SALMONELLA SURVEILLANCE ON FRESH PRODUCE AND INACTIVATION BY HIGH HYDROSTATIC PRESSURE

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submitted by **ELİF GÜNEL** in partial fulfillment of the requirements for the degree of **Master of Science in Food Engineering Department**, **Middle East Technical University** by,

Prof. Dr. Canan Özgen
Dean, Graduate School of **Natural and Applied Sciences** _____

Prof. Dr. Alev Bayındırlı
Head of Department, **Food Engineering** _____

Assist. Prof. Dr. Yeşim Soyer
Supervisor, **Food Engineering Dept., METU** _____

Prof. Dr. Hami Alpas
Co-Supervisor, **Food Engineering Dept., METU** _____

Examining Committee Members:

Prof. Dr. Hami Alpas
Food Engineering Dept., METU _____

Assist. Prof. Dr. Yeşim Soyer
Food Engineering Dept., METU _____

Assoc. Prof. Dr. Deniz Çekmecelioğlu
Food Engineering Dept., METU _____

Assist. Prof. Dr. İlkay Şensoy
Food Engineering Dept., METU _____

Assist. Prof. Dr. Dilek Avşaroğlu
Agricultural Bio-technology Dept., Ahi Evran University _____

Date: 11.02.2014

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

Name, Last name: Elif Günel

Signature :

ABSTRACT

SALMONELLA SURVEILLANCE ON FRESH PRODUCE AND INACTIVATION BY HIGH HYDROSTATIC PRESSURE

Günel, Elif
M.Sc., Food Engineering Department
Supervisor: Assist. Prof. Dr. Yeşim Soyer
Co-Supervisor: Prof. Dr. Hami Alpas

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In this study, 248 fresh produce samples including tomato, parsley and lettuce (i.e. iceberg and greenleaf lettuce) were collected from supermarkets and local bazaars in Ankara for investigating the presence of *Salmonella*. *Salmonella* was detected in 1.2% (3/248) of samples by conventional culturing method with molecular confirmation conducted through polymerase chain reaction (PCR). For further characterization of isolates, serotyping, multilocus sequence typing (MLST) of seven housekeeping genes (*aroC*, *thrA*, *purE*, *sucA*, *hisD*, *hemD* and *dnaN*) and pulsed-field gel electrophoresis (PFGE) were performed. *S. enterica subsp. enterica* serotypes Anatum, Charity and Mikawasima were isolated from two parsley samples and one lettuce sample respectively. MLST resulted in 3 sequence types (STs) for our isolates, including one novel ST for serotype Mikawasima. Similarly, PFGE revealed three different *XbaI* PFGE patterns.

The effect of high hydrostatic pressure (HHP) treatment on the viability of *Salmonella* isolates, artificially inoculated onto fresh produce samples which they were isolated from, was evaluated at 500 MPa for 5 min at 25^{o}C. *Salmonella* was not detected in any of the HHP treated samples. Shelf life analysis (7 days at 25^{o}C and 4^{o}C) revealed growth only for serotype Anatum which was stored at 25^{o}C, thus indicating the presence of injured cells after HHP treatment.

The results of this survey, obtained by the most common subtyping methods (i.e. serotyping, MLST and PFGE) worldwide, contributes to the development of national database in Turkey, which is essential for investigating evolutionary pathways, geographical distribution and genetic diversity of *Salmonella* strains. In addition, in this study HHP treatment, as an alternative technique to heat processing, represents an applicable tool for control of *Salmonella* in fresh produce.

Keywords: *Salmonella*, fresh produce, pulsed-field gel electrophoresis, multilocus sequence typing, high hydrostatic pressure

ÖZ

TAZE SEBZELERDE *SALMONELLA* İNCELENMESİ VE YÜKSEK HİDROSTATİK BASINÇ İLE İNAKTİVASYON

Günel, Elif
Yüksek Lisans, Gıda Mühendisliği Bölümü
Tez Yöneticisi: Yard. Doç. Dr. Yeşim Soyer
Ortak Tez Yöneticisi: Prof. Dr. Hami Alpas

Şubat 2014, 108 sayfa

Bu çalışmada domates, maydonoz ve marul (iceberg ve kıvırcık marul) içeren 248 taze sebze örneği *Salmonella* tayini amacıyla Ankara'nın süpermarket ve pazarlarından toplanmıştır. Klasik kültürel yöntem ile analiz edilen ve moleküler olarak Polimeraz Zincir Reaksiyonu ile doğrulanan örneklerin %1.2'sinde (3/248) *Salmonella* saptanmıştır. *Salmonella* pozitif izolatların ileri düzeyde tanımlanması amacıyla serotiplendirme ile yedi referans genin (*aroC, thrA, purE, sucA, hisD, hemD* ve *dnaN*) çoklu lokus dizilim analizi (MLST) ve vuruşlu alan jel elektroforezi (PFGE) yöntemleri uygulanmıştır. *Salmonella enterica subsp. enterica* serotipleri Anatum, Charity ve Mikawasima sırasıyla iki adet maydonoz ve bir adet iceberg maruldan izole edilmiştir. MLST sonucunda izolatlarımız için, Mikawasima serotipi için yeni olmak üzere, 3 farklı sekans tipi bulunmuştur. Benzer şekilde PFGE yöntemi, *XbaI* enzimi kullanılarak üç farklı PFGE modeli açığa çıkarmıştır.

Yüksek hidrostatik basınç uygulamasının, izole edildikleri taze sebze örneklerine yapay olarak ekimi yapılmış *Salmonella* izolatlarının canlılıkları üzerine etkisi, 500 MPa, 5 dakika ve 25^{o}C'de değerlendirilmiştir. Yüksek basınç uygulanan örneklerde *Salmonella* saptanmamıştır. Raf ömrü analizi (7 gün, 25^{o}C ve 4^{o}C) sonucunda yalnızca 25^{o}C'de saklanan Anatum serotipi için gelişme olduğu görülmüş ve bu durum yüksek basınç uygulaması sonrasında zedelenmiş hücrelerin varlığını göstermiştir.

Bu araştırma sonucunda, dünyada en yaygın uygulanan alt tiplendirme yöntemleri olan serotiplendirme, MLST ve PFGE ile elde edilen sonuçlar ile, *Salmonella* suşlarının evrimsel süreçlerini, coğrafik dağılımlarını ve genetik çeşitliliklerini araştırmak açısından gerekli olan ulusal bir veri ağının Türkiye'de geliştirilmesine katkıda bulunacaktır. Ayrıca ısıl işleme alternatif olan yüksek hidrostatik basınç uygulaması, bu çalışmada taze sebzelerde *Salmonella* kontrolüne yönelik uygulanabilir bir yöntemi temsil etmektedir.

Anahtar kelimeler: *Salmonella*, taze sebze, vuruşlu alan jel elektroforezi, çoklu lokus dizilim analizi, yüksek hidrostatik basınç uygulaması

To my beloved family

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INTRODUCTION

1.1 The Genus *Salmonella*

Salmonella is an important pathogen for both humans and animals. As having widespread distribution and being isolated from a wide range of sources; raw meats, poultry, poultry products, raw milk, pasteurized milk, and ready-to-eat vegetables, it has a significant role in the global food chain. It can cause mild to severe illnesses, sometimes leading to life-threatening cases and deaths. *Salmonella* has caused some outbreaks, involving large numbers of people, with high rates of morbidity and mortality in the past and it is likely to continue to be one of the major food safety concerns for public and health authorities.

1.1.1 General characteristics

Salmonella, the genus of the family Enterobacteriaceae, is Gram-negative, non-spore forming, facultatively anaerobic and generally motile bacilli which may cause diseases in both human and animals. Species of *Salmonella* live in the intestinal tract of birds, reptiles and mammals and can be transmitted from their feces (Bauman, 2007).

Some biochemical characteristics of salmonellae are summarized in Table 1.1. They are able to grow at temperatures ranging between 4 and 48^{o} C (Anderson et

al., 2001), in pH 4-9 and above 0,94 water activity (aw) conditions (Hanes, 2003). Best growth of *Salmonella* is at moderate temperatures (35-37^{o}C) and pH around neutrality. They have not resistance to high salt concentrations. As being heat sensitive, there is no need to high temperatures to kill them, especially in foods with a high water activity (e.g. ≥ 0.98).

Table 1.1 Some biochemical characteristics of salmonellae (Bell et al., 2000)

Characteristic	**Usual reaction**
Catalase	+
Oxidase	-
Acid produced from lactose	-
Indole	-
Urease produced	-
Methyl Red	-
Voges-Proskauer	-
Lysine decarboxylase	+

However, in foods with a low water activity, inactivation is achieved through higher temperatures (Bell et al., 2000). Cooking the egg thoroughly above 70^{o}C (CDC), pasteurization of milk (71.7^{o}C, 15 seconds) and fruit juices (70-74^{o}C, ≤20 seconds) provides total elimination of *Salmonella* (Gray et al., 1990).

1.1.2 Nomenclature and taxonomy

The taxonomy of *Salmonella* is a complex and still evolving system. The genus was first discovered by an American bacteriologist D. E. Salmon in 1884 and called initially as *Bacillus choleraesuis* (Jay et al., 2005). Ligniéres changed the name of the organism as *Salmonella choleraesuis* in 1900. Subsequently new isolates were studied and identified based on differentiation of the antigens on the

cell surface. The first Kauffmann-White scheme, including 44 serotypes, was published in 1934. Kauffmann's proposal was standing on the assumption that each serovar was a different species. In 1973, DNA relatedness studies demonstrated that all *Salmonella* strains were closely related, thus classified as subgroups belonged to single species. Interpretation of the seven subgenera as species (subspecies I, II, IIIa, IIIb, IV, V and VI) was proposed by Le Minor & Popoff in 1987. Afterwards *S.bongori*, previously known as subspecies V, was approved as second species due to different DNA-DNA hybridization.
Following the request of Le Minor & Popoff , the name *Salmonella choleraesuis* changed as *Salmonella enterica* due to avoid misunderstanding between the species and the serovar already named as *Salmonella choleraesuis* subsp. *choleraesuis*.

According to current nomenclatural system used by Centers Disease Control and Prevention (CDC), *Salmonella* consists of two species; *S. enterica* and *S. bongori*. *S. enterica* is divided into six subspecies distinguished by different biochemical characteristics (Fig 1.1). The six subspecies are *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*. The number of *Salmonella* serotypes identified is above 2600 (Dieckmann et al., 2011). The majority (%59) of the serotypes is included within *S. enterica* subsp. *enterica* (Brenner et al., 2000).

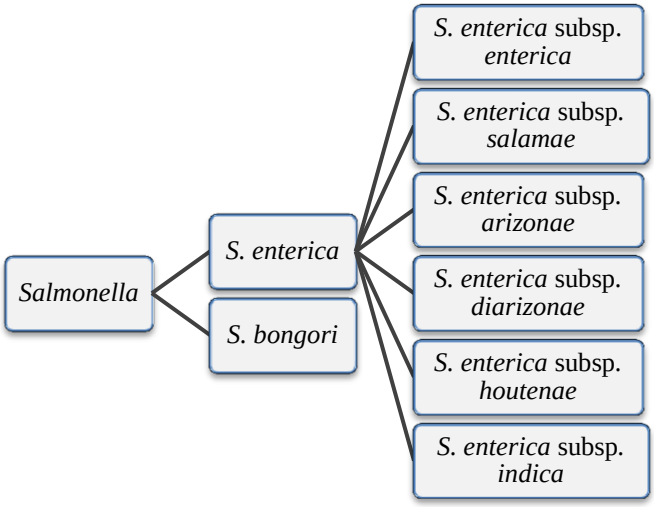

Figure 1.1 The species and subspecies of *Salmonella*

Throughout the years, there have been various proposals and controversies for the taxonomy of *Salmonella*. However, the current nomenclatural CDC system gained wide acceptance through scientists, health officials and public. This system is based on the concept that *Salmonella* includes two subspecies as *S. enterica* and *S. bongori* as mentioned above. The subspecies are referred to by a Roman numeral and name (I, *S.enterica* subsp. *enterica*; II, *S.enterica* subsp. *salamae*; IIIa, *S.enterica* subsp. *arizonae;* IIIb, *S.enterica* subsp. *diarizonae*; IV, *S.enterica* subsp. *houtenae*; and VI, *S.enterica* subsp. *indica*).

General method used for *Salmonella* differentiation beyond the level of subspecies is serotyping. *Salmonella* serotypes have been defined based on the variability of somatic (O) antigens, flagellar (H) antigens and capsular (Vi) antigens (Uzzau *et al.*, 2000). They are classified by antigenic analysis according to the Kauffman-White scheme. The somatic O antigen is crucial for serological differentiation. *Salmonella enterica* subsp. *enterica* includes over 2500 serotypes. These serotypes are classified within 67 different O-antigen groups. Although

each group was initiated to be named by letters, the current classification continues numerically as there is not enough letter.

The H antigens of *Salmonella* have been encoded by two types of genes, *fliC* and *fljB*. *fliC* gene, which is present in all *Salmonella* serotypes, expresses the phase 1 H antigen, whereas *fljB* gene expresses the phase 2 H antigen only in *S. enterica* subspecies I, II, IIIb, and VI. In rare cases, some *Salmonella* serotypes express a capsuler antigen, which is described as Vi antigen. These uncommon R phase was first reported for Typhi by Kauffman (Grimont et al., 2007).

In the Kauffmann-White scheme, 114 H antigen types have been identified (McQuiston et al., 2011). Antigenic formula of some *Salmonella* serotypes according to the Kauffman-White scheme are given in Table 1.2.

Table 1.2 Antigenic formula of some *Salmonella* serotypes (Grimont et al., 2007)

Group	Serotype	Somatic (O) antigen	Flagellar (H) antigens	
			Phase 1	Phase 2
O:2 (A)	Paratyphi A	1,2,12	a	[1,5]
O:4 (B)	Typhimurium	1,4,[5],12	i	1,2
O:7 (C_1)	Choleraesuis	6,7	c	1,5
O:8 (C_2-C_3)	Kentucky	8,20	i	z_6
O:9 ($D_{1)}$	Miyazaki	9,12	l,z_{13}	1,7

1.1.3 Salmonellosis

Salmonellosis is a foodborne disease caused by *Salmonella*. It is estimated that each year 80.3 million non-typhoidal foodborne salmonellosis caused by non-Typhi seroypes occur globally (Majowicz et al., 2010). According to data collected by CDC between 2009 and 2010, salmonellosis was the second most

commonly reported infection with 30% of outbreaks and also caused the most outbreak-related hospitalizations (49%) (CDC, 2014). Although *Salmonella* has been recovered from nearly all types of foods, the primary sources of salmonellosis are of animal origin, especially meat and poultry products and also animal feces contaminated foods.

Salmonella is generally transmitted through consumption of contaminated food or water. Following consumption, it passes through the stomach and enter the small intestine. The organisms attach to the surface of intestinal cells with their fimbriae, start to colonize and subsequently invade the intestinal tissues. This invasion causes destruction of the intestinal mucosa and inflammation (gastroenteritis) (Bell, 2010). Most healthy people recover within few days without specific treatment. However, host adapted organisms can pass through the blood stream and/or the lymphatic system and cause more severe illnesses.
The majority of the serotypes that cause diseases in human and other warm-blooded animals, are included in *S. enterica* subsp. I . Despite the high genetic similarity, *S. enterica* serotypes have significant differences in host range and the types of the disease. Uzzau et al. (2001) classified the serotypes into three groups as host restricted, host adapted and un-restricted. Host restricted serotypes are associated with diseases only in single host species; for example Typhi, Paratyphi A,B,C and Sendai in humans, Gallinarum in poultry and Abortusequi in the mare. Serotypes which are generally isolated from single host species but can also infect other host species are host adapted; for example Dublin which is associated with cattle, can rarely cause diseases in pigs and humans. Furthermore, un-restricted serotypes such as Typhimirium and Enteridis are both disease agents for a variety of animals such as cattle, poultry, sheep, pigs, horses and wild rodents and also dominant serotypes in human salmonellosis.

Based on clinical syndromes on human, *Salmonella* serotypes are grouped in two types; typhoid and non-typhoid *Salmonella*. *S. enterica* subsp. *enterica* serotypes Typhi and Paratyphi are typhoid serotypes causing enteric fever whereas the remaining strains are specified as non-typhoid which have broad disease

spectrum. Enteric fever also known typhoid fever is defined with fever, malaise, abdominal pain and constipation. Non-typhoid salmonellosis generally results in self-limited gastroenteritis, however bacteremia and focal extraintestinal infections can occur. Gastroenteritis is qualified by watery diarrhea, abdominal pain, nausea, and sometimes vomiting and fever. Bacteremia has been related with highly invasive serotypes such as *Cholerasuis* or *Dublin* (Portillo, 2010). It is characterized by prolonged fever and positive blood culture and may result in life threatening results especially in adults (Li et al., 2012). Some characteristics of typhoid and non-typhoid *Salmonella* are summarized in Table 1.3.

Table 1.3 Comparison of typhoid and non-typhoid salmonellosis (Sánchez-Vargas et al., 2011)

Characteristic	**Typhoid *Salmonella***	**Non-typhoid *Salmonella***
Serotypes	*S.* Typhi *S.* Paratyphi	Remaining strains
Reservoir	Humans	Animals
Transmission	Predominantly water	Predominantly food
Location	Developing countries	Worldwide
Disease	Systemic	Local or systemic
HIV infection risk	No higher risk	Increased risk
Carrier rate	1-4%	<1%

1.2 Fresh produce

Fresh produce are major components helping the diet healthier and more nutritious due to providing essential vitamins, minerals, and fiber. They reduce the risk of cardiovascular diseases and cancer. According to the recommendation of World Health Organization (WHO) and Food and Agriculture Organization (FAO), the minimum daily intake of fruit and vegetables should be 400 g (WHO, 2003).

On the other hand, the increasing number of foodborne outbreaks associated with consumption of fresh fruits and vegetables lead to increasing concern about the safety of these foods. As there is no elimination step for elimination of pathogens due to consumption uncooked, fresh produce have a potential to be a source of illnesses.

1.2.1 Fresh produce production and consumption worldwide

In recent years, consumption of fresh produce has increased worldwide due to increased public awareness of the health benefits and nutritional values that fresh produce provide. There is an average 4.5% increase of global fruit and vegetable consumption between the years 1990 and 2004 (EU, 2007).

In response to the growing consumer demand, the production, distribution and trade of fresh produce have rised in developing countries over the past 30 years, especially in Asia (FAOSTAT, 2012). The global production per annum (p.a.) of fruit and vegetables increased by 94% from 1980 to 2004 (FAO/WHO, 2008). In Figure 1.2 and 1.3, the amounts of fruits and vegetables produced (2000-2010) worldwide are seen respectively.

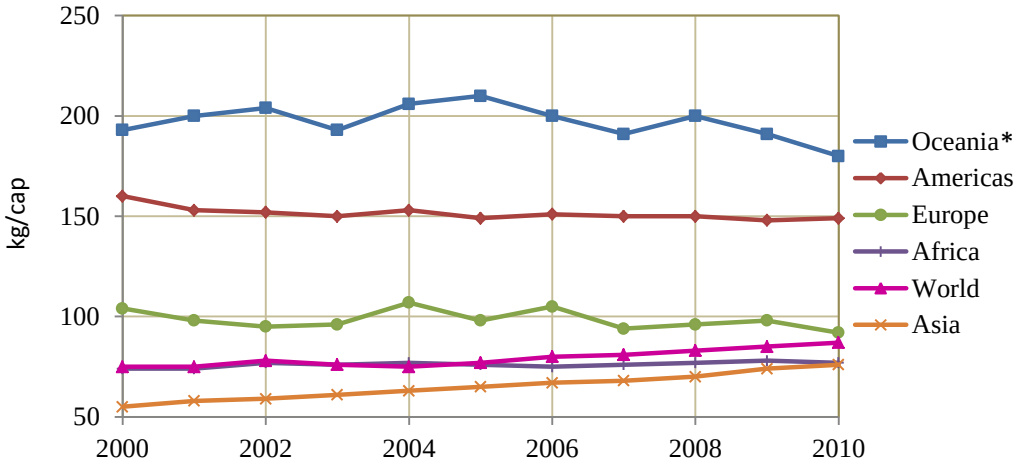

Figure 1.2 Per capita global fruit production between 2000-2010 (FAOSTAT, 2013) *Oceania including the islands of the Pacific Ocean and seas around them

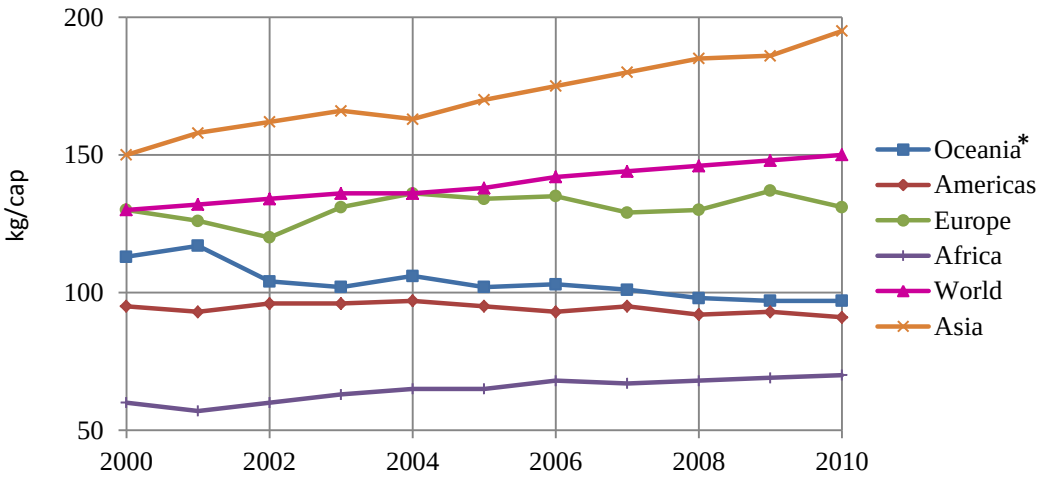

Figure 1.3 Per capita global vegetable production between 2000-2010 (FAOSTAT, 2013) *Oceania including the islands of the Pacific Ocean and seas around them.

Global fruit and vegetable production has grown at an annual rate of about 3 percent over the last decade (FAO, 2013). The lists of top 10 fresh fruit and vegetable producers are shown in Table 1.3 and Table 1.4.

Table 1.4 Top vegetable producers and their productivity (FAOSTAT, 2013)

	Area		Yield		Production			
	total	p.a. growth	total	p.a. growth	total		p.a. growth	
	thousand ha	%	thousand hg/ha	%	thousand tonnes	thousand tonnes	%	%
	2010	2000-10	2010	2000-10	2009	2010	1990-99	2000-10
China	23458	2.6	230	0.8	522686	539993	8.5	4.3
India	7256	2.9	138	0.5	90635	100405	4.0	3.3
U.S.	1120	-2.2	318	1.2	37289	35609	2.5	-1.0
Turkey	1090	0.9	238	-0.4	26702	25901	3.9	0.5
Iran	767	3.3	261	2.2	18421	19995	7.3	5.5
Egypt	775	2.2	251	0.5	21350	19487	4.9	2.7
Italy	537	-1.2	265	-0.3	15082	14201	0.8	-1.5
Russia	759	-1.4	175	2.0	14827	13283		0.6
Spain	348	-1.1	364	1.5	13457	12679	1.3	0.4
Mexico	681	0.4	184	1.6	11727	12515	5.3	2.0

Table 1.5 Top fruit producers and their productivity (FAOSTAT, 2013)

	Area		Yield		Production			
	total	p.a. growth	total	p.a. growth	total		p.a. growth	
	thousand ha	%	thousand hg/ha	%	thousand tonnes	thousand tonnes	%	%
	2010	2000-10	2010	2000-10	2009	2010	1990-99	2000-10
China	11316	2.2	108	2.7	115858	122350	12.6	6.7
India	6403	5.3	117	0.4	68975	75121	5.8	5.7
Brazil	2383	-0.0	163	0.5	37155	38793	2.3	0.5
U.S.	1145	-1.3	229	-1.0	27448	26181	0.4	-2.2
Italy	1277	-0.7	132	0.1	18364	16908	0.1	-0.6
Philippines	1228	2.7	132	1.4	15980	16182	2.2	4.1
Spain	1601	-1.3	97	0.9	14497	15456	1.5	-0.4
Mexico	1227	1.3	125	0.1	15890	15368	3.1	1.5
Indenosia	607	1.9	240	3.6	17577	14598	3.8	5.7
Turkey	1088	0.8	128	1.7	14223	13946	1.7	2.5

1.2.2 Fresh produce production in Turkey

As seen in Table 1.3 and 1.4, Turkey is a major world producer of fruits and vegetables. According to the latest forecasts of Turkish Statistical Institue (TurkStat) statistics, around 56% of Turkey's total vegetative production derives from the fruit and vegetables sector. The production of major fresh fruit and vegetables and the diversity of production is shown in Table 1.4.

According to the TurkStat, total fresh fruit and vegetable production has risen 4.9% in 2011, particularly due to significant increase in tomato production. Tomatoes have an important place in Turkish agricultural sector with the highest amounts of production and exportation among all fresh fruit and vegetables.

Table 1.6 The Production of Major Fresh Fruits & Vegetables in Turkey (1,000 tons) (TurkStat, 2012)

PRODUCTS	2010	2011	Annual Change %	Share 2011 (%)
Tomatoes	10,052	11,003	9,5	24,6
Watermelons&melons	5,294	5,512	4,1	12,3
Grapes	4,255	4,296	1,0	9,6
Apples	2,600	2,680	3,1	6,0
Onions, dry	1,900	2,141	12,7	4,8
Peppers	1,986	1,975	-0,6	4,4

The increase in production of tomatoes between 2010 and 2012 is seen in Figure 1.4.

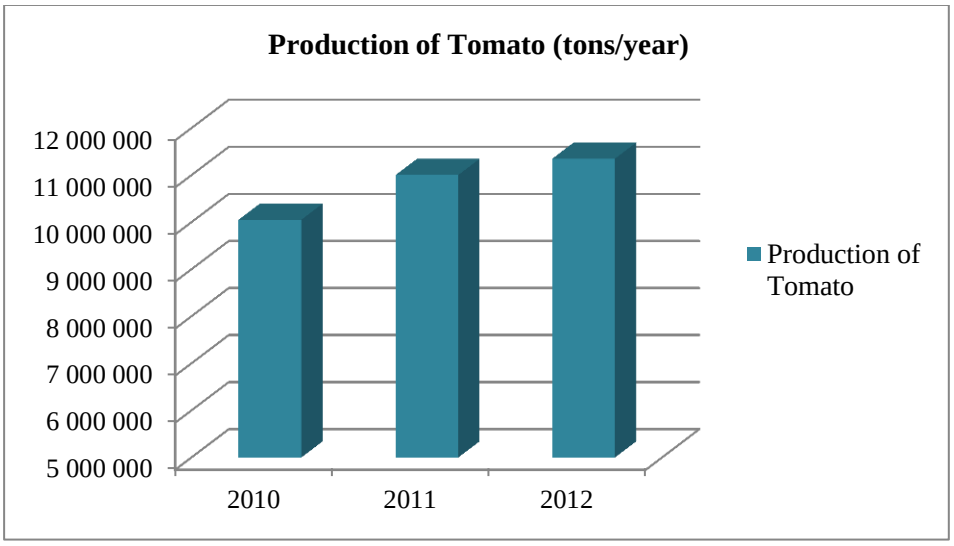

Figure 1.4 The amounts of tomato produce in Turkey between 2010-2012 (TurkStat, 2012)

1.2.3 Food Safety Concerns in Fresh Produce

Food safety of fresh produce is a continuing concern with increasing recalls and foodborne outbreaks linked to fresh produce. Bacterial pathogens, viruses, pesticide residues, mycotoxins are the major food safety concerns for fresh produce. According to the results of workshop organized in 2011, pathogens were considered as the most crucial food safety issue among the food safety experts from different organizations, institutions and companies, due to socio-economic and health effects (Boxstael et al., 2013).

European Commission's Rapid Alert System for Food and Feed (RASFF) is one of the basic information databases of food safety issues. In Table 1.7, it is seen that notifications due to bacterial pathogens share only 3.9% whereas notifications for pesticide residues share 39.2% of total notifications between the years 2008-2010. There is a significant increase in the share of RASFF

notifications linked to bacterial pathogens in 2011. This increase also indicates the importance of foodborne pathogens for fresh produce.

Table 1.7 Notifications to the European commission RASSF system for the categories 'fruits and vegetables' during the period 2008-2010 and 2011 ((Boxstael et al., 2013)

	Fruits and vegetables	
	2008-2010 (n=1338)	2011 (n=669)
Pesticide residues	39.2%	45.7%
Mycotoxins	18.5%	13.6%
Bacterial pathogens	3.9%	16.7%[a]
Additives	9.0%	3.9%
Hygiene/quality hazard	12.5%	7.8%
Physical hazards	4.9%	2.1%
Heavy metals	1.8%	1.3%
Viruses	1.0%	1.2%
Chemical hazard	4.9%	3.0%
Parasites	0.0%	0.0%
Unauthorized colour	0.0%	0.0%
Other	4.4%	4.6%
Total	**100.0%**	**100.0%**

[a]11.8% is linked to alerts on *Salmonella* in paan leaves; 4.9% is linked to other alerts such as VTEC in sprouted seeds, *Salmonella* in melons or Campylobacter in baby corn.

The increase of large foodborne outbreaks related to fresh produce, thus severe outcome, mortality and huge economic losses, generated increasing considerations about the foodborne pathogens. One of the recent large outbreaks, VTEC O104:H4 outbreak (Germany, 2011) created anxiety among public as a result of 908 hospitalizations, 50 death and 15 affected countries (EFSA, 2011). The outbreak was related most likely to consumption of fresh produce (i.e., fenugreek sprout through contaminated seeds), therefore concerns for microbial safety of fresh produce have arisen. Pathogens related to fresh produce of greatest current concern are *Salmonella* particularly on tomatoes, seed sprouts and spices, and *Escherichia coli* O157:H7 on leafy greens; spinach and lettuce (Olaimat et al.,2012).

1.2.4 *Salmonella* Outbreaks Related to Fresh Produce Worldwide

Although foodborne illnesses from *Salmonella* is generally linked to consumption of poultry and meat products, fresh produce are common vehicles for transmission (Sivapalasingam et al., 2004).

One of the largest *Salmonella* outbreaks, a multi-state *Salmonella* Saintpaul outbreak (2008) associated with the consumption of jalapeño peppers, occurred in 43 states in the U.S. and Canada, and caused 1442 cases (CDC, 2008). Furthermore, there are over 78 salmonellosis outbreaks linked to fresh produce reported by Centers for Disease Control and Prevention (CDC) in USA and Canada between the years 2006-2011 . Some recent outbreaks occured in the world are shown in Table 1.8.

Table 1.8 Some recent outbreaks associated to fresh produce worldwide (CDC/ECDC)

				Cases	
Serotype	**Year**	**Country**	**Fresh produce**	**Hospitalizations**	**Deaths**
Berta	2006	USA	tomatoes	4	0
Typhimurium	2006	USA	lettuce, unspecified; tomato, unspecified	4	0
Newport	2006	USA	tomato, unspecified	8	0
Oranienburg	2006	USA	fruit salad	7	0
Typhimurium	2006	USA	tomato, unspecified	24	0
Thompson	2006	USA	peanuts	3	0
Javiana	2006	USA	iceberg lettuce, unspecified	7	0
Typhimurium	2007	USA	lettuce, unspecified; spinach	4	0
Newport	2007	USA	tomato, beefsteak	11	0
Litchfield	2007	USA	cantaloupe	17	0
Braenderup	2008	USA	green salad; tomato, unspecified	5	0
Javiana	2008	USA	watermelon	31	0
Saintpaul	2008	USA	peppers, jalapeno; peppers, serrano; tomato, unspecified	308	2
Saintpaul	2009	USA	tomatoes	7	0
Typhimurium	2009	USA	alfalfa sprouts	2	0
Newport	2009	USA	lettuce; roast beef	6	0
Carrau	2009	USA	melon	4	1
Saintpaul	2010	USA	watermelon	11	0
Javiana	2010	USA	potato salad	5	0
Javiana	2010	USA	tomatoes	8	
I 4,[5],12:i:-	2010	USA	alfalfa sprouts	31	0
Muenchen	2011	USA	clover sprouts	4	0

Table 1.8 Some recent outbreaks associated to fresh produce worldwide (CDC/ECDC) (continued)

				Cases	
Serotype	**Year**	**Country**	**Fresh produce**	**Hospitalizations**	**Deaths**
Typhimurium	2011	USA	multiple salads	3	0
Saintpaul	2011	USA	cucumber; tomato, unspecified	2	0
Enteritidis	2011	USA	salad, unspecified	2	0
Typhimurium	2011	USA	watermelon	2	0
Hartford	2011	USA	lettuce; roast beef	5	0
Panama	2011	USA	cantaloupe	3	0
Agona	2011	USA	papaya	10	
Newport	2011	USA	tomatoes	3	0
Uganda	2011	USA	cantaloupe	4	0
Enteritidis	2011	USA	alfalfa sprouts	3	0
Enteritidis	2011	USA	Turkish pine nuts	2	0
Enteritidis	2011	UK, Germany	ready-to-eat sliced		1
Strathcona	2011	Denmark, Germany, Austria	tomato	40	
Braenderup	2012	USA	mango	33	0
Typhimurium Newport	2012	USA	cantaloupe	94	3

In EU countries excluding Spain, totally 37 salmonellosis outbreaks linked to consumption of food of non-animal origin including fresh produce have been reported between 2007 and 2011 (EFSA, 2013).

As seen in Table 1.5, different fresh fruits and vegetables can be the sources of salmonellosis outbreaks. However, *Salmonella* spp. are more frequently reported with sprouted seeds and leafy greens eaten raw as salads (EFSA,2013). The frequency may depend on the ability of *Salmonella* to attach or internalize into these produce items. Contaminated water which is used to irrigate and wash produce crops, and contaminated manure or animal wastes are common

environmental sources for transmission of the organisms into fresh produce (Olaimat et al., 2012).

1.3 Detection of *Salmonella*

The detection of *Salmonella* by regulatory agencies is basically performed with conventional cultural methods which may take up to 5 days to confirm the results (Bhagwat, 2006). However, a rapid pathogen detection method is required to identify source of pathogen during outbreak investigation. Advances in biotechnology have permitted more rapid identification and surveillance of pathogens (Feng, 1997). Polymerase chain reaction (PCR)-based methods have emerged as valuable tools for investigating foodborne outbreaks and identifying the responsible etiological agents.

1.3.1 *Salmonella* Detection by Polymerase Chain Reaction (PCR)

PCR is one of the most efficient analytical methods for confirming the identification of foodborne pathogens isolated from food. A single segment of DNA can be amplified several millionfold in few hours by PCR.

There are 3 major steps in PCR; denaturation of DNA template, primer annealing and extension of the annealed primers from the 3'-ends of both DNA strands by DNA polymerase activity (Fig 1). These 3 major steps are repeated generally for 30-40 times. This results in exponential amplification of the specific target DNA sequence.

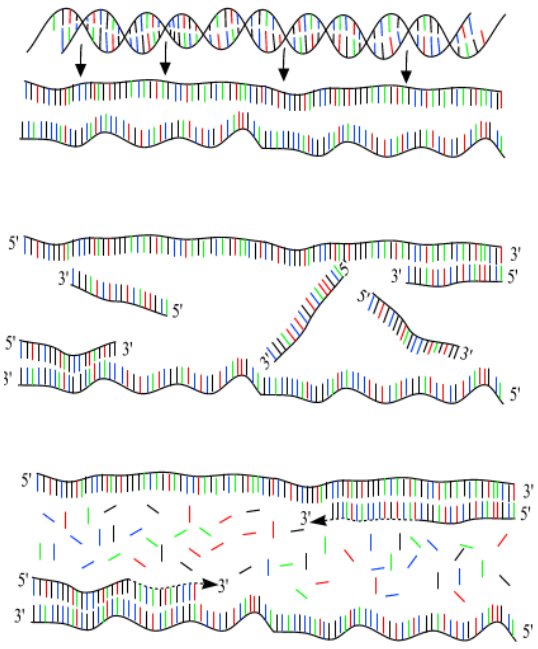

Fig. 1.5 Three major steps in PCR
(adapted from http://users.ugent.be/~avierstr/principles/pcr.html)

There are many virulence genes and virulence-enhancing genes identified for *Salmonella*. The current number of *Salmonella* specific genes that have been used for the polymerase chain reaction is over 30 (Levin, 2010). The researchers generally choose highly conserved genes as *invA* gene, *his* gene, fimbriae protein-encoding genes and also 16S rDNA genes for identification of *Salmonella* . Specific PCR primer pairs can be used to describe target genes that are particular to species or strain.

1.4 *Salmonella* Subtyping

Subtyping of *Salmonella* is essential for outbreak investigation, source identification, diagnosis, treatment and epidemiological surveillance of salmonellosis. Subtyping of baterial pathogens can be divided into two groups;

phenotypic and genotypic subtyping methods. The most common techniques used for subtyping of *Salmonella* are detailed.

1.4.1 Phenotypic methods for *Salmonella* subtyping

Most phenotypic methods have been in use for many decades for *Salmonella* subtyping such as serotyping, phage typing and antimicrobial susceptibilities. These methods are based on comparisons of phenotypic characteristics of bacterial strains.

1.4.1.1 Serotyping

Serotyping is a basic method used in epidemiological surveillance and outbreak investigations of *Salmonella*.The serotypes are determined based on the antigenic structure of three antigens which are present in the cell surface; somatic (O), capsular (Vi) and flagellar (H) antigens. The Kauffmann-White scheme, the list including antigenic formulae of all the *Salmonella* serotypes, is used for the designation of the serotypes (CDC, 2011).

The O-antigen is a polysaccharide which is built of repeating units of oligosaccharides containing 3-6 sugar units. The structure of O-antigens varies among strains of *Salmonella* due to difference in sugar composition, arrangement of sugar units, the linkeages between O subunits and modifications of side groups (Ellermeier, 2006). In the Kauffman-White scheme, there are currently over 60 serogroups defined on the basis of o-antigen structure (Grimont et al., 2007). For *S. enterica*, the enzymes required for the biosynthesis of O-antigens are encoded by the genes located in *rfb* gene cluster. There may be significant differences in composition and sequences of genes in *rfb gene* clusters of distinct *S.enterica* serogroups. For example, rhamnose synthesis is common in *S.enterica* serotypes and encoded by the genes *rfbA*, *rfbB*, *rfbC*, and *rfbD*. Despite the similarity of these genes in serogroups A, B, C2 and D1, the expression of *rfbD* gene differs considerably from that of other groups. The galactosyl transferase gene *rfbP* was

found and expressed in serogroups A, D1, E1, and C2, whereas in other strains from groups A, B, C2, C3, D1, D2, E1, E2, E3, E4, and 54, the gene was not present. The *rfb* genes of strains from the same serogroups were identified very similar or identical (Xiang et al., 1993).

Flagellar (H) antigens are heat-labile proteins found on the flagella of the motile *Salmonella* strains. They can be encoded by two different genes; *fliC and fljB.* The *fliC* gene is expressed in many motile enteric bacteria including *Salmonella* and *Escherichia*, while the *fljB* is unique to *Salmonella* enterica subspecies I, II, IIIb, and VI . Serotypes which express both flagellar antigens are termed as diphasic; those, including subspecies IIIa, IV, VII and *S. bongori*, with only one flagellar antigen type are monophasic. Serological differences between the flagellar antigens constitute the further part for classification according the Kauffman-White scheme.

S. Typhi, *S.* Paratyphi C and some strains of *S.* Dublin express a capsular polysaccharide called Vi antigen. The biosynthetic and export genes for Vi antigen are arranged in specific chromosal region called *viaB* locus. The *viaB* locus of *S.* Typhi consists of 10 genes; *tviA, tviB, tviC, tviD* and *tviE* for the synthesis of the capsule, and *vexA, vexB, vexC, vexD* and *vexE* for the export of capsule (Virlogeux et al., 1995).

Salmonella serotyping is conducted through a series of tests. Isolates are first characterized to the genus and species level. For identification of the subspecies, biochemical tests are applied. The O, H and Vi antigens are detected in independent agglutination assays using antisera that react with groups of related antigens or a single antigen. As the procedure requires testing with a complete set of antisera, it is time-consuming, expensive and technical expertise is needed to perform tests. Such drawbacks prompted the researchers to find alternative molecular methods for *Salmonella* identification. However, it is still an essential method for salmonellosis surveillance and outbreak detection, as both the virulence and host range of isolates can be serotype specific.

1.4.1.2 Phage typing

Salmonella serotypes can be subcharacterized by defining the sensitivities of bacterial strains against the group of bacteriophages. The method can be applied for common *Salmonella* serotypes such as as *S.* Typhimirium and *S.* Enteritidis. However it is an inexpensive, rapid and accurate method. Because of the necessity of maintenance of sets of typing phages, only some reference laboratories can perform phage typing. Technical experience is also essential for the interpretation of results. In addition, phage can change the phenotype of the bacterial cell. This modification is called lysogenic conversion. Change in the genome of the bacterial cell is one of the main disadvantages of phage typing (Cho et al., 2008). Despite some disadvantages, phage typing, especially when used in conjuction with other subtyping methods, is useful for characterization of *Salmonella* strains. (Barco et al., 2012).

1.4.1.3 Antimicrobial Resistance Typing

Antimicrobial susceptibility of *Salmonella* strains can differ due to different genetic characteristics. This method yields antimicrobial resistance patterns of *Salmonella* strains against the panel of antimicrobials, therefore provides subcharacterization. Besides being economical, there is no need to use specific equipment. However, use of appropraite procedure is very important for ensuring the uniform interpretation and repruducibility of the results.

Although some *Salmonella* strains are naturally resistant to certain types of antibiotics, some genetic mutations and gene transfer enable the susceptible strains to acquire antimicrobial resistance. As a consequence of these genetic mechanisms, distinct strains may develop similar resistance profile thus reducing the discriminating power. On the other hand, the isolates taken over a period of time from the same *Salmonella* strain may differ in resistance profiles for some specific antibiotics due to acquisition of resistance.

Comparison studies revealed that phenotypic subtyping methods have less discriminatory power for *Salmonella* than molecular methods such as multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE) and repetitive sequence-based PCR (Rep-PCR) (Foley et. al, 2006).

1.4.2 Genotypic subtyping methods for *Salmonella*

In addition to the phenotypic methods, many DNA-based genotyping methods can be used to discriminate *Salmonella* isolates beyond species and subspecies level. These molecular methods can be divided into three basic groups; DNA binding pattern, DNA sequencing and DNA hydridization-based methods. The methods in the first group differentiate the strains by seperating DNA fragments by size. These DNA fragments are yielded by PCR amplification or cleavage of the target DNA sequence by restriction enzymes. The methods in the second group are based on the sequencing of specific genomic DNA. The third group includes macroarray and microarray studies (Li et. al, 2009).

The genotypic methods which are commonly used by European surveillance and health agencies worldwide laboratories for *Salmonella* subtyping are specified below.

1.4.2.1 Pulsed Field Gel Electrophoresis (PFGE)

PFGE is generally referred as the "gold standard" due to discriminating ability of genetic differences and lineage among bacterial strains of the same species (Levin, 2010).

The method is principally based on the use of low-frequency restriction enzymes to generate large DNA fragments, and resolution of these fragments depending on their size in an agarose gel by using two alternating electric fields at reoriented directions. Large fragments elongate in the direction of the electric field. When the direction of electric field is changed, DNA fragment changes its

conformation and reorients, then linearizes in the direction of new electric field. Currently PFGE is conducted in an advanced system involving multiple electrodes located in a hexagonal array. The system, which is referred to as clamped homogenous electric field (CHEF), gives homologous electric fields with an angle of 120^{o}C (Levin, 2010).

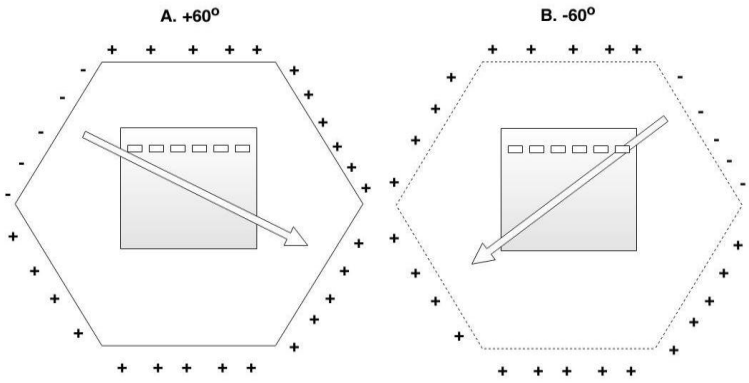

Figure 1.6. Homogeneous electric fields applied at +60^{o} and -60^{o} angle by CHEF Mapper system (Bio-Rad) (adapted from http://www.aesociety.org/areas/pfge.php)

In many of the epidemiological studies of *Salmonella*, PFGE has been the method of choice for subtyping. In salmonellosis outbreak occured in 1998, PFGE was used to confirm that the genetically indistinguishable strain of *S.* Javiana was transmitted from restaurant food handlers to leftover food and customers, who were epidemiologically related to the outbreak (Lee et al., 1998). Five distinct serotypes of *S. enterica* were involved in a large outbreak associated to fresh tomatoes served at gas station deli counters in Pennsylvania and neighboring states in 2004. *S.* Anatum, one of the serotypes, was isolated from both tomatoes and patients. PFGE patterns revealed that the *S.* Anatum strains isolated from tomatoes were identical to ones isolated from patients. Furthermore, among 146

S. Javiana isolates, the main cause of the outbreak, 132 of them have identical PFGE patterns. PFGE provided differentiation of outbreak-related isolates of serotoypes epidemically linked the outbreak, from unrelated sporadic isolates (Sandt et al., 2006).

PFGE offers many advantages such as interpretation of the entire bacterial genome in a single gel, high discriminiation, reproducibility, typability and high degree of standardization (Adley, 2006). These advantages facilitate the widespread and consistent use of the method. Surveillance laboratories all over the world apply standardized PFGE protocols described on PulseNet (Swaminathan et al., 2006; Soyer et al., 2010). However, PFGE has also some disadvantages. High level of technical expertise and labour is necessary to carry out the procedure. In addition, typeability may not be excellent for some serotypes because of DNA degradation. An outbreak of *S. enterica* serotype Panama, transmitted by contaminated breast milk, was reported in Taiwan (Chen et al., 2005). As *S. enterica* serotype Panama possessed intracellular DNase activity that degraded the genomic DNA, PFGE was not the chosen method for discrimination. Besides ribotyping was performed and revealed identical patterns for the isolates collected from the infant's blood, cerebral spinal fluid and from the mother's breast milk.

However, DNA degradation can be prevented by addition of thiourea to electrophoresis gel buffer (Silbert et al., 2003). In some cases, PFGE has been recognized as too discriminatory due to revealing distinct PFGE patterns for isolates that show recent common ancestry (Soyer et al., 2010). This result is undesirable for the source attribution studies. On the other hand, discrimination power of PFGE may not be enough for some strains with highly similar genetic backgrounds, such as *S.* Enteritidis (Boxrud et al., 2007), since it may reveal identical PFGE patterns for epidemiologically unrelated isolates.

Differences in PFGE banding patterns depend on the size and shape of resolved bands. Tenover et al. (1995) proposed a criteria for interpretation of PFGE data

by considering the genetic mechanisms that cause the alteration of PFGE patterns of DNA. This alteration may occur owing to a point mutation or a frame shift mutation involving the insertion or deletion of one or more nucleotides. When there is such a random genetic event , PFGE pattern of an isolate differs from the reference strain generally by two to three bands, therefore they can be interpreted to be closely related. An isolate and the reference strain are considered as possibly related if there are four to six band differences. Moreover, if the PFGE patterns of an isolate and the reference strain differ by seven or more bands, it indicates that they are unrelated.

1.4.2.2 Multiple-locus variable-number tandem repeat analysis (MLVA)

Variable number tandem repeats (VNTR) are repeated DNA sequences which have common dispersion in the genetic malterial of an organism. VNTRs can differ in copy numbers in the genome. The method is based on determination of size polymorphisms in multiple variable number tandem repeats (VNTR) loci which is amplified by PCR (Li et al., 2009). When compared to PFGE, MLVA seems to have significant benefits for surveillance of *Salmonella.* Because it is cheaper and needs less time and labour than PFGE. Furthermore, MLVA is carried out through complete automation.. It provides data that can be easily analysed and shared between laboratories. This method usually yields a higher degree of discrimination than PFGE (Torpdahl et al., 2007). On the other hand, as each organism generally requires different MLVA assay for discrimination, application can be limited for *Salmonella* strains due to high variability of serotypes of this genus (Ross et al., 2011). MLVA assays for *Salmonella* were only developed for few serotypes, thus further research is needed for protocol development for different *Salmonella* serotypes.

One of the main concerns that may limit the utility of MLVA is the uncertainty about the stability of the targeted loci examined. Lindstedt (2005) demonstrated that the tandem repeats may evolve very rapidly, thus can lead to yield unreliable

results in an investigation of genetic relationships among *Salmonella* strains. Although there have been some concerns, MLVA has been an useful tool for determining the potential sources of human diseases particularly when it is used in conjuction with PFGE (Best et al., 2007).

1.4.2.3 Ribotyping

Ribotyping subcharacterizes microorganisms by using rRNA-based probes that target conserved regions of rRNA genes. There are flanking regions of rRNA operon that leads to variations in ribotypes (Bauchet et al., 2008). Conventional ribotyping is performed respectively on the basis of extraction of bacterial genomic DNA, DNA-cutting with restriction enzymes and hybridization of probes and targeted region of rRNA. The restriction fragment length polymorphism provides determination of differences between targeted DNA regions of the strains. Conventional ribotyping is time-consuming and needs high level of technical expertise. The main limitation for utility of conventional ribotyping is difficulty in comparing the results between different laboratories because any change in the procedure applied can affect the results (Pavlic and Griffiths, 2009). Conventional ribotyping is generally not an useful tool for surveillance and source attribution studies when differentiation of a large number of *Salmonella* strains is required . An automated protocol (RiboPrinter™) was developed to prevent these limitations (Clark et al., 2003). Automated ribotyping provides subtyping of *Salmonella* isolates without a need of highly technical expertise. It also obtains results that can be easily standardized between different laboratories (Clark et al., 2003). However, automated system for ribotyping is expensive, thus limiting the common use of the method.

1.4.2.4 Multi-locus Sequence Typing (MLST)

MLST discriminates the isolates of bacterial species based on identifying the sequences of multiple genetic loci which is located in seven housekeeping genes. Seven housekeeping genes are highly conserved genes and crucial for microbial metabolism due to essential functions of the proteins they encode (Li et al., 2009). The principle of the method basically consists of PCR amplification and DNA sequencing of seven housekeeping genes. MLST has many advantages such as reproducibility, high typeability power and reliability. The main advantage of MLST is that allelic profiles of *Salmonella* can readily be compared to those in a MLST database via internet. Sequence data can be interpreted clearly by different laboratories. For each of seven housekeeping genes, the different sequences are represented as alleles and the alleles at the loci give an allelic profile. Strains can be identified according to these allelic profiles .Since differences in sequences are determined depending on single nucleotide base changes, high quality of sequencing data is very important to make reliable comparison (Foley et al., 2009).

A number of previous studies demonstrated that the discriminatory power of MLST was not adequate when it was applied for subcharacterization of the isolates of the same serotype serovar (Alcaine et al., 2006; Torpdahl et al., 2005; Foley et al., 2006). Low discrimatory power of MLST limits the use of this method particularly for source attribution studies.

1.5 *Salmonella* Surveillance Studies in Turkey

Salmonella serotypes are common causes of foodborne diseases in Turkey. There is limited information on foodborne dieseases, since there is not an active national foodborne pathogen surveillance system that collect and obtain information on isolates in Turkey. Having not a *Salmonella* reference center, the only sources to reach the data are the research papers and publications. In 2013, Toreci et al. reviewed all the publications, libraries and research articles in Turkey about *Salmonella* isolation studies. They gathered all the data and prepared the list of *Salmonella* serotypes isolated from Turkey up to the end of 2011. According to this review, a total of 129 different *Salmonella* serotypes have been isolated from Turkey. Among these serotypes, 53 of them were collected from humans, 38 from humans and non-human samples, and 38 from non-human samples (Toreci et al., 2013). It is mentioned that *S.* Istanbul (Özek et al., 1969) and *S.* Adana (Ewing, 1986) were the only serotypes that were isolated firstly in Turkey.

1.6 Novel Approach in Food-Processing: High Hydrostatic Pressure (HHP) Treatment

Hydrostatic pressure is a novel food processing technique that has been recognized in food industry for pasteurization of foods without undesirable effects of heat treatment. Conventional heat treatment often affects structural and sensory characteristics of foods. However, HHP can eliminate pathogenic bacteria in foods and retain quality and freshness of foods.

Effect of HHP on quality and shelf life of some food samples was firstly examined in 1899 by Hite. The study showed that HHP enhanced shelf life of the foods (i.e. milk, fruit and other foods). However, applicability of HHP in the food industry is much more recent and has been considerably developed in the past two decades (Considine et al., 2008; Devlieghere et al., 2004). Industrial

application of HHP is currently widespread for a range of pressures between 100 and 800 MPa, for a variety of foods (e.g. milk, fish, seafood, fruit juices) . In 2010, a total annual production of more than 200,000 tonnes of HPP-treated products was reported in the food industry, with the approximate distribution shown in Figure 1.6. (Ortega-Rivas, 2012).

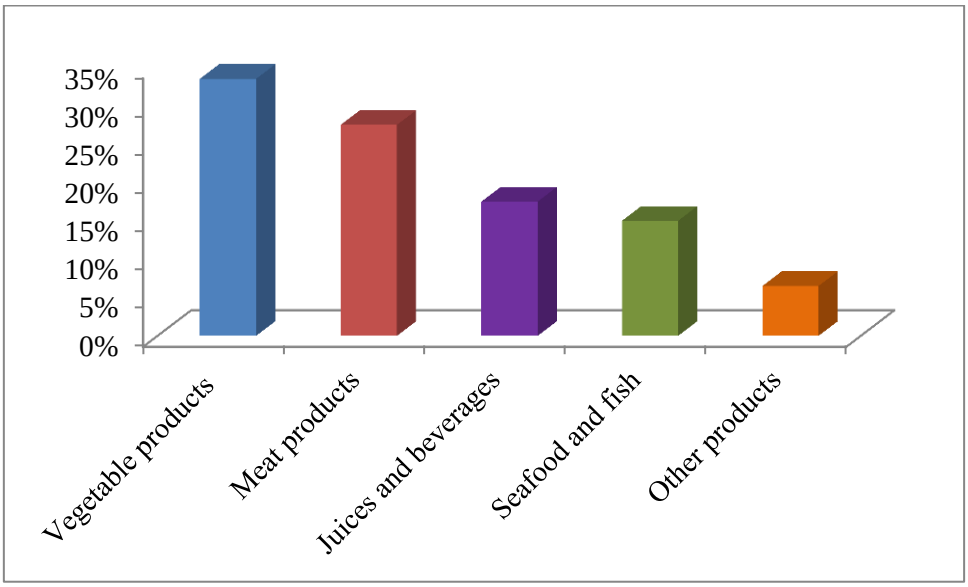

Figure 1.7. Utilization of HHP processing preservation on different segments of the food industry (Ortega-Rivas, 2012)

Some advantages of HHP treatment on foods are reduced processing time, minimal heat damage problems, well-retained freshness, flavor, texture and color and no loss of vitamin C (Cheftel, 1995; Farr, 1990; Knorr, 1995).

1.6.1 Mechanism of HHP

HHP process is isostatic and principally applied to food through instantaneous and uniform transmission (independent of size, shape and the composition of the food). Temperature changes slightly with increasing pressure (approximately 3^{o}C

per 100 Mpa) (Smelt, 1998). Based on Le Chatelier's principle, any phenomenon in food systems, accompanied by a decrease in volume, can be enhanced by pressure. The principle of HHP is illustrated in Figure 1.7.

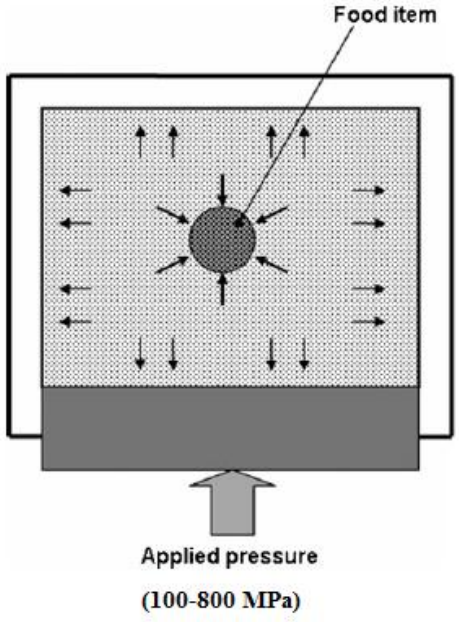

Figure 1.8. The principle of isostatic pressure (adapted from Ortega-Rivas, 2012)

1.6.2 Microbial inactivation by HHP treatment

When foods are exposed to extremely high pressures, microorganisms are eliminated in the same way as when heat treatment is performed. HHP treatment affects the cellular structure of the microorganisms. It inactivates bacterial cells by hampering the basic cellular functions essential for reproduction and survival. HHP can destruct cell membranes of the microorganisms, thus hampering the transport of nutrients and wastes. Vital cellular processes change if crucial enzymes are inhibited or selective permeability of the membrane is diminished

(Wang et al., 2013). Microbial cells are killed when multiple parts of a cell are destructed. When damage in cell is too much, cells can not repair themselves and cell death occurs. Occasionally the injured cells can repair themselves where the posttreatment conditions facilitate the recovery (Follonier et al., 2012).

The parameters that are affecting sensitivity of microorganism by pressurizing are magnitude of pressure, pressurization time and temperature, type of microorganism, antimicrobial substances, such as bacteriocins and lysozyme, pH, cell growth phase and the characteristics of the suspending media. (Alpas and Bozoglu, 2000a). Gram negative bacteria and the cells in the exponential growth phase are determined to be the less sensitive than Gram positive bacteria and the cells in stationary phase (Cheftel 1995; Mackey et. al. 1995). Pressure resistance of the bacteria also vary between the strains of a specific species at moderate temperatures, however as the temperature rises up to 50°C, the resistance factor becomes ineffective (Alpas et. al. 1999).

1.6.2.1 Inactivation of *Salmonella* by HHP

Several studies have been carried out to investigate the efficiency of HHP treatment on *Salmonella* in both food and the media. As it is shown in Table 1.9, the HHP efficiency differs depending on the serotype of *Salmonella*, pressurization time and temperature, the magnitude of pressure and the substrate which the organism is pressurized in. Some serotypes may be pressure-resistant while others are susceptible to HHP.

Table 1.9 Viability loss of S*almonella* strains by HHP with different time, temperature and pressure combinations

Serotype	Substrate	P (MPa)	Time (min)	T (^{o}C)	Inactivation	Reference
Senftenberg	Strained baby food	340	10	23	<2 log cfu/g	Metrick et. al., 1989
Enteritidis	Broth	345	10	35	8 log cfu/ml	Alpas et al., 2000
Enteritidis	Broth	550	10	25	8 log cfu/ml	Lee et al., 2010
Newport Javiana Braenderup Anatum	Broth	550	2	20	8 log cfu/ml	Maitland et al., 2011
Braenderup	Diced tomato	550	2	20	3,7 log cfu/g	Maitland et al., 2011
Baildon	Orange juice	300	2	6	0.4 log/ml	Whitney et al., 2007
Saintpaul	Jalapeño peppers Serrano peppers	500	2	20	3,5 log cfu/g 5,1 log cfu/g	Neeto et al., 2012

Table 1.9 Viability loss of S*almonella* strains by HHP with different time, temperature and pressure combinations (continued)

Serotype	Substrate	P (MPa)	Time (min)	T (^{o}C)	Inactivation	Reference
Enteritidis	Dry-cured ham	600	5	12	4.3 log cfu/g	Alba et al., 2011
Enteritidis	Green onion	450	2	20	3,5 log cfu/g	Neeto et al., 2011
Enterica	Spanish potato omelette	600	5 8	21	5.9 log cfu/g 6.5 log cfu/g	Toledo et al., 2012

1.7 Aim of the study

As being one of the world's largest producer and exporter of fresh produce, Turkey needs a strong surveillance system for early detection of potential foodborne outbreaks. However surveillance studies are inadequate and there is no national network or database for foodborne pathogens in Turkey. In the first part of this study, it was aimed to determine the prevalence of *Salmonella* in fresh produce and to find out phenotypic and genotypic diversity of the isolates. Serotyping, MLST and PFGE, the most commonly applied subtyping methods for *Salmonella* in the world, were chosen as for their potential applicability for foodborne outbreak investigations in Turkey.

The objective of the second part of this study was to investigate the effectiveness of HHP treatment on inactivation of *Salmonella* in fresh produce. The treatment (500 MPa, 25 °C, 5 min) was determined according to the previous studies on *Salmonella* inactivation. Shelf life was studied at 25^{o}C and 4^{o}C to examine the presence of injured cells in HHP treated fresh produce.

For ensuring microbial safety of fresh produce, monitoring, surveillance and control of *Salmonella* are essential through all the steps of farm-to-consumer continuum. This study aims to contribute to build strong surveillance system for ensuring safe fresh produce and enforce further investigations in Turkey.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Fresh produce samples for *Salmonella* isolation

248 fresh produce samples, including tomato (62), parsley (62), iceberg lettuce (62) and greenleaf lettuce (62) were collected from local bazaars and supermarkets of 3 different districts in Ankara during August-November 2012. The list of the fresh produce samples and their suppliers is given in Appendix A. Local bazaars and supermarkets are coded as below;

Table 2.1 Coding system for fresh produce suppliers*

	District 1	District 2	District 3
Bazaar	B1	B2	B3
Supermarket 1	S1.a	S2.d	S3.g
Supermarket 2	S1.b	S2.e	S3.h
Supermarket 2	S1.c	S2.f	S3.i

***:**
B: Bazaar, S: Supermarket, First number indicates the district and small capture letters indicate different supermarkets

2.1.2 Buffers and solutions

All of the buffers and solutions that were used through the analyses are listed with their suppliers in Appendix B.

2.1.3 Growth media

Appendix C desribes the preparation of growth media that were used for the analyses.

2.2 Methods

2.2.1 Isolation of *Salmonella*

The procedure for isolation of *Salmonella* was carried out according to the techniques recommended by the International Organization for Standardization (ISO 6579, 2002).

25 g of fresh produce sample was weighed and mixed with 225 ml buffered peptone water and incubated at 37°C for 16-20 h. 0.1 ml of the pre-enrichment sample was transferred to 10 ml Rappaport Vassiliadis soy peptone (RVS) broth with duplicate and incubated at 41.5 ± 1°C for 24 ±3 h. 10 µl of inoculum in RVS broth was added onto the XLD and BGA agar and incubated at 37 ± 1°C for 24 ±3 h. All suspective *Salmonella* colonies (colonies with slightly transparent zone of reddish color and a black centre on XLD agar and grey-reddish/pink colonies on BGA agar) were inoculated on BHI agar and incubated at 37 ± 1°C for 24 ±3 hours.

PCR targeting the *invA* (F: 5’ - GAA TCC TCA GTT TTC AGT TTC - 3’, R: 5’- TAG CCG TAA CAA CCA ATA CAA ATG - 3’) gene of *Salmonella* was used to confirm the identity of the presumptive *Salmonella*. PCR confirmation was performed according to the technique developed by Kim et al. (2007).

For DNA preparation**,** single colony for each isolate of *Salmonella* from BHI agar was scraped into PCR tube which contained 95 µL sterile dH_2O. Lysis of cells occurred by exposing the prepared mixture to microwaving for 30 seconds in a microwave.

The reagents and their quantities used for preparation of PCR master mix were given in Table 2.2.

Table 2.2 The master mix reagents used for PCR amplification of *invA* gen (Kim et al., 2007)

Master Mix Reagents [Concentration]	**Vol (µl) for 1 reaction**
dH_2O	31
5X Go Taq Flexi Buffer	10.0
$MgCl_2$ [25mM]	3.0
dNTPs [10mM]	1.0
invA- F [12.5 mM] 5' - GA TCC TCA GTT TTC AGT TTC - 3'	2.0
invA – R [12.5 mM] 5'- TAG CCG TAA CAA CCA ATA CAA ATG - 3'	2.0
Go Taq DNAPolymerase	0.25
TOTAL	49.25

49 µl of the master mix and 1 µl of *Salmonella* raw DNA were put into a 0.2 ml PCR tube. For positive control, 49 µl of the master mix and 1 µl DNA from a *Salmonella* reference bacteria culture were put into a 0.2 ml PCR. For negative control, 49 µl of the master mix and 1µl of dH_2O were put into a 0.2 ml PCR.

T100 Thermal Cycler (Bio-Rad) which is shown in Figure 2.1, was used for PCR amplification. 49 µl of the master mix and 1 µl of *Salmonella* isolate were mixed in 0.2 ml PCR tube. This was repeated for each isolate, then all tubes were put into thermal cycler. The PCR conditions consisted of an initial denaturation at 94°C for 8 min, which was followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec, with a final extension at 72°C for 5 min.

Figure 2.1 Thermal Cycler for PCR amplification

After amplification completed, 5 µl of each PCR product was mixed with 1 µl 6X loading buffer and then loaded on a 1.5% agarose gel to run electrophoresis for 30 min at 110 V (Bio-Rad). DNA-size marker (Thermo Fisher Scientific) was used to determine the size of the DNA bands. Agarose gel was stained for 5 min

in ethidium bromide solution (0.5 μgml-1), then stained for 30 min in dH_2O, visualized and photographed under UV light (Bio-Rad).

2.2.2 Freezing *Salmonella* isolates

Salmonella isolates, confirmed by PCR amplification, were frozen at -80^{o}C in 15 % glycerol solution for further analyses. For freezing, each *Salmonella* isolate was streaked onto BHI agar and incubated at 37 ± 1°C overnight. Subsequently, one colony of each isolate was inoculated into 5 ml BHI broth and incubated at 37^{o}C overnight. After incubation, 850 μl culture broth and 150 ml of 15% glycerol solution were mixed and put into tubes. The isolates were labeled according to coding system of Food Safety Laboratory of Food Engineering Department (FDE), Middle East Technical University (METU) Database. For example, ID codes for isolates from this study were given as METU-S1-408, METU-S2-409 and METU-S1-410 with all the information, then frozen at -80^{o}C. In Food Safety Laboratory of Food Engineering Department (FDE), Middle East Technical University (METU) Database information of isolates were also saved, such as source, location, isolatation date, subtypes, etc.

2.2.3 Serotyping

Salmonella isolates were serotyped in the laboratory of Public Health Agency of Turkey, in Ankara. Serotyping of isolates was performed according to the White-Kauffmann-Le Minor Scheme, then *Salmonella* isolates were also confirmed by using biochemical tests was also performed.

2.2.4 Multi-locus Sequence Typing (MLST)

2.2.4.1. Purification of DNA

For DNA preparation, single colony of each *Salmonella* isolate was transferred from BHI Agar to BHI and cultures were incubated overnight at 37°C. Spin column-based DNA isolation was carried out with NanoBiz Bacterial Genomic DNA Isolation Kit.

2.2.4.2 PCR Amplification of 7 housekeeping genes

PCR amplification of 7 characteristic housekeeping genes (*aroC, thrA, purE, sucA, hisD, hemD and dnaN*) of *Salmonella* were carried out according to the protocol of *Salmonella enterica* MLST Database of Environmental Research Institute (ERI) within the body of University College Cork (UCC) (available on http://mlst.ucc.ie/mlst/dbs/Senterica).

The primer pairs used to amplify the targeted genes are given in Table 2.3.

Table 2.3 Nucleotide sequences of forward and reverse primers for each gene (source: http://mlst.ucc.ie/mlst/dbs/Senterica)

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

The reagents and their quantities used for preparation of PCR master mix are given in Table 2.4.

Table 2.4 The master mix reagents used for PCR amplification of seven house keeping genes

Master Mix Reagents [Concentration]	Vol (µl) for 1 X 100 µl reaction
dH_2O	35,75
10X Go Taq Flexi Buffer	5.0
$MgCl_2$ [25mM]	3.0
dNTPs [10mM]	1.0
Primer- F [12.5 mM]	2.0
Primer – R [12.5 mM]	2.0
Go Taq DNA	0,25
TOTAL	49

For PCR amplification, T100 Thermal Cycler (Bio-Rad) was used. 49 µl of the master mix and 1 µl of *Salmonella* isolate were mixed in 0.2 ml PCR tube. This was repeated for each isolate, then all tubes were put into thermal cycler. The PCR conditions consisted of an initial denaturation at 94°C for 10 min, which was followed by 35 cycles of denaturation at 94°C for 1 min , annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 7 min.

After amplification completed, electrophoresis was run and gel picture was visualized as described above, section 2.2.1.

2.2.4.3 DNA sequencing

For purification of PCR products and DNA sequencing, PCR products of three isolates were sent to Macrogen Inc. (Geumchon-gu, Seoul, Korea). Capillary sequencing technology based on Sanger method was applied for genome sequencing.

2.2.4.4 Nucleotide analysis

All sequences were trimmed, proofread and assembled by using SeqMan and SeqBuilder software (DNAStar, Madison, USA). In accordance with the UCC MLST Database, trimmed sequences of certain length from *aroC* (501 nt), *dnaN* (501 nt), *hemD* (432 nt), *hisD* (501 nt), *purE* (399 nt), *sucA* (501 nt), *thrA* (501 nt) were aligned by Clustal W algorithm using MegAlign software (DNAStar, Madison, USA). Assignment of gene alleles was implemented in compliance with the allelic numbers specified in the UCC MLST Database. As the combination of seven allelic types, allelic type profiles of the isolates were formed.

2.2.5 Pulsed-field gel electrophoresis (PFGE)

PFGE analyses were carried out according to the CDC PulseNet protocol (Ribot et al., 2006).

2.2.5.1 Preparation of PFGE plugs

Frozen *Salmonella* stock cultures from -80^{o}C were used, a loop of each culture was transferred onto BHI agar and incubated at 37 ± 1°C for 14-18 h. After growing on BHI, each isolate culture was added to 5 mL cell suspension buffer solution by using a sterile cotton swab. 1.3 mL of cell suspensions were transferred to cuvettes and adjusted to an OD_{610} of between 1.3 and 1.4 (UV-1700 PharmaSpec UV-Visible Spectrophotometer, Shimadzu). The cell suspensions were held in ice-bath until the agarose plugs were prepared. 400 µL of adjusted cell suspensions were transferred into eppendorf tubes and incubated at 37°C water bath for 10 min. 20 µL proteinase K (end concentration of 20 mg/mL) was added to each eppendorf tube. 400 µL Seakem Agarose (1%)-SDS solution was added to each sample. They were mixed with pipette for 2-3 times to ensure that solution and cell suspension mixed well. Each sample including agarose/SDS solution was transferred to the PFGE-molds with a special care to avoid bubble formation. During this step, in order not to solidify the agarose, the mixture was prepared in the waterbath (55ºC). The PFGE plugs were kept at room temperature for at least15 min to be cooled.

2.2.5.2 Lysis of cells in agarose plugs

The agarose plugs were removed from the mold by using sterile thin spatula and transferred to 5 mL cell lysis buffer containing 25 µl proteinase K (Roche) in tubes. For lysis of the cells, they were incubated at 54ºC for 1.5-2 h in shaking waterbath (170 rpm).

2.2.5.3 Washing agarose plugs after cell lysis

Before washing process started, sterile double-distilled water (ddH_2O) and TE buffer solution were pre-heated at 50ºC water bath. The plugs were washed at 50ºC in shaking incubator (70 rpm). Washing was repeated with 10 ml ddH_2O twice at 10 min intervals and with 10 ml TE buffer solution for four times at 15 min intervals. After washing process, the plugs were stored in 5 ml TE buffer solution at 4ºC.

2.2.5.4 Restriction Digest of DNA in Agarose Plugs with Xba1

The plugs were cut by using a scalpel to 2 mm slices and transferred into eppendorf tubes containing 200 μL of H-Buffer solution. The slices were incubated in H-buffer solution in 37°C water broth for 10 min. After H-buffer solution was removed, 200μL of *Xba1* enzyme solution was added to the slices. Slices were incubated at 37^{o}C in waterbath for 5 h 45 min. DNA of agarose plugs were digested by *Xba1* enzyme through the incubation period.

2.2.5.5 Casting Pulsed Field Agarose Gel

The Seakem Agarose (1%)-TBE solution was prepared. 8 mL ddH_2O was added and and microwaved until the 8 mL is evaporated. Agarose was cooled in 55°C water bath for at least 10 minutes, then left to cool at room temperature at least 5 minutes. Gel cast was leveled with bubble. Agarose was casted into cleaned gel mold. The gel is covered with plastic container to avoid dust. It was kept at room temperature for 20 min until the gel solidified.

2.2.5.6 Electrophoresis, Staining and Documentation of Pulsed Field Agarose Gel

Pulsed field chamber (CHEF-DR II, Variable Angle System, BioRad), which is shown in Figure 2.2, was filled with 2.2 L of running buffer (including 0.11 L 10XTBE buffer solution and 2,09 L ddH_2O).

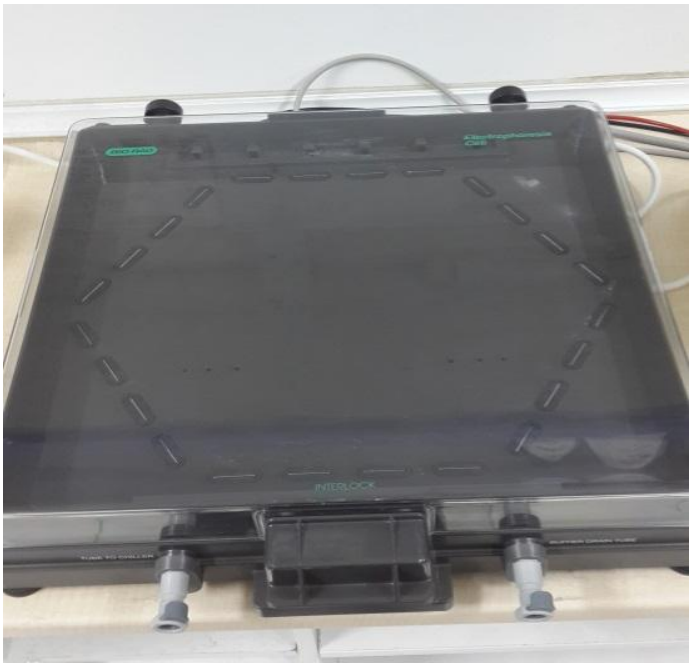

Figure 2.2 Pulsed field chamber used for PFGE analysis

Pump speed was set to 70 (0.75 L/min) for 30 min before running, then PFGE system was cooled to 14ºC. At this time, *Xba1* enzyme solution was removed from the 2 mm slices. Sealing agarose was prepared and microwaved. Sealing agarose was cooled in 55°C water bath at least 10 minutes. The slices were loaded into gel. 836µL of 10mg/1mL thiourea solution was added to running buffer. Electrophoresis was performed under the conditions mentioned in Table 2.5.

Table 2.5 Electrophoresis conditions for PFGE analysis

Low KB	30KB
High KB	700KB
% Agarose	1%
Gradient	6.0 V/cm
Run Time	19 h
Included Angle	120°
Initial Switch Time	2.2s
Final Switch Time	1.03m.80s
Pump Speed	70
Initial Current:	0

Later, the gel was stained with ethidium bromide solution (0,1µg/mL) for 45 min. The chamber was washed for 30 min with 2.1 L of dH_2O. The gel was destained with dH_2O for 30 min. PFGE pattern was documented under UV light by Bio-Rad Universal Hood II.

2.2.6 High Hydrostatic Pressure (HHP) Treatment

Artificially *Salmonella* contaminated fresh produce samples were treated with HHP in multiple steps as detailed below.

2.2.6.1 Obtaining growth curves of *Salmonella* isolates

Growth curve analyses were carried out to determine the early stationary phases of *Salmonella* isolates, which were used for artificial inoculation. *Salmonella* colonies of each isolate was grown in BHI broth at 37ºC overnight. 100µl of each culture was transferred to 100ml BHI in each 250ml Erlenmeyer flask. Flasks were incubated at 37ºC at rotary shaker (120 rpm) incubator up to 18h. Duplicate sample of flasks were taken from the incubator at 37ºC every hour and Optical Density at 600nm (OD_{600nm}) was measured with a spectrophotometer. Average

OD data versus incubation time was calculated then growth curve was plotted for the strain.

Simultaneously, 1ml of the culture was taken from the flasks in every 2 hours for enumeration of colony forming units per mL (CFU/mL). Each tube contains 9 ml of BPW. 1 ml of bacterial culture was transferred to 900µl BPW then spread plated with serial dilutions to calculate number of colony on the TSA. Pipette 100µl 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.

2.2.6.2 Sample preparation for HHP treatment

Fresh parsley and iceberg lettuce were purchased from a supermarket in Ankara. After being transported to the laboratory, they were stored at 4^{o}C and used within 24 h. Samples were sorted to eliminate damaged leaves and cut into small portions (10 g). Each portion was washed with tap water for 1 min and then dried at room temperature for 5 min. The samples were made ready to be used for inoculation procedure.

2.2.6.3 Inoculation of *Salmonella* isolates on fresh produce

From frozen *Salmonella* stock cultures (-80^{o}C), a loop of each culture was transferred onto BHI agar and incubated at 37 ± 1°C for 24 ±3. Single colony from each incubated culture was transferred to 10 ml BHI broth and incubated at 37 ± 1°C overnight. After incubation, 100 µL of each BHI broth culture was

transferred to 90 ml TSB. The isolates were grown to obtain about ~10^9-10^{10} colony forming units (cfu)/ml at 37 ± 1°C for approximately 14 h.

Small portions (10 g) of fresh parsley and iceberg lettuce samples were immersed into the cultures of isolates (at their early stationary phase) in TSB to obtain about 10^7 colony forming units (cfu)/ml.

The fresh produce with bacteria were dispensed in 2-ml portions in sterile plastic vials(Simport Plastic, Canada) avoiding air bubbles as much as possible. The vials were vortexed for 2min and vacuum-sealed in sterile plastic bags. After the HHP treatments, Samples were held in ice and all the measurements were done in 1h.

2.2.6.4 High Hydrostatic Pressure Treatment

High Hydrostatic Pressure was applied by a 760.0118 type industrial high pressure system (SITEC CH-8124, Zürich, Switzerland) which is shown in Figure 2.3. In order to apply high pressure magnitudes isothermally, temperature should be stable during treatment (Rastogi, Raghavarao, Balasubramaniam, Niranjan & Knorr, 2007). The volume and length of the vessel is 100 ml with ID 24 mm and 153 mm respectively. Ethylene glycol was used for a cooling / heating that was circulated around the jacketed pressure vessel. The maximum design pressure was 700 MPa at an operating temperature of -10 to 80°C. A built-in heating-cooling system (Huber Circulation Thermostat, Offenburg, Germany) was used to maintain and control the required temperature which was measured by a thermocouple type K. Samples were pressurized at 500 MPa at 25 °C for 5 min. Temperature increase due to adiabatic heating was estimated as 4-5°C during the time period of pressurization.

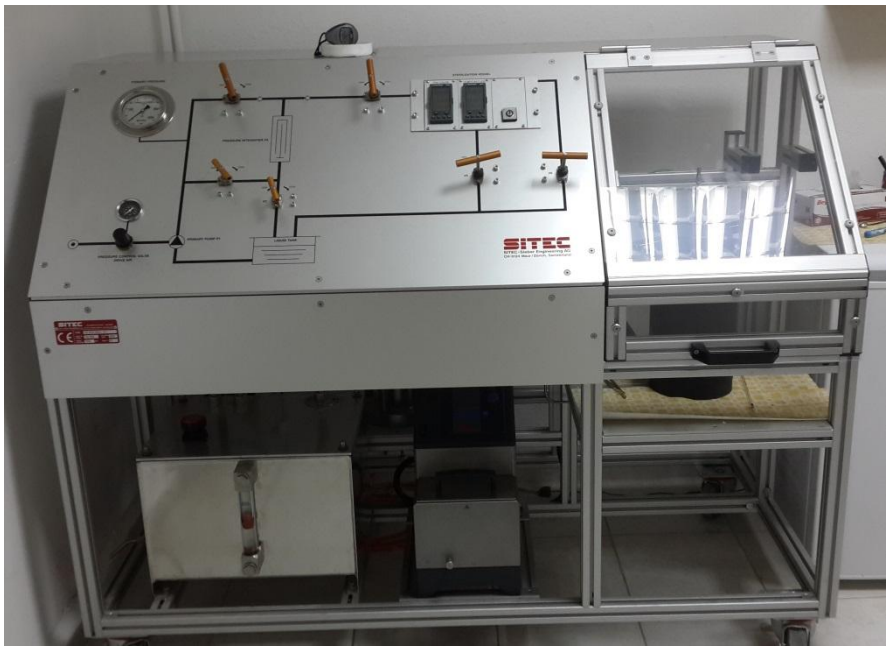

Fig 2.3 Laboratory scale HHP equipment

2.2.6.5 Shelf life analysis

HHP treated (500 MPa, 25 °C, 5 min) fresh produce samples were stored at two different temperatures; 25^{o}C and 4^{o}C . 10 g portions of 2 greenleaf lettuce and 1 parsley samples were kept at 25^{o}C and 4^{o}C for a week. After the storage period, they were put into sterile plastic bags together with 90 ml BPW. They were mixed thorougly. 100 µL of each sample was transferred to TSA. Samples were incubated at 37 ± 1°C for 24 ±3 hours for enumeration of plate counts.

CHAPTER 3

RESULTS AND DISCUSSION

The aim of this study was to investigate the prevalence of *Salmonella* in fresh produce including tomato, parsley and lettuce and to obtain phenotypic and genotypic characterization of the isolates. Isolates were firstly differentiated by phenotypic characterization (i.e. serotyping), then further subtyping was conducted by MLST and PFGE. After the *Salmonella* isolates were subcharacterized, their resistance to HHP treatment (500 MPa, 25 °C, 5 min) in fresh produce was examined. Shelf life study was also done to determine whether *Salmonella* was totally eliminated or sublethally injured.

3.1 Cultural and PCR Assays for fresh produce samples

A total of 248 fresh produce samples including tomato (n=62), parsley (n=62), greenleaf lettuce (n=62) and iceberg lettuce (n=62) were collected from 9 different supermarkets and 3 local bazaars in Ankara between August-November 2012. The number of samples by suppliers is given in Table 3.1.

Table 3.1 Sampling of fresh produce by suppliers, August-November 2012

	Number of samples			
Supplier	Tomato	Parsley	Greenleaf lettuce	Iceberg lettuce
S1.a	6	6	6	6
S1.b	6	6	6	6
S1.c	6	6	6	6
S2.d	5	5	5	5
S2.e	5	5	5	5
S2.f	5	5	5	5
S3.g	5	5	5	5
S3.h	4	4	4	4
S3.i	4	4	4	4
B1	5	5	5	5
B2	6	6	6	6
B3	5	5	5	5
TOTAL	62	62	62	62

B: Bazaar , S: Supermarket. First number indicates the district, small capture letters indicate the different supermarkets.

51 presumptive *Salmonella* colonies were obtained by traditional biochemical culturing method which was carried out according to the standard method ISO 6579/2002. PCR for confirmation of presumptive colonies was evaluated in terms of discrimination of *Salmonella* from non-*Salmonella* strains. Each presumptive colony was subjected to *Salmonella*-specific *invA* primers. Out of 51 presumptive colonies, 3 of them were confirmed as *Salmonella* by PCR (Table 3.2). *Salmonella* positive isolates, including positive control, generated a single 284-bp amplified DNA fragment on 1.5% agarose gel (Figure 3.1). The rate of isolation was 3,2% for parsley and 1,6% for iceberg lettuce. *Salmonella* was not detected from neither tomato nor greenleaf lettuce samples.

Table 3.2 The results of traditional culturing method and PCR confirmation

Sample	Supplier	Supply date	Culturing Result	PCR Result
Parsley	S2.d	06.08.2012	+	-
Greenleaf lettuce	S2.d	06.08.2012	+	-
Greenleaf lettuce	S2.e	06.08.2012	+	-
Iceberg	S2.e	06.08.2012	+	-
Parsley	S2.f	06.08.2012	+	-
Iceberg	S2.f	06.08.2012	+	-
Parsley	B1	06.08.2012	+	-
Greenleaf lettuce	B1	06.08.2012	+	-
Parsley	B2	06.08.2012	+	-
Parsley	S3.g	10.08.2012	+	-
Greenleaf lettuce	S3.h	10.08.2012	+	-
Iceberg	S3.h	10.08.2012	+	-
Tomato	S3.h	10.08.2012	+	-
Iceberg	S3.h	04.09.2012	+	-
Parsley	S3.i	04.09.2012	+	-
Tomato	S1.c	08.09.2012	+	-
Greenleaf lettuce	S1.c	08.09.2012	+	-
Tomato	B3	11.09.2012	+	-
Iceberg	B3	11.09.2012	+	-
Iceberg	B2	11.09.2012	+	-
Parsley	B1	11.09.2012	+	-
Parsley	S2.f	11.09.2012	+	-
Parsley	S2.d	11.09.2012	+	-
Greenleaf lettuce	S2.d	11.09.2012	+	-
Iceberg	S2.d	11.09.2012	+	-
Parsley	S1.a	08.09.2012	+	+
Greenleaf lettuce	S1.b	08.09.2012	+	-
Iceberg	S1.b	24.09.2012	+	-
Iceberg	S2.d	25.09.2012	+	-
Iceberg	S2.e	25.09.2012	+	-
Iceberg	S2.f	25.09.2012	+	-
Iceberg	B2	01.10.2012	+	-
Iceberg	B1	01.10.2012	+	+
Tomato	S1.b	10.10.2012	+	-
Iceberg	S1.a	10.10.2012	+	-
Iceberg	S2.f	14.10.2012	+	-
Parsley	S2.d	14.10.2012	+	+
Parsley	B3	14.10.2012	+	-
Greenleaf lettuce	S1.c	02.11.2012	+	-

*: Red color indicates the *Salmonella* positive samples

Table 3.2 The results of traditional culturing method and PCR confirmation (continued)

Sample	Supplier	Supply date	Culturing Result	PCR Result
Iceberg	S3.i	02.11.2012	+	-
Tomato	S3.g	02.11.2012	+	-
Tomato	S3.h	02.11.2012	+	-
Iceberg	S3.h	02.11.2012	+	-
Iceberg	S2.f	04.11.2012	+	-
Iceberg	S1.a	08.11.2012	+	-
Tomato	S1.b	08.11.2012	+	-

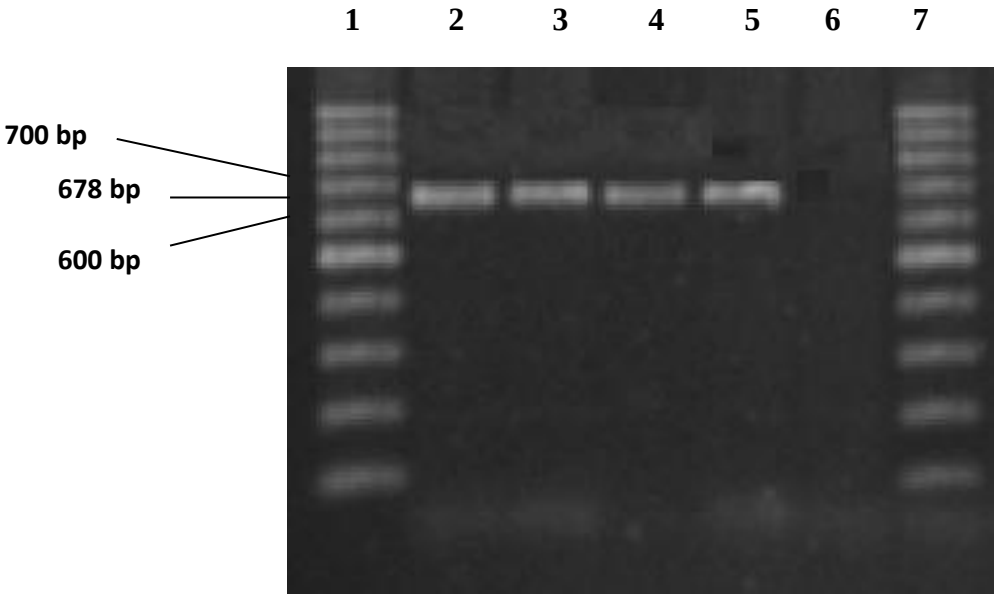

Fig 3.1 Electrophoresis of PCR products on 1.5% agarose gel stained with ethidium bromide: 100 bp molecular weight marker (lanes 1 and 7); positive control (lane 2); isolates collected from S1a, B1 and S2d (lanes 3, 4 and 5); negative control (lane 6).

Details for samples contaminated with *Salmoenlla* are given in Table 3.3.

Table 3.3 Positive samples representing Lane 3-5

Gel Lane No.	Source	Supplier	Supply date
3	Parsley	S1.a	08.09.2012
4	Iceberg	B1	01.10.2012
5	Parsley	S2.d	14.10.2012

PCR amplification of *invA* gene has been demonstrated as an useful tool for confirmation of *Salmonella* isolates (Malorny et al., 2003). The *invA* target gene is placed on *Salmonella* pathogenicity island 1 (SPI-1), which is essential for the invasion of epithelial cells. This gene is highly conserved in almost all *Salmonella* serotypes and has been used as a potential target for *Salmonella* detection. In this part of the study, the specificity of *invA* primers for *Salmonella* was verified and amplification of *invA* gene provided successfull discrimination of *Salmonella* strains from non-*Salmenolla* strains.

To avoid false-positive results due to either potential detection of nonviable cells or mispriming of nucleic acid sequences that are similar to target DNA (Feder et al., 2001), PCR was not used alone for detection of *Salmonella.* Since DNA is stable and can persist in a sample long after the target organism has died, it may lead revealing of false-positive results (Drahovska et al., 2001). Therefore, traditional culturing method and PCR confirmation was employed respectively in this study and they provided detection only viable cells of *Salmonella* on fresh produce in addition to avoid potential mispriming of DNA sequences of another organisms'. Conventional PCR method has been commonly used as a confirmatory test following bacterial isolation (Feder et al., 2001).

In our study it was obtained that two *Salmonella* positive isolates for parsley and one for iceberg lettuce out of 248 total fresh produce samples. *Salmonella* was not detected in neither tomato nor greenleaf lettuce samples. Two parsley samples were purchased from different supermarkets located in two distinct districts of Ankara at different times, thus not representing any relatedness between the isolates which were further serotyped as different strains. In addition, the iceberg lettuce that contained *Salmonella* was obtained from a local bazaar in Ankara.

In this study, the proportion of fresh produce samples that obtained *Salmonella* was determined as 1.2% (3/248). This result corresponds to the results of the surveys conducted worldwide based on investigating the prevalence of *Salmonella* in fresh produce. In 2007, *Salmonella* was detected in approximately 0.3% of produce-related samples in the European Union (Westrell *et al*., 2009). According to the large surveys carried out in the UK, Ireland, Germany and the Netherlands (2007), the proportion of fresh produce samples containing *Salmonella* in these studies ranged from 0.1% to 2.3% (Westrell *et al*., 2009). Table 3.4 summarizes the surveys conducted worldwide on some leafy green vegetables investigating the prevalence of *Salmonella.*

Table 3.4 *Salmonella* isolation from some leafy green vegetables in the world

Produce Item	Country	Sampling target	Positive sampled	% prevalence	Reference
Cabbage	India	fields	4/33	12.1	Rai et al., 2007
	India	street vendors	2/8	25.0	Viswanathan and Kaur, 2001
	Ireland	Supermarkets	0/4	0	McMahon and Wilson, 2001
	Mexico	Supply Station	1/100	1.0	Quiroz-Santiago et al., 2009
	U.S.	packing sheds, southern U.S.	0/109	0	Johnson et al., 2006
	U.S.	farms, organic, conventional, semiorganic	0/291	0	Mukherjee et al., 2004, 2006
Lettuce	Canada	retail distribution centers/farmers' markets	1/530	0.2	Arthur et al.,2007
	Ireland	Supermarkets	0/8	0	McMahon and Wilson , 2001
	Italy	producers	2/62	3.2	De Giusti et al., 2010
	Korea	department store, supermarket, restaurant	1/30	3.3	Seo et al., 2010
	Mexico	markets, supermarkets	10/75	1.3	Castaneda,-Ramirez et al., 2011
	Norway	producers, organic	0/179	0	Loncarevic et al., 2010
	Spain	farms, organic, conventional	0/72	0	Oliviera et al., 2010
	Spain	retail establishments	1/29	3.4	Abadias et al., 2008
	U.S.	farms, organic, conventional, semiorganic	0/261	0	Mukherjee et al., 2004,2006
	U.S.	supermarkets, farmers' markets	0/10	0	Thunberg et al., 2002
	U.S.	markets and wholesale distribution centers	2/5453	0.04	USDA(2007,2008,2009
Mixed salads/vegetables	Brazil	retailers	1/21	4.8	Fröder et al., 2007
	Cyprus	production sites, retail outlets	6/294	2.0	Eleftheriadou et al., 2002
	Korea	department store, supermarket, restaurant	1/129	0.8	Seo et al., 2010
	Malaysia	wet markets	40/112	35.7	Salleh et al., 2003

As it seen in Table 3.4 , a higher prevalence of *Salmonella* occurs in leafy greens in developing countries than developed countries indicating the differences in sanitary practice standards.

Salmonella can contaminate fresh produce at any steps through farm-to-consumer continuum (growth, harvest, processing, packaging, transportation, handling and retail). However, there is a large amount of uncertainty associated with the contamination and colonization on fresh produce, particularly leafy vegetables on the field. Internalization has been found to be possible during pre-harvest under experimental conditions, but only after exposure of young plants (seedlings) to high pathogen loads. There is no evidence reflecting that internalization is significant in practice, particularly when Good Agricultural Practices (GAP) is implemented (WHO/FAO, 2008). In a comprehensive study, investigating the prevalence, distribution and diversity of *Salmonella enterica* in environment of a major agricultural region of California, Gorski et al. reported (2011) no detection of *Salmonella* in fresh produce (i.e. lettuce and spinach) whereas prevalence was observed on environmental samples; 7.1%, 4.2% and 2.6% of water, wildlife and soil/sediment samples respectively were tested positive.

As a consequence, identifying reservoirs and transmission routes of *Salmonella* in important leafy green production regions of Turkey is critical to improving food safety and public health.

3.2 Serotyping

Serological analysis revealed three different serotypes; *S. enterica* subsp. *enterica* serotypes Anatum, Mikawasima and Charity. Antigenic formula of each serotype is given in Table 3.2.

Table 3. 5 Antigenic formula of *Salmonella* isolates

Serotype	Antigenic formula	Serogroup	Isolated Source
Anatum	3,10,15;e,h;1,6	E1	Parsley
Mikawasima	6,7,14;y;e,n,z	C1	Iceberg
Charity	1,6,14,25,d;e,n,x	H	Parsley

S. Anatum, generally associated with meat and poultry products, has a widespread distribution worldwide (Sallam et al., 2013; Favier et al., 2012). According to the annual report of United States Department of Agriculture (USDA), it is estimated within the 10 most common serotypes identified in meat and poultry products in 2011. In RASSF Portal, there are 55 notifications linked to *S.* Anatum listed between 1997-2013. Among 55 notifications, notifications for fresh produce share approximately 6% (3/55) of total notifications linked to *S.* Anatum. The different kinds of fresh produce contaminated with *S.* Anatum notified to RASSF were sesame seeds from India and Bolivia, fresh margosa from Thailand. *S.* Anatum also caused few foodborne outbreaks previously. For example, in 2006 an infection of enterotoxigenic *E. coli* and *S.* Anatum, which affected around 200 people, occured after a high-school dinner in Denmark. It is reported that imported fresh basil used for preparation of the pesto was the most likely source of contamination (Pakalniskiene et al., 2006). Since *S.* Anatum is generally associated with animals, in our study contamination of *S.* Anatum to parsley is most likely to occur in field because of animal feces.

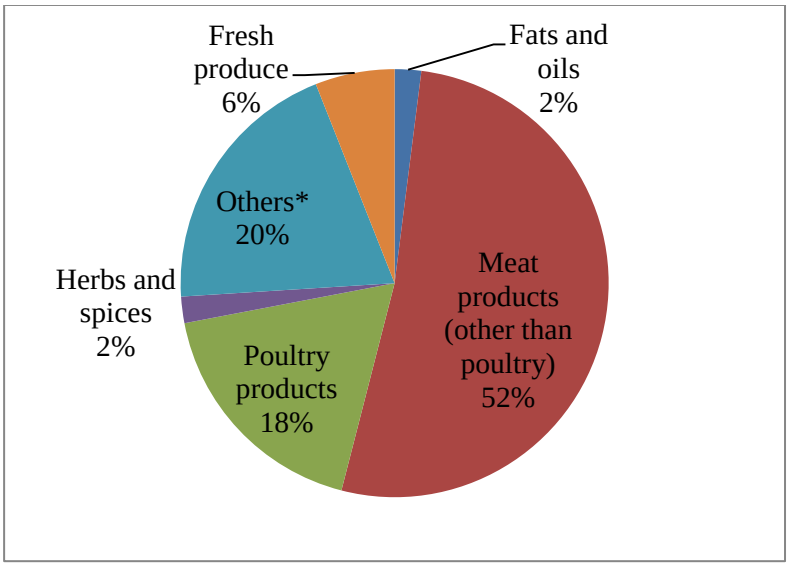

Fig 3.2 Percentage of notifications of due to *S.*Anatum in RASSF Portal
*Others: Animal feed, pet food, infant food

S. Anatum was also previously isolated from animals, i.e. sheep in Turkey (Töreci et al., 2013) but our isolate is the first serotype isolated from fresh produce in Turkey.

S. Charity is a rarely found serotype. It has not been any information in literature about this serotype causing an outbreak previously. According to RASSF Portal, there is only one notification for *S.* Charity (2003) and it was linked to chili powder. In Turkey, *S.* Charity was only isolated from tortoise up to end of 2011 (Özek et al., 1965).

S. Mikawasima is also an uncommon serotype, however there is an increasing concern for this serotype in the world, especially in European Union due to unusual increase in *S.* Mikawasima infections in humans since 2013 (ECDC, 2013). It was first isolated from tortoises in Turkey in 1967 (Özek et al., 1967) and first reported relating to pigs in EU in 1976 (Edel et al., 1976). Afterwards, it was isolated from environmental samples (fresh waters) in north-east Spain (Polo et al., 1999) and more recently isolated from pigs and wild boars in southern and northern Spain (Gomez-Laguna et al., 2011; Navarro-Gonzalez et al., 2012). A

recent retrospective study from the Czech Republic reported several sporadic cases of *S.* Mikawasima in six regions of the country in 2012 (Petra et al., 2013). In 1992, an outbreak of *S.* Mikawasima causing human gastroenteritis was reported in UK. Epidemiological investigations identified doner kebabs as a probable source (Synnott et al., 1993). A search in the Rapid Alert System for Food and Feed (RASFF) database resulted in one notification posted in 2005 related to *S.* Mikawasima presence in frozen squid tentacles from India.

During 2004—2012 (including some data from 2013), 120 isolates of *S.* Mikawasima were reported from food, feed and animals in eight Member States and one Non-Member State of EU (Table 3.3). Most of the isolations were reported from animals. However, *S.* Mikawasima was also isolated from various food sources, such as vegetables, sausages meat and nuts (ECDC, 2013).

S. Mikawasima which was isolated from iceberg lettuce in our study, has been published first in Turkey related to fresh produce.

Table 3.6 Isolations of *S.* Mikawasima in the European Union (ECDC, 2013)

Source	Number of isolations in 2004-2009	Number of isolations in 2010-2011	Number of isolations in 2012-2013*	Total number of isolations in 2004-2013
Animals, in total	36	44	11	91
Gallus gallus (fowl), in general	12	26		38
Broilers	1	7	10	18
Laying hens	3	7	1	11
Pigs	10	3		13
Ducks	2	1		3
Poultry, in general	2			2
Cattle	4			4
Domestic solipeds	2			2
Food, in total	10	7	4	21
Food of non-animal origin (unspecified category)	5			5
Vegetables	1	4 (tomatoes)		5
Fruit	1			1
Sweets	1			1
Sausages	2			2
Bovine meat				1
Rice salad		1		1
Frock legs		1		1
Broiler meat		1	2	2
Wild boar meat			1	1
Almonds			1	1
Feed	1	6	1	8
Totals	47	57	16	120

3.3 MLST Results

Allelic profiles and sequence types of *S.* Anatum, *S.* Mikawasima and *S.* Charity were obtained based on the UCC MLST Database (Table 3.3).

Table 3. 7 MLST allelic profiles and sequence types of three *Salmonella* isolates detected in fresh produce samples in our study

Serotype	Allelic profile							Sequence type (ST)
	aroC (501 b.p.)	dnaN (501 b.p.)	hemD (432 b.p.)	hisD (501 b.p.)	purE (399 b.p.)	sucA (501 b.p.)	thrA (501 b.p.)	
Anatum	10	14	15	31	25	20	33	64
Mikawasima	14	2	331	7	105	19	12	1815 (Novel)
Charity	125	63	17	62	12	58	3	383

When UCC MLST Database is searched for the strains of *S.* Anatum, it has been seen that allelic profile of our isolate *S.* Anatum is identical with %96 (23/24) of the allelic profiles of all *S.* Anatum strains isolated from different countries (e.g. USA, Germany, Australia, Denmark and South Africa). The predominant sequence type among the *S.* Anatum strains is ST 64. The data demonstrates that *S.* Anatum has a widespread geographical distribution and adaptability to different environments and host organisms. The only different allelic profile is for the strain isolated from food in Morocco. Among all the sources of isolations, humans (5/24) and environmental sources (5/24) constitute the major proportions. *S.* Anatum strains share %0.4 (24/6180) of total strains in UCC MLST Database The detailed information for allelic profiles of *S.* Anatum is given in Appendix D.

In UCC MLST Database, there were only 3 *S.*Charity strains, indicating that the serotype is not common worldwide.The sequence type of our isolate, *S.*Charity, is identical with 2 of the 3 different strains isolated in different countries (i.e. Sweden, the other one is unknown).

*S.*Charity isolated from Sweden was detected on food, however the source was not specified. The detailed information for allelic profiles of *S.* Charity is given in Appendix E.

Our study yielded a novel sequence type of *S.* Mikawasima. According to data saved previously on UCC MLST Database, there were 3 *S.* Mikawasima strains with 3 different sequence types. Among 3 strains of *S.* Mikawasima excluding our isolate, isolation sources of two strains are unknown (USA). The other strain was isolated from reptile in Japan (1937). These data indicates that allelic profiles vary for the strains of *S.* Mikawasima in different regions of the world. The detailed information for allelic profiles of *S.* Mikawasima is given in Appendix F.

3.4 PFGE Subtyping

Salmonella isolates were analysed for their PFGE profiles. For our 3 isolates, 3 different *XbaI* PFGE patterns were obtained.

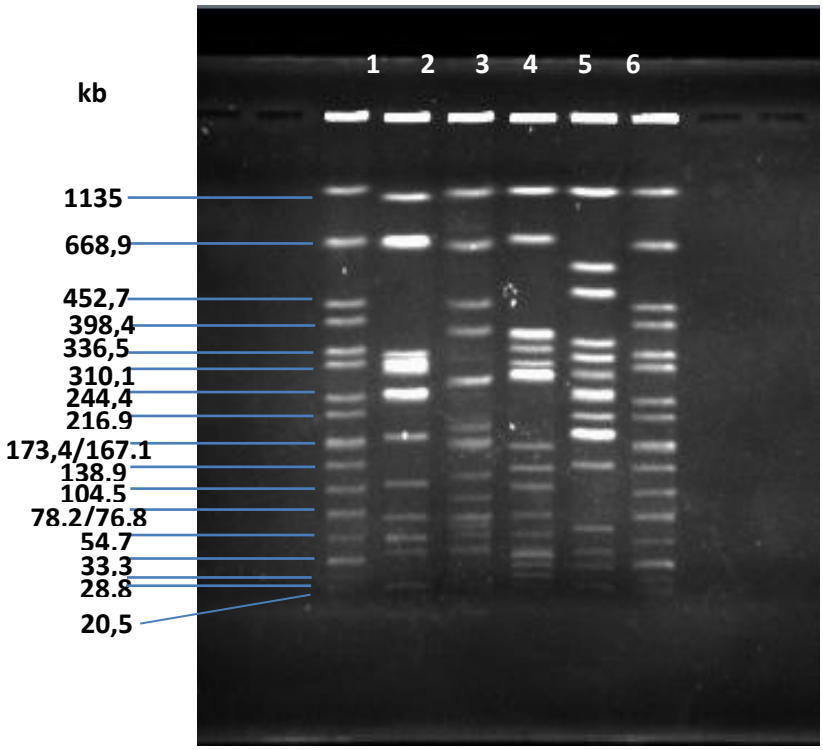

Fig 3.3 PFGE profiles of *Salmonella* isolates

Table 3.8 The lanes representing the *Salmonella* serotypes on PFGE

Lane	METU ID	Isolate Source	Serotype
1	MET-S1-717	Reference strain	Braenderup*
2	MET-S1-411	Red Pepper	Enteritidis**
3	MET-S1-408	Parsley	Anatum
4	MET-S1-409	Iceberg lettuce	Mikawasima
5	MET-S1-410	Parsley	Charity
6	MET-S1-717	Reference strain	Braenderup*

**S.* Braenderup as reference strain was obtained by Food Engineering Department (FDE) of Middle East Techical University (METU)

***S.* Enteritidis was isolated in red pepper by the study of investigatigating prevalence of *Salmonella* in fresh peppers by Gözde Polat (2012), carried out simultaneously with this study in FDE, METU.

Since PFGE patterns represent different subspecies, the differences in banding patterns were not compared. However, our study revealed comparable and sharable data that can be used both nationally and internationally in other researchs. The PFGE profiles of our isolates were saved in the database of Food Engineering Department (FDE) of Middle East Technical University. These data can be used for outbreak traceability or other epidemiologic investigations, it is needed.

3.5 HHP Treatment

3.5.1 Growth curves of the *Salmonella* isolates

The results of growth curve analyses for *S.* Anatum, *S.* Mikawasima and *S.* Charity are given in Appendix G, Appendix H and Appendix I respectively. Early stationary phases of isolates were determined according to the growth curves.

3.5.2 Viability of *Salmonella* isolates after HHP treatment

S. Anatum, *S.* Mikawasima and *S.* Charity isolates were artificially inoculated into parsley, iceberg lettuce and parsley respectively on their early stationary phase. The samples which have initial *Salmonella* content approximately 7 log cfu/g, were pressurized under 500 MPa at 25^{o}C for 5 min. The microbiological results of the treatment and shelf life analysis are given in Table 3.9.

Table 3.9. Viability of *Salmonella* isolates after HHP treatment (500 Mpa-25^{o}C-5 min)

		Viability		
		Direct enumeration	Shelf life analysis (7 days)*	
Isolate	**Sample**		**4^{o}C**	**25^{o}C**
S. Anatum	parsley	-	-	+
S. Mikawasima	iceberg lettuce	-	-	-
S. Charity	parsley	-	-	-

* "-" indicates no growth, "+" indicates growth

After HHP treatment in samples directly enumarated on TSA, no growth was observed. In addition shelf analyis at 4^{o}C for 7 days resulted in no growth of *Salmonella* on samples. However after 7 days at 25^{o}C, *S.* Anatum grew on TSA. It indicates the prescence of injured *S.* Anatum cells after HHP treatment.

The effect of HHP on serotypes Mikawasima and Charity were firstly examined by this study. HHP eliminated these serotypes efficiently. However for serotype Anatum, total elimination of the cells could not be provided under 500 MPa-5min-20^{o}C HHP treatment . The viability of *S.* Anatum after HHP treatment was previously studied on diced and whole tomatoes (Maitland et al., 2011). Maitland et al. reported that *S.* Anatum was the most pressure sensitive serotype when compared to other serotypes, i.e. Newport, Javiana and Braenderup. Although Anatum was represented as pressure sensitive in that study, it is the most pressure resistant serotype among the three serotypes in our study.

Control of *Salmonella* in the food chain requires careful selection of treatments compatible with product characteristics and production processes.

As fresh produce are perishable foods and not have a pasteurization step due to

raw consumption, HHP can be applicable to these foods.

The distinctive effects of HHP on the cell membrane integrity and ultrastructure of *Salmonella* have not yet been thoroughly described. There is a need of more studies for better understanding of the mechanism of inactivation of HHP on the cells.

CHAPTER 4

CONCLUSION

Foodborne outbreaks related to fresh produce, in particular leafy green vegetables have increased worldwide. *Salmonella* outbreaks constitute a significant portion of all fresh produce related outbreaks. Despite *Salmonella* has been reported from both human and animal sources in Turkey, there are very few studies have been carried out on fresh produce. Considering that Turkey is a major world producer of fresh produce, this study revealed important data for phenotypic and genotypic diversity of *Salmonella* isolated from fresh produce. The results of our study obtained by the most common subtyping methods (i.e. serotyping, MLST and PFGE) will contribute to development of national database, which is located in Food Engineering Department at METU. This database is publicly available reference national database, which also includes the isolates that can be used for further characterization analysis. In addition HHP treatment, which is a novel food processing technology alternative to heat treatment, efficiently eliminated *Salmonella* in fresh produce. Further studies are required for preserving shelf life of HHP treated fresh produce

CHAPTER 5

RECOMMENDATIONS

As being one of the largest exporters and producers of fresh produce in the world, Turkey needs a powerful surveillance system for ensuring safety of fresh produce. For this purpose, this study provided epidemiologic data for *Salmonella* on fresh produce. However, further studies, based on investigating the prevalence and subtyping of *Salmonella*, should be done for better understanding of evolutionary pathways, geographical distribution and genetic diversity of *Salmonella* strains in Turkey. As PFGE is an efficient subtyping tool and commonly used worldwide, particularly in outbreak investigations, the use of PFGE will obtain sharable and comparable data of genotypic characteristics of *Salmonella* strains. Therefore more PFGE-based studies should be carried out in Turkey. *Salmonella* can contaminate fresh produce at any steps through farm-to-consumer continuum (growth, harvest, processing, packaging, transportation, handling and retail). Source tracking based studies and comprehensive epidemiological surveys should be prompted as they contribute to understanding of potential contamination points, thus developing control measures for preventing the contamination.

The effect of high pressure on inactivation of *Salmonella* can vary depending on both the resistance of serotype to HHP and the food content. As a recommendation, more studies can be carried out to improve understanding the behaviours of different *Salmonella* serotypes in different fresh produce samples under HHP treatment.

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APPENDIX A

COLLECTION OF FRESH PRODUCE SAMPLES AND ISOLATION OF *SALMONELLA*

Table A.1 Fresh produce samples by supplier, sampling date and results of isolation

Supplier code	Fresh produce	Sampling date	Result
S1.a	Tomato	01.08.2012	-
S1.a	Parsley	01.08.2012	-
S1.a	Greenleaf lettuce	01.08.2012	-
S1.a	Iceberg lettuce	01.08.2012	-
S1.b	Tomato	01.08.2012	-
S1.b	Parsley	01.08.2012	-
S1.b	Greenleaf lettuce	01.08.2012	-
S1.b	Iceberg lettuce	01.08.2012	-
S1.c	Tomato	01.08.2012	-
S1.c	Parsley	01.08.2012	-
S1.c	Greenleaf lettuce	01.08.2012	-
S1.c	Iceberg lettuce	01.08.2012	-
S2.d	Tomato	06.08.2012	-
S2.d	Parsley	06.08.2012	-
S2.d	Greenleaf lettuce	06.08.2012	-
S2.d	Iceberg lettuce	06.08.2012	-
S2.e	Tomato	06.08.2012	-
S2.e	Parsley	06.08.2012	-
S2.e	Greenleaf lettuce	06.08.2012	-
S2.e	Iceberg lettuce	06.08.2012	-
S2.f	Tomato	06.08.2012	-
S2.f	Parsley	06.08.2012	-
S2.f	Greenleaf lettuce	06.08.2012	-
S2.f	Iceberg lettuce	06.08.2012	-
B1	Tomato	06.08.2012	-
B1	Parsley	06.08.2012	-
B1	Greenleaf lettuce	06.08.2012	-
B1	Iceberg lettuce	06.08.2012	-
B2	Tomato	06.08.2012	-
B2	Parsley	06.08.2012	-
B2	Greenleaf lettuce	06.08.2012	-
B2	Iceberg lettuce	06.08.2012	-
S3.g	Tomato	10.08.2012	-

Table A.1 Fresh produce samples by suppliers and sampling date (continued)

Supplier code	Fresh produce	Sampling date	Result
S3.g	Parsley	10.08.2012	-
S3.g	Greenleaf lettuce	10.08.2012	-
S3.g	Iceberg lettuce	10.08.2012	-
S3.h	Tomato	10.08.2012	-
S3.h	Parsley	10.08.2012	-
S3.h	Greenleaf lettuce	10.08.2012	-
S3.h	Iceberg lettuce	10.08.2012	-
S3.i	Tomato	10.08.2012	-
S3.i	Parsley	10.08.2012	-
S3.i	Greenleaf lettuce	10.08.2012	-
S3.i	Iceberg lettuce	10.08.2012	-
B3	Tomato	14.08.2012	-
B3	Parsley	14.08.2012	-
B3	Greenleaf lettuce	14.08.2012	-
B3	Iceberg lettuce	14.08.2012	-
B2	Tomato	14.08.2012	-
B2	Parsley	14.08.2012	-
B2	Greenleaf lettuce	14.08.2012	-
B2	Iceberg lettuce	14.08.2012	-
S3.g	Tomato	04.09.2012	-
S3.g	Parsley	04.09.2012	-
S3.g	Greenleaf lettuce	04.09.2012	-
S3.g	Iceberg lettuce	04.09.2012	-
S3.h	Tomato	04.09.2012	-
S3.h	Parsley	04.09.2012	-
S3.h	Greenleaf lettuce	04.09.2012	-
S3.h	Iceberg lettuce	04.09.2012	-
S3.i	Tomato	04.09.2012	-
S3.i	Parsley	04.09.2012	-
S3.i	Greenleaf lettuce	04.09.2012	-
S3.i	Iceberg lettuce	04.09.2012	-
S1.a	Tomato	08.09.2012	-
S1.a	Parsley	08.09.2012	+
S1.a	Greenleaf lettuce	08.09.2012	-
S1.a	Iceberg lettuce	08.09.2012	-
S1.b	Tomato	08.09.2012	-
S1.b	Parsley	08.09.2012	-
S1.b	Greenleaf lettuce	08.09.2012	-
S1.b	Iceberg lettuce	08.09.2012	-
S1.c	Tomato	08.09.2012	-

Table A.1 Fresh produce samples by suppliers and sampling date (continued)

Supplier code	Fresh produce	Sampling date	Result
S1.c	Parsley	08.09.2012	-
S1.c	Greenleaf lettuce	08.09.2012	-
S1.c	Iceberg lettuce	08.09.2012	-
B3	Tomato	11.09.2012	-
B3	Parsley	11.09.2012	-
B3	Greenleaf lettuce	11.09.2012	-
B3	Iceberg lettuce	11.09.2012	-
B2	Tomato	11.09.2012	-
B2	Parsley	11.09.2012	-
B2	Greenleaf lettuce	11.09.2012	-
B2	Iceberg lettuce	11.09.2012	-
B1	Tomato	11.09.2012	-
B1	Parsley	11.09.2012	-
B1	Greenleaf lettuce	11.09.2012	-
B1	Iceberg lettuce	11.09.2012	-
S2.f	Tomato	11.09.2012	-
S2.f	Parsley	11.09.2012	-
S2.f	Greenleaf lettuce	11.09.2012	-
S2.f	Iceberg lettuce	11.09.2012	-
S2.d	Tomato	11.09.2012	-
S2.d	Parsley	11.09.2012	-
S2.d	Greenleaf lettuce	11.09.2012	-
S2.d	Iceberg lettuce	11.09.2012	-
S2.e	Tomato	11.09.2012	-
S2.e	Parsley	11.09.2012	-
S2.e	Greenleaf lettuce	11.09.2012	-
S2.e	Iceberg lettuce	11.09.2012	-
S1.b	Tomato	24.09.2012	-
S1.b	Parsley	24.09.2012	-
S1.b	Greenleaf lettuce	24.09.2012	-
S1.b	Iceberg lettuce	24.09.2012	-
S1.c	Tomato	24.09.2012	-
S1.c	Parsley	24.09.2012	-
S1.c	Greenleaf lettuce	24.09.2012	-
S1.c	Iceberg lettuce	24.09.2012	-
S1.a	Tomato	24.09.2012	-
S1.a	Parsley	24.09.2012	-
S1.a	Greenleaf lettuce	24.09.2012	-
S1.a	Iceberg lettuce	24.09.2012	-
S2.d	Tomato	24.09.2012	-

Table A.1 Fresh produce samples by suppliers and sampling date (continued)

Supplier code	Fresh produce	Sampling date	Result
S2.d	Parsley	24.09.2012	-
S2.d	Greenleaf lettuce	24.09.2012	-
S2.d	Iceberg lettuce	24.09.2012	-
S2.e	Tomato	25.09.2012	-
S2.e	Parsley	25.09.2012	-
S2.e	Greenleaf lettuce	25.09.2012	-
S2.e	Iceberg lettuce	25.09.2012	-
S2.f	Tomato	25.09.2012	-
S2.f	Parsley	25.09.2012	-
S2.f	Greenleaf lettuce	25.09.2012	-
S2.f	Iceberg lettuce	25.09.2012	-
B2	Tomato	01.10.2012	-
B2	Parsley	01.10.2012	-
B2	Greenleaf lettuce	01.10.2012	-
B2	Iceberg lettuce	01.10.2012	-
B1	Tomato	01.10.2012	-
B1	Parsley	01.10.2012	-
B1	Greenleaf lettuce	01.10.2012	-
B1	Iceberg lettuce	01.10.2012	+
S3.g	Tomato	10.10.2012	-
S3.g	Parsley	10.10.2012	-
S3.g	Greenleaf lettuce	10.10.2012	-
S3.g	Iceberg lettuce	10.10.2012	-
S1.b	Tomato	10.10.2012	-
S1.b	Parsley	10.10.2012	-
S1.b	Greenleaf lettuce	10.10.2012	-
S1.b	Iceberg lettuce	10.10.2012	-
S1.c	Tomato	10.10.2012	-
S1.c	Parsley	10.10.2012	-
S1.c	Greenleaf lettuce	10.10.2012	-
S1.c	Iceberg lettuce	10.10.2012	-
S1.a	Tomato	10.10.2012	-
S1.a	Parsley	10.10.2012	-
S1.a	Greenleaf lettuce	10.10.2012	-
S1.a	Iceberg lettuce	10.10.2012	-
B1	Tomato	14.10.2012	-
B1	Parsley	14.10.2012	-
B1	Greenleaf lettuce	14.10.2012	-
B1	Iceberg lettuce	14.10.2012	-
S2.f	Tomato	14.10.2012	-

Table A.1 Fresh produce samples by suppliers and sampling date (continued)

Supplier code	Fresh produce	Sampling date	Result
S2.f	Parsley	14.10.2012	-
S2.f	Greenleaf lettuce	14.10.2012	-
S2.f	Iceberg lettuce	14.10.2012	-
S2.d	Tomato	14.10.2012	-
S2.d	Parsley	14.10.2012	+
S2.d	Greenleaf lettuce	14.10.2012	-
S2.d	Iceberg lettuce	14.10.2012	-
S2.e	Tomato	14.10.2012	-
S2.e	Parsley	14.10.2012	-
S2.e	Greenleaf lettuce	14.10.2012	-
S2.e	Iceberg lettuce	14.10.2012	-
B2	Tomato	14.10.2012	-
B2	Parsley	14.10.2012	-
B2	Greenleaf lettuce	14.10.2012	-
B2	Iceberg lettuce	14.10.2012	-
S3.h	Tomato	14.10.2012	-
S3.h	Parsley	14.10.2012	-
S3.h	Greenleaf lettuce	14.10.2012	-
S3.h	Iceberg lettuce	14.10.2012	-
S3.i	Tomato	14.10.2012	-
S3.i	Parsley	14.10.2012	-
S3.i	Greenleaf lettuce	14.10.2012	-
S3.i	Iceberg lettuce	14.10.2012	-
B3	Tomato	14.10.2012	-
B3	Parsley	14.10.2012	-
B3	Greenleaf lettuce	14.10.2012	-
B3	Iceberg lettuce	14.10.2012	-
S1.b	Tomato	02.11.2012	-
S1.b	Parsley	02.11.2012	-
S1.b	Greenleaf lettuce	02.11.2012	-
S1.b	Iceberg lettuce	02.11.2012	-
S1.c	Tomato	02.11.2012	-
S1.c	Parsley	02.11.2012	-
S1.c	Greenleaf lettuce	02.11.2012	-
S1.c	Iceberg lettuce	02.11.2012	-
S1.a	Tomato	02.11.2012	-
S1.a	Parsley	02.11.2012	-
S1.a	Greenleaf lettuce	02.11.2012	-
S1.a	Iceberg lettuce	02.11.2012	-
B3	Tomato	02.11.2012	-

Table A.1 Fresh produce samples by suppliers and sampling date (continued)

Supplier code	Fresh produce	Sampling date	Result
B3	Parsley	02.11.2012	-
B3	Greenleaf lettuce	02.11.2012	-
B3	Iceberg lettuce	02.11.2012	-
S3.i	Tomato	02.11.2012	-
S3.i	Parsley	02.11.2012	-
S3.i	Greenleaf lettuce	02.11.2012	-
S3.i	Iceberg lettuce	02.11.2012	-
S3.g	Tomato	02.11.2012	-
S3.g	Parsley	02.11.2012	-
S3.g	Greenleaf lettuce	02.11.2012	-
S3.g	Iceberg lettuce	02.11.2012	-
S3.h	Tomato	02.11.2012	-
S3.h	Parsley	02.11.2012	-
S3.h	Greenleaf lettuce	02.11.2012	-
S3.h	Iceberg lettuce	02.11.2012	-
B1	Tomato	04.11.2012	-
B1	Parsley	04.11.2012	-
B1	Greenleaf lettuce	04.11.2012	-
B1	Iceberg lettuce	04.11.2012	-
S2.f	Tomato	04.11.2012	-
S2.f	Parsley	04.11.2012	-
S2.f	Greenleaf lettuce	04.11.2012	-
S2.f	Iceberg lettuce	04.11.2012	-
S2.d	Tomato	04.11.2012	-
S2.d	Parsley	04.11.2012	-
S2.d	Greenleaf lettuce	04.11.2012	-
S2.d	Iceberg lettuce	04.11.2012	-
S2.e	Tomato	04.11.2012	-
S2.e	Parsley	04.11.2012	-
S2.e	Greenleaf lettuce	04.11.2012	-
S2.e	Iceberg lettuce	04.11.2012	-
B2	Tomato	04.11.2012	-
B2	Parsley	04.11.2012	-
B2	Greenleaf lettuce	04.11.2012	-
B2	Iceberg lettuce	04.11.2012	-
B3	Tomato	08.11.2012	-
B3	Parsley	08.11.2012	-
B3	Greenleaf lettuce	08.11.2012	-
B3	Iceberg lettuce	08.11.2012	-
S1.c	Tomato	08.11.2012	-

Table A.1 Fresh produce samples by suppliers and sampling date (continued)

Supplier code	Fresh produce	Sampling date	Result
S1.c	Parsley	08.11.2012	-
S1.c	Greenleaf lettuce	08.11.2012	-
S1.c	Iceberg lettuce	08.11.2012	-
S1.a	Tomato	08.11.2012	-
S1.a	Parsley	08.11.2012	-
S1.a	Greenleaf lettuce	08.11.2012	-
S1.a	Iceberg lettuce	08.11.2012	-
S1.b	Tomato	08.11.2012	-
S1.b	Parsley	08.11.2012	-
S1.b	Greenleaf lettuce	08.11.2012	-
S1.b	Iceberg lettuce	08.11.2012	-
S3.g	Tomato	08.11.2012	-
S3.g	Parsley	08.11.2012	-
S3.g	Greenleaf lettuce	08.11.2012	-
S3.g	Iceberg lettuce	08.11.2012	-

APPENDIX B

COMPOSITION OF BUFFERS AND SOLUTIONS

Table B.1 Composition of Buffers and Solutions

0,25 N HCl Solution

Formula	
5 N HCl	12.5 mL
Sterile dH_2O	247.5 mL

0,5 M EDTA, pH 8

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

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

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

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

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 (1%)-TBE

Formula	
Seakem Agarose	1 g
0.5X TBE solution	100 mL

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 m

APPENDIX C

COMPOSITION OF MEDIAS

Table C.1 Composition of Medias

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	

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	

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	

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	

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	

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	

Tryptone Soy Agar

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

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 D

ALLELIC PROFILES AND SEQUENCE TYPES OF *S.* ANATUM STRAINS IN UCC MLST DATABASE

STRAIN	aroC	dnaN	hemD	hisD	purE	sucA	thrA	ST	SEROTY	HOST_[	COUNT	YEAR
842/03	10	14	15	31	25	20	33	64	Anatum	Human	Portugal	2003
M264	10	14	15	31	25	20	33	64	Anatum	monophasic	Canada	2001
M44	10	14	15	31	25	20	33	64	Anatum		USA	2004
M73	10	14	15	31	25	20	33	64	Anatum		South Afri	2001
84K	10	14	15	31	25	20	33	64	Anatum	Duck	USA	1919
899/04	10	14	15	31	25	20	33	64	Anatum	Human	Portugal	2004
98-00485	10	14	15	31	25	20	33	64	Anatum	Snake	Germany	1998
99-01212	10	14	15	31	25	20	33	64	Anatum	Reptile	Germany	1999
M255	10	14	15	31	25	20	33	64	Anatum		Canada	2001
3203	10	14	15	31	25	20	33	64	Anatum	Human ca	Germany	1985
3229	10	14	15	31	25	20	33	64	Anatum	Human ca	Germany	1985
3232	10	14	15	31	25	20	33	64	Anatum	Human ca	Germany	1985
78K	10	14	15	31	25	20	33	64	Anatum	Poultry	U.S.A.	1918
RM_145	10	14	15	31	25	20	33	64	Anatum	River	USA	2006
RM_473	10	14	15	31	25	20	33	64	Anatum	River	USA	2008
SARB2 (Fi	10	14	15	31	25	20	33	64	Anatum	Human	USA	None
SARB2	10	14	15	31	25	20	33	64	Anatum	Human	U.S.A.	None
RM_226	10	14	15	31	25	20	33	64	Anatum	River	USA	2006
RM_392	10	14	15	31	25	20	33	64	Anatum	River	USA	2008
RM_370	10	14	15	31	25	20	33	64	Anatum	River	USA	2008
M133	10	14	15	31	25	20	33	64	Anatum		Australia	2001
M145	10	14	15	31	25	20	33	64	Anatum		Germany	2001
M180	10	14	15	31	25	20	33	64	Anatum		Denmark	2001
SSM4211	10	14	15	121	25	20	33	1694	Anatum		Morocco	None

Fig D.1 Allelic profiles and sequence types of *S.* Anatum in UCC MLST Database

APPENDIX E

ALLELIC PROFILES AND SEQUENCE TYPES OF *S.* CHARITY STRAINS IN UCC MLST DATABASE

STRAIN	aroC	dnaN	hemD	hisD	purE	sucA	thrA	ST	SEROTY	HOST	COUNT	YEAR
8073/93	125	63	17	62	12	58	3	383	Charity	Food	Sweden	1993
599K	125	63	17	62	12	58	3	383	Charity			None
R350	125	63	208	62	12	58	3	862	Charity	Reptile	Australia	2001

Fig E.1 Allelic profiles and sequence types of *S.* Charity in UCC MLST Database

APPENDIX F

ALLELIC PROFILES AND SEQUENCE TYPES OF S. MIKAWASIMA STRAINS IN UCC MLST DATABASE

STRAIN	aroC	dnaN	hemD	hisD	purE	sucA	thrA	ST	SEROTYPE	HOST_[	COUNT	YEAR
1220K	106	101	89	7	105	39	12	294	Mikawasima		U.S.A.	1960
1406K	106	101	217	7	105	39	12	921	Mikawasima		USA	1959
45K	106	289	122	7	105	39	12	922	Mikawasima	Rat	Japan	1937
MET_S1_4	14	2	331	7	105	19	12	1815	Mikawasima	Iceberg le	Turkey	2012

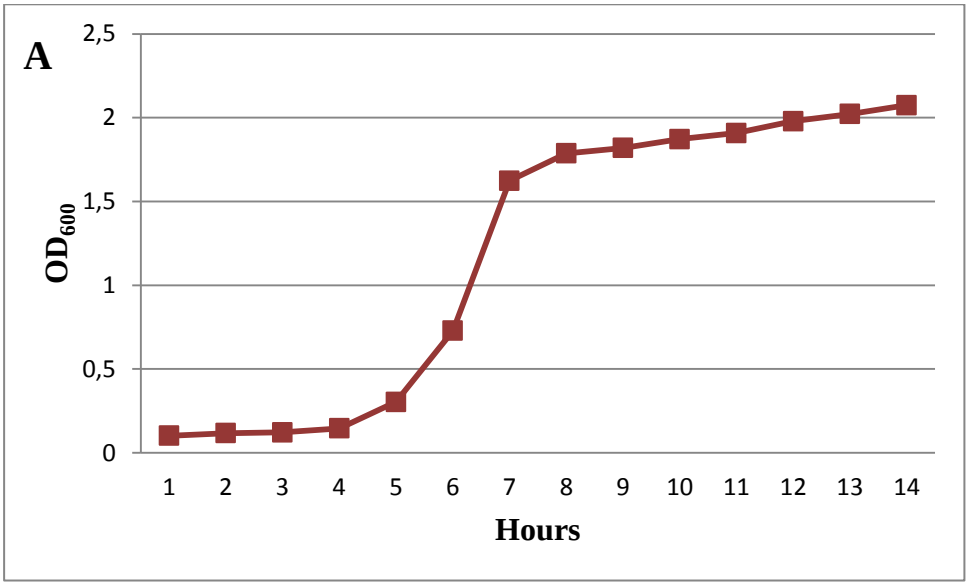
Fig F.1 Allelic profiles and sequence types of *S.* Mikawasima in UCC MLST Database

APPENDIX G

RESULTS OF GROWTH CURVE ANALYSIS OF *S.* ANATUM

Table G.1 Simultaneous quantification of *S.* Anatum by plate counting and measuring optical density at 600 nm

		TSA Plate Count									
Hours	**OD_{600}**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**Average Log N (cfu/ml)**
1	0,1025										
2	0,1179	>300	231	107	17						5,477266
3	0,1218										
4	0,1459	>300	232	186	43						5,328516
5	0,3037										
6	0,7291				295	126	62				6,860338
7	1,6233										
8	1,7866				>300	>300	270				8,431364
9	1,8199										
10	1,8717						>300	>300	>300		9,477121
11	1,9088										
12	1,9791						>300	>300	>300		10,43933
13	2,0217										
14	2,0750							>300	>300	280	11,44716



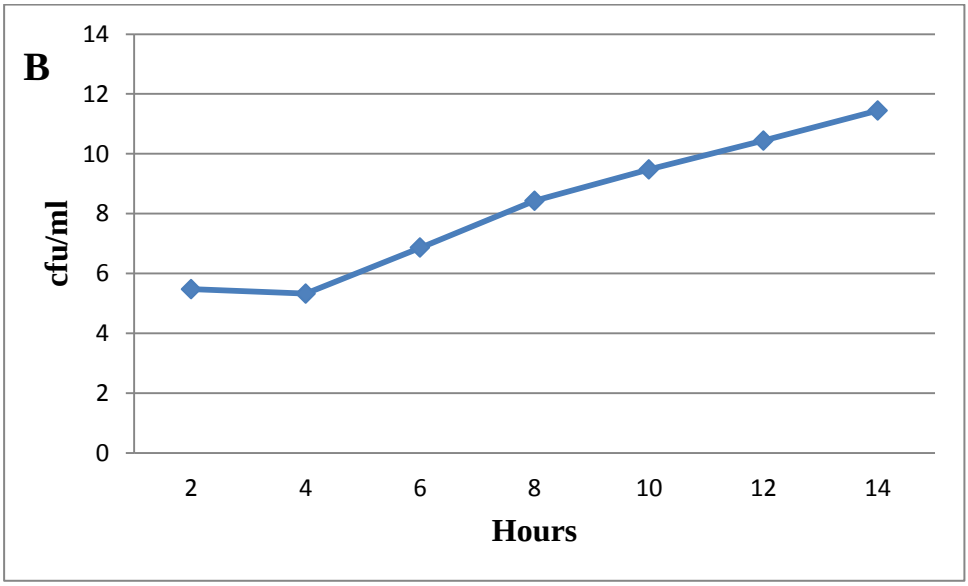


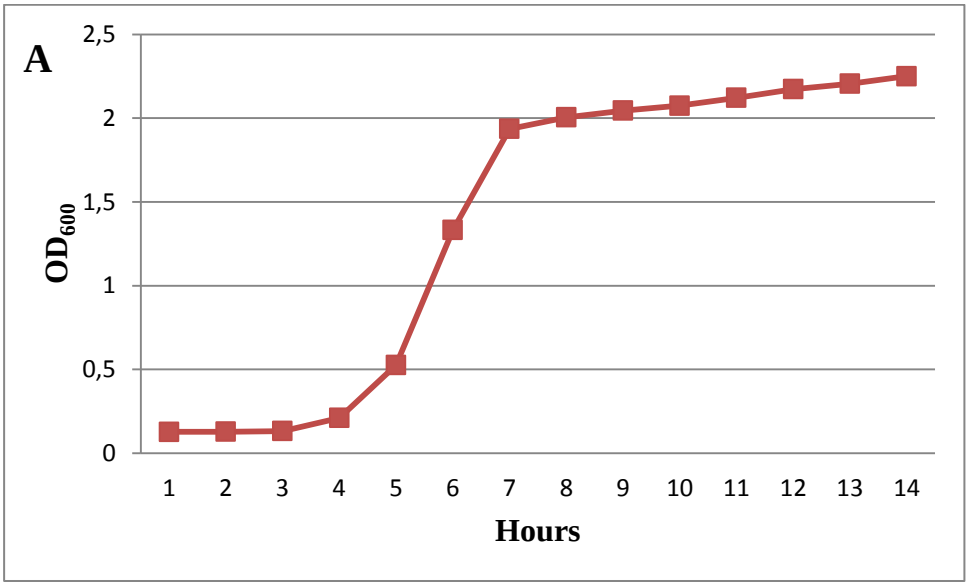
Fig G.1 Growth curves of *S.* Anatum using OD_{600} measurements (A) and cfu/ml counting method (B)

APPENDIX H

RESULTS OF GROWTH CURVE ANALYSIS OF *S.* CHARITY

Table H.1 Simultaneous quantification of *S.* Charity by plate counting and measuring optical density at 600 nm

		TSA Plate Count									
Hours	**OD_{600}**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**Average Log N (cfu/ml)**
1	0,1278										
2	0,1285		>300	243	43	16					6,3566
3	0,1332										
4	0,211		>300	269	61	34					6,15422
5	0,5259										
6	1,3324			202	167	60					6,91593
7	1,9357										
8	2,0052			>300	>300	265					8,42325
9	2,0447										
10	2,0750				>300	>300	104				9,01703
11	2,1210										
12	2,1727					>300	240	161			9,91222
13	2,2054										
14	2,2507						270	228	125		10,7004



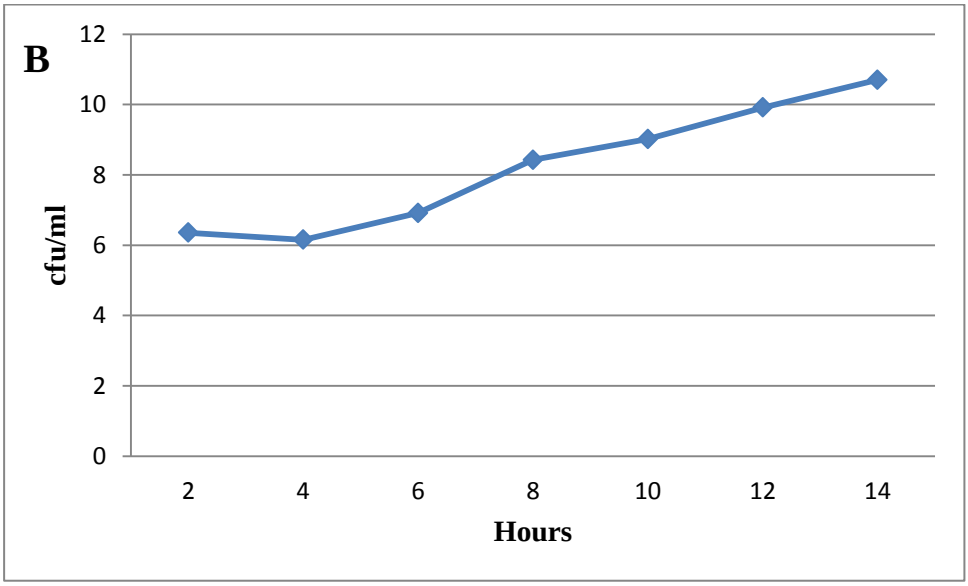


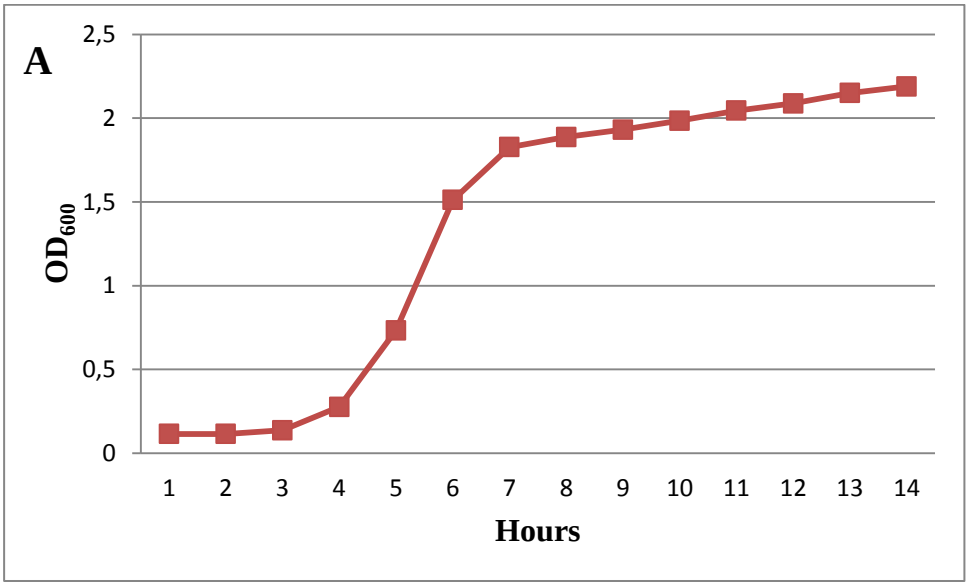
Fig H.1 Growth curves of *S.* Charity using OD_{600} measurements (A) and cfu/ml counting method (B)

APPENDIX I

RESULTS OF GROWTH CURVE ANALYSIS OF *S.* MIKAWASIMA

Table I.1 Simultaneous quantification of *S.* Mikawasima by plate counting and measuring optical density at 600 nm

		TSA Plate Count									
Hours	**OD_{600}**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**	**Average Log N (cfu/ml)**
1	0,1157										
2	0,1158	>300	>300	239	68						5,96332
3	0,1375										
4	0,2767		>300	248	56						5,60638
5	0,7333										
6	1,512			>300	265	183					8,02015
7	1,8268										
8	1,8877				>300	>300	265				8,42325
9	1,9310										
10	1,9843					>300	>300	104			9,44716
11	2,0447										
12	2,0876						>300	>300	178		10,25040
13	2,1497										
14	2,1887							270	211	179	10,82990



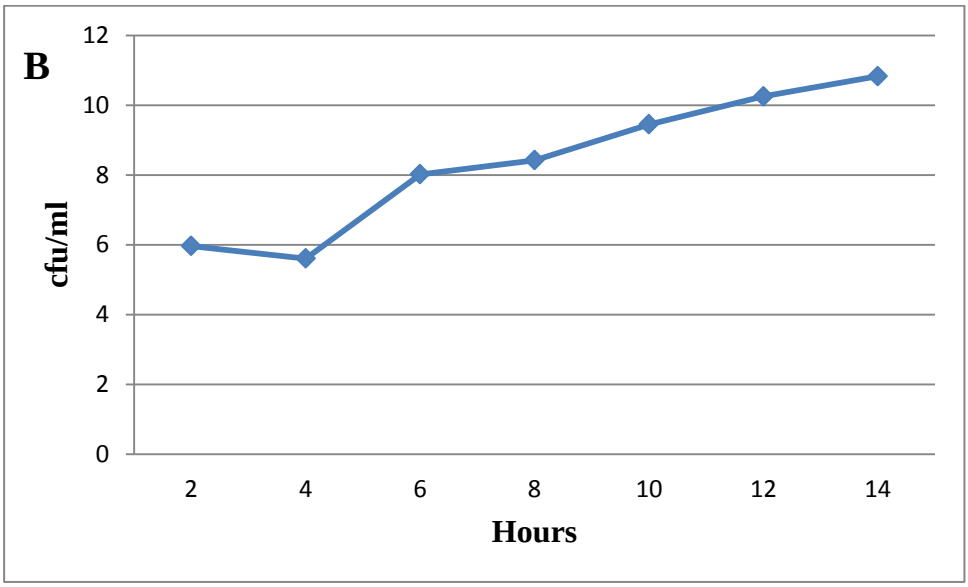


Fig I.1 Growth curves of *S.* Mikawasima using OD_{600} measurements (A) and cfu/ml counting method (B)