SURVEILLANCE STUDY OF *SALMONELLA* IN FRESH PEPPERS (*CAPSICUM ANNUUM L.)* AND INACTIVATION BY HIGH HYDROSTATIC PRESSURE TREATMENT

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SURVEILLANCE STUDY OF *SALMONELLA* IN FRESH PEPPERS (*Capsicum annuum L.)* AND INACTIVATION BY HIGH HYDROSTATIC PRESSURE TREATMENT

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ABSTRACT

SURVEILLANCE STUDY OF *SALMONELLA* IN FRESH PEPPERS (*Capsicum annuum L.*) AND INACTIVATION BY HIGH HYDROSTATIC PRESSURE TREATMENT

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The main objective of this study was to investigate distribution of *Salmonella* in fresh peppers in Turkey. To serve this purpose, a total of 255 fresh pepper samples (green, kapya, bell, mazamort and charleston) were collected from 3 districts within 9 supermarkets and 3 bazaars in Ankara, Turkey. *Salmonella* suspected colonies was confirmed by using polymerase chain reaction (PCR) of *Salmonella* specific gene, *invA*. One of isolate was assigned as *Salmonella*, which was isolated from kapya pepper. Confirmed *Salmonella* isolate was characterized using phenotypic (serotyping) and genotypic (multilocus sequence typing, MLST and pulsed field gel electrophoresis, PFGE) methods. In MLST analysis, seven gene (*aroC, dnaN, hemD, hisD, purE, sucA* and *thrA*) MLST method designed by University College Cork were used. Within the scope of PFGE, total number of 4 *Salmonella* isolates was analyzed to compare with our *Salmonella* isolate and molecular subtyping of our strain exhibited different PFGE pattern.

For the last section of this study High Hydrostatic Pressure (HHP) was carried out to inoculated sliced peppers. *Salmonella* was not detected in 1 ml of suspension from

HHP treated (500 MPa, 25°C, 5 min) peppers. HHP treated fresh pepper samples stored at 25°C and 4°C for 7 days. After 7 days storage there was no any colony was count on TSA at 4°C but *Salmonella* colonies were re-growth at 25°C. This result demonstrated *Salmonella* cells were injured with the 500 MPa pressure treatments. HHP treated (500 MPa) fresh peppers can be safely consumed at refrigeration temperatures.

Keywords: *Salmonella,* fresh pepper, MLST, PFGE, High Hydrostatic Pressure

ÖZ

TAZE BİBERLERDE (*Capsicum annuum L.) SALMONELLA*
ARAŞTIRILMASI VE YÜKSEK HİDROSTATİK
BASINÇ İLE İNAKTİVASYONU

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Şubat 2014, 104 sayfa

Bu çalışmanın ana amacı Türkiye' deki taze biberlerde *Salmonella* dağılımını araştırmaktır. Bu amaçla, Ankara, Türkiye'den 9 süpermarket ve 3 pazar ın bulunduğu 3 bölgeden toplam 255 taze biber örneği (yeşil, kapya, dolma, köy, çarliston) toplanmıştır. Şüpheli *Salmonella* kolonileri, *Salmonella*'ya özgü bir gen ait olan *invA ile* polimeraz zincir reaksiyonu (PZR) kullanılarak doğrulanmıştır. Kapya biberden izole edilen izolatlardan biri *Salmonella* olarak tanımlanmıştır. *Salmonella* olarak tanımlanan izolat fenotipik (serotipleme) ve genotipik (genotipik çoklu bölge sekans tipleme, MLST ve vuruşlu alan jel elektroforezi, PFGE) methodları kullanılarak karakterize edilmiştir. MLST analizinde, College Cork Üniversitesi tarafından dizayn edilen 7 gen (*aroC, dnaN, hemD, hisD, purE, sucA* ve *thrA*) MLST metodu kullanılmıştır. PFGE çalışması kapsamında elde edilen *Salmonella* izolatıyla karşılaştırmak için toplamda 4 *Salmonella* izolatı kullanılmış ve suşumuzun moleküler tiplemesinde diğerlerinden farklı bir PFGE patern gözlenmiştir.
Çalışmanın son bölümünde inoküle edilmiş, dilimlenmiş biberlere yüksek hidrostatik basınç (YHB) uygulanmıştır. Yüksek hidrostatik basınca tabi tutulan (500

MPa, 25°C, 5 dk) örneklerin 1 ml süspansiyonlarında *Salmonella* saptanmamıştır. Yüksek basınç uygulanan taze biber örnekleri 25°C ve 4°C'de 7 gün muhafaza edilmiştir. 7 gün muhafazadan sonra 4°C'deki örneklerde TSA üzerinde hiçbir koloni gözlemlenmemiş fakat 25°C'de *Salmonella* kolonileri gelişmiştir. Bu sonuç gösteriyor ki *Salmonella* hücreleri 500 MPa'da zarar görüyor ve uygun koşullarda tekrar büyüyor. YHB (500 MPa) uygulanmış taze biberler buzdolabı sıcaklığında tutularak güvenle tüketilebilir.

Anahtar Kelimeler: *Salmonella*, taze biber, MLST, PFGE, Yüksek Hidrostatik Basınç

To

My father, mother, sister,

&

My husband Melih

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LIST OF ABBREVIATIONS

°C	Degree Celcius
AFLP	Amplified Fragment Length Polymorphism
BGA	Brilliant Green Agar
BHI	Brain Heart Infusion
BPW	Buffered Peptone Water
CDC	Centers for Disease Control and Prevention
CLB	Cell Lysis Buffer
CSB	Cell Suspension Buffer
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
EDTA	Etilendiamin tetraasetik asit
EFSA	European Food Safety Authority
EU	European Union
HHP	High Hydrostatic Pressure
HUS	Hemolytic Uremic Syndrome
h	Hour
min	Minute
MLST	Multilocus Sequence Typing
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
RVS	Rappaport-Vassiliadis Soy Peptone
s	second

SDS	Sodium dodecyl sulfate
SKG	SeaKem Gold
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

CHAPTER 1

INTRODUCTION

Turkey is one of the major agricultural product exporters in the world. During the last two decades, the number of outbreaks caused by foodborne pathogens associated with fresh produce consumption has increased. The common pathogen linked to the fresh produce outbreaks is *Salmonella*.

In 2013, the volume of fresh vegetable export was 1.2 millions tons; 5.6 % of it was fresh pepper. As being one of the major exporters, Turkey needs a strong surveillance and early warning system for early detection of potential foodborne outbreaks and for national, regional and global health security. However, investigations are inadequate and no active network system has been detected to share the results of existing studies among researchers in Turkey. To fill this gap, this study focuses on surveillance of common pathogen; *Salmonella* in fresh produce. To serve this purpose, a total of 255 fresh pepper samples (green pepper, kapya pepper, bell pepper, mazamort pepper, charleston) were collected from 3 districts within 9 supermarkets and 3 bazaars in Ankara, Turkey. The samples were analyzed for the presence of *Salmonella*. Then, suspected colonies were confirmed as *Salmonella* by using polymerase chain reaction (PCR) with the *invA* which is the specific gene of *Salmonella*. Following this, confirmed colonies was characterized by phonotype based method, serotyping and two genotypic methods. Serotyping analysis was performed in Türkiye Halk Sağlığı Kurumu, Ankara. For the genotypic analysis part, Multi Locus Sequence Typing (MLST) and Pulsed Field Gel Electrophoresis (PFGE) were performed.

The aim of MLST is to provide a portable, accurate and highly discriminating subtyping system that is useful for the detection of foodborne outbreaks. Amplification and sequence analysis of seven house-keeping genes (*thr*A,

*pur*E, *suc*A , *his*D, *aro*C, *hem*D and *dna*N) was performed as stated in the 7 gene *Salmonella* MLST analysis according to University College Cork, Environmental Research Institue, *Salmonella enterica* MLST Database (http://mlst.ucc.ie/mlst/dbs/Senterica).

Currently considered gold standard method for characterization of many pathogens, pulsed field gel electrophoresis (PFGE), was used to characterize *Salmonella* isolate. For this purpose, United States Centere for Disease Control and Prevention (CDC) protocol and *Xba*I as a restriction enzyme were used.

Additionally, the efficiency of High Hydrostatic Pressure (HHP) - an emerging technology proposed as an alternative to thermal processing for inactivation of foodborne pathogen on inactivation of selected pathogen in fresh pepper samples was investigated. Overall, this study showed how the most common foodborne pathogens are distributed in fresh peppers in Turkey and also improved our understanding of the most common foodborne pathogen biology, ecology and transmission.

CHAPTER 2

LITERATURE SURVEY

2.1 Foodborne Diseases

Foodborne diseases are the illnesses related to eating contaminated food or beverages. Foodborne pathogens are the bacteria that cause illnesses. Common foodborne pathogens are *Escherichia coli, Salmonella, Listeria monocytogenes*. EFSA and ECDC 2013 reported that the biggest percentage (26.6 %) of food-borne outbreaks source was *Salmonella*, followed by bacterial toxins (12.9 %), Campylobacter (10.6 %) and viruses (9.3 %).

Salmonellosis is an infection that caused by *Salmonella*. *Salmonella* infections generally causes diarrhoea, headache, abdominal pain, vomiting and fever 2 to 24 hours after infection (Bhunia, 2008).

Salmonella was the most frequently isolated pathogen from food materals which cause outbreaks in European Union. For instance, in 2011 a total of 97,897 salmonellosis cases were reported by the 27 European Union Member States, with 95,548 confirmed cases. The rate of notification is 20.7 cases per 100,000 population in EU. The rate of death of human among confirmed cases was 0.12 % in 2011(EFSA and ECDC, 2013).

Approximately 42,000 salmonellosis cases are recorded in the United States each year and nearly 400 people die every year within these cases (CDC, 2012d). According to CDC 48 million people get sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases in every year (CDC, 2013a). Annually 1.2 million *Salmonella* cases are recorded in the United States and 49 *Salmonella* outbreaks has been recorded since 2006, which is the most common cause of hospitalization and

death is tracked by FoodNet (CDC, 2013d). Characteristic of some foodborne pathogens are given in Table 2.1.

Table 2.1 Characteristics of some food-borne pathogens (modified from Harris *et al.,* 2003)

Bacteria	Typical Incubation Period	Symptoms	Infectious dose (Number of cells)	Source
Clostridium botulinum	12 to 36 hours	Nausea, vomiting, fatigue, dizziness, dryness of mouth and throat, muscle paralysis, difficulty swallowing, double or blurred vision, drooping eyelids, and breathing difficulties	intoxication, growth and toxin production in food	soil, lakes, streams decaying vegetation reptiles
Escherichia coli O157:H7	2 to 5 days	Bloody diarrhea, abdominal pain. Can lead to hemolytic uremic syndrome and kidney failure especially in children and the elderly	10 to 1000	animal feces, especially cattle, deer and human cross contamination from raw meat

Bacteria	Typical Incubation Period	Symptoms	Infectious dose (Number of cells)	Source
Salmonella spp.	18 to 72 hours	Abdominal pain, diarrhea, chills, fever, nausea, vomiting	10 to 100,000	animal and human feces; cross contamination from raw meat, poultry, or eggs
Shigella spp.	1 to 3 days	Abdominal pain, diarrhea, fever, vomiting	Around 10	human feces
Listeria monocytogenes	1 day to 5 or more weeks	Febrile gastroenteritis in healthy adults; may lead to spontaneous abortion or stillbirth in pregnant women; severe septicemia and meningitis in neonates and immuno compromised adults; mortality may be 20 to 40 %.	unknown, dependent uponhealth of individual	soil, food processing environments

2.2 *Salmonella*, the Most Common Bacterial Pathogen

Salmonella serotypes can grow in a large scale of food materials. Environmental and ecological factors can affect the growth of *Salmonella*. For instance minimum conditions of *Salmonella* growth for some serotypes can occur at pH 3.8 - 3.9, 5.2°C and 0.93 aw. On the other hand some serotypes can continue to grow at pH 9.5, 46.2°C and > 0.99 aw. Optimum growth conditions of *Salmonella* spp. is pH 7 - 7.5, 35-43°C and 0.99 aw (FSANZ, 2013).

Salmonella is gram negative and rod shape bacteria, classified under the family of Enterobacteriaceae (Darwin & Miller, 1999). The genus *Salmonella* divided into two species; *Salmonella enterica* and *Salmonella bongori*. *S. enterica ser. Enteritidis* has 6 subspecies which are explained in the Figure 2.1 (Winn Jr. *et al.*, 2006).

Among these subspecies *S. enterica* subspecies *enterica* serotype Enteriditis is the most common pathogen linked to the foodborne diseases (Hadjinicolaou *et al.*, 2009). *S.enterica* species has more than 2,600 serovars which are most common human cases in the EU (European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC), 2013). Typhodial *Salmonella* serovars which are *S.* Typhi and *S.* Paratyphi A,B,C causing enteric fever (Darby & Sheorey, 2008).

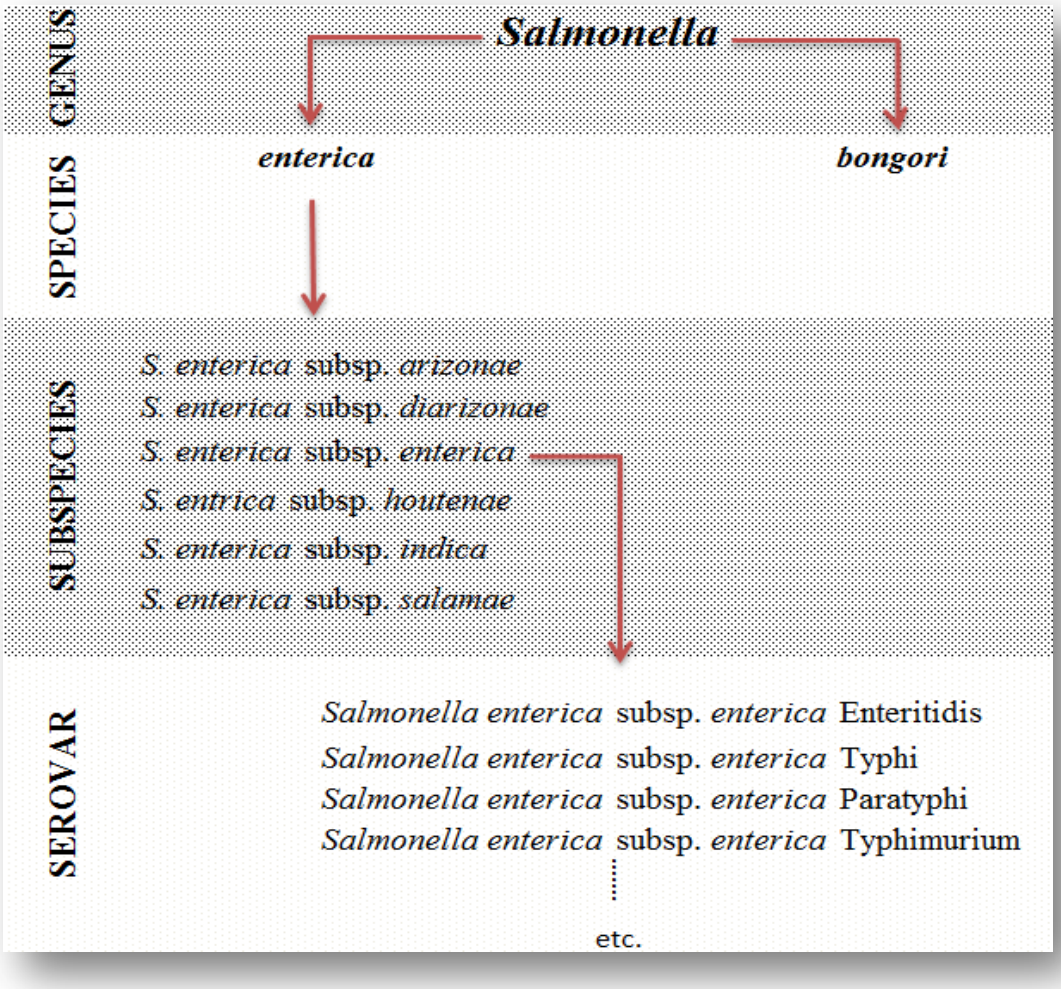


Figure 2.1 Genus of *Salmonella*

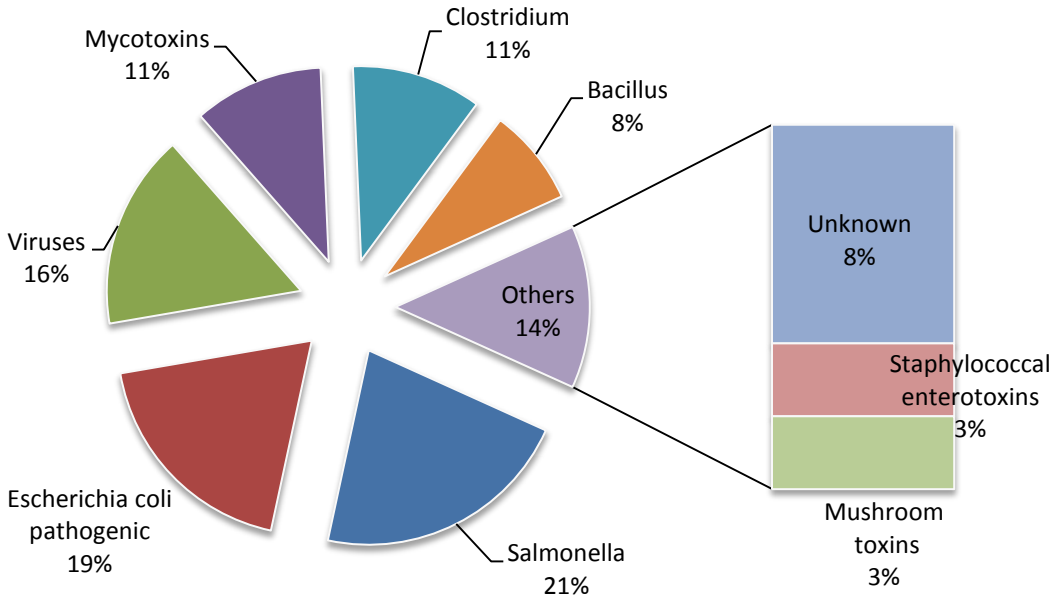
2.3 Foodborne Outbreaks Related to Fresh Produces

Generally the source of *Salmonella* infections are related to animal origin. However, the number of outbreak linked to the fresh produce has increased during the last two decades due to the increased number of meals eaten away from home or consumption of raw and minimally processed fruits and vegetables (Warriner *et al.*, 2009). In recent years eating habits have changed due to consumer's willingness to eat raw and minimally processed vegetable products such as packed salads. The growth in consumption of ready-to-eat foods can be linked to increase in foodborne diseases associated to the fresh produces. For example reported foodborne outbreaks related

to the fresh produces has increased 1970s to 6 % in the 1990s to 13 % in the 2000s, and to 33 % in 2011, in the United States (Kisluk & Yaron, 2012).

Salmonella Javiana was the first foodborne pathogen that was related to the first large multistate outbreak of *Salmonella* infections in fresh produce, caused 176 illnesses in four states of USA in 1990. The source of outbreak was contaminated tomatoes (Hedberg *et al.*, 1999).There has been increased number of outbreaks after this incidence; outbreaks of illness linked to the fresh produce from 2005 to 2013 are summarized in Table 2.2. It was seen from the table that the common pathogen linked to the fresh produce outbreaks is *Salmonella*. The most common cause of foodborne outbreak is associated with the serovar *S.* Enteritidis. *Salmonella* infections are generally linked to meat and dairy products. However, there have been numerous *Salmonella* outbreaks linked to raw, fresh produce and large salmonellosis outbreaks being linked to tomatoes, sprouts, cantaloupes, peppers (Table 2.2). Although fresh produces have not been directly related to foodborne outbreaks, many researchers have reported to *Salmonella* as an ingredient of raw food products such as salad mix. Even the source of contamination is minor, outbreaks show us ingredients can cause large scale of foodborne outbreaks. An important, multistate peppers (serrano and jalapeño) associated salmonellosis outbreak occurred in 2008. Because of consumption of pepper the largest outbreak occured in Mexico which covered more than 1414 persons had infections and 286 persons were hospitalized with 2 deaths (CDC, 2008a).

Many types of fresh fruit and vegetables including tomatoes, cantaloupes, sprouts, peppers are recorded as *Salmonella* outbreak source (CDC, 2013c). It has been reported by EFSA and ECDC, 2013 that 37 numbers of outbreaks linked to the vegetables have been occurred. *Salmonella* (21 %) was the most isolated pathogen from the vegetables among 37 outbreaks (Figure 2.2).



Note: Data from 37 outbreaks included: Belgium(1), Denmark(7), Finland(2), France(2), Germany(5), Hungary(1), Netherlands(2), Poland(4), Spain(8), Sweden(1) and United Kingdom(4)

Figure 2.2 Distribution of strong evidence outbreaks caused by vegetables by causative agent in the EU, 2011 (modified from EFSA and ECDC, 2013).

Table 2.2 Outbreaks related to fresh produce from 2005 to 2013.

Location	**Year**	**Pathogen**	**Produce**	**Cases (deaths)**	**References**
Canada	2005	*Salmonella* Enteritidis	Mungbean sprouts	592	Rohekar *et al.*, 2008
USA	2005	*Salmonella* Newport	Tomatoes	459	CDC, 2007
USA	2006	*E. coli* O157:H7	Spinach	199 (3)	CDC, 2006b
USA, Canada	2006	*Salmonella* Oranienburg	Fruit salad	41	Landry *et al.*, 2007
USA	2006	*Salmonella* Typhimurium	Tomatoes	183	CDC, 2006a
USA	2006	*E. coli* O157:H7	Lettuce	81	FDA, 2007
Australia	2006	*Salmonella* Saintpaul	Cantaloupe	115	Munnoch *et al.*, 2008
USA	2006	*E. coli* O157:H7	Spinach	22	Grant *et al.*, 2006
Europe	2007	*Salmonella* Java	Baby spinach	354	Denny *et al.*, 2007
North America, Europe	2007	*Salmonella* Senftenberg	Basil	51	Pezzoli *et al.*, 2007
Australia, Europe	2007	*Shigella sonnei*	Baby carrots	230	Lewis *et al.*, 2009
Europe	2007	*Salmonella* Weltevreden	Alfalfa sprouts	45	Emberland *et al.*, 2007

Table 2.2 Outbreaks related to fresh produce from 2005 to 2013(continued)

Location	**Year**	**Pathogen**	**Produce**	**Cases (deaths)**	**References**
USA, Canada	2008	*Salmonella* Saintpaul	jalapeño and serrano peppers	1442 (2)	CDC, 2008a; Mody *et al.*, 2011
USA, Canada	2008	*E. coli* O157:H7	Lettuce	134	Warriner and Namvar, 2010
UK	2008	*Salmonella* Senftenberg	Basil	32	Elviss *et al.*, 2009
USA	2008	*Salmonella* Litchfield	Cantaloupe	51	CDC, 2008b
USA, Canada	2008	*Salmonella* Typhimurium	Peanut butter	714 (9)	CDC, 2009b
USA	2009	*Salmonella* Saintpaul	Alfalfa sprouts	235	CDC, 2009a
USA	2010	*E. coli* O145	Lettuce	26	CDC, 2010a
USA	2010	*Salmonella* Newport	Alfalfa sprouts	44	CDC, 2010b
USA	2010	*L. monocytogenes*	Fresh cut produce (celery)	10(5)	FDA, 2010
USA	2011	*Salmonella* I 4,[5],12:i:-	Alfalfa and mixed sprouts	140	CDC, 2011b
USA	2011	*Salmonella* Panama	Cantaloupe	20	CDC, 2011c
USA	2011	*Salmonella* Agona	Papaya	106	CDC, 2011d

Table 2.2 Outbreaks related to fresh produce from 2005 to 2013(continued)

Location	**Year**	**Pathogen**	**Produce**	**Cases (deaths)**	**References**
Europe	2011	*E. coli* O104:H4	Vegetable sprouts	3911(47)	ECDC, 2011; EFSA, 2011
USA	2011	*L. monocytogenes*	Cantaloupe	146(31)	CDC, 2011e
USA	2011	*E. coli* O157:H7	Strawberries	15(1)	FDA, 2011
USA	2011	*E. coli* O157:H7	Lettuce	60	CDC, 2011a
USA	2012	*Salmonella* Typhimurium and *Salmonella* Newport	Cantaloupe	261(3)	CDC, 2012b
USA	2012	*Salmonella* Braenderup	Mangoes	127	CDC, 2012a
USA	2012	*E. coli* O157:H7	Organic Spinach and Spring Mix Blend	33	CDC, 2012c
USA	2013	*Salmonella* Saintpaul	Cucumbers	84	CDC, 2013b

2.4 Export Value of Fresh Fruit and Vegetables from Turkey

Turkey is one of the major agricultural product exporters in the world. In 2012, the volume of fresh vegetable export was 1.1 millions tons; 6.5 % of it was pepper while fresh fruits were 0.7 millions tons (Türkiye Yaş Meyve Sebze İhracatçı Birliği, 2013). Fresh produce is exported from Turkey to more than 50 countries. According to Turkish Exporters Assembly; the most exported fresh produce in 2013 was tomato with the value of 486126 ton, cucumber (78860 ton), pepper (68815 ton). Turkish Exporters Assembly reported that most of fresh produce was exported to Russia, followed by Iraq (Table 2.3). The reason of this if any outbreak is occurred in Russia, the source can be exported fresh produce from Turkey. Strong surveillance system is crucial for Turkey to obtain whether source comes from Turkey or not. According to the USDA's Economic Research Service more than 15 % of food consumed in the US in 2005 was imported. Vegetable market has been shared 13 % of these commodities and 32 % of them is for fruit market (Fatica & Schneider, 2011).

Table 2.3 Countries that Imported Fresh Produce from Turkey in 2012 (Türkiye Yaş Meyve Sebze İhracatçı Birliği, 2013)

Country	**Ton**
Russia	413247
Iraq	238726
Syria	155763
Bulgaria	85741
Ukraine	57599
Germany	37972
Georgia	36489

2.5 Surveillance Network Systems in the World

As being one of the major exporters, Turkey needs a strong surveillance and early warning system for early detection of potential foodborne outbreaks and for national, regional and global health security. However, investigations are inadequate and no active network system has been detected to share the results of existing studies among researchers: such as; USA Foodborne Diseases Active Surveillance Network (FoodNet) tracks an important foodborne illness, which provides crucial informations about outbreaks for food safety policy and prevention methods (Scallan & Mahon, 2012). The PulseNet International, the international molecular subtyping network for food-borne disease surveillance molecular subtyping network internationally in Africa, Asia Pasific, Canada, Europe, Latin America & Carribean, Middle East and USA. Some of the advantages of these network systems are all of the laboratories in its network work with same procedures and a standard, possible to compare fingerprints into their database, identify the source and allows investigators to spend their time on specific bacteria, outbreaks are tracked globally and the most important think is the early warning detection system.

2.6 *Salmonella* Subtyping Methods

Subtyping methods are used to determine differentiation of isolates beyond the species and subspecies level. The aim of the subtyping in outbreak investigations is to characterize relationship of isolates then determine share common ancestor or not. Subtyping methods are divided into two major groups; phenotype based methods such as serotyping, phage typing and genotype based methods such as plasmid profiling, insertion sequence (IS) subtyping, ribotyping, randomly amplified polymorphic DNA analysis (RAPD), amplified fragment length polymorphism (AFLP), pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). There are some criteria to choose subtyping method which should provide discriminatory power, typeability, reproducibility, ease of interpretation, easy to use and low cost (Avşaroğlu, 2007). In recent years DNA

based methods have been used to differentiate unrelated strains following to their discriminatory power. *Salmonella enterica* isolates can be identified by many methods. Each method has it's own advantages but molecular subtyping methods are rapid, high discriminatory power, efficient for the surveillance (Wattiau *et al.*, 2011). An ideal subtyping method should be reproducible, has high discriminatory power, easy to use, low cost and has unambiguous positive result (EFSA, 2013).

2.6.1 Phenotype Based Subtyping Methods

2.6.1.1 Serotyping

Salmonella species can be divided into over 2,500 serovars according to the Kauffmann-White Scheme (Grimont and Weill, 2007). Serotyping is based on the detection of somatic (O) and flagellar (H) antigens expressed by these bacteria. Each serotype has specific antigenic formula. Some of the *Salmonella enterica* subsp. *enterica* serotypes are given in Table 2.4. As we see from the table that there are some Arabic Numbers and Lower case letters within the antigenic formula of serotypes. Somatic(O) antigens are denoted by Arabic numbers, the Phase 1 - antigens by lower case letters and the Phase 2 antigens again by Arabic numbers (Grimont and Weill, 2007).

Table 2.4 Antigenic formulas of selected *Salmonella enterica* subsp. *enterica* serotypes according to Kaufmann-White scheme (Grimont & Weill, 2007).

	Somatic (O)	**Flagellar (H) Antigen**	
Serotype	**Antigen**	**Phase 1**	**Phase 2**
S. Enteritidis	1, 9, 12	g,m	-
S. Typhimurium	1, 4, 5, 12	i	1, 2
S. Dublin	1, 9, 12, [Vi]	g, p	-
S. Heidelberg	1, 4, [5], 12	r	1, 2
S. Infantis	6, 7, 14	r	1, 5

Some abbreviation has been used by Centers for Disease Control and Prevention (CDC) and other authorities to designate the name of the bacteria for the ease of reading. For instance, complete name is *Salmonella enterica* subspecies *enterica* serovar Typhimurium, CDC designation is only *S. enterica* ser. Typhimurium. During the thesis complete serotype designaton name *S. enterica* subspecies *enterica* serovar Enteritidis was replaced by *S. enterica* ser. Enteritidis which is internationally accepted abbreviation.

2.6.1.2 Phage Typing

Bacterial pathogens are differentiated beyond strains and subspecies level relied on their susceptibility to lysis by set of bacteriophages (Chen *et al.*, 2011). Phage typing has been used for the strain characterization of *Salmonella* mid.-1950s in surveillance (Avşaroğlu, 2007). The largest outbreak of *Salmonella* infection was occured in Denmark in 2008, in which *Salmonella* Typhimurium phage type U292 was detected from foods. Almost 1500 isolates were characterized by phage typing (Baggesen & Wegener, 1994). Phage typing was applied according to the World Health Organization (WHO) Collaborative Centre, phage typing of *Salmonella* (Health Protection Agency (HPA), Colindale, United Kingdom) for surveillance of *S.* Enteritidis and *S.* Typhimurium in humans, food and food production animals within Denmark outbreak (Baggesen *et al.,* 2010). There are some of the disadvantages of this method such as needs biologically active phages

which is available at the National Reference Centers, many strains are non-typeable, limited discriminatory power, on the other hand phage typing is cheap and less labour-intensive method. (Avşaroğlu, 2007; Baggesen *et al.,* 2010; Chen *et al.,* 2011).

2.6.1.3 Multilocus enzyme electrophoresis (MLEE)

MLEE is based on the characterization of bacterial strains by differences in the electrophoretic mobilities under electrophoresis of a set of metabolic enzymes. Soluble enzymes are obtained from cell and separated by size in starch gels. Almost all strains can be typed by this method. Whereas MLEE has excellent reproducibility and ease of interpretation, standardization of the method is difficult in the most laboratories and also discriminatory power of the MLEE is less than some other subtyping methods (Wiedmann, 2002).

2.6.2 Genotype Based Subtyping Methods

2.6.2.1 Randomly Amplified Polymorphic DNA Analysis (RAPD)

RAPD is a PCR based technique commonly used for subtyping of bacterial pathogens (Chen *et al.,* 2011). This method is performed with arbitrary short (10 bases) random primers. The primers are arbitrary because PCR products of RAPD are uncertain. Amplification of the multiple fragments of the bacterial DNA is carried out by these random primers. As PCR methods, randomly amplified fragments are then analyzed with gel electrophoresis. (Avşaroğlu, 2007; Chen *et al.,* 2011; Kumar & Gurusubramanian, 2011).

2.6.2.2 Amplified Fragment Length Polymorphism (AFLP)

This method is based on digection of whole DNA with two restriction enzymes after that double-stranded adaptors are ligated to the end of the restriction fragments. Subsequently, PCR amplification is performed (Sabat *et al.,* 2013; Chen *et al.,* 2011). The main disadvantages of this method are labour-intensive and equipments are expensive (Sabat *et al.,* 2013).

2.6.2.3 Pulsed Field Gel Electrophoresis (PFGE)

PFGE is one of genotype based methods that has been known "gold standard" technique within the other molecular subtyping methods (Sabat *et al.,* 2013). PulseNet is a international network system which has been created by Centers for Disease Control and Prevention (CDC) and Local Health Departments. The aim of PulseNet is to standardize PFGE protocol and sharing PFGE datas in all the PulseNet (http://www.cdc.gov/pulsenet/) participants via Internet (Swaminathan *et al.,* 2001).

PFGE provide us to compare large genomic DNA fragments after digestion with restriction enzyme. After restriction digestion of whole DNA, several linear molecules of DNA fragments (ranging from 40 to 600kb) have been yielded. DNA fragments are size-seperated by the electrophoresis technique through an agarose gel (Wiedmann, 2002). According to Tenovar *et al*. (1995) interpretation of PFGE banding patterns have following criterias;

Indistinguishable isolate: There are no any band differences,

Closely related isolate: 2-3 band differences,

Possibly related isolate: 4-6 band differences,

Different isolate: $\geq$ 7 band differences, compared with outbreak pattern.

If there are no any band differences compared with ourtbreak pattern, our isolate is genetically indistinguishable. 2-3 band differences in PFGE pattern shows us probably this isolate is probably part of outbreak and 4-6 differences indicates that possibly outbreak strain. Finally, if our isolate have seven or more band differences, isolate is considered unrelated to the outbreak strain (Tenovar *et al*., 1995).

2.6.2.4 MLST (Multilocus Sequence Subtyping)

First MLST study was developed with the scheme of the Neisseria meningitidis in 1998. After that it becomes very popular method on the molecular evaluation of pathogens (Sabat *et al.,* 2013). MLST is nucleotide sequence-based subtyping method based on the partial sequencing of housekeeping genes (Wattiau *et al.,* 2011). Nucleotide differences in the individual genes are combined and used to determine the differentiation of strains (Yan *et al.,* 2003). Approximately 450-500 bp of seven housekeeping genes are amplified and housekeeping genes are sequenced. According to the differences of the sequences, a unique (arbitrary) allele number is assigned (Figure 2.3) and allelic profile of each isolate are determined (Pagotto *et al.,* 2005)

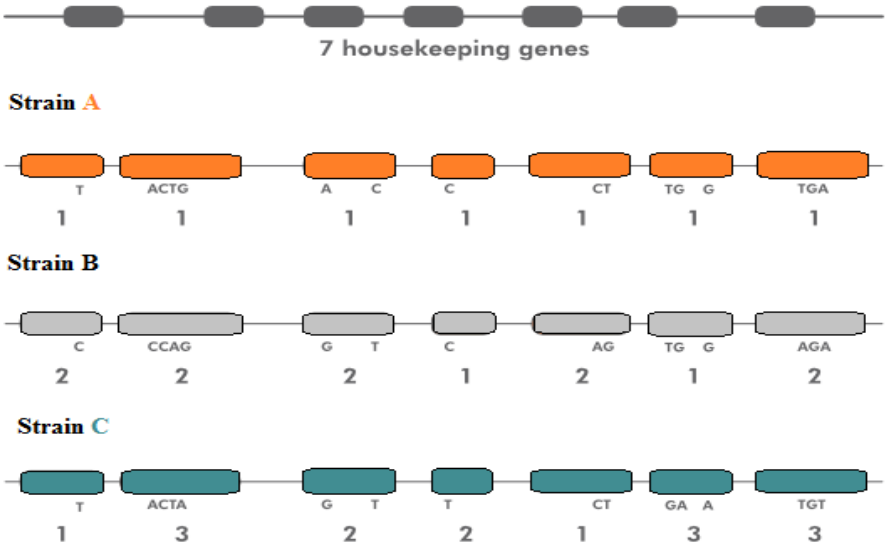


Figure 2.3 Numerical Allelic profiles of the strains

Internet accessible databases for MLST information is advantageous sources for this study in order to compare the subtypes found in the different geographical regions to trace back the source of foodborne pathogens (http://www.mlst.net). The Sequencing informations have been collected in MLST database website which is hosted at the ERI University College Cork, Ireland (http://mlst.ucc.ie/mlst/dbs/*Senterica*). A main advantage of MLST is to have high discriminatory power

among subtyping methods. Other than this; portable method which provide us to disseminate of the approach (Maiden, 2006). On the other hand the cost of MLST is still high (Sabat *et al.,* 2013).

2.7 High Hydrostatic Pressure (HHP) Technology

High Hydrostatic Pressure is an alternative method to thermal processing and HHP treatment of minimally processed foods has been increased in last years for the goal of microbiologically safe and healthy food (Alpas, 2000). A major function of high pressure processing of food is destruction of microorganisms. Food preservation is relied on the inactivation, growth delay or prevention of spoilage and pathogenic microorganisms (Palou, 1998).

2.7.1 General Principle and Mechanism of HHP

Basically, there are two principles of HHP such as Le Chatelier Rule and Isostatic Principle. According to Le Chatelier Principle; if a system is disturbed in equilibrium by changes in determining factors, for instance; temperature, pressure, and concentration of components, the system will tend to shift its equilibrium position then new equilibrium is formed. This phenomenon allied with increasing pressure is in a volume decrease or lowers the pressure by increasing the volume. This means that pressure treatments result in a decrease in volume (Nguyen & Balasubramaniam, 2011). Secondly, in accordance with Isostatic rule pressure is uniformly transferred independent of size and geometry of the food. The pressure is applied to the every direction of the food equally and when the pressure release food sample then food sample preserve its shape (Buzrul, 2003).

The system of high pressure is divided into following parts; a high pressure vessel and its closure, a temperature control device, a pressure generation system, a material handling system (Buzrul, 2003).

There are two general pressure application methods: direct and indirect compression. As we seen from Figure 2.4 a piston coaxial with the container is required for direct compression method which is suitable only in laboratory-scale plant since there is a sealing problem between the piston and the internal surface of

the container. The most common method is indirect method which consists of a pressure booster to pump the liquid from the pressure medium tank to the sample cell (Figure 2.5). Pumping stops when the target pressure is reached (Bertucco & Spilimbergo, 2001).

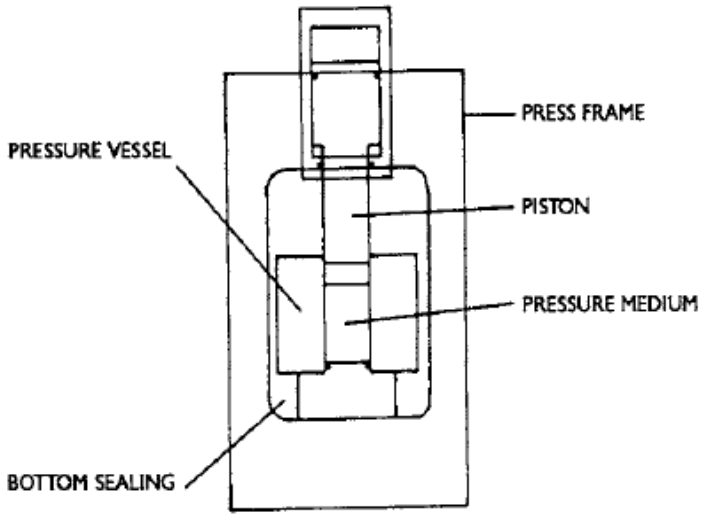


Figure 2.4 Schematic diagram for HHP which uses the direct method (Adapted from Bertucco & Spilimbergo, 2001)

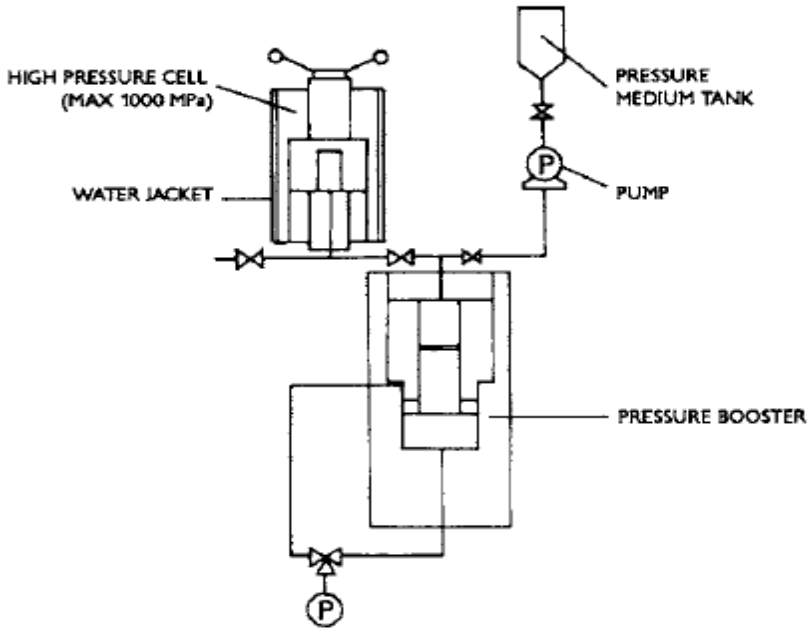


Figure 2.5 Schematic diagram for the HHP using the indirect method (Adapted from Bertucco & Spilimbergo, 2001)

2.7.2 Effect of HHP on microorganisms

Inactivation of pathogens by HHP requires 300-700 MPa pressure (Black *et al.*, 2011). Some researchers found that some of the strains of *Salmonella* are more or less pressure resistant than other strains of the species. Such as, *Salmonella* Typhimurium E 21274 was less pressure resistant than *Salmonella enteritidis* FDA (Alpas, 2000). Neetoo & Chen, (2012) demonstrated that 500 MPa pressure was enough for decontaminating soaked Jalapeño and Serrano peppers from *Salmonella* within their study. Application of pressure to the inoculated food samples shows us increases death rate of microorganisms (Black *et al.*, 2011).

Many authors have demonstrated that *Salmonella* has the ability to grow in pepper extracts (Nutt *et al.*, 2003), chopped bell pepper (Liao *et al.*, 2010) and chopped Jalapeño peppers (Black *et al.*, 2010; Liao *et al.*, 2010) as far as survive on intact (whole) bell and Jalapeño peppers (Black *et al.*, 2010; Ma *et al.*, 2010). Furthermore Liao *et al.*, (2010) and Ma *et al.*, (2010) explained that *Salmonella*

inoculated onto Jalapeños have the ability to grow at low storage temperatures of 12°C.

After HHP treatment of the samples some of the microbial population may be injured, depending on the pressure level. This can cause overestimation of the HHP inactivation (Black *et al.*, 2011). Bozoglu *et al.*, (2004) observed that two types (I1 and I2) of injuries occured on the cell of *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7 and *Salmonella* Enteritidis after HHP treatment at 350, 450, and 550 MPa within 4 week.

CAHPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Fresh Pepper Samples

During the study period (from July–October 2012), a total of 255 fresh pepper samples (green pepper, kapya pepper, bell pepper, mazamort pepper, charleston) were collected from 3 districts within 9 supermarkets and 3 bazaars in Ankara, Turkey. The selection of supermarkets and bazaars were based on to provide 5 different pepper samples during the period and their geographical location, situated in western, northern and central Ankara. According to availability of the peppers, 3 bazaars and 9 supermarkets (3 districts) were visited to collect 5 different fresh pepper samples and analyzed for the presence of *Salmonella* per week. Analysed material, date, place and results informations were given in appendix A. Coding system of samples are also explained in Table 3.1. Analysis was done within 12 hours of sample collection.

Table 3.1 Sample coding system

	Purchased Places			
Districts	Supermarket 1	Supermarket 2	Supermarket 3	Bazaar
Çankaya	A	B	C	D
Yenimahalle	E	F	G	H
Keçiören	I	K	L	M

As we see from Figure 4.2; 3 different closely districts were choosen in Ankara city because Ankara 2012 population cencus result shows us most of the population lives in Yenimahalle, Çankaya and Keçiören districts (Figure 3.1). Sampling districts are shown in Ankara disticts map (Figure 3.2).

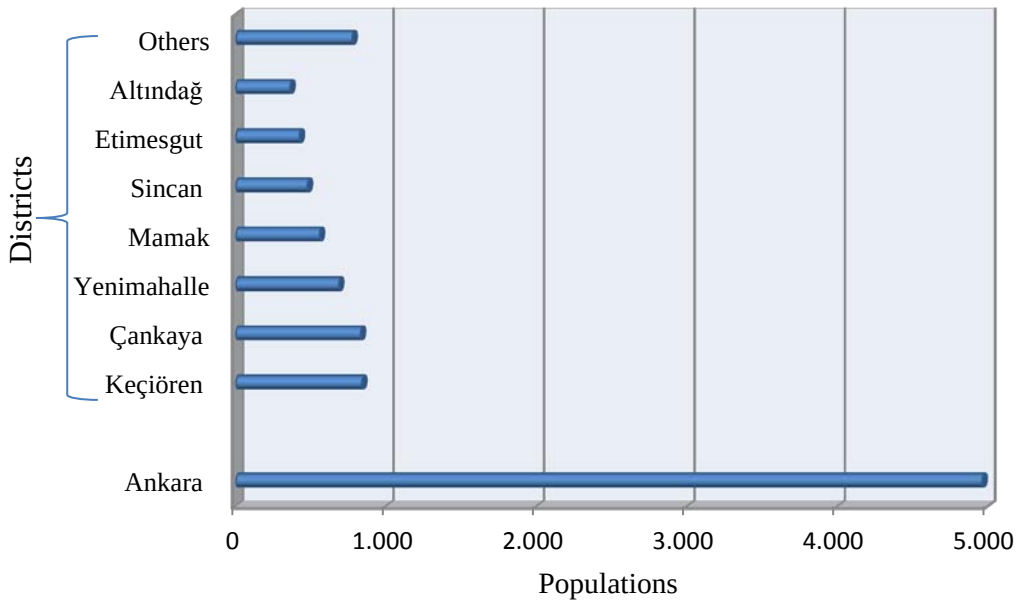


Figure 3.1 2012 Population of Ankara and Districts (http://www.yerelnet.org.tr/iller/il.php?iladi=ANKARA)

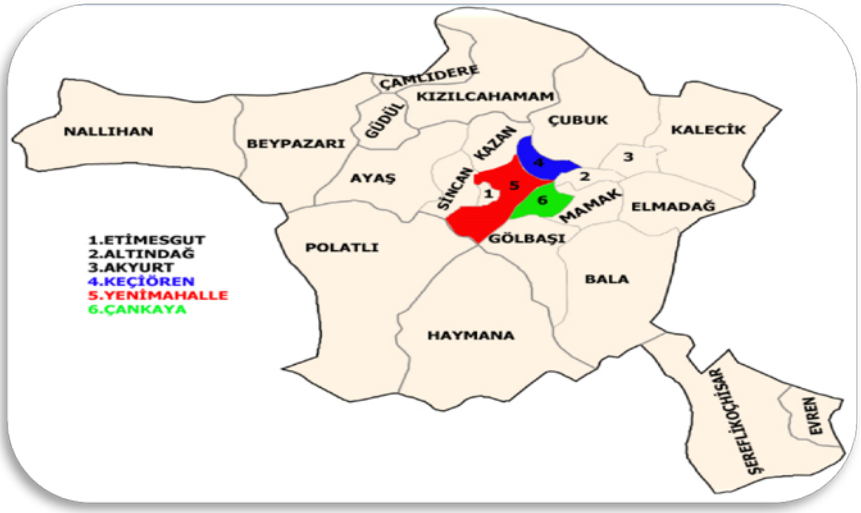


Figure 3.2 Ankara districts map. Colours indicates sampling districts; red for Yenimahalle, blue for Keçiören, green for Çankaya.

3.1.2 Enzymes, chemicals and primers

Enzymes, chemicals and primers used in this study were obtaine from Genoks, Ankara, Sigma, USA, Roche, Germany and NanoBiz, Ankara.

3.1.3 Buffers, solutions and medias

The preparation of buffers, solutions and medias used are given in Appendix C and D.

3.2 Methods

3.2.1 *Salmonella* Isolation & Identification from Fresh Pepper Sample

Salmonella was isolated according to ISO 6579:1993 standart.

3.2.1.1 Sample Preparation

Analysis was done within 12 hours of sample collection.

3.2.1.2 *Salmonella* Isolation

Salmonella was isolated according to ISO 6579:2002 standart. 225 ml samples was added to 225 ml of buffered peptone water (BPW) (Oxoid Ltd., UK) and mixed with a bag mixer (Interscience, France) for 60 s then incubated for 18h at 37°C. Each 0.1ml of pre-enriched samples were transferred to two of 10 ml Rappaport-Vassiliadis soy peptone (RVS) (Oxoid Ltd., UK) broth then incubated the tubes at 41.5 °C overnight (18-24 hours). After 24 hours enriched RVS broths were streaked on both Xylose lysine desoxycholate (XLD) (Oxoid Ltd., UK) and Brilliant Green Agar (BGA) (Oxoid Ltd., UK) agars and incubated 24 hours at 37°C. Presence of typical *Salmonella* colonies from the agar medias were examined such as grey/reddish and slightly convex on BGA agar plate and slightly transparent zone of reddish color colonies with black centers on XLD agar.

3.2.1.3 Confirmation of *Salmonella* with Polymerase Chain Reaction (PCR)

Suspected colonies were confirmed by PCR (Kim *et al.,* 2007).

3.2.1.3.1 DNA Extraction

Suspected *Salmonella* colonies on BHI agars (Oxoid Ltd., UK) were transferred by a sterile toothpick scrape into a PCR tube, which contains 98 µL sterile distilled water. The tubes were vortexed (Velp Scientifica, Europe) then microwaved (Arçelik - MD 554) for 30 seconds in order to lyse cells.

3.2.1.3.2 PCR Preparation

The reaction mixture (master mix) was prepared in a total volume of 49µl containing; 10µl of 5X Go Taq Flexi Buffer (Genoks, Ankara), 3 µl of 25 mM $MgCl_2$ (Genoks, Ankara), 1µl of 10 mM each deoxynucleoside triphosphate (dNTPs) (Thermo Fisher Scientific Inc., USA) 0.25 µl of Taq DNA Polymerase, 2 µl of 12.5 mM each primers (Nanobiz, Ankara) and 1 µl samples of extracted bacterial DNA. 49 µl of the master mix was trasferred into each 24.5 ml PCR tube and added 1 µl of *Salmonella* purified DNA. 1 µl DNA from a *Salmonella* reference

bacteria culture was used as a positive control, and 1µl of dH_2O was used as a negative control. The master mix of the reaction is given in Table 3.2. Sequence of primers are given in Table 3.3.

Table 3.2 PCR Master Mix Reagents (Kim *et al.,* 2007).

Master Mix Reagents [Concentration]		**Vol (µl) for 1 X**
dH_2O	……	31
5X Go Taq Flexi Buffer	……	10.0
$MgCl_2$ [25 mM]	……	3.0
dNTPs [10 mM]	……	1.0
invA- F [12.5 mM]	……	2.0
invA–R[12.5 mM]	……	2.0
Go Taq DNA Polymerase	……	0.25
	TOTAL	49.25

Table 3.3 Sequences of forward and reverse primers (Kim *et al.,* 2007)

Primer	**Sequence 5' – 3'**
invA – F	GAA TCC TCA GTT TTT CAA CGT TTC
invA – R	TAG CCG TAA CAA CCA ATA CAA ATG

3.2.1.3.3 PCR Amplifications

PCR involved 35 cycles of denaturation (94°C, 30 s), primer annealing (60°C, 30 min) and primer extension (72°C, 30 s). The primer extension step (72°C, 5 min) followed the final amplification cycle. Amplification cycles are also explained in Figure 3.3.

PCR amplification conditions:

One hold at 94°C for 8 minutes

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35 cycles of the following:

94°C for 30 seconds

60°C for 30 seconds

72°C for 30 seconds

One hold at 72°C for 5 minutes

One hold at 4°C until you stop the reaction.

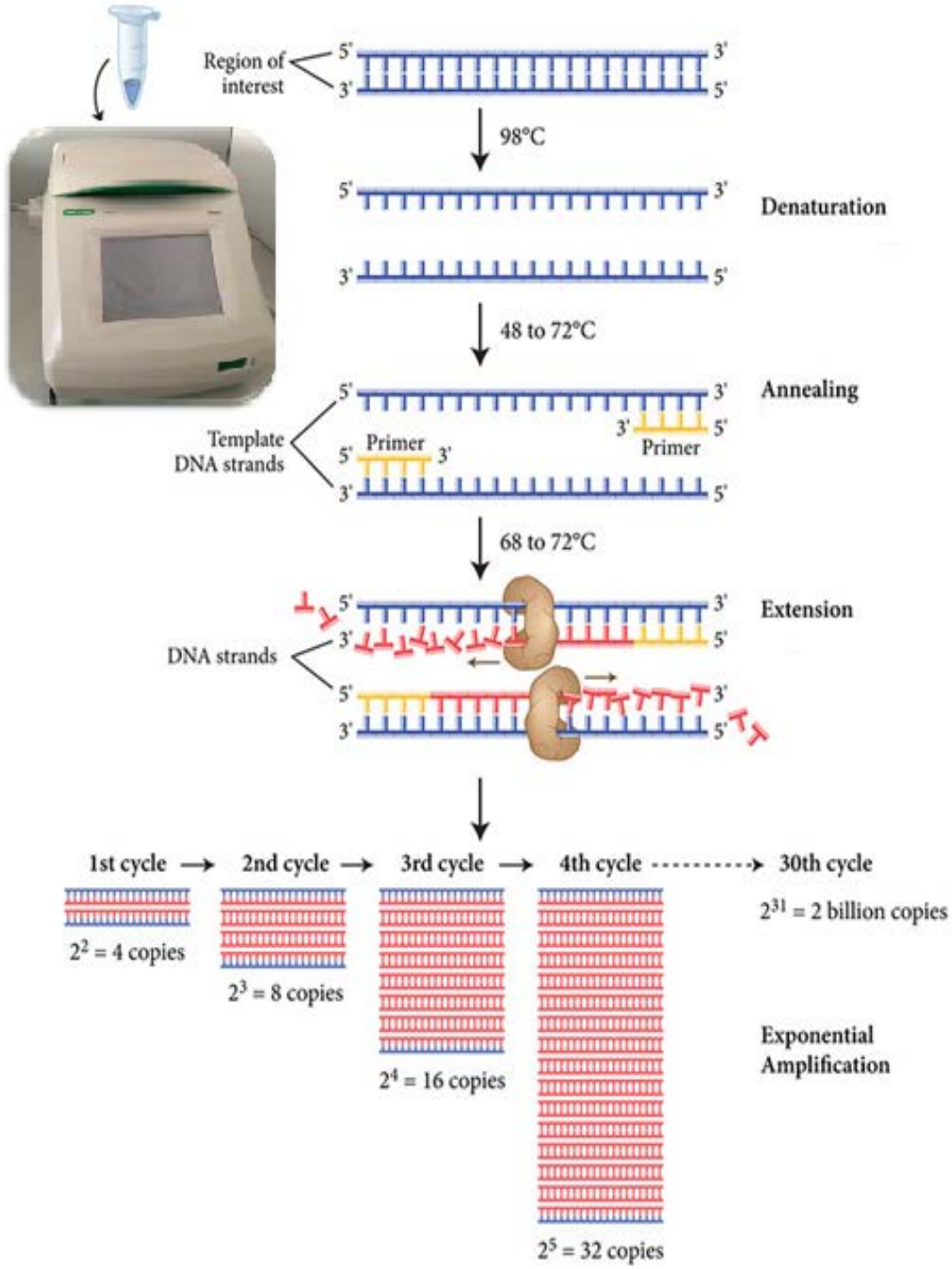

Figure 3.3 PCR Amplification (Adapted from website of Biolabs, New England)

3.2.1.3.4 Agarose Gel Electrophoresis and Gel Documentation

PCR products were confirmed by electrophoresis. Agarose gel was put into EtBr solution and stained it for 2 minutes. Then Agarose gel was transfer into dH_2O for staining for 5 minutes. Finally the gel was photographed under the UV light. Preparation of the TBE Buffer, Agarose Gel and Ethidium Bromide are explained below.

TBE Buffer Preparing

50 ml 0.5X TBE buffer (Genoks, Ankara) were prepared. 0.5X TBE buffer working solution (54 g Tris base 15.5 ml 85 % phosphoric acid [1,679g/ml] 40 ml 0.5 M EDTA [pH:8]) was prepared.

Agarose Gel Preparing

0.75 g agarose was weighted into a glass beaker and added 50 ml 0.5X TBE buffer. Solution was microwaved for 2 min to dissolve of the agarose. Then left solution to cool for 5 minutes at 55°C. Comb was placed into the tank to create a hole for DNA samples. The gel was poured slowly into the tank then allowed to solidify at room temperature for 30 minutes. Then comb was removed gently. 5 µl from each PCR products with 1.5 µl marker were added into each hole in the tank. A current of 110 V was applied to each gel.

Ethidium Bromide Preparing

To prepare stock solution, dissolve ethidium bromide (Sigma, USA) in a light-protected container at room temperature in water at 10 mg/ml to give a red solution. For staining 10mg/ml EtBr solution, 20 µl of EtBr stock solution (10 mg/ml) was added into 100 mL dH_2O.

3.2.1.3.5 Examination and Evaluation

Band formation around the 678 bp region confirms that *invA* is present and isolated organism belongs to *Salmonella* genus.

- ✓ -/- for a negative
- ✓ -/+ for a weak positive
- ✓ +/+ for a strong positive

3.2.1.4 Freezing Isolates

Confirmed *Salmonella* isolate was streaked onto BHI Agar (Oxoid, UK) then incubated at 37°C overnight. One colony on the BHI agar was inoculated into 5mL BHI broth, incubated at 37°C overnight. The vial was labeled as METU-S1-411. Vials are labeled like; Isolate number (e.g. METU-S1-001), genus name, date and your initials. 850 µl isolate suspension and 150 pre-sterilized glycerol solution were added to a 2 ml screw-cap vial then mixed the vial gently by up and down. *Salmonella* glycerol solution was frozen at –80°C (Wan *et al*., 2000).

3.2.2 Serotyping

Serotyping Analysis was performed in Ankara Türkiye Halk Sağlığı Kurumu according to the White-Kauffman-Le Minor scheme, as most recently described by Grimont and Weill (2007).

3.2.3 Pulsed Field Gel Electrophoresis (PFGE)

PFGE of the pepper isolate was performed according to the standardized CDC PulseNet protocol with restriction enzyme *Xba*I (Roche, Germany). Three main procedures are involved in the PFGE protocol. They are; (i) Plug preparation, (ii) Restriction digestion and (iii) Electrophoresis. First of all, the *Salmonella* isolates were streaked on BHI agar with an ino

3.2.3.1 PFGE Plugs Preparation from Agar Cultures

4ml of Cell Suspension Buffer (CSB) (100 mM Tris, 100 mM EDTA [pH 8.0]) was distributed in red capped tubes. A sterile cotton swab which was moistened with the cell suspension buffer was used to gently rub the bacterial cultures on the BHI agar and transferred to the 4 ml CSB red capped tubes. They were then mixed thoroughly in the capped tube with the swab. To determine the approximate cell concentrations, we used spectrophotometer. 1300 microlitres of the cell suspension was transferred in a cuvette to determine the absorbance of the solution with spectrophotometer (Shimadzu, UV-1700PharmaSpec) at a wavelength of 610 nm. Ideal absorbance should be between an optical density of 1.3 and 1.4. If the solution does not fall within this range, adjustment is done with by adding more bacterial cells or adding more of the CSB. After this procedure, 400 microlitres of adjusted CSB was put into labelled eppendorf tubes. A freshly prepared 20 mg/mL Proteinase-K solution was added directly to each eppendorf tube. It was mixed by gently flicking it several times with fingers. 1% SeaKem Gold (SKG) Agarose and 1% Sodium dodecyl sulfate were prepared. These were used for the plugs. For the preparation of the SKG Agarose, 0.25 grams of SKG was added to 23.5 ml of TE Buffer in a 300 ml flask. They were put in a microwave (Arçelik MD554) until they form a transparent agarose mixture. The agarose mixture was allowed to cool in a water bath at 55°C for 10 minutes. Before the 20 % SDS was added, it was also pre-warmed in 55°C water bath for at least 10 minutes. 1.25 ml of the 20 % SDS was added to the Agarose and mixed gently by shaking the bottle. 400 microliters of the equilibrated agarose mixture were added to each cell suspension and mixed gently by pipetting up and down two to three times before immediately dispensing into the wells of reusable or disposable PFGE plug molds. The plugs were allowed to cool so that they can solidify at room temperature for 15 minutes.

3.2.3.2 Lysis of Cells in agarose Plugs

After the plugs have been solidified, they were taken from the molds and placed in 5 ml of Cell lysis Buffer/Proteinase K solution contained in falcon tubes. Cell Lysis Buffer contains (CLB; 50 mMTris, 50 mM EDTA [pH 8.0] and 1 % Sarcosyl). They were then incubated at 54°C at a rate of 170 per minute for 2 hours with constant and vigorous agitation.

3.2.3.3 Washing of Agarose Plugs

After lysis of the plugs, the lysis buffer was poured away and they were quickly washed with 10ml of double distilled water for 10 minutes. This was done at 50°C at a rate of 70 rpm. The same procedure (of washing with distilled water) was repeated again under the same conditions. After washing with water, they were washed with 10 ml of TE buffer at 50°C and 70 rpm but for 15 minutes. This same procedure (of washing with TE Buffer) was repeated three times more. All these washing were done to remove the residual lysis buffer coating the plugs and the inside of the walls of the tubes. After the last wash, 5 mL of sterile TE buffer (room temperature) were added to each tube to serve as storage media for the plugs. The plugs were restricted immediately or stored in TE buffer at 4°C until needed. They were now ready to undergo restriction digestion with *Xba*I restriction enzyme.

3.2.3.4 Restriction Enzyme Digestion with *Xba*I

The restriction digestion process was started by the preparation of the restriction buffer or H buffer. In each of the labelled eppendorf tubes, 200 microlitres of the H buffer was added. The H buffer solution contains 20 microlitres of the H buffer and 180 microlitres of double distilled water for each isolate. The plugs were cut into 2 mm slices with a single edge razor blade or scalpel and immersed into the H buffer. The DNA size standard strain (*Salmonella* ser. Braenderup H9812; Hunter *et al.*, 2004) was also cut into 3 slices and immersed into H buffer. They were then incubated in a 37°C water bath for 10 minutes. After the incubation, the *XbaI* enzyme solution was prepared. This consists of 175 microlitres of double distilled

water, 20 microlitres of H buffer and 5 microlitres of the *XbaI* enzyme. The restriction buffer was poured away and replaced with 200 microlitres of the *XbaI* enzyme solution to each of the slices. The slices were incubated in the enzyme solution in a 37°Cwater bath for 5 hours 45 minutes. After incubation, it was ready for electrophoresis.

3.2.3.5 Elctrophoresis conditions and casting of the agarose gel

This stage was began by first preparing the SeaKem Gold agarose (SKG gel).The 1 % SKG agarose gel was prepared using a 10-well comb (Bio-Rad, UK) in the standard casting stand or 15-well comb in the wide or long casting stand (Bio-Rad, UK). 1.5 grams of SKG was added to 7.5 ml of 10X TBE buffer and 142.5ml of double distilled water. 8ml of double distilled water was added to the solution and was microwaved until it was evaporated. This extra 8 ml was added to make losses for the solution we will lose through vaporization so as to maintain the actual volume needed. After they were well mixed, the agarose was cooled in a 55°C water bath for 10 minutes. Then it was cooled at room temperature for at least 5 minutes. The comb was placed in the gel casting mold so that the teeth of the comb and the plug slices are flush with the bottom of the casting mold. The agarose was cast in a very clean gel mold. Dust was avoided from getting into it by covering it with a plastic container. The gels were allowed to polymerize for approximately 30 min at room temperature.

A running buffer which consists of 110 ml 10X TBE and 2090 ml of double distilled water was poured into the chamber. The pump speed was set at 70 and the PFGE system cooled to 14°C. The *XbaI* solution buffer was removed from the slices. Then, the slices were loaded into the gels in their respective lanes. A sealing agarose which had been cooled in a 55°C water bath for 10 minutes was added onto the lanes with the plugs to keep the plugs in the agarose during electrophoresis. After the agarose was put into the PFGE chamber with the running buffer, 836 microlitres of 10 mg/1ml thiourea solution was added to the running buffer. The resulting electrophoresis conditions are as follows: low KB - 30 KB, High KB – 700 KB, one percent agarose, at a gradient of 6.0 v/cm, a running time of 19 hours,

an initial switch time of 2.2 seconds, a final switch time of 1.03 minutes and 80 seconds, a pump speed of 70 and an included angle of 120 degrees.

Image acquisition: After the electrophoresis was completed, the gels were stained with 400 mL of ethidium bromide solution (40 g/mL) for 45 min. The gels were then de-stained with 400 mL of distilled water for 30min.The banding pattern was observed under ultraviolet (UV) illumination and a digital image (that can be converted to the JPEG of TIFF format) of the PFGE patterns is acquired using the Gel Doc system (Bio-Rad) following the saturation and integration parameters recommended by the manufacturer.

3.2.4 Multi Locus Sequence Typing (MLST)

According to protocol the amplification and DNA isolation steps of the 7 characteristic house-keeping genes of *Salmonella* with PCR amplification, including;

1) DNA preparation
2) PCR analyses steps
3) DNA sequencing
4) DNA sequence analysis of seven housekeeping gene

3.2.4.1 DNA Preparation

Single colony of *Salmonella* isolate was selected from BHI Agar by a sterile toothpick. Selected colony was grown in a BHI Broth overnight at 37°C. Spin column-based DNA isolation was carried out with Bacterial Genomic DNA Isolation Kit (NanoBiz, Ankara). Liquid cell culture prepared by BHI Broth, was centrifuged for 1-2 minutes at maximum speed in 1.5 ml microcentrifuge tubes. The supernatant forming was discarded after centrifuged and put 600 µl tampon-bg 1 solution until homogenation of the pellet with the liquid, continue pipetting. During this time, because of the low temperature levels, crystallization may occur. Thus, put tampon bg-1 solution at 37°C for 5-10 minutes and the solution was mixed. Then tube was incubated at 65 °C for 10 minutes. 300 µl tampon-bg 2 was added to tubes and by upturning the tubes, mixed the solutions. Tube was put in the freezer

part of the refrigerator for 5 minutes then centrifuged at 13,000 rpm for 10 minutes. 400-500 µl volume of supernatant was taken and transferred it to a new microcentrifuge tube. Tampon-bg 3 solution was added on this supernatant at 1.5 volumetric rates and by upturning mixes the tubes. 600 µl from the mixture was taken and transfered it to spin column. During 1 minute, centrifuged the tube at 10,000 rpm. The liquid forming at the bottom was discarded. The centrifuging was repeated for the liquid left. 500 µl tampon-bg 4 was added and centrifuged it for 1 minute at 10,000 rpm. The liquid forming at the bottom was discarded again. Note that tapon-bg4 is concentrated. Before usage, the required amount of ethanol was added as stated on the label of the bottle. In order to increase efficiency of the purification of DNA, 500 µl tampon-bg 4 was added and centrifuged it for 1 minute at 10,000 rpm again then the liquid was discarded. The tube at 13,000 rpm was centrifuged for 2 minutes in order to remove the ethanol of tampon bg-4. The tube under the spin column was thrown, and placed the spin column on a new sterile 1.5 ml centrifuge tube. 30-50 µl water at 65°C was added and kept the column at room temperature for 2 minutes. In order to gain the genomic DNA, centrifuged at 6,000 rpm for 1 minute. Isolated Sequences of forward and reverse primers for each gene has been shown in Table 3.4.

Table 3.4 Sequences of forward and reverse primers for each gene for MLST

Gene	Primer sequence 5' –3'	Amplified region, bp
aroC-F	GGCACCAGTATTGGCCTGCT	826
aroC-R	CATATGCGCCACAATGTGTTG	
thrA-F	GTCACGGTGATCGATCCGGT	852
thrA-R	CACGATATTGATATTAGCCCG	
purE-F	ATGTCTTCCCGCAATAATCC	510
purE-R	TCATAGCGTCCCCCGCGGATC	
sucA-F	AGCACCGAAGAGAAACGCTG	643
sucA-R	GGTTGTTGATAACGATACGTAC	
hisD-F	GAAACGTTCCATTCCGCGC	894
hisD-R	GCGGATTCCGGCGACCAG	
hemD-F	ATGAGTATTCTGATCACCCG	666
hemD-R	ATCAGCGACCTTAATATCTTGCCA	
dnaN-F	ATGAAATTTACCGTTGAACGTGA	833
dnaN-R	AATTTCTCATTCGAGAGGATTGC	

Adjustment of the concentration of primer and other reagents;

According to the mass value in picomoles (pmol) or nanomoles (nmol) written on the package of the primer in the lyophilized form, the amount of double-distilled sterile dH_2O was calculated, needed to make a solution of 100 μM. 12.5 μM working solution was prepared from 100 μM stock solution.

3.2.4.2 PCR Analysis Steps

PCR was analyzed with the same main principle of the confirmation of *Salmonella* by PCR of *invA* gene. As explained before this method consists of four main steps, these were;

- PCR Preparation
- PCR Amplifications
- Agarose Gel Electrophoresis and Gel Documentation
- Examination and Evaluation

3.2.4.2.1 PCR Preparation

The reaction mixture(master mix) was prepared in a total volume of 49µl containing; 35.5 µl dH_2O, 5µl of 10X Go Taq Flexi Buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM each deoxynucleotide triphosphate (dNTPs), 0.25 µl of Taq DNA Polymerase, 2 µl of 12.5 mM each primers and 1 µl samples of extracted bacterial DNA. 49 µl of the master mix was transferred into each 0.2 ml PCR tube, and 1 µl *Salmonella* DNA was added. 1 µl DNA from a *Salmonella* reference bacteria culture was used as a positive control, and 1µl of dH_2O was used as a negative control.

3.2.4.2.2 PCR Amplifications

Thermocycler (Bio-Rad, UK) was turned on and confirmed that was being functioned properly. PCR tubes were placed in the wells of Thermocycler. PCR involved 35 cycles of denaturation (94°C, 1 min), primer annealing (60°C, 1 min) and primer extension (72°C, 1 min). The primer extension step (72°C, 7 min) followed the final amplification cycle.

PCR amplification conditions:

One hold at 94^{o}C for 10 minutes [1X]

---

35 cycles of the following:

94^{o}C for 1 minute

60^{o}C for 1 minute [35X]

72^{o}C for 1 minute

One hold at 72^{o}C for 7 minutes

Final hold at 4^{o}C

3.2.4.2.3 Agarose Gel Electrophoresis and Gel Documentation

PCR products were confirmed by electrophoresis. 50 ml 0.5X TBE buffer were prepared. 0.5 X TBE buffer working solution (54 g Tris base 15.5 ml 85% phosphoric acid [1,679g/ml] 40 ml 0.5 M EDTA [pH:8]) was prepared. 1.5 g agarose was weighted into a glass beaker and added 100 ml 0.5X TBE buffer. Solution was microwaved for 2 min to dissolve of the agarose. Then, the solution was left to cool down about 55^{o}C. While the agarose was cooling, gel tray was prepared by sealing ends with tape. The comb was placed in gel tray about 2.5 cm from one end of the tray and positioned the comb vertically such as the teeth are about 1-2 mm above the surface of the tray. The gel was poured slowly into the tank then allowed to solidify at room temperature for 30 minutes. Then comb was removed gently and placed tray in electrophoresis buffer as used previously. 5 µl from each PCR products with 1.5 µl marker were added into each hole in the tank. A current of 110 V was applied to each gel for 30 min.

Agarose gel was put into EtBr solution and stained it for 2 minutes. Then Agarose gel was transfered into dH_2O for staining for 5 minutes. Finally the gel was photographed under the UV light.

3.2.4.2.4 Examination and Evaluation

Band formation around the mentioned regions in Table 3.4 for each gene confirms that the gene is present in the examined *Salmonella* isolates.

- ✓ -/- for a negative
- ✓ -/+ for a weak positive
- ✓ +/+ for a strong positive

For test results to be considered valid the following criteria must be met:

- ✓ The negative control must show a negative result.
- ✓ The positive control must show a positive result.
- ✓ The bands on the gel must be clean and not smeared.

3.2.4.3 DNA Sequence Analysis

DNA samples were sent to the Macrogen Inc. (Seoul, Korea) for purification and capillary sequencing. DNA was sequenced by capillary electrophoresis.

3.2.4.4 DNA sequence analysis of seven housekeeping gene

Seqman Pro and MegAlign tools of DNAStar software (Madison, United States) were used for the sequences analysis. Raw sequence files, as .abi files provided by the sequencing company were visually checked for any defects in the chromatographic sequence data by SeqMan Pro tool. Start and end fragments in the each sequence belonging to each housekeeping gene were trimmed. Then, these .abi files were converted to Fasta format. Final adjusted forms of each gene in Fasta format were entered into UCC MLST *S. enterica ser. enterica* databank.

Allelic type (AT) of each gene was attained through the database. As a result of seven-number allelic profile of the isolate, sequence type (ST) was acquired. In order to find out the phylogenetic relationship of isolate with other certain isolates in the databank, sequences of each gene at certain length (*aroC* (501 bp), *dnaN* (501 bp), *hemD* (432 bp), *hisD* (501 bp), *purE* (399 bp), *sucA* (501 bp), *thrA* (501 bp)) was concatenated one by one, and aligned and compared with other isolates via

MegAlign tool. Finally, phylogenetic tree of the isolates were constructed with the same tool.

3.2.5 Application of High Hydrostatic Pressure to Peppers

The second set of experiments was designed to determine optimum HHP parameters to inactivate of *Salmonella* from sliced fresh peppers samples. The effect of HHP on the isolated pathogen was evaluated on the inoculated sliced fresh pepper samples. The objective of HHP treatment of this study is to determine the pressure resistance of isolated *Salmonella enterica* Enteritidis which was the only isolate from a total of 255 fresh pepper samples in our study. High hydrostatic pressure treatments of sliced peppers were analysed by HHP equipment in Food Engineering Department (FDE) in METU. Samples was pressurized with 760.0118-type pressure equipment supplied by SITEC CH-8124-Sieber Engineering AG, Zurich, Switzerland which is shown in Figure 3.4. The main parts of the HHP equipment were pressure vessel, a pressure pump, temperature control device and other system controls. Temperature inside the vessel was controlled at the intervals of 10-90°C by the temperature control device. The liquid inside the device was warmed before pressurization to the desired temperature. Up to 250-300 MPa pressure was created in the pressure vessel. Pressure increase rate was approximately 300 MPa/min, and pressure come-down time is 15 sec. The pressure level and the temperature of pressurization was controlled by device automatically.

Figure 3.4 HHP Equipment (SITEC CH-8124, Zürich, Switzerland)

Our aim of HHP treatment is to determine proper pressure, temperature and time to inactivate *Salmonella* on the sliced inoculated pepper samples to identify the efficacy of HHP to inactivate *Salmonella* from fresh peppers.

3.2.5.1 Growth Curve of *Salmonella*

One day before the experiment 100 ml BHI in 2 of 250 ml Erlenmeyer flasks, 1000 ml TSA (Tryptic Soy Agar) and 1000 ml BPW were sterilized for 15 min at 121°C. After sterilization 2 erlenmayer flasks and BPW were kept in 4ºC and TSA was placed into a waterbath set at 50°C with heat-protective gloves. 15 min later TSA was taken from the waterbath then it was poured into the plates with aseptic conditions. TSA plates were kept in 4ºC. Confirmed *Salmonella* colony was grown in BHI at 37ºC overnight and 100 µl sample was inoculated to 100 ml BHI in each 250 ml Erlenmeyer flask. Flasks were incubated at 37ºC at rotary shaker (120 rpm) incubator up to 18 h.

Duplicate sample of flasks were taken from the incubator at 37ºC every hour and Optical Density at 600 nm ($OD_{600\ nm}$) was measured with a spectrophotometer (Shimadzu, UV-1700PharmaSpec) (Figure 3.5). Average OD data versus incubation time was calculated then growth curve was plotted for the strain.

Figure 3.5 Spectrophotometer in laboratory

At the same time 1 ml of the culture was taken from the flasks in every 2 h to calculate the number of colony forming units per mL (CFU/mL). Each tube contains 9 ml of BPW. 1ml of bacterial culture was transferred to 900 µl BPW then spread plated with serial dilutions to calculate number of colony on the TSA. Pipette 100 µl of the each serial diluted (1:10, 1:100, 1:1000, 1:10000…) bacterial culture was spread plated onto the center of a TSA plate using with L-shaped glass rod. The spreader was immersed in ethanol for every step of plating. Inverted TSA plates were incubated at 37ºC, 24±3h. The day after experiment plates were taken from the incubator and 30-300 colonies were calculated on the each plate. Average values were calculated for every 2 hours. Average colony forming units per mL (CFU/mL) versus incubation time was calculated then growth curve was plotted for the strain.

Growth curve analysis information was used to determine cells at early stationary growth phase before high hydrostatic pressurization.

3.2.5.2 Sample Preparation for HHP Analysis

Culture of *Salmonella* Enteritidis from frozen stock was plated onto BHI Agar which was incubated 37ºC overnight. One colony on the BHI Agar was inoculated to duplicated 100 ml BHI Broth and incubated 37ºC overnight.

One day before the experiment 500 ml BHI in 2 of flasks, 1000 ml TSA (Tryptic Soy Agar) and 1000 ml BPW were sterilized for 15 min at 121°C. After sterilization 2 erlenmayer flasks and BPW were kept in 4ºC and TSA was placed into a waterbath set at 50°C with heat-protective gloves. 15 min later TSA was taken from the waterbath then it was poured into the plates with aseptic conditions. TSA plates were kept in 4ºC.

Salmonella colony was grown in BHI at 37ºC overnight and sample was inoculated to 500 ml BHI in each flasks which were incubated at 37ºC at rotary shaker (120 rpm) incubator up to 12 h to have 10^{11} cfu·mL^{-1} of *Salmonella* Enteritidis suspension. 5 pepper samples were analysed within HHP Analyses. 10 g of each sliced pepper samples were weighted. 8 sliced samples (Figure 3.6) were analysed for each pepper. 1 negative control, 1positive control, 4 slices for shelf life analyse and rest 2 for HHP inactivation analysis. 40 slices pepper samples which were shown in Figure 3.6, were analysed within HHP analysis.

Figure 3.6 Sliced pepper samples for HHP Analysis

Sliced peppers were immersed in a bacterial suspension 10^{11} cfu·mL^{-1} of *Salmonella* Enteritidis at room temperature for 30 min. Pepper slices were removed and air dried at room temperature for 10 min at room temperature.

High hydrostatic pressurization of pepper slices were carried out 500 MPa for 5 min at 25°C. 10 g samples was added to 100 ml of buffered peptone water (BPW) and mixed with a stomacher for 60 s then 0.1 ml of samples were spread plated onto TSA Agar plate. Plates were incubated at 37ºC overnight.

3.2.5.3 Shelf Life Analysis of Inoculated Peppers

Shelf life analysis was fulfilled to see the growth of *Salmonella* on the HHP treated slices after 1 week storage of different temperatures. 2 sets of pepper samples were performed within the same experiment of HHP inoculation.

HHP treated pepper samples were storage at 4ºC and 25ºC. After 1 week storage samples were analysed for the presence of *Salmonella*.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Result of Isolation

A total of 255 fresh pepper samples (green pepper, kapya pepper, bell pepper, mazamort pepper, charleston) were collected from 3 districts within 9 supermarkets and 3 bazaars in Ankara, Turkey. 3 supermarkets and 1 bazaar were visited within the 3 months sampling period in 2012. The samples were analyzed for the presence of *Salmonella*. Then, suspected colonies were confirmed as *Salmonella* by using polymerase chain reaction (PCR) with the *invA* gene which is a specific gene of *Salmonella*. 24 suspected colonies were PCR analysed for the confirmation of *Salmonella* and one *Salmonella* strain was isolated from kapya pepper, was confirmed by PCR. These results demonstrate that fresh kapya pepper from the bazaars of Ankara was an important source of *Salmonella* contamination.

According to appendix A, total of 83 fresh produce were collected from A-B-C markets and D bazaar in Cankaya District, 86 fresh produce were collected from E-F-G markets and H bazaar in Yenimahalle District, 86 fresh produce were collected from I-K-L markets and M bazaar in Keçiören District. Every sample has specific code such as KEP-R0110; first part of codes (KEP) gave us district and supplier informations, second part is the fist letter of sample, R-Kapya pepper and following numbers were related to purchased date of our sample as explained Figure 4.1. Figure 4.2 also shows us distribution of collected fresh pepper samples within 3 districts in Ankara. Total of 53 bell pepper, 53 green pepper, 53 kapya pepper, 53 mazamort pepper, 43 charleston pepper samples were analysed (Figure 4.3).

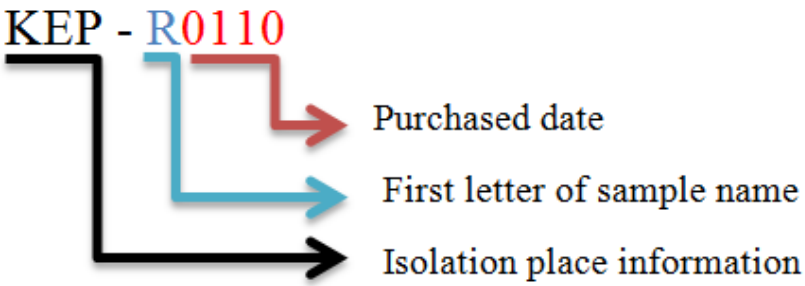

Figure 4.1 Explanations of Sample Codes

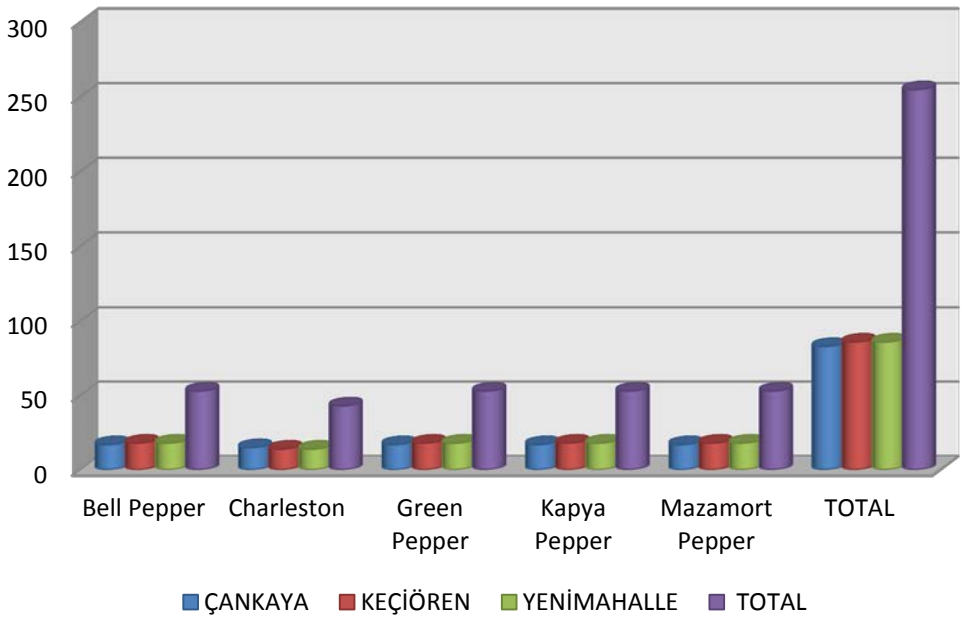

Figure 4.2 Distribution of collected fresh pepper samples within 3 districts in Ankara

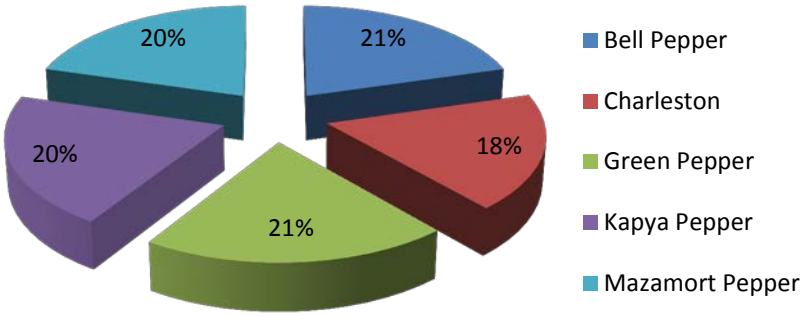

Figure 4.3 Distribution of analysed pepper samples

4.1.1 PCR Analysis Result

24 suspected colonies were PCR Analysed and one positive sample was confirmed by PCR (Figure 4.4) which was purchased from Keçiören district M bazaar. Details of *Salmonella* positive isolate was given in Table 4.3.

Table 4.3 PCR Positive Sample

No	Sample Code	Sample Name	District in Ankara	Supplier	Date of Purchase	PCR Result
200	KEP-R0110	Kapya pepper	Keçiören	M	01.10.2012	+

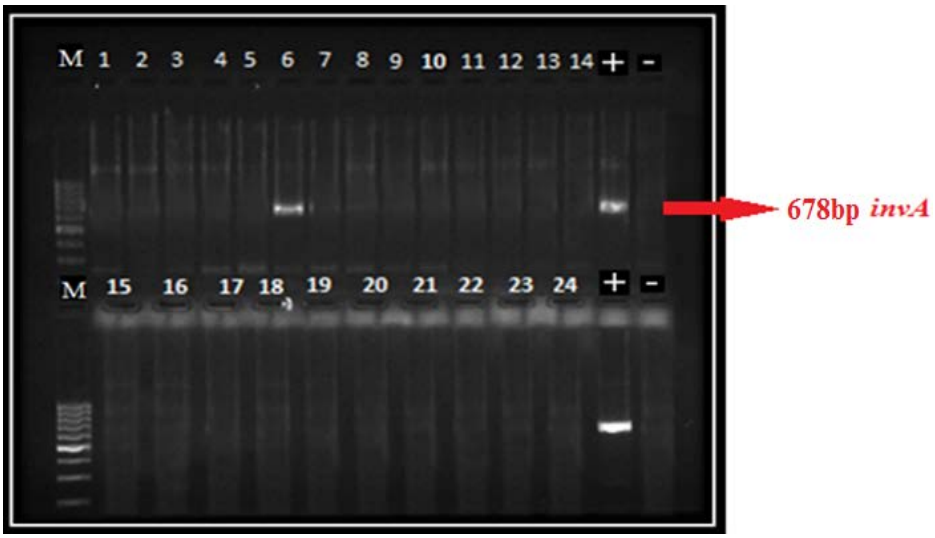

Figure 4.4 PCR Result of 24 suspected samples. M is marker, + positive control and – negative control.

Detection of pathogens in samples of fresh ready to eat foods such as fresh pepper is required for two main purposes to identify contaminated foods. The result of our study indicated that isolation rate of *Salmonella* from Turkish bazaar was high. On the other hand this study showed fresh peppers in Turkey are slightly contaminated with *Salmonella*. Outbreaks are increasingly related to consumption of fresh produce and fresh produce are needed to control salmonellosis although composition of the fresh pepper among isolated strains was not highly variable. Generally foodborne illness from *Salmonella* is linked to consumption of poultry (Greig & Ravel, 2009); however, fresh produce has proven to be a frequent vehicle (Sivapalasingam *et al.*, 2004). In 2008 a large outbreak of salmonellosis occurred in 43 states in the US and Canada, which involved 1442 illnesses that were linked to the consumption of hot peppers (Mody *et al.*, 2011; CDC, 2008b)

4.2 Results of Subtyping

4.2.1 Result of serotyping

Serotyping result is given table Table 4.4. Our antigenic formula was matched with Rimont & Weill‘s (2007) research as *S.* Enteritidis.

Table 4.4 Serotyping result of the isolated strain.

	Somatic (O)	Flagellar (H) Antigen	
Serotype	Antigen	Phase 1	Phase 2
Enteritidis	1, 9, 12	g,m	-

4.2.2 Result of PFGE

Three more serotype was analyzed with our strain to see the band differences of our isolated strain. These serotypes were isolated from fresh produces within the same period of time and also same districts in Ankara, details were given Table 4.5. Isolate details indicate that purchased place -M- of the serotype of *S.* Mikawasima isolated from iceberg, was the same as our strain (Günel *et al.*, unpublished date). We can easily understood that presence of *Salmonella* in Ankara bazaar is higher than market.

Table 4.5 The details of isolates, used in PFGE.

METU ID	**Serotype**	**Sample Name**	**District**	**Purchased Place**	**Date of Purchase**
MET-S1-411	***S.* Enteritidis**	**Kapya pepper**	**Keçiören**	**M**	**01.10.2012**
MET-S1-408	*S.* Anatum	Parsley	Keçiören	I	14.10.2012
MET-S1-409	*S.* Mikawasima	Iceberg	Keçiören	M	01.10.2012
MET-S1-410	*S.* Charity	Parsley	Çankaya	X	08.09.2012

Table 4.6 PFGE profile groups of the strains

No.	METU ID	Source	Serotype	PFGE Pattern
1	-	-	*S.* Braenderup	Reference
2	**MET-S1-411**	**Pepper**	***S.* Enteritidis**	**PT1**
3	MET-S1-408	Parsley	*S.* Anatum	PT2
4	MET-S1-409	Iceberg	*S.* Mikawasima	PT3
5	MET-S1-410	Parsley	**S.** Charity	PT4
6	-	-	*S.* Braenderup	Reference

PFGE profile of the strains detail were given in Table 4.6. Our strain was number 2 on the band profile (Figure 4.5). Our isolated subtype was found to be different from the other isolates.

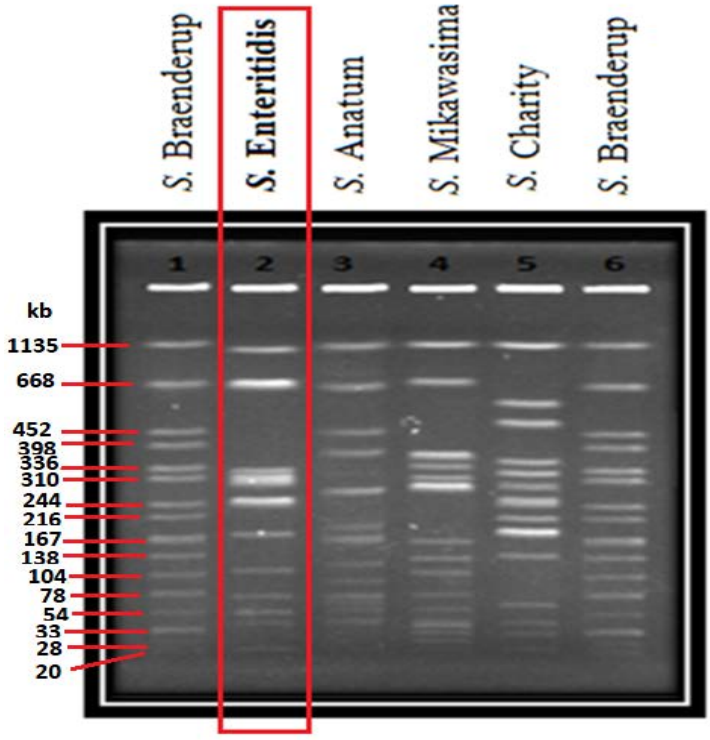

Figure 4.5 Pulsed-field gel electrophoresis (PFGE) images of *S.enterica* strains.

In fact, PFGE has been successfully applied to determination of the molecular epidemiology of numerous organisms including Enteritidis (Barrett *et al.*, 1994).

S. Enteritidis is one of the most common foodborne pathogen cause acute gastroenteritis the reason of that this pathogen needs effectively monitoring system (Philips & George, 1994). *S.* Enteritidis was commonly subtyped with the DNA-based methods like DNA restriction pattern and plasmid profile analysis (Hickman-Brenner *et al.,* 1991). This is a possible alternative method to identify *Salmonella* serotype using with hierarchical analysis of PFGE. On the other hand recents studies show that supervised random forest classification analysis supplies more effective than conventional hierarchical cluster analysis to obtain *Salmonella* serotypes (Gaul *et al.,* 2007).

4.2.3 Result of MLST

MLST Result of S.Enteritidis isolate is given on Table 4.7. Nucleotides of genes representing sequence type (ST), ST 11 is given in appendix Table D. Our strain was genetically characterized by MLST database following the guidelines described in http://mlst.ucc.ie/mlst/dbs/Senterica, and the results indicate that it belongs to the sequence type (ST) 11. According to MLST database around 20 *S.* Enteritidis ST 11 were isolated from foods in Brazil, Japan and China. It has been shown that ST 11 includes the majority of the *Salmonella* Enteritidis isolates uploaded to the *S. enterica ser. Enteritidis* MLST database. MLST is increasingly being used for characterization of foodborne pathogens (Aanensen and Spratt, 2005). Harbottle *et al.* (2006) investigated that discriminatory power of seven housekeeping genes of MLST is sufficient for *Salmonella* isolates; on the other hand it may not be suitable to use MLST when we want to see the differences of the closely related strains.

Table 4.7 MLST Result of the strain

METU ID	Serotype	Sequence Type (ST)	Isolate Source
MET-S1-411	*S.* Enteritidis	11	Kapya pepper

Table 4.7 MLST Result of the strain (continued)

Houskeeping Genes	**Allelic Type**	**Length (b.p.)**
aroC (501 b.p.)	5	613
sucA (501 b.p.)	6	587
thrA (501 b.p.)	11	765
dnaN (501 b.p.)	2	799
hemD (432 b.p.)	3	667
purE (399 b.p.)	6	476
hisD (501 b.p.)	7	762

4.3 Effect of High Hydrostatic Pressure

The viability loss data by pressurization, 500 MPa, 5 min and 25°C for *S.* Enteritidis which was isolated from kapya pepper, was given in Table 4.8. Different range of pressure was applied to the sliced fresh peppers to determine optimum parametres of treatment. These were also given in Appendix F.1, F.2, F.3 respectively.

Table 4.8 Viability loss of *Salmonella* strain by pressurization, 500 MPa, 5 min and 25°C.

	Log_{10} cfu/ml			
Samples	*Salmonella* Solution	Positive Control	Negative Control	After Pressurization
Kapya pepper	11.27	8.39	ND	ND
Charleston	11.27	8.64	ND	ND
Mazamort pepper	11.27	9.16	ND	ND
Bell Pepper	11.27	8.44	ND	ND
Green Pepper	11.27	9.10	ND	ND

**ND, cfu was not detected in 0.1 ml of suspension from tested samples*

It was obtained that S. Typhimurium and *S.* Enteritidis achieved limited resistance to pressures more than 600 MPa (Rendueles *et al.*, 2010). A major function of high pressure processing of food is destruction of microorganisms.The death rate of the strain of pathogen is similar to those reported by other researchers (Rendueles *et al.* 2010; Alpas, 2000; Neetoo & Chen, 2012). The principle of HHP treatment is the fast and uniform pressure distribution among the food size, shape and the composition (Farkas & Hoover, 2000).

4.3.1 Growth Curve Result of *S.* Enteritidis

Average results of OD 600 nm readings for *S.* Enteritidis is given in Figure 4.6. Early stationary phase was determined for the HHP analysis. Average logN(cfu/ml) of *Salmonella* was given in Figure 4.7.

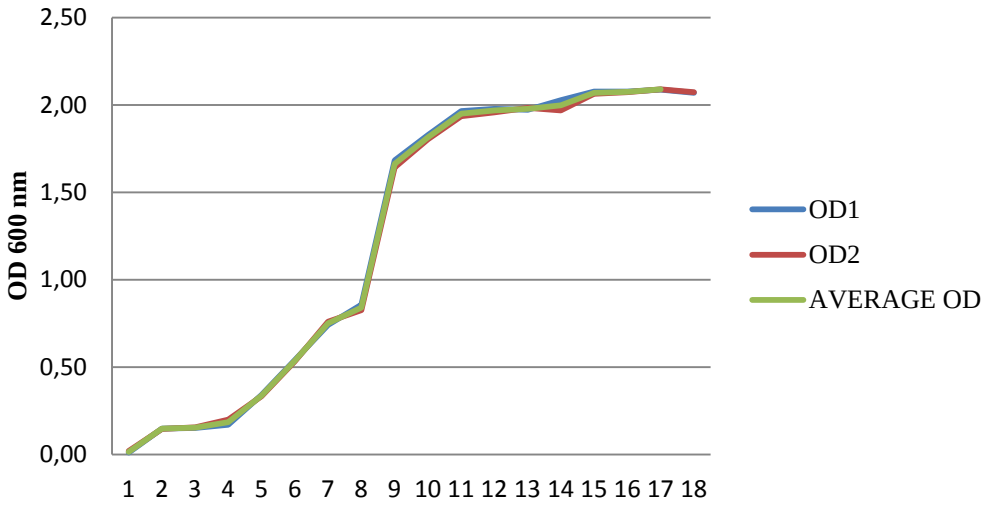

Figure 4.6 Spectrophotometric growth curve of *Salmonella* Enteritidis at 37°C.

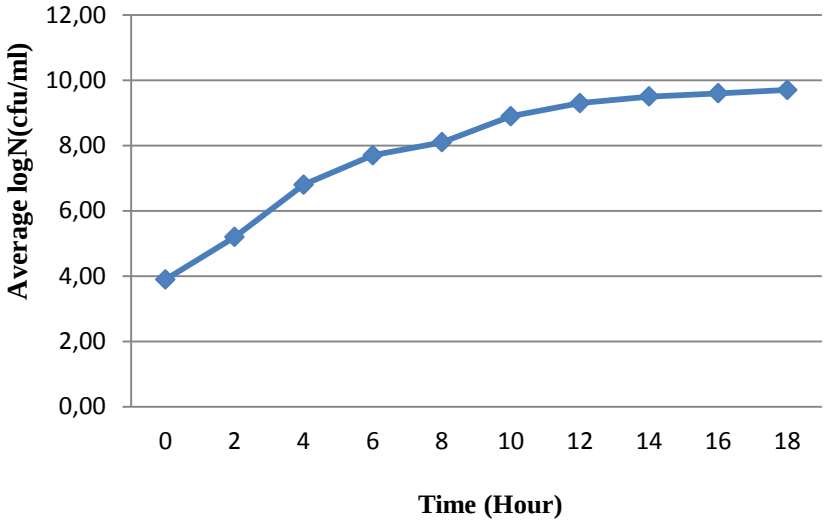

Figure 4.7 Growth of *Salmonella* Enteritidis at 37°C.

4.3.2 Shelf Life Analysis

Heat treatment is one of the most common methods used in food processing to inactivate microorganisms. Environmental impacts like temperature and relative humidity have a large effect on the quality of fruits and vegetables (Allen, 2003).

An important step for HHP analysis is the determination of cells surviving after pressure treated samples. High levels of (re)growing might cause illness, if fresh produce is consumed. To deal with this possibility, it is necessary to have information concerning the growth kinetics of injured cells that were subject to shelf life analysis of the inoculated then pressure treated samples. Viability loss of *S. enterica ser. Enteritidis*, at 25°C 1 week storage, Table 4.9 shows us *Salmonella* cells were damaged with the 500 MPa pressure treatment then re-growth when they find optimum conditions. On the other hand there was no any colonies were counted on TSA agars with 1 week storage at 4°C.

Table 4.9 Viability loss of *S.* Enteritidis, at 25°C and 4°C after 1 week storage.

Inoculated Samples	1 week storage at 4°C	1 week storage at 25°C
Kapya pepper	ND	detected
Charleston	ND	detected
Mazamort pepper	ND	detected
Bell Pepper	ND	detected
Green Pepper	ND	detected

**ND, cfu was not detected in 0.1 ml of suspension from tested samples*

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The main objective of this study was to investigate distribution of *Salmonella* in fresh peppers in Turkey and find out optimum conditions to inactivate isolated *Salmonella* by high hydrostatic pressure treatment.

It was found that that prevalence of *Salmonella* in Turkish bazaars is higher than markets. PFGE data and serotyping data of *S.* Enteritidis isolate will be guide for future experiments. Multilocus sequence typing (MLST) analysis indicated that the strain belongs to ST 11. There is no any data has been recorded related to isolation of *S.* Enteritidis ST 11 from fresh peppers in literature. MLST and PFGE data has limitations in terms of tracking *Salmonella* strains in Turkey. There must be governmental instution to perform surveillance of *Salmonella* such as in Europe and other countries.

Second part of this study was shown that HHP treatment at 4°C can be used to inactivate *S.* Enteritidis for fresh peppers at 500 MPa. However, storage conditions and temperature should carefully be optimized to increase the safety of HHP treated fresh peppers. Further research may be done with more fresh produce samples within country and will improve our understanding transmisson of foodborne pathogens. More research is required on pressure effect on fresh produces. Sensory analysis of fresh produce should be done in order to determined how various pressure treatment condition will affect the quality and sensorial characteristics of the fresh produce.

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APPENDIX A

ANALYSED MATERIAL, DATE, PLACE AND RESULTS

Table A.1 Analysed material, date, place and results

No	Sample Code	Sample Name	District in Ankara	Purchased Place	Date of Purchase	Result
1	KA-G0508	Green	Keçiören	I	05.08.2012	-
2	KA-R0508	Kapya	Keçiören	I	05.08.2012	-
3	KA-C0508	Charleston	Keçiören	I	05.08.2012	-
4	KA-B0508	Bell	Keçiören	I	05.08.2012	-
5	KA-V0508	Mazamort	Keçiören	I	05.08.2012	-
6	KS-G0508	Green	Keçiören	K	05.08.2012	-
7	KS-R0508	Kapya	Keçiören	K	05.08.2012	-
8	KS-C0508	Charleston	Keçiören	K	05.08.2012	-
9	KS-B0508	Bell	Keçiören	K	05.08.2012	-
10	KS-V0508	Mazamort	Keçiören	K	05.08.2012	-
11	KR-G0508	Green	Keçiören	L	05.08.2012	-
12	KR-R0508	Kapya	Keçiören	L	05.08.2012	-
13	KR-C0508	Charleston	Keçiören	L	05.08.2012	-
14	KR-B0508	Bell	Keçiören	L	05.08.2012	-
15	KR-V0508	Mazamort	Keçiören	L	05.08.2012	-
16	YP-G0508	Green	Yenimahalle	H	05.08.2012	-
17	YP-R0508	Kapya	Yenimahalle	H	05.08.2012	-
18	YP-C0508	Charleston	Yenimahalle	H	05.08.2012	-
19	YP-B0508	Bell	Yenimahalle	H	05.08.2012	-
20	YP-V0508	Mazamort	Yenimahalle	H	05.08.2012	-

Table A.1 Analysed material, date, place and results (continued).

No	Sample Code	Sample Name	District in Ankara	Purchased Place	Date of Purchase	Result
21	KEP-G0508	Green	Keçiören	M	05.08.2012	-
22	KEP-R0508	Kapya	Keçiören	M	05.08.2012	-
23	KEP-C0508	Charleston	Keçiören	M	05.08.2012	-
24	KEP-B0508	Bell	Keçiören	M	05.08.2012	-
25	KEP-V0508	Mazamort	Keçiören	M	05.08.2012	-
26	YM-G0908	Green	Yenimahalle	E	09.08.2012	-
27	YM-R0908	Kapya	Yenimahalle	E	09.08.2012	-
28	YM-C0908	Charleston	Yenimahalle	E	09.08.2012	-
29	YM-B0908	Bell	Yenimahalle	E	09.08.2012	-
30	YM-V0908	Mazamort	Yenimahalle	E	09.08.2012	-
31	YK-G0908	Green	Yenimahalle	F	09.08.2012	-
32	YK-R0908	Kapya	Yenimahalle	F	09.08.2012	-
33	YK-C0908	Charleston	Yenimahalle	F	09.08.2012	-
34	YK-B0908	Bell	Yenimahalle	F	09.08.2012	-
35	YK-V0908	Mazamort	Yenimahalle	F	09.08.2012	-
36	YH-G0908	Green	Yenimahalle	G	09.08.2012	-
37	YH-R0908	Kapya	Yenimahalle	G	09.08.2012	-
38	YH-C0908	Charleston	Yenimahalle	G	09.08.2012	-
39	YH-B0908	Bell	Yenimahalle	G	09.08.2012	-
40	YH-V0908	Mazamort	Yenimahalle	G	09.08.2012	-
41	ÇM-G1108	Green	Çankaya	A	11.08.2012	-
42	ÇM-R1108	Kapya	Çankaya	A	11.08.2012	-
43	ÇM-C1108	Charleston	Çankaya	A	11.08.2012	-
44	ÇM-B1108	Bell	Çankaya	A	11.08.2012	-
45	ÇM-V1108	Mazamort	Çankaya	A	11.08.2012	-
46	ÇP-G1108	Green	Çankaya	C	11.08.2012	-
47	ÇP-R1108	Kapya	Çankaya	C	11.08.2012	-

Table A.1 Analysed material, date, place and results (continued).

No	Sample Code	Sample Name	District in Ankara	Purchased Place	Date of Purchase	Result
48	ÇP-C1108	Charleston	Çankaya	C	11.08.2012	-
49	ÇP-B1108	Bell	Çankaya	C	11.08.2012	-
50	ÇP-V1108	Mazamort	Çankaya	C	11.08.2012	-
51	ÇÇ-G1108	Green	Çankaya	B	11.08.2012	-
52	ÇÇ-RR1108	Kapya	Çankaya	B	11.08.2012	-
53	ÇÇ-C1108	Charleston	Çankaya	B	11.08.2012	-
54	ÇÇ-B1108	Bell	Çankaya	B	11.08.2012	-
55	ÇÇ-V1108	Mazamort	Çankaya	B	11.08.2012	-
56	ÇEP-G1108	Green	Çankaya	D	11.08.2012	-
57	ÇEP-R1108	Kapya	Çankaya	D	11.08.2012	-
58	ÇEP-C1108	Charleston	Çankaya	D	11.08.2012	-
59	ÇEP-B1108	Bell	Çankaya	D	11.08.2012	-
60	ÇEP-V1108	Mazamort	Çankaya	D	11.08.2012	-
61	KEP-G1108	Green	Keçiören	M	11.08.2012	-
62	KEP-R1108	Kapya	Keçiören	M	11.08.2012	-
63	KEP-C1108	Charleston	Keçiören	M	11.08.2012	-
64	KEP-B1108	Bell	Keçiören	M	11.08.2012	-
65	KEP-V1108	Mazamort	Keçiören	M	11.08.2012	-
66	YP-G2608	Green	Yenimahalle	H	26.08.2012	-
67	YP-R2608	Kapya	Yenimahalle	H	26.08.2012	-
68	YP-B2608	Bell	Yenimahalle	H	26.08.2012	-
69	YP-V2608	Mazamort	Yenimahalle	H	26.08.2012	-
70	ÇEP-G2608	Green	Çankaya	D	26.08.2012	-
71	ÇEP-R2608	Kapya	Çankaya	D	26.08.2012	-
72	ÇEP-B2608	Bell	Çankaya	D	26.08.2012	-

Table A.1 Analysed material, date, place and results (continued).

No	Sample Code	Pepper Samples	District in Ankara	Purchased Place	Date of Purchase	Result
73	ÇEP-V2608	Mazamort	Çankaya	D	26.08.2012	-
74	ÇM-G2708	Green	Çankaya	A	27.08.2012	-
75	ÇM-R2708	Kapya	Çankaya	A	27.08.2012	-
76	ÇM-C2708	Charleston	Çankaya	A	27.08.2012	-
77	ÇM-B2708	Bell	Çankaya	A	27.08.2012	-
78	ÇM-V2708	Mazamort	Çankaya	A	27.08.2012	-
79	ÇP-G2708	Green	Çankaya	C	27.08.2012	-
80	ÇP-R2708	Kapya	Çankaya	C	27.08.2012	-
81	ÇP-C2708	Charleston	Çankaya	C	27.08.2012	-
82	ÇP-B2708	Bell	Çankaya	C	27.08.2012	-
83	ÇP-V2708	Mazamort	Çankaya	C	27.08.2012	-
84	ÇÇ-G2708	Green	Çankaya	B	27.08.2012	-
85	ÇÇ-R2708	Kapya	Çankaya	B	27.08.2012	-
86	ÇÇ-C2708	Charleston	Çankaya	B	27.08.2012	-
87	ÇÇ-B2708	Bell	Çankaya	B	27.08.2012	-
88	ÇÇ-V2708	Mazamort	Çankaya	B	27.08.2012	-
89	ÇM-G0509	Green	Çankaya	A	05.09.2012	-
90	ÇM-R0509	Kapya	Çankaya	A	05.09.2012	-
91	ÇM-C0509	Charleston	Çankaya	A	05.09.2012	-
92	ÇM-B0509	Bell	Çankaya	A	05.09.2012	-
93	ÇM-V0509	Mazamort	Çankaya	A	05.09.2012	-
94	ÇP-G0509	Green	Çankaya	C	05.09.2012	-
95	ÇP-R0509	Kapya	Çankaya	C	05.09.2012	-
96	ÇP-C0509	Charleston	Çankaya	C	05.09.2012	-
97	ÇP-B0509	Bell	Çankaya	C	05.09.2012	-

Table A.1 Analysed material, date, place and results (continued).

No	Sample Code	Pepper Samples	District in Ankara	Purchased Place	Date of Purchase	Result
98	ÇP-V0509	Mazamort	Çankaya	C	05.09.2012	-
99	ÇÇ-G0509	Green	Çankaya	B	05.09.2012	-
100	ÇÇ-R0509	Kapya	Çankaya	B	05.09.2012	-
101	ÇÇ-C0509	Charleston	Çankaya	B	05.09.2012	-
102	ÇÇ-B0509	Bell	Çankaya	B	05.09.2012	-
103	ÇÇ-V0509	Mazamort	Çankaya	B	05.09.2012	-
104	YM-G0509	Green	Yenimahalle	E	05.09.2012	-
105	YM-R0509	Kapya	Yenimahalle	E	05.09.2012	-
106	YM-C0509	Charleston	Yenimahalle	E	05.09.2012	-
107	YM-B0509	Bell	Yenimahalle	E	05.09.2012	-
108	YM-V0509	Mazamort	Yenimahalle	E	05.09.2012	-
109	YK-G0509	Green	Yenimahalle	F	05.09.2012	-
110	YK-R0509	Kapya	Yenimahalle	F	05.09.2012	-
111	YK-C0509	Charleston	Yenimahalle	F	05.09.2012	-
112	YK-B0509	Bell	Yenimahalle	F	05.09.2012	-
113	YK-V0509	Mazamort	Yenimahalle	F	05.09.2012	-
114	YH-G0509	Green	Yenimahalle	G	05.09.2012	-
115	YH-R0509	Kapya	Yenimahalle	G	05.09.2012	-
116	YH-C0509	Charleston	Yenimahalle	G	05.09.2012	-
117	YH-B0509	Bell	Yenimahalle	G	05.09.2012	-
118	YH-V0509	Mazamort	Yenimahalle	G	05.09.2012	-
119	KEP-G1009	Green	Keçiören	M	10.09.2012	-
120	KEP-R1009	Kapya	Keçiören	M	10.09.2012	-
121	KEP-B1009	Bell	Keçiören	M	10.09.2012	-
122	KEP-V1009	Mazamort	Keçiören	M	10.09.2012	-

Table A.1 Analysed material, date, place and results (continued).

No	Sample Code	Pepper Samples	District in Ankara	Purchased Place	Date of Purchase	Result
123	YP-G1009	Green	Yenimahalle	H	10.09.2012	-
124	YP-R1009	Kapya	Yenimahalle	H	10.09.2012	-
125	YP-B1009	Bell	Yenimahalle	H	10.09.2012	-
126	YP-V1009	Mazamort	Yenimahalle	H	10.09.2012	-
127	KA-G1009	Green	Keçiören	I	10.09.2012	-
128	KA-R1009	Kapya	Keçiören	I	10.09.2012	-
129	KA-C1009	Charleston	Keçiören	I	10.09.2012	-
130	KA-B1009	Bell	Keçiören	I	10.09.2012	-
131	KA-K1009	Mazamort	Keçiören	I	10.09.2012	-
132	KS-G1009	Green	Keçiören	K	10.09.2012	-
133	KS-R1009	Kapya	Keçiören	K	10.09.2012	-
134	KS-C1009	Charleston	Keçiören	K	10.09.2012	-
135	KS-B1009	Bell	Keçiören	K	10.09.2012	-
136	KS-V1009	Mazamort	Keçiören	K	10.09.2012	-
137	KR-G1009	Green	Keçiören	L	10.09.2012	-
138	KR-R1009	Kapya	Keçiören	L	10.09.2012	-
139	KR-C1009	Charleston	Keçiören	L	10.09.2012	-
140	KR-B1009	Bell	Keçiören	L	10.09.2012	-
141	KR-V1009	Mazamort	Keçiören	L	10.09.2012	-
142	ÇEP-G2409	Green	Çankaya	D	24.09.2012	-
143	ÇEP-R2409	Kapya	Çankaya	D	24.09.2012	-
144	ÇEP-B2409	Bell	Çankaya	D	24.09.2012	-
145	ÇEP-V2409	Mazamort	Çankaya	D	24.09.2012	-
146	KEP-G2409	Green	Keçiören	M	24.09.2012	-
147	KEP-R2409	Kapya	Keçiören	M	24.09.2012	-

Table A.1 Analysed material, date, place and results (continued).

No	Sample Code	Pepper Samples	District in Ankara	Purchased Place	Date of Purchase	Result
148	KEP-B2409	Bell	Keçiören	M	24.09.2012	-
149	KEP-V2409	Mazamort	Keçiören	M	24.09.2012	-
150	YM-G2409	Green	Yenimahalle	E	24.09.2012	-
151	YM-R2409	Kapya	Yenimahalle	E	24.09.2012	-
152	YM-C2409	Charleston	Yenimahalle	E	24.09.2012	-
153	YM-B2409	Bell	Yenimahalle	E	24.09.2012	-
154	YM-V2409	Mazamort	Yenimahalle	E	24.09.2012	-
155	YK-G2409	Green	Yenimahalle	F	24.09.2012	-
156	YK-R2409	Kapya	Yenimahalle	F	24.09.2012	-
157	YK-C2409	Charleston	Yenimahalle	F	24.09.2012	-
158	YK-B2409	Bell	Yenimahalle	F	24.09.2012	-
159	YK-V2409	Mazamort	Yenimahalle	F	24.09.2012	-
160	YH-G2409	Green	Yenimahalle	G	24.09.2012	-
161	YH-R2409	Kapya	Yenimahalle	G	24.09.2012	-
162	YH-C2409	Charleston	Yenimahalle	G	24.09.2012	-
163	YH-B2409	Bell	Yenimahalle	G	24.09.2012	-
164	YH-V2409	Mazamort	Yenimahalle	G	24.09.2012	-
165	ÇM-G2409	Green	Çankaya	A	24.09.2012	-
166	ÇM-R2409	Kapya	Çankaya	A	24.09.2012	-
167	ÇM-C2409	Charleston	Çankaya	A	24.09.2012	-
168	ÇM-B2409	Bell	Çankaya	A	24.09.2012	-
169	ÇM-V2409	Mazamort	Çankaya	A	24.09.2012	-
170	ÇP-G2409	Green	Çankaya	C	24.09.2012	-
171	ÇP-R2409	Kapya	Çankaya	C	24.09.2012	-
172	ÇP-C2409	Charleston	Çankaya	C	24.09.2012	-

Table A.1 Analysed material, date, place and results (continued).

No	Sample Code	Pepper Samples	District in Ankara	Purchased Place	Date of Purchase	Result
173	ÇP-B2409	Bell	Çankaya	C	24.09.2012	-
174	ÇP-V2409	Mazamort	Çankaya	C	24.09.2012	-
175	ÇÇ-G2409	Green	Çankaya	B	24.09.2012	-
176	ÇÇ-R2409	Kapya	Çankaya	B	24.09.2012	-
177	ÇÇ-C2409	Charleston	Çankaya	B	24.09.2012	-
178	ÇÇ-B2409	Bell	Çankaya	B	24.09.2012	-
179	ÇÇ-V2409	Mazamort	Çankaya	B	24.09.2012	-
180	YP-G0110	Green	Yenimahalle	H	01.10.2012	-
181	YP-R0110	Kapya	Yenimahalle	H	01.10.2012	-
182	YP-B0110	Bell	Yenimahalle	H	01.10.2012	-
183	YP-V0110	Mazamort	Yenimahalle	H	01.10.2012	-
184	YM-G0110	Green	Yenimahalle	E	01.10.2012	-
185	YM-R0110	Kapya	Yenimahalle	E	01.10.2012	-
186	YM-C0110	Charleston	Yenimahalle	E	01.10.2012	-
187	YM-B0110	Bell	Yenimahalle	E	01.10.2012	-
188	YM-V0110	Mazamort	Yenimahalle	E	01.10.2012	-
189	YK-G0110	Green	Yenimahalle	F	01.10.2012	-
190	YK-R0110	Kapya	Yenimahalle	F	01.10.2012	-
191	YK-C0110	Charleston	Yenimahalle	F	01.10.2012	-
192	YK-B0110	Bell	Yenimahalle	F	01.10.2012	-
193	YK-V0110	Mazamort	Yenimahalle	F	01.10.2012	-
194	YH-G0110	Green	Yenimahalle	G	01.10.2012	-
195	YH-R0110	Kapya	Yenimahalle	G	01.10.2012	-
196	YH-C0110	Charleston	Yenimahalle	G	01.10.2012	-
197	YH-B0110	Bell	Yenimahalle	G	01.10.2012	-

Table A.1 Analysed material, date, place and results (continued).

No	Sample Code	Pepper Samples	District in Ankara	Purchased Place	Date of Purchase	Result
198	YH-V0110	Mazamort	Yenimahalle	G	01.10.2012	-
199	KEP-G0110	Green	Keçiören	M	01.10.2012	-
200	KEP-R0110	Kapya	Keçiören	M	01.10.2012	+
201	KEP-B0110	Bell	Keçiören	M	01.10.2012	-
202	KEP-V0110	Mazamort	Keçiören	M	01.10.2012	-
203	KA-G0110	Green	Keçiören	I	01.10.2012	-
204	KA-R0110	Kapya	Keçiören	I	01.10.2012	-
205	KA-C0110	Charleston	Keçiören	I	01.10.2012	-
206	KA-B0110	Bell	Keçiören	I	01.10.2012	-
207	KA-V0110	Mazamort	Keçiören	I	01.10.2012	-
208	KS-G0110	Green	Keçiören	K	01.10.2012	-
209	KS-R0110	Kapya	Keçiören	K	01.10.2012	-
210	KS-C0110	Charleston	Keçiören	K	01.10.2012	-
211	KS-B0110	Bell	Keçiören	K	01.10.2012	-
212	KS-V0110	Mazamort	Keçiören	K	01.10.2012	-
213	KR-G0110	Green	Keçiören	L	01.10.2012	-
214	KR-R0110	Kapya	Keçiören	L	01.10.2012	-
215	KR-C0110	Charleston	Keçiören	L	01.10.2012	-
216	KR-B0110	Bell	Keçiören	L	01.10.2012	-
217	KR-V0110	Mazamort	Keçiören	L	01.10.2012	-
218	YM-G1110	Green	Yenimahalle	E	11.10.2012	-
219	YM-R1110	Kapya	Yenimahalle	E	11.10.2012	-
220	YM-C1110	Charleston	Yenimahalle	E	11.10.2012	-
221	YM-B1110	Bell	Yenimahalle	E	11.10.2012	-
222	YM-V1110	Mazamort	Yenimahalle	E	11.10.2012	-

Table A.1 Analysed material, date, place and results (continued).

No	Sample Code	Pepper Samples	District in Ankara	Purchased Place	Date of Purchase	Result
223	ÇP-G1110	Green	Çankaya	C	11.10.2012	-
224	ÇP-R1110	Kapya	Çankaya	C	11.10.2012	-
225	ÇP-C1110	Charleston	Çankaya	C	11.10.2012	-
226	ÇP-B1110	Bell	Çankaya	C	11.10.2012	-
227	ÇP-V1110	Mazamort	Çankaya	C	11.10.2012	-
228	ÇÇ-G1110	Green	Çankaya	B	11.10.2012	-
229	ÇÇ-R1110	Kapya	Çankaya	B	11.10.2012	-
230	ÇÇ-C1110	Charleston	Çankaya	B	11.10.2012	-
231	ÇÇ-B1110	Bell	Çankaya	B	11.10.2012	-
232	ÇÇ-V1110	Mazamort	Çankaya	B	11.10.2012	-
233	KEP-G1110	Green	Keçiören	M	11.10.2012	-
234	KEP-R1110	Kapya	Keçiören	M	11.10.2012	-
235	KEP-B1110	Bell	Keçiören	M	11.10.2012	-
236	KEP-V1110	Mazamort	Keçiören	M	11.10.2012	-
237	YP-G1110	Green	Yenimahalle	H	11.10.2012	-
238	YP-R1110	Kapya	Yenimahalle	H	11.10.2012	-
239	YP-B1110	Bell	Yenimahalle	H	11.10.2012	-
240	YP-V1110	Mazamort	Yenimahalle	H	11.10.2012	-
241	KA-G1410	Green	Keçiören	I	14.10.2012	-
242	KA-R1410	Kapya	Keçiören	I	14.10.2012	-
243	KA-C1410	Charleston	Keçiören	I	14.10.2012	-
244	KA-B1410	Bell	Keçiören	I	14.10.2012	-
245	KA-V1410	Mazamort	Keçiören	I	14.10.2012	-
246	KS-G1410	Green	Keçiören	K	14.10.2012	-
247	KS-R1410	Kapya	Keçiören	K	14.10.2012	-

Table A.1 Analysed material, date, place and results (continued).

No	Sample Code	Pepper Samples	District in Ankara	Purchased Place	Date of Purchase	Result
248	KS-C1410	Charleston	Keçiören	K	14.10.2012	-
249	KS-B1410	Bell	Keçiören	K	14.10.2012	-
250	KS-V1410	Mazamort	Keçiören	K	14.10.2012	-
251	KR-G1410	Green	Keçiören	L	14.10.2012	-
252	KR-R1410	Kapya	Keçiören	L	14.10.2012	-
253	KR-C1410	Charleston	Keçiören	L	14.10.2012	-
254	KR-B1410	Bell	Keçiören	L	14.10.2012	-
255	KR-V1410	Mazamort	Keçiören	L	14.10.2012	-

APPENDIX B

ANALYSED PEPPER SAMPLES PHOTOS

Figure B.1 Analysed Pepper Samples Photos

APPENDIX C

COMPOSITION OF BUFFERS AND SOLUTIONS

Table C.1 Composition of Buffers and Solutions used.

0,25 N HCl Solution

Formula	mL
5 N HCl	12.5
Sterile dH_2O	247.5

0,5 M EDTA, pH 8

Formula	
EDTA	93,05 g
Sterile dH_2O	450 mL
NaOH	12 g

pH was adjusted 8.0 by using 12g NaOH and the solution was autoclaved (121^oC/15 min).

0,5 N NaOH Solution

Formula	mL
5 N NaOH	25
Sterile dH_2O	225

1 M Tris-HCl, pH 8

Formula

Trizma-base 24.22 g
Sterile dH_2O 200 mL

pH was adjusted by using 5 M HCl and the solution was autoclaved (121^{o}C/15 min).

10X Tris-Borat-EDTA (TBE) Stock Solution

Formula

(0.9 M Trizma-base, 0.9 M Boric acid, 0.02 M EDTA)
Tris-Base 108 g
$Na_2EDTA.2H_2O$ 9.3 g
Boric acid 55 g

Solution was prepared in 1000 mL distilled water and sterilized in autoclave at 121^{o}C for 15 min.

20 % SDS Solution

Formula

SDS 2 g
Sterile dH2O 10 mL

Cell Lysis Buffer Solution

Formula

1 M Tris-HCl, pH 8 25 mL
0,5 M EDTA, pH 8 50 mL
Sarcosyl 5 g
Sterile dH_2O 425 mL
Proteinase K (20 mg/mL) 2.5 mL

Cell Suspension Buffer Solution

Formula

(100 mM Tris-HCl, 100 mM EDTA, pH 8)	
1 M Tris-HCl, pH 8	10 mL
0.5 M EDTA, pH 8	20 mL
Sterile dH2O	70 mL

Solution sterilized in autoclave at 121^{o}C for 15 min.

Seakem Agarose (1 %)-SDS

Formula

Seakem Agarose	0.25 g
Tris-EDTA solution (TE)	23.5 mL
20 % SDS solution	1.25 mL

Seakem agarose was molten in TE solution in microwave. Then, in the waterbath (Indem Tesisat ve Ticaret A.Ş., Ankara), it was cooled down to 50^{o}C and mixed with 1.25 mL of pre-warmed (50^{o}C) 20% SDS solution.

Seakem Agarose (1%)-TBE

Formula

Seakem Agarose	1 g
0.5X TBE solution	100 mL

Seakem agarose was molten in 0,5X TBE solution in microwave and cooled down to 50^{o}C in waterbath (Indem Tesisat ve Ticaret A.Ş., Ankara) before poring into the gel tray.

Tris-EDTA (TE) Buffer Solution

Formula	
(10 mM Tris-HCl, 1 mM EDTA, pH 8)	
1 M Tris-HCl, pH 8	10 mL
0,5 M EDTA, pH 8	2 mL
Sterile dH2O	988 mL

Solution sterilized in autoclave at 121^{o}C for 15 min.

APPENDIX D

COMPOSITION OF MEDIAS

Table C.1 Composition of Medias used.

Xylose Lysine Desoxcholate (XLD) Agar, (Oxoid Ltd., UK - CM0469)

Typical Formula	**gm/litre**
Yeast extract	3.0
L-Lysine	5.0
Xylose	3.75
Lactose	7.5
Sucrose	7.5
Sodium deoxycholate	1.0
Sodium chloride	5.0
Sodium thiosulfate	6.8
Ferric ammonium citrate	0.8
Phenol red	0.08
Agar	12.5

pH 7.4 ± 0.2 @ 25°C

53 g of the medium was suspended in 1 litre of distilled water. The medium was boiled but it was not overheat. The solution then ransferred immediately to a water bath at 50°C. When the medium was cooled, it was poured into sterile Petri dishes.

Brillant Green Agar (Modified) - (BGA), (Oxoid Ltd., UK -CM0329)

Typical Formula	**gm/litre**
`Lab-Lemco' powder	5.0
Peptone	10.0
Yeast extract	3.0
Disodium hydrogen phosphate	1.0
Sodium dihydrogen phosphate	0.6
Lactose	10.0
Sucrose	10.0
Phenol red	0.09
Brilliant green	0.0047
Agar	12.0

pH 6.9 ± 0.2 @ 25°C

52 g medium was suspended in 1 litre of distilled water. It was boiled then cooled to 50°C in a waterbath then poured to the plates.

Rappaport-Vassiliadis Soy Broth, (Oxoid Ltd., UK -CM0866)

Typical Formula	**gm/litre**
Soya peptone	4.5
Sodium chloride	7.2
Potassium dihydrogen phosphate	1.26
Di-potassium hydrogen phosphate	0.18
Magnesium chloride (anhydrous)	13.58
Malachite green	0.036

pH 5.2 ± 0.2 @ 25°C

26.75 g medium was suspended in 1 litre of distilled water and heated gently to dissolve. The solution was dispensed 10ml volumes into tubes and sterilised by autoclaving at 115°C for 15 minutes.

Brain Heart Infusion Broth, (Oxoid Ltd., UK -CM1135)

Formula	**gm/litre**
Brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Glucose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5

pH 7.4 ± 0.2 @ 25°C

37 g medium was dissolved in 1 litre of distilled water. Mixed well and distributed into final containers. Sterilized by autoclaving at 121°C for 15 minutes.

Xylose Lysine Desoxcholate (XLD) Agar, (Oxoid Ltd., UK)

Typical Formula	**gm/litre**
Enzymatic digest of casein	10.0
Sodium chloride	5.0
Disodium hydrogen phosphate (anhydrous)	3.5
Potassium dihydrogen phosphate	1.5

pH 7.0 ± 0.2 @ 25°C

20 g of Buffered Peptone Water (ISO) was added to 1 litre of distilled water. Mixed well sterilised by autoclaving at 121°C for 15 minutes.

Brain Heart Infusion Agar, (Oxoid Ltd., UK)

Formula	**gm/litre**
Brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Sodium chloride	5.0
Glucose	2.0
Disodium phosphate	2.5
Agar Bacteriological, OXOID UK (LP0011)	15.0
pH 7.4 ± 0.2 @ 25°C	

37 g BHI Broth with 15 g Agar Bacteriological were dissolved in 1 litre of distilled water. Mixed well and sterilized by autoclaving at 121°C for 15 minutes.

Tryptone Soy AGAR

Formula	**gm/litre**
Agar Bacteriological, OXOID UK (LP0011)	15g
Tryptone Soy Broth, (Lab M Ltd., UK)	30g

30g TS Broth with 15g Agar Bacteriological were dissolved in 1 litre of distilled water. Mixed well and sterilized by autoclaving at 121°C for 15 minutes.

Tryptone Soy Broth, (Lab M Ltd., UK)

Formula	**gm/litre**
Tryptone (casein digest U.S.P)	17.0
Soy Peptone	3.0
Sodium Chloride	5.0
Dipotassium hydrogen phosphate	2.5
Dextrose	2.5

APPENDIX E

HOUSEKEEPING GENE SEQUENCES OF *S.* Enteritidis

Table E.1 Housekeeping Gene Sequences *S.* Enteritidis: MET_S1_411

MET_S1_411 (ST 11)
S1_*aroC*_411: (501 b.p) – *aroC*5
GTTTTTCGTCCGGGACACGCGGATTACACCTATGAGCAGAAATACGGCC TGCGCGATTAC CGTGGCGGTGGACGTTCTTCCGCGCGTGAAACCGCGATGCGCGTAGCGG CAGGGGCGATC GCCAAGAAATACCTGGCGGAAAAGTTCGGCATCGAAATCCGCGGCTGC CTGACCCAGATG GGCGATATTCCGCTGGAGATTAAAGACTGGCGTCAGGTTGAGCTTAATC CGTTCTTTTGT CCCGATGCGGACAAACTTGACGCGCTGGACGAACTGATGCGCGCGCTG AAAAAAGAGGGC GACTCCATCGGCGCGAAAGTGACGGTGATGGCGAGCGGCGTGCCGGCA GGGCTTGGCGAA CCGGTTTTTGACCGACTGGATGCGGACATCGCCCATGCGCTGATGAGCA TCAATGCGGTG AAAGGCGTGGAGATCGGCGAAGGATTTAACGTGGTGGCGCTGCGCGGC AGCCAGAATCGC GATGAAATCACGGCGCAGGGT

Table E.1 Housekeeping Gene Sequences *S.* Enteritidis: MET_S1_411 (continued).

MET_S1_411 (ST 11)
S1_*dnaN*_411: (501 b.p) – *dnaN*2
ATGGAGATGGTCGCGCGCGTTACGCTTTCTCAGCCGCATGAGCCGGGCG CCACTACCGTG CCGGCGCGGAAATTCTTTGATATCTGCCGCGGCCTGCCGGAGGGCGCGG AGATTGCCGTT CAGTTGGAAGGCGATCGGATGCTGGTGCGTTCTGGCCGTAGCCGCTTCT CGCTGTCTACG CTGCCTGCCGCCGATTTCCCGAATCTTGACGACTGGCAAAGCGAAGTTG AATTTACGCTG CCGCAGGCCACGATGAAGCGCCTGATTGAAGCGACCCAGTTTTCGATGG CTCATCAGGAT GTGCGCTACTACTTAAACGGTATGCTGTTTGAAACGGAAGGTAGCGAAC TGCGCACTGTC GCGACCGACGGCCACCGCCTGGCGGTGTGCTCAATGCCGCTGGAAGCG TCTTTACCCAGC CACTCGGTGATTGTGCCGCGTAAAGGCGTGATTGAACTGATGCGTATGC TCGACGGCGGT GAAAACCCGCTGCGCGTGCAG

Table E.1 Housekeeping Gene Sequences *S.* Enteritidis: MET_S1_411 (continued)

MET_S1_411 (ST 11)

S1_*hemD*_411: (432 b.p) - *hemD*3

GCGACACTGACGGAAAACGATCTGGTTTTTGCCCTTTCACAGCACGCCG
TCGCCTTTGCT
CACGCCCAGCTCCAGCGGGATGGCCGAAACTGGCCTGCGTCGCCGCGCT
ATTTCGCGATT
GGCCGCACCACGGCGCTCGCCCTTCATACCGTTAGCGGGTTCGATATTC
GTTATCCATTG
GATCGGGAAATCAGCGAAGCCTTGCTACAATTACCTGAATTACAAAATA
TTGCGGGCAAA
CGCGCGCTGATTTTGCGTGGCAATGGCGGCCGCGAACTGCTGGGCGAA
ACCCTGACAGCT
CGCGGAGCCGAAGTCAGTTTTTGTGAATGTTATCAACGATGTGCGAAAC
ATTACGATGGC
GCGGAAGAAGCGATGCGCTGGCATACTCGCGGCGTAACAACGCTTGTT
GTTACCAGCGGC
GAGATGTTGCAA

S1_*hisD*_411: (501 b.p) - *hisD*7

ATTGCGGGATGTCAGAACGTGGTTCTGTGCTCGCCGCCGCCCATCGCTG
ATGAAATCCTC
TATGCGGCGCAACTGTGTGGCGTGCAGGAAATCTTTAACGTCGGCGGCG
CGCAGGCGATT
GCCGCTCTGGCCTTCGGCAGCGAGTCCGTACCGAAAGTGGATAAAATTT
TTGGCCCCGGC
AACGCCTTTGTAACCGAAGCCAAACGTCAGGTCAGCCAACGCCTCGAC
GGCGCGGCTATC
GATATGCCAGCCGGGCCGTCTGAAGTACTGGTGATCGCCGACAGCGGC
GCAACACCGGAT
TTCGTCGCTTCTGACCTGCTCTCCCAGGCTGAGCACGGTCCGGATTCGC
AGGTGATTCTG
CTGACGCCTGATGCTGACATTGCCTGCAAGGTGGCGGAGGCGGTAGAA
CGTCAACTGGCA
GAACTGCCGCGCGCGGACACCGCCAGGCAGGCCCTGAGCGCCAGTCGT
CTGATTGTGACC
AAAGATTTAGCGCAGTGCGTC

Table E.1 Housekeeping Gene Sequences *S.* Enteritidis: MET_S1_411 (continued)

MET_S1_411 (ST 11)

S1_*purE*_411: (399 b.p) - *purE* 6

AGCGACTGGGCTACCATGCAATTCGCCGCCGAAATTTTTGAAATTCTGG
ATGTCCCGCAC
CATGTAGAAGTGGTTTCCGCCCATCGCACCCCCGATAAACTGTTCAGCT
TCGCCGAAACG
GCGGAAGAGAACGGATATCAAGTGATTATTGCCGGCGCGGGCGGCGCG
GCGCACCTGCCG
GGAATGATTGCGGCAAAAACGCTGGTCCCGGTACTCGGCGTGCCGGTA
CAAAGCGCTGCG
CTAAGCGGCGTGGATAGCCTCTACTCCATTGTGCAGATGCCGCGCGGCA
TTCCGGTGGGT
ACGCTGGCGATCGGTAAAGCCGGTGCCGCTAACGCCGCCCTGCTCGCCG
CGCAGATTCTG
GCGCAACACGACGCGGAACTGCATCAGCGCATTGCCGAC

S1_*sucA*_411: (501 b.p) - *sucA*6

AAACGCTTCCTGAACGAACTGACCGCCGCTGAAGGGCTGGAACGTTATC
TGGGTGCCAAA
TTCCCGGGTGCGAAACGTTTCTCGCTCGAGGGGGGAGATGCGCTGATAC
CCATGCTGAAA
GAGATGGTTCGCCATGCGGGTAACAGCGGCACTCGCGAAGTGGTGCTG
GGGATGGCGCAC
CGCGGTCGCCTGAACGTGCTGATCAACGTACTGGGTAAAAAACCGCAG
GATCTGTTCGAC
GAATTTGCCGGTAAGCATAAAGAACATCTGGGTACCGGCGACGTGAAG
TATCACATGGGC
TTCTCGTCAGATATCGAAACCGAAGGCGGTCTGGTTCACCTGGCGCTGG
CGTTTAACCCA
TCGCATCTGGAAATTGTGAGCCCGGTGGTGATGGGCTCCGTGCGCGCCC
GTCTGGACAGA
CTGGACGAACCGAGCAGCAACAAAGTGTTGCCGATCACTATTCACGGC
GACGCCGCGGTG
ACCGGCCAGGGCGTGGTTCAG

Table E.1 Housekeeping Gene Sequences S. Enteritidis: MET_S1_411 (continued)

MET_S1_411 (ST 11)
S1_*thrA*_411: (501 b.p) - THRA11
GTGCTGGGCCGTAATGGTTCCGACTATTCCGCCGCCGTGCTGGCCGCCT GTTTACGCGCT GACTGCTGTGAAATCTGGACTGACGTCGATGGCGTGTATACCTGTGACC CGCGCCAGGTG CCGGACGCCAGGCTGCTGAAATCGATGTCCTACCAGGAAGCGATGGAA CTCTCTTACTTC GGCGCCAAAGTTCTTCACCCTCGCACCATTACGCCCATCGCCCAGTTCC AGATCCCCTGT CTGATTAAAAATACCGGTAATCCGCAGGCGCCAGGAACGCTGATCGGC GCGTCCAGCGAC GATGATAACCTGCCGGTTAAAGGGATCTCTAACCTTAACAACATGGCGA TGTTTAGCGTC TCCGGCCCGGGAATGAAAGGGATGATTGGGATGGCGGCGCGTGTTTTCG CCGCCATGTCT CGCGCCGGGATCTCGGTGGTGCTCATTACCCAGTCCTCCTCTGAGTACA GCATCAGCTTC TGTGTGCCGCAGAGTGACTGC

APPENDIX F

VIABILITY LOSS OF *SALMONELLA* STRAIN

Table F.1 Viability loss of *Salmonella* strain by pressurization, 400 MPa, 3 min and 25°C

	Log10 cfu/ml			
Samples	*Salmonella* Solution	Positive Control	Negative Control	After Pressurization
Kapya pepper	8.10	7.20	ND	2.1
Charleston	8.10	6.90	ND	1.1
Mazamort pepper	8.10	7.25	ND	1.3
Bell Pepper	8.10	7.33	ND	2.4
Green Pepper	8.10	7.40	ND	1.1

**ND, cfu was not detected in 0.1 ml of suspension from tested samples*

Table F.2 Viability loss of *Salmonella* strain by pressurization, 420 MPa, 10 min and 25°C

	Log10 cfu/ml			
Samples	*Salmonella* Solution	Positive Control	Negative Control	After Pressurization
Kapya pepper	8.80	7.39	ND	ND
Charleston	8.80	7.10	ND	ND
Green Pepper	8.80	7.76	ND	ND

**ND, cfu was not detected in 0,1 ml of suspension from tested samples*

Table F.3 Viability loss of *Salmonella* strain by pressurization, 500 MPa, 5 min and 25°C.

	Log10 cfu/ml			
Samples	*Salmonella* Solution	Positive Control	Negative Control	After Pressurization
Mazamort pepper	8.80	7.45	ND	ND
Bell Pepper	8.80	7.66	ND	ND

**ND, cfu was not detected in 0.1 ml of suspension from tested samples*