COMPARATIVE ANALYSIS OF *SALMONELLA* IN CHICKEN MEAT

By

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NUST201463479MSCEE65214F

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

In

Environmental Science

Institute of Environmental Sciences and Engineering(IESE)

School of Civil and Environmental Engineering (SCEE)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

2016

CERTIFICATE

It is certified that the contents and forms of the thesis entitled

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Dedicated

To

Mom

For being my first teacher and the reason of what I become today

Papa

For supporting and encouraging me to believe in myself

ACKNOWLEDGEMENTS

All acclamation and appreciation for **ALMIGHTY ALLAH** the Benevolent and Clement, who directed my thoughts and emboldened my ambitious in the form of this dissertation.

I deem it great honor and utmost pleasure to pay my gratitude to my worthy supervisor **Dr. Imran Hashmi**, Associate Dean & Professor (IESE, SCEE, NUST). I may never have enough words of thanks to him for his great help, suggestions, sincerity, encouragement and guidance during my whole work. He has been my inspiration during my MS journey.

I am highly thankful to my Guidance and Examination Committee members

Dr. Muhammad Arshad and **Dr. Urma Mahrosh** who had always been very considerate and supportive in my research work. I am indebted to **Dr. Saud Ahmad Khan** (PIDE), **Ms. Sahaab Farooq**, Lab Demonstrator (IESE, NUST) and **Ms. Hira Waheed** (IESE, NUST) for their never ending help. This thesis would not have been possible without their support and motivation.

I feel my great pleasure to express my gratitude to my friends especially **Tehseen Tahir, Tania Ayub, Zaib-un-Nisa**, **Anza Javaid** and **Nida Batool**. Special thanks to Tehseen and Tania for their guidance and support and to bear my jibber-jabbering through the entire research phase.

No acknowledgement could ever adequately express my obligation to my **PARENTS** and **SIBLINGS** whose endless prayers and support gave me audacity and hope. They are my nearest surrounds and have provided me with a strong love shield that always surround me and never let any sadness enter inside. May Allah the Almighty bless them with good health and prosperous long life.

UM-E-HABIBA

Contents

LIST OF ABBREVIATIONS

dw	Distilled Water
NA	Nutrient Agar
SS	Salmonella Shigella Agar
XLD	Xylose Lysine Deoxycholate Agar
BGA	Brilliant Green Agar
TSA	Tryptic Soy Agar
LB	Lauria Bertani
OD	Optical Density
PBS	Phosphate Buffered Saline
Soln.	Solution
Spp.	Species
Subsp.	Subspecie
CFU/g	Colony Forming Unit per gram
rRNA	Ribosomal Ribonucleic Acid
DNA	Deoxyribonucleic Acid
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
APHA	American Public Health Association
FAO	Food and Agricultural Organization
QS	Quorum Sensing
AIs	Autoinducers
AHLs	Acyl Homoserine Lactones
ISO	International Organization for Standardization

USDA	U.S. Department of Agriculture
EU	European Union
CDC	Center for Disease Control and Prevention
WHO	World Health Organization
FDA	U.S. Food and Drug Administration

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ABSTRACT

Consumption of *Salmonella*-contaminated foods, such as poultry and fresh eggs, is known to be one of the main causes of salmonellosis. The aim of the study was to identify *Salmonella spp.* in freshly cut and frozen chicken meat to protect consumer's health and investigate effect of temperature and quorum sensing in *Salmonella* and microbial contamination within chicken meat in Islamabad. Samples were analyzed under low temperature (4 °C) storage time and conditions to predict the potential growth and survival of *Salmonella* in meat. *Salmonella* counts were observed to increase after 48 h. Heterotrophic plate count (HPC) in frozen chicken meat (6-7.71 log CFU/g) was low as compared to that of freshly cut chicken meat (8.1-11.07 log CFU/g) samples. Microbial and *Salmonella* counts within chicken meat samples was low in frozen chicken meat however both types of meat exceeded World Health Organization (WHO) 3 log CFU/g and International Commission on Microbiological Specifications for Food Standards (ICMSF) permissible limit of 10^3 CFU/g. D-values for *Salmonella* cocktail were 4.05, 3.28, 1.89, 1.65 and 1.38 min at 50, 55, 60, 65 and 70 °C respectively and obtained Z-value was 40.7 °C. All *Salmonella spp.* isolated from chicken meat lack AI-1 mechanism but biofilm formation during crystal violet assay showed that QS is via AI-2 mechanism. The maximum biofilm formation ability was 0.321 ± 0.001 and 0.318 ± 0.001 by *Salmonella kentucky* and *Salmonella Heidelberg* respectively.

Chapter 1

INTRODUCTION

1.1 Background

Contaminated food is the root cause of outbreak of many diseases. With increasing global trade in past century, occurrence of such illnesses has also increased. These widespread food-borne diseases lead to high rates of morbidity and mortality, affecting both developed and developing countries (Kuchenmüller *et al*., 2013).

It is difficult to determine global incidences of food borne diseases, yet researchers generally agree that estimated percentage of population suffering from such illnesses each year could be upto 30% in developed countries and could be even worse in developing countries (Germini *et al*., 2009). Typhoid fever, tuberculosis and cholera were some of the most common diseases a hundred years ago but at present day, vomiting, diarrhea or bloody diarrhea, headache, abdominal pain, fever, chills, blurred vision, numbness of skin, weakness, dizziness and paralysis, Reiter's syndrome, shigellosis and botulism etc. are common illnesses caused by contaminated food and water. The causative agents that cause such illness include *Campylobacter*, *Salmonella*, *E. coli O157:H7*, *Shigella*, *Clostridium botulinium* etc.

Reports from Centers for Disease Control and Prevention, USA revealed that 90% of all illnesses are due to these pathogens and *Salmonella* alone affects about 1.4 million people each year in United States with about 16,000 hospitalizations and more than 500 deaths annually. The U.S Department of Agriculture (USDA) Economic Research Service evaluated total costs for medical care and lost

productivity, resulting from food-borne *Salmonella* infections and it was between 3.5-6 billion dollars annually. They concluded that production cost was high because of control measures like biosecurity practices, cleaning and disinfecting of facilities, rodent control programs, testing and vaccination.

Hence *Salmonella* serotypes are major cause of food-borne diseases with various sources such as raw or undercooked eggs, meat, milk, raw fruits and vegetables and contaminated water. But among them, poultry is considered as one major source of *Salmonella* (Salehi *et al*., 2005).

Poultry is a term given to domestic fowls particularly raised for food and laying eggs such as chickens, turkeys, ducks and geese. But chicken meat is considered as the most common poultry meat. It is very popular food around the world and its consumption has been increased during past decade. Currently, 30% of world's meat is comprised of chicken meat (del Rio *et al*., 2007).

The low cost, low fat content and high nutritional value makes chicken meat preferable and desirable among consumers (Uzmay *et al*., 2013). However, frozen meat is getting popularity among consumers these days because of high degree of safety, same nutritional value, sensory quality and convenience. Additionally, this type of meat is considered to have less microbial population as compared to that of freshly cut meat. Yet chicken meat is a perishable food that provides ideal medium for microbial growth as is highly nutritious has a favorable pH and normally lightly salted or not salted at all (Aral *et al*., 2013; Elnawawi *et al*., 2012; El-Rahman *et al*., 2010; Karaboz and Dincer, 2002).

The chief importance for industry, consumers, and public health officials is microbial safety of meat and poultry products because associated foodborne diseases

remains a great concern worldwide in terms of economic losses and health effects (Bohaychuk *et al*., 2006; Hernández *et al*., 2005). It is considered that consumption of poultry products is a vehicle for transmission of foodborne pathogens such as *Campylobacter jejuni*, *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* and *Shigella* (Sackey *et al*., 2001). The relative risk of foodborne diseases transmission through poultry is high since contamination with different pathogenic organisms may occur at several production levels (Moore, 2004).

The contamination of chicken meat by *Salmonella spp.* is the goal of constant research and control in several countries due to high prevalence, health risks to consumers and economic costs. *Salmonella spp.* is present in environment, and around 2,300 different serotypes of this microorganism may contaminate intestines of animals, water and foods in general. The list of the most susceptible foods to contamination by *Salmonella spp.* is long, and includes meats in general (Fortuna *et al*., 2012).

According to FAO food quality standards, there should be no *Salmonella* contamination in 25 g of chicken meat. In developing countries, *Salmonella enterica* serotype *typhi* isolated from both freshly cut and frozen chicken meat is most common for public health problems (Jyoti *et al*., 2010).

1.2 Significance of the study

There are about 80% food poisoning pathogens while 50% food poisoning cases are reported by *Salmonella*. Thus it is an important cause of enteric fever, gastroenteritis, and septicemia. A number of local and international surveys revealed presence of *Salmonella* in various foods. This problem has been increased in

countries like Pakistan with poor meat storage due to interruption of electricity; a very common issue in country. Hence there is a need for data to provide awareness about nature and magnitude of problem to improve food safety during slaughtering and production processes, transportation, storage and retail sale. Furthermore, this study will help to determine either freshly cut and frozen chicken meat is the most contaminated with *Salmonella* or various *Salmonella* serovars are to be commonly found in chicken meat.

1.3 Objectives

The objectives of study were:

a. Estimation of bacterial load and isolation of *Salmonella* from chicken meat

b. Species identification of isolated *Salmonella*

c. Determination of biofilm formation ability of microbial strains

<div align="right">

Chapter 2

</div>

LITERATURE REVIEW

Key focus of this chapter is to provide the detailed information related to the food borne illness, contamination of fresh and frozen chicken meat and importance of food safety.

2.1 Overview of *Salmonella*

2.1.1 Historical perspective

In 1880, rod-shaped organisms in lymph nodes and spleens of typhoid patients were observed by Karl Joseph Eberth. He suggested it as *Eberthella typhi* and is credited with uncovering of serovar *Typhi* organism (Eberth, 1880). The first positive cultivation of *Salmonella* serovar *typhi* was achieved by George Gaffky from German patients in 1884 (Hardy, 1999). In 1885, *Salmonella* was originally described by a technician named Theobald Smith; however, it was named after the technician's research leader, Daniel Elmer Salmon who was a veterinarian (FDA/CFSAN, 2008). Originally, the organism was called "*Bacillus choleraesuis*," which was changed to "*Salmonella choleraesuis*" by French scientist Joseph Leon Lignieres in 1900 (Hui Su and Chiu, 2007). Over the decades of time, Salmonella was isolated from both animals and plants (Getenet, 2008).

2.1.2 Taxonomy

Salmonella is a gram negative, mostly non-lactose fermenter, facultative, anaerobic, non-spore forming, rod-shaped, mesophilic heterotrophs that produce acid and gas from glucose; belongs to the *Enterobacteriaceae* family (Karmi, 2013; Al-Mogbel *et*

al., 2015). *Salmonella* has more than 2500 serotypes (Barbour *et al*., 2015; Medeiros *et al*., 2014; Akbar and Anal, 2014; Hsieh, 2014; Fashae *et al*., 2010; Nagappa *et al*., 2007; WHO, 2005). It is divided into two species, *enterica* and *bongori*. *Salmonella enterica* is further divided into six subspecies*: Salmonella enterica* subsp. *enterica*, *Salmonella enterica* subsp. *salamae*, *Salmonella enterica* subsp. *arizonae*, *S*almonella *enterica* subsp. *diarizonae*, *Salmonella enterica* subsp. *houtenae* and *Salmonella enterica* subsp. *Indica* (WHO, 2003a). Among them *Salmonella enterica* serovar *typhimurium* and *Salmonella enterica* serovar *enteritidis* are the most common and isolated serovars (Jamshidi *et al*., 2009; Kaushik *et al*., 2014).
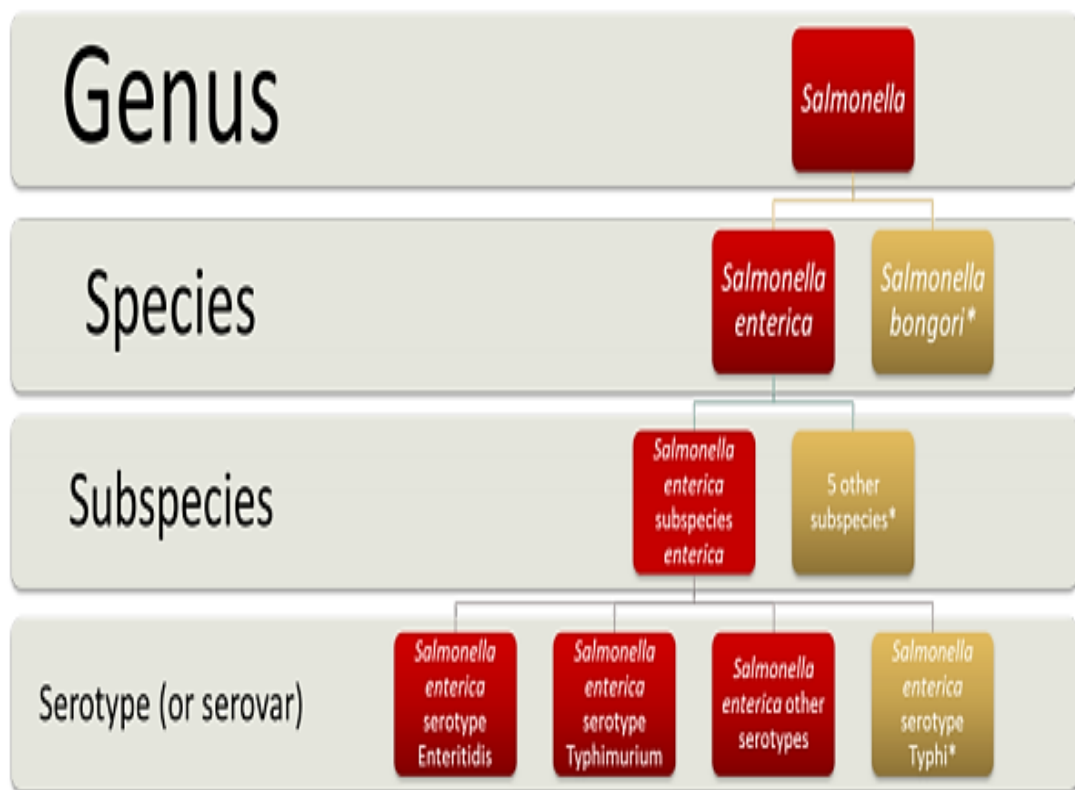
Fig. 2.1: Nomenclature within the *Salmonella* genus (Bedale and Milkowski,

2015)

*Salmonella **categories not typically associated with foodborne disease are denoted with asterisks

2.2 Situation of Salmonellosis

During the last two decades, food borne diseases has emerged as an important and serious growing public health and economic problems in many countries of the world (Rocourt *et al*., 2003; Hur *et al*., 2011). Hendriksen (2010) conducted a research study that food-borne diseases are causing illness up to 76 million people in the United States annually. If these data were extrapolated, this would equal one fourth of the people affected in the developed world per year.

Salmonellosis is the leading disease worldwide (Cheng *et al*., 2008). Recent studies estimated that there are about 1.3 billion cases of salmonellosis worldwide with 3 million deaths annually (El Hussein *et al*., 2010) and is imposing serious threats to human health (EFSA, 2010). In EU alone, 192,703 incidences of salmonellosis were reported (Forshell and Wierup, 2006). Presently, incidences of non-typhoidal salmonellosis have doubled in United States. Hendriksen *et al*. (2011) isolated 15 serovars of *Salmonella* from humans from 37 different countries. They found out 43.5% *S. enteritidis* and 17.1% *S. typhimurium*. Interestingly, serovars reported from developed countries depicted consistency in their spectrum and distribution over the years as compared to that of developing countries. Some of the incidences of salmonellosis in different part of the world are shown in Figure 2.2.

Norway (2006)
Salmonellosis
notification rat: 39.1
per 100,000 population
(Norwegian Instaitute
of Public Health, 2007)

Pakistan (2008)
Typhoid fever
incidence rate:
451.7 per 100,000
population (Kothari
et al., 2008)

China (2008)
Typhoid fever
incidence rate:
15.3 per 100,000
population
(Kothari et al.,
2008)

Vietnam (2008)
Typhoid fever
incidence rate: 21.3
per 100,000
population (Kothari et
al., 2008)

United States
(2006)
Salmonellosis
notification rate:
13.6 per 100,000
population
(Hindriksen,2010)

Malaysia (2006)
Typhoid and
paratyphoid fever
incidence rate: 0.77
per 100,000
population (Hamdan
et al., 2008)

California
(2008)Typhoid
fever incidence
rate: 0.21 per
100,000
population
(CDPH, 2008)

Singapore (2006)
Salmonellosis
notification rate: 4.7
per 100,000
population
(Ministry of Health
Singapore, 2008)

India (2008)
Typhoid fever
incidence
rate:214.2 per
100,000
population
(Kothari et al.,
2008)

Australia
(2006)
Typhoidnotific
ation rat: 39.1
per 100,000
population
(Queensland
Health, 2006)

Sauth Africa
(2006)
Salmonellosis
notification rate:
1.9 per 100,000
population(Hindrik
sen,2010)

Ind
(2008)Typhoid
fever incidence
rate: 81.7 per
100,000 population
(Kothari et al., 2008)

New Zealand (2009)
Salmonellosis
notification rate:
26.2per 100,000
population (ESR,
2009)

Fig. 2.2: Rate of enteric fever and salmonellosis in different parts of the world

(Pui *et al*., 2011)

2.2.1 Salmonellosis in Pakistan

Salmonella infection is mostly common in those countries that have poor standards of hygiene in food preparation, handling and lack of sanitary disposal of sewage. It mainly occurs in tropics, sub-tropics in Africa, India, Pakistan, South East Asia and South America (WHO, 2003b; Senthikumar and Prabakaran, 2005).

Poultry is one of the reasons of salmonellosis in Pakistan and preventive measures are taken to implement hygienic conditions in this industry (Soomro *et al*., 2010). It is the widest spread food borne disease in the whole world but the cases are more common in developing countries like Pakistan. According to WHO, Pakistan is

included in those countries that have the highest incidences of *Salmonella* i.e., 412 per 100,000 person per year (Raza *et al*., 2014; Sultan *et al*., 2013).

An average of 14% and 10% samples were positive for *Salmonella enteritidis* and *Salmonella typhimurium* from 100 suspected patients. It was then concluded that poultry meat and eggs played a vital role in transmission of *Salmonella enteritidis* and *Salmonella typhimurium* to those affected persons and cause food borne diseases like diarrhea, fever and vomiting etc. (Younus *et al*., 2011). Nusrat *et al*. (2012) conducted a study in which 11.1% of the food samples were positive for *Salmonella* in peri urban areas of Rawalpindi and Islamabad. Pakistan Institute of Medical Sciences (PIMS) observed that about 600 *Salmonella* infected patients were admitted to hospitals of Rawalpindi and Islamabad in January 2014. Among them 290 cases were related to gastrointestinal illness while 310 were related to *Salmonella* induced typhoid fever (Mehmood *et al*., 2014).

Albeit the specific data of food borne diseases associated with *Salmonella* in poultry meat is limited yet it is considered to be an important issue. Because it has not only affected the human and animal health but also has a negative impact on economy and trade of a country. In recent past, the contamination of chicken meat led to the rejection of large consignments of raw poultry meat (WHO, 2009; FAO, 2009). It causes negative economic impacts due to surveillance investigation, illness treatment and prevention.

The determination of prevalence of microorganisms is one of the basic steps to control safety and quality of food. It is necessary to meet safety standards of food products and risk management by developing new, fast and reliable identification methods for the minimization of bacterial contamination.

2.2.2 *Salmonella* in nature

Salmonella is found in water, soil, vegetation and normal intestinal flora of many animals, including humans. The primary habitat is the intestinal tract of animals such as birds, reptiles, farm animals and humans. It has been isolated from water, soil, plant surfaces, animal feces, eggs, raw meats, raw poultry, and raw seafood (Maqsood, 2012).

Steneroden *et al*. (2011) estimated the prevalence of *Salmonella* in animals in Colorado. Among thirty two animals, 28% were positive for *Salmonella* contamination. The study hypothesized that increased level of *Salmonella* in those areas is because of presence and proximity of livestock facilities such as beef feed lots or dairy operations.

The surveys conducted by WHO (2013) proved that domestic and wild animals have *Salmonella*. It occurs in food animals, for example, poultry, cattle; and in pets, like birds, cats and dogs. *Salmonella* contamination in the food-chain may occur at any point from crop, farm, livestock feed, through food manufacturing, processing and retailing, as well as during food preparation at home, in restaurants and canteens (Aldapa *et al*., 2012) and becomes the reason of food borne diseases.
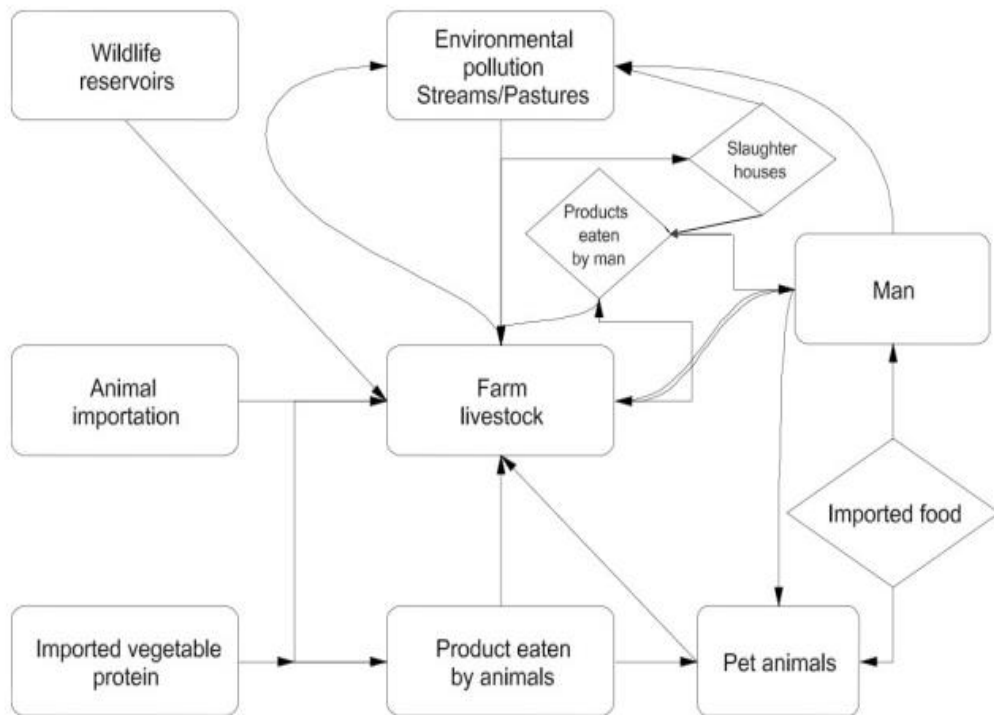
Fig. 2.3: *Salmonella* transmission cycle (Giaccone, 2012)

2.3 *Salmonella* contamination in chicken meat

2.3.1 Chicken meat: a source of valuable nutrients

Poultry is a significant industry because of the requisite of securing food supply for increasing population in terms of quality and quantity, need of animal proteins, health problems due to nutrition, customers' awareness to maintain a healthy and balanced diet. Albeit there is reduction in demand and fall in prices due to the economical crisis and last bird flu epidemic but still it is a growing sector (Aral *et al.,* 2013) as it has many advantages over other meats.

Nutritionally, chicken meat is a source of proteins, vitamins and minerals, and has a relatively low fat content (Bošković *et al*., 2010; Grashorn, 2007). In addition, fat present in chicken meat is of unsaturated type, which protects from heart diseases

(Farrell, 2015). It is also a good source of Vitamin B3 (niacin) helps in metabolism, B6 essential for immune system and blood sugar level, Vitamin B7 (biotin) helps cell growth and B12 is involved in nerve cells and red blood cell maintenance. It contains zinc which protects immune system and DNA synthesis as well (NCC, 2012).

2.3.2 Consumption and contamination of fresh and frozen chicken meat

The average consumption of chicken meat is 12.5 kg while in 2013, it was about 95,156,000 tons worldwide. By continents, the highest consumption rate is 39% in Asia, 38% in America, 18% Europe and 5% Africa between 2008-2010 (Uzmay *et al.*, 2013). Meanwhile in Pakistan, annual production of chicken meat is 953.600 metric tons and consumption is only 5.5 kg (Pakistan Poultry Association, 2015).

The chicken meat consumption is affected by a number of factors that has an important place in human nutrition. These include income level, socio economic and demographic factors, seasons, food quality, personal habits, consumer preferences and product price that affects demand for chicken meat. In countries like Slovenia, Germany and Belgium, researches were conducted to determine the tendency of consumption of chicken meat. The results depicted that country of origin, frozen or fresh meat preference, product's brand plays a vital role in purchasing process (Aral *et al.,* 2013).

Infections caused by *Salmonella* are about >30 million per year. In 2012, CDC reported that *Salmonella* is the leading cause of bacterial foodborne. Fig 2.4 depicts the percentage contamination of *Salmonella* in different foods.
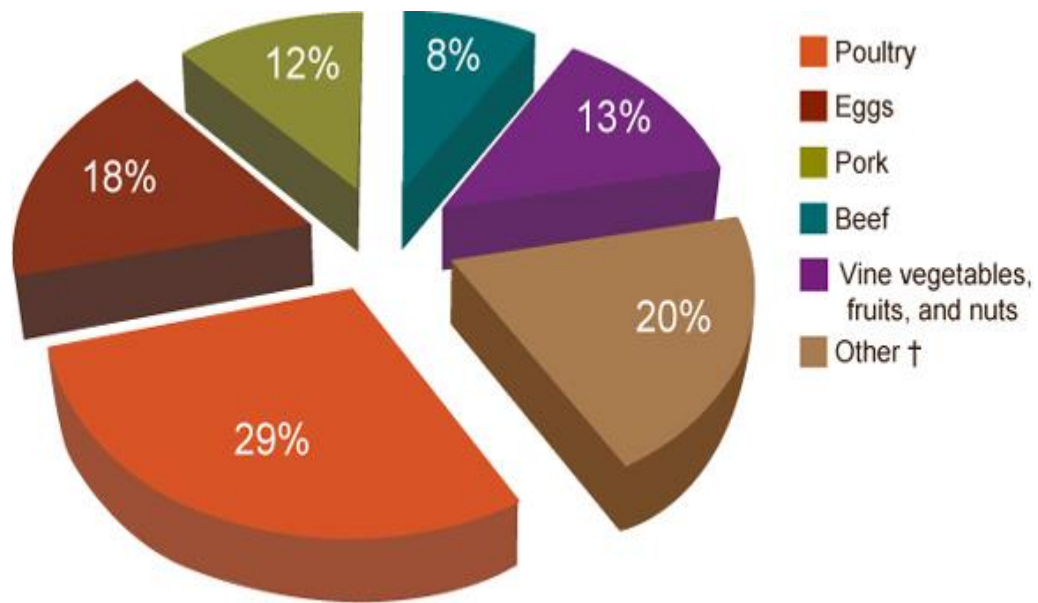
Fig. 2.4: Foods associated with *Salmonella* outbreak (FDA/CDC, 2010)

Where other includes: leafy greens, fish, roots, grain beans, shell fish, oil sugar and dairy

The report collected cases from 2008 (as it was the most recent year on which data was available) and concluded that major cause of *Salmonella* outbreak was poultry (Gould *et al*., 2011) and consumption of poultry products especially chicken meat (Tennant *et al*., 2015; Thai *et al*., 2012).

Now a day, frozen chicken meat is getting popular because of urbanization, hurried way of living and changing life style of individual (Biesalski, 2005). The production and consumption of frozen poultry product has increased because of its high demand on different occasions and events (Nwachukwu and Nnamani, 2013). Ready-to-eat (RTE) food is a great risk to consumer health due to the presence of *Salmonella* in it. Although, level of *Salmonella* in such foods must be 0 Cfu/ml but reports proved that RTE exceeds these limitations. Handling, processing and storage are some of the factors that affect RTE foods (Roy *et al*., 2011). There is a very limited data related to the prevalence of *Salmonella* in frozen chicken meat.

Recently, frozen chicken meat has been identified as a risk factor for *Salmonella* (Currie *et al*., 2008). Within the last decade, it was reported that salmonellosis outbreaks in Australia, British Columbia - Canada (MacDougall *et al*., 2004) and Minnesota – United States are due to raw, frozen chicken nuggets and strips. These products seem to be cooked and thus make them potentially dangerous for consumers; who only reheat them before consumption according to epidemiological investigations of recent outbreaks (Smith *et al*., 2008).

Adeyanju and Ishola (2014) performed a study on frozen samples obtained from Ibadan, Oyo state and compared with commercial Nigerian-registered poultry company having a broiler processing plant. Out of one 152 frozen meat samples, *Salmonella* was identified from 99 frozen meat samples. El Nasri *et al*., (2015) conducted a research in which samples were collected randomly from different markets situated at Kharoum state. Different microbes like *E.coli* (63%), *Salmonella* (10%), *Staphylococcus aureus* (7%) were ovserved. Moreover out of 70 samples, it was observed that 30 whole carcass and 40 cut samples exceeded permissible limit for frozen meat.

Dominguez and Schaffner (2009) identified frozen chicken meat as a source of Salmonellosis. About 8 Salmonellosis outbreaks were related to undercook frozen chicken nuggets, strips and entrees from 1998-2008. It was then concluded that if the frozen poultry product are improperly cooked it may pose an infection risk.

Hassanein (2011) undertaken a study to determine prevalence of *Salmonella*. The total 75 samples including frozen chicken fillets and leg pieces bought from super markets in Assiut, Egypt were collected and experiment was performed. From 75 samples, *Salmonella* was detected in 9 (36%) of frozen chicken leg and 13 (52%) of frozen chicken fillet. *Salmonella entrica* subsp. *entrica* serovar *enteritidis* and

Salmonella entrica subsp. *entrica* serovar *kentucky* were found to be the two dominant serotypes of *Salmonella*. The public health related to *Salmonella* and suggestive measures were given to protect the consumers and improve quality of chicken meat and its products.

Taking into account these studies, it is obvious that high level of *Salmonella* is present in both fresh and frozen chicken meat and becomes a challenge for for food industry. However, this contamination may be due to the cross contamination or from bacteria whose presence is to be believed in processing premises.

2.4 Effect of temperature on removal of bacterial contamination

Removal of bacterial contamination in chicken meat is a challenge for food industry as it is highly perishable and promotes the growth of microbes especially *Salmonella* because of favourable conditions like pH, moisture content etc. For the elimination of Salmonella from meat, several disinfectants have been used in past such as 1% sodium hypochlorite, 70% ethanol, 2% glutaraldehyde, iodine-based disinfectants, phenolics and formaldehyde but Beatriz and coworkers (2012) reported that conventional disinfectant techniques are not significant for the complete removal of this microorganism.

Among several handling practices during cooling, raw cooked food, cleaning and cooking, cooking plays a vital role in controlling food-borne disease (Kennedy *et al*., 2011; Luber, 2009; Medeiros *et al*., 2001; Taché and Carpentier, 2014). But about 30% population of world consume undercook meat (Angelillo *et al*., 2001; Phang and Bruhn, 2011). Moreover, 50-80% people think visual inspection is enough and satisfactory to determine either meat is properly cooked or microbes are killed during cooking process (Lazou *et al*., 2012; Redmond and Griffith, 2003);

questionnaires were filled during different surveys and it was concluded that visual indication played a vital role for 93% of consumers (Redmond and Griffith, 2003; Sampers *et al*., 2012).

The recommended temperature for *Salmonella* is 68-70 °C. Nevertheless, time-temperature required to reduce the *Salmonella* level depends upon many factors such as heat resistance of microorganism, transfer rate of heat and distribution of *Salmonella*. Product composition is also very important for the effectiveness of heat treatment on *Salmonella* (fat content, NaCl, pH and water activity) and geometry (volume and size) (Roccato *et al*., 2015; Bermudez-Aguirre and Corradini, 2012; de Jong *et al*., 2012; Silva and Gibbs, 2012; Juneja, 2007; NACMCF, 2007; Juneja and Eblen, 2000).

2.5 Quorum sensing

It was said that bacteria have the basic and simplest mechanism but recently Moghaddam and coworkers (2014) provided interesting and complex behavior that is similar to that of multicellular organisms. This mechanism is controlled by quorum sensing that facilitates intra and inter species communication, which involves in terms of biofilm formation and stress environmental conditions etc.

Quorum sensing coordinated processes like spoilage of food and other infections, which are regulated by the activity of small molecules called autoinducers (AIs). To activate the process of QS, it is necessary for AIs to reach a certain threshold. The expression of a variety of genes is modulated by AI receptors that control the behavior of bioluminescence, sporulation, conjugation, swarming motility and biofilm formation (Annous *et al*., 2009; Skandamis and Nychas, 2012; Solano *et al*., 2014; Wu *et al*., 2014).

Gram negative bacteria use primary signals to sense cell density known as acyl-homoserine lactone (AHL) molecules, composed of a homoserine lactone ring (HSL) with an acyl chain (Figure 2.5). Generally, acyl-chain length varies from C4 to C18 (Marketon *et al*., 2002) and this can be modified by a 3-oxo substituent, or in some cases, a 3-hydroxy substituent, a terminal methyl branch, or varied degrees of unsaturation (Thiel *et al*., 2009). AHL synthases are the enzymes that synthesize AHLs. Once produced, the AHLs diffuse in and out of the cell by active and passive transport mechanisms. The concentration of AHL ultimately reaches a sufficiently high concentration at a given threshold cell number or bacterial "quorum", and it is then recognized by a receptor protein, which is the second component of the system. The AHL-responsive receptors include a wide variety of transcriptional regulators called "R proteins", such as LuxR or LasR (Churchill and Chen, 2012). The binding of the AHL by most of the characterized R-proteins initiates the activation and repression of target genes, and in some cases AHL binding leads to target gene derepression (Wagner *et al*., 2003; Schuster *et al*., 2003; von Bodman *et al*., 2003).
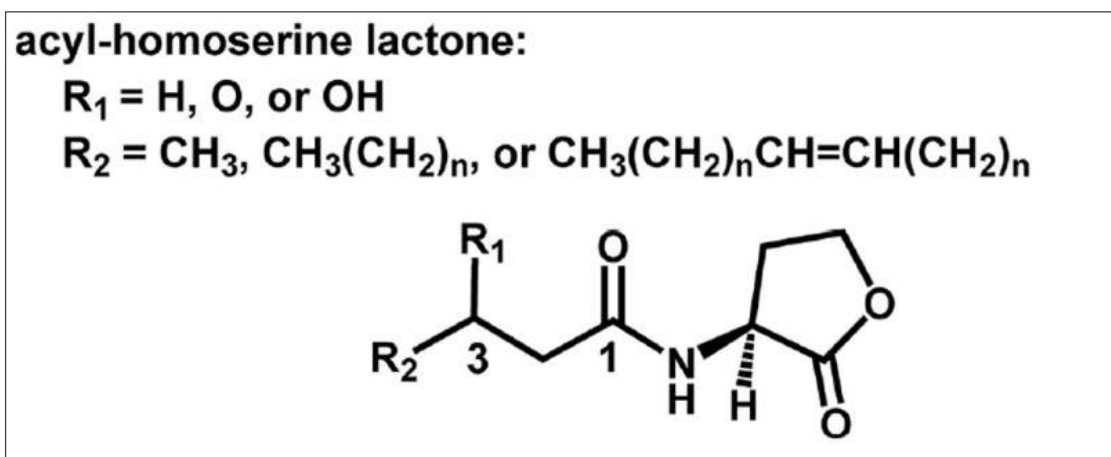
Fig. 2.5: Structure of acyl homoserine lactones (AHLs) (Churchill and Chen, 2012)

The acyl-homoserine lactones (AHLs) found in Proteobacteria vary by substitution at the C3 position (R1) and the length and unsaturation at the C1 position indicated by R2.

2.5.1 Biofilm formation in food industry

The group of bacteria which assembled to form a slimy mass over any surface is known as biofilm (Tiwari *et al*., 2016). The process of biofilm formation has been a concern for food industry including dairy processing, sea food processing, poultry and meat processing; hence a critical hindrance to the food preservation system (Srey *et al*., 2013; Solano *et al*., 2014). Researches are trying to gain insight associations between biofilm formation, persistence ability and virulence of foodborne pathogens (Hanna and Wang, 2003).

It is widely accepted that in a biofilm, the cells are enclosed in matrix which forms multiple layers. The estimated composition is about 15% cells and 85% matrix by volume (Agle, 2002) protected by extracellular polysaccharides and proteins (Sutherland, 2001; Stewart and Costerton, 2001), extracellular DNA, and dead cells (Webb *et al*., 2003; Yarwood, *et al*., 2004). In the case of foodborne pathogens of interest, such as *S. typhimurium* and *E. coli*, cellulose has been shown to be a crucial component of the extracellular matrix (Zogaj *et al*., 2001; Solano *et al*., 2002). L. monocytogenes produces extracellular polymeric substances (EPS) and can readily use EPS produced by other bacteria species to form biofilms (Hanna and Wang, 2003).

Recent scientific reviews (Høiby *et al*., 2010; Bridier *et al*., 2011) have scrutinized latest findings regarding resistance of bacterial biofilms to disinfectants. Altogether, elucidated biofilm conditions and exposure of bacterial cells to concentration gradients of disinfectants has been hypothesized to trigger various processes within bacterial cells, namely, adaptation responses of specific phenotypes, upregulation of bacterial genes involved in the oxidative stress response, efflux pumps, and cell-to-cell communication (quorum sensing) mechanisms. Furthermore, biofilms may

constitute an optimum environment for bacterial cells to exchange genetic elements at an increased rate, possibly allowing for the acquisition of new genes for antibiotic or biocide resistance, virulence, and other environmental survival abilities (Watnick and Kolter, 2000). In short, it is believed that once bacteria adhere to a surface and form biofilm, they become more resistant to cleaning and sanitation treatment and removal strategies; furthermore, cells detaching from the biofilm could further turn into the source of persistent contamination (Chae and Schraft, 2000; Hanna and Wang, 2003). It remains unclear how the interactions that take place in multi-species biofilms might contribute to synergistic relationships among bacterial species.

2.5.2 Biofilm formation in *Salmonella*

There were several single culture experiments that provide evidences that *Salmonella* has ability of biofilm formation on materials commonly found in food processing environment. Jun *et al*. (2010) evaluated microbial biofilms on common food contact surface materials including stainless steel, white high-density polyethylene, formica-type plastic, and polished granite. The authors reported that *Salmonella* adhered and grew well on stainless steel, high-density polyethylene, and granite.

Joseph *et al*. (2001) studied sensitivity of hypochlorite and iodophor of the biofilm cells of *Salmonella* isolated from poultry. Biofilm was observed on plastic, cement and stainless steel and cells were exposed to sanitizers at different concentrations. Biofilm cells on stainless steel were most sensitive to the sanitizers whereas those on plastic were most resistant.

Stepanović *et al*. (2004) experimented on 122 *Salmonella* spp. strains, isolated from humans, animals or food, to determine the effect of the growth media on biofilm formation. The suitable media supported production of biofilms of all tested strains.

Stepanović *et al.* (2003) suggested that the best environment for *Salmonella* biofilm formation is microaerophilic and CO_2-rich conditions and is resistant to dry conditions. Iibuchi *et al.* (2010) evaluated that *Salmonella* survived on polypropylene discs under desiccation conditions and may survive for more than 200 days at 28°C. It concluded that strain with high biofilm productivity can survive under dry conditions longer as compare to that of low biofilm productivity.

Milan and coworkers (2015) studied *Salmonella enterica* subsp. *enterica* isolated from chicken meat to determine biofilm formation ability on the surface of different equipment that is used in food processing industries. A total of 20 isolated strains were used for this purpose. The results showed that *Salmonella* has ability to form biofilm on aluminum, polyethylene and glass surfaces and other commonly used materials and equipment in industry. When concentration of these microbes reached to higher level than the disinfection techniques used for the elimination or reduction of this strain is less effective.

2.6 Related work done at IESE, NUST

A study has been done at the Institute of Environmental Sciences and Engineering (IESE) regarding environmental microbiology, especially for the food microbiology and *Salmonella*. This includes:

Author	Study	Microbe	Findings
Khaula Aisha Batool, 2014	Distribution of *Salmonella typhimurium* in environemental samples	*Salmonella enterica* serotype *typhimurium*	HPC exceeding WHO permissible limit 90 % lettuce contaminated with *Salmonella*

Chapter 3

MATERIALS AND METHODS

The purpose of this chapter is not only to describe the experimental setup of present study but to also confirm either contamination of chicken meat is one of the pathways for the spread of foodborne illness. The chicken meat was selected as a model because of easy access, availability round the clock, essential for human diet and favorable for growth of foodborne pathogens especially *Salmonella*.

3.1 Study area

Samples were collected from different areas and the reasons of selecting current study areas are their presence at road side, overly crowded areas, availability of plentiful offices and food chain restaurants. Moreover it provide chicken meat to majority of population of Islamabad.
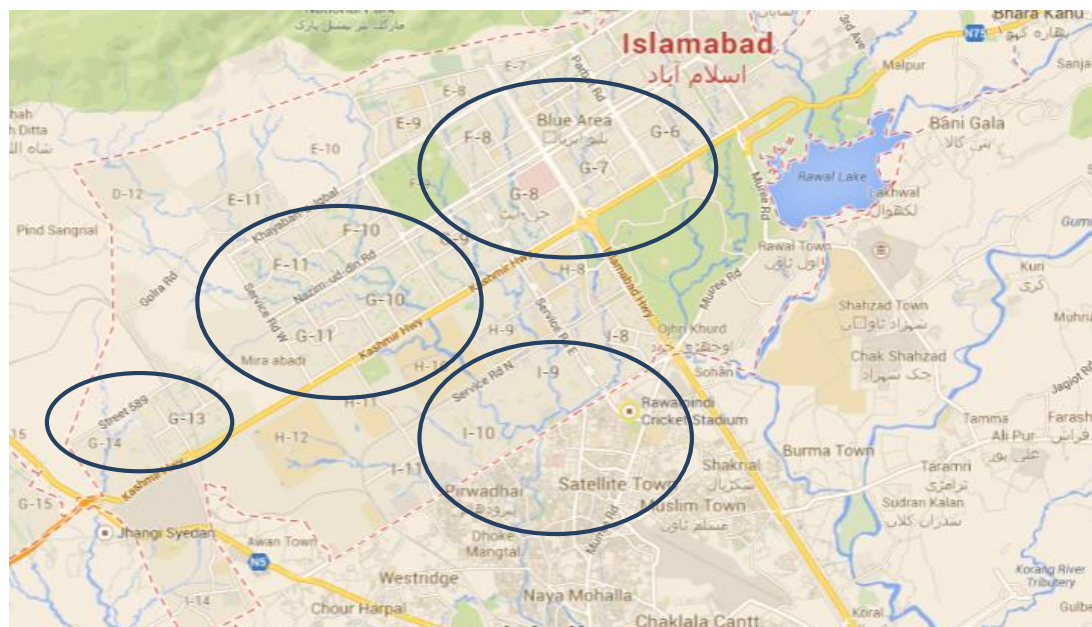
Fig. 3.1: Number of sampling sites

Some of the few sites are shown on above map from where samples were collected.

Table 3.1: Sampling sites

Sampling Sites (Sectors)	No. of samples
F	15
G	10
I	12

3.2 Sample collection

A total of 52 samples including 37 freshly cut and 15 frozen chicken meat samples were collected from local markets and frozen meat's outlets respectively; situated in different sectors of Islamabad, Pakistan. The samples were put into plastic bags, cooled in ice box and immediately transported to laboratory. This study was conducted during a period of six months i.e., from September 2015 to March 2016.

3.3 pH measurement

The pH value of meat samples was determined according to Melo *et al*., 2012. Briefly, 10 g of chicken meat was homogenized in 90 mL of distilled water. The measurements were carried out at 0 (immediately after the transportation of samples to laboratory), 2, 4, 24, 48 and 72 h with the help of pH meter (HANNA HI 2211) and performed in triplicate.

3.4 Microbiological analysis

3.4.1 Preparation of agar plates

For preparation of agar plates, petri plates were autoclaved for 15 min at 121 °C (K-AC-60) and oven dried. Nutrient agar (Oxoid, UK) was used for the experimental setup. The media was prepared as instructed by company. Once media prepared, conical flask was sealed with aluminum foil and autoclaved. Molten agar was then poured in oven dried petri plates in sterile conditions of laminar flow hood (CB-100, Korea) and incubated for 24 h at 37 °C.

3.4.2 Serial dilution technique

10 g of chicken meat sample was homogenized in 90 mL of sterile buffered peptone water and filtrate was collected. Serial dilutions were made up to 10^{-10} as method described in FAO manual (1992). Briefly, by using sterile pipette, 1 mL of sample was transferred into test tube having 9 mL autoclaved distilled water. 1 mL of 10^{-1} dilution was mixed in 9 mL of autoclaved distilled water to from 10^{-2} dilution. 1 mL of 10^{-2} dilution was mixed in 9 mL of autoclaved distilled water to get 10^{-3} dilution. Similarly, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} dilutions were made for each of the chicken meat samples. 0.2 mL aliquot from each dilution was spread on nutrient agar plates with the help of sterilized glass spreader. The agar plates were incubated at 37 °C for 24 h. The plates showing countable colonies (30-300) were taken and counted with the help of colony counter (Suntex CC560, Taiwan).

3.4.3 Isolation of *Salmonella*

Salmonella was isolated from chicken meat samples by following standard method ISO-6579:2007. Briefly, 25g sample of chicken meat was homogenized in 225 mL of buffered peptone water in a sterile sample bottle and incubated at 37 °C for 24 h.

0.1 and 1 mL of pre-enriched sample was added in 10 mL Rappaport-Vassiliadis Soya peptone broth (RVS; Oxoid) and Muller-Kauffmann Tetrathionate-Novobiocin broth (MKTT-n; Oxoid); incubated at 41.5 °C and 37 °C respectively. After 24 h, one loopful from each broth was streaked in Xylose Lysine Deoxycholate agar (XLD; Oxoid), *Salmonella* and *Shigella* Agar (SS; Oxoid) and Brilliant Green Agar (BGA; Oxoid). It was then incubated at 37 °C for 24 h. The plates were scrutinized for the presence of distinctive Salmonella colonies i.e. orange-red colonies with black centers on XLD agar, red colonies on BGA and transparent colonies with black centers on SS agar. The suspected colonies were streaked on nutrient agar plates with further incubation at 37 °C for 24 h.

Non-selective pre-enrichment
25 g food in 225mL of 10% buffered peptone water 37°C, 24 h

Selective enrichment
0.1 mL in 10 mL RV broth 41.5°C, 24 h
1 mL in 10 mL MKTTn broth 37°C, 24 h

Isolation
SS, XLD and BGA agar plates with an inoculation loop 37°C, 24 h

Colony Purification
Selected colonies were streaked on nutrient agar for purification 37°C, 24 h

Biochemical confirmation 37°C, 24 h
A number of biochemical tests to determine the biochemical properties of selected colonies

Fig. 3.2: Horizontal detection method for isolation of *Salmonella* ISO 6579:2007

3.5 Morphological study

Morphology of a colony was investigated by observing their size, color, shape, elevation, margin, texture, odor, opacity and gram reaction using standard technique while gram staining was performed to determine cell morphology.

Table 3.2: Traits to study colony morphology

Sr. No.	Morphological Traits	Description
1.	**Appearance**	Shiny, Dull
2.	**Shape**	Circular, Irregular, Filamentous, Rhizoid
3.	**Elevation**	Flat, Raised, Umbonate (having a knobby protuberance), Crateriform, Convex, Pulvinate (Cushion-shaped)
4.	**Size**	Punctiform, Small, Medium, Large
5.	**Margin**	Entire (Smooth with no projection), Undulate (Wavy), Lobate (Lobed), Filamentous, Rhizoid
6.	**Texture**	Dry, Wet, Thick, Moist, Smooth, Rough, Brittle, Wrinkled, Having concentric rings
7.	**Color**	Green, Pink, Red, White, Off white, Yellow etc.
8.	**Opacity**	Transparent, Opaque, Translucent, Iridescent (Changing colors in reflected light)
9.	**Gram Staining**	Gram positive or negative

3.5.1 Gram staining

Gram staining was performed as per method described in standard method to identify cell morphology (APHA, 2012). It is the most common identification method, depends on cell wall structure. In case of gram negative bacteria, plasma is surrounded by a thin layer of peptidoglycan and an outer layer of phospholipids (Alfred, 2011).

1. 24h fresh colony was spread in 1 drop of dw	**2. Air dried and heat fixed it**	**3. Crystal violet was applied**
4. Washed with distilled water after 1 min	**5. Iodine soln. was applied**	**6. Washed with distilled water after 1 min**
7. Decolorizing agent was used so that smear was decolorized	**8. Then treated with Safranin and left for 1 min**	**9. After 1 min, washed it with distilled water**
10. A drop of oil emulsion was subjected and observed under microscope		

Fig. 3.3: Pictorial view of method of gram staining

3.6 Biochemical confirmatory tests

The selected colonies showed a wide range of difference in their morphology so a person cannot depend on only these tests. Hence for more precise result, following biochemical tests were carried out to differentiate closely related microbes.

3.6.1 Motility test

Hanging drop technique was used to examine cell motility. A drop of distilled water was placed on coverslip, bacterial colony was introduced and mixed gently. Then coverslip was overturned so that smear drop hanged in hollow depression of slide. A drop of oil emulsion was applied and observed under 100X resolution through light microscope (LEICA).

3.6.2 Oxidase test

A loop full inoculum of 24 h fresh colony was placed on filter paper. Then one drop of N, N, N, N-dimethyl-p-phenylenediamine dihydrochloride (Sigma-Aldrich, US) solution was added in it. Appearance of purple color indicated oxidase positive and no color change showed oxidase negative.

3.6.3 Catalase test

A 24 h fresh inoculum of colony was placed on a slide with the help of sterilized wire loop. A drop of 3% hydrogen peroxide was added in it. Appearance of bubbles confirmed that the catalase test was positive.

3.6.4 Simmons citrate

Simmons' citrate agar is used to distinguish gram negative bacteria on the basis of citrate utilization i.e. microbes use citrate as their carbon and energy source. Simmons' citrate agar (Oxoid, UK) was prepared according to manufacturer's instructions, poured in test tubes so that slants were made. It was then incubated at

37 °C. After 24 h, bacterial colony was streaked and again incubated for 24 h at 37 °C. A positive result indicated by growth on slants with an intense blue color.

3.6.5 MacConkey agar

MacConkey agar is selective agar that is used for the identification of gram negative bacteria. It inhibits the growth of gram positive bacteria. For this purpose, MacConkey agar (Oxoid, UK) was prepared by following company's directions. An isolated bacterial colony was streaked and plates were incubated at 37 °C. After 24 h period, colonies turned pink denoted lactose fermenters. It must be noted here that it was the bacteria which ate lactose and turned pink nit the media.

3.7 Molecular characterization

3.7.1 16s rRNA sequencing analysis

Bacteria isolated from carcass and frozen chicken meat was wiped gently with distilled water and inoculum was transferred to eppendorf tubes. Tubes were centrifuged at 2000 rpm for 10 min to separate supernatant. After removal of supernatant, 1 mL of 50% glycerol and 3 mL of 30% nutrient broth were added in it and samples were preserved at -20 °C. For 16s rRNA gene sequencing, preserved samples were sent to Genome Analysis Department Macrogen Inc. Korea.

3.7.2 Phylogenetic analysis

Phylogenetic is the study of relationship between ancestors and descendants. Phylogenetic tree is an evolutionary branched diagram that depicts a projected image; suggests genetic linkage among organisms (Brinkman & Leipe, 2001).

The obtained sequences were analyzed using BLAST search at National Center for Biotechnology Information (NCBI) databases. Once CLUSTALW was used for the alignment of sequences after complete removal of mismatch sequence, MEGA 7 software was used to develop phylogenetic tree. It exhibits phylogenetic connection and linkage of identified bacterial strains with strain selected from GenBank (NCBI).
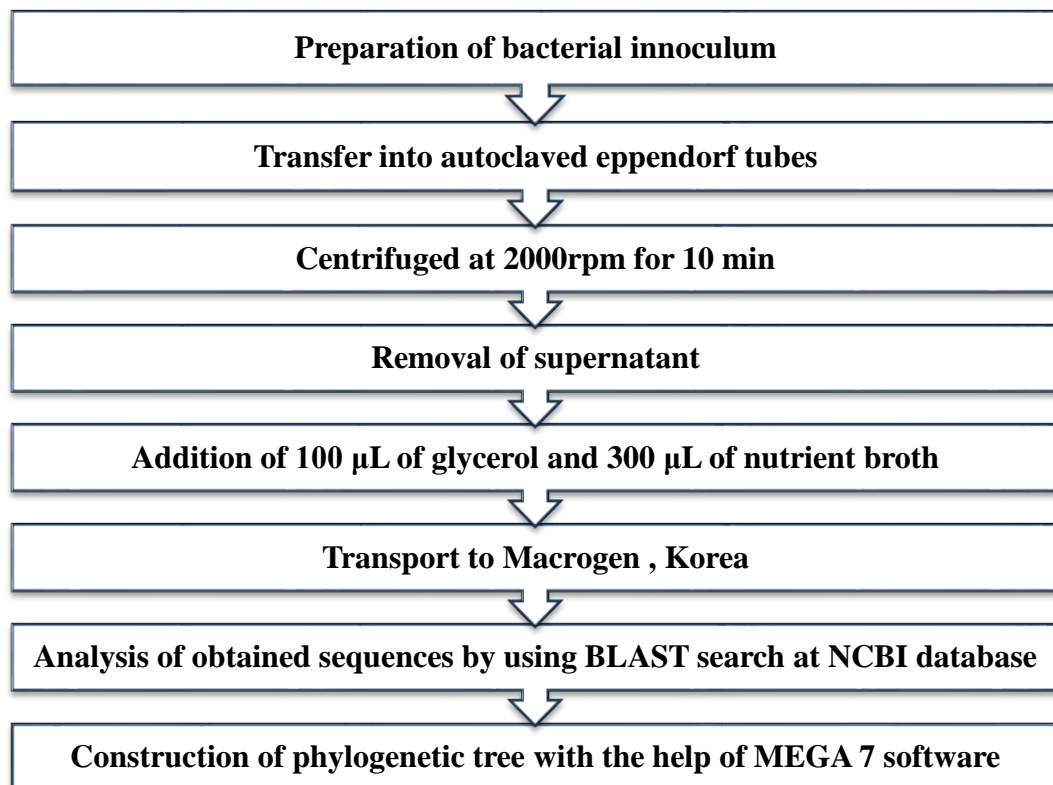
```
┌─────────────────────────────────────────────────────────────┐
│              Preparation of bacterial innoculum              │
└─────────────────────────────────────────────────────────────┘
                              ↓
┌─────────────────────────────────────────────────────────────┐
│            Transfer into autoclaved eppendorf tubes          │
└─────────────────────────────────────────────────────────────┘
                              ↓
┌─────────────────────────────────────────────────────────────┐
│              Centrifuged at 2000rpm for 10 min               │
└─────────────────────────────────────────────────────────────┘
                              ↓
┌─────────────────────────────────────────────────────────────┐
│                    Removal of supernatant                    │
└─────────────────────────────────────────────────────────────┘
                              ↓
┌─────────────────────────────────────────────────────────────┐
│     Addition of 100 μL of glycerol and 300 μL of nutrient broth │
└─────────────────────────────────────────────────────────────┘
                              ↓
┌─────────────────────────────────────────────────────────────┐
│                   Transport to Macrogen , Korea              │
└─────────────────────────────────────────────────────────────┘
                              ↓
┌─────────────────────────────────────────────────────────────┐
│   Analysis of obtained sequences by using BLAST search at NCBI database │
└─────────────────────────────────────────────────────────────┘
                              ↓
┌─────────────────────────────────────────────────────────────┐
│   Construction of phylogenetic tree with the help of MEGA 7 software │
└─────────────────────────────────────────────────────────────┘
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Fig. 3.4: Flow chart of molecular characterization of bacterial isolates

3.8 Effect of temperature on inactivation of *Salmonella*

3.8.1 Organisms

A number of five isolated serotypes of *Salmonella* individually and its cocktail were used. Theses strains were preserved at -20 °C in vials containing tryptic soy broth (Oxoid, UK) supplemented with 10% glycerol.

3.8.2 Culture preparation

Vials were partially thawed at room temperature to propagate the cultures. 0.1 mL of thawed culture was transferred to 10 mL Luria Bertani (LB) broth (Scharlau) and incubated for 24 h at 37 °C. 0.1 mL inocula were used to make two consecutive 24 h transfers. These cultures were maintained in LB broth for 2 weeks at 4 °C.

The inocula used for heating studies, were prepared a day before experiment, by transferring 1 mL of culture to LB broth (100 mL) in 250 mL flasks and incubated aerobically for 18 h at 37 °C, to provide late stationary phase cells.

3.8.3 Thermal inactivation and bacterial enumeration

Thermal inactivation studies were carried out as per methods described by Yadav *et al*., 2016. Stated briefly, the experiment was conducted in a water bath (HH-S6) stabilized at 55, 60, 65 and 70 °C. Time and temperature values were chosen according to preliminary tests. Chicken meat samples inoculated with *Salmonella* serotype cocktail were first transferred in sampling bags containing 20 mL sterile water. Bags for each replicate were removed at different time intervals during thermal treatment and placed into ice slurry until analysis (approximately within 30 min).

Chicken meat samples were transferred to a new filter bag containing 20 mL PBS (pH 7.4) for determination of the number of surviving bacteria and mixed for 2 min. Sterile 0.1% peptone water was used for the preparation of ten-fold serial dilutions and appropriate dilutions were spread on nutrient agar (Oxoid, UK) and tryptic soy agar (Oxoid, UK) plates. When low numbers were expected, 0.1 and 1.0 mL of undiluted suspension were plated. All plates were incubated at 37 °C for 48 h for bacterial enumeration. Two independent experiments were done as a replicate for each temperature.

3.8.4 D-values and Z-values

In microbiology, D-value refers to decimal reduction time and is the time required at a given condition (e.g. temperature), or set of conditions, to kill 90% (or 1 log) of the exposed microorganisms. Thus after a colony is reduced by 1 D, only 10% of the original organisms remain, i.e., the population number has been reduced by one decimal place in the counting scheme. Generally, each lot of a sterilization-resistant organism is given a unique D-value. D-value determination is often carried out to measure a disinfectant's efficiency to reduce the number of microbes present in a given environment.

Z-value is a term used in microbial thermal death time calculations. It is the number of degrees the temperature has to be increased to achieve a tenfold (i.e. 1 log10) reduction in the D-value. The z-value is a measure of the change of the D-value with varying temperature, and is a simplified version of an Arrhenius equation. It is useful when examining the effectiveness of thermal inactivations under different conditions, for example in food cooking and preservation.

3.9 Quorum sensing

3.9.1 Selection of QS bacteria

Genetically modified organisms, *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* A136 (received from Nanyang Technological University, Singapore) were used as biosensors for screening of quorum sensing bacteria responsible for spoilage of chicken meat among all isolated strains.

3.9.2 Fresh culture of A136

1 mL of A136 and CV026 were added in 20 mL LB broth in a test tube. 100 μL spectinomycin and 20 μL tetracycline in case of A136 and 100 μL kanamycin in case of CV026 were added. These test tubes were then incubated at 28 °C to obtain fresh cultures of A136 and CV026.

3.9.3 Bioassay for QS bacteria

Bioassay, consisting of an indicating agar plate and bacteria to be tested, was carried out as per method demonstrated by Waheed *et al*., 2015. In order to prepare indicating agar plate, a fresh culture of CV026 or A136 and LB agar were mixed in a ratio of 1:9. Kanamycin (20 mg/mL) was added in indicating agar plates for CV026, and spectinomycin (50 mg/mL) and tetracycline (4.5 mg/mL) were supplemented along with X-gal for A136 bioassay, respectively. All strains that produced purple or blue pigmentation in presence of CV026 and A136 were considered as QS bacteria.

3.9.4 Analysis of biofilm formation

Microtiter plate assay was used to observe biofilm formation ability of selected strains. Stated briefly, six-well or 96-wells micro plate were filled with LB broth to

perform conditioning at room temperature. After 1 h, wells were emptied, bacterial suspension along with LB agar was added to each well at a ratio of 1:100 ml and sealed with parafilm. Under static condition, plates were incubated at 28 °C for 28, 48 and 72 h.

The quantification of biofilm was determined by using a classical crystal violet assay. The biofilm absorbed the amount of crystal violet was extracted with 200 mL ethanol (95%), per well for 1 h, and OD_{595nm} was assessed with a UV-Vis spectrophotometer. A well containing ethanol served as a control.

3.10 Statistical analysis

The analysis of results was done by using statistical tools (mean, standard deviation and t-test) available on Microsoft Office Excel 2010 package (Microsoft Corporation).

Total bacterial load and Salmonella count were normalized by log transformation before any analysis. T-test was used to determine the significance of difference of total HPC and Salmonella from carcass and frozen chicken meat. Standard errors, variance and mean were calculated using Microsoft office excel 2010. Results of analysis are at $p < 0.05$ level of significance.

BioEdit (Hall 1999) software package was used to obtain consensus sequence and MEGA 7 software was used to generate a phylogenetic tree.

The D-values for each temperature were calculated by plotting log10 number of survivors against time using Microsoft Office Excel Software (Microsoft Corporation). Linear regression was used to determine, with five values in the straight-line portion of plots descending more than or equal to 5 log10 cycles.

The z-values (temperature in °C required to change D-value by 1 log10 scale) were calculated by linear regression of mean log10 D-values versus their corresponding heating temperatures using Microsoft Office Excel Software (Microsoft Corporation).

Chapter 4

RESULTS AND DISCUSSION

4.1 pH value

The pH plays a vital role in growth of microbes as they only grow at a specific range of pH and cannot grow above and below that pH. In present study, the initial average pH of freshly cut chiken meat samples was 6.35 and maximum value of 8.5 was reached on 72 h and in case of frozen samples it was 5.74-6.30. The chicken meat stored under 4°C i.e., aerobiosis, enriched in proteins and free of amino acids shows increase in pH level because it has high rate of proteolytic activity with the passage of days. Thus high the number of microorganisms, more the spoilage of meat occurred (Melo *et al*., 2012). The pH range for the growth of *Salmonella* lies between 4-9.5 (Ordonez *et al*., 2011; Food Safety Authority of Ireland, 2011). Salmonella spp. survival strategies within the host gastrointestinal tract. Microbiology. 157: 3268–3281.. The data in Figure 4.1 shows that sudden increase in pH occurred on 72 h which proved the fact that there was high level of microorganisms resulting in the spoilage of meat.
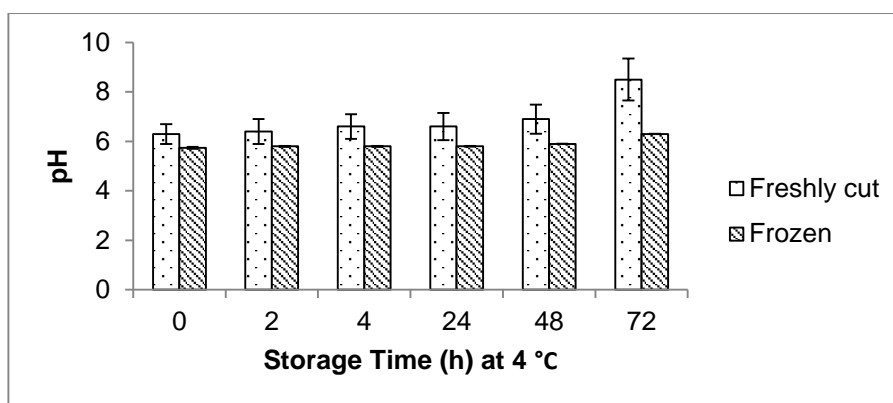
Fig. 4.1: pH value of freshly cut and frozen chicken meat samples collected from different sectors

All pH values of freshly cut meat were significantly higher ($p < 0.05$) than frozen chicken meat (Bradeeba and Sivakumaar, 2013). The frozen chicken meat has low pH (min. 5.74) as compared to that of freshly cut chicken meat (min. 6.3). There are many reasons of it. Firstly organic acids are applied during its processing and storage. Secondly, water is needed for the growth of bacteria but freezing alters the available water into solid ice crystals. This loss of fluid from meat tissue may cause an increase in the concentration of solutes, which results in a decrease in pH (Akhtar *et al*., 2013).

4.2 Microbiological analysis

4.2.1 Total heterotrophic plate count

The total heterotrophic plate count detected in freshly cut samples ranged from 8.1-11.07 log CFU/g while 6-7.71 log CFU/g in frozen chicken meat samples.
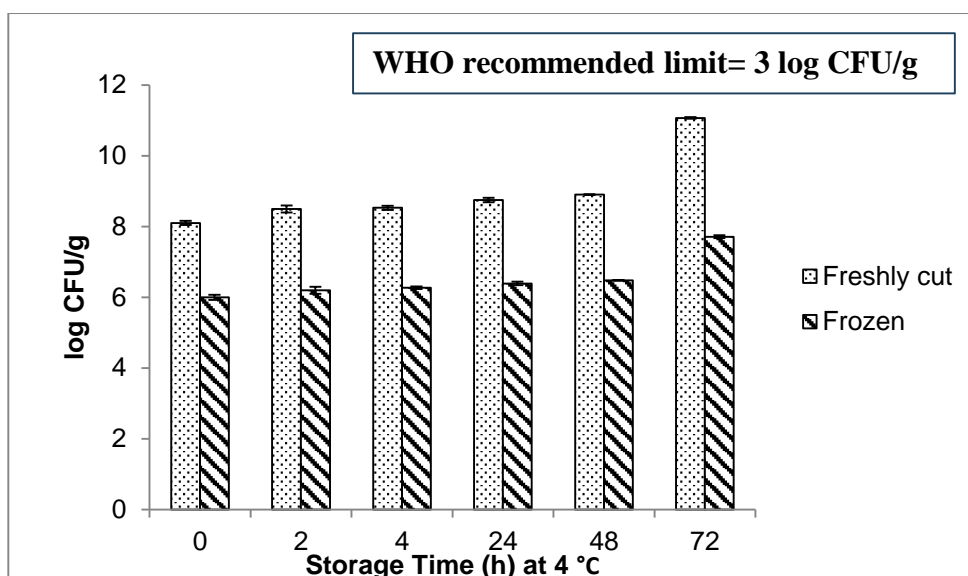
Fig. 4.2: Heterotrophic plate count (HPC) of chicken meat

Fig. 4.2 shows that with the passage of time the microbiological population of chicken meat has increased and it was significantly high (p< 0.05) in freshly chicken meat as compare to that of frozen meat samples. Furthermore, both freshly cut and frozen chicken meat had exceeded the quality standards of 10^3 CFU/g of International Commission on Microbiological Specifications Food (ICMSF) and 3 log CFU/g of World Health Organization (WHO).
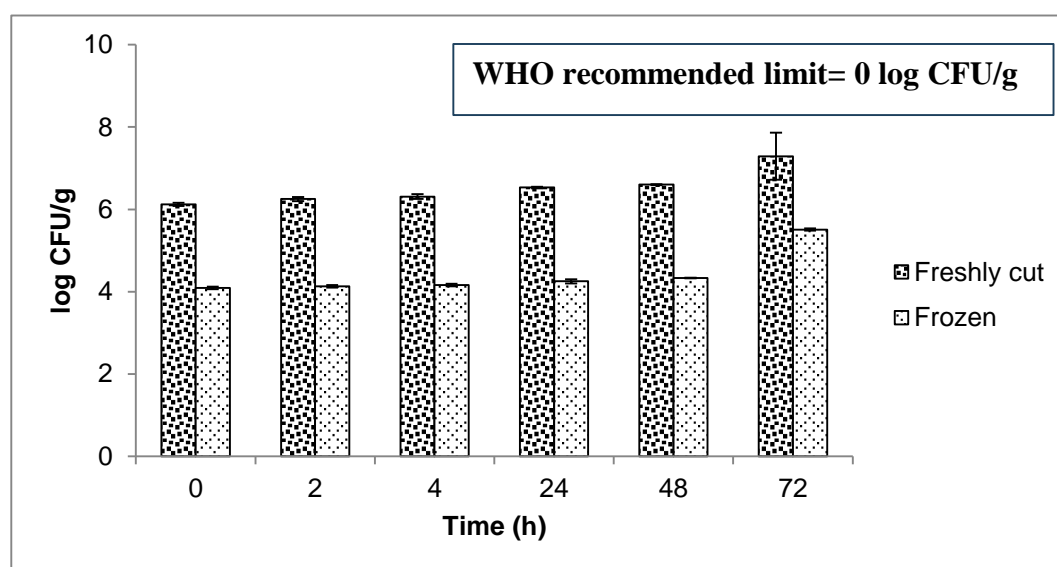
4.2.2 *Salmonella* count

The data in Table 4.1 shows the *Salmonella* contamination in different samples collected from different sectors and K&Ns outlets. It shows that most contaminated meat with *Salmonella* is present in sector F among targeted sectors. Several factors may include in its contamination including road-side shops, traffic and overly crowded areas.

Table 4.1: Prevalence of *Salmonella* in chicken meat

Location	Samples collected n=52		*Salmonella* positive	
	No	**%**	**No**	**%**
F	15	28.8	14	93.3
G	10	19.2	6	60
I	12	23.1	9	75
K&Ns outlets	15	28.8	7	46.7

Freshly cut chicken samples were more contaminated (Garedew et al., 2015) than frozen chicken meat samples. Mean *Salmonella* counts was 6.15-7.29 log CFU/g in fresh and 4.09-5.51 log CFU/g in frozen meat. However according to WHO, there must be 0 log CFU/g of *Salmonella* in both fresh and frozen chicken meat.

Fig. 4.3: *Salmonella* count of chicken meat

The low *Salmonella* conatmination level in frozen meat as compared to that of freshly cut meat is basically because of the usage of various organic acids during processing and storage. Above all, this high contamination depicts the lack of food monitoring authorities in meat processing industries and shops.

4.3 Identification of bacterial strains

4.3.1 Identified isolated species from chicken meat

Eight different strains HD1-HD8 were isolated from fresh and frozen chicken meat samples. After their comprehensive morphological and biochemical analysis, they were selected for 16s RNA gene sequencing. This was performed at Genome Analysis Department Macrogen Inc. Korea.

Predominant species identified from chicken meat along with their accession numbers are given in Table 4.2.

Table 4.2: 16s rRNA Gene sequencing results of selected isolates

SeqID	Isolation source (Chicken meat)	Organism	Accession numbers
Seq1	Fresh	*Proteus mirabilis*	KU978817
Seq2	Fresh	*Salmonella enterica* subsp. *enterica* serovar *Typhimurium*	KU978818
Seq3	Frozen	*Salmonella enterica* subsp. *enterica* serovar *Heidelberg*	KU978819
Seq4	Frozen	*Salmonella enterica*	KU978820
Seq5	Fresh	*Salmonella enterica* subsp. *enterica* serovar *Kentucky*	KU978821
Seq6	Fresh	*Morganella morganii*	KU978823
Seq7	Fresh	*Salmonella enterica* subsp. *enterica* serovar *Hadar*	KU978824
Seq8	Fresh	*Stenotrophomonas maltophilia*	KU978825

A phylogenetic tree, assembled through MEGA 7 software demonstrates the phylogenetic relatedness and linkage among identified strains, shown in Figure 4.4.
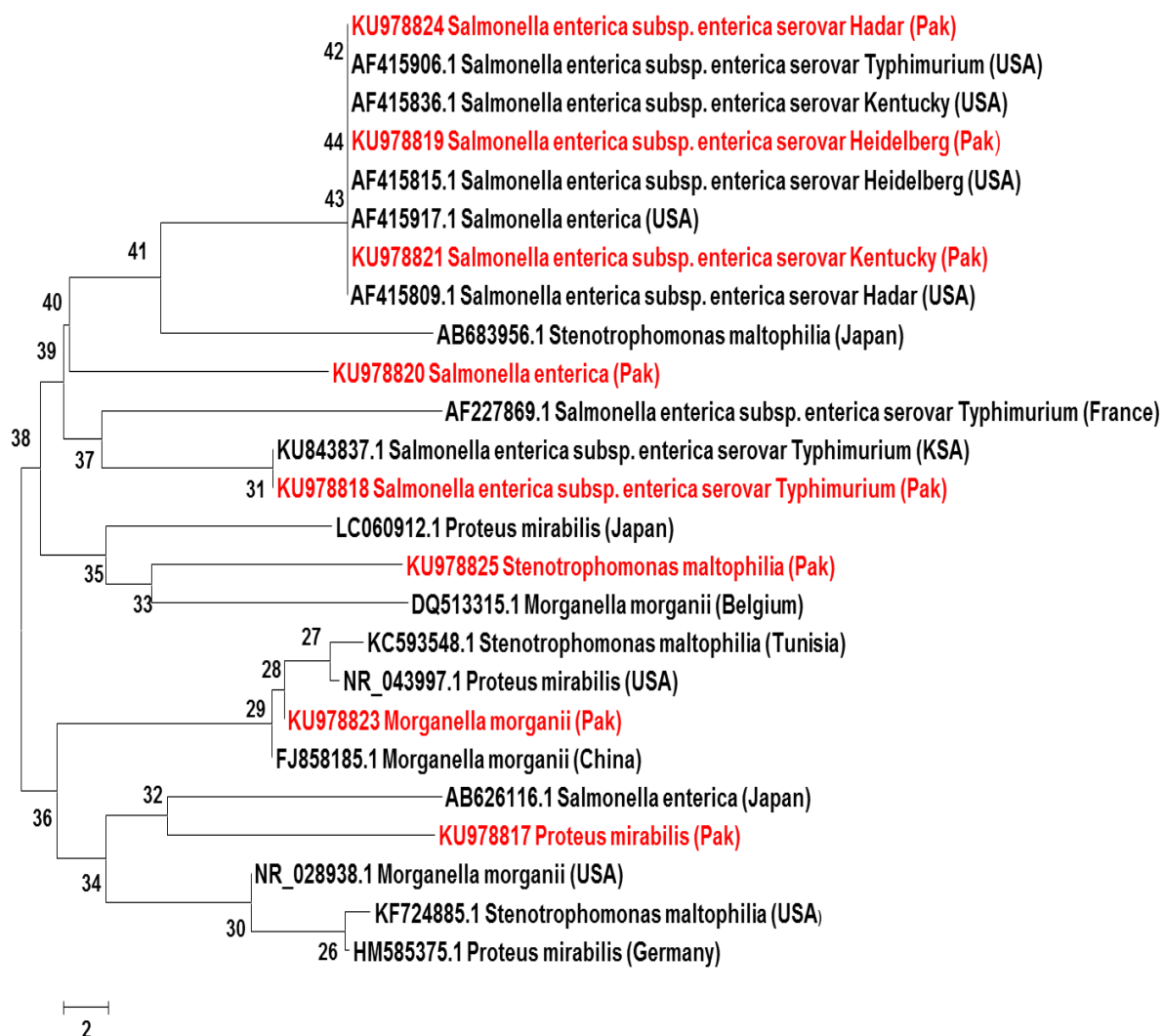
Fig. 4.4: Phylogenetic tree demonstrating the relatedness and linkage of

bacterial strains

In above fig. 4.4, horizontal lines represent amount of genetic change. It basically signifies the evolutionary shift over time. The larger the branch length in horizontal direction, the greater the amount of change. The scale for this is provided by the bar at the bottom of the figure. In the above phylogenetic tree, the line segment with the number '2' shows the branch length that represents an amount of genetic change of '2'. The units of branch length are usually nucleotide substitutions per site that is the number of changes or 'substitutions' divided by the length of the sequence (although they may be given as % change, i.e., the number of changes per 100 nucleotide

sites). The vertical dimension in this figure has no meaning and is used simply to lay out the tree visually with the labels evenly spaced vertically. The vertical lines therefore simply tells that which horizontal line connects to which and how long they are irrelevant.

4.4 Thermal inactivation of *Salmonella spp*.

The inactivation kinetics of cocktail of *Salmonella spp.* (*Enterica, Typhimurium, Hadar, Heidelberg,* and *Kentucky)* was modeled by using linear regression. All studied temperature (50, 55, 60, 65 and 70°C) illustrated that resulting curves had coefficient of determination (r^2) values > 0.95. This study showed that with increase in heating time, *Salmonella* survivors decreased in a linear manner in all samples. D-values for *Salmonella* cocktail were 4.05, 3.28, 1.89, 1.65 and 1.38 min at 50, 55, 60, 65 and 70°C respectively in fresh and frozen chicken meat samples.

The data obtained in this study was compared with the published literature on the heat resistance of *Salmonella spp*. Similar D-values were reported by other researchers for *Salmonella* serovars in chicken products. Bucher *et al*. (2008) reported D-values of 6.87, 1.51, and 0.69 min for *S. Enteritidis*, 4.50, 0.96 and 0.39 min for *Salmonella heidelberg* and 4.49, 1.19 and 0.39 min for *Salmonella kentucky* in chicken nuggets/strips that had pH values of 5.78 at temperatures of 55, 58 and 60 °C, respectively. Mazzotta (2000) reported D-values for a cocktail of *Salmonella* serovars (*Montevideo, Thompson, Heidelberg, Mbandaka, Typhimurium ATCC 13311, Enteritidis NFPA N-4016 and Enteritidis ATCC 13076*) of 3.2 and 0.6 min at 56 and 60 °C, respectively, in ground chicken.

In contrast, other researchers have reported higher D-values for *Salmonella* serovars in chicken meat than those reported in the current study. Murphy *et al*. (2000) reported D-values of 30.1, 12.9 and 5.88 min, at temperatures of 55, 57.5 and 60 °C, respectively, for a cocktail of *Salmonella* serovars (*Senftenberg, Heidelberg, Typhimurium, Montevideo, California and Mission*) in ground chicken breast.
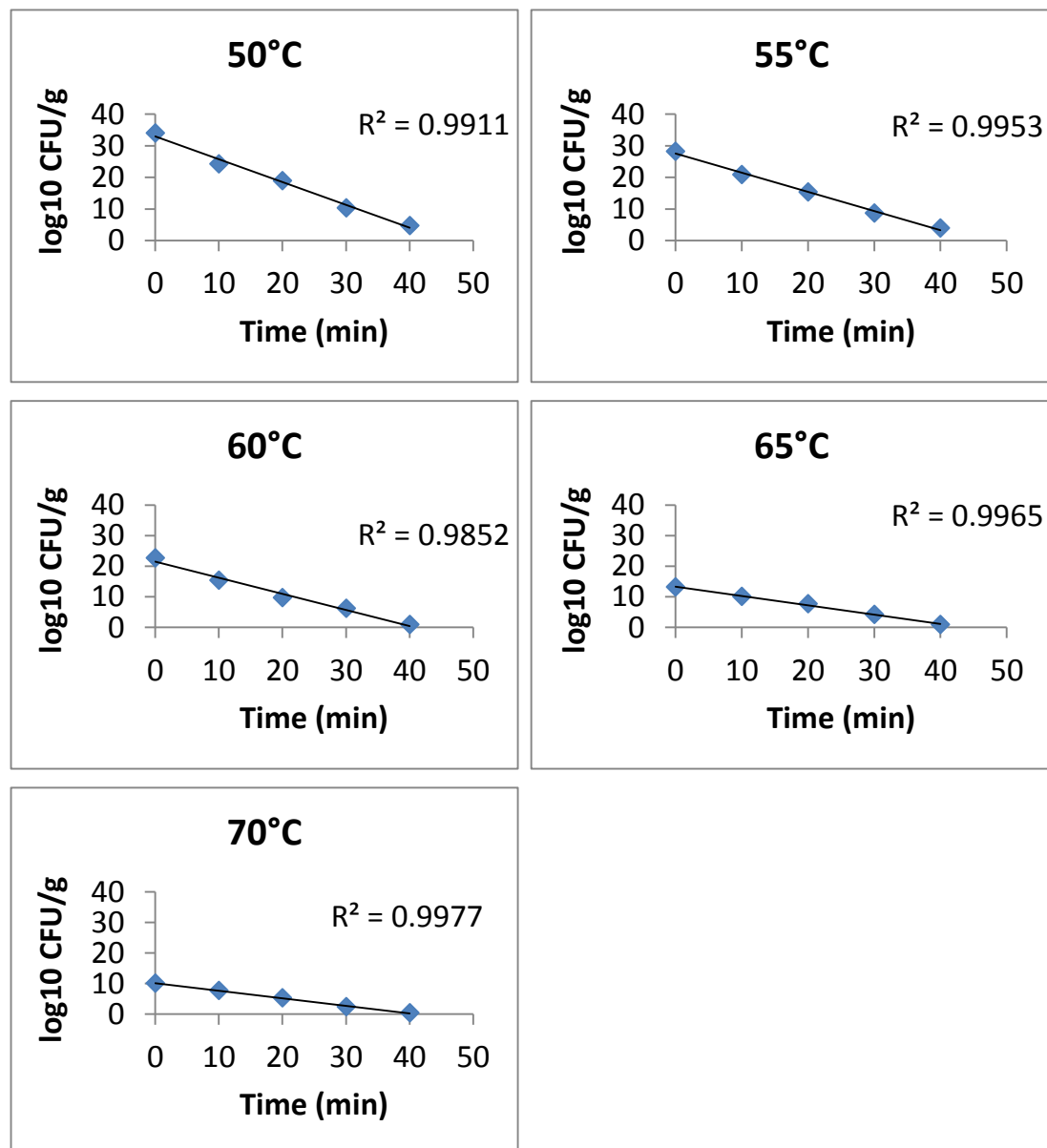

Fig. 4.5: Survival curves of cocktail of *Salmonella* at 50, 55, 60, 65 and 70 °C

The z-value obtained from this study was 40.7°C which is higher to many other researchers as that of Bucher *et al*., 2008 who reported 4.10-5.17°C *Salmonella* serovars in chicken nuggets/strips. Similarly Murphy et al. (2004) reported a z-value of 5.34 °C for a cocktail of *Salmonella* serovars in ground chicken thigh meat. Mazzotta (2000) reported 5.7 °C for a cocktail of *Salmonella* serovars in chicken breast, while Osaili *et al*. (2006) and Juneja *et al*. (2001) reported 6.0 °C and 6.1 °C for cocktails of *Salmonella* serovars in ready-to-eat chicken-fried beef patties and ground chicken respectively. Higher z-values mean that a higher temperature is required to obtain a 5-fold reduction in the D-value and current study indicated that larger changes in temperature are required to cause 90% reduction in D-value when a *Salmonella spp*. cocktail is evaluated in meat (Osaili *et al*., 2013).
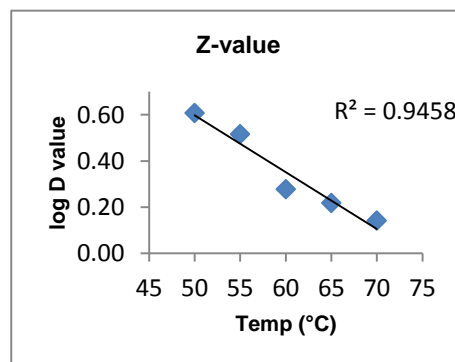

Fig. 4.6: Z-values for 5 *Salmonella* serotype cocktail over the temperature range 50 to 70 °C

The differences between z-values reported in the present study and those reported in previous studies maybe related to the differences in bacterial strains, the physiological state of bacterial serovars or untested differences in product composition, pH and fat content of the heating menstruum, and methodology used for enumeration of survivors (Juneja et al., 2012; Waldroup, 1996).

4.5 Quorum sensing

4.5.1 Screening of AHLs producing bacteria

Albeit there are a number of signal molecules such as oligopeptides in gram positive bacteria and AHLs in gram negative bacteria and AI-2 for interpecies bacterial communication, this study was aimed on AHLs based QS as all strains are found to be gram negative. According to the findings of a number of researches, AHLs have a variety of acyl chains and each bacteria use its own AHL to initiate QS (Yeon *et al*.,2009; Li *et al*., 2014 and Martins *et al*., 2014). Therefore, two biosensor systems, A136 and CV026, were used for the screening of short and medium/long chain AHLs producing strains. A total of 8 strains (*Salmonella spp., Proteus mirabilis, Stenotrophomonas maltophilia and Morganella morganii*) were used and not even a single strain developed blue or purple pigmentation. It did not mean the absence of QS in these bacteria but it showed they are non-AHLs producers and QS occurred via other mechanisms like AI-2 etc.

4.5.2 Relationship between bacterial growth rate and biofilm formation ability

Most of the bacteria are known to establish a complex and highly organized communities in the form of biofilm depending on the growth rate and environmental conditions. The development of matured biofilms highly depends on the bacterial ability for survival and colonization on surfaces. This study was done to estimate the relationship between bacterial growth rate and their biofilm formation ability using crystal violet assay. After 72 h of incubation, maximum growth rate of 0.56 ± 0.04 and 0.55 ± 0.04, was observed in *Salmonella enterica* and *Salmonella kentucky*.
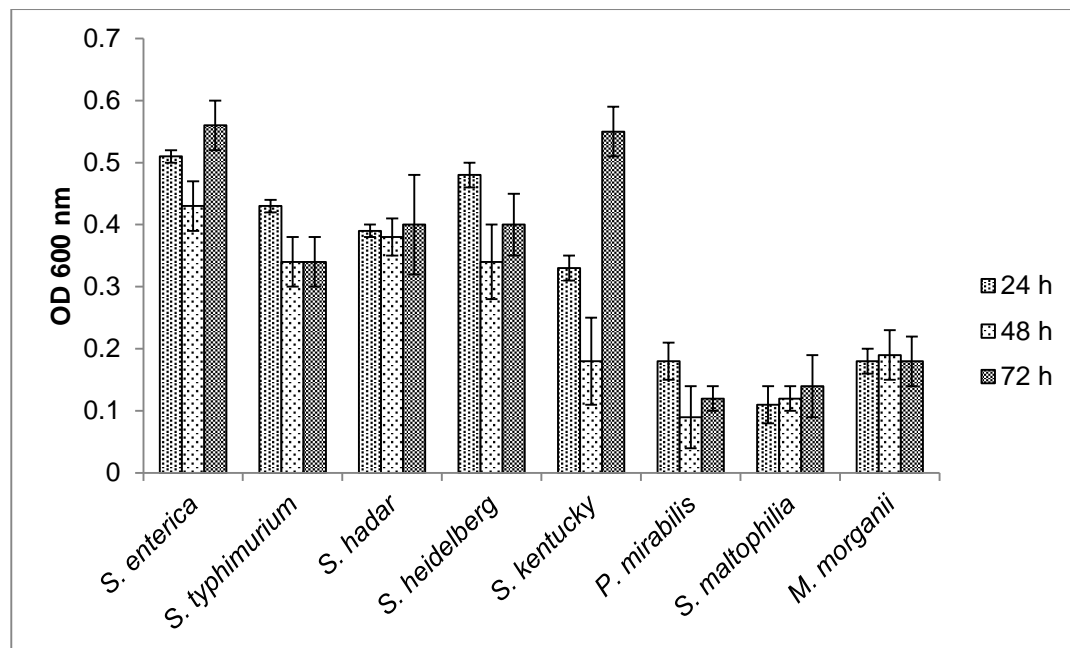
Fig. 4.7: Bacterial growth rate of isolated bacteria from chicken

The maximum biofilm formation ability was observed in 0.321 ± 0.001 and 0.318 ± 0.001 by *Salmonella kentucky* and *Salmonella heidelberg* (Fig. 4.8). Biofilm formation of non-AHL producing species in the microtiter plate may explain the role of other mechanisms like AI-2 etc. in QS.
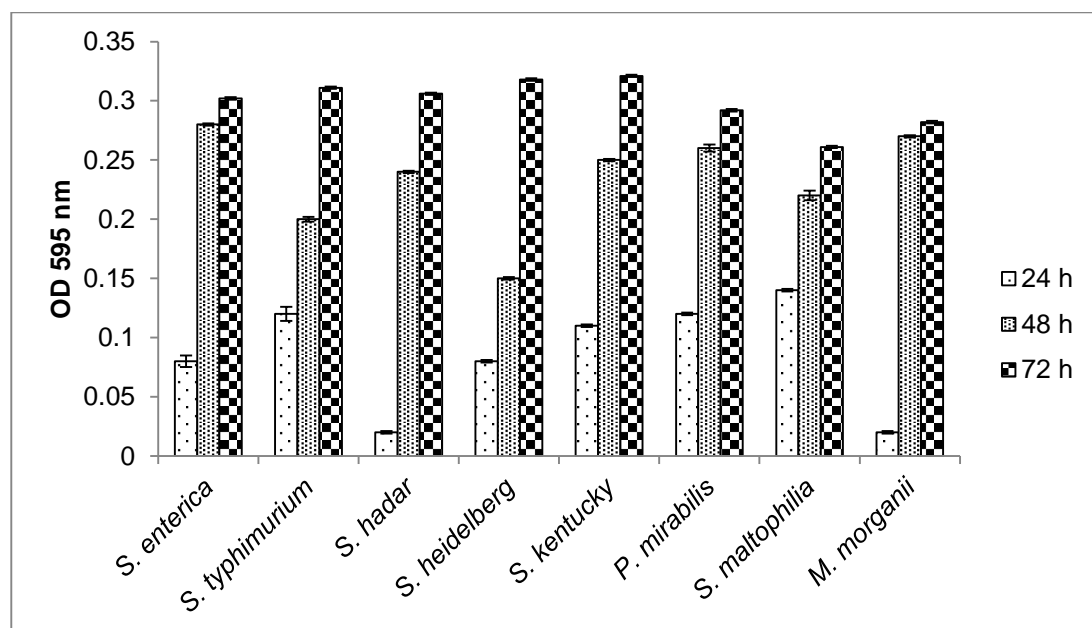

Fig. 4.8: Biofilm formation assay of isolated bacterial strains from chicken meat

The different amount of biofilm depicts that strains seem to have different biofilm forming tendencies, nonetheless growth rate. Thus, it may be concluded that the rate of biofilm formation does not always depend on the bacterial growth, rather there are some other agents or chemical signals responsible for evaluating population density (Shrout and Nerenberg, 2012).

Fig 4.7 and 4.8 may correlate with each other. Growth studies showed that microbial growth rate was high during 24 h and low during 48 h and then there was a significant increase during 72 h. On the other hand, biofilm formation rate was increased with increase in incubation time. The probable resaon of higher growth rate during 24 h of incubation is excessive amount of nutrients are present in broth that provide enough energy for the growth of bacteria. During 48 h, the substrate started to degrade and there is not sufficient energy to carry on microbial metabolism for growth and reproduction. Moreover, an increase in incubation time also resulted into depletion of the substrate. As a result, microbes would form ultramicrocells and enter a physiological state known as "starvation-survival". This physiological state results in metabolic arrest which permits the organisms to survive for longer periods of time without sufficient energy for growth and reproduction (Ferrocino et al., 2009).

Thus, it is assumed that prolonged starvation may recover the signal molecules-based QS. As described by Waheed *et al*. (2015), the bacteria release more AHLs or other signal molecules possibly to adapt to harsh environmental conditions by enhancing the cell to cell communication during the formation of biofilm. Therefore, many species including *Salmonella, Enterobacter, Klebsiella, Staphylococcus aureus* and *Pseudomonas* witnessed the same mechanism even after 72 h of

incubation. Furthermore, it has also been reported that some bacteria may modify their behavior in a coordinated fashion, via chemical signals. Current results of biofilm formation are in agreement of the hypothesis that in the period of prolonged starvation, the microorganisms would emit more AHLs (in AI-1 mechanism) and other chemical molecules (in AI-2 mechnaism) thereby protecting themselves to resist against the starvation.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

With the passage of time, increase in pH values of samples were observed. In case of freshly cut meat, it was increased upto 8.5 while in frozen chicken meat it was 6.35. Albeit HPC was low in frozen chicken meat samples but still both fresh and frozen meat were found to exceed the permissible WHO limit of 3 log CFU/g, the possible reason of it might be cross contamination and handling during storage and transportation. More than 90 % chicken meat samples collected from sector F were contaminated with high concentration of *Salmonella spp*. Gene sequencing confirmed *Salmonella* species including *Salmonella enterica, Salmonella enterica* subsp. *enterica* serovar *hadar, Salmonella enterica* subsp. *enterica* serovar *typhimurium, Salmonella enterica* subsp. *enterica* serovar *heidelberg* and *Salmonella enterica* subsp. *enterica* serovar *kentucky*. At 4 °C, meat storage is not safe for more than 48 h because after that time period, pH, HPC and *Salmonella* count was found to be increased. With the increase in heating temperatures, *Salmonella* survivors decreased in linear manner. D-values for *Salmonella* cocktail were 4.05, 3.28, 1.89, 1.65 and 1.38 min at 50, 55, 60, 65 and 70 °C respectively. The Z-value obtained was 40.7 °C. Thus cooking meat at high temperature (70 °C) significantly reduced *Salmonella* count by 90% and this reduction occurred after 1.38 min. All isolated strains of *Salmonella spp*. and other species (*Proteus mirabilis, Stenotrophomonas maltophilia and Morganella morganii*) from chicken

meat did not produce AHLs which means AI-1 mechanism was absent. But the biofilm formation ability in microtiter plate showed that QS by other mechanisms like AI-2 might occur in these strains. The maximum biofilm formation ability was observed by *Salmonella kentucky* (0.321 ± 0.001) and *Salmonella heidelberg* (0.318 ± 0.001).

5.2 Recommendations

Following recommendations are proposed for future research:

1. Similar investigation may be done for food processing industry, restaurants and hotels.

2. AI-2 mechanism in *Salmonella* may be studied.

3. *Salmonella* contamination might be identified in others types of meat including mutton and beef being produced in Rawalpindi and Islamabad.

4. Food preservatives from approved class I and II (in other words organic acids) may be analyzed for their antimicrobial efficacy.

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APPENDICES

Table A1: Mean pH values with standard deviations

Type of meat	Time (h)					
	0	**2**	**4**	**24**	**48**	**72**
Freshly cut	6.3±0.4	6.4±0.5	6.6±0.5	6.6±0.55	6.9±0.59	8.5±0.85
Frozen	5.74±0.04	5.8±0.02	5.8±0.02	5.8±0.01	5.9±0.01	6.3±0.006

Table A2: Total heterotrophic plate count (log_{10} CFU/g)

Type of meat	Time (h)					
	0	**2**	**4**	**24**	**48**	**72**
Freshly cut	8.1±0.06	8.5±0.1	8.53±0.06	8.57±0.06	8.9±0.01	11.07±0.02
Frozen	6±0.07	6.2±0.1	6.27±0.04	6.39±0.05	6.48±0.01	7.71±0.04

Table A3: Total Salmonella count (log_{10} CFU/g)

Type of meat	Time (h)					
	0	**2**	**4**	**24**	**48**	**72**
Freshly cut	6.12±0.04	6.25±0.05	6.31±0.06	6.53±0.02	6.6±0.01	7.29±0.57
Frozen	4.09±0.03	4.13±0.03	4.16±0.03	4.25±0.05	4.33±0.01	5.51±0.03

Table B: Colony morphology of species isolated from chicken meat samples

Isolates	Shape	Elevation	Margin	Color	Odor	Surface	Gram reaction	Structure	Opacity	Shine
1	Circular	Flat	Entire	Black centered	Smelly	Smooth	Pink	Bacillus	Translucent	No
2	Circular	Raised	Entire	Black	Smelly	Smooth	Pink	Bacillus	Translucent	No
3	Circular	Flat	Entire	Black centered	Smelly	Smooth	Pink	Bacillus	Translucent	No
4	Circular	Flat	Entire	Black centered	Smelly	Smooth	Pink	Bacillus	Opaque	No
5	Circular	Flat	Entire	Creamy white	Smelly	Smooth	Pink	Bacillus	Opaque	No
6	Circular	Flat	Entire	Black centered	Smelly	Smooth	Pink	Bacillus	Opaque	No
7	Circular	Flat	Entire	Pink	Smelly	Smooth	Pink	Bacillus	Opaque	No
8	Circular	Flat	Entire	Black centered	Smelly	Smooth	Pink	Bacillus	Translucent	No

Appendix C

Table C1: Heat resistance (expressed as D-values in min and Z-value in °C) for

Salmonella spp. **in chicken broth**

Meat	Temp (°C)	Linear regression		Log D-value	Z-value (°C)(r²) [c]
		D-value (min) [a]	(r²) [b]		
Chicken	50	4.05±0.02	0.998	0.61	40.7 (0.95)
	55	3.28±0.01	0.997	0.52	
	60	1.89±0.01	0.985	0.28	
	65	1.65±0.00	0.995	0.22	
	70	1.38±0.01	0.991	0.14	

[a] D-values are the mean ± standard deviation of two replicates and were obtained by linear regression using Excel

[b] Correlation coefficient

[c] Z-value was determined by the means of replicate D-values obtained in chicken broth and based on survivors on the recovery medium

Table C2: Bacterial growth rate of isolated bacteria from chicken

Time (h)	Bacterial species							
	S. enterica	*S. typhimurium*	*S. hadar*	*S. heidelberg*	*S. kentucky*	*P. mirabilis*	*S. maltophilia*	*M. morganii*
24	0.51±0.01	0.43±0.01	0.39±0.01	0.48±0.02	0.33±0.02	0.18±0.03	0.11±0.03	0.18±0.02
48	0.43±0.04	0.34±0.04	0.38±0.03	0.34±0.06	0.18±0.07	0.09±0.05	0.12±0.02	0.19±0.04
72	0.56±0.04	0.34±0.04	0.4±0.08	0.4±0.05	0.55±0.04	0.12±0.02	0.14±0.05	0.18±0.04

Table C3: Biofilm formation assay of isolated bacteria from chicken

Time (h)	Bacterial species							
	S. enterica	*S. typhimurium*	*S. hadar*	*S. heidelberg*	*S. kentucky*	*P. mirabilis*	*S. maltophilia*	*M. morganii*
24	0.08±0.005	0.12±0.006	0.02±0.001	0.08±0.001	0.11±0.001	0.12±0.001	0.14±0.001	0.02±0.001
48	0.28±0.001	0.2±0.002	0.24±0.001	0.15±0.001	0.25±0.001	0.26±0.003	0.22±0.004	0.27±0.001
72	0.30±0.001	0.31±0.001	0.306±0.001	0.318±0.001	0.321±0.001	0.292±0.001	0.261±0.001	0.282±0.001

Microbiological analysis
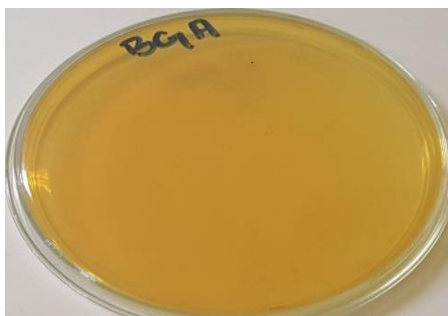
Salmonella count
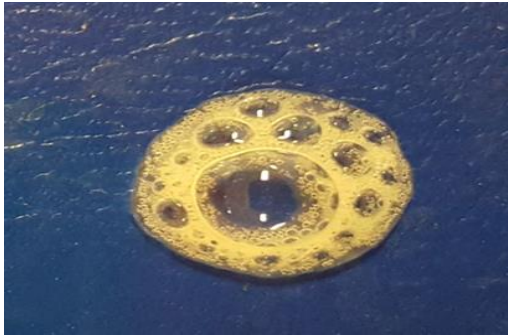
Salmonella appears as black colonies on S.S agar
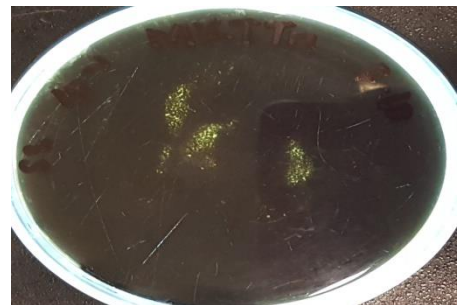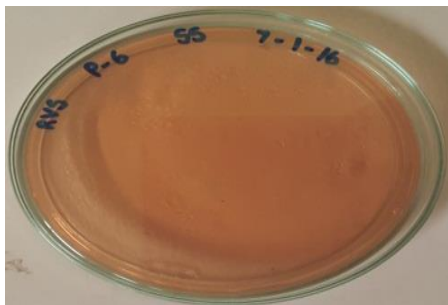
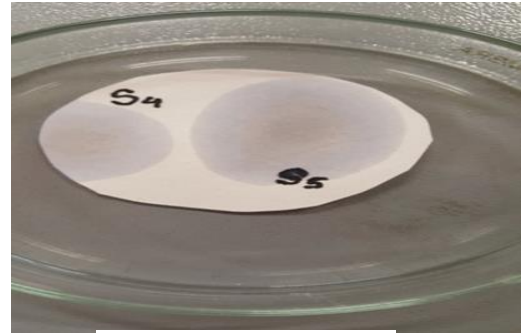XLD turns reddish-pink for *Salmonella* positive sample

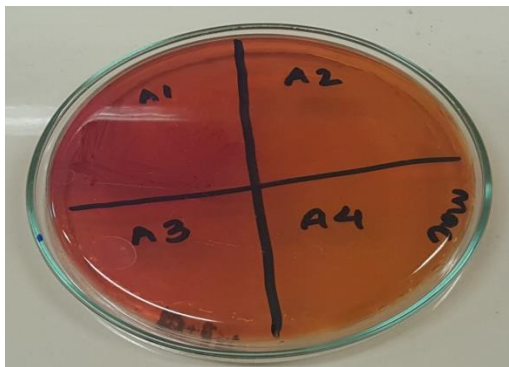BGA turns pink for *Salmonella* positive sample

Biochemical Confirmatory Tests
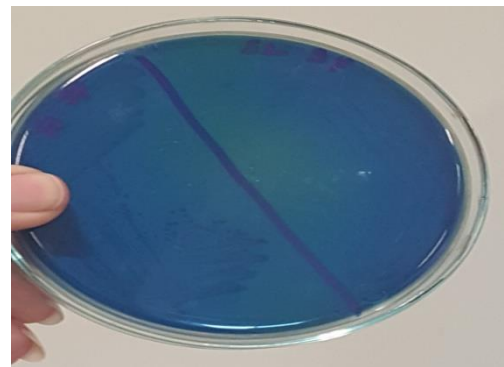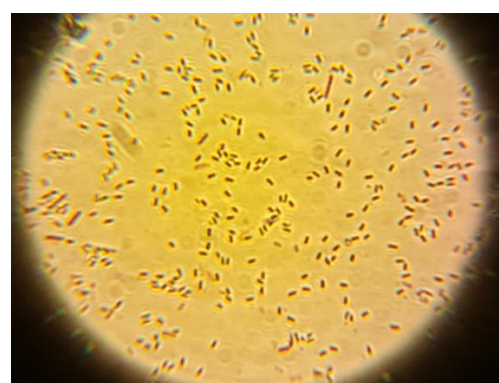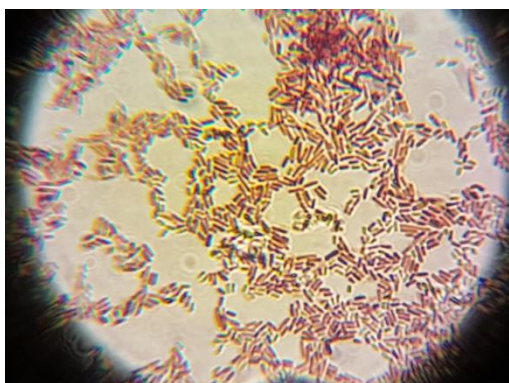

Catalase test

Oxidase test

MacConkey agar

Simmons citrate

Results of gram reaction