bottles were used to transporting the frozen food samples to the laboratory of Microbiology Department of University of Benin for isolation and identification of different bacteria.

3.1.2Sterilization of Materials.

Materials such as petri dishes, pipettes, glass containers {conical flasks, round bottom flasks etc.} and bottles were washed, drained, and dried. They were wrapped in aluminium foil and sterilized in a hot-air oven at 160°C for an hour. They were allowed to cool at about 40°C after sterilization. An antiseptic working environment was achieved with the use of a spirit lamp and ethanol for swabbing the working desk.

3.2 Culture Media Preparation:

The preparation of culture media was done according to the labelled requirements given on the bottle. For the ingredients of each media, separate flasks were used, and the ingredients were mixed by heating and shaking. Then the flask was covered with aluminium foil and autoclaved. After autoclave, the media flasks were kept cooling. The culture media used in this study include Nutrient agar, Eosin Methylene Blue agar, Plate Count agar (PCA) and Salmonella Shigella agar (SSA)

3.2.1 Nutrient Agar

28.0g of Nutrient agar powder was weighed out using the weighing balance. It was the suspended in 1 litre (1000ml) of distilled water in a conical flask. The solution was mixed by stirring to dissolve the agar. The mixture was boiled over a Bunsen burner flame. This helped to dissolve the agar completely. The boiling process closely monitored to avoid charring the agar. The conical flask containing the boiled/mixed nutrient agar suspension was transferred to the autoclave and sterilized at 121 degrees Celsius at 15 psi (or 15 lbs. of pressure) for 15 minutes in the autoclave.

3.2.2 Eosin Methylene Blue Agar (EMB)

Eosin Methylene Blue Agar (EMB) is a differential microbiological medium, which slightly inhibits the growth of Gram-positive bacteria and provides a colour indicator distinguishing between organisms that ferment lactose (e.g., E. coli) and those that do not (e.g., Salmonella, Shigella).

We suspended 35.96g in 1L (1000ml) distilled water. We mixed the solution by stirring to dissolve the agar. The mixture was boiled over a Bunsen burner flame which helped to dissolve the agar completely. The boiling process closely monitored to avoid charring the agar. The conical flask containing the boiled/mixed nutrient agar suspension was transferred to the autoclave and sterilized at 121 degrees Celsius at 15 psi (or 15 lbs. of pressure) for 15 minutes in the autoclave.

3.3 Sample processing

Serially diluted chicken faeces were inoculated onto nutrient agar, EMB agar and Salmonella Shigella agar using pour plate method, for detection of different bacterial colony. The petri

dishes were kept in reverse position at 37℃ for 24 hours and after incubation period, colonies were formed. Thus, the number of colonies should give the number of bacteria that can grow under the incubation conditions employed.

3.3.1 Purification

The colonies on Plate Count agar, Salmonella-shigella agar and Eosin Methylene Blue agar were obtained and identified based on morphology and biochemical properties. For obtaining of pure colonies, subculture was carried out of already isolated colonies and kept in incubator at 37℃ for 24 hours.

3.4 Morphological Identification:

3.4.1 Colonial identification:

After 24 hours incubation bacterial growth was occurred in colonial form. Based on colony, surface texture, colour, size, elevations, shapes, and edges different bacterial colonies were identified. Further morphological identification was done through Gram-staining technique.

3.4.2 Gram staining

The Gram staining technique was used to differentiate Gram-positive from Gram-negative bacterial stains according to (Mac and Jean, 1980). A drop of sterile distilled water was placed on a neat and clean glass slide containing a single isolated colony of 24hours. The smear was made by spreading the culture. This smear was air dried and fixed by rapidly passing the slide three times over the flame. It was then flooded with crystal violet for 1minute and then washed off with distilled water. Then Grams iodine solution was added to the smear and the glass slide was left for one minute and rinsed with distilled water. This step was followed by the application of decolorizing agent (ethanol) for 30 seconds. Decolorizing agent was immediately washed with distilled water and the smear was counter stained with

safranin for one minute. The slide was washed with distilled water, air dried and was observed under the microscope using oil immersion objective (*100) (Cheesebrough, 1987).

3.5 BIOCHEMICAL TESTS

Biochemical tests refer to the chemical identification of unknown substances within a living thing. Such tests are used to determine organisms quantitatively and qualitatively like bacteria, fungi, and substances like enzymes within the blood. A biochemical test can be used to diagnose a given disease and can also be used to ascertain for their metabolic properties. The biochemical tests carried out for the identification of according to the methods described by Mac and Jean (1980) include:

3.5.1 Catalase test

This test was carried out to differentiate between a catalase enzyme-producing bacterium such as Staphylococcus and non-catalase enzyme producing bacteria such as Streptococcus. Two millilitres (2 ml) of 3% hydrogen peroxide solution were measured and transferred into test tube. Using a sterile glass rod, several colonies of the test organisms were removed and immersed in the hydrogen peroxide solution. Immediate bubbling in the tube shows a positive catalase test.

3.5.2 Coagulase test

This test was used to differentiate coagulase-producing Staphylococcus from the nonproducing ones. A drop of distilled water was placed on each end of a clean slide. Colonies of the test organisms were emulsified in each of the drops to make two thick suspensions. A loopful fresh human plasma was added to one of the suspensions and mixed gently. Clumping of the organisms within 10 minutes indicates a positive coagulase test.

3.5.3 Oxidase test

Oxidase test strip (Oxoid, England) was used. This test was used to differentiate between oxidase-positive and oxidase-negative bacteria. Several colonies of the test organisms were rubbed on the strip using sterile glass rod. Formation of purple coloration within 5 seconds indicates a positive oxidase test.

3.5.4 Urease test

The bacterial isolates were inoculated into slants of urea medium and incubated at 37^0C for 24 to 48 hours. Urease positive cultures produced a red pink colour due to changes in the colour of the indicator (Cheesbrough, 2005). $NH_2CO.NH_2 + H_2O \rightarrow 2NH_3 + CO_2$ (Mac and Jean, 1980)

3.5.5 Indole Test

This test was used to determine which of the isolates can split indole from tryptophan present in buffered peptone water. The test is usually used as an aid in the differentiation of gram-negative bacilli especially those of the Enterobacteriaceae. Peptone water was prepared and about 3ml of it was dispensed in test tubes using a sterile pipette. Then, fresh sterile loops were used to pick a well-isolated colony of bacteria and inoculated into the test tubes, thereafter, the tubes were incubated at 37ºC for 48hours.After incubation, 0.5ml of Kovac's indole reagent was added to the inoculated bijoux tubes. The tubes were subjected to gentle shaking and examined for red colour in the surface layer within 10 minutes. A red ring on top of the tube indicates indole positive reaction (Mac and Jean, 1980).

3.6 Determination of Phenotypic Virulence

Virulence factors, such as ability to degrade DNA, produce gelatinase, hemolysin, urease and lipase were carried out on the bacterial isolates. A 24-hour bacterial cell was standardized

(1.5x108 cells/ml) and spread on DNAse agar plates (for DNAse activity). 5% sheep blood agar plates were used to culture isolated bacteria for their ability to produce hemolysin.

3.7 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing was performed on bacterial isolates using Kirby-Bauer Disc diffusion method. The following antibiotics (codes and concentration) used in the study include MEM -Meropenem (10 µg), ERY - Erythromycin (15 µg), MET- Metronidazole (50 µg), AMC-Amoxicillin/clavulanic acid (20/10 µg), CL-Clindamycin (20 µg), CN-Gentamicin (10 µg), CIP-Ciprofloxacin (5 µg), and TET-Tetracycline (10 µg). Briefly, an 18 h. culture in broth was standardized using 0.5 McFarland scale and streaked on Mueller Hinton agar plates. With the aid of a sterile forceps, the discs were impregnated into the cultured plates. The medium was incubated for 24 h. at 37 °C. The plate was examined after incubation for zones of inhibition around each of the antibiotics. After then, the diameter of the inhibitory zone was measured in millimetres (mm).

CHAPTER FOUR

RESULTS

The results of the bacteriological assessment of the frozen food obtained from different locations in Benin city are given below:

Table 1 shows the total heterotrophic bacterial count of the frozen food samples gotten from the various location. The mean heterotrophic bacterial count ranged from 7.0×10^3 to 2.8×10^4 cfu/g for the samples. Figure 1. shows a bar chart of the total heterotrophic bacterial count (Log10 cfu/g) gotten from the results of the frozen food.

Table 2 shows the cultural, morphological, and biochemical characteristics of the bacteria species isolated from the samples. The bacteria species isolated from the frozen food samples included *Proteus mirabilis, Staphylococcus aureus* and *Serratia marcescens*.

Table 3 shows the phenotypic virulence determinants of bacterial isolates from meat samples. Table 4 shows the antibiotic susceptibility of bacterial isolates from meat samples while figure 2. shows Multiple antibiotic resistance index of bacterial isolates from meat samples.

However, table 5 shows the frequency of occurrence of the various isolates identified from the frozen food samples. The frequency talks about how often a particular microorganism occurs in the various frozen food samples. Figure 3. shows the percentage of the frequency of occurrence of the various isolates in the frozen food samples. Figure 4. shows the percentage of bacterial reduction that occurs due to freezing.

Table 1: Total Heterotrophic Bacterial Count

Samples	count1	count2	dilution	vol. inoculum	cfu/g	cfu/g	mean	SD
Chicken1	54	36	100	0.2	27000	18000	22500.00	6363.96
Chicken2	48	67	100	0.2	24000	33500	28750.00	6717.51
Turkey1	63	35	100	0.2	31500	17500	24500.00	9899.49
Turkey2	60	32	100	0.2	30000	16000	23000.00	9899.49
Fish 1	13	15	100	0.2	6500	7500	7000.00	707.11
Fish 2	35	26	100	0.2	17500	13000	15250.00	3181.98

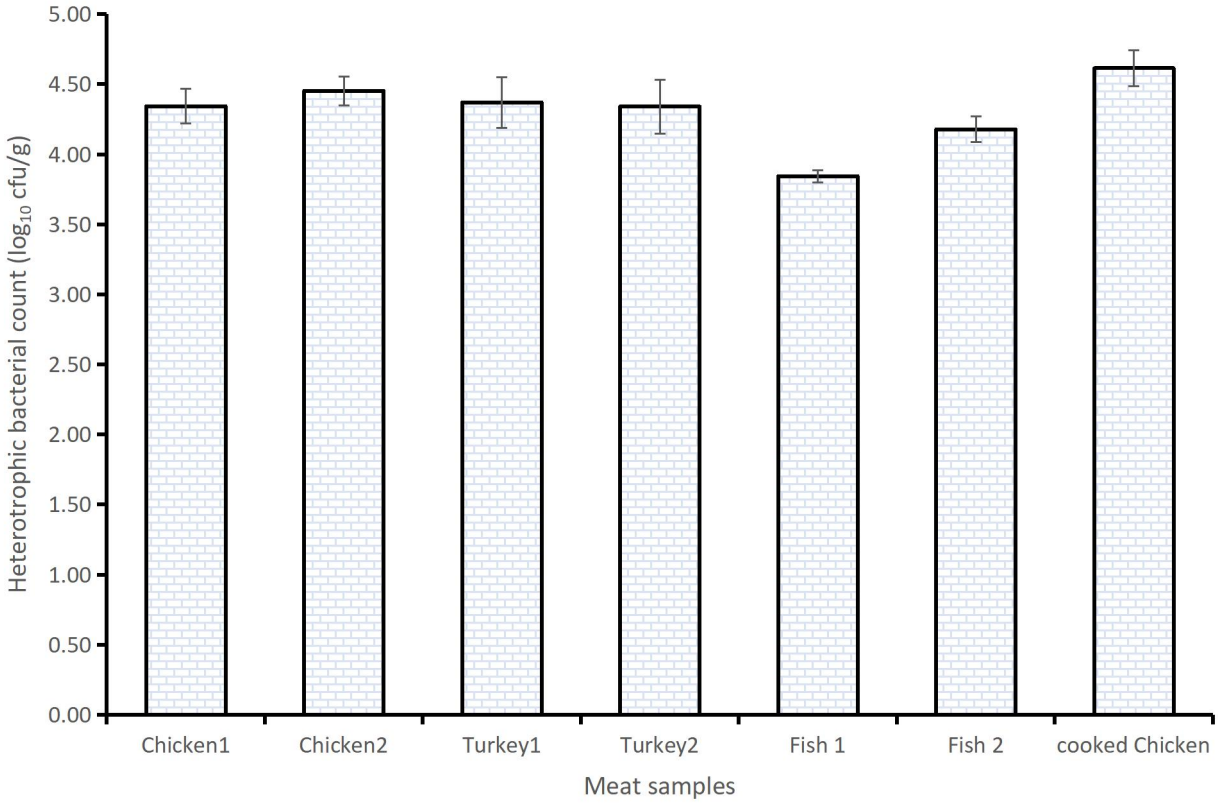

Figure 1. Total Heterotrophic Bacterial Count (Log10 cfu/g) of samples.

Table 2: Cultural, Morphological and Biochemical Characteristics of Isolates

Morphological	**Isolate 1**	**Isolate 2**	**Isolate 3**
Elevation	Flat	Raised	Raised
Margin	Entire	Entire	smooth
Color	Cream	Cream	Cream
Shape	Circular	Circular	Irregular
Size	Medium	Medium	Small
Gr. diff. agar	SSA	EMB	MSA
Colour	black	opaque	Yellow
Staining			
Gram stain	-	-	+
cell type	rod	rod	Cocci
Arrangement	disperse	disperse	clusters
Color	pink	pink	purple
Spore staining	-	-	-
Biochemical			
KOH test	+	+	-
Catalase	+	+	+
Indole	-	-	-
Citrate	+	+	+
Oxidase	-	-	-
Motility	+	+	-
Urease	+	-	+
Glucose	+	+	+
Sucrose	-	+	+
Lactose	-	-	+
Mannitol	-	+	-
Gas formation	+	-	-
H2S formation	+	-	-
Identity	*Proteus mirabilis*	*Serratia marcescens*	*Staphylococcus aureus*

Table 3. Phenotypic virulence determinants of bacterial isolates from meat samples

Isolates	Hemolysin	DNAse	Gelatinase	Lipase
S. aureus	β 10(100)	10(100)	10(100)	10(100)
Enterobacter cloacae	β 0(0)	5(100)	0(0)	5(100)
Proteus mirabilis	β 0(0)	1(25)	4(100)	4(100)

Table 4: Antibiotic susceptibility of bacterial isolates

1	No.	CIP	CRO	RL	E	TET	MEM	AMC	CN
Staphylococcus	10	8(80)	6(60)	4(40)	4(40)	2(20)	9(90)	7(70)	8(80)
Serratia	5	4(80)	4(80)	2(40)	2(40)	2(40)	5(100)	4(80)	5(100)
Proteus	4	4(100)	3(75)	1(25)	2(50)	1(25)	4(100)	3(75)	3(75)

Legend: CIP; Ciprofloxacin (5µg), CRO; Ceftriaxone (30 µg), RL; Sulfamethoxazole (1.25/23.75 µg), E; Erythromycin (15 µg), TET; tetracycline (30 µg), MEM; Meropenem (10 µg), AMC; Amoxicillin/clavulanic acid (20/10 µg), CN; Gentamicin (10 µg).

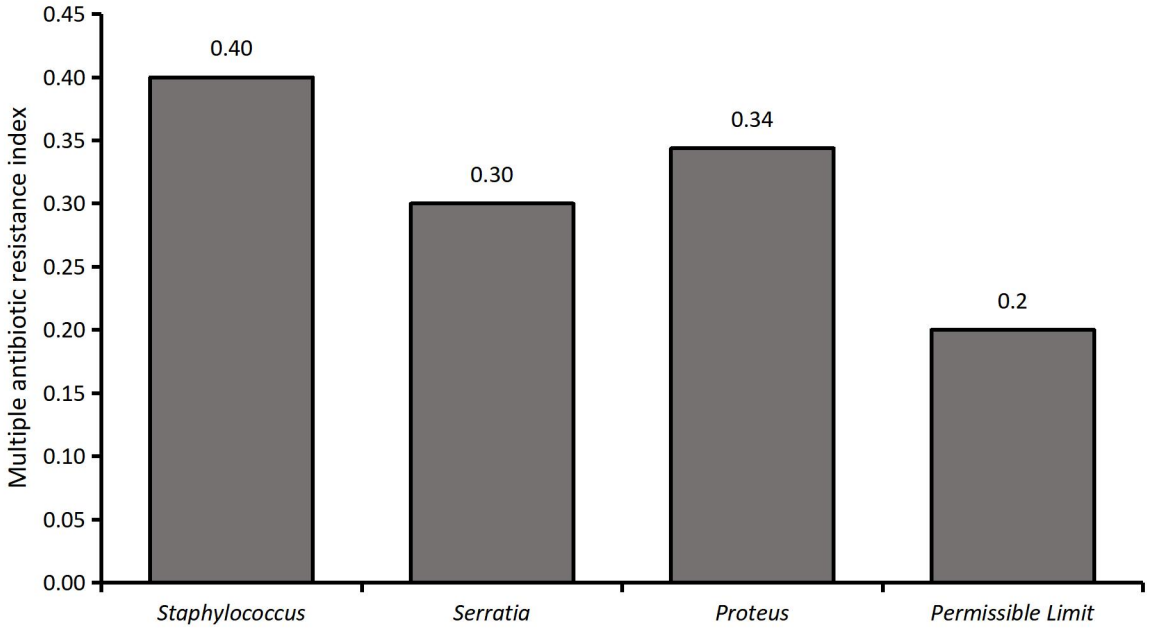

Figure 2. Multiple antibiotic resistance index of bacterial isolates

Table 5: Frequency of Occurrence of Isolates in samples.

Isolates	**total**	**Frequency**	**Percentage**
Proteus mirabilis	18	2	11.11
Serratia marcescens	17	4	23.53
Staphylococcus aureus	21	12	57.14

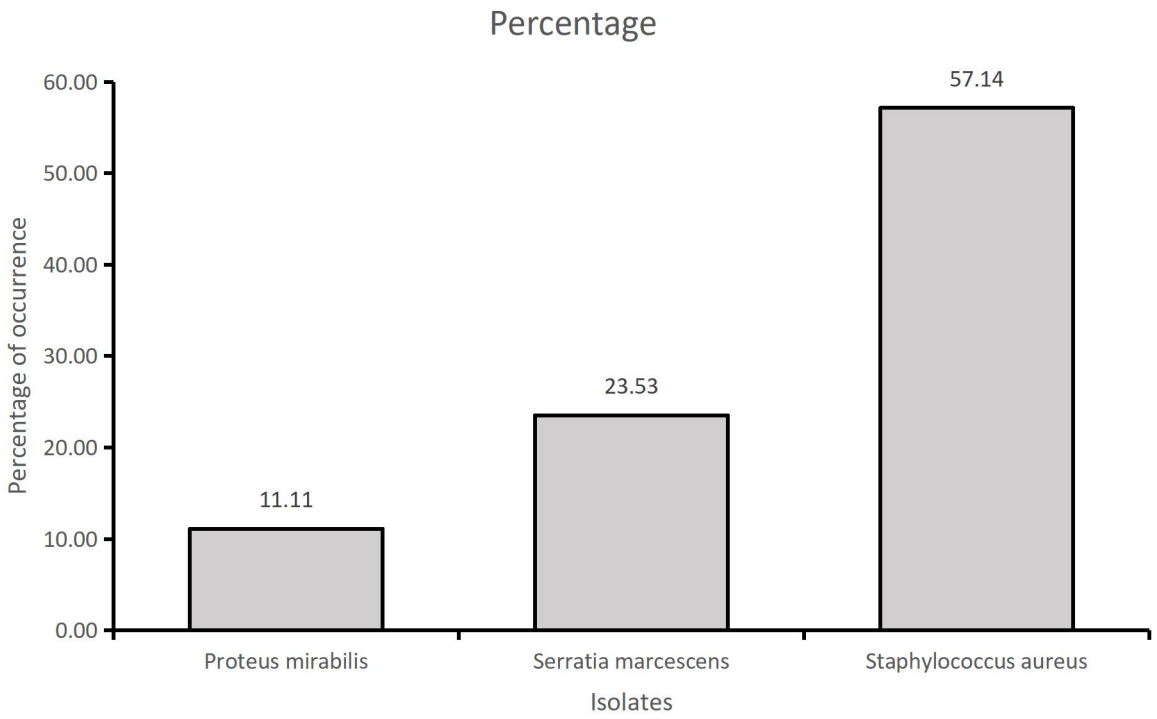

Figure 3. Percentage of Frequency of Occurrence of Isolates in Samples.

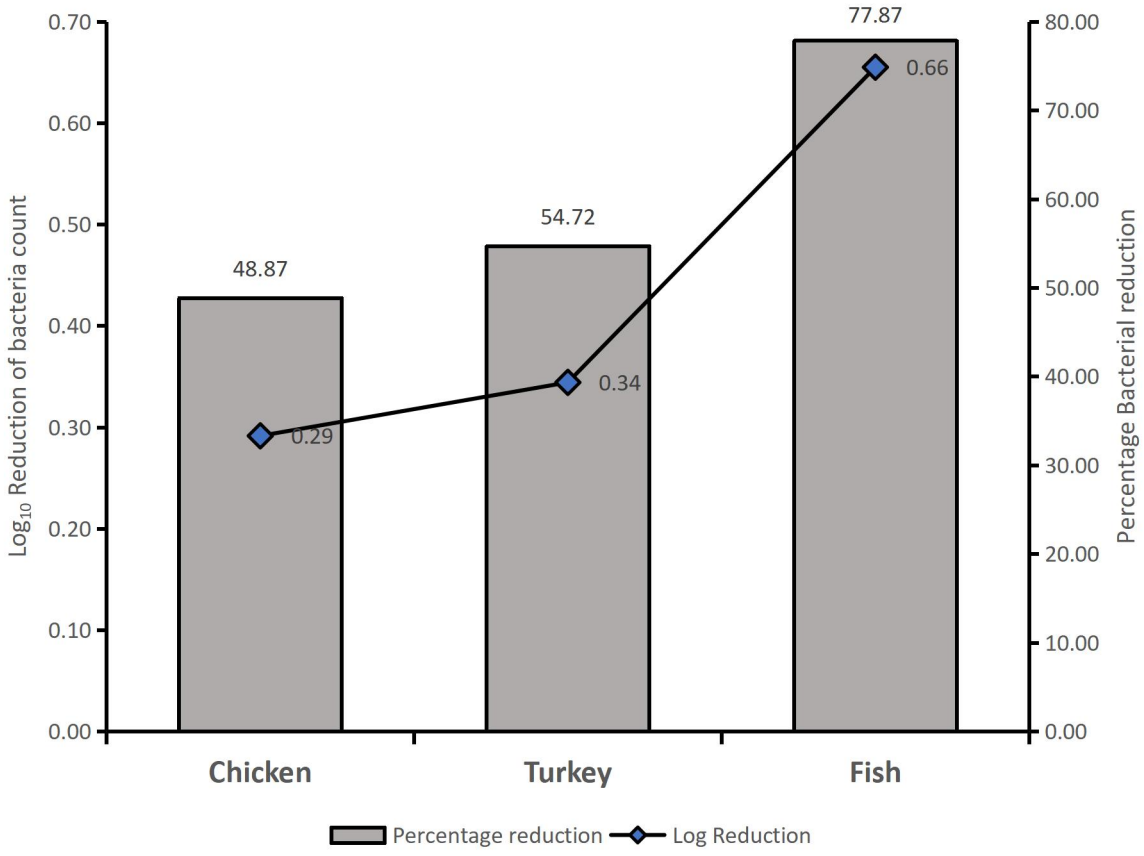

Figure 4. Percentage Bacterial Reduction due to Freezing

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

The results of the microbial assessment of the frozen foods to isolate, identify and enumerate *Staphylococcus aureus*. From the table 1 the total heterotrophic bacterial counts revealed that the population density of the microorganisms varied from one sample to another. The mean total heterotrophic bacterial count ranged from 7.0×10^3 to 4.2×10^4 cfu/g for the samples.

The isolates were identified using colonial morphology and biochemical test. The results revealed the isolates as *Serratia marcescens, Proteus mirabilis* and *Staphylococcus aureus*. which indicated a diversity of the microbial species found in the frozen food samples. From the results obtained in this study, the bacterial isolates present in the frozen food samples were found to harbour certain enzymes and factors, which contributes to their virulence factors which are molecules synthesized by certain bacteria that increases their capacity to infect or damage human tissues which agrees with the study carried Stewart, 2003; Simon and Sanjeev, 2007. Virulence factors may be coded within the bacterial genome, thus being inherent aspects of the organism's structure, or may be coded within transmissible genetic elements and thus acquired from the environment and by extension, makes the isolates to be of public health significance.

Based on the results, it was revealed that *Staphylococcus aureus* had the highest prevalence amongst the isolates found in the frozen food samples with a percentage occurrence of 57.14% followed by *Serretia marcescens* with a percentage occurrence of 23.53% and *Proteus mirabilis* being the least prevalent with a percentage occurrence of 11.11%. This is in agreement with the study carried out by Simon and Sanjeev, 2007 where they stated *Staphylococcus aureus* was the most frequent isolate.

The result of the antibiotic test against these isolates revealed a generally high resistance, characterized by average MAR index of 0.40. This high MAR index reflects a potential public health threat should a disease be established by this cause. Also, described that the MAR index highlights the pathogens' importance in health threat and its origin and exposure to antibiotics in concordance with the study of Stewart, 2003. Although, the sources of these pathogens may not be fully ascertained, with a few references to cross contamination and self-contamination of the sample culture, these contaminants are more likely of direct biological origin or with biological activities diffused in the air.

Based on the results gotten from the percentage bacterial reduction, during freezing, 80 to 90% of the Gram-negative bacteria die out and the residual bacteria cannot grow in the temperature of frozen storage. So, during freezing preservation of frozen food, there is no bacterial poisoning. The relation between microbial growth and temperature is still considered as complex phenomena governed by inter-related factors such as substrate composition, freezing rate, microbial type. According to Prosapio, 2017 as the temperature falls, bacterial growth rate is reduced, and the lag period is extended until the minimum temperature limit is reached when growth ceases. Some microbes will cease growing at 0^0C or even higher while others continue to grow below the freezing point of food. Growth rate below 0^0C is very slow which agrees with the results obtained in this study.

5.2 CONCLUSION

This study revealed that freezing as a means of preservation can reduce bacteria contamination. It does also reveal that the frozen foods are contaminated with bacteria isolates of public health significance thus the usefulness of proper freezing and preservation cannot be overemphasized.

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