DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* IN ENVIRONMENTAL SAMPLES

By

Khaula Aisha Batool

(NUST201261040MSCEE65212F)

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

Master of Science

In

Environmental Sciences

Institute of Environmental Sciences and Engineering (IESE)
School of Civil and Environmental Engineering (SCEE)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan
(2014)

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* IN ENVIRONMENTAL SAMPLES

By

Khaula Aisha Batool

(NUST201261040MSCEE65212F)

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

Master of Science

In

Environmental Sciences

Institute of Environmental Sciences and Engineering (IESE)
School of Civil and Environmental Engineering (SCEE)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan
(2014)

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

DISTRIBUTION OF *SALMONELLA TYPHIMURIUM* IN ENVIRONMENTAL SAMPLES

Submitted by

Khaula Aisha Batool

Has been found satisfactory for the requirements of the degree of
Master of Science in Environmental Sciences

Supervisor: _____
Dr. Imran Hashmi
Professor
IESE, SCEE, NUST

Member: _____
Dr. Ishtiaq A. Qazi
Associate Dean & Professor
IESE, SCEE, NUST

Member: _____
Dr. Muhammad Arshad
Assistant Professor
IESE, SCEE, NUST

External Examiner:_____
Dr. Yesim Soyer
Assistant Professor
Middle East Technical University, Ankara
Turkey

This thesis is dedicated to my Family

For their endless affection, support and encouragement

ACKNOWLEDGEMENTS

This dissertation would not have been possible without the guidance and the help of several individuals who, in one way or another, contributed in the preparation and completion of this study.

First and foremost, utmost gratitude is due to my supervisor, Professor ***Dr. Imran Hashmi*** *(IESE, SCEE, NUST) whose technical assistance, sincerity and encouragement I will never forget.* ***Dr. Hashmi*** *has been my inspiration as I cleared all the obstacles in the completion of this research work and my MS journey. Many thanks also go to* ***Dr. Ishtiaq A. Qazi,*** *Associate Dean and Professor and* ***Dr. Muhammad Arshad,*** *Assistant Professor, IESE, NUST, who had always been very considerate and supportive in my research work.*

I am also thankful to ***Dr. Yesim Soyer,*** *Assistant Professor, Middle East Technical University, Ankara, Turkey, for her unfailing support. My special gratitude goes to* ***Ms. Sara Qaiser (IESE****), Lab Demonstrator and batch fellow* ***Ms. Arish Naseem*** *(IESE) whose self-less technical assistance regarding various microbiological techniques and instrument utilization took me out of difficult situations more than once.*

I cannot express my gratitude in words to my sister ***Madiha*** *and my* ***brothers*** *for their unconditional support and encouragement during every difficult phase of my research.*

I would also like to extend my thanks to all of my friends especially, ***Hira Waheed, Saira Khalid,*** *and* ***Amina Khalid****. Finally, to my dear friend* ***Mehreen Khan****, without whose support and understanding it would have taken a much longer time to finish this work.*

KHAULA AISHA BATOOL

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

API	Analytical Profile Index
BGA	Brilliant Green Agar
CDC	Centre of Disease Control
CFU/G	Colony Forming Unit Per Gram
DNA	Deoxy-Ribonucleic Acid
EFSA	European Food Safety Authority
GAP	Good Agricultural Practices
GHP's	Good Healthy Practices
GRAS	Generally Recognized as Safe
HPC	Heterotrophic Plate Count
ICMF	International Commission on Microbiological Specification for Food Standards
IHD	Ischaemic Heart Diseases
IND	Indole
ISO	International Standards Organization
MKTT-n	Muller Kauffman's Tetrathionate Novobiocin Broth
NPK	Nitrogen Phosphorus and Potassium
PCR	Polymerase Chain Reaction
RNA	Ribo-Nucleic Acid
RVS	Rapport Vassiliadis Soya Peptone Broth
SQCA	Standard Quality Control Authority
TDH	Tryptophan Deaminase
USEPA	United States Environment Protection Agency
w/v	Weight/ Volume
WHO	World Health Organization

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Contaminated leafy greens are occasionally involved in disease outbreaks through enteric pathogens. In order to control the contamination it is important to understand the factors that influence enteric pathogen survival. The study aimed to examine the effect of temperature and application of food grade reagents (sodium benzoate, acetic acid and potassium sorbate) on the survival of *Salmonella* and microbial contamination within lettuce produced in Rawalpindi and Islamabad. Samples were analyzed under low (4°C) and high temperature (41.5 °C) storage conditions to predict the potential growth and survival of *Salmonella* in lettuce. *Salmonella* count were observed to increase significantly ($P < 0.05$) at low temperature (3.4 – 4.1 log cfu/g) whereas it decreased at high temperature storage conditions (3.4– 0.36 log cfu/g; $P < 0.05$). Heterotrophic plate count (HPC) in lettuce ranged from 5.6 – 5.971 log cfu/g; $P < 0.05$ at 4°C and 5.6 – 3.18 log cfu/g; $P < 0.05$ at 41.5 °C. Microbial and *Salmonella* count within lettuce samples exceeded the World Health Organization (WHO) 2.3 log cfu/g and International Commission on Microbiological Specifications for Food standards (ICMSF) permissible limit of 10^3 cfu/g. Among the different organic acid treatments, acetic acid proved to be more reliable as it reduces HPC count by 69 % (5.6 – 1.7 log cfu/g) and *Salmonella* count by 64 % (3.4 – 1.1 log cfu/g). Potassium sorbate exhibited 29 % HPC reduction (5.6 – 4.1 log cfu/g) and 19 % *Salmonella* count (3.4 - 2.8 log cfu/g) whereas sodium benzoate showed a reduction of HPC by 27 % (5.6 – 3.9 log cfu/g) and *Salmonella* count by 11 % (3.4 – 3.1 log cfu/g).

Chapter 1

INTRODUCTION

1.1. Background

Food borne diseases are the important cause of morbidity and mortality worldwide. The world has become more vulnerable to outbreaks of disease caused by contaminated food because of growing global trade (Kuchenmüller *et al.,* 2013). Food borne diseases associated with leafy greens have marked an increase of 38.6% since 1996-2005 (Crandall *et al.,* 2011).

Leafy greens is a term given to vegetables including lettuce, cabbage, collard green, mustard green, endive, spinach, turnip greens, kale, broccoli, escarole, and spinach (Economic Research Service, 1998). These are considered to be ready to eat after minimal processing (Sandeep *et al.,* 2013; Longchamp *et al., 2009*) and providing a diversified flavored, low caloric and micro nutrient rich diet. Leafy greens are perceived to be of optimum quality within 24 hours of its harvesting. Consumer's preference is linked to them because of their sufficient nutrient content and freshness (Gomez- Govea *et al., 2012).*

According to estimation, insufficient raw vegetable intake causes 27 million annual deaths, with a share of 19 % in gastro-intestinal cancers, about 31 % in ischemic heart diseases (IHD) and 11 % in strokes. These are significantly linked with lung/pharyngeal/laryngeal/oral cancers, type-II diabetes, bone-health and micronutrient deficiency. Low vegetable intake is marked as the sixth main factor for mortality in the world (WHO, 2009).

Consumer demand for vegetables has increased round the globe; for the very reason alternative options are being explored to sufficiently maintain the demand and supply chain (Juning *et al.,* 2014). Though the availed alternative options increase the yield but they keep the health issues at bay. This attitude provides a major pathway to food borne diseases causing pathogens to enter human bodies through daily consumption of contaminated vegetables.

Globally, research is being carried out to control food contamination introduced via food borne pathogens. Common perception is that multiple washing of vegetables with tap water is enough to remove any contaminants from them but research has proven that it is not sufficient in reducing bacterial contamination rather it increases the chances of cross contamination (Jensen *et al.,* 2015). FAO and WHO has approved various chemicals from class I and II including sorbates, benzoates, sulfites, salt, sugar, vinegar etc. as preservatives to be used as disinfectants. Salt and sugar are common preservatives used domestically but are not as effective against pathogen growth in vegetables as certain chemicals especially organic acids (Ravishankar and Juneja, 2014). People, however, have concerns about organic acids as food preservatives because of expected chemical reactions (Hamid *et al.,* 2012).

Pakistan is a developing country where agriculture is considered as the backbone of economy. It is contributing 45 % of total exports, 26 % of GDP and providing a source of livelihood to 52 % population. Various exported vegetables of Pakistan were and are being rejected currently due to pathogen contamination. This contamination is basically linked with poor agricultural activities practiced by farmers to increase the crop yield within their limited resources. In order to fulfill the nutrient and water requirements of crop, use of wastewater for irrigation and animal dung as manure are

common practices. More than 10 % of the world population consumes food produced from wastewater irrigation (WHO, 2006).

In Rawalpindi and Islamabad (twin cities) commercial farming (most commonly referred as inorganic farming) is carried out by using wastewater (Lei nala as the main source) for irrigation purposes. The water from Lai not only compensates the nutrient requirements of the crop but is quite helpful for the farmers during the dry seasons when market prices of vegetables rise due to shortage of production. Lei nala (running throughout the year) contaminated with sewage and animal waste enables farmers (small scale) to diversify their cropping practices. Though Lai prove to be lucrative for farmers it is a substantial contributor of potential health problems by providing entry route for pathogens like *Salmonella,* E. *coli, Shigella* etc. Direct use of wastewater for irrigation purposes, prior to any treatment is a potential threat to health quality and standard of the irrigated vegetables for consumers (Ensink *et al.,* 2003).

Organic vegetables are also being produced in Rawalpindi by using bio-fertilizer in the form of animal dung, (domestically produced manure), though full of nutrients (NPK; Nitrogen, Phosphorus, Potassium) but do carry a lot of microbes along, which are the main cause of gastro intestinal infections (Carr, 2003).

Due to food contamination not only food borne diseases spread but the country's exports are also affected. In 2002, 122 Pakistani food products were rejected by United States, Australia and European Union because of microbiological contents, unauthorized additives, traces of rodent etc. In 2007, the list of rejected products reached to 169 items (Ministry of Commerce Pakistan, 2014). According to FAO food quality standards there should be no *Salmonella* contamination in vegetables preferably consumed in raw form. Currently at national level there is no active national food and

safety authority in Pakistan whereas at provincial level authorities like Punjab Food Safety and Standard Authority (FSSA) , KPK FSSA etc. are present and are playing their parts by weekly inspecting vegetables markets i.e. Sunday bazar, Tuesday bazar, main vegetable markets etc.

The current research was aimed to understand the degree of contamination in vegetables being produced in Rawalpindi and Islamabad. Lettuce was selected as a model plant to carry out the study. Lettuce is considered to be most suitable due to its raw consumption, supplementary component of daily human diet as salads and principle constituent of fast foods, availability round the year, potential source of targeted pathogen and easy access to organic and inorganic production sources.

1.2. Present Study

In the present study, it was intended to analyze the microbiological quality of the lettuce (organic/inorganic) being produced in twin cities. The efficiency of organic acids as antimicrobial agents was studied. Study was also focused on determining the impact of environmental factors (soil, water and temperature) on the level of *Salmonella* contamination and its survival in lettuce.

1.3. Objectives

The objectives of the study were to:

- Assess the bacteriological quality of lettuce (organic and inorganic) being produced in Rawalpindi and Islamabad.
- Specie identification of the *Salmonella* spp. extracted from lettuce leaves using Polymerase Chain Reaction (PCR).
- Determine significant treatment for *Salmonella* and bacterial log reductions.

Chapter 2

LITERATURE REVIEW

2.1. Importance of Fresh Produce

Globalization of food supply has made the availability of fresh fruits and vegetables possible round the clock. Technological improvements in the area of production, yield and distribution as well as flexible trade policies have resulted in transportation of food to the farthest destination with no or minimal spoilage (Beuchat, 1996). Public awareness and concerns about healthy diet and consumption of fruits and vegetables as an integral part of daily intake has increased over the last few decades. Regular consumption of vegetables in the form of salads has proved to be vital against reducing the risk of obesity, cancer and cardiovascular diseases (Legnani *et al.,* 2010). Consumer's demand of fresh produce consumption is linked with increasing awareness about the health benefits associated with nutrient rich contents of fruits and vegetables. These are the fundamental sources for the immediate provision of necessary dietary nutrients (vitamin, antioxidant) on regular basis, promoting a better and healthy lifestyle by providing important ingredients that are necessary to fight against fatal health diseases (Barak and Schroeder, 2012). This approach has led to an attitude where consumers are more than willing to pay for the products that are minimally processed and are produced indigenously, retaining their maximum freshness (Murchie *et al.,* 2005).

2.1.1. Fresh Produce Contamination

According to Centre of Disease Control and Prevention (CDCP) USA, fresh produce related illnesses are continuously increasing since the early 1970's. <1 % increase in

fresh produce related infections was observed in 1970 compared to 1969, <6.5 % in 1996 (Sumathi *et al.,* 2004) and <13 % in 2009 (Barati *et al.,* 2010). This sharp and continuous increase in diseases is a challenge to the food industry which needs to be addressed on urgent basis. Centre of Diseases Control and Prevention estimates that 10 % reduction in food borne diseases will save 5 million infected humans annually. Leafy greens are perceived as one of the safest products by consumers in their daily diet and it is difficult to convince the masses that these nutritious greens can be highly contaminated with pathogens. Leafy greens are known as the most convenient vehicles and vector agents for pathogen transmission in human food chain as they are susceptible to contamination at all stages of their production (CDCP, 2011). Contamination may occur during growth, harvesting in farms or during distribution. Possible sources of contamination in farms include untreated manure, use of untreated wastewater for irrigation purposes, unhygienic tools and instruments used in harvesting and packaging; sources of contamination during transport include unhygienic transportation vehicles (Minhas *et al*., 2005). Absence of any pathogen inactivation mechanism in vegetables has led to a higher risk of foodborne illness in humans. Table 2.1 shows the food borne outbreaks due to leafy greens in the last 4 decades.

Table 2.1: Foodborne outbreaks associated with leafy greens (Scallan *et al.*, 2011)

Food-borne Outbreaks linked with Leafy Greens (1973-2010)	
Outbreaks	4.8%
Illnesses	6.5%
Deaths	4.0%
Salmonella Outbreaks	10.4%

2.1.2. Lettuce Consumption and Contamination

Lettuce (*Lactuca sativa L. var. longifolia*), as a Mediterranean vegetable, is the major salad crop to be cultivated and commercialized internationally. It is grown as a leafy vegetable, having high nutritional value and is usually consumed in raw form as part of salads, burgers and a number of other dishes. Lettuce being a leafy vegetable has an important role in human nutrition and is a good source of minerals. It is a cold season vegetable with a worldwide production area of 1,110,720 ha and production of 24,324,716 tons in 2011 (FAO, 2013).

A research survey was conducted by Obayagobana and coworkers (2014) in Okada, Nigeria to determine the bacteriological quality of vegetable salads sold at restaurants. For this 18 salad samples were collected from 3 main restaurants of Okada. The mean heterotrophic and coliform count recorded for the salad samples ranged from 1.46×10 - 2.80×10 cfu/g and 1.46×10 - 2.84×10 cfu/g respectively. The high cfu's in the salad samples indicated that the microenvironment within vegetables was serving favorable conditions for microbial growth and survival.

Oliveira and coworkers (2010) conducted a research study to determine the microbiological quality of lettuce being produced in Spain by collecting lettuce from organic and conventional production technologies. The lettuce samples from 18 farms were examined for the presence of yeasts and molds, psychrotrophic microorganisms, aerobic mesophilic, *Pseudomonas* spp. enterobacteriaceae, mesophilic lactic acid bacteria, *Listeria monocytogenes*, presumptive *Escherichia coli* and, *Salmonella* spp. All the samples from 18 farms were found positive for *E. coli* and *Salmonella* spp.

Siele and coworkers (2014) set up an experiment to analyze the microbiological quality and safety assessment of lettuce in Brazil by enumeration of hygiene indicators;

enterococci enteric pathogens *Salmonella* and *E.coli* O157:H7 and coliforms. The results of their study revealed that 20 % samples were contaminated with *Salmonella spp*.

Lettuce is a vector agent for number of microbiological contaminants especially enterohemorrhagic *Salmonella enterica* (Anderson *et al.,* 2011). *Salmonella* is a negative, motile (except for *S. gallinarum* and *S. pullorum*) facultative, flagellated rod shaped anaerobic bacterium with a size 2-3 x 0.4 -0.6 μm (Montville and Matthews, 2008). *Salmonella* is most common foodborne pathogen causing infections worldwide. The genus *Salmonella* consists of two species; *Salmonella enterica* and *S. bongori*. *Salmonella enterica* is responsible for major food borne diseases. Salmonellosis is a food borne zoonosis caused by *Salmonella typhimurium* (sub-specie of *Salmonella enterica*), contributing 10 % of overall foodborne outbreaks (Scallan *et al.,* 2011). Most people who are infected with *Salmonella* suffer from abdominal cramps, fever and diarrhea accompanying chills, headache, nausea, and vomiting within 12 to 72 hours of exposure. *Salmonella* is also responsible for Reiter's syndrome which is characterized by painful urination, joints problems specially pain and eye irritation. If Reiter's syndrome prevails for few months or a year it leads to chronic arthritis. Treatment of salmonellosis is difficult because currently there is no vaccine available against it.

Round the globe there are 16 million cases of typhoid fever, approximately 3 million deaths and 1.3 million gastro intestinal cases annually due to *Salmonella*. It is continuously imposing unacceptable threats to the human health via foodborne pathogen across the globe (European food safety authority, 2010). The main sources of lettuce contamination are highlighted in Figure 2.1. The major documented fresh produce borne outbreaks are; *Salmonella* associated with alfalfa sprouts, *Salmonella typhimurium* linked to spinach and romaine lettuce, *Salmonella Braenderup* infections

from mangoes and *Salmonella typhimurium* linked to *Salmonella litchfield* from cantaloupes tomatoes (US-CDCP).

2.2. Supporting Environmental Conditions for *Salmonella* Survival

The supporting agents for the dynamics of *Salmonella* infection are affected by the living styles, human behaviors, eating habits, travelling choices, industrial preferences and commerce etc. (Foley *et al.,* 2008). *Salmonella* spp. is expected to be present in any raw food product since this pathogen is widely disseminated in nature (e.g., water, soil, plants, and animals). *Salmonella* spp. is able to survive for weeks in water and for years in soil if environmental conditions such as temperature, humidity, and pH are favorable (Betts, 2007). Some species of *Salmonella* have the tendency to grow at temperatures as low as 2℃ (El-Safey, 2013).

Salmonella spp. is resistant to cold temperature and this psychotropic attribute has intensified concerns regarding low temperature (cold conditions bacteriostatic) as food safety technique. *Salmonella* have optimum growth in the pH range of 6.5 - 7.5 with a water activity of 0.93 or above (Silva, 2010). The ability of *Salmonella* to resist bactericidal food process and the propensity to survive at low, high and ambient temperature is highlighting a serious health and food safety concern.

In a healthy growing state microorganism contains 80 % of water which they get from the food they inhabit. Treating the food with high temperature evaporates the water and deprives food spoilage bacteria from their basic requirement of nourishment. All microorganisms are not resistant to high temperatures, as some bacteria are thermophilic and high temperature conditions are ideal for their growth. Therefore, in order to cope with the potential health hazards associated with consumption of

vegetables, it is vital to gain insight into the interactions between foodborne-pathogens, environmental conditions and vegetables (Leveau, 2009).

2.3. Efficacy of Washing and Disinfection Treatments

Lettuce is considered to be contaminated with pathogens even after several washes. Conventional techniques of vegetable washing and disinfection are not significant in complete removal of microorganisms (Beatriz *et al.,* 2012). These techniques are only useful in reduction of experimentally introduced pathogens. Rinsing vegetables with water is a simple disinfection technique but is useful in cases of minor contaminations, resulting in reductions of less than 1 log10 cfu/g (Nguyen and Carlin, 1994), whereas in cases of higher contamination, use of an effective disinfectant is essential.

2.3.1. Efficacy of Organic Acids Against Bacterial Load

Scientists have explored a number of ways to reduce the *Salmonella* contamination from poultry but minimum information is available in case of effective disinfectants for vegetable contamination. Chlorine chill tanks were once perceived as the most effective disinfectants against any *Salmonella* spp. contamination but it had a number of disadvantages that reduce its effectiveness including the production of toxic by-products (Odabasi, 2008) and less efficacy in case of any organic debris remnant (Ricke *et al.,* 2005).

Due to less efficacy of chlorine in organic vegetable contamination and no attached strings of significant disadvantages, customers' interest has been increased in organic acids for food processing applications. A number of organic acids and their salts are being used as disinfectants, food additives and preservatives. Organic acids are given GRAS (generally recognized as safe) status. Several organic acids like lactic acid and

acetic acid have efficient antimicrobial activity even in the presence of organic matter unlike chlorine (Ricke, 2003).

Organic acids have numerous putative antimicrobial mechanisms, including osmotic stress, disruption of intra-cellular pH, and newer concepts like membrane perturbation (Hirshfield *et al.,* 2003). Some FDA and WHO approved organic acids do not restrict pathogen growth by increasing the acidity; these rather immobilize the pathogens by affecting their cellular membranes that ultimately lead towards bacterial population decline.

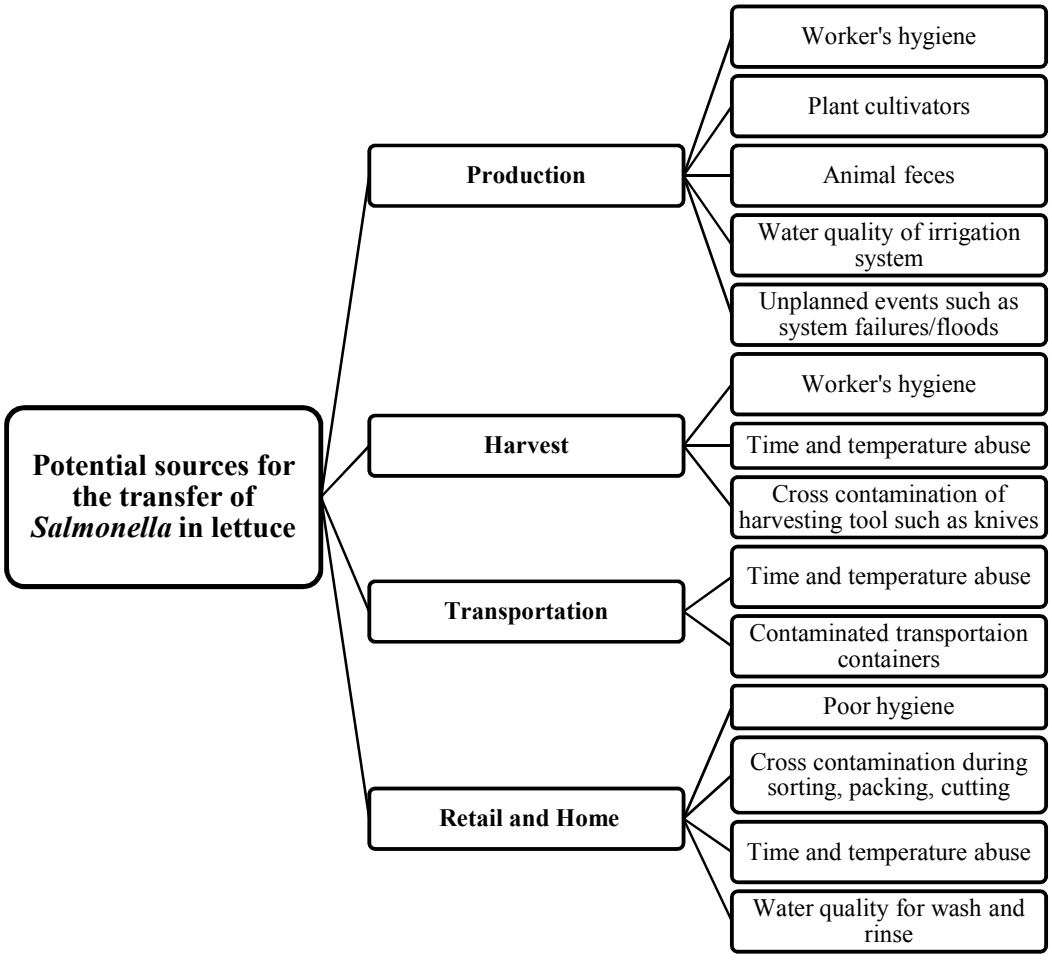


Figure 1.1 Potential sources for *Salmonella enterica* contamination in lettuce

2.4. Pakistan and Salmonellosis

In Pakistan Salmonellosis is usually traced back to poultry and remedial measures are taken to implement hygienic conditions only in that industry (Soomro *et al.,* 2010) while vegetables are ignored although being an important vehicle of the pathogen transmission. Vegetables that are simply rinsed with water only are falsely considered safe for raw consumption. Fresh produce outbreaks are commonly linked with transfer of pathogen from vegetables due to fecal contamination (Gajraj *et al.,* 2012). Fecal contamination is traced back with poor agricultural practices for high crop yield e.g. use of unprocessed bio-fertilizer (animal dung) and wastewater. Raw consumption of salad vegetables is the potential pathogen transfer pathway in food chain causing gastrointestinal diseases that might lead to dangerous stages of Salmonellosis i.e. Reiter's syndrome and chronic arthritis (Hurd *et al.,* 2002).

According to bacteriological study of food in Pakistan's peri urban areas of Rawalpindi and Islamabad, 11.1 % of the food samples were positive for *Salmonella*. Salad vegetables had 1.1 $x10^3$ - $2.3x10^3$ cfu/g *Salmonella* count whereas 2.3 x 10^8 - 4.4 x 10^8 cfu/g for HPC against the permissible limit of 10^5 cfu/g (Nusrat *et al.,* 2012).

According to Mehmood and coworkers (2014) , a survey conducted by Pakistan Institute of Medical Sciences (PIMS) declares that during January 2014, 600 *Salmonella* infected patients were admitted in hospitals of Rawalpindi and Islamabad. 290 of the admitted patients were given treatment against gastrointestinal diseases while 310 were cases of *Salmonella* induced typhoid fever. According to authors out of 744 cases of diarrhea in goats of Islamabad 10 % were caused by *Salmonella.*

The analysis of food products for presence of pathogenic microorganisms is one of the basic steps to control safety and quality of food. Development of new, fast and reliable

identification methods for biological threats and significant disinfectants to minimize the bacterial contamination are necessary to meet the safety standards of food products and risk management.

To protect the health of masses it is not enough to check only the sanitary quality of drinking water consumed by human beings, it requires assessment of the environmental and health risks associated with the use of untreated wastewater, primary treatment of wastewater before its release for irrigations purposes, innovation of practices to make animal manure less contaminated and free of pathogen. Development and implementation of good food safety guidelines that are coupled with risk prevention equally beneficial for small and large scale farmers are necessary to pursue Good Agricultural Practices (GAP's) and even Good Handling Practices (GHP's) while food transportation.

Chapter 3

MATERIALS AND METHODS

The rationale of the research was to determine the contamination level of vegetables and analyze whether these are serving as one of the main pathway for the spread of foodborne diseases in twin cities (Rawalpindi and Islamabad) of Pakistan.

Lettuce was selected as a model vegetable due to number of reasons which include:

- Availability round the clock
- Supplementary component of daily human diet
- Easy access to organic and inorganic products
- Potential vector for the targeted foodborne pathogen

Microbiological quality of lettuce samples from different irrigation sources and harvesting mechanisms were taken into consideration. This study was focused to determine food preservatives to reduce bacterial contamination as well as to suggest ways to restrict pathogen growth in fresh produced. Three FAO allowed food grade reagents were also examined to check their antimicrobial efficiency without harming the quality and taste of lettuce.

This study also noticed the impact of cold and warm temperature storage conditions on the microbiological quality of the lettuce.

3.1. Study Area

The experiments were performed on the samples taken from different commercial (vegetables shops, local and weekly markets) areas providing lettuce to majority of

people of Rawalpindi and Islamabad as well as from domestic points (backyards of houses and farm houses) of twin cities. Basis of organic and inorganic categorization is given in Table 3.1

Table 3.1: Organic/ inorganic categorization of lettuce

Characteristics	Organic Lettuce	Inorganic Lettuce
Fertilizer	Bio-fertilizer (animal manure)	Inorganic Fertilizer
Irrigation Water	Wastewater	Tap Water
Source	Domestic	Grocery Stores, Daily and Weekly Vegetable Markets

Table 3.2 Sampling sites

Residential sites	No. of samples	Commercial sites	No. of samples
G-6, G-9,I-10	18 (IL*)	F-6, I-9, F,7	15 (OL)
Askari 14	8 (OL**)	Scheme 3	12 (IL)
Mini-mart(NUST)	5(IL)	Kohsar market	12 (OL)
Bani gala	15 (OL)	Weekly vegetable markets	15 (IL)

3.2. Sample Collection

A total of 100 lettuce (50 organic and 50 inorganic) samples were collected from different markets of Rawalpindi and Islamabad at early morning hours during a period of 3 months (November 2013 to January 2014). Samples were collected aseptically in sterilized sampling bags and were immediately transported to laboratory for analysis.

3.2.1. Sample Analysis

Samples were introduced in to the experimental setups within 2 hours of their collection. Damaged outermost leaves of the lettuce were aseptically removed to

avoid any remnants of pesticide, soil and surface microbes. The subsequent layers of leaves were detached and used for the experiments. Experimental samples were stored for 24 hours at different temperature conditions (4 and 41.5 °C) and control samples were kept at ambient temperature (10-15°C).

3.3. Research Design

The current study was carried out in three main phases with different tasks to be completed in each phase (Figure 3.1).

3.4. Microbiological load

To measure the microbiological quality of lettuce, microbial load was determined using heterotrophic plate count technique. 10 g lettuce sample was homogenized with 90 ml of sterile peptone water and filtrate was collected. Serial dilutions of samples were prepared upto 10^{-8} according to the method described in FAO manual (1992). 0.1 ml aliquot of each dilution was plated on nutrient agar (Oxoid, UK) and petri plates were incubated at 37°C for 24 h. After 24 h bacterial load inhabiting the fresh lettuce was measured using colony counter.

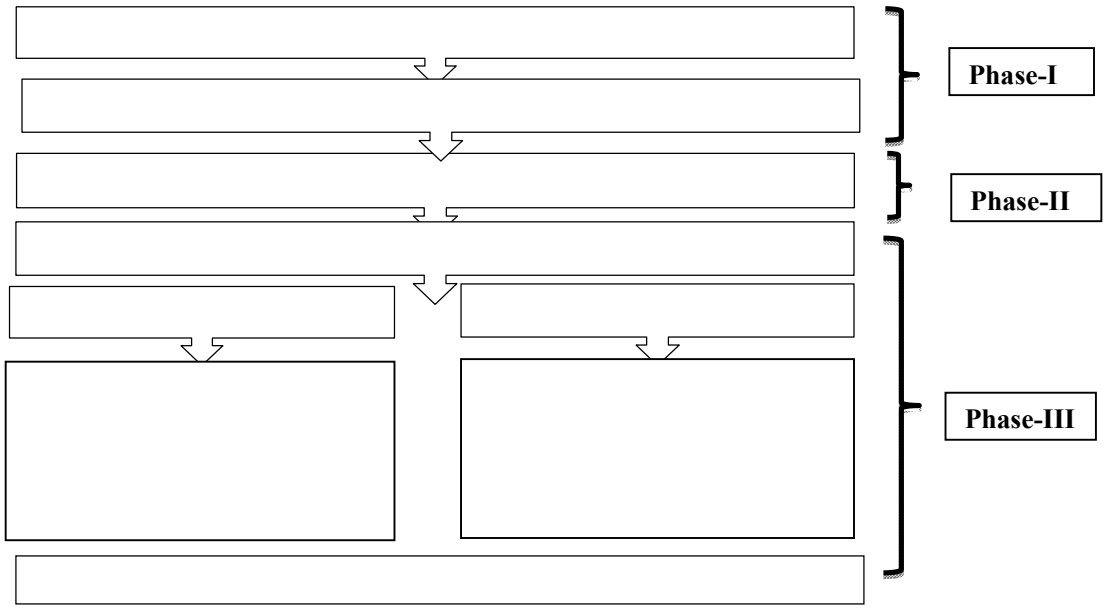


Figure 3.1 Research Methodology

3.4.1. IS0-6579: 2002 Horizontal Detection Method for Isolation of *Salmonella*

USEPA standard IS0-6579:2004 (Fig 3.2) was followed for the enrichment and isolation of *Salmonella* from the collected lettuce samples. Non selective enrichment of samples stored at ambient temperature were carried out by adding 25 g of lettuce and 225 ml of 10 % buffered peptone water in a sterile sample bottle and was incubated for 24 hours. Selective enrichment was carried out by adding 100 and 1000 µl of pre-enriched sample in 10 ml Rappaport-Vassiliadis Soya peptone broth (RVS) and Muller-Kauffmann Tetrathionate-Novobiocin broth (MKTT-n) respectively.

The sample with RVS broth was incubated for 24 hours at 41.5 °C and whereas MKTT-n broth was incubated for 37°C for 24 hours. The samples were then plated out on Xylose Lysine Deoxycholate agar *(*XLD) and Brilliant Green Agar (BGA) after 24 hours. Streaking was performed from BGA and XLD plates on nutrient agar medium after 24 hours. Streaked nutrient agar plates were further incubated for 24 hours at a temperature of 37 °C. On XLD agar plates positive samples would have orange - red colonies and at BGA agar plates red colonies would be present.

3.4.2. Morphological Characteristics

The selected strains were screened were made separated for the identification process. Identification of selected strains was carried out with the help of cell morphology and colony morphology.

Morphology of separated colony was studied by noting their color, form, elevation, size, margins, gram reaction using gram staining techniques. Gram staining was performed as per methods described in standard methods (APHA, 2012).

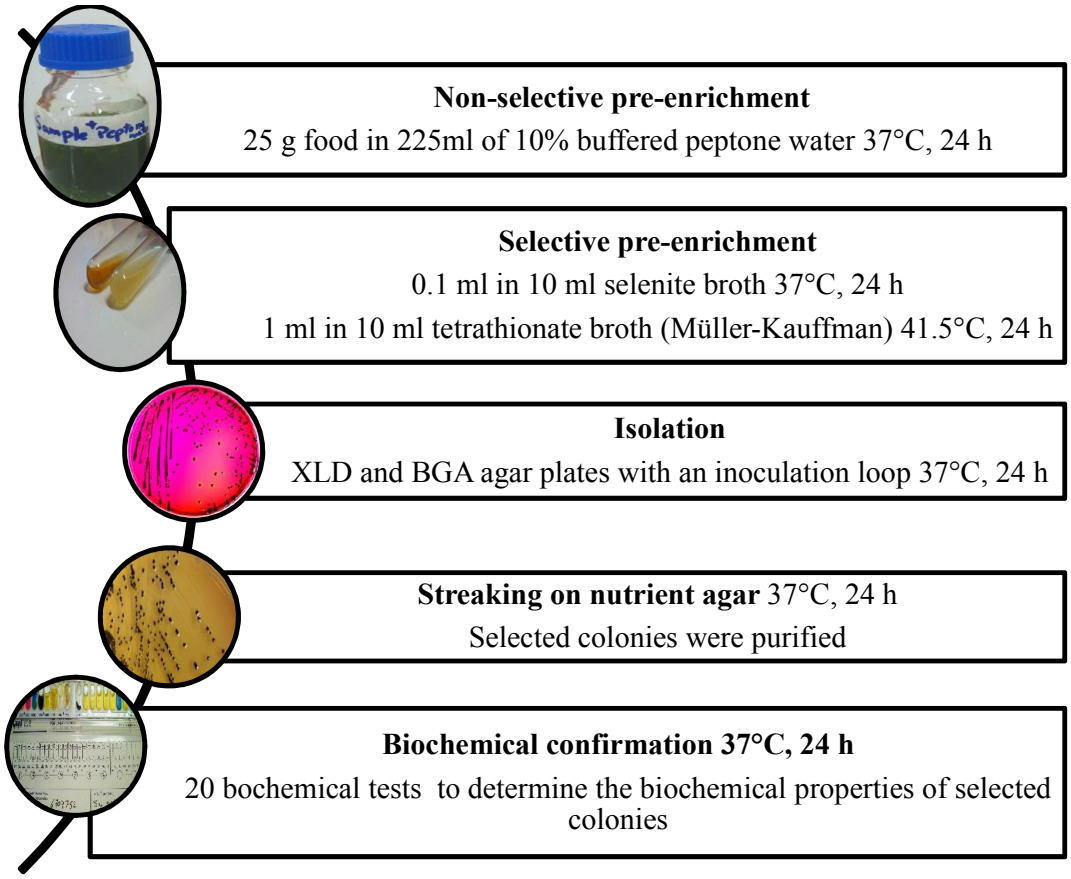


Figure 2.2 ISO-6579:2004 for *Salmonella* Isolation

3.5. Biochemical Confirmation and Genus Identification

3.5.1. Analytical Profile Index (API)

Isolated pathogens were confirmed using biochemical tests and were counter checked by using API kits. Strip consists of 20 individual microtubes that are filled with hydrated substrates and specific medium for biochemical characterization. For performing the test a saline suspension (0.85 % NaCl) was prepared and autoclaved. Saline suspensions were prepared for fresh culture; the suspensions were added in the microtubes upto the mark except for citrate utilization (CIT), voges–proskauer (VP) and gelatin liquefaction (GEL), where the cupule was filled completely. A drop of mineral oil was added in the cupules filled to neck to avoid

drying out. The strips were covered with the lids provided and placed in incubator overnight and color changes were noted. Following incubation, identification of the organism was made by means of computer assisted system called PRS (Profile Recognition System). PRS includes an API coder, profile register and selector.

Figure 3.3 API 20-E strip

3.6. Specie Identification

3.6.1 Polymerase Chain Reaction (PCR)

The PCR method is a sensitive and delicate approach for the specie identification as well as detection of particular species in variant samples. It amplifies specific DNA sequence from a single particle or single virus to a detectable limit within in brief period. After purifying the *Salmonella* isolated from lettuce leaves, these pure cultures were used for DNA extraction. Optimization was done by varying different parameters like $MgCl_2$ concentration, template DNA amount, concentration of primers and annealing temperature depending on the Tm of the primers (Innis *et al.,* 1999).

3.6.2 Maintenance and Cultivation of Bacterial Strains

3.6.2.1. Washing of Fresh Culture

Salmonella typhimurium strains isolated from the lettuce samples were inoculated in nutrient agar slants and kept in incubator at 37°C for 24 hours. After 24 hours incubation the grown cultures from slants were washed with 0.85% saline solution

(dissolve 0.85 g NaCl in 100 ml of distilled H_2O). This resulted in a bacterial suspension of 10^8 cfu/ml with a McFarland Turbidio-metric index scale of 0.5.

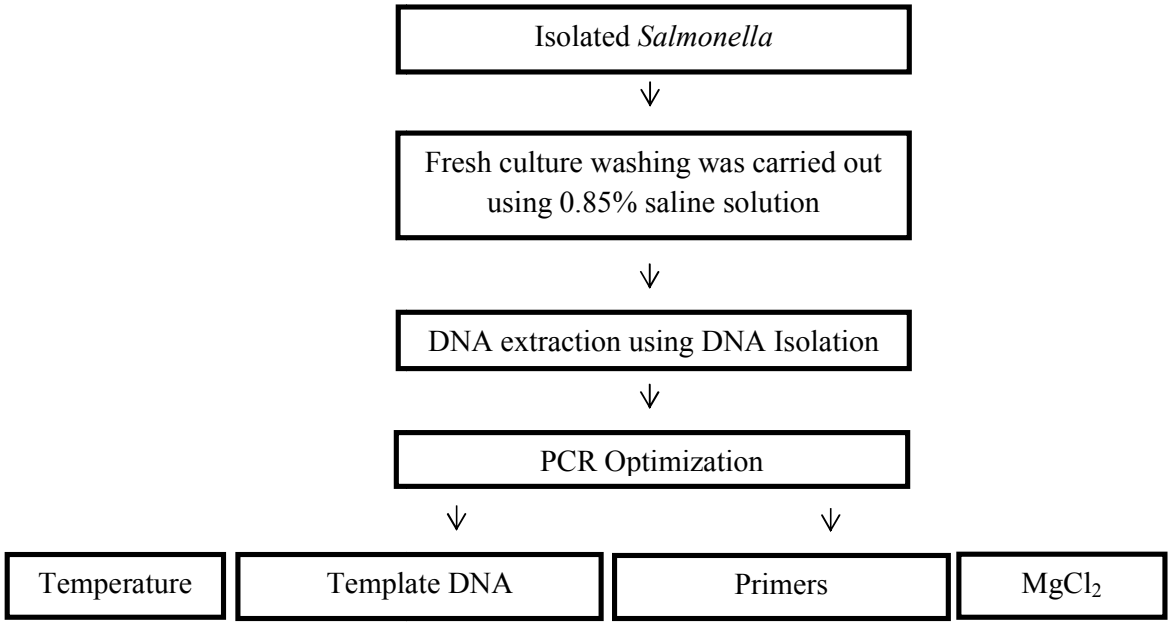


Figure 3.4: PCR reaction mixture protocol for *Salmonella* spp. identification

3.6.2.2. DNA Extraction

Bacterial suspension obtained after washing of culture was used for the DNA extraction of the microbes. DNA Isolation Kit (Norgen, Canada) and the provided protocol were used for DNA extraction as per manufacturer's instructions. Four important steps of DNA extraction were;

- Lysate Preparation
- Binding Column
- Column Wash
- DNA Elution

Figure 3.5: DNA extraction

3.6.3. Primer Selection

Primers were designed using the Genebank data base sequence of NCBI through Basic Local Alignment Search Tool (BLAST) program (http://www.ncbi.nlm.nih.gov/). The targeted genes, primers and PCR conditions used are listed in Table 3.3.

Table 3.3 Primer sequence for *Salmonella spp.*

Primers	Primers Sequence (5' - 3')	Target Specie	Target gene	Product length (bp)	Annealing temp. (°C)
SirA2-F	GCCGTACTAACGCCGTTGAC	*Salmonella*	SirA	284	62.7
SirA2-R	TAGCGATAGCTGTTCACCGT				

3.6.4. PCR Amplification

For the DNA template amplification a complete PCR mixture protocol was formulated. All the ingredients required for PCR mixture and PCR tubes were kept on ice which was prepared by mixing the ingredients in the exact order provided in the Table 3.4 and were vortexed. Taq polymerase was added few seconds before using PCR because of its ultra-heat sensitivity.

3.6.5. Agarose Gel Electrophoresis

In order to analyze the amplified DNA, Gel Electrophoresis was carried out. It included gel preparation and loading process.

Gel Preparation

1 % (w/v) agarose gel was prepared by mixing 0.6 g of agarose with 60 ml of 1x TBE buffer, Whereas 1x TBE is composed of 10.8 g of Tris base, 5.5 g of boric acid and 0.93 g of EDTA dissolved in 1 L of distilled water. 0.5 µg/ml of ethidium bromide

was added to Gel solution before it was poured into the casting tray of electrophoresis with well making combs.

Loading Process

Solidified gel was immersed in the electrophoresis machine. Loading dye was added to tract the amplified DNA and was loaded into the wells. Total amount of 6 µl was loaded in each well; includes 4 µl sample and 2 µl loading dye. 1x TBE running buffer was used as a base to perform the Electrophoresis at 100 volts for 30 minutes. The DNA bands were visualized with Geldoc system (Micro Doc cleaver scientific UK) on UV illuminator.

Table 3.4 PCR mixture for *Salmonella typhimurium*

Ingredients	Volume (µl)	Purpose
$MgCl_2$	2.5	Provide ionic conditions in reaction mixture for binding of primers with DNA template.
dNTPs	2	Basic building blocks that follow standard base pairing rule and helps to synthesize new DNA strand.
Taq buffer	2.5	Provides suitable chemical environment, optimal pH and salt conditions for the optimal activity of DNA polymerase.
Primers	2	For each target sequence at the end of the DNA, both strands are copied simultaneously in both directions.
DNA template	5	Contains the targeted DNA region needed to be amplified.
Taq polymerase	0.3	Thermo-stable DNA polymerase that produces an enzyme. This enzyme synthesizes the DNA strand from template.
PCR water	5.7	Maintains the final volume.

Table 3.5 Thermal cycler PCR conditions for *Salmonella typhimurium*

PCR steps	Temperature (^{o}C)	Time (min)	Number of cycles
Denaturation	95	10	1
Annealing	95	30	40
	64	30	
	72	30	
Extension	72	4	1

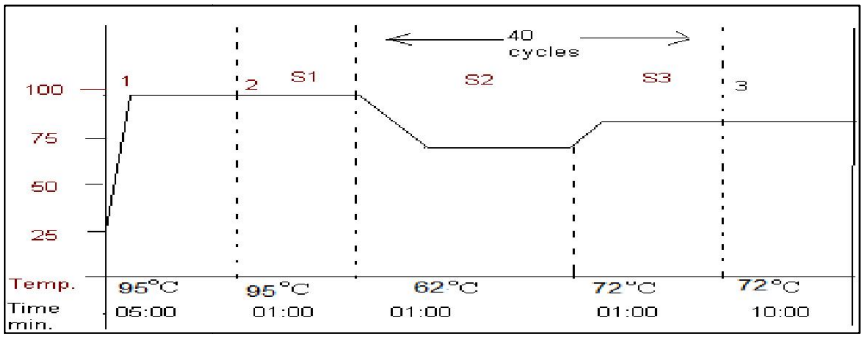


Figure 3.6 PCR programme for *Salmonella spp.*

3.7. Variable Temperature Treatments

In order to analyze the impact of high and low temperature storage condition on microbial load and survival of *Salmonella*, experimental samples of lettuce were stored at 4 and 41.5 °C. These were subjected to standard method of *Salmonella* isolation from food (IS0-6579:2004) and HPC to determine the microbial load (FDA, 2001).

3.7.1. High Temperature Treatment

To determine the impact of warm storage conditions, lettuce samples were stored in incubator at 41.5°C for 24 hours. The experimental samples from the incubator were

analyzed for bacterial load via HPC and *Salmonella* presence by standard method ISO 6579:2004 for isolation of *Salmonella* from foods.

3.7.2. Low Temperature Treatment

Effect of low temperature storage conditions on microbiological quality of lettuce was assessed by storing experimental lettuce samples in refrigerator at 4°C for 24 hours. These experimental samples were then analyzed after 24 hours to determine the bacterial load via HPC and *Salmonella* presence by standard method ISO 6579:2004 for isolation of *Salmonella* from foods.

3.8. Chemical Treatment

Decontamination is an important step to influence the shelf life safety as well as quality of fresh produce (Ölmez and Kretzschmar, 2009). The quantity of disinfectant introduced must go with the allowed limits in order to prevent any harmful effect (Parish *et al.,* 2003).

To compare the antimicrobial efficiency of food grade reagent FDA and WHO allowed class I (acetic acid/synthetic vinegar, CH_3COOH) and class II (sodium benzoate, NaC_7H5O_2: potassium sorbate, $C_6H_7KO_2$) preservatives were analyzed (Table 3.6 and 3.7).

Table 3.6 Targeted organic acid (Class-1 Preservative)

Class-1 Preservative	
Chemical	Acetic acid
Occurrence	Naturally occurring substances,
FAO/WHO allowed limit	No limitation on use
Used in research	2% v/v (100 ml/50g of sample)
Consumer acceptability	Constituent element of daily human diet

Table 3.7 Targeted organic acids (Class-2 Preservative)

Class-2 Preservatives		
Chemicals	**Sodium Benzoate**	**Potassium Sorbate**
Occurrence	Manmade chemical substances	
FAO/WHO allowed limit	328mg/day	875mg/day
Used in research	20mg/day	
Consumer acceptability	Not very popular	

3.8.1. Chemical Preparation

Antimicrobial efficiency of food grade reagents was compared by preparing 2 % (w/v) solutions of synthetic vinegar (CH_3COOH), sodium benzoate (NaC_7H5O_2) and potassium sorbate ($C_6H_7KO_2$) were prepared using distilled water. 25 g of lettuce was transferred to 50 ml 2 % (v/v) solution of synthetic vinegar for 10 min. For further analysis HPC and *Salmonella* count were recorded. Similar procedure was repeated for sodium benzoate and potassium sorbate (Chang and Fang, 2007).

3.9. Data Analysis

The results were analyzed using statistical tools (ANOVA and t-test) and Microsoft Office Excel 2010 (Microsoft Corporation).

Total bacterial load and *Salmonella* count were normalized by log transformation before analysis of variance (ANOVA). ANOVA was applied to compare the total bacterial load and *Salmonella* in lettuce against different temperature and organic acid treatments. T-test was used to determine the significance of difference of total HPC and *Salmonella* in lettuce from organic and inorganic sources considering the assumption of variances as equal or not. Standard errors, variance and mean were calculated using Microsoft office excel 2010. Results of analysis are at $p < 0.05$ level of significance.

Chapter 4

RESULTS AND DISCUSSION

4.1. Microbiological Quality of Lettuce

4.1.1. Total Heterotopic Plate Count in Lettuce Samples

The total heterotrophic plate count observed in lettuce ranged from 5.45-7.23 log cfu/g in inorganic and 3.81- 5.11 log cfu/g in organic lettuce (Table 4.1)

Table 4.1: Microbiological quality of lettuce being produced in Rawalpindi and Islamabad

Lettuce source	Total Heterotrophic plate count (Log_{10} cfu/g)	
Inorganic	Range (N-50)	5.45-7.23
	Geometric mean	6.54 (± 0.50)*
Organic	Range (N-50)	3.81-5.11
	Geometric mean	4.67 (± 0.36)*

*** Standard errors**

Mean heterotrophic plate count in inorganic lettuce was significantly higher ($P < 0.05$) than those in organic lettuce being produced in Rawalpindi and Islamabad. The HPC in organic and inorganic lettuce samples were exceeding the quality standard of 10^3 cfu/g provided by International Commission on Microbiological Specifications for Food (ICMSF) and 2.3 log cfu/g by WHO.

4.1.2 *Salmonella spp.* in Lettuce Samples

Significant concentration of *Salmonella* was present in 90 % of organic and inorganic lettuce samples collected from Rawalpindi and Islamabad. The data in Figure 4.1 shows the *Salmonella* contamination in organic and inorganic samples collected from targeted sites. Samples of Rawalpindi were more contaminated than samples collected from Islamabad. *Salmonella* count in organic and inorganic lettuce produced in

Rawalpindi were 3.08 and 6.087 log cfu/g respectively whereas organic samples from Islamabad had 2.9 log cfu/g and inorganic samples had 5.4 log cfu/g *Salmonella* count. Mean *Salmonella* concentration in organic sample ranged from 2.9-3.9 log cfu/g, whereas in inorganic samples *Salmonella* concentration ranged from 5.4-6.1 log cfu/g (Figure 4.1).

Wastewater irrigation is a common practice in Rawalpindi and Islamabad which is one of the main causative agent behind pathogen contamination into fresh produce. Rawalpindi alone is generating 40 x 10^6 m^3/y of wastewater on daily basis in Lei nala which enters Soan River and vegetable farms. 65% of the city wastewater is directly discharged into Lai, which is a common irrigation water reservoir for city's agricultural activities. This further increases the level of contamination in inorganic samples that are directly irrigated with wastewater without any prior treatment. *Salmonella* contamination was found less in organic samples i.e. 4.67 log cfu/g as compared to inorganic i.e. 6.54 log cfu/g.

Such high *Salmonella* contamination can be related to lack of food monitoring authorities and continuous use of untreated wastewater and animal dung used in agricultural practices.

Salmonella contamination in organic samples is comparatively less. Organic lettuce is not being produced at large commercial scales and is produced only at domestic level as well as in some farms designated for organic farming. The main factor contributing *Salmonella* in organic samples is the raw usage of animal dung because people producing lettuce domestically are using tap water for irrigation. Use of commercial fertilizer is replaced with animal dung. Fresh animal dung is considered to be full of NPK (Nitrogen, Phosphorus and Potassium) nutrients and is transported

to farms immediately to be used as manure. Though usage of raw dung as manure is resulting in high crop yield but is also serving as a vector agent for the transfer of

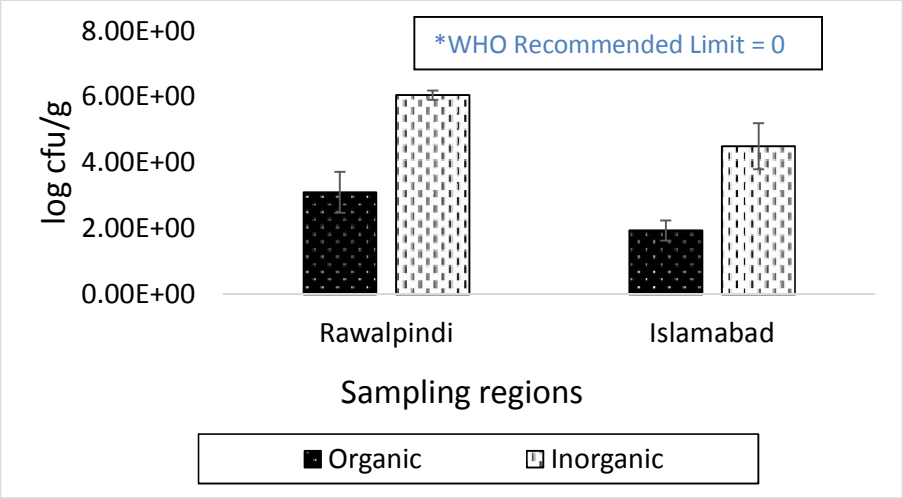


Figure 4.1 *Salmonella* contamination on lettuce sampled from different sites

Salmonella in organic lettuce being produced in Rawalpindi and Islamabad.

4.2 Specie Identification

4.2.1 Analytical Profile Index (API)

The selected gram negative strains were examined using API 20-E for biochemical identification. Approximately 92% of the isolates showed positive results for *Salmonella enterica* spp. (Table 4.2).

Table 4.2: API 20-E results of selected isolates

Lettuce sample	Isolates (Random)	Code Number	Confidence %	Organism
Inorganic	IL27	6704752	98.2 %	*Salmonella enterica* spp.
Organic	OL 17	6704552	86.4 %	

4.2.2 Specie Confirmation

PCR conditions were optimized using standard *Salmonella* (ATCC 14028) culture. For confirmation of *Salmonella* specie, 16 SrRNA sirA primers were detected at 284 bp size against 1 kb ladder which is specific for *Salmonella enterica*. Specie of the genus *Salmonella* present in the lettuce samples of Rawalpindi and Islamabad was confirmed as *Salmonella enterica* serotype *typhimurium* as supported by Figure 4.2.

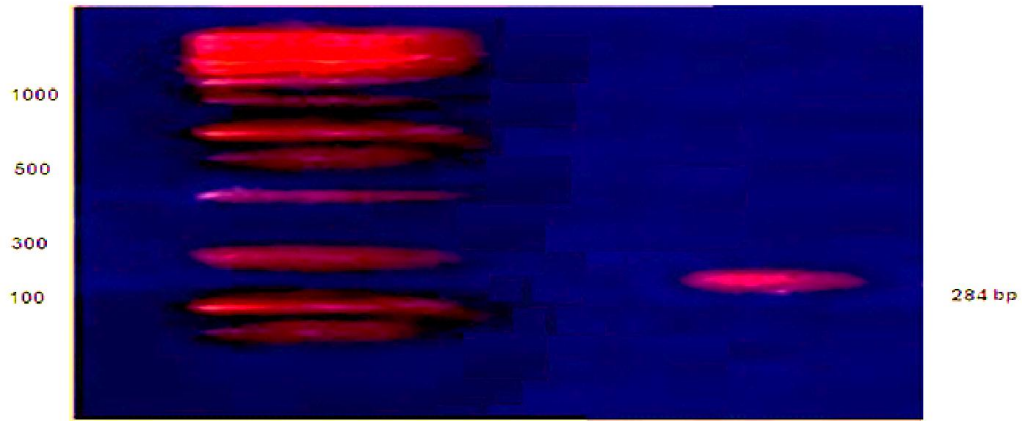


Figure 4.2: Agarose gel showing PCR product amplified from *Salmonella* strain. lane a: 1kb DNA ladder, lane c: PCR amplified product of Salmonella enterica serotype typhimurium

4.3. Effect of Temperature on Contamination Level

Significant change was observed in heterotrophic plate count (HPC) in experimental samples. The results indicated that the majority of bacterial load inhabiting lettuce is psychrophilic as their growth is favored by low temperature (4°C) conditions showing a log increase of 0.43 (6.54 - 6.99 log cfu/g) increase in HPC. This reveals that storing lettuce at low temperature will increase its contamination level. A log reduction of approximately 2.5 (6.54 - 3.99 log cfu/g) was observed in lettuce samples stored at high temperature (41.5°C) (Figure 4.3).

Table 4.3 Heterotrophic plate count against different temperature conditions

Lettuce source	Total Heterotrophic plate count (Log_{10} cfu/g)			
		4°C	10°C	41.5°C
Inorganic	Range (N-50)	5.81-7.75	5.45-7.23	4.34-6.71
	Geometric mean	6.97(± 0.41)	6.54 (± 0.50)	3.99(± 2.23)
Organic	Range (N-50)	4.17-5.51	3.8-5.1	2.31-3.22
	Geometric mean	4.97(± 0.27)	4.67 (± 0.36)	2.37(± 1.08)

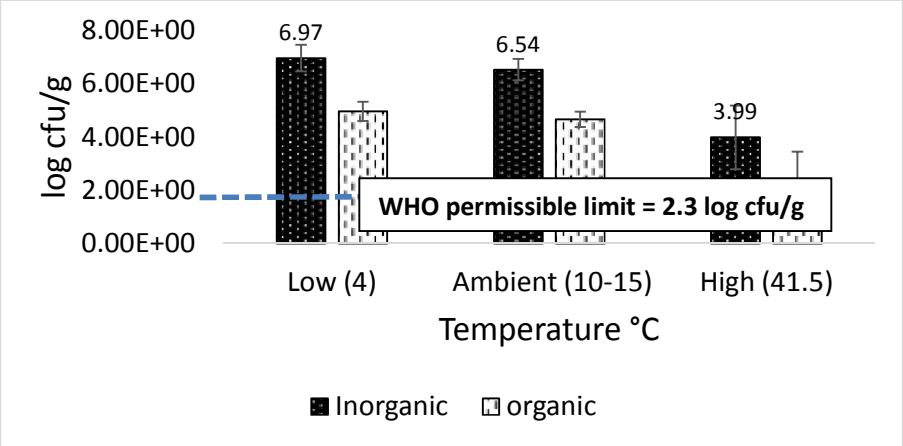


Figure 4.3 HPC at variable temperature conditions

A significant change in the *Salmonella* count was observed in experimental samples. Samples stored at 4°C showed an increase of approximately 0.7 log in *Salmonella typhimurium* count compared to the samples stored at 41.5 °C which showed 3.75 log reduction. These results indicate that *Salmonella typhimurium* being psychrotrophic in nature was considerably affected by temperature, which is also reported by Strauch (1991) and Ahmed and Sorensen (1995). High temperature proved to be growth

resistance factor (4.5- 0.29 log cfu/g) and low temperature conditions proved to be growth supporting factor (4.5.-4.8 log cfu/g) as illustrated in Figure 4.4.

Table 4.4 *Salmonella typhimurium* count against different temperatures

Lettuce source	Total *Salmonella typhimurium* count (Log_{10} cfu/g)			
		4°C	10°C	41.5°C
Inorganic	Range(N-50)	4.92-6.02	5.41-6.43	4.1-5.9
	Geometric mean	4.8(± 2.27)	4.5 (± 2.45)[a]	0.44(± 1.43)
Organic	Range(N-50)	2.04-3.90	3.6-4.4.27	2.21-2.31
	Geometric mean	3.4 (± 1.06)	2.3 (± 1.50)	0.29(±0.79)

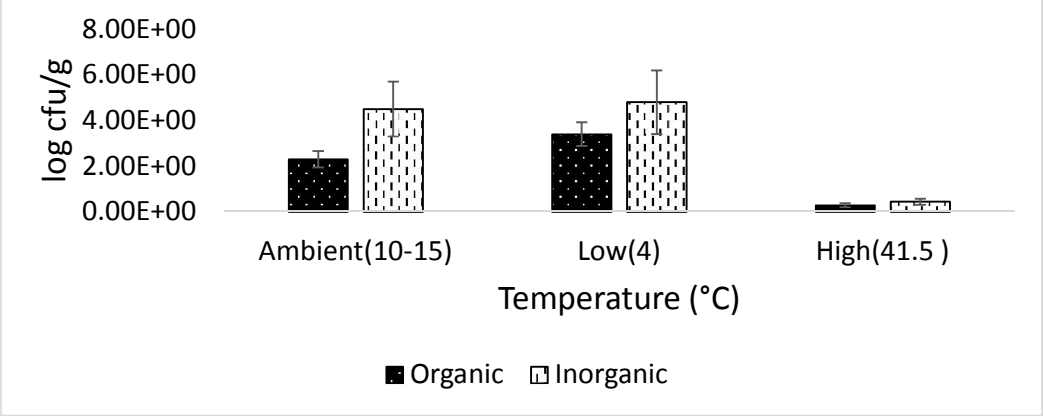


Figure 4.4 Effect of temperature on *Salmonella typhimurium*

4.4. Anti-Microbial Efficiency of Food Grade Reagents

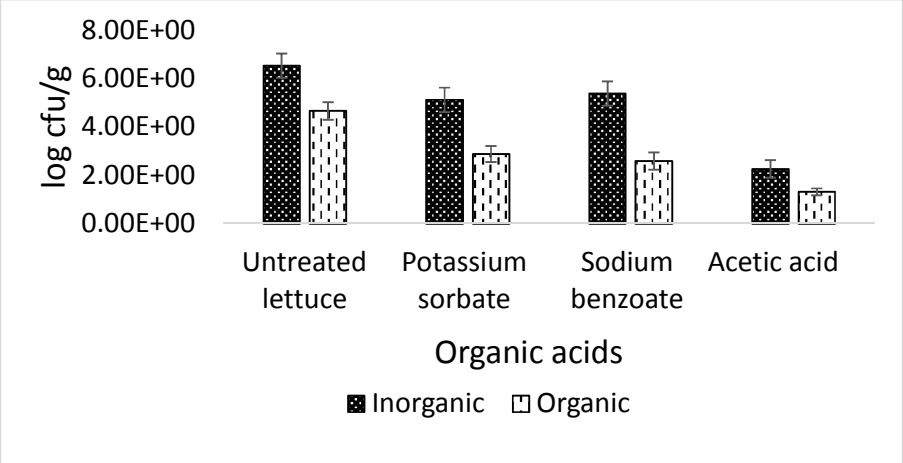
In order to increase the shelf life of food products organic acids were added to maintain pH ≤ 4.5 which decreases chances of food spoilage by creating an acidic environment. Majority of the food spoiling microorganisms were not adapted to acidic environment thus unable to survive. Mechanism of antimicrobial activity is based on

the principle of inhibition of membrane function, interfering with metabolism, enzyme activity, integrity of plasma membrane, overall microbial activity and nutrient transport. Food grade reagents have been studied for their effectiveness in removing pathogens from fruits and vegetables. Selected food grade reagents (acetic acid, sodium benzoate and potassium sorbate) were used to analyze their anti-bacterial action which focuses on, maintained acidic environment, interference with pathogens metabolism and with its plasma membrane. Organic acids are declared safe by FDA because human body readily metabolizes them (Eni *et al.,* 2010; Russell and Grahame, 2003).

Table 4.5 Efficacy of organic acid treatments against HPC

Lettuce source	Total HPC (Log_{10} cfu/g)			
	Untreated lettuce	$C_6H_7KO_2$	$NaC_7H_5O_2$	CH_3COOH
Inorganic	6.54	5.131	5.394	2.271
Organic	4.67	2.89	2.59	1.32

Changes in microbial load were observed when the lettuce was treated against 2% (w/v) synthetic vinegar, sodium benzoate and potassium sorbate for 10 min. Treatment with potassium sorbate resulted in 1 log reduction in HPC in inorganic lettuce and 2 log reductions in case of organic lettuce.



* Time: 10 min, Conc.: 2% w/v (acetic acid), 2% v/v (potassium sorbate & sodium benzoate)

Fig 4.5 Effect of organic acids on HPC

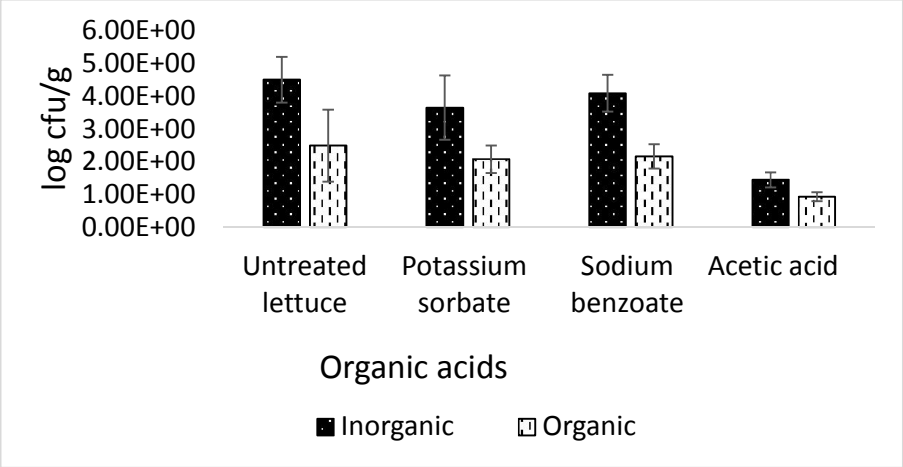
Sodium benzoate resulted in 1.2 log reduction in HPC in inorganic lettuce and 2.3 log reductions in case of organic lettuce while acetic acid treatment resulted in 4 log reduction in HPC in inorganic lettuce and 3 log reductions in case of organic lettuce. Significant antimicrobial effect was observed in samples treated with acetic acid. Treatment with acetic acid removed > 60% of the contaminants from the lettuce samples (Figure 4.5). Statistical analysis of the results showed that the effectiveness level of acetic acid is significant as a food reagent for lowering the pH, making it difficult for acid resistant food pathogens to survive, hence increasing the quality of fresh produce compared to the impact of other targeted food reagents. Oliveira *et al.,* (2012) showed that approximately 90% of the mesophilic bacteria (1.6×10^6- 3.08×10^4) were reduced when lettuce was treated with 2% acetic acid.

Salmonella typhimurium count were reduced when the samples were treated against 2% (w/v) acetic acid, sodium benzoate and potassium sorbate for 10 min. 1.15 and 0.4 log reduction of *Salmonella typhimurium* count in inorganic and organic lettuce was observed when treated with potassium sorbate. Treatment with sodium benzoate resulted in 0.4 log reduction of *Salmonella typhimurium* count in inorganic lettuce and

0.3 log reductions in case of organic lettuce whereas treatment with acetic acid resulted in 3 log reduction of *Salmonella* typhimurium count in inorganic lettuce and 1.3 log reductions in case of organic lettuce as indicated in the Figure 4.6.

Table 4.6 Efficacy of organic acid treatments against *Salmonella typhimurium*

Lettuce source	Total *Salmonella* count (Log_{10} cfu/g)			
	Untreated lettuce	$C_6H_7KO_2$	$NaC_7H_5O_2$	CH_3COOH
Inorganic	4.5	3.65	4.09	1.44
Organic	2.49	2.075	2.16	0.93



* Time: 10 min, Conc.: 2% w/v (acetic acid), 2% v/v (potassium sorbate & sodium benzoate)

Fig 4.6 Effect of organic acids on *Salmonella typhimurium*

FDA and WHO allowed Class I & II food preservatives were used because of their easy access and abundant use as safe preservative. Potassium sorbate is an approved class-II preservative by FDA for increasing the shelf life; maintenance of the taste and color of the food products but its use beyond the permissible limit (25mg/kg body

weight) may stimulate nausea, gastric uptakes and allergic reactions. Some people might develop skin allergies in case of contact with potassium sorbate (Rahul, 2012).

Although sodium benzoate (class-II preservative) is significant in increasing shelf life of fresh produce, it is not recommended as a disinfectant for vegetables due to its reported harmful effects on pregnant women, people with atopic allergies and those with respiratory tract problems because it damages mitochondria in cells which may lead to neuro-degenerative diseases.

Acetic acid is among those FDA and WHO permitted Class I food preservatives which is widely being used at domestic and commercial level for food storage with no daily intake limit when used in diluted form because of their natural origin. Human body has the ability to metabolize it easily, thereby, making it safe for human consumption as part of daily diet (Pundir and Jain, 2011).

With minimum negative health impacts associated with HPC and *Salmonella typhimurium* log reduction, efficacy of acetic acid was found to be more significant ($P < 0.05$) as compared to other food reagents. Acetic acid reduced *Salmonella* count to 1.14 - 4.5 log cfu/g Similar findings were reported by Park *et al*. (2011) confirming significant impact of acetic acid as anti-*Salmonella* reagent in lettuce leaves.

Chapter 5

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

1. HPC in all the Lettuce samples collected from Rawalpindi and Islamabad were found to exceed the permissible WHO limit of 2.3 log cfu/g, the possible reason of it might be use of untreated wastewater for irrigation and animal manure as bio fertilizer.
2. 90 % organic and inorganic lettuce samples collected from G-9, G-6, I-10, Bani gala, Mini-Mart (NUST), Askari-14 were contaminated with high concentration of *Salmonella spp* ranging from 2.9 - 6.4 log cfu/g.
3. PCR confirmed specie of *Salmonella*. as *Salmonella enterica* serovar *typhimurium.*
4. Storage of vegetables is not safe from health point of view because lettuce stored at 4°C showed increase in HPC by 13 % whereas *Salmonella* count was found to be increased by 7 %.
5. Cooking vegetables at raised temperature (< 40°C) significantly reduced the HPC by 53 % and *Salmonella* count by 44 %.
6. Among the different organic acid treatments, acetic acid (CH_3COOH) proved to be more reliable as it reduces HPC count by 69 % and *Salmonella* count by 64 % as compared to potassium sorbate (29 % HPC and 19 % *Salmonella*) and sodium benzoate (27 % HPC and 11 % *Salmonella*). This efficacy of acetic acid in bacterial and Salmonella log reduction is because of its undissociated forms. It passively diffuse

through the cell wall of bacteria and maintain a neutral internal pH which catalyze its dissociation into anions and protons. The release of protons helps in decreasing the internal pH values which generates an inhibitory effect on bacterial growth and survival.

5.2. Recommendations

Following recommendations are proposed for further research:

1. Microbiological quality of ready to eat foods may be analyzed with focus on *Salmonella* and *Listeria monocytogenes.*
2. *Salmonella* contamination might be identified in others vegetables being produced in Rawalpindi and Islamabad.
3. Food preservatives from approved class I and II like benzoic acid and sugars may be analyzed for their antimicrobial efficacy.

REFERENCES

Ahmed, A.U. and Sorensen, D.L. (1995). Kinetics of pathogen destruction during storage of dewatered bio-solids. Water Environmental Research. 67:143-150.

Anderson, M., Jaykus, L.A., Beaulieu, S., Dennis, S. (2011). Pathogen produce pair attribution risk ranking tool to prioritize fresh produce commodity and pathogen combinations for further evaluation. Food Control. 22: 1865-1872.

APHA. (2012). Standard Methods for the Examination of Water and Wastewater. 22nd ed., American Public Health Water Association, Washington.

Barak, J. D. and Schroeder, B. K. (2012). Interrelationships of food safety and plant pathology: The life cycle of human pathogens on plants. Annual Review of Phytopathology. 50:12.1-12.

Barati, H.A., Golmohammadi, A., Momeni, I. and Moradi, G. (2010). A cholera outbreak investigation in karaj district in 2008. Iranian Journal of Epidemiology. 6(3): 28-34.

Beatriz, A., Oliveira, A., Carolina, A., Tondo, E. and Cardoso, M. (2012). Comparison of different washing and disinfection protocols used by food services in southern Brazil for lettuce (lactuca sativa). Food and Nutrition Sciences. 3: 28-33.

Betts, R. (2007). Water, water, everywhere nor any drop to drink—the problem of *Salmonella* in low-moisture foods. IAFP Special Interest Session on *Salmonella* growth, persistence and survival in low-moisture foods and their environment Strategies for control. 94th IAFP Annual Meeting. 08.07.2007-11.07.2007. Buena Vista, Florida.

Beuchat, L. R. (1996). Pathogenic microorganisms associated with fresh produce. Journal of Food Protection. 59:204-216.

Brandl, M. and Sundin, G.W. (2013). Focus on food safety: Human pathogens on plants. Phytopathology. 103:304-305.

Carr, R. (2003). Safe use of wastewater in agriculture, WHO Guidelines. In: Workshop on "Integrated management of wastewater resources", World Health Organization, Switzerland. Available at http://www.who.int/water_sanitation_health/

CDCP (2013). Multistate food-borne outbreaks investigations. Available at: http://www.cdc.gov. Accessed July 21, 2014.

CDCP (2011). Investigation announcement: multistate outbreak of *E. coli* O157:H7 infections linked to romaine lettuce. Available at

http://www.cdc.gov/ecoli/2011/ecoliO157/romainelettuce/120711/index.html. Accessed July 2014.

Chang, J. M. and Fang, T. J. (2007). Survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovars *typhimurium* in iceberg lettuce and the antimicrobial effect of rice vinegar against *E. coli* O157:H7. Food Microbiology. 745-751.

Crandall, P. G., Neal, A., O'Bryan, C. A., Murphy, C. A., Marks, B. P. and Ricke, S.C. (2011). Minimizing the risk of *Listeria Monocytogens* in retail delis by developing employee focused, cost effective training. Agriculture, Food and Analytical Bacteriology. 1:159-174.

El-Safey, M. (2013). Incidence of *Salmonella Heidelberg* in some Egyptian foods. International Journal of Microbiology and Immunology Research. 1(2): 16-25.

Eni, A. O., Ibukunoluwa, A. O., Oranusi, U. S. (2010). Microbial quality of fruits and vegetables sold in Sango Ota, Nigeria. African Journal of Food Science. 4(5): 144-151.

Ensink, J. H. J., van der Hoek, W., Matsuno, Y., Munir, S. and Aslam, M.R. (2003). The use of untreated wastewater in peri-urban agriculture in Pakistan: risks and opportunities. IWMI Research Report no. 64, International Water Management Institute, Colombo, Sri Lanka. 22 pp

EFSA (2010). The community summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in the European Union in 2008. EFSA Journal. 8(1):1496.

FDA (2001). Analysis and evaluation of preventive control measures for the control and reduction/elimination of microbial hazards on fresh and fresh-cut produce. http://www.fda.gov/Food/FoodScienceResearch/SafePracticesforFoodProcesses/ucm091260.htm. Accessed July 2014

FAO (2013). Food and Agriculture Organization Statistical Database. http://faostat3.fao.org/home (Accessed in August, 2013).

FAO (1992). Manual of quality control: Microbial analysis IV, FAO, Rome.

Foley, S. L., Lynne, A. M., Nayak, R., (2008). *Salmonella* challenges: Prevalence in swine and poultry and potential pathogenicity of such isolates. Journal of Animal Science. 8, 149–162.

Gajraj, R., Pooransingh, S., Hawker, J. I., Olowokure, B. (2012). Multiple outbreaks of *Salmonella Braenderup* associated with consumption of iceberg lettuce. International Journal of Environmental Health Research. 22: 150-155.

Gomez-Govea, M., Solis-Soto, L., Heredia. N., Garcia, S., Moreno, G. and Tovar, O. (2012). Analysis of microbial contamination levels of fruits and vegetables at retail in Monterrey, Mexico. Journal of Food Agriculture and Environment. 10(1): 152-154.

Hamid, A., Ahmed, N. and Agboola R. (2012). Food: Its preservatives, additives and applications. International Journal of Chemical and Biochemical Sciences. 1: 36-47.

Hirshfield, I. N., S. Terzulli, and C. O'Byrne. (2003). Weak acids: panoply of effects on bacteria. Sci. Prog. 86:245–269.

Hurd, H.S., McKean, J.D., Griffith, R.W., Wesley, I.V. and Rostagno, M.H. (2002). *Salmonella* enterica infections in market swine with and without transport and holding. Applied and Environmental Microbiology, 68, 2376-2381.

Innis, M., Gelfand, D. and Sninsky, J. (1999). PCR Applications: Protocols for functional genomics. Academic Press, San Diego, CA.

Jensen, A., Loretta, M., Friedrich, B., Linda, J., Harris, C., Michelle, D., Danyluk, B. and Donald, W. (2015). Cross contamination of *Escherichia coli* O157:H7 between lettuce and wash water during home-scale washing. Food Microbiology. 46: 428-433.

Juning, Su., Jiebing and Wu., Chenguang, Liu. (2014). Research on coordination of fresh produce supply chain in big market sales environment. The Scientific World Journal. 12-15.

Kuchenmüller,T., Abela, B., Corrigan, T. and Tritscher A. (2013). WHO initiative to estimate the global burden of foodborne diseases. Review of Science Tech off International Epiz. 32 (2), 459-467.

Legnani, E., Legnani, R.F.S., Barbosa, V.C., Krinski, K., Elsangedy, H.M., de Campos, W., da Silva, S.G. and Lopes, A.D. (2010). Factors associated with overweight in students from tri-border region: Argentina, Brazil and Paraguay. Arch. Latinoam. Nutr. 60: 340–347.

Leveau, J. (2009). Microbiology life on leaves. Nature. 461-741.

Longchamp, J., Barry-Ryan, C. and Devereux, M. (2009). Identification of volatile quality markers of ready-to-use lettuce and cabbage. Food Research International. 42: 1077-1086.

Matthews, K.R. (2006). Microorganisms associated with fruits and vegetables. In: microbiology of fresh produce (edited by K.R. Matthews). Washington DC: ASM Press.

Mehmood, A. K., Khan, M. S., Khan, M. A., and Bilal, M. (2014). Prevalence of *Salmonella* in diarrheic adult goats in field conditions. The Journal of Animal and Plant Sciences. 24(1): 98-102.

Minhas, P. S., Sharma, N., Yadav, R. K. and Joshi, P. K. (2005). Prevalence and control of pathogenic contamination in some sewage irrigated vegetable, forage and cereal grain crops. Bioresource Technology, 97, 1174-117

Ministry of Commerce Pakistan. (2014). Contaminated export products. Available at: http://www.dawn.com/news/768407/samples-of-vegetable-fruit-found-highly-contaminated.

Murchie, L. W., Curz-Romero, M., Kerry, J. P., Linton, M. and Patterson, M. F. (2005). High pressure processing of shellfish: a review of microbiological and other quality aspects. Innovative Food Science and Emerging Technologies 6(3):257-270.

Nguyen, C. and Carlin, F. (1994). The microbiology of minimally processed fresh fruits and vegetables. Food Science Nutrition. 34:371-401.

Nusrat, Y., Jafar, K., Noureen, S., Zia, I., Rashid, A. and Noor, S. (2012). Bacteriological study of food in the Pakistan's peri urban areas of Rawalpindi and Islamabad. African Journal of Biotechnology. 11(39): 9445-9451.

Obayagobana, O.N., Osamwonyi, O.U, Aborishade, W., Olisaka, F., Uwadiae, E. and Igiehon, O.N. (2013). Bacteriological quality of vegetable salads sold at restaurants within Okada Town, Edo State, Nigeria. African Journal of Basic and Applied Sciences 5 (1): 37-41.

Odabasi, M. (2008). Halogenated volatile organic compounds from the use of chlorine-bleach-containing household products. Environment, Science and Technology. 42:1445–1451.

Oliveira, A., Ritter, A., Tondo, E. and Cardoso, M. (2012). Comparison of different washing and disinfection protocols used by food services in southern Brazil for lettuce (Lactuca sativa). Food and Nutrition Sciences. 3: 28-33.

Oliveiraa, M., Usallb, J., Viñasa, I., Anguerab, M., Gatiusc, F. and Abadias, M. (2010). Microbiological quality of fresh lettuce from organic and conventional production. Food Microbiology. 27: 679-684.

Ölmez, H. and Kretzschmar, U. (2009). Potential alternative disinfection methods for organic fresh-cut industry for minimizing water consumption and environmental impact. Swiss Society of Food Science and Technology. 42:686-693.

Parish, M. E., Beuchat, L. R., Suslow, T.V., Harris, L.J., Garrett, E.H., Farber, J.N. and Busta, F.F. (2003). Methods to reduce/eliminate pathogens from fresh

and fresh-cut produce. Comprehensive Reviews in Food Science and Food Safety. 2:161-173.

Park, S.H., Choi, M.R., Park,J.W., Park, K.H., Chung, M.S., Ryu, S. and Kang, D.H. (2011). Use of organic acids to inactivate *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Listeria monocytogenes* on organic fresh apples and lettuce. Journal of Food Science. 76(6): 293-298.

Pundir.R. K. and Jain, P. (2011).Evaluation of five chemical food preservatives for their antibacterial activity against bacterial isolates from bakery products and mango pickles. Journal of Chemical and Pharmaceutical Research. 3(1): 24-31.

Rahul, P. (2012). Food Preservatives. (Chapter 2). Ghandinagar, India

Ravishankar, S. and Juneja, K. (2014). Preservatives: traditional preservatives – sodium chloride. Encyclopedia of Food Microbiology. 2: 131-136.

Ricke, S. C., M. M. Kundinger, Miller, D. R. and Keeton, J. T. (2005). Alternatives to antibiotics: chemical and physical antimicrobial interventions and foodborne pathogen response. Poultry Science. 84:667-675.

Ricke, S. C. 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry Science. 82:632–639.

Russell, J. N. and Grahame, W. G. (2003). Food Preservatives. (2nd Ed.). (Chapter 1). New York: Springer.

Sandeep, S., Tilak, S. and Ruchi, S. (2013). Increasing fruit and vegetable consumption: challenges and opportunities. Indian Journal of Community Medicine. 38(4): 192-197.

Scallan, E. H. R., Angulo, F.J., Tauxe, R.V., Widdowson, M.A. and Roy, S.L. (2011). Food-borne illness acquired in the United States-major pathogens. Emerging Infectious Diseases. 17-23.

Siele, C., Claudia, T. H., Rochele, Q. R., Sabrina, B., Eduardo, C. T. and Mieke. U. (2014). Microbiological quality and safety assessment of lettuce production in Brazil. International Journal of Food Microbiology. 181: 67-76.

Silva, N. (2010). Manual of Methods for Microbiological Analysis of Food and Water.

Soomro, A. H., KhaskhelI, M., Bhutto, M. B., Shah, G., Memon, A. and Deewani, P. (2010). Prevalence and antimicrobial resistance of *Salmonella* serovars isolated from poultry meat in Hyderabad, Pakistan. Turkish Journal of Veterinary and Animal Science. 34(5): 455-460.

Strauch, D. (1991). Survival of pathogenic micro-organisms and parasites in excreta, manure and sewage sludge. Review of Science Tech off International Epiz. 10: 813-846.

Sumathi, S., Cindy, R. F., Linda, C. and Robert, V. T. (2004). Fresh produce: A growing cause of outbreaks of food-borne illness in the United States, 1973 through 1997. Journal of Food Protection. 67:2342-2353.

U.S. Food and Drug Administration. (2008). Guidance for industry: guide to minimize microbial food safety hazards of fresh-cut fruits and vegetables. Available at: http://www.fda.gov/food/guidancecomplianceregulatoryinformation/guidancedocuments/produceandplanproducts/ucm064458.htm. Accessed July 2014.

U.S. Department of Agriculture. Economic Research Service. 1998. Leafy greens: foundation of the vegetable industry. Available at: http://webarchives.cdlib.org/sw1s17tt5t/http:/ers.usda.gov/publications/agoutlook/jan1998/ao248b.pdf.

WHO. (2009). Global health risks: Mortality and burden of disease attributable to selected major risks. Geneva, Switzerland.

WHO. (2006). Guidelines for the safe use of wastewater, excreta and grey water: Wastewater use in agriculture (Volume 2). Report of a WHO scientific group. WHO technical report series 778. World Health Organization, Geneva, Switzerland.

US Centers for Disease Control and Prevention. (2011). CDCP estimates of foodborne illness in the United States. Atlanta, GA. Available at: http://www.cdc.gov/foodborneburden/2011- foodborne-estimates.html.