4. Effect of temperature and solute (sucrose) treatment on growth of Salmonella in Eggs

4.1. Introduction

It is well known that Salmonella infections are responsible for a large number of bacterial food borne diseases (Mead et al., 1999). Salmonella species have had a long historical association with contaminated eggs and egg products. S. Enteritidis is reported as one of the most frequently isolated serotypes and is closely associated with eggs and egg products (McKellar and Knight, 2000). S. Enteritidis, S. Heidelberg and S. Typhimurium have been isolated from the ovaries of naturally infected chickens (Barnhart et al., 1991). S. Enteritidis has been isolated from yolk, albumen, and shell of naturally infected intact eggs (Humphrey et al., 1989). When hens were experimentally infected with S. Enteritidis, the organism was found in various organs, including the ovary (Gast and Beard, 1990; Timoney et al., 1989). These birds can produce eggs with S. Enteritidis contaminated yolk and albumen.

Because of the public health significance and the economic importance of food borne Salmonella a high research priority has been directed at the reduction of salmonellae in food supply. Foods of animal origin such as meat, poultry, eggs and dairy products are widely recognized as the primary vehicles for salmonellosis outbreaks (Roberts, 1990). Based on epidemiological evidence, Gast and Beard (1992) suggested that egg associated human S. Enteritidis outbreaks are generally a result of a series of three independent events. Firstly, S. Enteritidis contaminated eggs must be produced by infected hens. Secondly, contaminated eggs must be subjected to improper food handling practices that permit multiplication of S. Enteritidis to infectious levels. Thirdly, S. Enteritidis contaminated eggs must be undercooked or
consumed raw. Many products have been implicated in *S*. Enteritidis outbreaks because they typically receive little or no heat treatment prior to consumption. It is therefore important to assess the behavior of *S*. Enteritidis in eggs and egg dishes (Baker *et al*., 1998).

Proper cooking is essential if viable *S*. Enteritidis inside egg is to be eliminated. Cooking remains a primary means of eliminating pathogens from eggs and therefore, serves to protect against food borne disease. Hard-cooked eggs are assumed to be free of viable *S*. Enteritidis but have been epidemiologically associated with many outbreaks of salmonellosis (Caceres *et al*., 1996, CDCP 1997). Whether these outbreaks are caused by *S*. Enteritidis that was inside eggs when they were laid by the chicken and survived the cooking process or contamination after cooking is not known. Baker *et al*., (1990) reported that *S*. Enteritidis can survive in egg albumin for only a few days but can live and grow in egg yolk. Moreover certain traditional egg cooking practices such as sunny-side' frying, soft poaching, marginal hard-cooking may be inadequate to kill *Salmonella* in eggs (St.Louis *et al*., 1988). Long-term control of *S*. Enteritidis infection will require an examination of the ecology of this organism in poultry flocks and may depend ultimately on either its elimination from flocks or on the pasteurization of shell eggs or liquid eggs. During the 1940’s and 1950’s the thermal treatment of shell eggs to prevent embryonic growth in fertile eggs to reduce the incidence of spoilage during long - term storage and to maintain internal quality received considerable research attention.

Accordingly, teams of investigators have conducted thermal inactivation studies of different *Salmonella* serotypes in aqueous media and foods. The primary means of ensuring the microbiological safety of egg products is the use of appropriate egg pasteurization process. However heat pasteurization can affect the quality of the...
egg content. Low dose irradiation is an alternative process that can be used to solve this problem. Ionizing radiation at medium doses can eliminate non-spore forming pathogens such as Salmonella in food products (Radomyski et al., 1994).

Owing to the widespread occurrence of Salmonella food poisoning, considerable attention has been given in recent years to destruction or control of the responsible microorganisms. High temperatures used in cooking and in pasteurization processes have been regarded as the treatment of choice for the destruction of Salmonella in eggs, milk and meat products.

4.2. Literature Review

The inside of a hen’s egg once considered sterile, is now known to occasionally harbor S. Enteritidis. Lysozyme, ovotransferrin and the alkaline pH appear to be of primary importance of the defense to the egg. Ovotransferrin deprives the microorganism of iron, preventing their multiplication (Mayes and Takeballi, 1983). The pH of albumin immediately after lay is \(~7.4\), but increases upon storage up to \(\geq 9\) (Silversides and Scott, 2001) which is beyond the maximum tolerated by many microorganisms (Mayes and Takeballi, 1983). Substantial public and private resources have been invested in control programs to reduce the occurrence of S. Enteritidis infections in laying flocks and to improve storage and preparation practices for egg and egg-containing foods (Hogue et al., 1998). Detecting internal contamination of eggs with Salmonella enterica serovar Enteritidis (S. Enteritidis) is an important aspect of efforts to identify infected laying flocks.

The exact mechanism by which grade A shell eggs are contaminated with Salmonella is not clear, although it is generally accepted that transovarian transmission
is one likely route. S. Enteritidis, S. Heidelberg and S. Typhimurium have been isolated from the ovaries of naturally infected chickens (Barnhart et al., 1991). The contents of hen’s egg may become contaminated before (horizontal transmission) and after lay (vertical transmission) (Cox et al., 2000). In the later case, the albumen gets contaminated when the cuticle, shell and shell membranes fail to prevent microbial invasion in the albumin. Several reports indicate that S. Enteritidis can occur in the internal portion of eggs as a result of deposition in the oviduct before shell formation.

Studies revealed that S. Enteritidis is capable of penetrating the egg shell and subsequently can reach the egg yolk (Todd, 1996; Koen, 2001). Manson (1994) reported that 0.01% of eggs in the retail market may be internally contaminated, whereas 0.5 to 8.3% of eggs produced by an infected flock may contain S. Enteritidis. The number of S. Enteritidis cells present is generally less than 20 per egg (Humphery, 1991). If internally contaminated eggs are subjected to temperature abuse, high numbers of Salmonella can result (Schoeni, 1995), and some cells may survive the hard-cooking process if not conducted correctly.

Baker (1990) reported that S. Enteritidis can survive in egg albumen for only a few days but can live and grow in egg yolk. In addition, the heat resistance of S. Enteritidis in yolk is greater than in albumen because of difference in pH, fat and water content. Proper cooking is essential if viable S. Enteritidis inside eggs to be eliminated. Hard-cooked eggs are assumed to be free of viable S. Enteritidis but have been epidemiologically associated with outbreaks of salmonellosis (Caceras 1996; CDCP 1997). Chantarapanont et al. (2000) studied six strains of S. Enteritidis to determine the heat resistance, when heated in liquid egg yolk and egg white using a capillary tube method, and also evaluated the two methods for hard cooking shell.
eggs for their effectiveness in killing S. Enteritidis inoculated into the yolk. Their observations confirm that heat resistance of S. Enteritidis is higher in yolk than in albumen. They also reported that the time required to kill S. Enteritidis in egg yolk of shell eggs increase with an increase in size of eggs and a decrease in initial temperature of eggs. With regard to the method for hard cooking eggs, considering the time elapsed after egg are exposed to boiling water, the American Egg Board method is clearly most effective in killing S. Enteritidis.

Egg and egg dishes are important vehicles for S. Enteritidis, S. Heidelberg and S. Typhimurium, which can be isolated from chicken ovaries and feces, have been implicated in approximately 50% of the food borne salmonellosis outbreaks in the United States. Jean et al. (1995) conducted a study for the growth of these three organisms, inoculated into the yolk and the albumin was compared at 4, 10 and 25°C. Regardless of whether 10^2 cfu/g or 10^4 cfu/g was inoculated into yolk or albumen, populations of all strain increased 3 logs or more in number in one day when incubated at 25°C. Maximum numbers of Salmonella ranged from 10^8 to 10^10 cfu/g. All strains grow at 10°C, but peak numbers were lower and occurred later than those at 25°C. Populations of the three Salmonella strains inoculated into eggs stored at 4°C grew sporadically; in some test groups populations declined.

Jean et al. (1995) also examined the potential for shell penetration by Salmonella, which might be present in feces. At 25°C growth of salmonella in feces was observed throughout the test period; feces may have provided the required nutrients. All three strains of Salmonella penetrated the shell and could be isolated from egg contents by day 3. Penetration was reduced by incubating eggs at 4°C. At this temperature, S. Typhimurium penetrated into the membrane but not to the contents. S. Enteritidis and S. Heidelberg were not recovered from the inner shells,
membranes, or contents of eggs stored at 4°C. Their studies also emphasized the need to rapidly remove any fecal contamination from eggs to decrease potential for penetration of *Salmonella* into contents. The need for properly controlled storage, cooking and serving procedures is critical.

Limited studies have examined the potential for growth of *Salmonella* in albumen. It has been reported that the albumen is more frequently contaminated by *S. Enteritidis* than yolk, in eggs from naturally infected hens (Humphrey *et al.*, 1991; Gast and Beard, 1990) found that 71% of albumin samples from eggs, laid in the first week after hens were administered *S. Enteritidis*, were contaminated by the organism. These researchers found that *S. Enteritidis*, *S. Typhimurium* and *S. Heidelberg* were able to grow well in albumin at 25°C, while slight growth was observed in several cases at 10°C and 4°C. Clay and Board (1991) found that *S. Enteritidis* inoculated into the air cell rapidly contaminated and grew in the albumen and yolk of eggs stored at 25°C, but not when stored at 4°C or 10°C. Humphrey (1990) observed no growth of *S. Enteritidis* and *S. Typhimurium* in the albumen of inoculated shell eggs at 8°C and 10°C. Differences in results may be partially due to strain variation and age of eggs.
4.2.1 Growth of S. Enteritidis in eggs at refrigeration and storage temperatures

Although *Salmonella* can survive or slowly multiply in egg albumen (Chen *et al.*, 2005; Guan *et al.*, 2006; Kang *et al.*, 2006; Murchie *et al.*, 2008), rapid multiplication occurs in egg yolk (Humphrey and Whitehead, 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000). Accordingly, egg refrigeration must achieve growth-inhibiting internal temperatures more rapidly when bacteria are present in the yolk than when they are found in the albumen. Because high levels of contaminants have seldom been reported in freshly laid eggs, initial *Salmonella* deposition inside nutrient-rich yolks appears to be relatively uncommon.

Refrigeration of eggs during transportation and storage at an ambient temperature of 7°C has been proposed and instituted as a means of preventing small initial numbers of *S*. Enteritidis contaminants from multiplying to levels more likely to cause human illness (USDA, 1998; President’s council on Food Security, 1999). A study conducted by Humphrey (1990) revealed that the storage of eggs below 10°C might have significant public health benefits in preventing the multiplication of salmonellas including *S*. Enteritidis. However, conventional refrigeration technologies may require several days to, lower internal egg temperatures sufficiently to restrict bacterial growth (Curtis *et al*., 1995; Thompsan *et al*., 2000). Although this cooling period has few adverse consequences if *S*. Enteritidis contaminants remain in the albumen, the initial deposition of *S*. Enteritidis within yolks or subsequent bacterial penetration into yolks (Humphry and Whitehead, 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000) could allow multiplication of *S*. Enteritidis to reach more dangerous levels during the early stages of refrigeration.
Refrigeration of eggs is often identified as one of the most critical issues in minimizing the risk associated with *Salmonella* contamination of eggs (President’s Council on Food Safety, 1999). Because freshly laid eggs are very rarely reported to harbor more than a few hundred *S.* Enteritidis cells (Humphrey *et al.*, 1989; Chen *et al.*, 2002) prompt refrigeration to growth-inhibiting internal temperature (7.2°C or lower) can reduce the probability that consumers will be exposed to doses of the pathogen sufficient to cause disease. However, the prospective protective efficiency of refrigeration can be heavily influenced by the location of *Salmonella* contaminants within eggs. Although *Salmonella* can persist or even grow slowly in albumen (Cogan *et al.*, 2001; Messens *et al.*, 2004), egg yolk supports rapid bacterial multiplication (Gast and Holt, 2005). Accordingly, the degree of urgency for rapidly attaining growth inhibiting internal temperatures inside eggs is directly related to the likelihood that the pathogen is present inside the yolk. Infected hens have been reported to deposit *S.* Enteritidis in either yolk or albumen of developing eggs, perhaps as a consequence of colonization of different regions of the reproductive tract (Humphrey *et al.*, 1989; Gast and Holt, 2000). Detailed examination of eggs from experimentally infected hens has suggested that *S.* Enteritidis is deposited more often in association with the yolk (vitelline) membrane than in the interior contents of the yolk (Gast and Holt, 2001).

Even under normal storage conditions, over time the ability of the egg to prevent microbial growth decreases and allows the growth of bacteria to occur. When *S.* Enteritidis exists in eggs, the storage temperature of the eggs is important for prevention of growth of *S.* Enteritidis Gast and Beard (1992) have shown that storage at 25°C for 7 days increase *S.* Enteritidis in naturally contaminated eggs, although storage at 7°C for 7 days does not alter the contamination level. Schoeni *et al.*, (1995) reported that *S.* Enteritidis grow during 1 day storage at 25°C but not at 10°C for 7 days.
Numbers of *Salmonella* in the egg contents tend to be low. Gast and Beard (1992) reported that in 4 of 132 fresh eggs laid by hens experimentally infected with *S. Enteritidis*, the average level of contamination was 5.5 cfu/ml. In a study conducted by Humphrey *et al.* (1989), *S. Enteritidis* positive eggs contained less than 10 cfu /ml. Hence it is crucial to prevent the organisms from growing during storage of the contaminated eggs. Previous research has not clearly indicated whether *Salmonella* strains commonly found in eggs are likely to penetrate through yolk membranes and begin multiplying inside egg yolks during 36 h of unrefrigerated storage. The objective of the study by Humphrey *et al.* (1989) was to determine whether small numbers of 4 strains of *S. Enteritidis* and 4 strains of *S. Heidelberg* were able to migrate through the yolk membrane to reach the yolk contents during 36 h of incubation at either 20 or 30°C in an *in vitro* egg contamination model.

Infected hens can deposit *Salmonella* in either the yolk or albumen of developing eggs because of the colonization of different regions of the reproductive tract (Bichler *et al.*, 1996; Gast and Holt, 2000; Gast *et al.*, 2007). In a study by Gast and Holt (2001) of eggs from experimentally infected hens, *S. Enteritidis* was isolated far more often in association with the yolk (vitelline) membrane than with the interior contents of the yolk. However, even if *Salmonella* deposition occurs on the exterior surface of the yolk membrane or in the adjacent albumen, migration through that membrane to reach the yolk contents could then allow rapid and extensive bacterial multiplication (Braun and Fehlhaber, 1995; Gast and Holt, 2000; Gast *et al.*, 2005; Murase *et al.*, 2005). Declining vitelline membrane integrity during non refrigerated storage could promote this process (Chen *et al.*, 2005).

During storage of shell eggs the pH of the albumen increases from ca.7.6 to 9.7 (Powrie and Nakai, 1986). *Salmonella* has been reported to be tolerant of pH values...
ranging from 4 to 9.5 (D’Aoust, 1991), this change in the albumen pH may not inhibit Salmonella growth until later in storage. Therefore, the chemical defenses of albumen towards Salmonella are limited. Yukiko et al. (2001) studied the effects of laying season and eggshell cracks on the ability of egg album into support the growth of S. Enteritidis in eggs. The findings demonstrated that S. Enteritidis can grow more preferably in albumen of cracked eggs than intact eggs, indicating that storage conditions should be considered more intensively for cracked eggs than intact eggs from the view of the ability of albumen to support S. Enteritidis growth. Although very little bacterial multiplication occurs in egg albumen, S. Enteritidis can persist there at supportive temperatures (Lock and Board, 1992; Boron et al., 1997; Gast and Holt, 2000). However, egg yolk can support rapid and extensive growth of S. Enteritidis, especially at storage temperatures above 20°C (Bradshaw et al., 1990; Clay and Board, 1991; Humphrey and Whitehead, 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000; Caudill et al., 2010). Holding eggs at room temperature for several hours has been implicated as a factor in outbreaks associated with S. Enteritidis (Lin et al., 1988).

4.2.2 Survival of Salmonella in pasteurized eggs

The egg products industry must provide egg products free of viable salmonellae. Various methods have been recommended to achieve this goal. The most efficient processes used involve the application of heat. The treatment of liquid whole egg at 140°F for 3.5 min as a means of killing salmonellae was recommended over 15 years ago (Goresline et al., 1951) and has had successful application for these many years. Advances in methods of pasteurizing egg whites allow the industry to guarantee egg whites free of viable Salmonella. Pasteurization is a heat treatment process that aims the inactivation of harmful microorganisms and this process must
be carefully designed to cause minimal damage to the lipoprotein components of the liquid food. In a study, conducted by Ignacio et al. (2006) investigated the effect of ionizing radiation in combination with thermal treatment on the survival of *Salmonella* serovars. Of the six *Salmonella* serovars tested, *S. Senftenberg* was the most resistant to radiation \( (D_T = 0.65 \text{ kGy}) \) and heat \( (D_{55^\circ C} = 11.31 \text{ min}, \ z = 4.9^\circ C) \). Their results revealed that irradiation followed by thermal treatment at 55 or 57°C improved the pasteurization process.

Many products, such as casesar salad dressing, hollandaise sauce, eggnog and home-made ice-cream, have been implicated in *S. Enteritidis* outbreaks because they typically receive little or no heat treatment prior to consumption. Moreover, certain traditional egg cooking practices may be in adequate to kill *Salmonella* in eggs (St.Louis et al., 1988). Pasteurization of whole eggs or egg yolk is legally required in many countries, mainly because of the high risk of contamination with members of the Enterobacteriaceae, including *Salmonella* spp., and members of the Micrococcaceae (proposed pasteurization conditions for whole eggs, egg yolk, and salted egg yolk: 61 to 63°C for 1.75 to 6.2 min). However, when pasteurization is carried out at too high a temperature \( (>70^\circ C) \), some functional properties of egg yolk, such as emulsifying capacity, are at least partly lost. A common method for stabilizing pasteurized egg yolk against the outgrowth of various types of bacteria is the addition of a large amount of salt or a combination of a lower amount of salt and preservatives. It is well known that members of the Enterobacteriaceae cannot grow in medium with more than 8% salt (Matches et al., 1972).

While the microflora of the hen's egg has been the subject of several reviews (Haines 1939; Brooks and Taylor 1955; Board 1969), relatively little attention has been given to the microbiology of pasteurized egg products. Pasteurization at a...
temperature not lower than 64.4°C for at least 2.5 min was introduced in Great Britain as a statutory requirement in 1963 to destroy any salmonellas that may be present in the liquid whole egg. Various pasteurization processes for the destruction of viable *Salmonella* in egg white have been described in previously (Cunningham *et al.*, 1965; Kline *et al.*, 1966).

It is well known that *Salmonella* infections are responsible for a high number of bacterial food borne diseases (Mead *et al.*, 1999). Since *S*. Enteritidis reported as one of the most frequently isolated serotypes and is closely associated with eggs and egg products and it is important to assess the behavior of *S*. Enteritidis in eggs and egg dishes. The percentage of eggs contaminated with *Salmonella* in the retail market is estimated as 0.01%, and 0.5-8.3% of the egg produced by an infected flock may be contaminated. Though the number of *S*. Enteritidis cell is generally quite low (<20/eggs) (Humphrey *et al.*, 1991), but temperature abuse can lead to a very high number of *Salmonella* especially in the yolk (Schoeni *et al.*, 1995). Adding to the risk is the fact that *Salmonella* bacteria appear to have a higher heat resistance in liquid eggs and liquid egg yolks than in pure albumen (Muriana *et al.*, 1996; Doyle and Mazzotta., 2000).

Long-term control of *S*. Enteritidis infections will require an examination of the ecology of this organism in poultry flocks and may depend ultimately on either its elimination from the flocks, or on the pasteurization of shell eggs or liquid eggs. Hou *et al.* (1996) documented the feasibility of combining water-bath and hot air oven processing to reduce viable *S*. Enteritidis populations within inoculated intact shell eggs. They reported that a combination of water bath heating (25 min at 57°C) followed by hot-air heating (60 min at 55°C) reduced the viable *S*. Enteritidis population within inoculated shell eggs.
Salmonella species have had a long historical association with contaminated egg and egg products. Several studies have documented the thermal resistance of Salmonella spp. in liquid whole egg (Foegeding et al., 1990; Shah et al., 1991; Bartlett et al., 1995). In recent years, research efforts aimed at more accurately defining the microbiological safety of current egg pasteurization processes have been initiated.

The effects of S. Enteritidis inactivation potential of 57°C and 58°C water bath immersion heat treatments for fresh, intact shell eggs were evaluated by Suhuman et al. (1997). They also evaluated the effects of selected immersion heat treatments on albumen functionality and egg interior quality attributes. The effects of water-bath immersion heat treatment on the inactivation of S. Enteritidis with intact shell eggs were evaluated. Six pooled strains of S. Enteritidis were completely inactivated with 50-57.5 min at a bath temperature of 58°C and within 65-75 min at 57°C.

Boiled eggs are very common type of egg dish in several countries of the world (Grijspeerdt et al., 1999) and three types of boiled eggs are generally distinguished: soft, medium and hard-boiled. While the risk of microbial contamination is minimal for hard-boiled eggs (Baker et al., 1983), but medium– and soft-boiled eggs are a potential risk factor for salmonellosis.

Eggs are very nutritious for humans and bacteria. In a study conducted by Michael et al. (2009) S. Typhimurium DT104 (ST DT104), were inoculated into the whole liquid egg, albumen, yolk with 10% sugar, or yolk with 10% salt. ST DT104 were greatly increased in whole egg and yolk with 10% sugar but tended to decline in albumen and yolk with 10% salt.

USDA prescribes minimum conditions of pasteurization for egg products sufficient to destroy harmful viable microorganisms. Many factors affect the thermal
resistance of *Salmonella* in eggs. Salt, pH, sugar, type of acid used to adjust the pH, and total solids are all important.

The review clearly identifies the *Salmonella* hazard associated with the shell eggs and the need to adopt temperature treatment procedures such as pasteurization and boiling to ward off this threat. Though there are several reports available on efficiency of such procedures from Europe and USA virtually no literature is available from Indian Scenario. Moreover, the survival of *Salmonella*, in particular *S*. Enteritidis, in the type of eggs available in different countries need to be studied as the procedures developed elsewhere might be inadequate as revealed by some of the researchers mentioned in the review of literature. Hence the present study has been taken up with the following objectives:

### 4.3 Objectives

1. To determine the growth of *S*. Enteritidis in albumen of hens egg
2. To determine the growth of *S*. Enteritidis in yolk of hens egg
3. To determine the heat resistance of *S*. Enteritidis in pasteurized liquid whole eggs
4. To determine the synergistic effect of solutes and temperature on growth of *S*. Enteritidis in liquid whole eggs
5. To find out the thermal inactivation of *S*. Enteritidis in eggs subjected to cooking in water
4.4 Materials and Methods

4.4.1 Determination of growth of *S. Enteritidis* in albumen and yolk of hen’s egg at different temperatures

4.4.1.1 Preparation of *S. Enteritidis* inoculum

For preparing the inoculum, a loopful of laboratory stock *S. Enteritidis* was inoculated into 10 ml sterile tryptic soy broth (TSB) and incubated overnight at 37°C. After incubation cells are harvested by centrifugation (3000 rpm for 10 minutes) and the supernatant was removed using a sterile micropipette. The cell pellet was resuspended in 10 ml sterile isotonic saline. These were centrifuged again and the whole process was repeated thrice. The cell density was determined by serial dilution and plating in nutrient agar. The plates were incubated at 37°C for 24 hours before taking the count and the cell density in each dilution was determined. Appropriate dilution was used to inoculate the pasteurized eggs.

4.4.1.2 Determination of growth of *S. Enteritidis* in albumen of egg at different temperatures

Large size eggs were bought from the retail outlet near medical college, Kottayam. The egg surface was sterilized by immersing the egg in 70% ethanol for 1 minute. The egg was aseptically removed, and dried in a laminar hood and using a sterile disposable syringe (Microlance), $10^4 - 10^5$ cells of *S. Enteritidis* was inoculated (the needle was inserted approximately 15mm) into the albumen of the egg. The hole on the egg was sealed using tape, placed in egg cartons and incubated at 4°C, 20°C and 30°C. The growth of *S. Enteritidis* was checked after 0 (immediately after inoculation), 1, 3, 7 and 14 days.
4.4.1.3 Determination of growth of *S.* Enteritidis in yolk of egg at different temperatures

A similar procedure was followed to find the growth of *S.* Enteritidis in yolk at 4°C, 20°C and 30°C over the 14 day period. The only difference was that the *S.* Enteritidis cells were inoculated into the yolk instead of the albumen.

4.4.1.4 Plating and Enumeration of *S.* Enteritidis

On the selected days for the examination of the bacterial growth, two inoculated eggs were randomly picked from the temperatures at which they were incubated (two eggs from 4°C, two from 20°C and two from 30°C). The surface of the egg was sterilized using 70% ethanol. The egg cracked (on the blunt end of the egg) and the yolk and the albumen were separated aseptically. The albumen or the desired portion of the egg was weighed and homogenized in TSB in 1:5 ratios. This homogenate was then serially diluted 100 times in 9ml, 0.1% peptone. In duplicates, 0.1ml of each dilution was spread plated onto HEA and incubated at 37°C for 24 hours. Typical *Salmonella* like colonies were counted. The identities of the typical colonies were confirmed by picking a few colonies and carrying out serological agglutination test on them. All the tests were conducted in duplicates.
4.4.2. Heat resistance of S. Enteritidis in pasteurized liquid whole egg

4.4.2.1. Preparation of pasteurized liquid whole egg microcosms

The fresh eggs were collected from a retail outlet and the contents were separated aseptically after disinfecting the shells by soaking in 70% ethanol for 15 minutes. The shell was cracked aseptically and the egg content was homogenized for 2 minutes in a sterile homogenizer. Then the contents were pasteurized by placing it in water bath at 60°C and heating for 3.5 minutes. These pasteurized eggs were then used for further tests.

4.4.2.2 Determination of Heat Resistance of S. Enteritidis in pasteurized liquid whole eggs at different temperatures

S. Enteritidis inoculum was prepared as per the procedure outlined earlier. Approximately, $10^7$ cells/ml was inoculated into 5ml of pasteurized egg, prepared as described in previous section (4.4.2.1) and vortexted for 10 seconds. Heat treatment was carried out on the eggs by submerging the eggs in a water bath set at 55°C. The eggs were subjected to heat treatment for 5, 10, 15, 20, 25, and 30 minutes. The tubes were removed from the water bath at the designated time interval and immediately cooled by placing the tubes in ice slurry. One ml of the test sample was pipetted; serially diluted using sterile 0.1% peptone water and 0.1ml of various dilutions were spread plated on sterile XLD agar plates. The plates were incubated at 37°C for 24 hours and typical Salmonella like colonies were counted. Heat treatment of pasteurized egg was also carried out at 60°C and 65°C respectively for 5, 10, 15, 20, 25, and 30 minutes and checked for growth on XLD agar plates.
4.4.2.3 Determination of growth of S. Enteritidis in pasteurized eggs with different sucrose concentration

Five ml of the pasteurized egg was taken in a sterile test tube. Pasteurized egg was selected because growth of the organism could be monitored with minimal interference from other microorganisms. Then the pasteurized egg microcosms were mixed with 250 mg, 500 mg and 750 mg of sucrose to get 5%, 10%, and 15% of sucrose concentration. S. Enteritidis (0.1 ml) was inoculated into the test organism, mixed well and overlaid with 1 ml sterile mineral oil (the purpose of the mineral oil was to minimize free oxygen transfer to more closely represent the environment in commercial egg packing). The tubes were then subjected to heat treatment by keeping submerged in water bath that were set at different temperatures such as 55°C, 60°C, and 65°C for different time intervals (5, 10, 15, 20, 25 and 30 minutes). The samples were drawn at specified time intervals from water bath and cooled immediately, and the growth was checked on hektoen enteric agar (HEA) plates by spread plate (0.1 ml) method.

4.4.3 Thermal inactivation of S. Enteritidis in egg during cooking in water

4.4.3.1. Inoculation of Egg

On the day of the experiment, large size eggs were bought from the retail outlet. Surface sterilization of the eggs was done by immersing the eggs in 70% ethanol for 15 minutes. S. Enteritidis inoculum is prepared as described under section 4.4.1.1 using a sterile disposable syringe, 0.1 of diluted cell suspension (approximately 10^2 cells/ml) of S. Enteritidis was inoculated by piercing the needle carefully through the yolk of the egg. Immediately after inoculation, the hole was sealed with wax.
4.4.3.2 Boiling and checking for growth of S. Enteritidis

The experimental eggs (3 in number) were placed in a pan with 400ml of water (the water level was maintained 2.5cm above the egg) and then heated to boiling (a thermometer set up was maintained for monitoring temperature). As soon as the water had started to boil, the pan was removed from the hot plate and the egg was left in the hot water. Eggs were drawn at different time intervals viz, 5, 10 and 15 minutes and homogenized in 0.1% peptone water, maintaining 1:9 ratios between sample and diluents. Homogenised samples (0.2 ml) were plated directly on HEA plates and were incubated at 37°C for 24 hours.

In another set of experiment, the eggs were inoculated with S. Enteritidis inoculum as stated under section 4.4.3.1. The inoculated eggs were then placed directly into boiling water instead of them being subjected to gradual boiling as carried out in the first set of experiment. Each boiled egg was drawn at 5, 10 and 15 minute for further analysis. Using the procedure described above (section 4.4.3.2) the eggs were checked for presence of S. Enteritidis.

4.4.4 Statistical analysis

The results were subjected to Two-Factor ANOVA without replication by using SPSS (Version: 11; SPSS Inc.1995).
4.5 Results

4.5.1 Growth of *S*. Enteritidis in the albumen of egg

The growth of *S*. Enteritidis in the albumen of LWE stored at 4\(^\circ\)C is presented in Figure 4.1. The cell numbers found to decrease as the storage time extended. The cells were not detected at the end of 7\(^{th}\) day.

![Survival curve of *S*. Enteritidis in the albumen of egg stored at 4\(^\circ\)C](image)

Figure 4.1 Survival curve of *S*. Enteritidis in the albumen of egg stored at 4\(^\circ\)C

Figure 4.2 represents growth of *S*. Enteritidis in the albumen of whole egg stored at 20\(^\circ\)C. The *S*. Enteritidis cells were showing rapid growth at these temperatures. The growth was rapid during the initial 24 hours during which the cell numbers of *S*. Enteritidis surged by nearly 2 log. There was a linear increase in cell numbers till 7\(^{th}\) day.
Chapter IV  

Effect of temperature and solute (sucrose) treatment on growth of Salmonella in Eggs

Figure 4.2. Survival curve of *S*. Enteritidis in the albumen of egg stored at 20°C

Figure 4.3 represents the growth of *S*. Enteritidis in the albumen of egg stored at 30°C. The growth pattern of *S*. Enteritidis in egg stored at 30°C was more or less similar to that at 20°C. The *S*. Enteritidis cells showed exponential growth till 3rd day and thereafter the cell numbers found to stagnate.

Figure 4.3 Growth of *S*. Enteritidis in the albumen of egg stored at 30°C

Relative growth curve of *S*. Enteritidis in the albumen of egg maintained at different temperature is represented at Figure 4.4. Results clearly revealed the
effectiveness of low temperature storage for prevention of growth of *S*. Enteritidis in albumen

Figure 4.4 Relative growth curve of *S*. Enteritidis in the albumen of egg maintained at different temperatures

Statistical analysis of the average counts of *S*. Enteritidis in the albumen of egg at 4°C than 20°C and 30°C revealed that, there is significant difference in the average counts of *S*. Enteritidis in the albumen of egg at 4°C, 20°C and 30°C (P<0.01). *S*. Enteritidis showed poor growth or lesser survival in the albumen at 4°C than 20°C and 30°C during the experimental period.

4.5.2 Growth of *S*. Enteritidis in the yolk of egg

Survival curve of *S*. Enteritidis in the yolk of egg stored at 4°C is represented in Figure 4.5. Though the cells showed significant reduction in the number of *S*. Enteritidis up to 7th day the cells showed acclimatization and growth after 7 days
Figure 4.5 Survival curve of *S*. Enteritidis in the yolk of egg stored at 4°C

Growth of *S*. Enteritidis cells in the yolk of egg stored at 20°C is presented in Figure 4.6. The *S*. Enteritidis cells showed rapid growth in the yolk till 3rd day of incubation after which the growth has slowed down.

Figure 4.6 Survival curve of *S*. Enteritidis in the yolk of egg stored at 20°C
Figure 4.7 Survival curve of *S. Enteritidis* in the yolk of egg stored at 30°C

Growth of *S. Enteritidis* cells in the yolk of egg stored at 30°C is presented in Figure 4.7. The cell numbers increased rapidly in the first 24 hours and thereafter the growth slowed down. From 3rd day onward *S. Enteritidis* cell numbers did not show any notable increase.

Relative growth curve of *S. Enteritidis* in the yolk of egg maintained at different temperatures is represented in Figure 4.8. The *S. Enteritidis* cells grow rapidly at 20 and 30°C though the rate of growth was higher at 30°C, indicating the preference of mesophilic temperature by *S. Enteritidis*.
There is significant difference in the relative growth of *S*. Enteritidis in the yolk of egg at 4°C, 20°C and 30°C. (P<0.01). *S*. Enteritidis showed poor growth or lesser survival in the yolk at 4°C than 20°C and 30°C during the experimental period.

Statistical analysis of comparison of growth of *S*. Enteritidis in yolk and albumen showed significant difference in the growth of *S*. Enteritidis in the albumen and yolk of egg at 4°C, (P<0.05). *S*. Enteritidis showed poor growth or lesser survival in albumen than in yolk. However there was no significant variation (p>0.05) of *S*. Enteritidis growth in yolk and albumen at 20°C and 30°C.
4.5.3 Heat resistance of *S. Enteritidis* in pasteurized liquid whole eggs

Growth of *S. Enteritidis* in pasteurized liquid whole eggs subjected to heat treatment at 55°C for different time intervals is given in Figure 4.9. The *S. Enteritidis* cells showed faster reduction in the initial 10 minutes after with the reduction was very gradual, indicating selection of temperature tolerant cells.

Figure 4.9 Heat Resistances of *S. Enteritidis* in pasteurized liquid whole eggs at 55°C

Growth of *S. Enteritidis* in pasteurized liquid whole egg subjected for temperature treatment at 60°C is represented in Figure 4.10. The *S. Enteritidis* cells reduce gradually with increase in time of temperature treatment. However after 25 minutes there was minor increase in number of *S. Enteritidis* indicating selection and growth of temperature tolerant cells.
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Figure 4.10 Heat resistance of *S*. Enteritidis in pasteurized liquid whole eggs at 60°C

At 65°C there was sharp decrease in *S*. Enteritidis cells during the first 5 minutes of temperature treatment. The reduction in the *S*. Enteritidis cells after further increase in time of temperature treatment was very gradual.

Figure 4.11 Heat resistance of *S*. Enteritidis in pasteurized liquid whole eggs at 65°C
Relative heat resistance of *S. Enteritidis* in pasteurized egg at 55°C, 60°C and 65°C for different time intervals are represented in Figure 4.12. Except for the steep reduction in the number of *S. Enteritidis* cells at 65°C, the death curve at different temperature, were more or less similar.

![Figure 4.12 Relative heat resistance of *S. Enteritidis* in pasteurized liquid whole eggs at different temperatures](image)

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4.5.4 Synergistic effects of solute concentration and temperature on growth of S. Enteritidis in pasteurized liquid whole eggs

Synergetic effect of 5% sucrose concentration and temperature treatment on reduction of S. Enteritidis in pasteurized liquid whole eggs is presented in Figure 4.17. The S. Enteritidis cells showed considerable reduction at all temperatures. When the pasteurized liquid whole egg was supplemented with 5% sucrose the synergetic effect was more pronounced when compared to temperature treatment alone.

Figure 4.13 The effect of 5% sucrose solution on the growth of S. Enteritidis in pasteurized liquid whole egg at different temperatures
Death curves of *S. Enteritidis* in pasteurized liquid whole egg supplemented with 10% sucrose and subjected to different temperatures is given in Figure 4.14. The pattern of reduction was more or less similar indicating a further increase in sucrose concentration beyond 5% do not have any significant effect on reduction of *S. Enteritidis* in pasteurized liquid whole egg.

![Figure 4.14](image)

Figure 4.14 Effect of 10% sucrose concentration on growth of *S. Enteritidis* in pasteurized liquid whole egg subjected to different temperature treatments
Figure 4.15 represents the death curves of *S*. Enteritidis in pasteurized liquid whole egg supplemented with 15% sucrose and subjected to different temperatures. The pattern of reduction was more or less similar to 10% sucrose. Beyond 5% sucrose do not have any significant effect on reduction of *S*. Enteritidis in pasteurized liquid whole egg.

![Death curves of *S*. Enteritidis in pasteurized liquid whole egg](image)

Figure 4.15. Effect of 15% solute concentration on growth of *S*. Enteritidis in pasteurized liquid whole egg subjected to different temperature treatments.
4.5.5 Thermal inactivation of *S. Enteritidis* during cooking in water

When the *S. Enteritidis* inoculated eggs were placed in boiling water there was one log reduction in the number of *S. Enteritidis* cells during the first 5 minutes. Further maintenance of egg in boiling water upto 15 minutes did not achieve any significant reduction of *S. Enteritidis* in shell eggs.

![Inactivation curve of *S. Enteritidis* in shell eggs when placed in boiling water for different time intervals](image)

Figure 4.16 Inactivation curve of *S. Enteritidis* in shell eggs when placed in boiling water for different time intervals

The result of inactivation curve of *S. Enteritidis* in shell eggs when placed in water and made to boiling for different time intervals revealed that the *S. Enteritidis* cells were completely destroyed after 5 minutes. There was no recovery of *S. Enteritidis* after 10 and 15 minutes indicating superiority of this treatment over placing the egg directly in boiling water for different time intervals.
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Figure 4.17 Inactivation curve of S. Enteritidis in shell eggs when placed in water and made to boiling for different time intervals

4.6. Discussion

Eggs can be contaminated with all types of Salmonella on the surface but if we discover a bacterium inside the egg itself, it is usually S. Enteritis. It is believed that S. Enteritis in yolk and egg white is the cause of the majority of the cases of salmonellosis. Studies revealed that S. Enteritidis is capable of penetrating the egg shell and subsequently can reach the egg yolk (Todd, 1996; Koen, 2001). Manson (1994) reported that 0.01% of eggs in the retail market may be internally contaminated, whereas 0.5 to 8.3% of eggs produced by an infected flock may contain S. Enteritidis. The pasteurization and dry heat treatment of intact shell-table eggs results in significant reduction of the high initial count of S. Enteritidis strains, regardless of their plasmid profile or density of contamination (Barbour et al., 2001).
The exact mechanism by which eggs are contaminated with *Salmonella* is not clear, although it is generally accepted that transovarian transmission is one likely route. *S. Enteritidis* has been isolated from yolk, albumen and shell of naturally infected intact eggs (Humphery *et al.*, 1989). The albumen gets contaminated before the yolk upon horizontal transmission except in those cases when the yolk gets into contact with the air cell (Clay and Board, 1991).

Numbers of *Salmonella* in the egg contents tend to be low. Gast and Beard (1992) reported that in 4 of 132 fresh eggs laid by hens experimentally infected with *S. Enteritidis*, the average level of contamination was 5.5 cfu/ml. In a study conducted by Humphery *et al.* (1989), *S. Enteritidis*-positive eggs contained less than 10 cfu/ml. If internally contaminated eggs are subjected to temperature abuse, high numbers of *Salmonella* can result (Schoeni., 1995), and some cells may survive the hard-cooking process if not conducted correctly. Hence, it is crucial to prevent the organisms from growing during storage of the contaminated eggs.

Even if the initial site of *Salmonella* deposition is the exterior surface of the vitelline membrane or in adjacent regions of the albumen, penetration of the membrane could lead to extensive multiplication inside the yolk. Penetration of *S. Enteritidis* through the vitelline membrane has been reported (Hammack *et al.*, 1993; Humphrey and Whitehead, 1993; Gast and Holt, 2001; Gast *et al.*, 2005). Although *Salmonella* can survive or slowly multiply in egg albumen (Chen *et al.*, 2005; Guan *et al.*, 2006; Kang *et al.*, 2006), rapid multiplication occurs in egg yolk (Humphrey and Whitehead, 1993; Braun and Fehlhaber, 1995; Gast and Holt, 2000). Accordingly, egg refrigeration must achieve growth-inhibiting internal temperatures more rapidly when bacteria are present in the yolk than when they are found in the albumen. However, conventional refrigeration technologies may require several days to, lower internal egg
temperatures sufficiently to restrict bacterial growth (Curtis et al., 1995; Thompsan et al., 2000).

Refrigeration of eggs is often identified as one of the most critical issues in minimizing the risk associated with Salmonella contamination of eggs (President’s Council on Food Safety, 1999). Because freshly laid eggs are very rarely reported to harbor more than a few hundred S. Enteritidis cells (Humphrey et al., 1989; Chen et al., 2002) prompt refrigeration to growth-inhibiting internal temperature (7.2°C or lower) can reduce the probability that consumers will be exposed to doses of the pathogen sufficient to cause disease.

In our study growth and persistence of S. Enteritidis strains were examined at two refrigeration temperatures, such as 4°C and 20°C and also at 30°C. The strains grew in egg contents and yolk at 20°C, although at slower rates than at 30°C. Growth at 4°C was sporadic however, the organism survived both in the yolk and albumen only for 7 days. Our results were similar to those reported by Schoeni et al. (1995). Torrey and Marth (1977) observed that home refrigerator temperatures ranged from 1.7°C to 20.2°C and Bradshaw et al. (1990) reported that it is common industry practice to store eggs at 13°C to 16°C. The result demonstrated by Gast et al. (2005) revealed that pathogen such as S. Enteritidis and S. Heidelberg can penetrate into and began to multiply inside the yolk of contaminated eggs during the first day of storage at warm temperatures.

Holding eggs at room temperature for several hours has been implicated as a factor in outbreaks associated with S. Enteritidis (Lin et al., 1988). Increasing populations of S. Enteritidis in yolks have been reported from 4°C (Saeed and Koons, 1993) to 37°C (Bradshaw et al., 1990). In contrast, Humphery et al., (1989) did not observe growth of S. Enteritidis at 4, 7, or 8°C. Baker (1990) reported that S.
Enteritidis can survive in egg albumen for only a few days but can live and grow in egg yolk. In addition, the heat resistance of *S*. Enteritidis in yolk is greater than in albumen because of difference in pH, fat and water content. Proper cooking is essential if viable *S*. Enteritidis inside eggs to be eliminated.

In the study area, the eggs are not kept under refrigeration at the retail or wholesale outlets. Refrigeration of eggs are not practiced at many households. The results of the study point to risk associated with such practices as *S*. Enteritidis showed very good growth in yolk and albumen at 30°C which is very close to the ambient temperature of the study area. Based on the result of the study we recommend mandatory refrigeration of the eggs, at individual households, retail vendors as well as at production facility.