SALMONELLA PREVALENCE ON EGGS AND PREVENTION STRATEGY BY BACTERIOPHAGE

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ABSTRACT

SALMONELLA PREVALENCE ON EGGS AND PREVENTION STRATEGY BY BACTERIOPHAGE

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Today, eggs and egg products are one of the biggest food sources in the World and Turkey is one of the biggest hen's egg producers in the world. In Turkey, there are 4 different poultry cultivation methods which are organic egg, free-range egg, cage-free egg and cage egg. *Salmonella* is one of the most common pathogen bacteria found in egg industry. Thus, protection of food sources from *Salmonella* like pathogen is very crucial for food safety requirements. In this study, *Salmonella* prevalence in eggs from cultivation methods of hen's were investigated and bacteriophage application, an alternative prevention method, was used to eliminate presence of *Salmonella.*

For this purpose, 250 different eggs were collected according to their cultivation methods (organic, free-range, cage-free-cage-village) and 17 *Salmonella* strains (6.8%) were isolated in this study. Prevalence of *Salmonella* in free-range egg was 12%, cage-free egg was 10% and cage egg was 12%. However, no *Salmonella* was found in commercial organic eggs and village eggs. From those 17 *Salmonella*

strains, 3 different serotypes *Salmonella* Infantis (88.2%), *Salmonella* Kentucky (5.88%) and *Salmonella* Telaviv (5.88%) were determined.

In the bacteriophage application study, 10^8 PFU/mL bacteriophage solution was used for egg samples which were initially contaminated with 2 different *Salmonella* solutions (10^7 cfu/mL & 10^5 cfu/mL) to test the ratio of the numbers of virus particles to the numbers of the host cells in a given infection medium which is known as multiplication of infection (MOI). After incubation of contaminated eggs at 4 °C for 7 days, *Salmonella* was counted. Respectively, 2.24 log reduction and 3.64 log reduction of *Salmonella* was obtained by 10 MOI and 10^3 MOI. Further studies should be conducted to increase the efficiency of bacteriophage treatment on commercial eggs and different food sources.

Keywords: *Salmonella*, Egg, Bacteriophage

ÖZ

YUMURTALARDA *SALMONELLA* YAYGINLIĞI VE BAKTERİYOFAJ İLE ÖNLEME STRATEJİSİ

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Bugün yumurta ve yumurta ürünleri dünyanın en büyük besin kaynaklarından biridir ve Türkiye dünyanın en büyük tavuk yumurtası üreticilerinden biridir. Türkiye'de organik yumurta, serbest dolaşan yumurta, kafessiz yumurta ve kafes yumurta olmak üzere 4 farklı kanatlı yetiştirme yöntemi bulunmaktadır. *Salmonella* yumurta endüstrisinde en yaygın bulunan patojen bakterilerden biridir. Bu nedenle gıda kaynaklarının *Salmonella* benzeri patojenlerden korunması gıda güvenliği gereksinimleri için çok önemlidir. Bu çalışmada, tavuk yetiştirme yöntemlerinden elde edilen yumurtalarda *Salmonella* prevalansı araştırılmış ve *Salmonella* varlığını ortadan kaldırmak için alternatif bir yöntem olan bakteriyofaj uygulaması kullanılmıştır.

Bu amaçla yetiştirme yöntemlerine göre (organik, serbest dolaşan, kafessiz-kafes-köy) 250 farklı yumurta toplanmış ve bu çalışmada 17 *Salmonella* suşu (%6,8) izole edilmiştir. Serbest dolaşan yumurtada *Salmonella* prevalansı %12, kafessiz yumurta %10 ve kafes yumurtası %12 olarak tespit edilmiştir. Bu çalışmada organik yumurtalarda ve köy yumurtalarında *Salmonella* bulunmamıştır. Elde edilen 17

Salmonella suşundan 3 farklı serotip *Salmonella* Infantis (%88,2), *Salmonella* Kentucky (%5,88) ve *Salmonella* Telaviv (%5,88) tespit edildi.

Bakteriyofaj uygulamasında, başlangıçta 2 farklı *Salmonella* solüsyonu (10^7 & 10^5 cfu/mL) ile kontamine olmuş yumurta örnekleri için 10^8 PFU/mL bakteriyofaj solüsyonu kullanılarak enfeksiyon çoğalması (MOI) olarak bilinen enfeksiyon ortamında virüs parçacıklarının sayısının konak hücre sayılarına oranları test edilmiştir. Kontamine edilen yumurtalar 4 °C' de 7 gün inkübe edildikten sonra *Salmonella* sayımı yapılmıştır. 10 MOI ve 10^3 MOI ile sırasıyla 2.24 log azalma ve 3.64 log azalma elde edilmiştir. Ticari yumurtalarda ve farklı gıdalarda bakteriyofaj tedavisinin etkinliğini artırmak için daha ileri çalışmalar yapılması gerekmektedir.

Anahtar Kelimeler: *Salmonella*, Yumurta, Bakteriyofaj

To my beloved family …

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CHAPTER 1

INTRODUCTION

In today's world, the poultry industry is one of the biggest food industries in worldwide. According to latest data, Turkey is 8th biggest hens egg producer in the world (Diker et al., 2020). Recently, consumers preferred more natural food products than processed food products and eggs and eggs products are known as natural food source for consumers. Because of nutritional value of egg and being natural food source, demand and consumption rate of egg are increased. Also, eggs are considered as a cheap food source for consumption. From the food safety aspect, providing sufficient sanitation and disinfection is very crucial for egg production. *Salmonella* is one of the most common pathogen bacteria in the world and this bacterium is found in egg industry very commonly. Most common source of *Salmonella* infection and outbreaks are eggs and egg products compared to other food sources (Diker et al., 2020). According to reported outbreaks in the world, main source of 22.6 % of the *Salmonella* outbreaks is consumption of raw eggs. Other than egg, meat, chicken, and dairy products might be the cause of *Salmonella* outbreaks. Thus, protection of food sources from Salmonella like pathogen is very crucial for food safety requirements. Contamination of eggs by *Salmonella* could be happened with two different ways. Eggs can be contaminated both internally and externally. However, prevalence of *Salmonella* contamination is higher on the surface (Savi et al., 2011).

There are many methods for sanitation for food production. In commercial hen's egg production, increasing shelf-life of egg is very important for consumers. For this purpose, many methods which are classified in three group; chemical, physical, and biological method were tried to inhibit microorganism growth and reducing existing microbial reproduction. Many of these methods are considered as expensive and

inadequate (Makalatia et al., 2018). Because of increasing number of antibiotic-resistance of bacteria, traditional methods for prevention like antibiotic treatment are out of the question (Henriques et al., 2013). Therefore, there has been searching for new application for controlling *Salmonella* infection in egg and egg production facilities.

Another aspect of hen's egg production is cultivation method. Today, there are four different methods were used in Turkey. Organic, free-range, cage-free and cage cultivation system is applied for egg production facilities (Yenilmez & Uruk, 2016). Differences in these breeding methods might affect the infection of pathogens and there and not enough research about these particular subjects.

Bacteriophage is known as bacterial viruses that only affect bacteria. they can only exist with bacterial presence and phages do not infect eucaryotic cells. Because of their host specificity, bacteriophage do not affect unrelated bacteria. Also, fast bacterial elimination, being specific to bacteria and self-replicability make bacteriophages most suitable for food safety applications (Spricigo et al., 2013). Application of bacteriophage therapy was implemented in different food products like milk, eggs, and seafood. At low storage temperature, bacteriophage application was achieved 3 log unit reduction against *Salmonella* (Thung et al., 2017). So, phage application can be implemented to the food industry with sufficient effort.

In this study, prevalence of *Salmonella* on different types of eggs were detected and to eliminate *Salmonella* on eggs by the help of bacteriophage. Isolation was performed from commercially collected eggs and village eggs. These commercial eggs were selected from different brands and cultivation methods. Then, phage therapy effect on contaminated eggs in a laboratory setting was performed. The effect of differences in cultivation methods on *Salmonella* contamination, natural contamination ratio and naturally contaminated *Salmonella* serovars were detected. Then, phage therapy effect on contaminated eggs in a laboratory setting was performed. These applications help for better understanding of which *Salmonella* serovars were found in eggs, natural contamination ratio of *Salmonella* in egg in

Turkey and give hints about effect of cultivation method on *Salmonella* infection on egg. Also, this study gives a preliminary information for bacteriophage applications to be adapted to egg production industry.

CHAPTER 2

LITERATURE REVIEW

2.1 *Salmonella*

Salmonella is facultative, Gram-negative rod shape bacterium in the family of *Enterobacteriaceae*. Most strains of *Salmonella* are motile with peritrichous flagella. *Salmonella* can be distinguished from other members of family by lack of fermentation of lactose and glucose with producing gas. The Centers for Disease Control and Prevention (CDC) is currently employed nomenclatural system for *Salmonella.* World Health Organization (WHO) also recommend this nomenclatural system. However, this system is still changing. According to this system, there are two main species for *Salmonella* genus. *Salmonella enterica* which is type species is classified into six subspecies. *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (III), *S. enterica* subsp. *diarizonae* (IV), *S. enterica* subsp. *houtenae* (V) and *S. enterica* subsp. *indica* (VI). *Salmonella enterica* subspecies *enterica* is most encountered subspecies. Almost 99 % of human infections is caused by this subspecies in human and warm-blooded animals. *Salmonella bongori* can be differentiated from *Salmonella enterica* by their 16S rRNA sequence analysis. *Salmonella bongori* and other five subspecies of *Salmonella enterica* generally can be seen in cold-blooded animals and in the environment. Human cases of *Salmonella bongori* and other subspecies of *enterica* are very rare (Eng et al., 2015). *S. enterica* subsp. *enterica* can growth in a very wide temperature range (4 - 47 °C) but the best growing temperature for *Salmonella* is 37 °C. *Salmonella* is one of the pathogenic bacteria that can cause human diseases. Generally, *Salmonella* is associated with food-borne outbreaks (Cox et al., 2000). *Salmonella* species are accepted as one of the most important foodborne pathogen in

the world (Jamshidi et al., 2010). *Salmonella* genus is divided into two group. Zoonotic non-typhoidal *Salmonella* serovars & human-adapted typhoidal *Salmonella* serovars. These two serovars significantly cause human infections and deaths (Hiyoshi et al., 2018). Two major human diseases which are localized gastroenteritis with an intact immune system and invasive bloodstream infection with a compromised immune system can be associated with non-typhoidal *Salmonella* serovars. For typhoidal *Salmonella* serovars, an invasive bloodstream infection with an intact immune system can be seen as a major human disease syndrome (Hiyoshi et al., 2018). Typhoidal fever is generally seen in the developing world. However, non-typhoidal Salmonellosis found worldwide. 93.8 million non-typhoidal *Salmonella* (NTS) infections result approximately 155,000 deaths every year. Animal base food products like dairy products, poultry and eggs are the reason for NTS transmission (Gal-Mor et al., 2014). Non-typhoidal *Salmonella* (NTS) are general reason for human infection because of contaminated foods and *S. enterica* subsp. *enterica* serovar Enteritidis is one of the most encountered serotype of NTS (Saravanan et al., 2015). It needs 8 to72 hours to incubate. *Salmonella* generally cause diarrhea, abdominal pain, meningitis and sometimes fever (Cox et al., 2000; Saravanan et al., 2015). More than 2,600 *Salmonella* serotypes were identified and most of the serotypes are known as human pathogens and 10 % of these serotypes were originated from poultry (Nagappa et al., 2007).

2.2 Transmission Route of *Salmonella*

There are many common mechanisms and vehicles for transmission of *Salmonella* to human beings. *Salmonella* can be found in nature very easily. Intestinal tract of any wild and domestic animal might have *Salmonella* strains. Therefore, there are many sources of *Salmonella* infection(Carrasco et al., 2012). Technological developments, industrial changes and human lifestyle can also be factor for *Salmonella* infection (Carrasco et al., 2012). Rare and raw meat, poultry and eggs are best known route for *Salmonella* to infect human beings. Also, any other food

can be cross contaminated by these best-known foods. Dairy products like unpasteurized milk, cheese and ice cream can be reason for the transmission of *Salmonella*. Transmission of *Salmonella* can be happened in the food preparation areas because of cross-contamination, unsuitable storage temperature and insufficient cooking (Carrasco et al., 2012). Other than these, contaminated water, contact with the feces of animals or infected animals are other reasons for *Salmonella* transmission to human beings. In order for the disease to be transmitted from person to person, the person who is sick must have diarrhea rather than a carrier state (Gantois et al., 2009). According to research, 1 out of 20,000 eggs could be contaminated with *Salmonella* over 200 million cases of produced eggs. These infections can cause big economic losses annually up to 1 billion dollar (Lakins et al., 2008). With modern poultry, there could be more changes for infection of poultry animals (Saravanan et al., 2015). The main source of *Salmonella* outbreak is thought to be of animal origin and food with animal sources like beef, poultry meat, milk and eggs. Even so, poultry product like eggs, egg products and poultry meat are considered as a primary source of this pathogen (Jamshidi et al., 2010). Eggs and poultry products are the main reason for infection of *Salmonella* Enteritidis (Carrasco et al., 2012). Eggs are known as reliable source of nutrition and eggs can supply more functions than many other products (Moosavy et al., 2015). Contamination of *Salmonella* was rarely encountered in commercial eggs. According to European Union data, 0.3 % of *Salmonella* contamination was observed in eggs. However, most important source of foodborne *Salmonella* outbreaks are originated from eggs and egg products (Makalatia et al., 2018); (Spricigo et al., 2013). Most of the *Salmonella* outbreaks which are caused by raw or undercooked eggs come from egg industry (Cox et al., 2000); (Jamshidi et al., 2010). After investigation of cases about *Salmonella* outbreaks, infection of this pathogen is generally caused by consumption of lightly cooked eggs, packed egg sandwich and scrambled eggs. This showed that naturally contaminated eggs can have big amount of cell count before cooking (Bustamante, 2019). Because of contaminated eggs with antimicrobial-resistant *Salmonella* serotypes, investigation

of these bacteria became very important (Saravanan et al., 2015). Abnormalities in the eggshell like small defects could be the first sign of the entry of foodborne pathogens into the eggs (K. K. Chousalkar & Roberts, 2012). According to the Center for Disease Control and Prevention, *Salmonella* Enteritidis outbreaks which were associated with raw or undercooked egg consumption were constituted 75 % of total *Salmonella* Enteritidis cases. For this reason, mandatory test for commercial egg producers and *Salmonella* Enteritidis cases were reduced 12 % (Lakins et al., 2008). *Salmonella* Enteritidis can be grew in egg yolk or albumen of developing egg by infected hens (Lakins et al., 2008). Even though *Salmonella* Enteritidis can grow in albumen, bacteriostatic and bactericidal characteristics of albumen proteins do not allow too much bacterial growth but egg yolk can provide very good nutritious medium for *Salmonella* (Lakins et al., 2008). The major *Salmonella* serotype of causing outbreak is *Salmonella* Enteritidis (Cox et al., 2000). Also, *Salmonella* Typhimurium is most common *Salmonella* serovar related with foodborne outbreaks in Australia (Crabb et al., 2019). Most common serovars which were isolated from humans are *Salmonella enterica* serovar Enteritidis and *S. enterica* serovar Typhimurium 46.6 % and 6.9 % respectively (Spricigo et al., 2013). These two serovars can be found in many parts of the world and can be carried by many animals. *Salmonella* Enteritidis is generally found in eggs and egg products. However, *Salmonella* Typhimurium is mostly found in pigs, cattle, poultry meat and other infected birds (Moosavy et al., 2015). *Salmonella* Entertidis and *Salmonella* Typhimurium are found in reproductive tract of hens. These pathogens infect hens and cause contamination during egg formation. However, *Salmonella* Entertidis can resist even after, eggs are laid (Jamshidi et al., 2010). Lysozyme which is naturally formed in eggs is very useful to kill gram-positive organisms. However, this method is not effective on *Salmonella.* So, first defense mechanisms of eggs against *Salmonella* are shell, shell membrane and albumen (Lakins et al., 2008). Also, microflora of eggshell is dominated by gram-positive bacteria. This situation gives an advantage to Gram-negative bacteria to surpass the antimicrobial defense of eggs (K. K. Chousalkar & Roberts, 2012).

2.3 Contamination Route of *Salmonella* in Eggs

In all around the world, eggs and egg products are consumed as a preferred food source. So, protection of eggs and egg products against pathogen like *Salmonella* is very important. Contaminated eggs can be produced during egg formation in the reproductive system of hens or the environmental conditions (Moosavy et al., 2015). Contamination of eggs by *Salmonella* could be happened with two different ways. Eggs can be contaminated both internally and externally (Savi et al., 2011).However, prevalence of *Salmonella* contamination is higher on the surface (Crabb et al., 2019).

2.3.1 Vertical Transmission

In the vertical transmission, contamination of eggs was originated from infected reproductive organs. In this case, yolk, albumen and eggshell were contaminated directly (Moosavy et al., 2015). Most likely, *Salmonella* is transmitted from infected hens. According to some researchers, laying hens can produce infected egg because of oral inoculation. *Salmonella* cannot be easily detected even the hens are infected. If inoculation of *Salmonella* is low, there is not big effect on hens. However, eggs are still contaminated. When higher doses of *Salmonella* are inoculated, this causes decreasing in egg production. In another research, oviduct of hens also can be contaminated. When, intravenous is inoculated, this situation caused colonization of *Salmonella* in the ovary. For this reason, contamination egg is produced in the oviduct. Except this, intravaginal inoculation of hens caused colonization only in the lower part of oviduct. Even so, eggs were internally contaminated. Also, eggs were contaminated by inoculated hen's ovary. In the same farm, hens which are not infected might be infected by inoculated hens. This cause laying infected eggs. Researchers explained that *Salmonella* inoculation could be through airborne by conjunctival route. (Cox et al., 2000).

2.3.2 Horizontal Transmission

In the horizontal transmission, pathogen like *Salmonella* which are source of contaminated feces, colonized gut or after oviposition penetrate from the eggshell (Moosavy et al., 2015). These *Salmonella* could be infected through farm environment, and this caused contaminated eggs. When the biology of the laying hens are examined, these animal has only one opening for all intestinal, urinary and reproductive system and this could cause external eggshell contamination because of contact between fecal material and eggshell cannot be prevented (K. K. Chousalkar & Roberts, 2012). In the farm environment, hens can bring feces and soil into their nest. These materials could contain *Salmonella.* By reducing contact between hens and increasing cleaning and disinfecting farm environments, the external contamination of egg could be decreased (Savi et al., 2011). Bacterial penetration of eggs is affected by pH, number of pores, egg quality and vapor pressure. Because of nest environment is wet and warm, *Salmonella* can easily grow. Even though, outer and inner shell of egg can provide some protection against bacteria, *Salmonella* can rapidly penetrate eggshell (Cox et al., 2000). *Salmonella* have ability to penetrate the eggshell and can reach the egg yolk easily (Savi et al., 2011). Also, increasing storage temperature caused increasing *Salmonella* number in eggs (Cox et al., 2000). Although its original source is not fully known, contamination of eggs can be affected by season to season, farm environment and hygiene problems. Also, they stated that, prevalence of egg contamination is higher at the onset of lay (Crabb et al., 2019). Beside these, cross-contamination can be one of the reasons for horizontal transmission. Cross-contamination contamination could be happened during processing, transportation or storage of shell eggs (Lakins et al., 2008). Although, it is not yet clear which route is most important, most of authors claimed that horizontal transmission is the most significant path for *Salmonella* contamination (Gantois et al., 2009).

2.4 *Salmonella* Outbreaks Linked to Eggs and Other Products

Even though all applied precautions in food industry, *Salmonella* outbreaks are second most common zoonosis disease (Spricigo et al., 2013). Annually, 93.8 million people approximately are diagnosed with *Salmonella* and almost 155,000 of those infected are fatal all around the world (K. Chousalkar et al., 2018). General causes of *Salmonella* outbreaks are animal origin contaminated food such as eggs and egg products. Different types of *Salmonella* Enteritidis and *Salmonella* Typhimurium are the main cause of human *Salmonellosis*. Nevertheless, *Salmonella* Enteritidis is very common when serovars are isolated from eggs and egg products in USA and UK (K. Chousalkar et al., 2018). Cooking will mostly destroy all the presence of bacteria including *Salmonella.* Presence of *Salmonella* is estimated 1 out of 20,000 from all microorganisms in egg. In United States, percentage of naturally contaminated eggs which are produced by commercially is very low. Egg contamination percentage of naturally infected flock is under 0.03 % (Lakins et al., 2008) (K. Chousalkar et al., 2018). Even though risk of infection from egg is very low, many consumers prefer raw or lightly cooked eggs to eat. So, risk of foodborne illness from egg rises (K. Chousalkar et al., 2018). Between 1985 and 1999, almost 80 % of *Salmonella* Enteritidis outbreaks were linked to eggs and egg products. Also, 62.5 % of all infection cases were caused by *Salmonella* Enteritidis and 12.9 % of them were caused by *Salmonella* Typhimurium (Moosavy et al., 2015). Besides, there are approximately 1.4 million non-typhoidal *Salmonella* (NTS) infections annually in the USA. 15,000 of them are hospitalized and 580 are dead every year according to estimations (Savi et al., 2011). In 2007, there were 1,195 recorded cases which were linked to *Salmonella* in Brazil and 22.6 % of these cases were originated from raw egg consumption (Savi et al., 2011). According to OZFoodnet report between 2003 – 2015, there were 275 egg related *Salmonella* outbreaks in Australia and New Zealand (K. Chousalkar et al., 2018).

There are many food products that can cause human salmonellosis such as meat and meat products, cheese, dairy products, fish and fish products, pig meat and their

products etc. Among them, egg and egg products have more strongest evidence for food-borne outbreaks. According to EFSA report about food-borne outbreaks in 2017 (Food & Authority, 2018).

Table 2. 1 Food types that cause salmonellosis (Food & Authority, 2018).

Food type	Number of evidence for food-borne outbreak	% Of total
Egg and egg products	99	36.8
Bakery products	45	16.7
Mixed food	34	12.6
Meat and meat products	22	8.2
Other foods	15	5.6
Pig meat and their products	12	4.5

According to EFSA report, there were many human *Salmonellosis* outbreaks in the European Union during 2013-2017 (Table 2.2). Like other food sources, egg and egg products caused many Salmonellosis cases. In the table below, salmonellosis cases which are linked to egg and egg products can be seen (Food & Authority, 2018).

Table 2. 2 Salmonellosis cases which are linked to eggs and egg products in EU countries (Food & Authority, 2018).

Year	Number of sampled units	Number of reporting countries
2013	30,283	19
2014	23,536	20
2015	9,768	19

Table 2.2 (continued)

2016	11,137	21
2017	17,315	23

According to Centers for Disease Control and Prevention (CDC) report about *Salmonella* outbreaks, there were some recent multistate outbreaks which were associated with egg and egg products in US (cdc.gov).

Table 2. 3 *Salmonella* outbreaks link to eggs and egg products.

Year	Case count	Effected states	Hospitalizations	Deaths
2010	1608	4	11	0
2016	8	3	2	0
2017	44	11	12	0
2018	45	10	11	0

Production of hen egg in Turkey is almost 1.25 billion tonnes in 2017. Turkey is the 8th largest hen egg producers in the world. According to a study conducted in Turkey, Most encountered *Salmonella* serotype was *S*. Infantis (14.7 %) within all serotypes (Diker et al., 2020). According to Republic of Türkiye Ministry of Agriculture and Forestry, most encountered *Salmonella* serotype was *S*. Enteritidis (70.5 %) within all serotypes. Prevalence of *S*. Typhimurium and *S*. Infantis were respectively 8.5 % and 4.4 % (Gıda ve Kontrol Genel Müdürlüğü, 2018).

2.5 Egg Production and Consumption in Turkey

The poultry industry is one of the most important food sectors in Turkey despite facing many problems such as microbial pathogens, high feed cost and the global

financial crisis. In 2017, Turkey's poultry meat and hen egg production reached to 2.1 million tonnes and 1.25 billion tonnes, respectively, ranking 8th in the world for hen egg production (Diker et al., 2020). Commercial egg production volume in Turkey is increasing. As a result, the number of eggs per capita has increased (Table 2.4). In addition, the consumption of eggs has increased as consumers have recently turned to a healthy and natural diet. With the establishment of new operations in egg production and the increase in volume, export volumes have also increased (Yumurta Tavukçuluğu Verileri 2018, 2018).

Table 2. 4 Egg poultry data in Turkey (Yumurta Tavukçuluğu Verileri 2018, 2018).

	2017	**2018**
Number of Chicken	121.294.047	127.372.689
Number of Operation	984	1080
Number of Poultry Houses	3063	3211
Imports of Breeder Laying Chicks	792.096	769.951
Commercial Egg Production	20,3 Billion	22,3 Billion
Informal Production	2,5 Billion	2 Billion
Organic Egg Production	93.041	160.893
Production Per Person	252	295
Consumption Per Person	214	224
Amount of Export (dollar)	376.607.865	430.725.307
Amount of Export	5.597.966.496	5.780.407.352
Population	80.810.525	82.003.882

Table 2. 5 Number of poultry chicken in Turkey (Yumurta Tavukçuluğu Verileri 2018, 2018).

	Piece (*1000) in 2017	**Percentage rate (%) in 2017**	**Piece (*1000) in 2017**	**Percentage rate (%) in 2017**
Number of Poultry	348 144	100,0	359 218	100,0
Chicken	342 801	98,5	353 561	98,4
Meat chicken	221 245	64,5	229 507	64,9
Egg Chicken	121 556	35,5	124 055	35,1

With the increase in commercial egg production in Turkey, egg consumption has gradually increased. In 2018, the number of eggs per capita was 294 per year (Table 2.6). In addition, in a study conducted in 2007, the annual egg consumption per capita in Turkey was stated as 9.08 kg. Egg consumption in the world has been announced as 8.57 kg, and Turkey is above this average. (Table 2.7). (Mizrak et al., 2012).

Table 2. 6 Commercial egg production per person in Turkey (Yumurta Tavukçuluğu Verileri 2018, 2018).

Years	**Production (million)**	**Population (million)**	**Production Per Person**
2010	12.737	73	174
2011	13.980	74	188
2012	15.677	75	207
2013	16.700	76	218
2014	17.600	77	226
2015	17.200	78	218
2016	18.655	79	233

Table 2. 6 (continued)

2017	20.254	80	252
2018	22.231	82	294

Table 2. 7 The annual per capita egg consumption in Turkey (Mizrak et al., 2012).

Country	Annually Egg Consumption (per person)
China	17.41 kg
USA	14.29 kg
European Union	12.44 kg
Turkey	9.08 kg
World Average	8.57 kg

Turkey increased its annual chicken egg export from 156.6 million dollars to 430 million dollars from 2010 to 2018, showing a growth of 275 % (Table 2.8). When the data of 2017 is examined, Italy takes the lead with 2,219,583 million dollars among the countries with the largest volume in egg export. Poland and France follow Italy with exports of 391,660 and 376,709 million dollars, respectively (Table 2.9). Turkey, on the other hand, has a very important position in the egg industry with an export of 376.7 million dollars in 2017 (Yumurta Tavukçuluğu Verileri 2018, 2018).

Table 2. 8 Egg export by years in Turkey (Yumurta Tavukçuluğu Verileri 2018, 2018).

Year	Export (Million Dollar)
2010	156,6
2011	285,6
2012	350,9
2013	406,7

Table 2. 8 (continued)

2014	404,1
2015	275,4
2016	290,4
2017	376,7
2018	430,7

Table 2. 9 Egg products imported by country in Turkey (Yumurta Tavukçuluğu Verileri 2018, 2018).

Country	**Imports (Dollars)**
France	376.709
Italy	2.219.583
Netherlands	271
Belgium	9.342
Ukraine	182.963
Poland	391.660
Germany	634
USA	186
India	68.711
Total	3.250.059

When the egg producing countries in the world are analyzed, Turkey ranks 8th with an annual production of 1,250,075 tons, while China is the leader in egg production with an annual production of 31,338,856 tons (Table 2.10). When the data are examined, it is observed that Turkey has an important position in egg consumption as well as egg production. When the data are examined, it is observed that Turkey has an important position in egg consumption as well as egg production. However, it is very important to take necessary food safety precautions during the production

of eggs. It is very important to apply the necessary sanitation methods to prevent any contamination or contamination (Yumurta Tavukçuluğu Verileri 2018, 2018).

Table 2. 10 Top 10 country in egg production (Yumurta Tavukçuluğu Verileri 2018, 2018).

Rank	Country	Production (Ton)
1	China	31.338.856
2	USA	6.258.795
3	India	4.847.500
4	Japan	2.601.173
5	Brazil	2.547.171
6	Mexico	2.171.198
7	Indonesia	1.527.135
8	Turkey	1.250.075
9	France	955.000
10	Ukraine	886.500

2.6 Antibiotic Resistance of *Salmonella* from Eggs

In today's world, usage of antibiotics fight against bacteria are very prevalent, but many bacteria have gained antibacterial resistance because of misusing or overusing of antibiotics. Also, this caused spreading of antibacterial resistance among bacteria. one of the reasons for spreading antibacterial resistance is using antibiotic in animal feed to increase their growth. During 1960s, first *Salmonella* resistance against antibiotic which was chloramphenicol was detected. Antibiotic resistance has increased rapidly since then (Eng et al., 2015). There are many *Salmonella* serotypes that are linked to eggs and poultry such as *Salmonella* Typhimurium, *Salmonella* indiana, *Salmonella* Enteritidis and *Salmonella* Kentucky and wide variety of antibiotics were used to inhibit infection of these pathogenic bacteria and support the growth of hens. According to research demonstrated that 36 different antibiotics

were detected in farms and percentage of usage of antibiotics in poultry farms was 19.6 %. Most common antibiotics used in poultry are β-Lactams, cephalosporins, and fluoroquinolone (Li et al., 2020). According to study conducted in India, 12 different antibiotics were used to observe the susceptibility of eggs and Novobiocin resistance was the highest from Salmonella that were isolated from eggs (Harsha, 2011).

In Turkey, antibacterial resistance of *Salmonella* serotypes that were isolated from laying hens were investigated. Results showed that all serotypes are highly resistant to some antibiotics such as sulfamethoxazole and nalidixic acid. In addition, most serotypes were found to be more than moderately resistant to antibiotics such as streptomycin, tetracycline and trimethoprim. Among these serotypes, *S.* Kentucky was resistant to ampicillin with a rate of 76.9 % and was the only serotype resistant to cefotaxime. Also, *S.* Infantis showed great resistance to all antibiotics except Cefotaxime. (Gıda ve Kontrol Genel Müdürlüğü, 2018).

Table 2. 11 Antibacterial resistance of *Salmonella* serotypes that were isolated from laying hens in Turkey (%).

	Ampicillin	**Cefotaxime**	**Streptomycin**	**Tetracycline**	**Gentamicin**	**Chloramphenicol**	**Ciprofloxacin**	**Nalidixic acid**	**Trimethoprim**	**Sulphamethaxozole**
***S.* Infantis**	44,4	0	66,6	77,7	33,3	33,3	44,4	88,8	44,4	100
***S.* Kentucky**	76,9	7,7	73,1	0	0	0	0	42,3	0	0
***S.* Enteritidis**	33,3	0	33,3	66,6	0	0	0	66,6	33,3	100
***S.* Senftenberg**	0	0	50	100	0	0	0	100	50	100
***S.* Mbandaka**	25	0	62,5	75	37,5	25	25	87,5	37,5	100
***S.* Agona**	25	0	75	50	25	25	0	75	50	100

2.7 Characterization of *Salmonella*

According to antigenic profile, every *Salmonella* species can show different syndrome and host specificity. So, classification of *Salmonella* serovars is very important (Jamshidi et al., 2010). For protection against this foodborne pathogen, fast, precise and sensitive methods should be used. Conventional cultural methods need long time. Also, these methods are laborious. According to microbial communities, *Salmonella* serovars are considered hard to detect and culture. Nowadays, DNA based methods which are ribotyping, RFLP (Restriction Fragment Length Polymorphism) and PFGE (Pulse Field Gel Electrophoresis) are used to isolate and identify the *Salmonella* serovars and these methods are considered to be better than conventional method in many ways (Nagappa et al., 2007).

2.8 Subtyping Methods of *Salmonella*

The aim of bacterial subtyping methods is identification and characterization of different kind of isolates. Also, these methods give an opportunity to decide ancestral relationship between isolates (Wiedmann, 2002b). Also, these methods are used for relevant isolated to differentiate each other from different isolates (Eberle & Kiess, 2012). Generally, bacterial subtyping methods are divided into two main groups which are phenotypic methods and genotypic methods. Phenotypic methods focus on specifying of characteristic expression. To detect foodborne pathogens, phenotypic subtyping methods have been used since a long time (Wiedmann, 2002b) (Eberle & Kiess, 2012). On the other hand, genotypic subtyping methods are very recent. Since genotypic methods have been introduced, ability to differentiation of bacterial subtyping increase. In genotypic methods, genetic elements of bacteria are analyzed o differentiate (Wiedmann, 2002b)(Eberle & Kiess, 2012).

2.8.1 Phenotypic Methods

2.8.1.1 Serotyping

Serotyping is one of the most common used phenotype-based classification methods that is used on wide range of foodborne pathogens such as *Salmonella, L. monocytogenes* and *E. coli*. Epidemiologically, strains which are same for isolates for both phenotypically and genotypically are known as smallest bacterial units and serotyping of *Salmonella* is one of the most common method that identifies strains(Steve Yan et al., 2003). Working principle of serotyping method is based on antigens which are carried on by different strains of bacteria on their surfaces can be identified by antibodies and antisera. These antigens vary according to the type of bacteria (Wiedmann, 2002a). In other words, this method depends on different cellular surface structure of microorganisms. These different surface structures can be identified by antibodies and antisera which are used for this purpose (Eberle & Kiess, 2012). Serotyping is generally used in epidemiologic studies, but this method has low discriminatory power for classification. Serotyping is time consuming, expensive and needs high quality antisera and skilled labors to perform. Also, it has low reproducibility (Jadhav et al., 2012) (Wiedmann, 2002a). Even though this method is very important for public health, it has limits and serotyping can only give essential information, therefore, more detailed and precise methods are needed (Steve Yan et al., 2003).

2.8.1.2 Phage Typing

Bacteriophages, bacteria infecting viruses are found in the nature. They have ability to lyse their host bacteria and closely related to their host bacterial species (Jadhav et al., 2012). Phage typing is using bacteriophage to classify specific *Salmonella* strains. Therefore, isolates of *Salmonella* can be differentiated. Bacteriophages have ability to infect different *Salmonella* isolates according to different phage and phage

receptors on the surface of bacteria. If bacteriophages find proper phage receptor on the cell surface, they infect bacteria and lyse the cell(Steve Yan et al., 2003). In another words, phage typing can be explained that specific phage lyses particular strain. Features of phage characteristics and receptors effect the capacity of infection and lysis. In phage typing, phage libraries are used (Ferrari et al., 2017). Even though this method is known as very quick, noncomplex implementation and high throughput typing, it has some limitations. Phage typing needs standardized reference phage to check and control (Wiedmann, 2002a).

2.8.2 Genotypic Methods

2.8.2.1 Pulse-Field Gel Electrophoresis (PFGE)

In 1984, pulse-field gel electrophoresis (PFGE) was introduced to the world for the first time. This method has been also used as a subtyping method for *Salmonella* since 1990s. For *Salmonella* serovars, PFGE is still most widely preferred method (Ferrari et al., 2017). This method has been preferred by health authorities and outbreak investigation committees worldwide. PFGE is generally used to classify *Salmonella* spp. from food products, human and animals that are used for food productions (Tang et al., 2019); (Ferrari et al., 2017). The main reasons for being highly preferred that PFGE has high discriminatory power and high reproducibility. Generally, other subtyping methods are crosschecked against PFGE and this method is also known as “Gold standard” method (Tang et al., 2019). With the development of PulseNet database which contains more than 350,000 PFGE pattern and 500 serotypes, success rate of PFGE is increased. However, PulseNet is only available for PulseNet participating laboratories (Ferrari et al., 2017). PFGE uses restriction enzymes like *Xba I, Sfi I, Spe I* and *Not I* to recognize chromosomal DNA. Among these enzymes, *Xba I* is specifically used for *Salmonella*. These enzymes cut DNA and create restriction fragments (10-30) which vary from 20 to 800 kb. These large fragments cannot be separated by standard gel electrophoresis (Wiedmann, 2002a).

they can only be split in a flat agarose gel which has electric current (pulse field) that is regularly changing and allow us to separate DNA according to their size. Then, fingerprint pattern which is specific for every isolate is obtained. Both Gram negative (*Salmonella, Escherichia coli, Shigella, Campylobacter jejuni*) and Gram positive (*L. monocytogenes*, *S. aureus*) bacteria can be classified by using PFGE with proper restriction enzyme and optimum electrophoresis condition provided for different bacterial species (Tang et al., 2019). PFGE procedure starts with seating bacterial cell into agarose gel (plug). Enzymes and RNases are applied to plug to remove undesirable proteins and RNA to get clean and pure chromosomal DNA. Then, the plug is divided into pieces and restriction enzymes are used to digest DNA into segments. After gel is ready, the plug is put into the wells of gel and it is covered with molten agarose. Afterwards, electric field which is switched periodically is performed (Eberle & Kiess, 2012). Even though, PFGE is considered as gold standard method, it has some disadvantages. PFGE is time consuming method to perform. It has a procedure that continues for 4 days. Additionally, this method needs high-level specialty to perform and it cannot be done automatically (Tang et al., 2019). Results of this method can change depend on the selected restriction enzymes and electrophoresis conditions. For this reason, comparability of results between laboratories can be difficult (Eberle & Kiess, 2012).

2.8.2.2 Whole Genome Sequencing (WGS)

With the development of technology, whole genome sequencing (WGS) that give much information from bacteria such as species, serovars, subtypes, antimicrobial resistance and virulence of bacteria is commonly used (Ibrahim & Morin, 2018). WGS is known as high-throughput sequencing technology and genomic resolution of WGS is very high compared the other methods (Vincent et al., 2018). Change of DNA sequencing for entire genome can be obtained by WGS method and these results can be used to investigate asses evolution and genetic relatedness of isolates (Tang et al., 2019). Identification and characterization of bacteria can be done more

detailed by WGS (Ferrari et al., 2017). Sequence data, identification of strains, determination of virulence and antimicrobial resistance genes are provided with more detailed and higher quality by using WGS method. Also, route of disease transmission and possible source of disease can be provided by WGS (Ibrahim & Morin, 2018). One of the many advantages of WGS is that only single nucleotide can be identified by this method and this helps the differentiation of clonality strains (Ferrari et al., 2017). In 2009, WGS was used for the first time to detect *Salmonella* outbreak in the United State. With many studies, WGS application for *Salmonella* classification and subtyping has been approved and number of studies is increasing day by day. Outbreak investigations and pathogen source tracking are some of the usage areas of WGS for these studies (Tang et al., 2019). High-resolution molecular subtyping can be possible for *Salmonella* classification by WGS technology (Ibrahim & Morin, 2018). Since 2019, more than 184,000 genome sequences data for *Salmonella enterica* can be found on NCBI and these data can be used off-line. Illumina, Ion Torrent, Pacific Biosciences (PacBio) and Oxford Nanopore Technologies are some of the sequencing platform that can be used for WGS (Tang et al., 2019). This method is alternative technology for identification of outbreaks clusters and to get the serotype data reliably and quickly (Ibrahim & Morin, 2018). In near future, WGS technology will be used for microbial hazard control, antimicrobial resistance prediction and contamination inquiry in the food industries (Tang et al., 2019). Additionally, discriminatory power of WGS is higher than PFGE and WGS technology is replacing PFGE quickly (Vincent et al., 2018). It is very effective method to identify outbreak source, prediction of antimicrobial resistance and clarifying the evolution of *Salmonella* subtypes. Some of the disadvantages of WGS technology are cost of WGS process is higher than other classification methods and bioinformatics analysis of WGS is restricted and expensive. Besides being an expensive method, WGS method needs expertise about bioinformatics analysis (Ferrari et al., 2017).

2.9 Cultivation Methods of Hens

Since 1930, cage system was used for egg production. This method is one of the most economic method systems. Using of cage system for egg production increased with the development of the egg production industry (Yenilmez & Uruk, 2016)(Baykal, 2015). However, chickens are unable to meet their natural needs in cage systems. Because of inadequate conditions in cage system, many health problems are faced. For this reason, many animal protection associations in Europe opposed the use of cage system (Yenilmez & Uruk, 2016) (Sözcü & İpek, 2016). In 2012, conventional cage system was banned in European Union (Hammeishøj, 2011). Because of that, new alternative systems were tried to replace conventional cage system (Baykal, 2015). Production of egg gain importance during 20th century. Even though, cage egg production cheap for consumers and give economical advantage to farmer, many different production methods have been tried over the years because of animal welfare concerns (Hammeishøj, 2011). As known, Turkey is in considering accession process to European Union. Therefore, alternative systems were also tried in Turkey. Because of economic crisis and high cost of red meat in turkey, daily protein requirement is provided from different sources like chicken meat or eggs (Baykal, 2015). Chicken has high reproduction rate and annual product quantity. Also, chicken meat and egg can provide high nutritious value. With these features, chicken products are very important food source for some countries which are insufficient to produce animal origin food production (Baykal, 2015). Therefore, production systems for chicken are very important issue in egg production industry. In Turkey and many other countries, 4 different systems are used in industry. Organic egg production system, free-range system, cage-free system and cage system are used in egg production industry.

- Cage system
- Cage-free system
- Free-range system
- Organic system

2.9.1 Cage System

Conventional cage system is widely used in poultry. In this system, different type of cage could be used. For example, there are cages where chickens are housed on their own. There are also group cages where 5-6 chickens are housed together. Apart from these cages, colony cages are used in cage system (Yenilmez & Uruk, 2016). Due to the advantage of high capacity in industrial poultry farming, today the most preferred cage types are 3-8 store systems. In cage system, there are also 12 floor cage system in industry (Baykal, 2015). At least 550 cm^2 space should be reserved for each chicken according to European Union standards (Yenilmez & Uruk, 2016; (Baykal, 2015). In the cages, groove type feeders are used and Drop type drinkers are widely preferred in cages. Also, cup-type drinker can be used in cages by EU standards (Baykal, 2015). However, chickens are restricted from many natural movements which are foraging, exercise, powder bath, self-grooming and nesting in cage systems. This affects the physical and mental health of chickens badly. Osteoporosis is a common disease in poultry due to insufficient space in the cage. If the disease progresses, it causes the bones to become brittle, paralyzed and deaths in the last stage (Yenilmez & Uruk, 2016).

2.9.2 Cage Free System

Because of effects of conventional cage systems on animal health, enriched cages systems are used in industry. This system is also known as cage free system or furnished cage system (Baykal, 2015). Enriched cages are equipped with many equipment that allows animals to exhibit their normal movement and behavior. Cages are enriched with materials such as nest, litter material and perch. Thus, chickens were provided with a more comfortable shelter in larger areas (Yenilmez & Uruk, 2016; Baykal, 2015). Enriched cages are divided into small, medium and large classes according to the number of chickens they contain. There are enriched cages that can accommodate 60 chickens. In enriched cages, 50 cm^2 wider area is

provided than the conventional cages for chickens. According to European Union, it is anticipated to use litter materials that allows chickens to peck and paw (Baykal, 2015). However, chickens are difficult to observe in the enriched cage systems and frequent breastbone deformation are the some of the problems of this system. Also, hygiene problems occur due to the defecation of chickens on top of each other and there are seen in cannibalism and plucking between chickens. Beside these, rate of broken and cracked eggs increase in cage free system (Yenilmez & Uruk, 2016).

2.9.3 Free Range System

In alternative new systems, the animals are tried to be provided with conditions in their natural environment. Chickens are provided to display their natural behavior to some extent (Sözcü & İpek, 2016). In free range system, chickens are hosted in closed shelters at night. In daytime, chicken have access to green fields outside the shelters. It is a system obtained by combining cage-free systems with open spaces (Baykal, 2015; Yenilmez & Uruk, 2016). In other word, chickens with the possibility to go to open areas away from artificial environment (Sözcü & İpek, 2016). Purpose of using shelter for this system is protecting the animals from bad weather conditions and ensuring night security. Mangers and leeches are provided to chickens in both shelters and open areas. Chickens can easily perform their natural movements in large green areas, and they can get enough sunlight (Baykal, 2015)(Yenilmez & Uruk, 2016). Most of open area should consist high percentage of green areas (Baykal, 2015). In green areas, chicken can also be fed with green plants, worms and insects other than chicken feed. So, feed consumption is not high in this system when it is compared with other methods. However, wild birds can involve the consumption of chicken feed. As a chicken feed, corn, wheat, barley and ready feed can be used. However, none of the chicken feed can contain GDO but natural additives such as enzymes, probiotics and prebiotics can be used. According to European union commission, chicken population should be such that a chicken per 4 m^2 (Yenilmez & Uruk, 2016) (Baykal, 2015). Inside the henhouse or shelters, density of chicken

must be 9 chicken per m^2 at most according to EU (Hammeishøj, 2011). Other than these, beak trimming is allowed in free-range systems (Hammeishøj, 2011).

2.9.4 Organic System

One of the most important systems is organic egg production. In this system, usage of any synthetic feed or chemical is forbidden. Chickens are fed and met their needs without disturbing physiology and natural behavior of animals (Yenilmez & Uruk, 2016). However, there is no specific rules for organic egg production system. Generally, rules for production organic eggs come from International Federation of Organic Agriculture Movements (IFOAM) and guidelines for organically produced food developed by FAO and WHO (Hammeishøj, 2011). There should be some differences between free-range and organic egg production method such as regulations and principles. In organic egg production chicken feed must be produced by organically according to European Union (Hammeishøj, 2011). Chemically produced or genetically modified any foods are not allowed for chicken diet. For organic egg production, chicken feeds are produced by 100 % organic agricultural principles. Any medical treatment such as antibiotics are used only with veterinary approval and beak trimming is forbidden in organic egg production. In the shelter, density of chicken must not exceed 6 hens per m^2 (Hammeishøj, 2011). Like any other methods, diet of organic hens is arranged according to provide all the nutrient needs of chicken and such as physical function of hens, body maintenance, and daily needed component for egg production. According to EU regulation, feed of organic hens must be originated from 100 % of organic ingredients (Hammeishøj, 2011). Mortality ratio is higher in organic egg production than any other production methods (14 % in organic production, 8 % in free-range and 4 % in cage production). Prohibition of beak trimming, synthetic medication and difficult diets for chickens are the main reasons for higher mortality rate.

In today's world, more healthy and safer foods are preferred from consumers. Also, demand for natural foods is higher. In Turkey, approximately 6-7 % of total

egg production is originated from rural areas and 20 million village chickens are grown in Turkey according to Ministry for Agriculture (Cicek & Kartalkanat, 2009). General opinion on village egg is that these eggs are considered as a natural and fresh foods. However, there are not enough experiment about village eggs. There are no rules, methods and features how they are produced. So, it is not known what affects the presence of *Salmonella.*

2.10 Prevention of *Salmonella* Contamination in Egg

Salmonella infection is still one of the biggest major public health problems in the world. (Eng et al., 2015). Even though, there are many technological developments, *Salmonella* contamination is still important problem for food industries. Therefore, control of *Salmonella* spp. to human is very important for human health (Callegari et al., 2015). The main transmission of *Salmonella* spp. to human is contaminated water and food sources. In recent years, *Salmonella* infection decreased with sanitation of food and water, pasteurization of dairy products. Providing drinkable water, efficient sewage disposal system and hygienic food production could reduce infection of *Salmonella* rate (Pal et al., 2015). *Salmonella* contaminations of food and water was drastically observed with the help of sanitation methods. When safe food, clean water and typhoid vaccines for *S.* Typhi are available for human, *Salmonella* infection rate decreases. Suitable cooking and usage of food products like poultry, eggs and dairy products eliminate *Salmonella* contamination risk (Eng et al., 2015). To avoid *Salmonella* infection, basic food safety prevention is very important. Preventing processed food from contamination, suitable cooking and refrigeration condition should be applied.

For shell eggs, maximum temperature for storage was determined as 7.2 °C which could strengthen egg's natural defenses against *Salmonella* Enteritidis by FDA in 2000 and growth rate of *Salmonella* Enteritidis would also be decreased (Galiş et al., 2013). According to European Commission (EC), storage and transportation condition for shell eggs should be at constant temperature and before

eggs are sold to final consumers, EC do not recommend refrigeration. The main reason behind this subject is that refrigerated eggs cannot be accepted as fresh by EC. As a result of scientific research, it was proven that ambient temperature is not suitable for storage of shell eggs because of multiplication and growth of *Salmonella* risk have increased with horizontal and vertical transmission. 20 °C is very convenient for *Salmonella* spp. to grow. However, this pathogen does not show a proper growth under 10 °C. Purchasing eggs that are stored at refrigeration temperature condition is recommended by FDA (Galiş et al., 2013).

2.11 Sanitation Methods Used in Commercial Eggs

In commercial egg production, extending shelf-life egg is very important for costumers. For this purpose, many methods were tried to inhibit microorganism growth and reducing existing microbial reproduction. As known, eggs are one of the most consumed and preferred high nutritious food. Besides, eggs can be easily contaminated. Some pathogens such as *Pseudomonas spp., Alcaligenes* spp. and *S.* Enteritidis can penetrate through the eggshell within 4-5 days. Also, some airborne bacteria and fungi can cause eggshell contamination in aviary and conventional caging systems. Based on these contamination problems, horizontal eggshell contamination is one of the biggest concern in egg-borne outbreaks (Makalatia et al., 2018). There are many sanitation methods for food preservation. To this date, many decontamination methods have been performed and studied to decontaminate shell eggs especially for *Salmonella* contamination. Generally, UV irradiation, steam and disinfection methods are used but these methods are considered as expensive, nonspecific and batch limited physical methods. Also, there are chemical methods used in decontamination. However, bacteria can gain resistance against chemical approaches (Makalatia et al., 2018). To prevent vertical transmission of bacteria, there are some traditional methods such as prebiotics, probiotics, vaccination and antibiotics. However, these methods is not useful for antibiotic-resistance bacteria (Henriques, AnaHenriques, A., & Sereno & Sereno, 2013). According to FDA, in

order for methods to be considered effective, minimum 5 log CFU/eggshell^{-1} reduction should be obtained by methods (Galiş et al., 2013). If a classification is to be made for these methods, classification for decontamination of shell eggs can be specified into 3 groups. There are chemical and procedure for sanitation. FDA and USDA approved some of the procedures that are used in commercially. But still, all of these procedures should be improved and novel technologies could be arisen (Galiş et al., 2013).

2.11.1 Chemical Methods

2.11.1.1 Egg Washing

Egg washing method is one of the chemical methods. Currently, this method is used in the USA, Canada, Australia and Japan. However, this method is not approved in the European Union (Hutchison & Sparks, 2003). In egg washing, chemical compounds that are used are classified as Generally Regarded As Safe (GRAS) (Galiş et al., 2013). For these chemical compounds, there are no specific limit for usage. In this procedure, the temperature of wash water should be higher than the internal inner temperature of the shell eggs and wash water is needed to change regularly for safety reasons. However, wash water temperature should not be greater than 45 °C to prevent cuticle damage or thermal cracking. Also, only potable water can be used as wash water to minimize the contamination risk (Hutchison & Sparks, 2003). Egg washing method provides reduction of microbial growth on the shell surface. Because of some chemical might adhere to the surface of egg after washing procedure, reduction of microbial load happens. Cross-contamination risk is decreased and risk of contamination of the egg content is decreased. However, the cuticle which is the first physical defense mechanism of eggs against contamination risk could be damaged by egg washing procedure. This is the main disadvantage of this method. Chemical components in the wash water might affect the structure of the eggshell and cause bacterial penetration (Galiş et al., 2013) (Hutchison & Sparks,

2003). Also, this method may generate detrimental by-products such as chloroform and trihalomethanes which have carcinogenic effect (Rivera-Garcia et al., 2019).

2.11.1.2 Hydrogen Peroxide Application

Hydrogen peroxide (H_2O_2) is used for bactericidal effect because of its toxicity (Galiş et al., 2013). Usage area of hydrogen peroxide is generally disinfectant, surface decontaminant and sterilant in the industrial scale (Sheldon & Brake, 1990). Hydrogen peroxide can be easily exterminated after use. It has no bad odor and can easily evaporate. This chemical should be used with caution because it is a strong oxidizing agent and it can damage skin, eyes and mucous membrane (Sheldon & Brake, 1990). Hydrogen peroxide was successfully used for decontamination of shell eggs by immersing eggs in hydrogen peroxide solutions of different concentrations in some experiments (Galiş et al., 2013).

2.11.1.3 Electrolyzed Water

Electrolyzed water (EW) technology is used as sanitizer in many areas such as disinfect medical supplies and ready-to-eat foods. It prevents contamination of food and microbial spoilage. Also, EW increases shelf-life of food products. EW contains mainly hypochlorous acid (HOCl) that provides bactericidal characteristic. In this method, NaCl is electrolyzed in water and HOCl is generated which is mentioned before has microbicide effect (Rivera-Garcia et al., 2019). This method is considered environmentally friendly because it can reverse to water and salt after reaction (Orejel & CanoBuendía, 2020). Although it has many advantages, electrolyzed water system has some limitations. If acidic electrolyzed water is used, it can cause corrosive effect on metal surfaces and after application of this method there can be salt residue on products which effects products' taste and texture (Orejel & CanoBuendía, 2020). According to study performed on shell eggs to eliminate *S.* Typhimurium by using electrolyzed water, EW completely remove all *S.*

Typhimurium. However, usage of EW is limited when low pH level are monitored (Galiş et al., 2013).

2.11.1.4 Ozone

Ozone (O_3) method is known as stronger sanitizer against all kind of microorganisms at low concentration. It has high reactivity and powerful antimicrobial agent and this method has been approved by US Food and Drug Administration (FDA) (Yüceer et al., 2016). Ozone is only produced according to demand because it cannot be stored due to low stability (Galiş et al., 2013). Also, there are no residual chemical after ozone treatment (Yüceer et al., 2016). In ozone method, bacterial structure such as membrane glycoproteins and glycolipids are attacked, and this causes cell death. this method is also considered as environmentally friendly. Ozone It does not cause pollution because of decomposition of ozone to O_2 automatically (Galiş et al., 2013). More than 5 log unit inactivation of *Salmonella* Enteritidis was achieved on surface of shell egg with high ozone concentration and in another study, *Salmonella* Enteritidis was reduced 3.1 log unit on eggshell by ozone treatment (Galiş et al., 2013).

2.11.1.5 Plant Extraction

With the changing world, the demands of the consumers are changing. In recent years, demand for organic and non-processed food increase. Therefore, natural source for sanitation of food is important. Plant extract is a method that meets this demand. Antimicrobial capacity of plant extract has several mechanisms such as preventing the pathogens from adherence to host cell and inhibition of protein, nucleic acids and cell wall synthesis of pathogen (Ullah et al., 2020). Also, phenolic compounds of plant extracts are considered as bactericidal effect of this method (Galiş et al., 2013). However, effect of plant extract on egg quality and other food products have not yet been investigated fully. According to study that Moraleco and

others (2019), plant extract did not have any influence on egg quality (Moraleco et al., 2019).

2.11.2 Physical Methods

2.11.2.1 Irradiation

Cold pasteurization or in other words food irradiation, which is non-thermal method destroys detrimental pathogens, increases shelf life of food product. Radiation from high-energy gamma rays, X-rays and accelerated electrons are approved sanitation methods of ionizing radiation (Galiş et al., 2013). In 2000, using ionizing radiation treatment on shell eggs as a sanitation method was accepted by US Food and Drug Administration (FDA). However, it has been limited up to 3 kGy. This method is one of the most efficient method to eliminate bacterial load for internally content of shell eggs and also, irradiation is accepted as very efficient method to eliminate *Salmonella* and other harmful pathogens for both externally and internally (Kim et al., 2011; Min et al., 2012). Because of egg components are oxidized by hydroxyl radicals during irradiation treatment, functional properties of egg white might be damaged significantly. Food products that are used eggs as an ingredient could lose their quality due to change in the physicochemical and functional properties of eggs during irradiation process (Min et al., 2012).

2.11.2.2 Microwave

Microwave which is electromagnetic frequency in the range of 300MHz to 300GHz effects pathogens in two ways. Generated heat in the microwave process is the cause of thermal inactivation. The second way to inactivation is nonthermal effects which is classified into four groups. Selective heating, electroporation, cell membrane rupture and magnetic field coupling are the nonthermal effects of microwave (Galiş et al., 2013; Lakins et al., 2008). To generate heat, dielectric materials are interacted

during the microwave process. Materials with high water and carbon content are considered as a good microwave absorbers (Lakins et al., 2008). According to Lakins and others, *S.* Enteritidis and other types of pathogen bacteria can be reduced 2 log units from eggshell by using directional microwave technology (Lakins et al., 2008).

2.11.2.3 Ultraviolet Light

Ultraviolet (UV) light contains wide range of wavelength which is between 200 nm (X-ray) and 400nm (visible light). For UV light to be lethal, range between 250 to 260 nm can be used for all kind of microorganisms (Galiş et al., 2013). UV light is applicable for most pathogen in air, water and even on hard surfaces (Turtoi & Borda, 2014). Inactivation of microorganisms during UV treatment starts with absorption of UV light which induces breaking cross-linking between pyrimidine dimers in the DNA. Therefore, mechanisms DNA replication and transcription are inhibited, and microorganisms become inactive. Eventually, this cause microbial cell death (Galiş et al., 2013) (Turtoi & Borda, 2014). During eggshell sanitation, cuticle damage is eliminated by UV treatment. Also, this method protects eggshell from internal contamination. 2.8 log unit reduction was obtained by exposing eggs direct UV light (Turtoi & Borda, 2014).

2.11.2.4 Pulsed Light

Pulsed light (PL) technology is another non-thermal physical method for sanitation of shell eggs. This method protects nutritional quality and sensory of the food products and PL can be used on wide range of microorganisms including food spoilage (Lasagabaster et al., 2011). In the application of PL technology, intense broad spectrum of light (200-1100 nm) is applied with short duration pulses. This method mainly damages DNA of microorganism, causes water vaporization inside the cell and the rupture of the membrane (Galiş et al., 2013; Lasagabaster et al.,

2011). In 2000, application of PL technology has been approved by the US Food and Drug Administration (Lasagabaster et al., 2011).

2.11.2.5 Gas Plasma

Gas plasma which is ionized gas consists of electron, photons, ions, free radicals and molecules. These particles permanently interact with each other's (Ragni et al., 2010). Gas plasma can be both thermal and non-thermal according to conditions. If pressure is high, thermal plasma is obtained and if pressure is low, non-thermal plasma is obtained (Galiş et al., 2013). OH and NO radicals are heavily bombarded on microorganisms during gas plasma process. During collision, internal energy of ions and reactive species are lost by surface or other particles that is the main microbial inhibition factor of gas plasma technology and there is no risk for egg quality during treatment (Galiş et al., 2013). According to study that Ragni and others (2010) performed, population of *S.* Enteritidis and *S.* Typhimurium were decreased on shell eggs. 4.5 log unit reduction was obtained during the study (Ragni et al., 2010).

2.11.2.6 Ultrasound

Ultrasounds (high-frequency sound waves) have been used in the food industries for quality measurement, preservation of food and microbial inactivation. Sanitation procedure can be shortened by ultrasound treatment because of transfer of acoustic energy instantly through the whole product. Cell membranes became thinner by the strike of ultrasound waves (Galiş et al., 2013). This shock waves cause functional and structural damages on cells and then, cell lysis occurs (Aygun & Sert, 2012). According to Sert and others (2012), bacterial load was dramatically decreased by using ultrasound method on both egg albumen and yolk. *Salmonella* spp. was reduced from 2.77 log unit to 1.16 log unit (Aygun & Sert, 2012).

2.12 Bacteriophage (Phage) – Phage Treatment as an Alternative Method

Presence of pathogenic microorganisms is the biggest problem of food industries. The first and foremost thing that food industries need to do is to ensure food safety. Until now, many methods have been tried to eliminate presence of bacteria and to ensure food safety. Even though these methods are suitable to use for food safety, they have few drawbacks. Therefore, more accurate and fast method for providing food safety and eliminating microorganisms is needed. In recent years, bacteriophage application is used instead of other methods. Bacteriophages is a viruses that inactivate bacteria in the food products and kill it (Vishweswaraiah et al., 2012).

2.12.1 Biology of Bacteriophage

The discovery of bacteriophage dated in 1915 and 1917. In 1915, the scientist named Frederick Twort has discovered bacteriophage. Independently from Frederick, Felix d'Herelle has also discovered bacteriophage (Michalczuk & Dolka, 2020). The mean of word of bacteriophage is microbe that can kill bacteria (Vishweswaraiah et al., 2012). In another words, bacteriophage is also known as viruses that infects bacteria. Bacteriophages are like all viruses; they contain genetic materials that surrounded by protein capsid. They have either DNA or RNA according to their type. Most of the bacteriophages have double-stranded DNA structure and few of them have single-stranded or RNA viruses. Also, bacteriophage can be double stranded or single stranded. Structure of bacteriophage consists of head, tail, tail fiber and contractile sheath and DNA or RNA. Genetic materials are packed in the head part which could be hexagonal or round. The tail part which interacts with bacteria and starts bacterial infection consists of fiber structure. It is known that bacteriophages have no harmful effect on human. They are considered as a non-pathogenic. Bacteriophages do not have any restrictions as habitats. They can fit many environments such as water, plant, and especially food. According to bacterial target, bacterial site, nucleic acid,

life cycle and morphology of bacteriophage, they can be classified (Michalczuk & Dolka, 2020). Depend on their shape and genetic material, vide variety of bacteriophage is found in the nature. There are more than 5000 surveyed phages types and there are 14 classified bacteriophage family according to International Committee on the Taxonomy of Viruses (ICTV) statement (Kassa, 2021). Also, some potential bacteriophage families are pending for classification. Classification of bacteriophages is made according to their nucleic acid content, their target bacterial species and morphology of bacteriophage. Also, area that bacteriophages can be specifically found is also a factor for classification (Principi et al., 2019). Bacteriophages are known as bacterial viruses. To reproduce new phages, bacteriophages must use bacteria. they are mandatory pathogens of bacteria. Other than prokaryotes, bacteriophage can target archaea. (Lewis & Hill, 2020; Michalczuk & Dolka, 2020). According to bacterium types and their life cycle, there are two types of bacteriophages which are lytic and lysogenic. In the lytic cycle, which is also known as virulent or productive, bacteria are infected by phages. Firstly, virion sticks to outer surface of the host cell. Only genetic material of phage is transferred into the cell, capsid of virion stays the surface of host cell. Then, phages respectively replicate inside the bacteria, kill prokaryotes by lysis and release themselves (Michalczuk & Dolka, 2020). Depend on the conditions and types of the bacteriophage, each one of the parent phage can form from 50 to 200 new daughter phages (Vishweswaraiah et al., 2012). In the lysogenic cycle, genetic material of bacteriophage is integrated into the host bacterial genome. Obtained new genomic material is named as prophage. This genomic material which has nucleic acid of bacteriophages can be transferred to the daughter cells by cell division. Prophage is known as dormant form of bacteriophage. It can make itself active and can pass into the lytic stage. Some of the bacteriophages can perform both lytic and lysogenic cycles (Michalczuk & Dolka, 2020; Vishweswaraiah et al., 2012). To eliminate bacterial pathogens, lytic bacteriophage is usually used. Only lytic bacteriophages are appropriate for phage treatment, which is used to treat bacterial infection because

to their restricted capacity to eliminate bacteria. Antibiotics are substantially less specific than bacteriophages (Michalczuk & Dolka, 2020).

2.12.2 Application of Bacteriophage on Pathogen Bacteria

In time, bacteria evolve, and they gain antibacterial resistance against antibiotics and this situation causes problems. Therefore, antibiotics are become insufficient against bacterial treatment. So, new novel method is needed for bacterial infections. For every bacterial species, there is bacteriophage that can exterminate it. In other words, bacteriophages have very specific feature that they have very narrow scope. If bacteriophage can match with pathogen bacteria, they can kill it. Even though antibiotics have larger domain of use, bacteriophages are more effective than antibiotics. Bacteriophages can exterminate multidrug resistant bacteria and resistant pathogenic bacteria (Kassa, 2021). Antibiotic usage can cause damage on the microbiome. In animal experiment, bacteriophage that specific for diarrhea associated *Escherichia coli* did not damage beneficial bacteria. Bacteriophages target specific bacteria species, and they have no harmful effect on animal cells. Because of surface properties of eukaryotic cells are different than prokaryotic cell, bacteriophages cannot affect eukaryotic cells. Because the bacteriophage concentration in the infection site increases quickly after the initial delivery, just a few doses are enough. According to studies, low dosage of bacteriophage has 100 % fatality rate against specific pathogen bacteria. Compared to antibiotics, bacteriophage application on pathogen bacteria is less expensive, higher safety, specific effect on specific bacteria species, higher tolerance and easy administration (Principi et al., 2019).

2.12.3 Phage Therapy Application in Food Industry

Escherichia coli, Salmonella, Listeria monocytogenes and *Campylobacter* are known as main pathogenic bacterium types for food industry. As an alternative

method for sanitation in commercial food production, phage-based method is preferred as one of the effective alternatives. While bacteriophage is killing pathogenic bacteria, it does not harm or effect animal cells during the therapy. Using of phage-based sanitation for ready to eat products have been approved by Food and Drug Administration (FDA). Recently, some phage-based food products to prevent *Listeria monocytogenes, Escherichia coli* and *Salmonella* serovars have been developed and have been release into the markets and bacteriophage application increased shelf-life of food products according to results (Sillankorva et al., 2012). For ready to eat foods and poultry products, Listeria-specific bacteriophage treatment have been applied since 2006 (Makalatia et al., 2018). In United States, phage-based products currently have been produced for food industry (Hong et al., 2016). For microbiological control, bacteriophage treatments have been used in chicken, pig skin, lettuce and sprout seeds on different kind of pathogens (Spricigo et al., 2013). Also, these applications have obtained very promising results to reduce *Salmonella* in different kind of food samples such as chicken breast, shell egg, egg liquid, lettuce and juice (Hong et al., 2016). In phage treatment, unwanted organisms are eliminated without damaging other important components in food samples. Also, it can evolve with pathogenic bacteria and provides a strong defense mechanism against antibiotic-resistant bacteria (Makalatia et al., 2018). Bacteriophage treatment is able to kill targeted specific bacteria rapidly and have ability to self-replicate. These features make phage-based application very useful for food safety applications (Spricigo et al., 2013). Today, bacteriophage application is used for *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* but phage treatments can be used in different types of bacteria in the future (Makalatia et al., 2018) (Hong et al., 2016). *Salmonella* is known as one of the most encountered pathogen bacteria in the poultry and one of the most significant zoonotic foodborne pathogens. Some methods like antibiotic treatment kills pathogenic bacteria as well as cause damage on intestinal microbiota. However, bacteriophage application kills specific bacteria according to their types and does not harm microbiota. General source of *Salmonella* outbreaks is mainly came from eggs and egg products (Michalczuk & Dolka, 2020).

Phage application as a sanitation method have been tried on many studies. Bacterial load were decreased approximately 3 log unit within 30 minute and reduction of bacterial load increased with time (Sonalika et al., 2020). In another study, reduction of *Salmonella* Typhimurium was conducted with using of bacteriophage application and 90 % of reduction was obtained after 15 minutes (Makalatia et al., 2018). According to Liu et al., used anti-*Salmonella* phage to eliminate *Salmonella* strains in tofu which is coagulated soy milk. 3.55 log cfu/mL reduction with 100 multiplication of infection (MOI) and 1.86 log reduction with the 1 MOI was obtained (Endersen & Coffey, 2020). The multiplicity of infection or MOI is the ratio of agents (phage or virus) to infection targets (cell). When referring to a group of cells inoculated with virus particles, the multiplicity of infection or MOI is the ratio of the number of virus particles to the number of target cells present in a defined space. In another experiment, Thung et al. used different kind of food products like fruit juice, beef, chicken and fresh eggs to eliminate pathogenic bacteria with anti-*Salmonella* enteritidis bacteriophage. According to their results, 2 log reduction was succeeded at refrigeration environment (4 °C) for 48 hours (Endersen & Coffey, 2020).

2.13 Aim of the Study

The aim of this study is to detect prevalence of *Salmonella* on different types of eggs and to investigate how efficient bacteriophage that were isolated in Turkey to eliminate *Salmonella*.

Commercially, there are four types of eggs in the markets and there is non-commercial village egg. The aim of isolation of *Salmonella* from eggs is to investigate effect of different cultivation methods and village eggs on prevalence of *Salmonella*.

For prevention of contamination in egg, different methods were applied on egg surface to exterminate pathogenic bacteria like *Salmonella*. Many methods are used

in the industry for the sanitation of the egg surface. These methods may affect eggs internally or externally. Therefore, alternative method for the sanitation of whole egg is needed. For this purpose, bacteriophage therapy levels were applied by using different multiplication of infection (MOI) as a sanitation method for eliminating *Salmonella* on eggs to investigate efficiency of bacteriophage therapy and effect of MOI during the bacteriophage application.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemical and Materials

For this study, all chemicals and materials were selected diligently. The information about chemicals and materials are given in the Table B.1.

3.1.2 Sample Collection for *Salmonella* Isolation

For sample collection, total 250 egg samples were collected for *Salmonella* isolation experiment and 126 egg samples were collected for phage treatment of *Salmonella* on contamination experiment commercially. Samples were obtained from Ankara region. To examine the all-egg types equally, 50 samples of each egg types were collected (organic, free-range, cage-free, cage and village egg). In this experiment, 24 different commercial egg brands were used and coded from 0 to 23. All egg samples were collected aseptically and transferred to the Food Safety Laboratory of Food Engineering Department, Middle East Technical University.

Table 3. 1 Types of egg production used in this experiment

Types of Egg Production	Number of Sample	Location
0 (organic egg)	50	Ankara
1 (free-range egg)	50	Ankara
2 (cage-free egg)	50	Ankara

Table 3.1 (continued)

3 (cage egg)	50	Ankara
4 (village egg)	50	Ankara

3.1.3 *Salmonella* Strains

Salmonella enterica Enteritidis, *Salmonella enterica* Infantis and *Salmonella enterica* Braenderup H9812 were provided from the Food Safety Laboratory of Food Engineering Department, Middle East Technical University. This Salmonella strains are coded respectively as MET S1-001, MET S1-056 & MET S1-713 in the database of the Food Safety Laboratory of Food Engineering Department. *Salmonella enterica* Enteritidis (MET S1-001) was used for Bacteriophage application on contaminated egg samples. *Salmonella enterica* Infantis (MET S1-056) was used as the standard for PCR confirmation of *Salmonella*. *Salmonella enterica* Braenderup H9812 (MET S1-713) was used in the PFGE experiment as a size standard for *Salmonella* strains after DNA of this strain was restricted by XbaI enzyme.

3.1.4 Bacteriophage Strain

Bacteriophage strain (*Salmonella* enterica Enteritidis) which was used in this experiment was specifically isolated from cow feces locally. It was coded as MET P1-001 in the database and provided from the Food Safety Laboratory of Food Engineering Department, Middle East Technical University. This strain has an influence on *Salmonella enterica* Enteritidis & *Salmonella enterica* Typhimurium (Table 3.5). Therefore, it was selected as a bacteriophage source for this experiment. Because *Salmonella enterica* Enteritidis (MET S1-001) was selected for bacteriophage application on contaminated eggs as a contamination source (Güzel, 2022).

3.2 Methods

3.2.1 Confirmation of *Salmonella* from Egg Samples

All provided eggs were broken in the sterile jar by shaking until it is homogenous. Each egg was put into sterile jar one by one. After egg sample were broken and homogenous, 25 ml sample was taken from jar by sterile pipette. 25 ml egg sample and 225 ml, Buffer Peptone Water (BPW) were mixed in stomacher bag by the help of stomacher for 1 minute. Dates and numbers were coded on the stomacher bags and all samples were incubated at 37 °C for 24 hours. After 24 hours of incubation, 1 ml of sample was pipetted with sterile pipette and transferred into the 9 ml Rappaport- Vassiliadis soy peptone (RVS) liquid medium which is known as a liquid selective enrichment medium in *Salmonella* analysis and incubated at 41 °C for 18-24 hours. Then, followed by transferring 1 ml to Brilliant Green Agar (BGA) and Xylose Lysine Deoxycholate (XLD) agar plates. XLD and BGA is used as a solid selective enrichment medium for standard *Salmonella* analysis. After incubation time of RVS finished, 1 ml liquid sample was transferred into the peptone water for dilution. Dilution was performed 3 times. Then, 10 µl of liquid sample of were transferred from both 10^2 and 10^3 dilution samples into the BGA and XLD agar by using a spread method. Purpose of dilution is to obtain single colonies. Then, BGA and XLD agar plates were incubated at 37 °C for 24 hours (Moosavy et al., 2015). In BGA, *Salmonella* colonies are pink color surrounded by a bright red zone. However, in XLD agar plate, *Salmonella* colonies are the same color as the medium, translucent, sometimes black centered. *Salmonella* colonies were detected according to information above and this isolation procedure was performed according to ISO 6579:2002.

Then, suspected *Salmonella* colonies were transferred into the Brain Heart Infusion Broth (BHI) agar plates for PCR (Polymerase Chain Reaction) verification. Incubation time for BHI agar plate is also same at 37 °C for 24 hours (Salm-surv & June, 2010).

3.2.2 Molecular Confirmation of *Salmonella* from Egg Samples

For DNA preparation, 95 µl sterile dH_2O was transferred into the PCR tube. Single *Salmonella* colony was selected from Brain Heart Infusion (BHI) agar by using sterile loop and then loop was scraped into the PCR tube which contains 95 µl sterile dH_2O. prepared PCR tubes were exposed to microwave for 30 seconds in microwave oven to lyse the cells of *Salmonella* bacteria. In the PCR preparation, master mix was prepared firstly. Master mix reagents were mixed at the calculated quantities for all samples in a sterile 1.5 ml tube. In addition to every sample, PCR tubes were prepared for positive and negative control. For conventional PCR, each prepared master mix sample were contained 10 µl buffer, 1 µl forward primer & 1 µl reverse primer (invA), 0.5 µl Taq polymerase and 36 µl ddH_2O.

Table 3. 2 Master mix reagents for invA PCR

Reagent	Volume (µl)
Template (Purified DNA)	1.5 µl
5X Go Taq Flexi Buffer	10 µl
Reverse Primer (invA) 12.5 mM	1 µl
Forward Primer (invA) 12.5 mM	1 µl
ddH_2O	36 µl
Taq DNA Polymerase	0.5 µl

48.5 µl master was pipetted from 1.5 ml tube which is contain master mix and transferred into the 0.2 ml PCR tube. Then, 1.5 µl *Salmonella* DNA which was lysed in microwave oven was added to the PCR tube. For positive control sample, 1.5 µl DNA from a *Salmonella* reference bacteria culture was used as a positive control. For negative control, 1.5 µl dH_2O is used. These prepared 0.2 ml PCR tubes were placed into the thermocycler. The conventional PCR protocol condition for *Salmonella* was adjusted like that.

Table 3. 3 PCR conditions

Cycles	Temperature	Hold time	Steps
1x	94 °C	8 min	Initial denaturation
	94 °C	30 sec	Denaturation
	60 °C	30 sec	Annealing
35x	72 °C	30 sec	Extension
1x	72 °C	5 min	Final extension

PCR amplification consists of parts. Firstly, initial denaturation occurs at 94°C for 8 minutes. Then, denaturation takes place at 94 °C for 30 seconds with 35 cycles. Temperature and time of annealing is 60 °C and 30 seconds. Extension of this procedure happens at 72 °C for 30 seconds and final extension happens at same temperature as extension, but final extension has longer time which is 5 minutes. Then all procedure can be held at 4 °C until it is stopped (Nagappa et al., 2007) (Moosavy et al., 2015) (Jamshidi et al., 2010).

0.5 X TBE buffer was obtained from 5 X TBE concentrated solution (54 g Tris base 15.5 ml 85 % phosphoric acid [1,679g/ml] 40 ml 0.5 M EDTA [pH:8]) by 1:10 dilution. 1.5 g agarose and 100 ml 0.5 X TBE buffer were mixed and put into the microwave oven to get dissolved and clear solution. After clean solution was obtained, solution was leaved to cool for 5 minutes until temperature of solution decrease to about 55 °C. comb was placed in gel tray about 2.5 cm from one end of the tray and position the comb vertically. The teeth of comb were about 1-2 mm above the surface of the tray. Then cooled gel was poured into the tank and bubble formation prevented. Gel was cooled for 30 minutes at room temperature to solidify. Solidified gel was transferred to the electrophoresis chamber and submerged into the with 0.5 X TBE electrophoresis buffer as it was used before. 5 µl was pipetted from

each PCR product and pipetted into the comb. Then, 1 µl of 6x loading dye for every 5 µl of PCR product was added. In this experiment, 3 µl "100 bp DNA Ladder Ready Load" was used as a ladder. Gel tank was closed, and gel was run at 110V for 50 minutes.

Ethidium bromide should be used very carefully because ethidium bromide is known as mutagenic and carcinogenic material. So, EtBr must be handled with gloves all the time. Preparation of ethidium bromide stock solution was done by dissolving ethidium bromide in water (10mg/ml). this procedure was performed until red solution was obtained. Also dissolving procedure was performed in a container protected from light at room temperature. Then, 20 µl was pipetted from stock solution and transferred into 100 ml dH_2O. agarose gel was waited for 5 minutes in 300 ml EtBr solution and waited for 30 minutes in dH_2O after EtBr solution carcinogenic. After 30 minutes in dH_2O, gel is ready for taking a photo under UV light. BIORAD Universal hood II (Biorad, SN 76 S, Milan, Italy) was used to take a picture of gel. Appearance of gen is like white band.

3.2.2.1 Freezing of *Salmonella* Isolates

Obtained all confirmed isolates were stored in the vials which were labeled according to sample numbers. All isolates were named with unique name. for coding the names, Middle East Technical University (METU) code was used. For every isolate, code name starts with MET. The following letter after MET indicate that person's last name who performed freezing procedure for isolates. The following number after letter, indicate that first 999 isolates were frozen by same person. In the last part, to make unique code for every isolate, different numbers were given for each isolate. During the freezing procedure, isolates that are confirmed by PCR were inoculated from BHI agar and then, they were transferred to 5 mL BHI broth for incubation at 37 °C for 24 hours. After incubation, 150 µL pure culture was added to the vials which were labelled. Then, 850 µL glycerol (15 % glycerol) was poured into the vials and it stored at -80 °C until further usage.

3.2.3 Molecular Subtyping of Non-typhoidal *Salmonella* Isolates by Pulse-Field Gel Electrophoresis (PFGE)

Strain variations of non-typhoidal *Salmonella* that were found in eggs were determined by using Pulsed Field Gel Electrophoresis (PFGE) and they were investigated through database system to detect strain variations. The protocol that was described by PulseNet USA was used for our experiment (Centers for Disease Control and Prevention., 2017).

3.2.3.1 Growing *Salmonella* Isolates

Salmonella isolates that were obtained from egg samples and *Salmonella enterica* Braenderup isolate which was used as a size standard were streaked on Brain Heart Infusion Broth (BHI) agar plates to incubate at 37°C for 14-18 hours to get fresh culture for our experiment. In our case, all our strains were taken from -80 °C storage units.

3.2.3.2 Casting PFGE Plugs

Cell Suspension Buffer (CSB) was used as blanked sample for spectrophotometer. 1.3 ml CSB was poured in cuvette. Cell samples on the surface of BHI agar were transferred by using sterile swab for each isolate to 10 ml test tube with 4ml CBS and then 1.3 ml cell suspension was transferred into a cuvette for every isolates. To obtain proper concentration level for each cell suspension, turbidity of cell suspension was adjusted. According to Center for Disease Control and Prevention, reading on the Microscan Turbidity Meter should be between 0.48-0.52 to adjust the absorbance value of cell suspension between 1.3-1.4 at 610 nm wavelength with a spectrophotometer (Ribot et al., 2006). To see visible DNA band pattern, the concentration needed is 10^9 cells/ml (Liu, 2014).Then, 400 µl prepared cell suspension is transferred into micro centrifuge tubes to incubate at 37°C for 10

minutes. Then, 20 µl of 20 mg/ml Proteinase K solution is transferred to each micro centrifuge tubes. The resulting mixture is mixed gently with pipetting method. For this experiment, 1 % SeaKem Gold agarose (SKG) and 1 % Sodium Dodecyl Sulfate (SDS) are used to make plug in Tris EDTA buffer (TE). 750 µl %20 preheated SDS solution (SDS) is added to completely dissolved 15 ml 1 % SKG Agarose (Ribot et al., 2006). Prepared solutions are microwaved until there is no particle in the solution. After homogenized solutions are obtained, solutions are cooled in 55 °C water bath for 10 minutes to stabilize the temperature. 400 µl of agarose SDS mixture is added to each sample in the micro centrifuge tube and mixed with pipetting method. After making sure that they are mixed, mixture is transferred into the plug mold. At least 15 minutes is necessary for plugs to solidify at room temperature and then, the plugs are removed from the plug molds. During the removing procedure of plugs, excess parts of plugs were separated.

3.2.3.3 Lysis of Cells in Agarose Plug

Each plug is transferred into the 50 ml labelled falcon tube contains 5mL of Cell Lysis Buffer (CLB) and 25µL Pro K solution (CLB/Pro K solution). The reason adding CLB & Pro K solution is to prevent degradation of DNA. Cells that are completely embedded in agarose were lysed by detergents in CLB & Pro K enzyme. The falcon tubes are then incubated at 54 °C for 1.5-2 hours and constant & vigorous agitation was applied during incubation with 170 rpm (Ribot et al., 2006).

3.2.3.4 Washing Agarose Plugs After Cell Lysis

After incubation, plugs are separated from lysis buffer. Washing procedure starts with 10 ml sterile deionize water which is preheated in 50 °C water bath for each plug sample. Then, falcon tubes are incubated at 54 °C for 10 minutes with 70 rpm. Washing with sterile deionize water is done 2 times back-to-back. When, washing with water part is finished, plugs are washed with Tris EDTA (TE) buffer which is

also preheated in 50 °C water bath. After sterile deionize water is removed from falcon tubes, 10 m TE buffer is added to the falcon tubes for each plug samples and falcon tubes are incubated at 54 °C for 15 minutes with 70 rpm. Washing with TE buffer part is repeated four times. During washing period, chromosomal DNA was protected by agarose gel structure and other cell components were removed (Tang et al., 2019)When fourth washing part is finished, plugs can be restricted immediately, or 5 ml TE buffer is poured into each falcon tube and plugs can be stored at 4 °C for months.

3.2.3.5 Restriction Digestion of DNA in Agarose Plugs with *XbaI*

After the washing process is finished, plugs were taken out from falcon tube that contained TE buffer with the help of sterile stainless-steel spatula. Then, all the plugs were placed onto sterile lame in order. Here, plugs were cut into 2 mm wide slices with sterile scalpel. 2mm plug slices were treansferred into 1.5 mL microcentrifuge tubes that contained 200 µL H Buffer solution (Ribot et al., 2006). For *Salmonella enterica* Braenderup H9812 that are used as a standard, three slices were cut and put into the microcentrifuge tube that containing 200 µL H Buffer solution. Remaining plugs were transferred back to the falcon tube that containing TE buffer and they are stored at 4 °C for further usage. The microcentrifuge tubes were incubated at 37 °C for 10 minutes. After 10 minutes, H buffer solution inside the microcentrifuge tube was removed and replaced with 200 µL XbaI solution. Then, microcentrifuge tubes that contained XbaI solution were incubated 37 °C for 4 hours for restriction digestion.

3.2.3.6 Agarose Gel Casting & Loading Restricted Plug Slices

1.5 g SeaKem Gold Agarose (SKG) and 150 mL 0.5X Tris-Borate EDTA (TBE) solution were mixed in 250 mL flask. The mixture was gently shaken and microwaved several times until all agarose dissolved in the solution. Then, mixture

was waited in the water bath at 55 °C for 10 minutes. Before pour the agarose, gel cast was leveled with bubble and cast comb was placed. Teeth of comb should not touch the platform of gel cast. To avoid any particles like dust, after pouring the agarose into the gel cast, it was covered with plastic container. After pouring the agarose, it was waited for half an hour at room temperature to solidify. After solidification, comb was removed from gel. All these procedures were performed before restriction digestion stage finished. Another SeaKem Gold Agarose (SKG) was prepared about 10 mL as a sealing agarose after plugs were placed into the wells of solidify agarose. Until usage, it was kept in the water bath at 55 °C.

After 4-hour restriction digestion part finished, XbaI solution was separated from microcentrifuge tube with pipet. Then, restricted plug slices were placed into the empty wells by sterile stainless-steel spatula. 1, 8 and 15. empty wells in the gel cast were used for *Salmonella enterica* Braenderup H9812 specifically. After all plug slices were placed into the wells, sealing agarose was poured to seal wells. During this stage of experiment, 2.1 L 0.5X Tris-Borate EDTA (TBE) solution was poured into the electrophoresis chamber as a running buffer. Pump power was set to 70 (1L/min) and cooling module of electrophoresis chamber was set to 14 °C.

3.2.3.7 PFGE Conditions

After sealing agarose solidified, agarose gel was removed from mold. Excess part of the agarose and side part of mold removed. Before gel and bottom platform of gel were placed into the electrophoresis chamber, black gel frame in the CHEF DR III electrophoresis system (Bio-Rad Laoratories, CA, USA) was placed into the chamber. then, gel with platform was placed into the chamber which contained 14 °C 0.5X TBE solution. Lastly, 836 µL of 10mg/ mL thiourea solution was poured into the running buffer. Then, cover of electrophoresis chamber was closed, and system is ready to start. Electrophoresis condition of this experiment was given in the table below (Table 3. 4).

Table 3. 4 Electrophoresis conditions

DNA size interval	**30 kb – 700 kb**
% Agarose	1 %
Voltage	6.0 v/ cm
Run time	19 h
Temperature set	14°C
Included angle	120°
Initial switch time	2.2 s
Final switch time	63.8 s
Pump speed	70 (1L/ min)

3.2.3.8 Staining and Destaining Gel

After 19 hours run time of electrophoresis finished, agarose gel was taken out from electrophoresis chamber. Before image of gel was taken, agarose gel firstly was immersed into ethidium bromide solution to get stained for 45 minutes. Then, agarose gel was immersed in distilled water and get destained for 60 minutes. All staining and destaining part finished, image of agarose gel was taken under UV light (Bio-Rad Universal Hood II gel imager). Then, obtained gel images were analyzed by using BioNumeric software.

3.2.3.9 Data Analysis

BioNumeric software uses similarities between each isolate. During analysis, band's locations were marked by software itself. Normalization of bands were adjusted according to bands that were in the database. In our case, normalization was set according to *Salmonella enterica* Braenderup H9812. In the case of incorrectly marked band, this can be fixed manually. In the BioNumeric software system, Dice's similarity coefficient was used. With a 2.5 percent band location tolerance and 1.5

percent optimization, Dice's similarity coefficient was utilized to calculate the similarity of each banding pattern. This means that this coefficient permitted displacement of a single band within a lane during band alignment and determined how much a lane with all bands could shift while still matching the bands (Ferris et al., 2004). To create dendograms, BioNumeric software operates unweighted pair group method with arithmetic means (UPGMA).

3.2.4 Phage Treatment of *Salmonella* on Contaminated Egg Samples

Commercially collected eggs were aseptically collected and transferred to to the Food Safety Laboratory of Food Engineering Department, Middle East Technical University for phage treatment experiment.

3.2.4.1 Preparation of *Salmonella* Solution (*Salmonella enterica* Enteritidis)

Bacterial strain *Salmonella* enterica Enteritidis isolate (MET S1-001) was used for contaminating which was stored at -80 °C. Isolate was inoculated on BHI (Brain Heart Infusion) agar plate by using streak plate technique. Then, it was incubated at 37 °C for 16 hours. Because of our host cell was in the mid-log phase, one of the pure *Salmonella* enterica Enteritidis colonies was collected with inoculation loop and transferred into the 100 mL BHI broth at 37 °C for 8 hours. Then, 1mL samples were transferred to the Eppendorf tubes by using pipette. Eppendorf tubes were placed into the MiniSpin Plus for 15 minutes at 5610 rpm. After 15 minutes, liquid parts were removed and 1 mL of 85 % NaCl solution was added to every tube and mix the sample in the Eppendorf tubes by pipetting method. To obtain desired titer (10^7 CFU/mL) *Salmonella* solution, optical density was adjusted to 0.103-0.105 at 600 nm (OD_{600}) with additional of 85 % NaCl solution. also, another *Salmonella* suspension that was prepared with 10^5 CFU/mL to use in this experiment.

3.2.4.2 Preparation of Bacteriophage Solution

Bacteriophage strain, used in this study, was isolated from cow feces in another study and stored at -80 °C for future study (Table 3.5). (Güzel, 2022).

For determination of bacteriophage titer and set its titer as a 10^8 PFU/mL, double plaque method was used. This method is also known as double-layer agar technique. *Salmonella enterica* Enteritidis (MET S1-001) was inoculated onto the petri dishes and incubated at 37 °C for 24 hours. Then, one of the colonies that growth on the BHI agar were selected and transferred to the 10 mL BHI broth by inoculation loop for another 24 hours at 37 °C. 500 µL sample from BHI broth and 100 µL from previously prepared bacteriophage lysate and stored 1 mL Eppendorf tubes at 4 °C were transferred into the 50 mL Luria-Bertani (LB) broth and incubated at 41.5 °C for 24 hours. During incubation, constant and vigorous agitation was applied with 170 rpm. Then, sample was poured into the falcon tubes and centrifuged at 8000 rpm for 10 minutes. After centrifugation, sample was filtered with 0.22 µm pour-size filters. Then, spot test was made to be sure that there was no bacterial residual. In this way, all bacterial residues were eliminated from mixture. 100 µL sample was taken from bacteriophage solution and transferred into the Eppendorf tube containing 900 µL 85% NaCl solution. Serial dilution for this solution was made up to 10^{-11} PFU/mL and last six dilution were used for double plaque assay. 100 µL from dilutions and 100 µL were taken from host bacteria were transferred into the 4 mL semi-solid LB broth (0.6 %) and poured onto the LB agar (1.5 %). After allowing 30 minutes at room temperature, samples were incubated at 37 °C for 24 hours. After 24 hours incubation, petri dishes were checked for clear zones which indicated the presence of bacteriophage (Henriques et al., 2013). Then, results were expressed as the number of plaques forming units per mL of sample. Titer of this bacteriophage stock solution was found to be 4.8×10^{11} PFU/mL. Therefore, titer of bacteriophage solution was diluted with 85 % NaCl solution to obtain 10^8 PFU/mL and the procedure described above is repeated until the desired titer is achieved (Sonalika et al., 2020). The bacteriophage strain (MET P1-001) was used in this experiment has

been characterized as having of host range of *Salmonella* Enteritidis. Also, this phage shows lytic characteristic which directly infect and kill the bacterial cell (Güzel, 2022).

Table 3. 5 Bacteriophage properties used in this experiment.

METUID	Genus	Species	Serotype	Isolate Source	City	Country	Year	Referance
MET P1-001	*Salmonella*	*enterica*	Entertidis	Cattle Farm	Adiyaman	Turkey	2020	(Güzel, 2022)

3.2.4.3 Contamination of Egg Samples

After desired bacterial suspension and bacteriophage solution were applied, commercially collected eggs were divided into 4 groups (Table 3.1). 1st Group was consisted of control group. 2nd Group was consisted of negative controls which were dipped into only 70 % of ethanol solution to eliminate any natural contamination on the shell egg surface. 3rd Group was positive control. According to titer of *Salmonella* suspension (10^7 or 10^5 CFU/mL), shell eggs were dipped into the *Salmonella* suspension after 70 % ethanol solution application. 4th Group were consisted of eggs that were sprayed by bacteriophage lysate (10^8 PFU/mL) after 70 % ethanol solution and bacterial suspension were applied. After, untreated eggs were separated, whole eggs were dipped into the ethanol solution (70 %) for 5 minutes to eliminate the unwanted contamination and sterilized egg surface before it was contaminated by us. Then, all egg samples were dried at room temperature in the bio-safety cabinet until they were all dry. Afterwards, sterilized shell eggs were dipped into the *Salmonella* suspension (10^7 and 10^5 CFU/mL) another five minutes. Then, shell eggs were incubated for 2.5 hours at room temperature in the bio-safety cabinet (Spricigo et al., 2013). Next, incubated shell eggs were evenly sprayed with 1 mL of bacteriophage solution (10^{-8} PFU/mL). In this experiment, 10 and 10^3 were used as a multiplication

of infection (MOI: the average number of virus particles infecting each cell) (Makalatia et al., 2018). After bacteriophage therapy, all shell eggs were placed into the sterile sample container and samples were held at 4 °C to mimic refrigerator environment for initial point (0 hour), 6 hours, 12 hours, 24 hours, 48 hours, 72 hours and 1 week. For initial point samples, selective plating and identification were applied right away to see any natural contamination (Hong et al., 2016).

3.2.4.4 Selective Planting and Identification

After every sample's incubation time at 4 °C finished, whole eggs were broken inside the sterile sample container by shaking the cups. Thus, shell, egg yolk and egg whites were mixed homogeneously. Then, 5 mL of liquid egg sample was mixed with 45 mL Phosphate Buffered Saline (PBS) at the rate of 1/10 in the falcon tube and samples were mixed with vortex mixer. Afterwards, 100 µL samples was transferred into the Eppendorf tubes containing 900 µL PBS (Hong et al., 2016). After Eppendorf tubes were mixed with vortex mixer, 100 µL sample was transferred into the labeled XLD agar by using spread plate technique and petri plates were incubated 37 °C for 24 hours. After incubation, petri plates were examined for *Salmonella* colonies which was observed as a red colony with black center. The results were expressed as the number of colonies forming units per mL (CFU/mL).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of *Salmonella*

For isolation of *Salmonella* from different type of eggs, 250 individual eggs were examined. For every different egg cultivation method, 50 eggs were used including village eggs (Organic, Free range, Cage free & Cage methods). In total, 24 different brands were used for this experiment. From 250 different egg samples, 17 *Salmonella* strains were isolated then they were confirmed by PCR. All obtained isolates were stored with triplicate at -80 °C in Food Safety Laboratory. All commercial egg brands that were used for this experiment were coded from 0 to 23 (Table 4.1).

Table 4. 1 Information of Isolated *Salmonella* Strains from Eggs

METU ID	Genus	Species	Source	Brand	Cultivation Method	Date
MET A2-138	*Salmonella*	*enterica*	Hen's egg	Brand 8	Free Range	25.09.2019
MET A2-141	*Salmonella*	*enterica*	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-144	*Salmonella*	*enterica*	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-147	*Salmonella*	*enterica*	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-149	*Salmonella*	*enterica*	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-153	*Salmonella*	*enterica*	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-156	*Salmonella*	*enterica*	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-159	*Salmonella*	*enterica*	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-162	*Salmonella*	*enterica*	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-165	*Salmonella*	*enterica*	Hen's egg	Brand 21	Free Range	2.07.2020

Table 4. 1 (continued)

MET A2-168	*Salmonella*	*enterica*	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-171	*Salmonella*	*enterica*	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-174	*Salmonella*	*enterica*	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-177	*Salmonella*	*enterica*	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-180	*Salmonella*	*enterica*	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-183	*Salmonella*	*enterica*	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-186	*Salmonella*	*enterica*	Hen's egg	Brand 1	Cage	2.07.2020

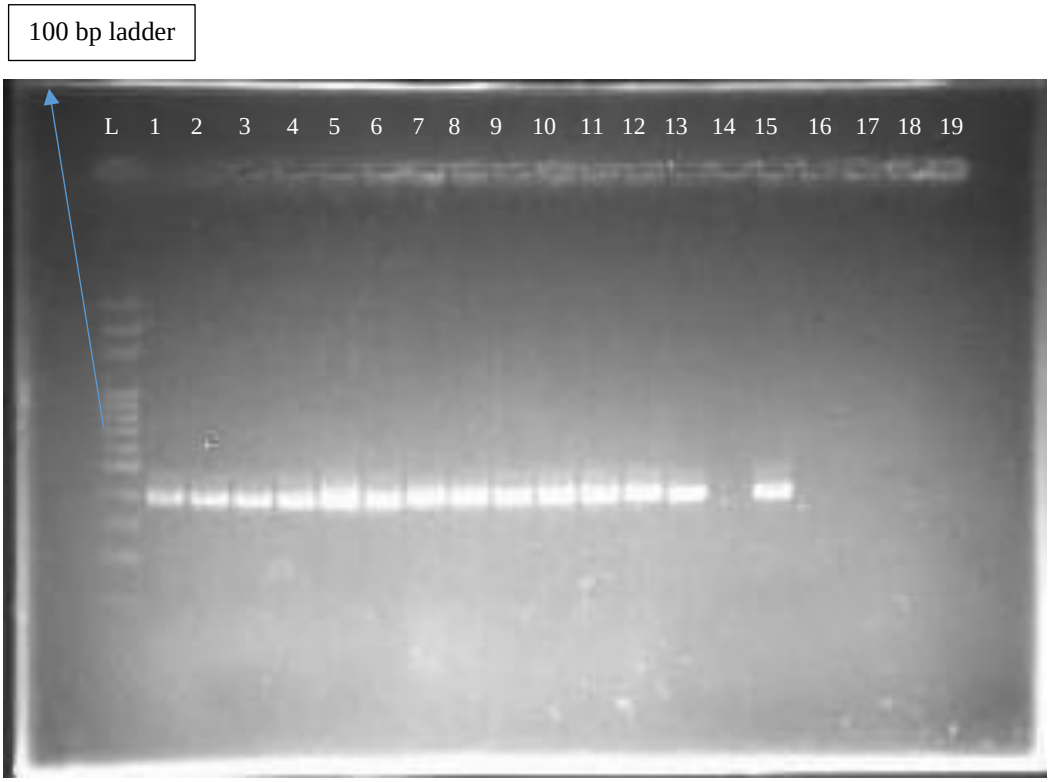

Figure 4. 1 PCR gel image of *Salmonella* isolates from eggs. L: DNA ladder; 15: MET S1-056; 14, 16,17,18,19: Negative Control; 1: MET A2-164, 2: MET A2-167; 3: MET A2-170; 4: MET A2-173; 5; MET A2-176; 6: MET A2-149; 7: MET A2-152; 8: MET A2-155; 9: MET A2- 158; 10: MET A2-161; 11: MET A2-179; 12: MET A2-182; 13: MET A2-185

If the isolates that were obtained from eggs were in the same band with control samples, this showed genus of isolates were proved to classify as a *Salmonella* (Figure 4.1). Among 250 different eggs, 17 eggs were confirmed as contaminated with *Salmonella*. In total 6.8 % of eggs was confirmed to be contaminated with *Salmonella* naturally from different egg from different cultivation methods. *Salmonella* was not found in organic eggs and village eggs. On the other hand, 6 each naturally contaminated eggs were detected in each of the free-range and cage eggs. The percentage of free-range and cage eggs naturally contaminated egg samples are 12 %. In cage-free cultivation method, 5 different eggs were found to be naturally contaminated by *Salmonella*. The percentage of naturally contaminated eggs for cage-free cultivation method is 10 %. *Salmonella* contamination was detected in 5 brands from 24 different commercial egg brands (Table 4.3). For free-range eggs, 10 different commercial brands were used and 2 different brands had naturally *Salmonella* contaminated eggs. On the market, less commercial brands produce cage-free eggs. In our experiment, 6 different brands were examined from cage-free eggs and 1 brand has naturally *Salmonella* contaminated eggs. When it comes to cage eggs, 10 different commercial brands were examined and naturally *Salmonella* contaminated eggs were detected from 2 different cage egg brands (Table 4.2).

When naturally contaminated 5 brands were examined, even though 4 different egg types were collected from Brand 0 only cage eggs were naturally contaminated with *Salmonella* (38 %). Free-range and cage eggs were collected from Brand 1 and *Salmonella* contamination was only detected in cage eggs (27 %). For Brand 21, free-range and cage-free eggs were collected from the markets and *Salmonella* contamination was detected only in free-range eggs (63 %). Thus, different cultivation methods of eggs in same brand might have effect on eggs according to our results (Table 4.2).

Table 4. 2 *Salmonella* Contamination according to brands.

Brand	Organic Eggs			Free-Range Eggs			Cage-Free Eggs			Cage Eggs			Total		
	Total	*Salmonella* Contaminated Eggs	%	Total	*Salmonella* Contaminated Eggs	%	Total	*Salmonella* Contaminated Eggs	%	Total	*Salmonella* Contaminated Eggs	%	Total	*Salmonella* Contaminated Eggs	%
Brand 0	17	0	0	11	0	0	7	0	0	8	3	38	43	3	7
Brand 1	-	-	-	5	0	0	-	-	-	11	3	27	16	3	19
Brand 6	-	-	-	-	-	-	23	5	22	-	-	-	23	5	22
Brand 8	-	-	-	3	1	33	-	-	-	-	-	-	3	1	33
Brand 21	-	-	-	8	5	63	4	0	0	-	-	-	12	5	42

According to Jamshidi et al., found out that percentage of naturally contaminated eggs with *Salmonella* genus is 1.6 % (Jamshidi et al., 2010). In a study conducted in Turkey, researchers reached the conclusion that 3.3 % of purchased eggs from different region of Turkey were contaminated naturally by *Salmonella* genus (Diker et al., 2020).

According to Moosavy et al., percentage of naturally contaminated eggs by *Salmonella* was found to be 1.33 % (Moosavy et al., 2015). According to study that were conducted by Singh et al., 5.6 % of commercially collected eggs were naturally contaminated by *Salmonella*. In another study showed that detection rate of naturally contaminated *Salmonella*-positive eggs were 6.6 % (Li et al., 2020). In Iran, study on *Salmonella* prevalence on eggs was conducted. According to their result, 7.49 % of commercial eggs were naturally contaminated by *Salmonella* and higher contamination occurred (Hosseininezhad et al., 2020).

When all the studies were reviewed, our result had similar percentages of naturally contaminated *Salmonella*-positive eggs (Table 4.2). In our experiment, no *Salmonella* contaminated eggs were found from village egg samples. This was unexpected for this study. Because of there are no specific cultivation method for village egg. According to study which was conducted in Turkey, naturally *Salmonella* contaminated egg percentage were found to be same for both commercial eggs and village eggs (2 %) (Yenilmez, 2020). In another study, while *Salmonella* prevalence of village egg was found to be 0.5 %, no *Salmonella* was detected from commercial egg samples (Karadal et al., 2018). Tirma et al. conducted similar experiment to see the different between commercial and village egg. According to their results, while the presence of *Salmonella* in village eggs is 10 %, this rate was determined as 21 % in commercial eggs (Tirma et al., 2008). The complex problem of *Salmonella* contamination of eggs is influenced by factors at every stage of the food manufacturing process. The literature is currently divided on the advantages of cage-free, caged, free-range, and organic production methods with relation to *Salmonella* contamination. The available research does, however, suggest that

producing eggs that are guaranteed to be *Salmonella*-free is not yet feasible. (Whiley & Ross, 2015).

According to many consumers, because of organic and free-range methods have more free space, special methods for diet and access to open air, prevalence of *Salmonella* is higher in traditional methods such as cage-free and cage chicken. However, there are very few studies about this subject. In this experiment, prevalence of *Salmonella* was observed on different cultivation methods. Production methods of eggs might affect the risk of salmonellosis, but still not enough survey had been conducted. In one study, organic methods such as organic and free-range method were found to be higher *Salmonella* prevalence than conventional systems such as cage-free and cage system. On the other hand, another study showed that there were no difference between these methods (Cardoso et al., 2021). Another aspect to evaluate the issue is that organic and free-range system are open to exposure of wild animals which might be cause of *Salmonella* infection. According to study about this subject, prevalence of *Salmonella* was higher in the free-range and organic system than conventional systems. Researchers approaches about the results were because of organic and free-range methods have access to open air, exposure risk of potential *Salmonella* infected wild animals were higher (Bailey & Cosby, 2005). According to another study, prevalence of *Salmonella* in organic eggs were found to be 20% and traditional methods had 5.4 % of *Salmonella* prevalence (Lee et al., 2013).

Even though consumers prefer organic or free-range eggs, this study and other studies showed that this assumption is not correct. Although organic and free-range systems are open air and have contamination risk from wildlife, cage and cage-free system have closed system and transmission risk one to another chicken or eggs are highly possible than organic methods (Cardoso et al., 2021).

Table 4. 3 Distribution of *Salmonella* prevalence by egg cultivation methods

Egg types	Number of Samples	Presence of *Salmonella*	% Of Presence of *Salmonella*
0 (Organic egg)	50	0	0 %
1 (Free range egg)	50	6	12 %
2 (Cage free egg)	50	5	10 %
3 (Cage egg)	50	6	12 %
4 (Village egg)	50	0	0 %
Total	250	17	6,8 %

4.2 Classification of *Salmonella* Isolates by PFGE Method

After PCR method was applied, isolated *Salmonella* samples were examined by using PFGE method to specify serovars. Pulsed-field gel electrophoresis has been considered as a gold standard method, and it is highly discriminated compared to other methods (Ferrari et al., 2017). In our study, the cluster analysis revealed 3 different *Salmonella* serotypes (Figure 4.4). Most of the serovars of isolated samples were found out to be *S. enterica* subsp. *enterica* serovar Infantis (*Salmonella* Infantis) (Table 4.3). 88.24 % of isolated shared this same pattern (MET A2-153, MET A2-156, MET A2-159, MET A2-162, MET A2-165, MET A2-168, MET A2-171, MET A2-174, MET A2-177, MET A2-180, MET A2-183, MET A2-186, MET A2-141, MET A2-144, MET A2-149) (Figure 4.2). Out of 17 isolates, MET A2-138 was determined as *Salmonella* Kentucky (5.88 %). Lastly, MET A2-147 found to be representing *Salmonella* Telaviv (5.88 %) (Figure 4.3).

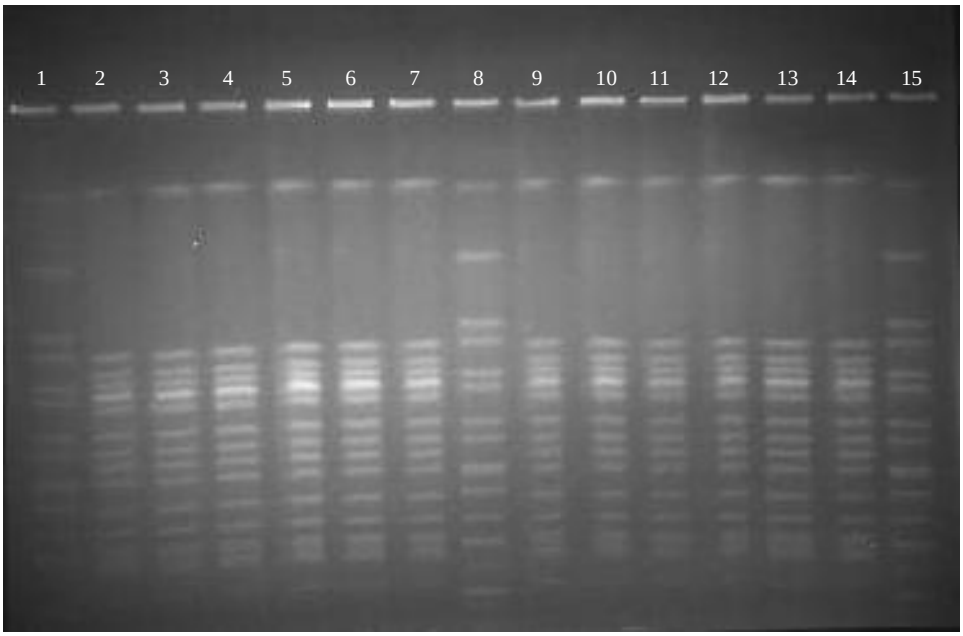

Figure 4. 2 PFGE gel picture of *Salmonella* isolates. 1,8, 15: MET S1-713 (*Salmonella* Braenderup as reference strain); 2: MET A2-153; 3: MET A2-156; 4: MET A2-159; 5: MET A2-162; 6: MET A2 165; 7: MET A2-168; 9: MET A2 171; 10: MET A2-174; 11: MET A2- 177; 12: MET A2-180; 13: MET A2-183; 14: MET A2-186.

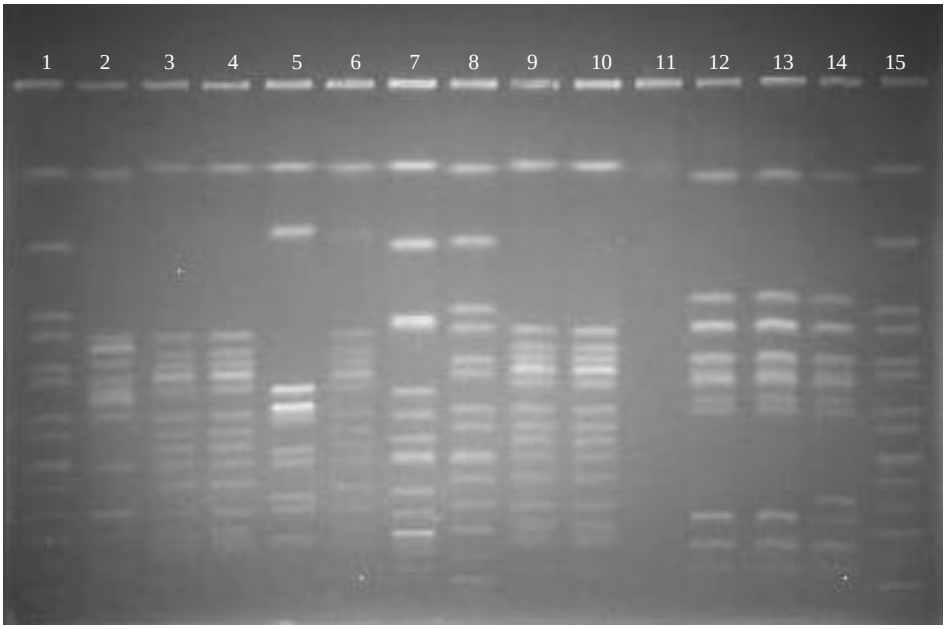

Figure 4. 3 PFGE gel picture of *Salmonella* isolates. 1,8, 15: MET S1-713 (*Salmonella* Braenderup as reference strain); 2: MET A2-138; 3: MET A2-141; 4: MET A2-144; 5: MET A2-147; 6: MET A2-149; 7, 9, 10, 11, 12, 13, 14: Belong to another study.

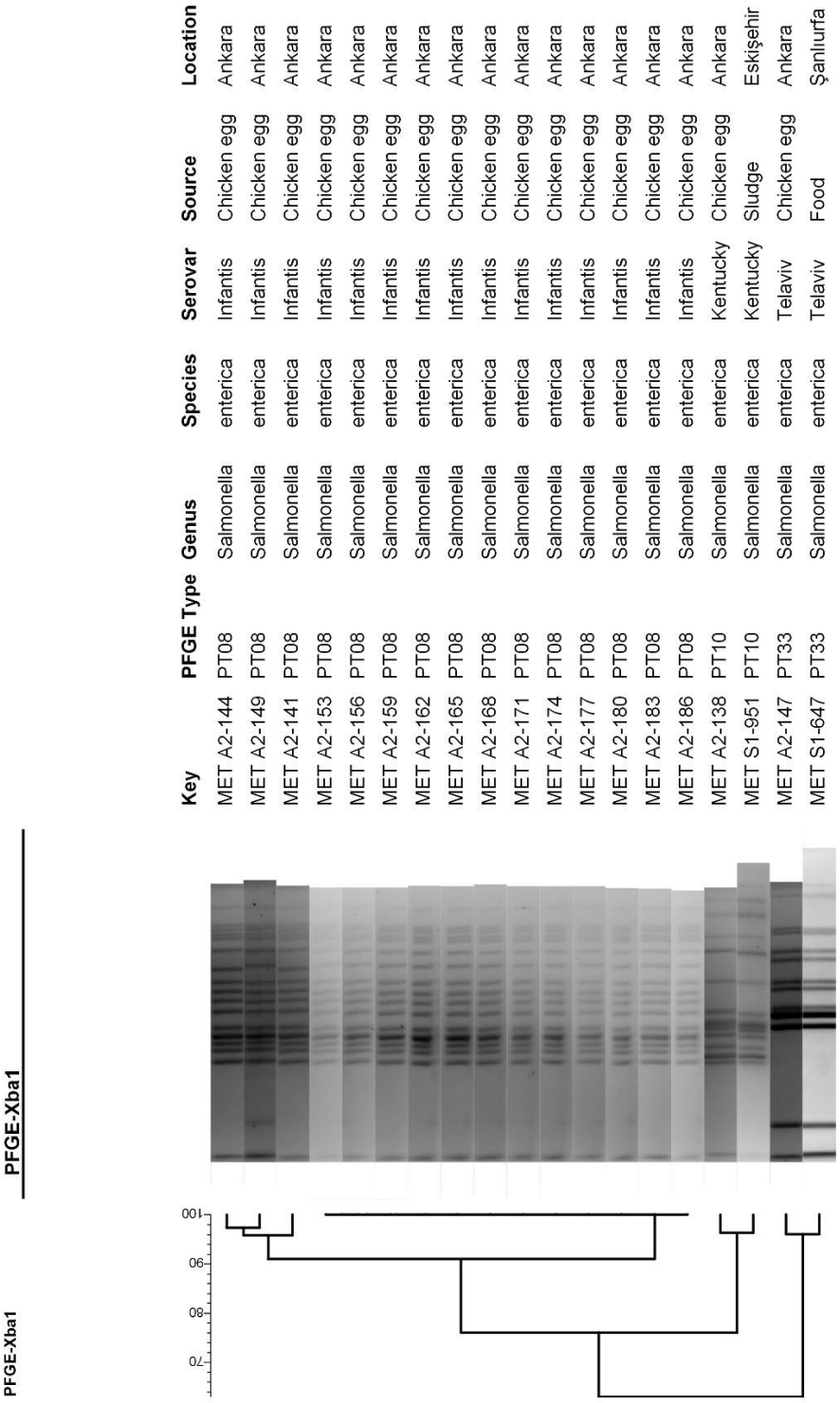

Figure 4. 4 Cluster analysis of *Salmonella* isolates from eggs

Salmonella Infantis is one of the most isolated serovar from poultry along with *Salmonella* Enteritidis in Turkey. (Sariçam & Müştak, 2018). According to study conducted in Turkey, *Salmonella* Enteritidis and *Salmonella* Infantis were the most encountered serovar of *Salmonella*. The prevalence of *Salmonella* Enteritidis and *Salmonella* Infantis increased over the year (Temelli et al., 2015). According to study conducted in Turkey, *Salmonella* prevalence in chicken eggs were examined and according to their results, all the *Salmonella* serovars were reported as *Salmonella* Enteritidis (Temelli et al., 2015). Aksoy et. al. reported that *Salmonella* Enteritidis, *Salmonella* Infantis and *Salmonella* Kentucky were frequently isolated from chicken meat and egg. However, *Salmonella* Telaviv was one of the rarest serovar (Aksoy & En, 2015). Durul et al. reported that out of 13 different food samples, 5 different food samples were contaminated by *Salmonella* Telaviv even though this serovar was rare to encounter (Durul et al., 2015).

According to Cardoso et. al, laying hens and chicken egg are the primary source of *Salmonella* strains that cause 42.4 % of all human cases of salmonellosis in Europe and 95.9 % of these cases were reported to have *Salmonella* Enteritidis which has been the main serovar for human cases. (Cardoso et al., 2021). According to EFSA Report in 2013, some of the most reported serotypes from chicken and eggs were *Salmonella* Enteritidis and *Salmonella* Infantis in the European Union (EU). Also, *S.* Enteritidis and *S.* Infantis had been also predominant serotype in Japan, United State and Saudi Arabia. According to another study in China, most common *Salmonella* serotypes that encountered in eggs were *S.* Typhimurium (24.5 %). Detection level of *Salmonella* Enteritidis was 11.4 % (Li et al., 2020). According to another study, prevalence of eight different *Salmonella* serotypes were analyzed in natural egg contamination during the study conducted in Iran and, *Salmonella* Enteritis was found to have the highest prevalence (Hosseininezhad et al., 2020).

Serotypes of *Salmonella* serotypes and their prevalence might show difference across the world. This actively demonstrates that geographical clustering of study affects the prevalence of serotypes in the different part of the world. Even though

predominant serotypes could change according to geographic clustering, most frequently seen *Salmonella* serotype in human cases is *Salmonella* Enteritidis in the world. Although, *Salmonella* Infantis was the most prevalent isolated serovar in our study, *Salmonella* Enteritidis is the most encountered serovar for human infection cases from eggs. Thus, *Salmonella* Enteritidis was selected to be used for bacteriophage application in this experiment.

Table 4. 4 Detailed information of *Salmonella* isolates from eggs

METU ID	Genus	Species	Serotype	Source	Brand	Cultivation Method	Date
MET A2-138	*Salmonella*	*enterica*	Kentucky	Hen's egg	Brand 8	Free Range	25.09.2019
MET A2-141	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-144	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-147	*Salmonella*	*enterica*	Telaviv	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-149	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-153	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-156	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-159	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-162	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-165	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-168	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-171	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-174	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-177	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020

Table 4. 3 (continued)

MET A2-180	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-183	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-186	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020

As mentioned before, usage of antibiotics against bacteria is very common in egg production industry. However, many bacteria have gained antibacterial resistance because of misusing or overusing of antibiotics. Also, this caused spreading of antibacterial resistance among bacteria including *Salmonella* spp. There are many *Salmonella* serotypes that are linked to eggs and poultry such as *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Kentucky and wide variety of antibiotics were used to inhibit infection of these pathogenic bacteria and support the growth of hens. According to study, *Salmonella* isolated from eggs showed high resistance against tetracycline (80 %) and Ampicillin (59.5 %). Also, relatively low (20 %) cefotaximine, nalidixic acid, ciprofloxacin and chloramphenicol resistance were reported (Kapena, 2020). In another study, Novobiocin and Vancomycin resistance was very high (100 %) among the *Salmonella* isolates from various types of eggs. Also, ampicilin (80 %) and tetracycline (50 %) resistance were reported (Ht et al., 2011). Our *Salmonella* isolates were checked for antibiotic resistance in another study (Konyalı, 2022). According to results, most of our isolates (N= 13) showed multidrug resistance (MDR). sulfisoxazole, azithromycin and pefloxacin were most encountered drugs isolates that were resistant against in our study (Table 4.6). only one isolates have susceptible against all the drugs studied. The presence of resistant *Salmonella* isolates in eggs can create serious public health concern given the consumption patterns of these table eggs (Konyalı, 2022). Thus, bacteriophage application could be an alternative option. Additionally, compared to antibiotics, bacteriophages are far more specific to bacteria. It should be emphasized that antibiotic treatment affects the normal gut microbiota in addition to killing

pathogenic bacteria, which could result in dysbiosis and immunosuppression (Michalczuk & Dolka, 2020).

Table 4. 5 Phenotypic and Genotypic antimicrobial resistance profiles of *Salmonella* isolates obtained by egg samples (Konyalı, 2022).

METUID	Serotype	Phenotypic AMRProfile	Genotypic AMR	Source
MET A2-137	Kentucky	AmpAzmPef	*qnrS parC mphA*	egg
MET A2-140	Infantis	Susceptible		egg
MET A2-143	Infantis	Amp		egg
MET A2-146	Telaviv	Sf		egg
MET A2-149	Infantis	AmpAzm	-	egg
MET A2-152	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-155	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-158	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-161	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-164	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-167	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-170	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-173	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-176	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-179	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-182	Infantis	SxtSfNAzmPef	*parC*	egg
MET A2-185	Infantis	SxtSfNAzmPef	*parC*	egg

Sxt: Trimethoprim-sulfamethoxazole, Sf: Sulfisoxazole, NA/N: Nalidixic acid, Azm: Azithromycin, Pef: Pefloxacin

4.3 Bacteriophage Therapy of *Salmonella* on Contaminated Eggs

For bacteriophage therapy, commercially collected whole eggs were firstly contaminated with MET S1-001 (*Salmonella enterica* Enteritidis) using 2 different *Salmonella* solution (10^7 CFU/mL and 10^5 CFU/mL) and then contaminated eggs were sprayed with 10^8 PFU/mL bacteriophage solution by using MET P1-001 (*Salmonella enterica* Enteritidis). Thus, different multiplication of infection (MOI) levels was examined during our experiment.

In the first part, 10^7 CFU/mL *Salmonella* solution and 10^8 PFU/mL bacteriophage solution were used (MOI: 10). Bacterial count at initial point for control samples which were only contaminated by *Salmonella* solution was 5,34±0,64 log CFU/mL. the contamination level was decreased to 3,41±0,79 log CFU/mL when bacteriophage solution was applied. At initial point, 1,93 log reduction was observed (Figure 4.5). After 7 days of incubation at 4 °C, *Salmonella* Enteritidis concentration for control group and phage therapy were respectively 5,33±0,55 log CFU/mL and 3,0,9±0,02 log CFU/mL. The reduction level increased to 2,24 log reduction. According to results, phage application showed that more than 2 log reduction was obtained from initial point to 7 days with 10 MOI (Table 4.5).

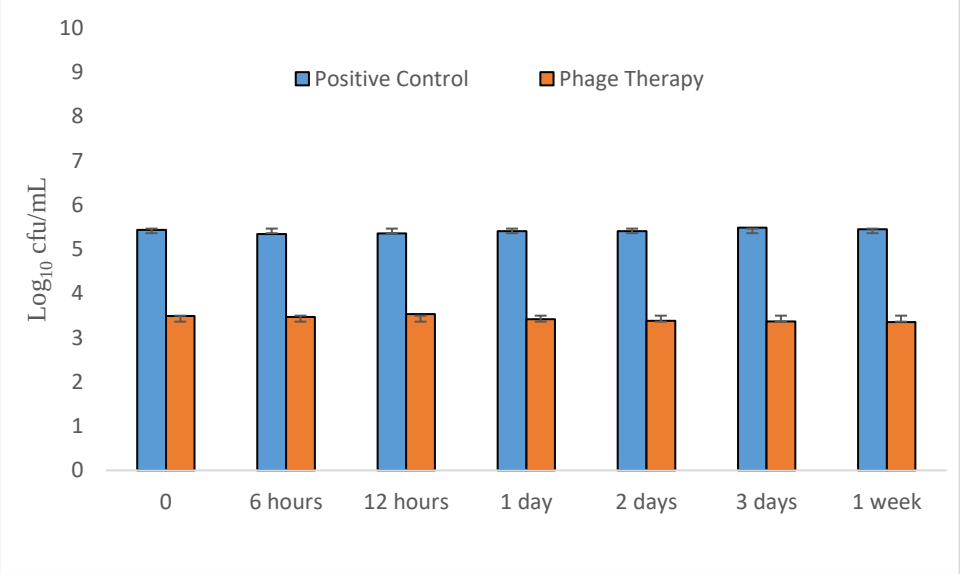

Figure 4. 5 Effect of P1-001 (10^8 PFU/mL) on its host S1-001 (10^7 CFU/mL).

Table 4. 6 Viable count of MET S1-001 (*Salmonella enterica* Enteritidis) after 0, 6, 12, 24, 48, 72 and 168 h at 4 °C with 10^1 MOI, samples dipped in *Salmonella* solution and samples with phage therapy.

Time (h)	*Salmonella* (log10 CFU/mL)		*Salmonella* Reduction (log10 CFU/mL)
	Salmonella Treated	Phage Treated	
0	5,34±0,64	3,41±0,79	1,93
6	5,38±0,47	3,33±0,47	2,05
12	5,38±0,77	3,30±0,43	2,08
24	5,24±0,64	3,27±0,17	1,97
48	5,42±0,82	3,18±0,18	2,24
72	5,33±0,5	3,29±0,67	2,04
168	5,33±0,55	3,09±0,02	2,24

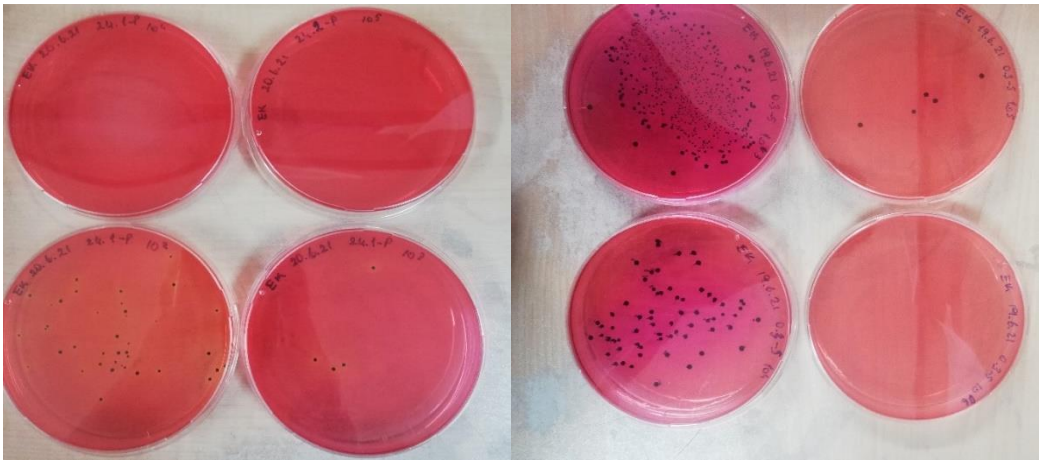

Figure 4. 6 According to dilution rate, presence of *Salmonella* on XLD agar after phage therapy & control samples with 10^1 MOI.

In the second part of our study, 10^5 CFU/mL *Salmonella* solution and 10^8 PFU/mL bacteriophage solution were used (MOI: 10^3). Bacterial count at initial point for control samples was 4,64±0,34 log CFU/mL. After bacteriophage therapy, 4,2 log reduction was observed at initial point (Figure 4.7). After 7 days of incubation at 4 °C, *Salmonella* Enteritidis concentration for control group were 4,11±0,61 log

cfu/mL. After bacteriophage therapy, 3,67 log reduction was observed after 7 days with 10^3 MOI (Table 4.6). After 7 days, 93 % reduction was observed.

Table 4. 7 Viable count of MET S1-001 (*Salmonella enterica* Enteritidis) after 0, 6, 12, 24, 48, 72 and 168 h at 4 °C with 10^3 MOI, samples dipped in *Salmonella* solution and samples with phage therapy

Time (h)	*Salmonella* (log10 CFU/mL)		*Salmonella* Reduction (log10 CFU/mL)
	Salmonella Treated	Phage Treated	
0	4,64±0,34	0,44	4,2
6	4,50±0,44	0,44	4,06
12	4,52±0,32	0,31	4,21
24	4,37±0,35	0,44	3,93
48	4,57±0,66	0,44	4,13
72	4,21±0,51	0,31	3,9
168	4,11±0,61	0,44	3,67

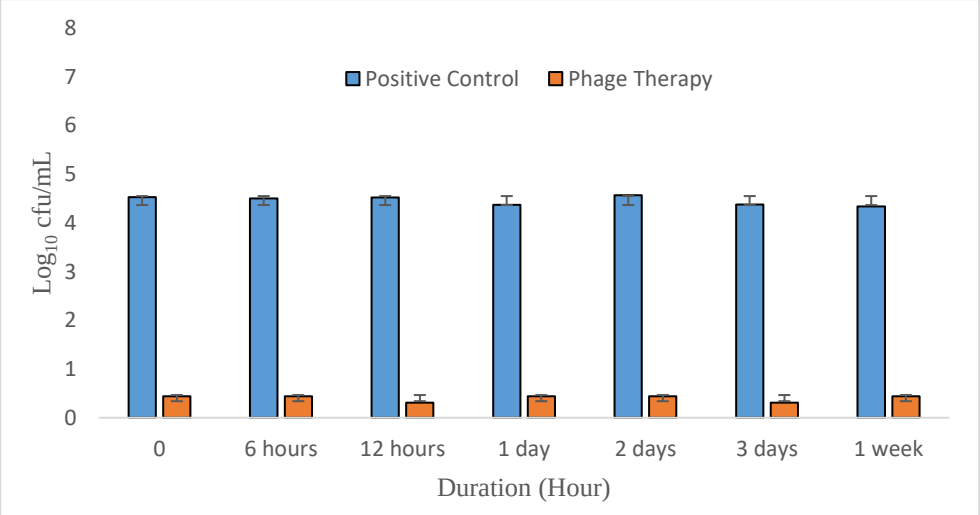

Figure 4. 7 Effect of P1-001 (10^8 PFU/mL) on its host S1-001 (10^5 CFU/mL).

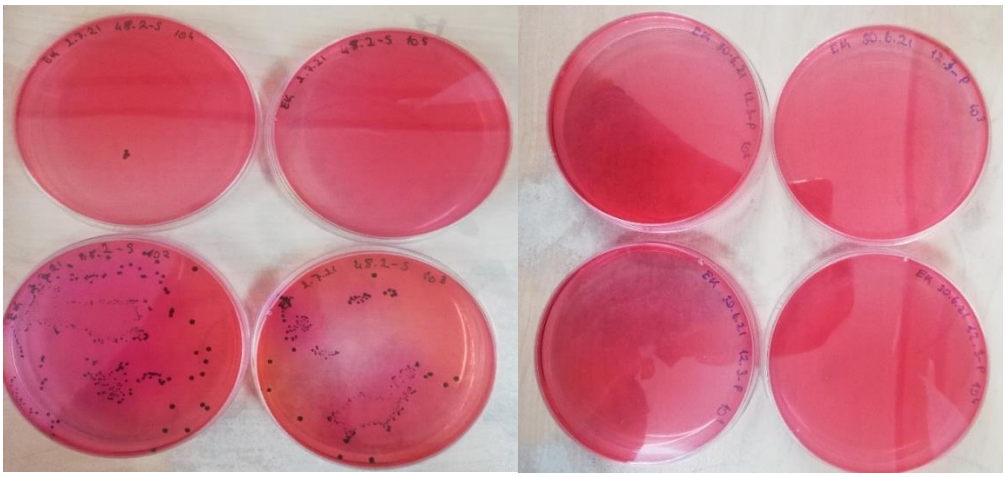

Figure 4. 8 According to dilution rate, presence of *Salmonella* on XLD agar after phage therapy & control samples with 10^3 MOI.

According to our results, bacteriophage application started to eliminate pathogenic bacteria rapidly from initial point. When multiplication of infection level was increased, reduction level of *Salmonella* has also increased. Almost 93 % of *Salmonella* was eliminated with 10^3 MOI.

According to another study, 3 log reduction within 30 minutes was obtained by phage application on fresh eggs with 0.01 MOI (Sonalika et al., 2020). Spricigo et, al. claimed that refrigeration temperature which reduces bacterial growth and higher dosage of multiplication of infection (MOI) can lead to better reduction level (Spricigo et al., 2013). Even though, lower level of phage concentration is enough to eliminate noticeable number of bacteria within 2 hours, higher level of phage concentration is enough to lyse pathogenic bacteria without multiplication. On the other hand, low concentration level of bacteriophage is more appropriate for mass production because of economic reason (Moye et al., 2018). Previous studies indicated that bacteriophage applications were more effective under refrigeration temperature (4 °C). Also, using higher multiplication of infection (MOI) level ensured better results to reduce the bacterial count within the first few minutes. (Hong et al., 2016).

According to study, 90 % of bacterial contamination was eliminated by bacteriophage therapy. This result was obtained in first 15 minutes and then, it was observed that it remained constant throughout the incubation period (Makalatia et al., 2018). In another study, almost 99 % of bacterial contamination was eliminated by phage therapy within 30 minutes (Sonalika et al., 2020). This actively demonstrates that our results showed similar outputs and obtained desired reduction level. Also, our results and other studies demonstrated that higher MOI level has better effect on elimination of bacterial load. However, the common feature of our study and these studies is that no study was able to obtain a complete elimination of *Salmonella* by using bacteriophage therapy. Thus, bacteriophage therapy might be used as an alternative sanitation method for decontamination of whole egg. When, bacteriophage application is compared to antibiotics, bacteriophage application has narrow spectrum to action. It provides higher safety and tolerability. Also, phage application is less expensive compared to antibiotics (Principi et al., 2019).

As a results, bacteriophage therapy is applicable method for fresh egg sanitation in the industry. However, further investigations are required for bacteriophage therapy method with other *Salmonella* serovars and other pathogenic bacteria.

CHAPTER 5

CONCLUSION

Egg is well known protein source and it has rich nutritional values and the consumption level of egg is very high worldwide. Besides being nutritionally rich, eggs and egg products are one of the main food sources of *Salmonella* which is one of the most common pathogens in the world. Thus, the elimination of *Salmonella* is very crucial in terms of food safety. In the commercial egg production, many regulations have been made recently. Today, 4 different egg production methods (organic, free-range, cage-free and cage) are applied in the world including Turkey. However, there is still uncommercial village eggs are consumed by consumers and many customers still prefer village eggs. Investigate the effect of these egg production methods on *Salmonella* prevalence poses significant importance. In commercial egg production, many sanitation methods are available but mostly egg washing is preferred. Egg washing and other methods generally used chemicals and it can damage the product and cause harmful effect to humans. Also, antibiotic usage is preferred in several steps of food production to fight against *Salmonella*. However, overuse and misuse of these substance causes spread of antibiotic resistance among bacteria. There is a need for alternative method without side-effects. Bacteriophages are promising biological entities and they can be used for in application to eliminate *Salmonella*.

In this study, commercial and village egg samples were collected from different region and different brands (n=250) in Ankara region. *Salmonella* isolation procedure was applied on these eggs. In total, 17 *Salmonella* strains were isolated. Form these 17 strains, 3 different *Salmonella* serotypes which are *Salmonella* Infantis (88.23 %), *Salmonella* Kentucky (5.88 %), and *Salmonella* Telaviv (5.88 %)

were obtained by using Polymerase Chain Reaction and Pulse Field Gel Electrophoresis (PFGE).

Another investigated parameter was the phage therapy used on contaminated egg samples to eliminate *Salmonella*. In total 126 eggs were used in this part. After phage therapy, almost 2 log reduction was achieved with 10 MOI. When 10^3 MOI was applied on egg surface, approximately 4 log reduction was observed after experiment and contamination level was reduced 93 %. For both experiments, reduction of *Salmonella* from initial time to 7 days remained almost constant. Thus, desired reduction of *Salmonella* was achieved by our experiment. However, our study as well as many other studies show that total eradication was not obtained.

This study investigated *Salmonella* isolates obtained from different types of eggs which have different production methods and help to understand their characteristics. Also, our study helped to understand bacteriophage application on eggs as an alternative method to eliminate *Salmonella.*

Still, further investigation is required to understand bacteriophage application. In our study, *Salmonella* Enteritidis was used because of its prevalence in the world. For future study, *Salmonella* Infantis should be selected for phage application and phage therapy with different food source should be investigated. Besides, future study should be conducted to find a way for total elimination for *Salmonella* by bacteriophage application and phage types must be well-defined by using phage GRASS in food safety applications.

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APPENDICES

A. LIST OF *SALMONELLA* ISOLATES USED

Table A. 1 List of *Salmonella* strains isolated in this study

METU ID	Genus	Species	Serotype	Source	Brand	Cultivation Method	Date
MET A2-137	*Salmonella*	*enterica*	Kentucky	Hen's egg	Brand 8	Free Range	25.09.2019
MET A2-138	*Salmonella*	*enterica*	Kentucky	Hen's egg	Brand 8	Free Range	25.09.2019
MET A2-139	*Salmonella*	*enterica*	Kentucky	Hen's egg	Brand 8	Free Range	25.09.2019
MET A2-140	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-141	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-142	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-143	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-144	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-145	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-146	*Salmonella*	*enterica*	Telaviv	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-147	*Salmonella*	*enterica*	Telaviv	Hen's egg	Brand 0	Cage	25.06.2020

Table A. 1 (continued)

MET A2-148	*Salmonella*	*enterica*	Telaviv	Hen's egg	Brand 0	Cage	25.06.2020
MET A2-149	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-150	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-152	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-153	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-154	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-155	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-156	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-157	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-158	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-159	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-160	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-161	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-162	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020
MET A2-163	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 6	Cage Free	2.07.2020

Table A. 1 (continued)

MET A2-164	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-165	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-166	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-167	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-168	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-169	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-170	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-171	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-172	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-173	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-174	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-175	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-176	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-177	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020
MET A2-178	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 21	Free Range	2.07.2020

Table A. 1 (continued)

MET A2-179	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-180	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-181	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-182	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-183	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-184	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-185	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020
MET A2-186	*Salmonella*	*enterica*	Infantis	Hen's egg	Brand 1	Cage	2.07.2020

Table A. 2 List of *Salmonella* strains isolated previously (2010-2015).

METUID	**Genus**	**Species**	**Serotype**	**Specified Source**	**Collection Date**	**City**
MET S1-024	*Salmonella*	enterica	Corvallis	Pistachio	29.12.2012	Şanlıurfa
MET S1-031	*Salmonella*	enterica	Salford	Pistachio	29.12.2012	Şanlıurfa
MET S1-035	*Salmonella*	enterica	Corvallis	Pistachio	29.12.2012	Şanlıurfa
MET S1-050	*Salmonella*	enterica	Infantis	Chicken meat	11.04.2012	Şanlıurfa
MET S1-056	*Salmonella*	enterica	Infantis	Chicken meat	11.04.2012	Şanlıurfa
MET S1-063	*Salmonella*	enterica	Telaviv	Offal	11.04.2012	Şanlıurfa
MET S1-065	*Salmonella*	enterica	Montevideo	Offal	11.04.2012	Şanlıurfa
MET S1-074	*Salmonella*	enterica	Telaviv	Offal	11.04.2012	Şanlıurfa

Table A.2 (continued)

MET S1-087	*Salmonella*	enterica	Othmarschen	Sheep ground meat	11.04.2012	Şanlıurfa
MET S1-088	*Salmonella*	enterica	Infantis	Chicken meat	11.04.2012	Şanlıurfa
MET S1-092	*Salmonella*	enterica	Infantis	Chicken meat	11.04.2012	Şanlıurfa
MET S1-103	*Salmonella*	enterica	Infantis	Chicken meat	11.04.2012	Şanlıurfa
MET S1-119	*Salmonella*	enterica	Telaviv	Unripened cheese	11.04.2012	Şanlıurfa
MET S1-142	*Salmonella*	enterica	Infantis	Chicken meat	11.04.2012	Şanlıurfa
MET S1-150	*Salmonella*	enterica	Infantis	Offal	11.04.2012	Şanlıurfa
MET S1-163	*Salmonella*	enterica	Hadar	Urfa cheese	11.04.2012	Şanlıurfa
MET S1-166	*Salmonella*	enterica	Newport	Cattle	09.04.2012	Şanlıurfa
MET S1-170	*Salmonella*	enterica	Montevideo	Cattle	06.05.2012	Şanlıurfa
MET S1-172	*Salmonella*	enterica	Montevideo	Cattle	06.05.2012	Şanlıurfa
MET S1-175	*Salmonella*	enterica	Montevideo	Cattle	06.05.2012	Şanlıurfa
MET S1-179	*Salmonella*	enterica	Montevideo	Chicken	08.05.2012	Şanlıurfa
MET S1-183	*Salmonella*	enterica	subsp. diarizonae	Sheep	15.05.2012	Şanlıurfa
MET S1-184	*Salmonella*	enterica	Paratyphi B	Human	17.04.2012	Şanlıurfa
MET S1-185	*Salmonella*	enterica	Typhimurium	Human	02.05.2012	Şanlıurfa
MET S1-186	*Salmonella*	enterica	Paratyphi B	Human	27.05.2012	Şanlıurfa
MET S1-187	*Salmonella*	enterica	Kentucky	Human	28.05.2012	Şanlıurfa
MET S1-188	*Salmonella*	enterica	Paratyphi B	Human	01.08.2010	Şanlıurfa
MET S1-189	*Salmonella*	enterica	Paratyphi B	Human	01.08.2010	Şanlıurfa
MET S1-190	*Salmonella*	enterica	Paratyphi B	Human	03.08.2010	Şanlıurfa
MET S1-191	*Salmonella*	enterica	Paratyphi B	Human	03.08.2010	Şanlıurfa
MET S1-192	*Salmonella*	enterica	Paratyphi B	Human	05.08.2010	Şanlıurfa

Table A. 2 (continued)

MET S1-193	*Salmonella*	enterica	Paratyphi B	Human	05.08.2010	Şanlıurfa
MET S1-194	*Salmonella*	enterica	Paratyphi B	Human	07.08.2010	Şanlıurfa
MET S1-195	*Salmonella*	enterica	Paratyphi B	Human	07.08.2010	Şanlıurfa
MET S1-196	*Salmonella*	enterica	Paratyphi B	Human	08.08.2010	Şanlıurfa
MET S1-197	*Salmonella*	enterica	Paratyphi B	Human	08.08.2010	Şanlıurfa
MET S1-198	*Salmonella*	enterica	Paratyphi B	Human	10.08.2010	Şanlıurfa
MET S1-199	*Salmonella*	enterica	Paratyphi B	Human	10.08.2010	Şanlıurfa
MET S1-200	*Salmonella*	enterica	Paratyphi B	Human	15.08.2010	Şanlıurfa
MET S1-201	*Salmonella*	enterica	Paratyphi B	Human	15.08.2010	Şanlıurfa
MET S1-202	*Salmonella*	enterica	Paratyphi B	Human	16.08.2010	Şanlıurfa
MET S1-203	*Salmonella*	enterica	Paratyphi B	Human	16.08.2010	Şanlıurfa
MET S1-204	*Salmonella*	enterica	Typhimuriu m	Human	19.08.2010	Şanlıurfa
MET S1-205	*Salmonella*	enterica	Paratyphi B	Human	22.08.2010	Şanlıurfa
MET S1-206	*Salmonella*	enterica	Paratyphi B	Human	14.09.2010	Şanlıurfa
MET S1-207	*Salmonella*	enterica	Typhimuriu m	Human	21.09.2010	Şanlıurfa
MET S1-208	*Salmonella*	enterica	Paratyphi B	Human	23.09.2010	Şanlıurfa
MET S1-211	*Salmonella*	enterica	Typhimuriu m	Human	28.09.2010	Şanlıurfa
MET S1-212	*Salmonella*	enterica	Paratyphi B	Human	29.09.2010	Şanlıurfa
MET S1-213	*Salmonella*	enterica	Paratyphi B	Human	02.06.2011	Şanlıurfa
MET S1-217	*Salmonella*	enterica	Enteritidis	Human	20.06.2011	Şanlıurfa
MET S1-218	*Salmonella*	enterica	Paratyphi B	Human	23.06.2011	Şanlıurfa
MET S1-219	*Salmonella*	enterica	Kentucky	Human	23.06.2011	Şanlıurfa
MET S1-220	*Salmonella*	enterica	Typhi	Human	26.06.2011	Şanlıurfa

Table A. 2 (continued)

MET S1-221	*Salmonella*	enterica	Enteritidis	Human	26.06.2011	Şanlıurfa
MET S1-223	*Salmonella*	enterica	Typhimurium	Human	13.09.2011	Şanlıurfa
MET S1-224	*Salmonella*	enterica	Paratyphi B	Human	15.09.2011	Şanlıurfa
MET S1-227	*Salmonella*	enterica	Othmarschen	Human	04.05.2012	Şanlıurfa
MET S1-228	*Salmonella*	enterica	Kentucky	Human	10.05.2012	Şanlıurfa
MET S1-231	*Salmonella*	enterica	Paratyphi B	Human	02.06.2012	Şanlıurfa
MET S1-232	*Salmonella*	enterica	Paratyphi B	Human	08.06.2012	Şanlıurfa
MET S1-233	*Salmonella*	enterica	Typhimurium	Human	12.06.2012	Şanlıurfa
MET S1-234	*Salmonella*	enterica	Typhi	Human	15.06.2012	Şanlıurfa
MET S1-235	*Salmonella*	enterica	Paratyphi B	Human	15.06.2012	Şanlıurfa
MET S1-236	*Salmonella*	enterica	Typhimurium	Human	18.06.2012	Şanlıurfa
MET S1-237	*Salmonella*	enterica	Othmarschen	Human	18.06.2012	Şanlıurfa
MET S1-238	*Salmonella*	enterica	Paratyphi B	Human	23.06.2012	Şanlıurfa
MET S1-239	*Salmonella*	enterica	Kentucky	Human	23.06.2012	Şanlıurfa
MET S1-240	*Salmonella*	enterica	Kentucky	Human	24.06.2012	Şanlıurfa
MET S1-241	*Salmonella*	enterica	Paratyphi B	Human	24.06.2012	Şanlıurfa
MET S1-242	*Salmonella*	enterica	Paratyphi B	Human	29.06.2012	Şanlıurfa
MET S1-243	*Salmonella*	enterica	Paratyphi B	Human	29.06.2012	Şanlıurfa
MET S1-248	*Salmonella*	enterica	Anatum	Sheep ground meat	18.07.2012	Şanlıurfa
MET S1-258	*Salmonella*	enterica	Anatum	Sheep ground meat	18.07.2012	Şanlıurfa
MET S1-272	*Salmonella*	enterica	Anatum	Sheep ground meat	18.07.2012	Şanlıurfa

Table A. 2 (continued)

MET S1-277	*Salmonella*	enterica	Montevideo	Sheep ground meat	18.07.2012	Şanlıurfa
MET S1-294	*Salmonella*	enterica	Anatum	Cow ground meat	18.07.2012	Şanlıurfa
MET S1-301	*Salmonella*	enterica	Anatum	Cow ground meat	18.07.2012	Şanlıurfa
MET S1-313	*Salmonella*	enterica	Kentucky	Cow ground meat	18.07.2012	Şanlıurfa
MET S1-324	*Salmonella*	enterica	Montevideo	Cow ground meat	18.07.2012	Şanlıurfa
MET S1-329	*Salmonella*	enterica	Infantis	Chicken meat	18.07.2012	Şanlıurfa
MET S1-345	*Salmonella*	enterica	Infantis	Chicken meat	18.07.2012	Şanlıurfa
MET S1-351	*Salmonella*	enterica	Infantis	Chicken meat	18.07.2012	Şanlıurfa
MET S1-360	*Salmonella*	enterica	Kentucky	Offal	18.07.2012	Şanlıurfa
MET S1-363	*Salmonella*	enterica	Reading	Offal	18.07.2012	Şanlıurfa
MET S1-372	*Salmonella*	enterica	Montevideo	Offal	18.07.2012	Şanlıurfa
MET S1-391	*Salmonella*	enterica	Telaviv	Urfa cheese	18.07.2012	Şanlıurfa
MET S1-392	*Salmonella*	enterica	Reading	Sheep	26.06.2012	Şanlıurfa
MET S1-393	*Salmonella*	enterica	Poona	Sheep	26.06.2012	Şanlıurfa
MET S1-394	*Salmonella*	enterica	Poona	Sheep	03.07.2012	Şanlıurfa
MET S1-395	*Salmonella*	enterica	Caracas	Sheep	03.07.2012	Şanlıurfa
MET S1-396	*Salmonella*	enterica	Montevideo	Cattle	16.07.2012	Şanlıurfa
MET S1-397	*Salmonella*	enterica	Montevideo	Cattle	16.07.2012	Şanlıurfa
MET S1-398	*Salmonella*	enterica	Caracas	Sheep	17.07.2012	Şanlıurfa
MET S1-399	*Salmonella*	enterica	Telaviv	Cattle	06.08.2012	Şanlıurfa
MET S1-400	*Salmonella*	enterica	Montevideo	Cattle	06.08.2012	Şanlıurfa
MET S1-401	*Salmonella*	enterica	Telaviv	Cattle	06.08.2012	Şanlıurfa
MET S1-402	*Salmonella*	enterica	Telaviv	Cattle	06.08.2012	Şanlıurfa
MET S1-403	*Salmonella*	enterica	Montevideo	Cattle	06.08.2012	Şanlıurfa

Table A. 2 (continued)

MET S1-404	*Salmonella*	enterica	Montevideo	Cattle	06.08.2012	Şanlıurfa
MET S1-405	*Salmonella*	enterica	Kentucky	Cattle	06.08.2012	Şanlıurfa
MET S1-406	*Salmonella*	enterica	Telaviv	Cattle	06.08.2012	Şanlıurfa
MET S1-407	*Salmonella*	enterica	Telaviv	Cattle	14.08.2012	Şanlıurfa
MET S1-408	*Salmonella*	enterica	Anatum	Parsley	14.10.2012	Şanlıurfa
MET S1-409	*Salmonella*	enterica	Mikawasima	Iceberg	01.10.2012	Şanlıurfa
MET S1-410	*Salmonella*	enterica	Charity	Parsley	08.09.2012	Şanlıurfa
MET S1-411	*Salmonella*	enterica	Enteritidis	Red pepper	02.10.2012	Şanlıurfa
MET S1-416	*Salmonella*	enterica	Chester	Sheep ground meat	07.11.2012	Şanlıurfa
MET S1-421	*Salmonella*	enterica	Anatum	Sheep ground meat	07.11.2012	Şanlıurfa
MET S1-430	*Salmonella*	enterica	Telaviv	Sheep ground meat	07.11.2012	Şanlıurfa
MET S1-439	*Salmonella*	enterica	Montevideo	Sheep ground meat	07.11.2012	Şanlıurfa
MET S1-440	*Salmonella*	enterica	Telaviv	Sheep ground meat	07.11.2012	Şanlıurfa
MET S1-443	*Salmonella*	enterica	Anatum	Cow ground meat	07.11.2012	Şanlıurfa
MET S1-456	*Salmonella*	enterica	Telaviv	Cow ground meat	07.11.2012	Şanlıurfa
MET S1-462	*Salmonella*	enterica	Reading	Cow ground meat	07.11.2012	Şanlıurfa
MET S1-465	*Salmonella*	enterica	Anatum	Cow ground meat	07.11.2012	Şanlıurfa
MET S1-482	*Salmonella*	enterica	Anatum	Cow ground meat	07.11.2012	Şanlıurfa
MET S1-485	*Salmonella*	enterica	Telaviv	Cow ground meat	07.11.2012	Şanlıurfa
MET S1-492	*Salmonella*	enterica	Infantis	Chicken meat	07.11.2012	Şanlıurfa
MET S1-498	*Salmonella*	enterica	Infantis	Chicken meat	07.11.2012	Şanlıurfa

Table A. 2 (continued)

MET S1-510	*Salmonella*	enterica	Infantis	Chicken meat	07.11.2012	Şanlıurfa
MET S1-512	*Salmonella*	enterica	Montevideo	Offal	07.11.2012	Şanlıurfa
MET S1-517	*Salmonella*	enterica	Montevideo	Offal	07.11.2012	Şanlıurfa
MET S1-528	*Salmonella*	enterica	Telaviv	Offal	07.11.2012	Şanlıurfa
MET S1-530	*Salmonella*	enterica	Telaviv	Unripened cheese	12.11.2012	Şanlıurfa
MET S1-536	*Salmonella*	enterica	Kentucky	Cow	17.09.2012	Şanlıurfa
MET S1-537	*Salmonella*	enterica	Kentucky	Cow	17.09.2012	Şanlıurfa
MET S1-538	*Salmonella*	enterica	Kentucky	Cow	17.09.2012	Şanlıurfa
MET S1-539	*Salmonella*	enterica	Kentucky	Cow	17.09.2012	Şanlıurfa
MET S1-540	*Salmonella*	enterica	Kentucky	Cow	17.09.2012	Şanlıurfa
MET S1-541	*Salmonella*	enterica	Montevideo	Cow	17.09.2012	Şanlıurfa
MET S1-542	*Salmonella*	enterica	Kentucky	Cow	17.09.2012	Şanlıurfa
MET S1-543	*Salmonella*	enterica	Telaviv	Cow	01.10.2012	Şanlıurfa
MET S1-544	*Salmonella*	enterica	Telaviv	Cow	01.10.2012	Şanlıurfa
MET S1-545	*Salmonella*	enterica	Telaviv	Cow	01.10.2012	Şanlıurfa
MET S1-548	*Salmonella*	enterica	Anatum	Sheep ground meat	07.12.2012	Şanlıurfa
MET S1-557	*Salmonella*	enterica	Telaviv	Sheep ground meat	07.12.2012	Şanlıurfa
MET S1-568	*Salmonella*	enterica	Newport	Cow ground meat	07.12.2012	Şanlıurfa
MET S1-579	*Salmonella*	enterica	Anatum	Cow ground meat	07.12.2012	Şanlıurfa
MET S1-581	*Salmonella*	enterica	Montevideo	Cow ground meat	07.12.2012	Şanlıurfa
MET S1-597	*Salmonella*	enterica	Infantis	Cow ground meat	07.12.2012	Şanlıurfa
MET S1-606	*Salmonella*	enterica	Infantis	Chicken meat	07.12.2012	Şanlıurfa
MET S1-611	*Salmonella*	enterica	Montevideo	Offal	07.12.2012	Şanlıurfa

Table A. 2 (continued)

MET S1-625	*Salmonella*	enterica	Typhimurium	Offal	07.12.2012	Şanlıurfa
MET S1-634	*Salmonella*	enterica	Montevideo	Offal	07.12.2012	Şanlıurfa
MET S1-637	*Salmonella*	enterica	Telaviv	Unripened cheese	07.12.2012	Şanlıurfa
MET S1-647	*Salmonella*	enterica	Telaviv	Unripened cheese	07.12.2012	Şanlıurfa
MET S1-653	*Salmonella*	enterica	Typhimurium	Bull	05.11.2012	Şanlıurfa
MET S1-654	*Salmonella*	enterica	Anatum	Sheep	05.11.2012	Şanlıurfa
MET S1-657	*Salmonella*	enterica	Typhimurium	Sheep	13.11.2012	Şanlıurfa
MET S1-658	*Salmonella*	enterica	Montevideo	Sheep	13.11.2012	Şanlıurfa
MET S1-660	*Salmonella*	enterica	Enteritidis	Sheep	13.11.2012	Şanlıurfa
MET S1-662	*Salmonella*	enterica	Newport	Sheep	03.12.2012	Şanlıurfa
MET S1-663	*Salmonella*	enterica	Typhimurium	Sheep	03.12.2012	Şanlıurfa
MET S1-664	*Salmonella*	enterica	Montevideo	Sheep	03.12.2012	Şanlıurfa
MET S1-665	*Salmonella*	enterica	Montevideo	Cattle	03.12.2012	Şanlıurfa
MET S1-666	*Salmonella*	enterica	Mbadaka	Egg surface	15.04.2012	Ankara
MET S1-667	*Salmonella*	enterica	Newport	Offal	2012	Ankara
MET S1-668	*Salmonella*	enterica	Infantis	Chicken breast	28.11.2012	Ankara
MET S1-669	*Salmonella*	enterica	Infantis	Chicken wing	12.12.2012	Ankara
MET S1-670	*Salmonella*	enterica	Newport	Chicken wing	12.12.2012	Ankara
MET S1-671	*Salmonella*	enterica	Infantis	Chicken breast	12.12.2012	Ankara
MET S1-672	*Salmonella*	enterica	Infantis	Chicken skin	19.12.2012	Ankara
MET S1-673	*Salmonella*	enterica	Infantis	Chicken wing	19.12.2012	Ankara
MET S1-674	*Salmonella*	enterica	Infantis	Chicken wing	19.12.2012	Ankara

Table A. 2 (continued)

MET S1-675	*Salmonella*	enterica	Kentucky	Chicken liver	14.11.2012	Ankara
MET S1-688	*Salmonella*	enterica	Infantis	Chicken Thigh	28.11.2012	Ankara
MET S1-702	*Salmonella*	enterica	Kentucky	Sheep	24.12.2012	Şanlıurfa
MET S1-703	*Salmonella*	enterica	Telaviv	Sheep	24.12.2012	Şanlıurfa
MET S1-704	*Salmonella*	enterica	Chester	Sheep	24.12.2012	Şanlıurfa
MET S1-705	*Salmonella*	enterica	Telaviv	Cattle	08.01.2013	Şanlıurfa
MET S1-706	*Salmonella*	enterica	Montevideo	Cattle	15.01.2013	Şanlıurfa
MET S1-707	*Salmonella*	enterica	Montevideo	Cattle	15.01.2013	Şanlıurfa
MET S1-708	*Salmonella*	enterica	Montevideo	Cattle	15.01.2013	Şanlıurfa
MET S1-709	*Salmonella*	enterica	Montevideo	Cattle	15.01.2013	Şanlıurfa
MET S1-710	*Salmonella*	enterica	Montevideo	Cattle	15.01.2013	Şanlıurfa
MET S1-711	*Salmonella*	enterica	NA	Sheep	15.01.2013	Şanlıurfa
MET S1-712	*Salmonella*	enterica	Montevideo	Cattle	15.01.2013	Şanlıurfa
MET S1-839	*Salmonella*	enterica	Hadar	Centrifuged Sludge	18.11.2015	Ankara
MET S1-844	*Salmonella*	enterica	Othmarschen	Centrifuged Sludge	18.11.2015	Ankara
MET S1-849	*Salmonella*	enterica	Hadar	Centrifuged Sludge	09.11.2015	Yozgat
MET S1-854	*Salmonella*	enterica	Bredeney	Centrifuged Sludge	18.11.2015	Ankara
MET S1-856	*Salmonella*	enterica	Abony	Centrifuged Sludge	18.11.2015	Ankara
MET S1-861	*Salmonella*	enterica	Infantis	Centrifuged Sludge	22.11.2015	Eskişehir
MET S1-864	*Salmonella*	enterica	Mbandaka	Centrifuged Sludge	18.11.2015	Ankara

Table A. 2 (continued)

MET S1-868	*Salmonella*	enterica	Bredeney	Centrifuged Sludge	18.11.2015	Ankara
MET S1-870	*Salmonella*	enterica	Bredeney	Centrifuged Sludge	18.11.2015	Ankara
MET S1-872	*Salmonella*	enterica	NA	Centrifuged Sludge	18.11.2015	Ankara
MET S1-877	*Salmonella*	enterica	NA	Centrifuged Sludge	18.11.2015	Ankara
MET S1-880	*Salmonella*	enterica	Kentucky	Centrifuged Sludge	22.11.2015	Eskişehir
MET S1-939	*Salmonella*	enterica	Kentucky	Centrifuged Sludge	18.11.2015	Ankara
MET S1-942	*Salmonella*	enterica	Infantis	Centrifuged Sludge	18.11.2015	Ankara
MET S1-946	*Salmonella*	enterica	Kentucky	Centrifuged Sludge	22.11.2015	Eskişehir
MET S1-950	*Salmonella*	enterica	Infantis	Centrifuged Sludge	22.11.2015	Eskişehir
MET S1-955	*Salmonella*	enterica	Kentucky	Centrifuged Sludge	22.11.2015	Eskişehir
MET S1-969	*Salmonella*	enterica	Mbandaka	Centrifuged Sludge	22.11.2015	Eskişehir
MET S1-975	*Salmonella*	enterica	Kentucky	Centrifuged Sludge	09.11.2015	Yozgat

B. CHEMICALS, REAGENTS AND DEVICES USED

Table B. 1 Chemicals, Reagents and Devices Used.

Chemical and Reagents	**Manufacturer**
Buffered Peptone Water	Merck
Peptone Water	Fluka
Rappaport-Vassiliadis Soy (RVS) Peptone Broth	LABM
Lauryl Sulfate Broth (LSB)	EMD
Xylose Lysine Deoxycholate (XLD) Agar	Merck
Brilliant-green Phenol-ted Lactose Sucrose (BPLS) Agar	Merck
E. coli (EC) Medium	LABM
Brean Heart Infusion (BHI) Broth	LABM
Agar No.1	LABM
Tris Base	LABM
Boric Acid	LABM
EDTA	LABM
5x MyTaq Red Reaction Buffer	Bioline
MyTaq DNA Polymerase	Bioline
Primers	Oligomer
100 bp DNA Ladder	Solis Biodyne
Agarose	Bioline
SeaKem Gold Agarose	Bioline
Sodium Dodecyl Sulfate	LABM
XbaI	LABM
Colistin and other antimicrobial agents	

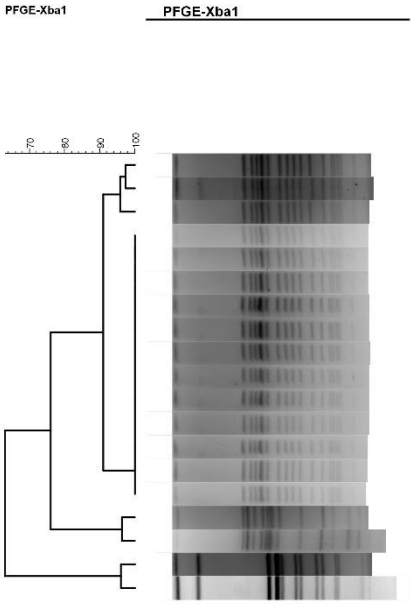
Table B. 2 Devices used in the study.

Devices Used	**Manufacturer-Model**
Microwave oven	Arçelik
Thermal Cycler	Bio-Rad T100
Gel Electrophoresis device	Bio-Rad Power Basic
PFGE system	Bio-Rad
Gel Screening System	Bio-Rad Universal Hood II (Biorad, SN 76 S, Milan, Italy)
Incubator	
-80 Deep Freezer	

Table B. 3 Egg brands used in this experiment.

Brands	**Code**
Carrefour	0
CP	1
Beypiliç	2
Eggy	3
Şişman Tavuk	4
Doğalım	5
Green Range	6
Çok Gezenti	7
Varda Çiftlik	8
Orvital	9
Raya	10
Yeşil Küre	11
Nova	12
Türe	13
Zeynep Hanımın Çiftliği	14
City Farm	15
Coşkun Yumurta	16
Türem	17
Yumurtacım	18
Metro Chef	19
Flotty	20
Keskinoğlu	21
Sorbey	22
Pure Organic	23

C. PFGE ANALYSIS OF *SALMONELLA*



Key	PFGE Type	Genus	Species	Serovar	Source	Location
MET A2-144	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-149	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-141	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-153	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-156	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-159	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-162	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-165	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-168	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-171	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-174	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-177	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-180	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-183	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-186	PT08	Salmonella	enterica	Infantis	Chicken egg	Ankara
MET A2-138	PT10	Salmonella	enterica	Kentucky	Chicken egg	Ankara
MET S1-951	PT10	Salmonella	enterica	Kentucky	Sludge	Eskişehir
MET A2-147	PT33	Salmonella	enterica	Telaviv	Chicken egg	Ankara
MET S1-647	PT33	Salmonella	enterica	Telaviv	Food	Şanlıurfa

Figure C. 1 PFGE analysis of recently collected *Salmonella* isolates from chicken samples purchased in Ankara.

D. PCR IMAGES FOR IDENTIFICATION OF *SALMONELLA*

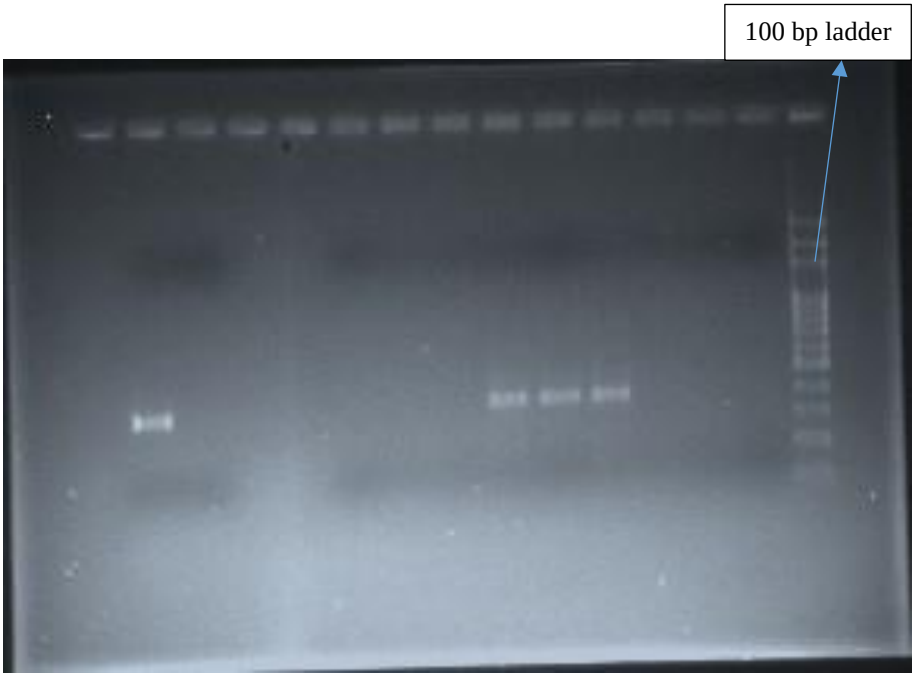


Figure D. 1 PCR gel image of *Salmonella* isolates from eggs. L: DNA ladder; 2: MET S1-056; 1,3,4,5,6,7,8,12,13,14: Negative Control; 9,10,11: MET A2-137.

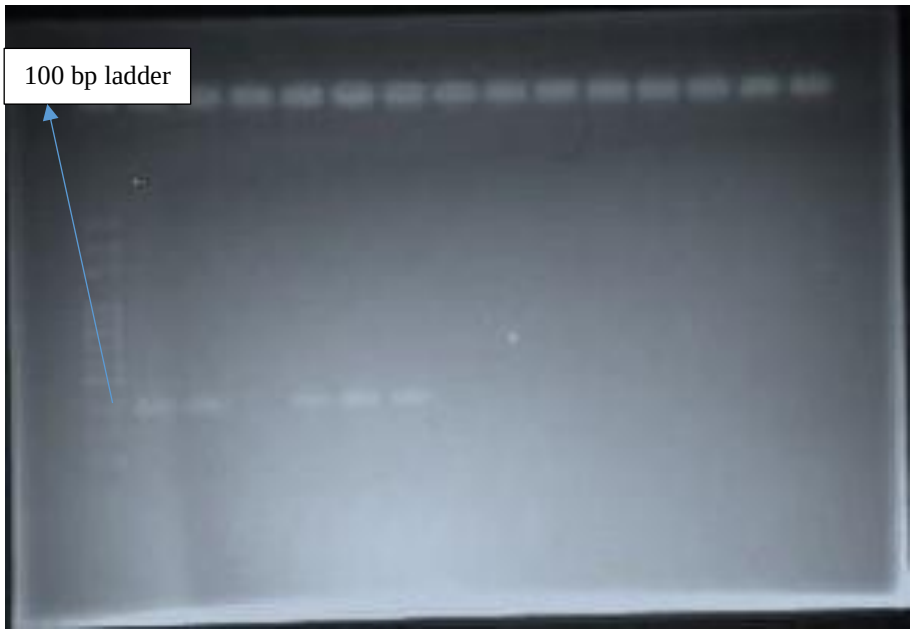


Figure D. 2 PCR gel image of *Salmonella* isolates from eggs. L: DNA ladder; 1,2: MET S1-056; 3,7,8,9,10,11,12,13,14: Negative Control; 4: MET A2-140; 5: MET A2-143; 6: MET A2-146.

E. STATISTICAL ANALYSIS TABLES

t-Test: Two-Sample Assuming Equal Variances		
Mean	5,346666667	5,41
Variance	0,003906667	0,00308
Observations	6	6
Pooled Variance	0,003493333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-1,85597858	
P(T<=t) one-tail	0,046563765	
t Critical one-tail	2,763769458	
P(T<=t) two-tail	0,09312753	
t Critical two-tail	3,169272673	

Figure E. 1 T-test two sample assuming equal variances (101 MOI).

t-Test: Two-Sample Assuming Equal Variances		
		3,49
Mean	3,243333333	3,42
Variance	0,008226667	0,00472
Observations	6	6
Pooled Variance	0,006473333	
Hypothesized Mean Difference	0	
df	10	
t Stat	-3,803218324	
P(T<=t) one-tail	0,001733742	
t Critical one-tail	2,763769458	
P(T<=t) two-tail	0,003467484	
t Critical two-tail	3,169272673	

Figure E. 2 T-test two sample assuming equal variances (103 MOI).

Anova: Two-Factor With Replication						
SUMMARY			Total			
1. parallel						
Count	3	3	6			
Sum	16,08092	10,12057	26,20149			
Average	5,360305	3,373525	4,366915			
Variance	0,000212	0,000804	1,184596			
2. parallel						
Count	3	3	6			
Sum	16,22655	10,04567	26,27222			
Average	5,408849	3,348558	4,378704			
Variance	0,000682	0,00052	1,273921			
3. parallel						
Count	3	3	6			
Sum	15,90309	10,12057	26,02366			
Average	5,30103	3,373525	4,337277			
Variance	0	0,000804	1,114905			
Total						
Count	9	9				
Sum	48,21055	30,28682				
Average	5,356728	3,365203				
Variance	0,00241	0,000688				
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,005467	2	0,002734	5,426767	0,020959	6,926608
Columns	17,84779	1	17,84779	35432,47	3,4E-22	9,330212
Interaction	0,013275	2	0,006637	13,17686	0,000938	6,926608
Within	0,006045	12	0,000504			
Total	17,87257	17				

Figure E. 3 Anova two factor with replicated for 0-hour samples.

Anova: Two-Factor With Replication						
SUMMARY			Total			
1. parallel						
Count	3	3	6			
Sum	16,33424	9,93922	26,27346			
Average	5,444748	3,313073	4,378911			
Variance	0,001131	0,002896	1,364822			
2. parallel						
Count	3	3	6			
Sum	16,3075	10,07962	26,38711			
Average	5,435832	3,359872	4,397852			
Variance	0,001135	0,002379	1,294289			
3. parallel						
Count	3	3	6			
Sum	16,19312	10,06258	26,25571			
Average	5,397708	3,354194	4,375951			
Variance	0,000302	0,001537	1,253521			
Total						
Count	9	9				
Sum	48,83486	30,08142				
Average	5,426096	3,34238				
Variance	0,00111	0,002192				
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,001694	2	0,000847	0,541902	0,595228	6,926608
Columns	19,53843	1	19,53843	12497,91	1,76E-19	9,330212
Interaction	0,005965	2	0,002982	1,907644	0,19082	6,926608
Within	0,01876	12	0,001563			
Total	19,56485	17				

Figure E. 4 Anova two factor with replicated for 6-hour samples.

Anova: Two-Factor With Replication						
SUMMARY			Total			
1. parallel						
Count	3	3	6			
Sum	16,12631	10,03842	26,16473			
Average	5,375435	3,34614	4,360788			
Variance	0,002261	0,003734	1,237809			
2. parallel						
Count	3	3	6			
Sum	16,34092	10,06588	26,4068			
Average	5,446973	3,355293	4,401133			
Variance	0,000241	0,000124	1,312684			
3. parallel						
Count	3	3	6			
Sum	16,10367	10,10037	26,20404			
Average	5,367889	3,36679	4,36734			
Variance	0,000114	0,001569	1,201992			
Total						
Count	9	9				
Sum	48,57089	30,20467				
Average	5,396766	3,356074				
Variance	0,002083	0,001437				
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,005625	2	0,002813	2,098378	0,165394	6,926608
Columns	18,7399	1	18,7399	13980,88	8,98E-20	9,330212
Interaction	0,006446	2	0,003223	2,404522	0,132382	6,926608
Within	0,016085	12	0,00134			
Total	18,76805	17				

Figure E. 5 Anova two factor with replicated for 12-hour samples.

Anova: Two-Factor With Replication						
SUMMARY			Total			
1. parallel						
Count	3	3	6			
Sum	15,76042	10,12057	25,881			
Average	5,253474	3,373525	4,313499			
Variance	0,00235	0,000804	1,061525			
2. parallel						
Count	3	3	6			
Sum	15,78675	10,00518	25,79193			
Average	5,26225	3,33506	4,298655			
Variance	0,001282	0,000962	1,115116			
3. parallel						
Count	3	3	6			
Sum	15,90309	10,00346	25,90655			
Average	5,30103	3,334487	4,317758			
Variance	0	0,00168	1,16086			
Total						
Count	9	9				
Sum	47,45026	30,12921				
Average	5,272252	3,347691				
Variance	0,001388	0,001237				
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,001207	2	0,000603	0,511489	0,612105	6,926608
Columns	16,66771	1	16,66771	14128,51	8,43E-20	9,330212
Interaction	0,005639	2	0,00282	2,390139	0,13375	6,926608
Within	0,014157	12	0,00118			
Total	16,68871	17				

Figure E. 6 Anova two factor with replicated for 24-hour samples.

Anova: Two-Factor With Replication						
SUMMARY			Total			
1. parallel						
Count	3	3	6			
Sum	16,22289	10,16979	26,39269			
Average	5,407631	3,389931	4,398781			
Variance	0,002251	0,002813	1,223361			
2. parallel						
Count	3	3	6			
Sum	16,13761	9,984977	26,12258			
Average	5,379202	3,328326	4,353764			
Variance	0,001317	0,000949	1,262735			
3. parallel						
Count	3	3	6			
Sum	16,20952	10,0607	26,27021			
Average	5,403172	3,353566	4,378369			
Variance	0,000675	0,002398	1,261494			
Total						
Count	9	9				
Sum	48,57002	30,21547				
Average	5,396669	3,357274				
Variance	0,001236	0,002259				
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,006097	2	0,003049	1,758417	0,213929	6,926608
Columns	18,71608	1	18,71608	10795,4	4,23E-19	9,330212
Interaction	0,00106	2	0,00053	0,305729	0,742157	6,926608
Within	0,020805	12	0,001734			
Total	18,74404	17				

Figure E. 7 Anova two factor with replicated for 48-hour samples.

Anova: Two-Factor With Replication						
SUMMARY			Total			
1. parallel						
Count	3	3	6			
Sum	16,13681	10,24428	26,38109			
Average	5,378937	3,414759	4,396848			
Variance	0,001661	0,000279	1,158174			
2. parallel						
Count	3	3	6			
Sum	16,24294	9,90309	26,14603			
Average	5,414313	3,30103	4,357671			
Variance	0,000872	0	1,340138			
3. parallel						
Count	3	3	6			
Sum	15,98498	9,943396	25,92837			
Average	5,328326	3,314465	4,321395			
Variance	0,000949	0,001059	1,217493			
Total						
Count	9	9				
Sum	48,36473	30,09076				
Average	5,373858	3,343418				
Variance	0,002271	0,003231				
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,017088	2	0,008544	10,63584	0,002201	6,926608
Columns	18,5521	1	18,5521	23094,76	4,43E-21	9,330212
Interaction	0,017293	2	0,008646	10,76355	0,002102	6,926608
Within	0,00964	12	0,000803			
Total	18,59612	17				

Figure E. 8 Anova two factor with replicated for 72-hour samples.

Anova: Two-Factor With Replication						
SUMMARY			Total			
1. parallel						
Count	3	3	6			
Sum	15,99449	9,953905	25,94839			
Average	5,331496	3,317968	4,324732			
Variance	0,000348	0,005745	1,218726			
2. parallel						
Count	3	3	6			
Sum	16,09982	10,04485	26,14467			
Average	5,366606	3,348284	4,357445			
Variance	0,001755	0,000867	1,223135			
3. parallel						
Count	3	3	6			
Sum	15,98498	9,922206	25,90718			
Average	5,328326	3,307402	4,317864			
Variance	0,000949	0,001044	1,226037			
Total						
Count	9	9				
Sum	48,07928	29,92096				
Average	5,342143	3,324552				
Variance	0,001101	0,002252				
ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0,005368	2	0,002684	1,504138	0,261278	6,926608
Columns	18,31803	1	18,31803	10265,55	5,72E-19	9,330212
Interaction	4,22E-05	2	2,11E-05	0,011831	0,98825	6,926608
Within	0,021413	12	0,001784			
Total	18,34486	17				

Figure E. 9 Anova two factor with replicated for 1-week samples.