MICROBIOLOGICAL QUALITY OF THE PACKAGED CHICKEN EGGS WITHIN AND AFTER SHELF LIFE

By

MUNIRAH BINTI AHMAD

Dissertation Submitted in Partial Fulfillment of the Requirement for the Degree of Bachelor of Health Science (Biomedicine)

June 2015

ACKNOWLEDGEMENT

This dissertation would not have been possible without the help of so many people in so many ways. Immeasurable appreciation and deepest gratitude for the help and support are extended to the following persons who have contributed in this study.

I take this opportunity to express my profound gratitude and deep regards to my supervisor, Dr. Noor Izani Noor Jamil for his guidance, monitoring and constant encouragement throughout the course of this project work. Sharing his knowledge and helped in the completion and success of this study.

I also take this opportunity to express a deep sense of gratitude to Mrs Siti Kurunisa Mohd Hanafiah for her support, invaluable information and guidance which helped me in completing this task through various stages.

I would also like to thank my final year project course coordinator, Dr. See Too Wei Cun for his guidance and support.

Finally, I thank almighty, my families and friends for their encouragement and understandings throughout my study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

-	To / until
%	Percentage
°C	Degree Celsius
E.coli	*Escherichia coli*
S.aureus	*Staphylococcus aureus*
C.jejuni	*Campylobacter jejuni*
L.monocytogens	*Listeria monocytogens*
B.cereus	*Bacillus cereus*
S.enteritidis	*Salmonella enteritidis*
S.virchow	*Salmonella Virchow*
S.thypimurium	*Salmonella thypimurium*
APEC	Avian pathogenic *Escherichia coli*
EC	European commission
Oz.	Ounce (ʒ)
EFSA	European Food Safety Authority
IgG	Immunoglobulin G
IgY	IgG- like immunoglobulin
GI	Gastrointestinal
mL	Milliliter
µL	Microliter

ABSTRAK

Telur merupakan makanan sihat dan berkhasiat yang dimakan oleh penduduk seluruh dunia. Walaubagaimanapun, isu keselamatan pengambilan telur menjadi kerisauan kerana telur dan produk dari telur telah dikaitkan dengan keracunan makanan. Kajian ini dijalankan untuk menentukan kualiti mikrobiologikal telur dalam tempoh dan selepas tempoh luput terutamanya kehadiran *E.coli* dan *Salmonella.* 30 biji telur dipilih secara rawak daripada pasaraya yang terpilih di sekitar Kubang Kerian, Kelantan. Keputusan menunjukkan ada kehadiran koliform najis (*E. coli*) tetapi tiada *Salmonella* spp. dijumpai daripada sampel. *E. coli* hadir pada kulit dan isi kandungan telur untuk kedua-dua kumpulan sampel. Bilangan sampel yang menunjukkan kehadiran *E. coli* lebih tinggi dalam kumpulan telur yang melepasi tarikh luput. Tambahan lagi, lapan spesis bakteria microflora telur (*Citrobacter freundii, Enterobacter aerogenes, E. coli, Klebsiella pneumonia, Proteus mirabilis, Proteus vulgaris, Serratia marcesens* dan *Shigella* spp.) dijumpai dari sampel kulit dan isi kandungan telur. Disimpulkan bahawa telur yang melepasi tarikh luput tidak selamat dimakan.

ABSTRACT

Eggs are healthy and nutritious food which are commonly consumed everywhere in the world. However issues of its safety for consumption and other uses remain doubtful since food poisoning associated to eggs and its products . This study was undertaken to provide information on the effect of storage time that is within shelf life and after the expiry date on the microbiological quality of the egg by detecting the presence of bacteria especially *E.coli* and *Salmonella* spp. 30 egg samples randomly collected from selected supermarket in Kubang Kerian, Kelantan. The result showed presence of fecal coliform (*E. coli*) but no *Salmonella* was isolated from the samples. *E. coli* is present in both the eggshell and egg content sample. More egg samples in the expiry category showed presence of *E. coli*. Additionally, eight egg microflora (*Citrobacter freundii, Enterobacter aerogenes, E. coli, Klebsiella pneumonia, Proteus mirabilis, Proteus vulgaris, Serratia marcesens* and *Shigella* spp.) were isolated from the samples. . It is concluded that eggs passed the expiry date are not safe for consumption.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Egg is a healthy and nutritious food which is consumed almost daily and is present in many food products. Poultry, especially chicken eggs are popular with people of all walks of life, inexpensive and fulfil the preference of all ethnic groups without religious restriction. However issues of its safety for consumption and other uses remain doubtful since eggs and its products are associated to food poisoning and human gastroenteritis worldwide. *Campylobacter* spp., *Salmonella* spp. *and Escherichia coli* are among the most common foodborne pathogens reported in poultry and poultry meat and eggs. Many studies have reported human campylobacteriosis, salmonellosis and *E. coli* infections are frequently due to consumption of poultry and poultry products (Wingstrand *et al.*, 2006). In many occasions, eggs and egg products have been implicated as sources of human infections with *Salmonella enteritidis* (Martelli & Davies, 2012) and *E. coli* (Loongyai *et al.*, 2011).

Foodborne diseases are the main public health problem in developing countries which cause illnesses, death and also give negative impact on economies around the world including Malaysia. "Foodborne disease can be defined as any illness resulting from the consumption of foods or beverages contaminated with one or more of the disease producing agents" (Sharifah *et al*, 2013). Ingestion of foodstuff contaminated with chemical or organisms can cause foodborne disease such as food poisoning cholera, typhoid fever, hepatitis A and dysentery. Food is the most important vehicle of disease transmission by microorganisms to human. Contamination of food may occur at any

stage of food source, production, handling, preparation, transporting and storage and environmental contamination may also be involved. Consuming raw or undercooked foods may definitely invite food poisoning. As egg is not only consumed cooked, it is also eaten raw or partially cooked which represent health risk to the consumer.

The number of foodborne disease (cholera, typhoid, dysentery, hepatitis A) in Malaysia from 2009 to 2013 is shown in Figure 1.1. Based on the statistics, in 2013 the number of hepatitis A cases showed an increase while cases of cholera, typhoid and dysentery showed a decrease compared to 2009. The 2013 statistics on the incidence rate and mortality rate of foodborne disease in Malaysia is shown in Table 1.1.

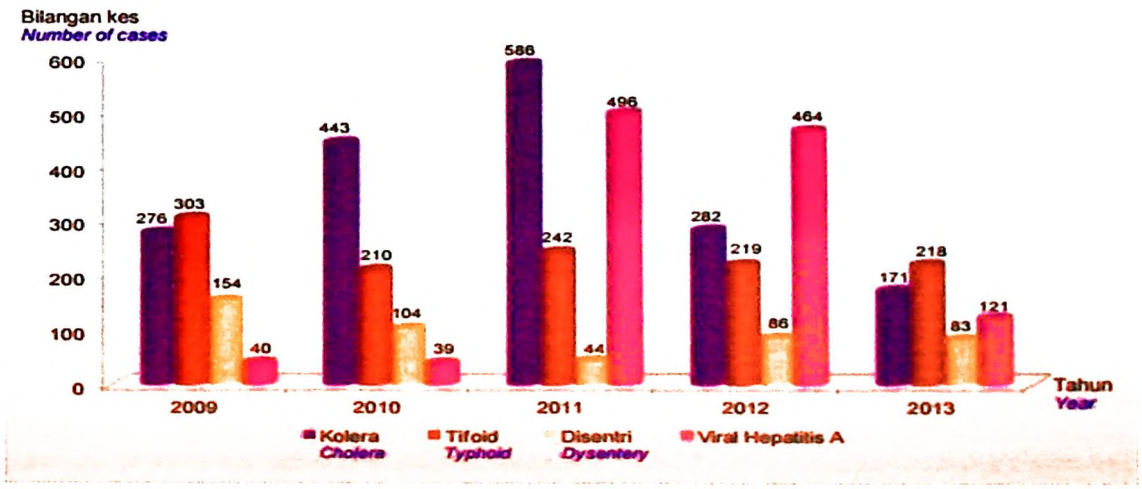

Figure1.1: Number of foodborne disease case in Malaysia 2009- 2013 (Department of statistic Malaysia, 2014)

Table 1.1: Incidence rate and mortality rate of foodborne disease in Malaysia 2013

Foodborne disease	Incidence rate	Mortality rate
Cholera	0.58	0.00
Dysentery	0.28	0
Food Poisoning	47.79	0.004
Hepatitis A	0.41	0
Typhoid	0.73	0.01

Source: Ministry of Health, 2014

Based on the statistics, the incidence rate of food poisoning is the highest compared to the incidence rate of the other types of foodborne disease. However, the mortality rate of typhoid case was highest with 0.01 compared to food poisoning, 0.004 and others recorded 0.00 mortality rate.

Figure 1.2 shows the incidence rate of food poisoning in Malaysia, 2013. Melaka recorded the highest incidence rate per 100000 population in 2013 which is at 83 and is followed by Perlis (75), Perak (72) and Kelantan (68).

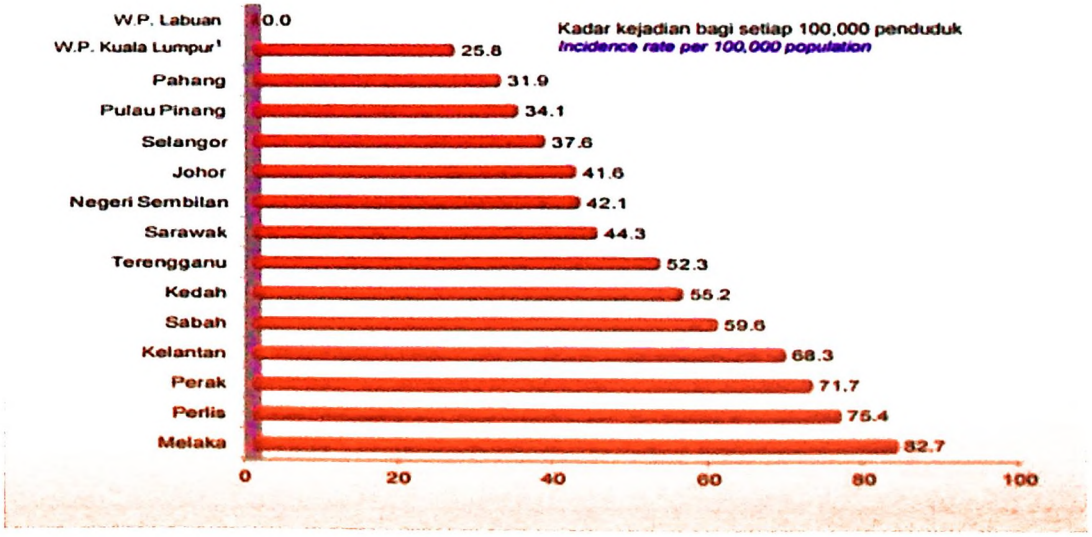

Figure 1.2: Incidence rate of food poisoning by state in Malaysia, 2013 (Department of Statistic Malaysia, 2014)

Food poisoning may be caused by several bacterias such as *Campylobacter, Salmonella* and *E.coli* which are present in various types of food category. Table 1.2 below showed the most common pathogens with food category pairs resulting in outbreak associated deaths in the United States, 2012.

Table 1.2: Pathogen- Food category pairs resulting in outbreak associated deaths in the United States, 2012

Pathogen	Food category	No. Outbreaks	No. Illnesses	No. Hospitalization	No. Deaths
Listeria monocytogenes	Dairy	1	23	21	5
Campylobacter	Chicken	4	37	3	4
***Escherichia coli*,** **Shiga toxin-producing**	Vegetable raw crops	8	136	34	1
Salmonella	Eggs	7	58	11	1
Salmonella	Friuts	6	446	55	1
Listeria monocytogenes	Fish	3	13	2	1
Clostridium botulinum	Sprout	1	6	4	1
Other chemical	Fish	1	3	3	1

Source:Centre for Disease Control and Prevention, 2012

Listeria monocytogenes in dairy product recorded the highest number of deaths and followed by *Campylobacter* spp. in chicken. While *Escherichia coli*, Shiga toxin-producing in vegetables had the highest number of outbreak in chicken and followed by *Salmonella* spp. Here, it showed that poultry and poultry products play an important role in food poisoning.

Poultry and poultry product are frequently contaminated with several bacterias such as *Campylobacter*, *E.coli*, *Klebsiella*, *Staphylococcus*, *Enterobacter*, *Proteus*, *Pseudomonas* and *Salmonella*, which can be transmitted to human through handling of raw poultry products and through consumption of undercooked poultry products like meat and egg. The egg can act as a vector in food poisoning since it is the most popular food among the consumer. The most common bacterias involve in egg contamination which reported in outbreaks of food poisoning are *Salmonella*, *C.jejuni*, *L.monocytogens* and *E.coli* (Cortés *et al*, 2004).

Salmonella is one of the major causes of foodborne disease around the world and it is the second most common cause of food poisoning after *Campylobacter* (Ghasemian *et al*., 2011). In brief, *Salmonella* is a facultative anaerobe, Gram negative and non-sporing bacteria which can cause salmonellosis and this bacteria belong to the family of Enterobacteriaceae. In addition, *Salmonella* is non-fastidious bacteria or in other word it is able to multiply under various environmental condition outside the living host and can survive in that environment for a long time. According to the study by Mokhtar (1996), since 1993, more that 30% of salmonellosis cases in human were due to *Salmonella enteritidis* infection. This disease is associated with the consumption of contaminated food products such as poultry, beef, pork and eggs. Salmonellosis can be divided into four different syndromes in human, namely enteric fever, gastroenteritis, bacteremia and nontyphoidal salmonellosis. The infectious dose of *Salmonella* depends on the serovar, bacteria strain, growth condition and host susceptibility (Pui *et al.*, 2011).

E.coli is a motile, facultative anaerobic and gram negative non spore forming bacteria which live in human and animal intestines. Shiga toxin– producing strain of *E.coli* is

responsible for most food related *E. coli* infection. Symptoms of *E.coli* infection usually begin after two to five days of infection and the symptoms include abdominal cramp, diarrhea and can cause serious illness particularly in vulnerable group.

Egg is rich in high quality protein, which is equivalent for protein of 1 oz. of red meat and also low in calories (Michele, 2013). Thus, egg is nutritious and an inexpensive meat substitute. According to Obi and Igbokwe (2009) fully mixed egg contains about 65% water, 12% protein and 11% fat. Eggs have important components source of nutrients in the human diet because they provide several vitamin and mineral including retinol (vitamin A), riboflavin (Vitamin B2), folic acid (Vitamin B9), Vitamin B6, Vitamin B12, choline, iron, calcium, phosphorus and potassium (King'ori, 2012)

The egg is made up of several structures and components, as stated by King'ori (2012) the proportions of components for fresh egg are 32% yolk, 58% albumen and 10% shell. Each structure and components of the eggs have their own function especially in defence mechanism against microbial invasion. The eggshell has a unique structure because it is made up of five layers, i.e. a mammillary layer, cone layer, palisade layer, vertical crystal layer and cuticle (EFSA, 2014). It was also stated that the eggshell is the highly mineralised structure which composed of calcium carbonate ($CaCO_3$) in the form of calcite. According to some researchers (Arias *et al.*, 2001; Hunton, 2005; Neospark, 2012; Nys *et al.*, 2004), the eggshell consists of 95% to 97% of calcium carbonate crystal, 0.3% of phosphorus and magnesium. They also claimed that there is presence of sodium, potassium, zinc, manganese, iron, copper and organic matter in eggshell. Presence of eggshell allows the exchange of gases and water between the interior and exterior of the egg and also act as first line defense to protect the egg from

microbial contamination. In addition, structure and composition of eggshell is also important in providing calcium for embryogenesis.

The effective barrier to bacterial penetration which is the inner part of the eggshell is called shell membrane. Like eggshell, shell membrane also has antibacterial properties which prevent the microbial invasion into the egg albumen. The shell membrane is made up of three layers that are the inner shell membrane, outer shell membrane and a homogenous third layer of electron dense material called the limiting membrane (Bruce and Drysdale, 1994). The structure which acts as barrier and microbial invasion into the inner part of egg is known as vitelline membrane. The viltelline membrane is built up of three layers that are the layer formed in the ovary, a continuous intermediate layer and the outer layer deposited in the oviduct (EFSA, 2014; Mann, 2008).

The egg components which surround the nutritious compartment of the egg (egg yolk), called egg albumen or also known as the egg white is rich with protein and provide protection for the egg yolk with the present of lysozyme and other antibacterial properties. Studies by Wand and Shelef (1991) and Ibrahim *et al.* (1996), showed presence of the enzyme (lysozyme) in egg albumen which express muramidase activity against Gram positive bacteria and also involve in bacterial membrane disruption. Egg white also contains avidin, which forms a complex with biotin thus making the vitamin unavailable to microbe (Obi and Igbokwe, 2009). The most inner part and the nutritious compartment of the egg is the egg yolk which consists the high fat value. Presence of antibodies such as IgY (IgG- like) in egg yolk help in limiting the growth of microbes (Bedrani *et al.*, 2013; Kovacs- and Mine, 2012; Rose *et al.*, 1974).

Although there are antimicrobial properties in each structure and component of the egg, it still can be contaminated by the bacterias due to the poor treatment and storage. This

can reduce the quality of the eggs and lead to the movement of bacteria in the egg and cause rotting when there is sufficient number of bacteria. There are two types of factors that are intrinsic factor and extrinsic factor which contribute to the microbial invasion in egg. Vaibhav *et al* (2014), had classified several factors or causes under intrinsic and extrinsic factor in their study. Intrinsic factor includes shell porosity, shell thickness and extent of cuticle present on the shell while under extrinsic factor, there are bacterial strain, temperature differential, moisture on the eggshell, number of microorganisms in the inoculum and the storage condition.

Moreover, there are three ways of the contamination which help the factors to cause the microbial invasion and multiplication in egg. EFSA (2014), had defined and explained about the ways of contamination known as primary contamination, secondary contamination and cross contamination. Contamination which occurs before shell formation is defined as primary contamination and also called vertical or ovarian transmission. While secondary contamination is the contamination that occur after the egg has been laid through eggshell penetration or also known as trans- shell contamination. Cross contamination occurs when bacteria are transferred from a contaminated surface to one that is not contaminated. Thus, not all of the contamination of egg caused by the hens and occur during egg formation, but also can be caused by the environmental factor such as temperature and humidity.

Storage can change or modify the characteristics of eggs, which can give effect on their microbiological quality. The modified characteristics include loss of water, carbon dioxide and changes in pH of the egg albumen (Decuypere *et al.*, 2001). Temperature control is important in preventing the microbial invasion and help in minimizing the incidence of food poisoning which caused by egg. The temperature needs to be

controlled which begin after the egg collection, transportation and storage. The best temperature for storage is below 10 °C or at refrigerator temperature because bacteria may not survive and will not multiply in such low temperature. But storage at low temperature can cause a decrease in water movement from egg albumen to egg yolk (Brake *et al.*, 1997). The best before date of egg is fixed that is not more that 28 days from laying while the sell by date is fixed for 21 days from laying (EFSA, 2014). Hasan and Aylin (2009) claimed that the strength of the vitelline membrane decreases when increasing the storage time and this may allow the bacteria to take nutrients from the egg yolk.

Since the egg is inexpensive and nutritious meal, it is popular among the consumer . Nowadays, consumption of raw or undercooked egg is a common habit because it is believed to improve the overall health especially recuperating from sickness. Hence, the quality of the eggs must be determined in order to avoid any foodborne disease because egg in particular has been described as the vehicles for the transmission of bacteria to human. The probability for the egg to get contaminated by the chicken is low because nowadays, vaccines and antibiotics against some microbes are given to the chicken. But egg still can be contaminated via cross- contamination which usually caused by extrinsic factors. Thus, this study was undertaken to provide information on the effect of storage time that is within shelf life and after the expiry date on the microbiological quality of egg by detecting the presence of bacterias especially *E.coli* and *Salmonella*.

1.2 Rationale of Study

Nowadays, eating raw and partially cooked eggs has become a common habit among the consumer . They believe that eating raw and undercooked eggs will give them more

health benefit. As more and more people are becoming health conscious, they believe that eating raw egg can help them improve their overall health . Unfortunately, without their awareness, eating raw and partially cooked eggs actually could lead to the foodborne disease if the egg is contaminated. Although the incidence of foodborne outbreak related to the egg has decreased, we still need to be cautious because contaminated egg could be health threatening.

After the introduction of vaccination and antibiotic in poultry industries, the incidence rate of foodborne disease associated with egg has declined and there is only very few studies today had successfully isolated several types of bacterias from eggs. But people should not feel comfortable because previous studies have proven the presence of pathogenic bacteria in eggs and there is a possible increase of foodborne disease caused by egg if there is no proper monitoring of the poultry, poor treatment and storage of the eggs.

People should be aware of the importance of proper treatment and storage of the eggs because poor treatment can cause the physiological and physical changes which increase the chances of contamination. Eggs should be stored at a suitable temperature for a certain period of time to avoid contamination and infection in human.

Infection in human usually causes diarrhea, nausea and abdominal pain and it will become worse if not treated. The most severe effect can be seen if elderly, infants and people with impaired immune system were infected. The infection may spread from the intestines to the blood stream and other body site (Ghasemian *et al.*, 2011). Late treatment for these patients could lead to death.

In other countries, various studies on eggs were performed but in Malaysia the study usually focuses on how to prevent the infection and how to improve the poultry quality.

To my knowledge, there are no reported studies as yet on the microbiological quality of packaged eggs during storage.

The purpose of this study is to provide information about the microbiological quality of packaged eggs within and after shelf life. Thus, the community will be more aware and alert toward food poisoning issues. And in the same time, they will practice good hygiene and handling food properly since most of the foodborne disease outbreak in Malaysia are due to unhygienic food handling. However, study on the extent of food poisoning from expired food is still scarce.

1.3 Objectives

General objective: To study the microbiological quality of packaged chicken eggs within and after shelf life.

Specific objectives:

1. To determine the fecal coliforms count of the eggshell and eggs within shelf life and after expiry date.
2. To determine the presence of *Salmonella* spp. in the eggshell and eggs within shelf life and after expiry date.
3. To determine the presence of microflora in the eggshell and eggs within shelf life and after expiry date.

1.4 Research Conceptual Framework

Figure 1 shows the research conceptual framework which has been used in this study. This laboratory experimental study includes the analysis of packaged chicken egg, which involve the enumeration and detection of microorganisms present on the surface

of the eggshell and the whole contents of the egg. These samples were analysed for the presence of *E.coli* and *Salmonella* which are commonly isolated from the fresh poultry product.

Microbiological methods used were based on the method recommended by the Malaysia Food Act 1985 and NFW Food Authority. Thirty samples (n = 28) were collected randomly over a period of two months from selected supermarket in Kubang Kerian.

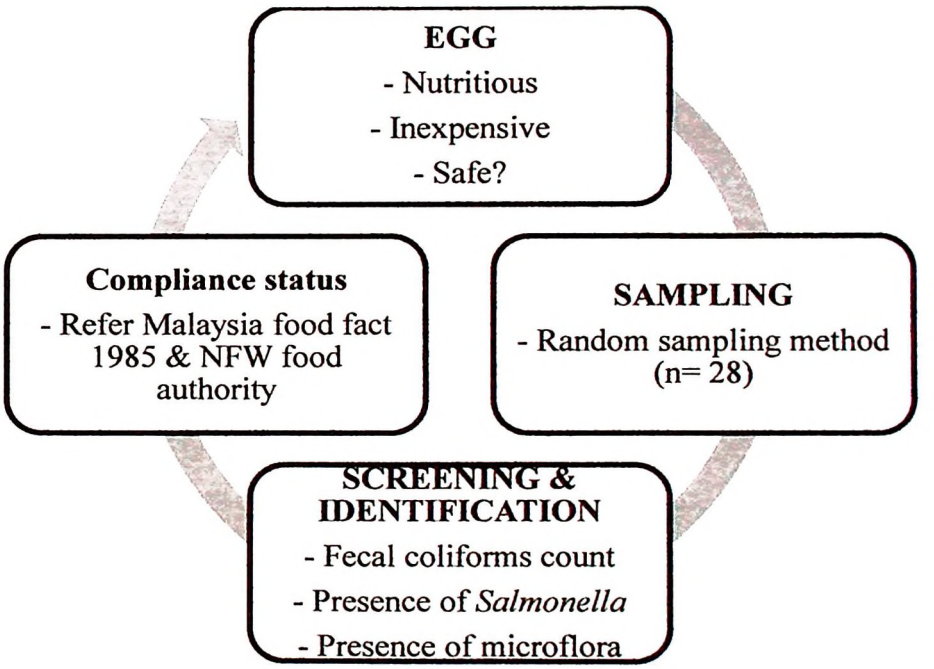

Figure 1.3: Research Conceptual Framework

CHAPTER 2

LITERATURE REVIEW

Eggs are healthy and nutritious food which contain high quality protein and offering many nutrients to consumers, especially for growing children, pregnant women and elderly. Malaysian consumed an average of 320 eggs per person per year, followed by the United States and India with 250 and 48 eggs per person per year respectively (Lee, 2011). It shows that Malaysians are among the world's largest egg consumers and may expose to the risk of egg contamination. Although there is a very low prevalence of pathogens in eggs, the number of cases can increas if there is no proper treatment and storage.

In Iran, from 100 eggs, 19 samples were contaminated by *E.coli*, 4 samples by *Proteus* and one sample by *Klebsiella* spp. (Ghasemian *et al.*, 2011). According to EFSA (2014), in year 2012, the most common food category reported in foodborne disease outbreaks are eggs and egg products that is 168 (22%) outbreaks which associate with *S. enteritidis, B. cereus* and *Staphylococcus* toxins. Study by Messens *et al.* (2006), showed that contamination of egg caused by *Salmonella* in Spain was 1%, Poland 5%, England 0 to 7% and India 1% to 8%.

Shane *et al.* (1986), reported that *C. jejuni* cannot infect the egg internally and fecal shedding *C. jejuni* in farm did not produce infected eggs. And Jones *et al.* (2004), reported that 0.5% of the eggshell sample were *Campylobacter* positive, 1.1% was *Salmonella* positive and 20% was *Listeria* positive.

The causative agents are involved in the outbreak shown in the figure 2.1. Data in Figure 2.1 included outbreaks in France, Germany, Netherlands, Poland, Solvakia, Spain and United Kingdom. More than half of the outbreaks were associated with *S. enteritidis* at 67% and 20.20% associated with other *Salmonella* spp. There are also two outbreaks caused by *Bacillus cereus* and *Staphylococcal* toxins.

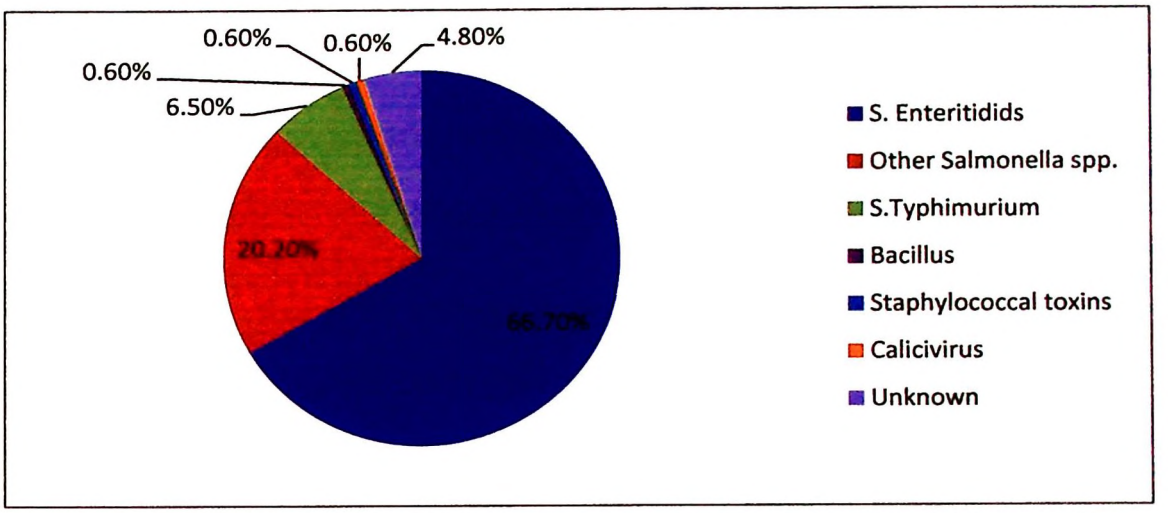

Figure 2.1: Causative agent isolated from outbreaks caused by eggs and egg products

Eggs and egg products are the most frequently reported sources of foodborne outbreaks caused by *Salmonella* in 2006 (EFSA, 2014) and products containing raw eggs such as Mayonnaise and custards are the example of food categories which expose the risk of salmonellosis to public health (EC, 2003). From an analysis, between the year 2009 to 2012, 610 foodborne outbreaks of human salmonellosis caused by eggs and egg products which resulted in 6339 cases, 1554 hospitalisation and 10 deaths (EFSA, 2014). Based on 'Turkey- Target Salmonella Attribution Model', 87% of the salmonellosis cases caused by *S. enteritidis*, 3.2% by *S. infantis*, *S. Virchow* 2.2% and *S.typhimurium* 1.8%. However, most of the cases are caused by serovars *S. enteritidis* and other serovars are rare.

Other than *S. enteritidis,* there also other pathogens involve in egg contamination such as Listeria monocytogenes and Campylobacter. In addition, fecal material promotes the growth of *E.coli* and *S. aureus* on the eggshell. Despite the colonization of *E. coli* in the intestines is possible, there is no outbreaks reported due to *E.coli*. But, *E.coli* able to penetrate the eggshell and cause contamination if there is sufficient number for contamination even in recommended storage temperature. Although most of the APEC isolated from chicken has low health threat to human, *E. coli* O157:H7 able to colonise in chicken intestine. *E. coli* O157:H7 can produce Shiga toxin which is pathogenic to human and can cause adverse effect if infected.

Staphylococci also one of the common bacteria in foodborne outbreaks because of the antibiotic- resistant *Staphylococcus* strains which can produce enterotoxins and cause severe effects to human. Egg foodborne outbreaks associated with *Staphylococcus* are uncommon but there are still some cases reported. In Britain, from 1969 to 1990, 3.5% of staphylococcal food poisoning were caused by eating contaminated egg (Wieneke *et al.*, 1993) while in France 11% cases are reported in 1999- 2000 (Haeghebaert *et al.*, 2002).

Bacillus Cereus also involve in the egg contamination, although in fact *Bacillus Cereus* cannot penetrate the egg andnd the contamination may occur during food handling process. There are two outbreaks were reported in 2011 (EFSA and ECDC, 2013). Although the prevalence of this bacteria is low but they still can reduce the microbiological quality of the eggs. *Listeria monocytogenes* is not frequently studied, but an investigation revealed that *L. monocytogenes* is present in 30% of caged hens (Chemaly *et al.,* 2008). This bacteria is able to contaminate the egg shell and transfer to

the egg product due to cross contamination during food preparation (Rivoal *et al.*, 2010).

There are studies on campylobacter and 1.1% of the sampled eggs were contaminated (Adesiyun *et al.*, 2005) and in Germany 4.1% of eggshell sampled were contaminated (Messelhäusser *et al.*, 2011). Campylobacter frequently found in caecum and this bacteria able to colonize other organ in chicken such as reproductive tract and liver (Cox et al., 2009) but there is no study talk about vertical transmission of this bacteria (Fonseca et al, 2006).

There are many factors contributing to the egg contamination such as storage time and temperature. These factors can cause changes to the egg structure and components, then result in decrease microbiological quality of the eggs. EFSA (2009) claimed that temperature and humidity affects the condensation process which could increase the risk of bacterial invasion into the eggs. They also conclude that cross contamination can occur at the processing level and depends on proportion of bacteria contaminated eggs and hygienic practices.

Each batch of packaged chicken eggs sold in the supermarket was labelled with best before date to ensure people only consume the eggs within that date. Because prolonged storage could lead to several changes in the eggs. Based on a study by EFSA (2014), prolonging of the storage time result in an increase in the number of illnesses but only for raw and partially cooked egg meal. This study also reported that by changing the best before date from 28 days to 35 days could increase the number of illnesses. And the number of illnesses could be high when the best before date is extended. This may caused by changes that occur in egg which lead to the bacterial invasion. Table 2.1

shows the mean number of illnesses when the best before date is changed for raw and partially cooked egg meals.

Table 2.1: Means number of illnesses per million serving of raw and undercooked egg meals with different best before date

Best before date	Raw egg meals	Undercooked egg meals
21 days	43.5	30.0
28 days	61.0	45.0
35 days	85.9	67.0
42 days	107.4	86.9

Source: EFSA, 2014

Temperature and humidity can cause excess loss of water from the egg through eggshell pores during the long time storage (Scott and Silversides, 2000). Increased the storage time and temperature can cause increases in albumen and yolk pH (Samli *et al*, 2005; Scholtyseek, S., 1981), flattening of egg yolk and decrease the vitelline membrane strength (Mertens *et al.*, 2010). Changes in albumen pH also can caused by loss of carbon dioxide through eggshell pores at ambient temperature (EFSA, 2014).

The cuticle is a layer of the surface of the eggshell which responsible to avoid bacterial penetration into the egg contents. It shows that cuticle acts as first line defense for eggs, which lowering trans shell penetration. Cuticle plug the eggshell pores, but it degrades over time and losing its defense properties. At ambient temperature, it lasts at least up to four days. However, trans shell penetration still can occur if there is a pressure gradient created when an egg is transferred from ambient temperature to lower temperature (Berrang *et al.*, 1999).

High storage temperature also can cause an increase in water movement from albumen to the yolk which lead to increase in egg yolk volume. Increase in yolk volume will result in decreased of its viscosity and weakening the vitelline membrane (EFSA, 2014). According to Chen *et al.* (2005) and Jones and Musgrove (2005), Vitelline membrane strength remains stable at 4 °C for 6 to 10 weeks. But its strength quickly reduced at ambient temperature (Jones and Musgrove, 2005).

Storage time and temperature also give effect on the survival of the bacteria on eggshell. Not all of the bacterias have ability to penetrate the eggshell and survive in internal egg compartments. According to Board (1994), gram negative bacteria are able to grow at low temperature and can survive since they have simple nutritional requirements. Moreover, high temperature and longer storage time can cause loss of membrane integrity which facilitate the movement of bacteria into the yolk by crossing the vitelline membrane.

There is study on changing temperature during storage by M. Okamura *et al.* (2007), which showed that different temperature of storage give different effects on bacterial growth. At 22 °C to 30 °C, bacterial counts increase and this may be cause by the decreased integrity of the vitelline membrane which allow the migration of bacteria into the yolk. And this situation had explained by Humphrey *et al.*, (1993) and claimed that "the fluctuation of temperature between 18 °C and 30 °C affects the integrity of the vitelline membrane rapidly, which accelerate a leakage of yolk contents and cause rapid growth of bacteria in albumen". While Chen *et al.* (2005), reported that storage at low temperature that is at 4 °C to 10 °C might maintain the integrity of vitelline membrane and inhibit the growth of bacteria in albumen.

Humphrey and Whitehead (1992), observed the bacterial invasion in eggs at room temperature and they believe that vitelline membrane inhibits yolk invasion by bacteria by delaying before yolk penetration and growth in the yolk. But vitelline membrane lost its integrity during storage and result in leakage of nutrients into albumen (EFSA, 2014).

Bacterial invasion in egg can be caused by several ways of contamination either during its formation or after the egg has been laid. Figure 2.2 shows the mechanism of the egg contamination by *Salmonella*.

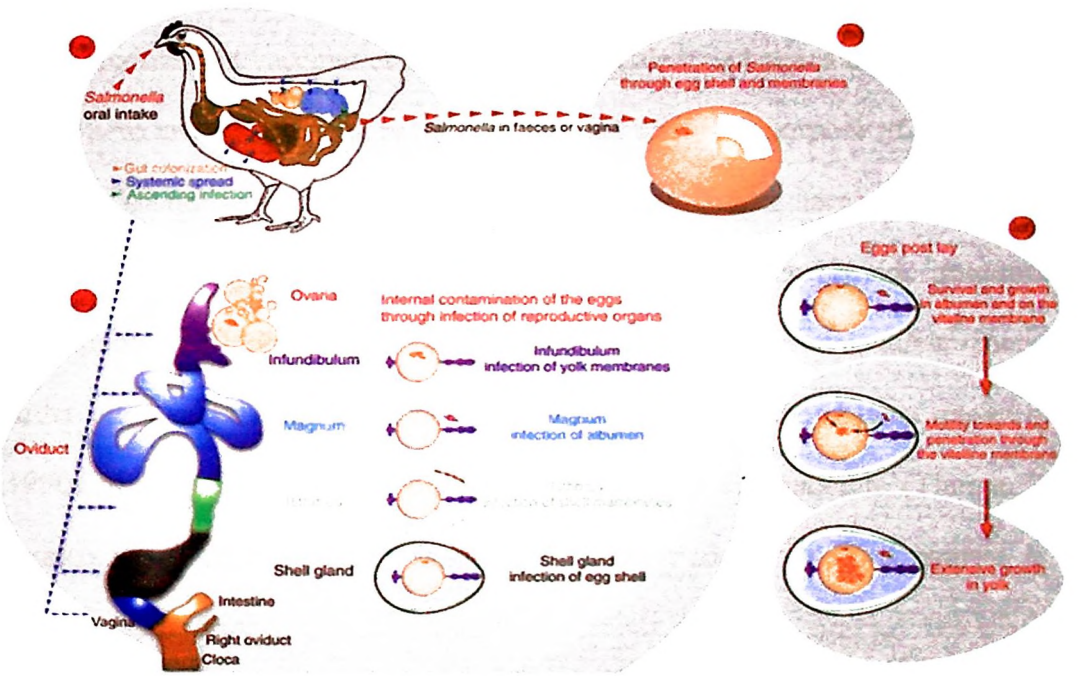

Figure 2.2: Mechanism of egg contamination by *Salmonella* (Gantois, 2009)

Figure 2.2 also can be used to explain the mechanism of egg contamination for bacteria other than *Salmonella*. This figure shows two types of contamination route that are primary contamination or transovarian transmission and secondary contamination or also known as trans shell contamination. The mechanism begins from the hen that is when the hen taken up *Salmonella* orally and enters the intestinal lumen and invade the

epithelial cells. As a result, macrophage encloses the *Salmonella* bacteria and allow it to multiply. Then the infected macrophage migrates to the internal organ and contaminate the egg during its formation. Infection of the reproductive tract can cause primary contamination or direct contamination of the yolk, vitelline membrane and albumen. While fecal contamination may result in surface contamination or secondary contamination. In this type of contamination, *Salmonella* penetrates the eggshell and shell membrane and multiply in internal part of the egg. The deposited *Salmonella* bacteria in the albumen survive and migrate to the yolk by penetrating the vitelline membrane. Then grow extensively after reaching the yolk.

Use of antibiotics and vaccination of laying hens against pathogens had been introduced to protect hens and eggs against bacterial infection. The main purpose of the vaccination and antibiotics are to prevent systemic infection and reduce fecal shedding (EFSA, 2014). There is a vaccine against *Salmonella* in poultry which provide immunization against typhoid (EFSA, 2014). The vaccine used are live oral and injectable inactivated, which have reduced the spread of infection. Vaccination for laying hens started during the 1990s in some countries because of the *S. Enteritidis* pandemic (Thorns, 2000). There also vaccination with the live *E. coli* vaccine to prevent and control *E. coli* infection in laying hens.

However, use of antibiotics should be monitored by the authorities to avoid the incidence of antibiotic resistant bacteria. The Consumer Association of Penang reported that studies on chicken meat found the antibiotic resistant bacteria in them. The spread of antibiotic resistant bacteria is alarming a serious health threat to consumers. Department of Veterinary Services in 2012 found that half of the chickens were resistant to sulphonamide, tetracycline and ampiciline. It's become worse when a study

found "13.5% Tetracycline-resistant *Salmonella*, 5.4% Polymixin B and Erythromycin-resistant *Salmonella* and 2.7% Chloramphenicol, Penicillin G and Trimethoprim-resistant *Salmonella* in local chicken" (The Poultry Site, 2014).

Because of the antibiotic resistant bacteria incidence, Europe has banned the use of antibiotic in poultry growth. To replace the use of antibiotic, there is research on probiotic which defined as live microbial feed supplements to improve the intestinal balance of the fowl. Study by Ho and colleagues have succesfully isolated and identified Lactobacillus strains from chicken intestines and "out of 42 isolated strains, three *Lactobacillus salivarius* strains demonstrated a high tolerance of stress conditions in the GI tract and a good capacity to adhere to intestinal epithelial cells" (The Poultry Site, 2014).

CHAPTER 3

METHODOLOGY

3.1 Study Design

The study design used in this study is the cross sectional study. In this study, 30 samples of packaged chicken egg were randomly selected from supermarkets in Kubang Kerian, Kelantan and were analysed for microbiological quality.

3.2 Apparatus and Material

Apparatus and materials used in this study were sterile swabs, hot plate, wire loop, disposable petri dish, hockey stick, stomacher bag, micropipette (100µl and 1000µl), 70% alcohol and test tubes. Incubators with various temperatures (42°C and 37°C) were used to incubate the cultured media and enrichment media. While water bath is used to incubate cultured MacConkey media at temperature 44.5 °C. Biosafety cabinet also had been used to protect personnel, product and the environment from exposure to biohazards and cross contamination during the procedures. In addition, to homogenize the sample, stomacher bag was used in the procedures.

To culture the selected microorganisms and bacterial isolation, several culture media such as MacConkey agar no.3, Xylose lysine deoxycholate agar (XLD agar), Eosin methylene blue agar (EMB) and Bismuth sulphite agar (BSA) were prepared based on the standard procedure. There are also other materials used for pre- enrichment and enrichment steps such as buffered peptone water, Rappaport-Vassiliadis broth (RV broth) and Mannitol Selenite Cystine broth (MSC broth). For analyzing the presence of *E.coli* and *Salmonella* , biochemical tests such as Triple Sugar Iron (TSI), Methyl Red (MR), Voges Proskauer (VP), motility, citrate and urease were performed and media for

these tests were prepared. Negative control used in this study is *Citrobacter freundii* which is the negative control for *Salmonella* sp.

3.3 Sample Collection

A total of 30 packaged chicken egg samples were collected within 3 months, which is from November 2014 until January 2015. The samples were collected from selected supermarkets in Kubang Kerian, Kelantan. 30 samples of the chicken eggs, was then divided into two groups. 15 samples were put into groups within the shelf life and other 15 samples were grouped into after the expiry date. All 30 samples were stored in the room temperature. Presence of *Escherichia coli* (*E.coli*) and *Salmonella* species were examined in this study.

3.4 Sample Processing

The weight and surface area of an egg was taken and recorded. Sterile swab was used to take samples from eggshell. The sterile swab was dip into buffered peptone water and swabbing around the whole eggshell. Then the swab was put into the test tube containing 10 ml of buffered peptone water and serial dilution up to 10^{-6} was made. 100 µl of the diluted sample was pipetted into the MacConkey agar and cultured. The test tube at dilution 10^{-1} was incubated at 37 °C for 16 hours to 20 hours before performing the *Salmonella* test.

A hole was made on the eggshell by using a sterile forceps and the whole egg content was put into stomacher bag which contain 225ml of 1.0 % buffered peptone water. Then, by using a stomacher bag mixer, the content was homogenized. 1ml of the mixture was pipetted into the tubes to make the serial dilutions up to 10^{-6} and duplicate cultured were made on MacConkey agar for each dilution to detect the presence of *E.*

coli. 100 µl of the diluted mixture was pipetted into each media. The cultured media were incubated in the water bath at temperature 44.5 °C for 18 hours to 24 hours.

While, for detection of *Salmonella* species, the homogenized mixture was incubated at 37°C for 16 hours to 20 hours before performing the serial dilution in selective enrichment broths that are RV broth and MSC broth. After 16 hours to 20 hours, 1 ml of the mixture was taken and put into the tubes for the serial dilution up to 10^{-6} in enrichment media. The dilutions then were incubated for 18 hours to 24 hours at 42°C for RV broth and 37°C for MSC broth. Same like the procedure for *E.coli*, duplicated cultured were made on XLD agar and BSA for each dilution by pipetting 100 µl of the diluted mixture into each media. Both types of the cultured media were incubated at 37°C but at different period of incubation. XLD agar was incubated for 18 hours to 24 hours while BSA was incubated for 48 hours. Negative control and positive control also were cultured on XLD agar and BSA.

3.5 Flow chart