MICROBIAL CONTAMINATION OF EGGSHELL AND EGGS CONTENT SOLD IN RETAIL AND FRESH MARKET IN PENANG

By

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LIST OF ABBREVIATIONS

Abbreviations	Definition
°C	Degree Celsius
%	Percentage
BAM	Bacteriological Analytical Manual
BPW	Buffered Peptone Water
BGLB	Brilliant Green Bile Broth
CDC	Centers for Diseases Control and Prevention
CFU	Colony forming unit
EC	*Escherichia coli* broth
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration
g	Gram
HEA	Hektoen Enteric Agar
L-EMB	Levine's Eosin-Methylene Blue
LIA	Lysine Iron Agar
LST	Lauryl Sulphate Tryptose Broth
MKTTn	Muller-Kauffmann Tetrathionate-novobiocin broth
MOH	Ministry of Health Malaysia
NA	Nutrient Agar
SC	Simmons Citrate
TSI	Triple Sugar Iron

PCA	Plate Count Agar
PDA	Potato Dextrose Agar
RVS	Rapport Vassiliadis Soy Broth
RPM	Revolutions per minute
spp.	Species
WHO	World Health Organization
XLD	Xlylose Lysine Deoxycholate

PENCEMARAN MIKROBI DALAM KULIT TELUR DAN ISI KANDUNGAN TELUR YANG DIJUAL DI KEDAI RUNCIT DAN PASAR SEGAR DI PULAU PINANG

ABSTRAK

Pencemaran telur oleh mikroorganisma akan mempengaruhi kualiti telur dan menyebabkan kerosakan dan keracunan telur kepada pengguna. Telur yang tercemar akan meningkatkan risiko penyakit. Gejala penyakit ini boleh berkisar dari ringan hingga parah. Tujuan kajian ini adalah untuk menentukan jumlah mikrob dan organisma penunjuk dan mengenal pasti spesies bakteria yang terpencil pada kulit telur dan kandungan telur yang dijual di kedai runcit dan pasar segar di Pulau Pinang. Dua puluh telur yang segar dikumpulkan secara rawak dan aseptik dari sepuluh pasar segar dan sepuluh telur dijual di kedai runcit di Pulau Pinang. Setiap kulit dan kandungan telur telah diperiksa untuk kandungan mikrobiologi mengenai jumlah kiraan plat, jumlah kiraan coliform, kiraan coliform najis, serta kiraan yis dan kulat. Jumlah kandungan mikroorganisma untuk kulit telur adalah daripada 3.90×10^4 hingga 2.82×10^7 CFU/ swab, dan kandungan telur adalah daripada $< 2.5 \times 10^3$ hingga 5.20×10^3 CFU/ mL. Seterusnya, jumlah kiraan coliform kulit telur adalah antara < 3 hingga > 1100 MPN/ g, dan kandungan telur adalah daripada < 3 hingga 210 MPN/ g. Jumlah kiraan yis bagi kulit telur adalah daripada $< 1.0 \times 10^3$ CFU/ swab hingga 4.90×10^6 CFU/ swab, sementara kandungan telur adalah $< 1.0 \times 10^3$ CFU/ mL. Jumlah kiraan kulat bagi kulit telur adalah daripada < 1.0 x 10^3 CFU/ swab hingga 3.30×10^4 CFU/ swab. Kemudian, bakteria dikultur pada media yang sesuai dan ujian mikrobiologi telah dijalankan untuk mengenal pasti organisma seperti *Salmonella* spp. dan *Escherichia coli*. Sebanyak 13 (65 %) dan 1 (5 %) daripada 20 sampel telur masing-masing positif untuk *Salmonella* spp. dan *E. coli*. Terdapat 10 (50 %) kulit telur dan 3

(15 %) kandungan telur dari 20 sampel daripada pasar segar dan kedai runcit positif untuk *Salmonella* spp., sementara hanya satu (5 %) kulit telur daripada 20 sampel positif untuk *E. coli*. Kulit telur mempunyai tahap pencemaran oleh mikroorganisma yang lebih tinggi daripada kandungan telur. Telur yang dijual di pasar segar mempunyai pencemaran oleh mikroorganisma yang tinggi berbanding telur yang dijual di kedai runcit.

MICROBIAL CONTAMINATION OF EGGSHELL AND EGGS CONTENT SOLD IN RETAIL AND FRESH MARKET IN PENANG

ABSTRACT

Egg contamination with microorganisms would impact the quality of eggs, which may lead to spoilage and cause intoxication to consumers, posing a public health risk. Contaminated eggs can increase the risk of illness, and this illness can vary from mild symptoms to life-threatening conditions. The purpose of this study was to determine the total microbial load and indicator organisms and identify the species of bacteria isolated from eggshells and egg contents sold in the retail and fresh markets at Penang. Twenty fresh table eggs were randomly and aseptically collected from ten fresh markets and ten retail markets in Penang. Each eggshell and egg content were examined for their microbiology contents regarding total plate counts, total coliform counts, fecal coliform counts and yeast and mold counts. The total plate counts of eggshells were ranged from 3.90×10^4 to 2.82×10^7 CFU/ swab, and the egg contents ranged from $< 2.5 \times 10^3$ to 5.20×10^3 CFU/ mL. Next, the total coliform of eggshells was ranged from < 3 to > 1100 MPN/ g and the egg contents ranged from < 3 to 210 MPN/ g. The total yeast counts of eggshells ranged from $< 1.0 \times 10^3$ CFU/ swab to 4.90×10^6 CFU/ swab, while the egg contents were $< 1.0 \times 10^3$ CFU/ mL. The eggshells ranged from $< 1.0 \text{ x } 10^3$ CFU/ swab to 3.30×10^4 CFU/ swab for the mold count. Then, the bacteria were cultured on suitable media and standard microbiology tests to identify isolated organisms such as *Salmonella* spp. and *Escherichia coli*. A total of 13 (65 %) and one (5 %) of 20 egg samples were positive for *Salmonella* spp., and *E. coli* respectively. About 10 (50 %) of eggshells and 3 (15 %) of egg contents out of 20 samples from fresh and retail markets were positive for *Salmonella* spp., while there were one (5 %) of eggshells out of 20 samples were

positive for *E. coli*. The eggshells had higher contamination levels of microorganisms than the egg contents. The eggs sold at fresh markets had higher contamination with microorganisms than the eggs sold at retail outlets.

CHAPTER 1: INTRODUCTION

1.1 Research Background

An egg is one of the important sources of food consumed worldwide to feed the world population. The egg contains a good source of nutrients such as proteins, vitamins, zinc and phosphorus; thus, it is a common and inexpensive source of proteins consumed by everyone (Munoz et al., 2015). The functional properties of eggs included emulsifying, binding agent, leavening agent, foaming agent, and others. These functional properties of eggs produce different types of foods, such as bakery products, mayonnaise, custard, and others.

Microorganisms that contaminate eggs have played a significant role in poultry production pathology. These microorganisms can survive on the eggshell's surface and penetrate the pores into the egg contents, such as yolk and albumen (Addo et al., 2020). The stable environment favours the growth of bacteria and subsequently colonizes the eggs. It can affect the quality of eggs, which may lead to spoilage and pathogen transmission. Besides that, the food-borne infection or intoxication occurs after consuming the contaminated eggs and causes economic loss as well. Several factors lead to egg contamination, such as improper handling during processing, storage condition, faeces, environment, transporting, and marketing.

On 20th March 2021, Singapore Food Agency (SFA) directed the importers to recall the eggs with the farm code CEM014 due to the *Salmonella* Enteritidis that can cause food-borne illness. The importers included An Hong Egg Supplies, Chuan Huat Poultry Farm Pte Ltd, Dasson Pte Ltd and FE Supply Pte Ltd. According to SFA, the Linggi

Agriculture Sdn Bhd eggs were contaminated with *S.* Enteritidis. On the same day, Aeon Malaysia immediately recalled the batches of eggs coded with CEM014 from 28 Aeon malls, 34 Aeon stores, and 22 Aeon Big.

Centres for Diseases Control (CDC) reported that approximately 1,200,000 food borne illnesses were due to *Salmonella* in the United States (Obe, 2017). Eggs and eggs products were the most common food category that caused the foodborne outbreak in Europe, 2012. There were 168 out of 763 outbreaks associated with eggs and egg products. About 66.7 % of salmonellosis outbreak is due to *S.* Enteritidis, and another two cases due to the *Bacillus cereus* and *Staphylococcal* toxins (EFSA and ECDC, 2014). In Malaysia, the food illness outbreak increased by 57 % in the first quarter of 2016 compared to 2015. The study found that 87 % of all reported cases of *Salmonella* were food-borne. The transmission of bacteria was common through food and eggs, including eggshells (Izyan, 2016). *S* Enteritidis is the major cause of foodborne salmonellosis outbreaks in humans, mostly associated with eggs and egg products such as pancake, mayonnaise, etc. Several outbreaks of salmonellosis are related to lightly cooked eggs and raw eggs (Moosavy et al., 2015).

Eggs have a good natural defense system capable of controlling bacterial invasion through the cuticle, calcium hard shell and shell membrane (Donald, 2016). The egg white consists of both bacteriostatic and bactericidal proteins that can contribute to the bacterial cell wall's degradation. It can reduce the movement of bacteria within the albumin (Spitzer, 2016). The penetration of bacteria through the eggshells and egg contents can easily occur when the eggshells are not fully developed. Microbial contaminations of eggs occur shortly after oviposition, transaction and till consumption (Indhu et al., 2014). The bacteria can contaminate the egg in two routes such as vertical transmission and horizontal transmission (Moosavy et al., 2015). The hens are known to be a common carrier of a variety of bacteria. In the vertical transmission, the eggs can be contaminated with microorganisms during egg formation in the hen's reproductive system. For the horizontal transmission, the eggs are contaminated through the environment, and faecal contamination penetrates through the eggshell. Hatch eggs that are contaminated with microorganisms result in poor hatchability and chick performance.

Besides that, the dust in barn and storerooms, cleanliness of shell or structure, storage conditions may affect the bacteria contamination (Mallet et al., 2010). Hence, it was very important for poultry producers to reduce the number of bacterial in hatcheries by maintaining the highest standard hygiene. Studies on the microbial contamination of eggshell and egg content have been focused on in recent years. There are many kinds of bacteria such as *Streptococcus, Staphylococcus, Bacillus, Escherichia, Micrococcus, Diplococci* and *Corynebacteria* are found on eggshells and egg contents. The fungal genus *Aspergillus* such as *A. flavus, A. niger* and *A. fumigatus*, also found in eggs (Salihu et al., 2015). Aflatoxins are poisonous substances produced by certain species of fungi. Aflatoxins can be life-threatening and damage our liver. The eggshell contamination with *B. cereus* cases rarely occurred in freshly laid eggs. In most cases, the organisms are absent from freshly newly laid eggs (Momani et al., 2017). However, the egg contaminated with different types of microorganisms was related to farmers' hygiene practices and environmental conditions such as dust, soil, and others (EFSA and ECDC, 2014).

Apart from that, the gram negative Enterobacteriaceae group is isolated as major source of contamination in eggs. *Salmonella* spp. are commonly found in eggs. *Salmonella* spp. is the most well-known bacterial likely to contaminate table eggs (Spitzer, 2016). *Salmonella* spp. is commonly found as normal flora for hens, but it can be pathogenic to humans. *E. coli* O157: H7, one of the Shiga toxin-producing, and a low occurrence of natural infection has been found in chickens in different geographic locations (Abdullah, 2010).

The good quality eggshells will prevent the penetration of bacteria into egg contents. However, the bacterial species on the eggshell's surface will penetrate the egg contents if there is presence a small defect on the eggshells (De Reu et al., 2006). Besides that, the storage of eggs in an open room without standard storage facilities will favour microbial growth because microbes grow within a range of 4 ℃ and 60 ℃ (Adzitey et al., 2011). The microbial growth on eggshells is very high since the rate of egg spoilage depends on nutrient availability, temperature, storage, and handling (Abdullah, 2010). Good hygiene practice storage can minimize the risk of contamination of eggs (Munoz et al., 2015).

1.2 PROBLEM STATEMENT

Today, so many people are concerned about food safety issues. Increased consumer awareness of food safety issues had changed the public's perception of the good quality of eggs. Consumers express concerns about eggs based on several risk factors such as bacterial contamination and storage behavior. However, the studies on microbial contamination of eggshell and egg content from Malaysia's retail and fresh market was rarely carried out. Therefore, this study is carried out to evaluate the total microbiology condition and status of eggs sold in Penang's fresh and retail markets. The indicator organisms such as coliform act as indicators to determine the hygienic status of processing equipment and environment.

The study on the type of microbial flora's presence on eggshell and egg content is crucial to determine public health risk. These microorganisms present on eggshells and egg contents can cause an outbreak of foodborne diseases worldwide. The *Salmonella* present on eggs can be emphasized to be spread from infect reproductive tissue to eggs, faecal contamination, improper handling, processing, storage, and careless working. Therefore, it is very important to know the dangers of consuming eggs with disregard to sanitary quality sold in retail and fresh market eggs.

1.3 OBJECTIVES

1. To determine the total microbial load and indicator organisms on eggshells and egg contents from retail and fresh market at Penang.
2. To identify the species of bacteria isolated on eggshells and egg contents sold in retail and fresh market at Penang.

CHAPTER 2: LITERATURE REVIEW

2.1 Eggshell and egg content: Structure and composition

The egg can be divided into three major components such as egg white, eggshell, and egg yolk. The composition of egg is shown in Table 2.1 and the Figure 2.1 shown the cross -sectional diagram of an egg.

Table 2.1: Composition of egg (Hincke, 2012)

Component	%	Water (%)	Protein (%)	Fat (%)	Ash (%)
Whole egg	100	65.5	11.8	11	11.7
Egg white	58-60	88	11	0.2	0.8
Egg yolk	31-33	48	17.5	32.5	2
Shell	9-12	-	-	-	-

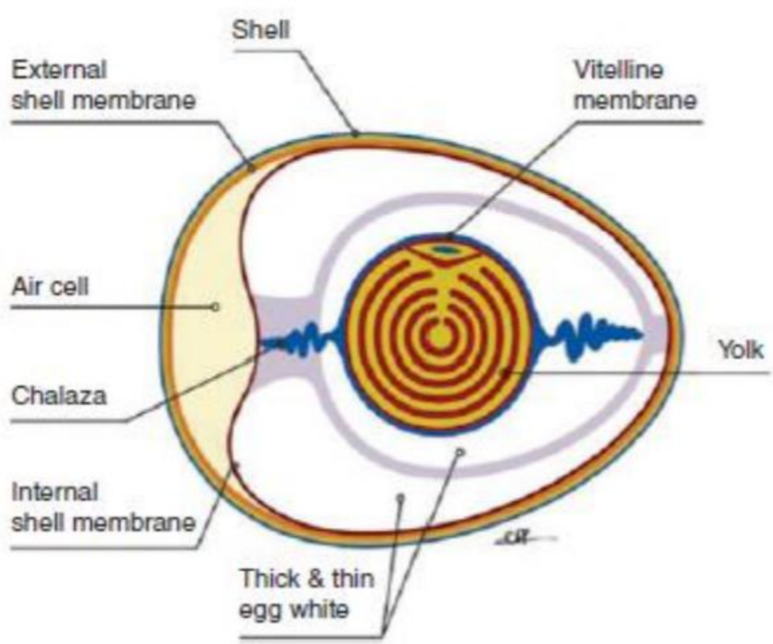

Figure 2.1: Cross sectional diagram of an egg (Hincke et al., 2012)

2.1.1 Eggshell

Eggshells compose of 94 % calcium carbonate ($CaCO_3$). The strength of the eggshell is determined by the mineral and vitamin content of the hen diet, primarily calcium, phosphorus, and vitamin D. The thickness of the eggshell is around 0.2 - 0.4 mm. Eggshell is the first defense layer against the entry of harmful bacteria into the interior of the egg. The number of pores on the eggshell varies and depends on the size of the egg, and the size of pores is approximately 12 to 20 μm in diameter (Nys and Guyot, 2011). The shell contains thousands of pores that allow moisture and carbon dioxide to move out and oxygen to move in.

The shell structure can be divided into four layers such as cuticle (bloom), spongy layer, mammillary layer, and pores. The shell is lined by a mucous layer known as a protective layer called a cuticle that cannot be washed. The cuticle layer comprises a protein-carbohydrate and contains a small amount of crystal complex hydroxyapatite - the outermost shell coating helps keep the dust and bacteria out and reduce moisture losses. Several factors influence the extension and composition of cuticles, such as the hen's age, the type of feed and the number of eggs laid (Messens et al., 2007).

Next, the spongy and calcareous layer is a matrix that contributes two-thirds of shell thickness below the thin cuticle. The mammillary layer consists of a thin layer of compressed, knob-like particles, with one side tightly attached to the spongy layer while the other side strongly attached to the outer surface of the shell membrane.

2.1.2 Eggshell membrane

The eggshell membrane comprises two layers: the inner shell membrane (ISM) and the outer shell membrane (OSM). The outer eggshell membrane sticks to the mammillary layer while the inside membrane sticks to the albumen. These two membranes are made up partly of keratin and prevent the penetration of bacterial. The thickness of eggshell membranes is approximately 70 µm, and the diameter of membrane fibers are about 0.4 to 3.6 µm (Nys and Guyot, 2011). The egg's contents cool and contract after the egg is laid, and the inner membrane will separate from the outer shell membrane to form the air cells. When the egg ages, the moisture and carbon dioxide will exit through the shell's pores, which allows the air to enter and replace them. As a result, the air cell will increase in size and become larger.

2.1.3 Egg albumen

Albumen, also known as egg white. It also consists of four distinct layers such as outer thin albumen (23 %), inner thick albumen (57 %), an inner thin layer (17 %) and a chalaziferous layer (3 %). There are 40 different proteins found in four alternating layers of thick and thin albumen such as ovalbumin, ovotransferrin, ovomucoid, ovoglobulin, lysozyme, ovomucins, flavoprotein, avidin and so on. The protein composition of egg albumen is shown in Table 2.2. These various proteins have different roles, such as prevent the degradation of the protein. Albumens have the function of protecting the yolk against microorganism and give the source of water, minerals, and protein to the embryo. When the microorganism penetrates the egg, the viscosity of the albumen will ensure the microorganism remains localized and combined action of chalazae, and albuminous sac prevents contamination of the yolk. Besides that, the ovotransferrin in the albumen chelates the iron and helps against bacteria's growth. (Board and Fuller, 2012).

Table 2.2: Protein composition of egg albumen (Hincke et al., 2012)

Protein content	Percentage (%)
Ovalbumin	55
Covalbumin	13
Ovomucoids	11
Ovoglobulin	8.5
Lysozyme	3.7
Ovomucins	1.5
Flavoprotein	0.8
Avidin	0.05

2.1.4 Egg yolk

Egg yolk comprises 33 % of lipid, 17 % of protein, and a trace amount of carbohydrate, minerals, and vitamins. The vitamins and minerals include vitamin A, vitamin D, iron, phosphorus, calcium, thiamine, and riboflavin. Besides that, the lecithin, which acts as emulsifier also found in the yolk.

The vitelline (yolk) membrane is a semi-permeable membrane that encloses the yolk and separates it from the egg albumen. It consists of two main layers, such as the inner layer and the outer layer. The inner layer formed in the ovary, while the outer layer is secreted in oviduct (Bellairs and Osmond, 2014). The vitelline membrane's outer layer consists of ovomucin, vitelline membrane outer layer protein I (VMOI) and lysosome. Ovomucin is responsible for forming the skeleton of an outer layer and preventing the penetration of microorganism. The salt soluble proteins lysozyme helps to maintain the integrity of the membrane.

The strand of fibers in outer layer membrane is extended into the chalazae (Bellairs and Osmond, 2014). The chalazae are twisted into chords and attach to either side of yolk. It holds the yolk in a central position. The more prominent the chalazae, the fresher the egg. The chalazae contain lysozyme, which acts as antimicrobial properties that prevent microbial spoilage of egg.

The yolk's pigmentation is primarily due to the direct transfer of pigments from feed or breed of hen, but certain endogenous pigments are used to colour the pigments. The yolk colour can be ranged from yellow to magnificent deep orange. The germinal disc is a small white spot found on the surface of the yolk. The geminal disc found in the nucleus of an egg is blastodisc (unfertilized). The embryo will develop from the germinal disc and send the blood vessels to the yolk and form the main nutrients for embryo development.

2.2 Type of microflora present on the eggshell and egg content

There are different types of microflora present on eggshells and egg contents. There are different types of microflora present on eggshells and egg contents. Gram negative bacteria such as *Pseudomonas, S.* Enteritidis, and *Alcaligenes* are most found in eggshells. The gram-positive *Staphylococcus* are found on eggshells such as *Staphylococcus equorum, Staphylococcus lentus,* and *Staphylococcus xylosus* (De Reu et al., 2007). They also examine that *Staphylococcus* is the most common microflora found in the air of poultry houses. The gram-positive bacteria are found on natural eggshells because they are tolerant to dry conditions. Most of the gram-positive bacteria originate from dust, soil, and faeces.

There are seven species that are commonly found in egg contents such as S*taphylococcus warneri, Acinetobacter baumanni, Alcaligenes* spp*., Serratia marcescens, Carnobacterium* spp., *Pseudomonas* spp., and *S.* Enteritidis (De Reu et al., 2004). *S.* Enteritidis, *Carnobacterium* spp., and *Serratia marcescens* are most frequently found in the egg contents of intact eggs. Mixed infection of gram-positive and gram- negative bacteria is found in rotten eggs such as *Micrococcus and Staphylococcus.*

A total of 160 egg samples that collected from 16 retail outlets are contaminated with bacteria (Salihu et al., 2015). There are 160 (100 %) eggshells, and 95 (59.4 %) of egg contents are contaminated with bacteria. Nine different genera have been isolated and identified from eggshells and egg contents. The bacteria genera included *E. coli*, *Salmonella* spp., *Shigella* spp., *Corynebacteria*, *Proteus* spp., *Bacillus* spp., *Staphylococcus* spp., and *Streptococcu*s spp.

Another study has analyzed the total of aerobes, yeasts, and mold, *Enterobacteriaceae* and *Pseudomonas* from eggshells and egg contents during the extended storage at 4 ℃ (Jones et al., 2004). The highest yeast and mold concentration of 0.7 log CFU/ mL are detected on week 8. The concentration of yeast and mold increase when the storage time increase. A low concentration of *Enterobacteriaceae* is detected in eggshells and egg contents. There are 5.7 % (28 out of 485) of washed and unwashed eggs positive for *Enterobacteriaceae.* The average concentration of *Pseudomonas* is less than 1 log CFU / mL.

Apart from that, there are varieties of *Enterobacteriaceae* and related organism from rinses of eggs that collected during processing in commercial shell egg processing plants (Musgrove, 2008). There are 12 eggs collected from each site along the processing line, such as accumulator, pre-wash rinse, sanitizer rinse and others. Both *E. coli* and *Enterobacter* spp. are found in nine egg processing plant visits. The other genera such as *Cedecea, Citrobacter, Erwinia, Hafnia, Klebsiella, Kluyvera, Leclercia, Morganella, Proteus, Providencia, Rahnella, Salmonella,* and *Serratia* also found in egg processing plants. The non-*Enterobacteriaceae* also isolated from the nine egg processing plants such as *Aeromonas, Chryseomonas, Listonella, Pseudomonas, Sphingobacterium, Vibrio,* and *Xanthomonas.*

2.3 Mechanism of microbial contamination of intact eggs

2.3.1 Vertical transmission, horizontal transmission, and cross contamination

There are three ways of microbial contamination of eggs, such as vertical transmission, horizontal transmission, and cross-contamination.

Vertical transmission originates from the infection of hens' ovaries through the systemic infection via oral or ascending infection from contaminated cloaca into the vagina and lower regions of the oviduct (De Reu et al., 2005). In vertical transmission, the yolk, egg albumen, egg membrane and eggshells are directly contaminated before oviposition. Besides that, the *Salmonella* also can penetrate via insemination of hen with infected sperm. Few serotypes might present in hens' reproductive tissue, such as *S.* Enteritidis, *S.* Gallinarum, *S.* Typhimurium, *S.* Heidelberg and, *S.* Arizonae. *S.* Enteritidis is the most common bacterium that contaminates the hen's egg in vertical transmission.

SEF14 fimbriae are found on *S.* Enteritidis and play a role in adherence to the reproductive tract tissue (Cogan and Humphrey, 2003).

In the horizontal transmission, the bacterial species can contaminate the egg by penetration through the eggshell. The surface contamination might occur due to infection of the lower reproductive tract or faecal contamination. As an egg is laid by a healthy hen, its bearing everts the vagina beyond the faecal alimentary tract to protect the egg from faecal contamination. Hence, the faecal contamination of eggs in healthy laying hen is unlikely to occur. When the wet egg enters an environment with a temperature of approximately 20 °C, below the hen's body temperature, the egg will cool immediately. The eggs are infected when they are cool because the egg content will contract and establish the negative pressure to facilitate microbial migration via the pores. *S.* Enteritidis will contaminate the egg contents such as albumen. *S.* Enteritidis has taken 58 hours to proliferate into 508 cells in albumen (Andreoletti et al., 2009).

Apart from that, the egg might contact any contamination surface such as dust, blood, insects, pets, nesting materials, shipping, and storage containers. The trans shell contamination depends on the cuticle of eggshells, dynamic stiffness value, shell quality, and contact time length (Messens et al., 2007).

2.3.2 Intrinsic and extrinsic factors

The intrinsic and extrinsic factor can influence the extent of *Salmonella* penetration into the egg. The intrinsic factors include shell porosity, shell thickness, distribution of cuticle and translucency. A few factors affect eggshell quality, such as hens' age, feeding regime, environmental temperature, stress, and diseases. The eggs that are laid by old hens get infected with bacterial more easily.

The eggshell consists of numerous pores, which range from 7000-17000 pores per egg. The egg's blunt pole has a high number of bacterial penetrations compared to the apex (Messeens et al., 2005). This is because most of the pores are located at the blunt end of the shell. A total of 155 egg samples, 72.9 % of bacterial penetrate at blunt pole whereas 52 % at the apex. Cuticle acts as a barrier for bacterial penetration, but the cuticle in older eggs is dehydrated, and shrinkage and the egg's pores are exposed more easily to the *Salmonella* penetration (Messens et al., 2005). The shell thickness also can prevent the penetration of bacteria into the eggs.

The cuticle is the first defense that prevents penetration of *Salmonella* through the eggshell. The cuticle in the freshly laid eggs is immature and moist and less effective prevent the penetration of bacteria. There is no relationship between the cuticle deposition and penetration of *Salmonella* through the eggshell (Messens et al., 2005). However, another study shows that bacterial penetration through the pores more easily in the absence of cuticle deposition (De Reu et al., 2006). Apart from that, the egg translucency will result from the penetration of *Salmonella*. In the early phase of eggshell formation, the changes in the mammillary layer and mammillary cores causes eggshell translucency. The egg translucency increases the cases of microcracks in eggshells and resulting bacterial penetration to occur (Chousalkar et al., 2010).

The extrinsic factor such as bacterial strain, temperature, moisture, level of initial inoculum and storage conditions can affect the eggshells and egg contents penetration by *Salmonella* spp. A positive temperature differential is present when the temperature of eggs (42 °C) is warmer than ambient temperature. *Salmonella* and *E. coli* are more easily colonize the eggshells and membrane. The egg contents will contract when cool, and the negative pressure is established and facilitate microbial migration via the pores. A combination of positive temperature differential with moisture will enhance the bacterial invasion through the eggshells and egg contents.

Moreover, storage time also one of the extrinsic factors that can influence the penetration of bacteria. The contamination of freshly laid eggs is 2.7 % in 15 out of 554 eggs, and the contamination of eggs will increase to 3.4 % during 21-days storage (De Reu et al., 2007). The highest egg contamination (5 - 10 %) with *S.* Enteritidis occurred during 8-days storage (Cogan et al., 2004).

2.4 *Salmonella* spp.

2.4.1 Taxonomy and characteristics of *Salmonella* spp.

Salmonella spp. are gram-negative, non-spore-forming rod-shaped bacteria in the family of Enterobacteriaceae. According to scientific studies, *Salmonella* has evolved for over 150 million years through genetic alteration, which has resulted in changes in pathogen ecology (Michaels, 2017). *Salmonella* strains can be divided into typhoid *Salmonella* and non-typhoid *Salmonella* (NTS). The non-typhoid *Salmonella* enteric fever, gastroenteritis or food poisoning, bacteremia with focal lesion and asymptomatic carrier stage are the four major syndromes caused by Salmonellae.

Salmonella can survive in the intestinal tract of human and warm or cold-blooded animals and capable of causing diseases. There are two common types of *Salmonella*, such as *S.* Bongori and *S.* Enteritidis (Michaels, 2017). *S.* Enteritidis is the most common foodborne illness (Crum-Cianflone, 2008). It can further subdivide into six subspecies such as enterica, salamae, arizonae, diarizonae, houtenae and indica based on biochemical and genomic modifications (Gul et al., 2016).

The genus of *Salmonella* can be subdivided into more than 2400 serotypes. Strains of *Salmonella* characterized serologically based on the somatic antigen (O), flagella antigens (H) and surface antigen (Vi). The H antigens are the slender threadlike structure that presents on flagella. These antigens are heat-labile and alcohol-labile. H antigens can be killed by the process of boiling or alcohol treatment but not formaldehyde. *Salmonella* spp. consist of two different genes that encode the flagellar protein. Flagella antigen can exist in two alternative phases, such as phase 1 and phase 2. Phase 1 antigens are known as a specific phase, while the phase 2 antigens are known as non-specific or group phase that widely shared by other serotypes (McQuiston et al., 2008). Agglutination pattern can be observed when mixing flagella-specific antisera with *Salmonella*. H antigen suspension forms large, loose, and fluffy clumps. The H antigen is highly immunogenic and stimulates the formation of high titers of antibody following infection.

Besides that, the O antigen is a complex of phospholipid-protein-polysaccharides that forms an integral part of the cell wall. The O antigen is located at the outer surface of the bacterial membrane and determined by the arrangement of specific sugar on the cell surface. The O antigen is heat stable, alcohol stable or weak acids. O antigen suspensions form compact, chalky, and granular clumps. O agglutination takes places at high temperature (50 - 55 °C), and the reaction is more slowly if compared to the H antigen. Then, the O antigen is less immunogenic if compared to the H antigen, and the titer of O antigen is induced after infection. *Salmonella* spp. can be categorized into a few groups according to the characteristics of antigens on the surface of bacteria. There are 67 of O antigens are identified.

Vi antigen is one of the superficial antigens that overlying the O antigen. The Vi antigen is present on *S.* Typhi, *S.* Paratyphi and *S.* Dublin (Eng et al., 2015). It is also a subtype of K antigen. Vi antigen is heat liable. It can separate bacteria by boiling method and centrifuging the bacteria from the Vi-containing fluid. Besides that, it is an acidic polysaccharide. Vi polysaccharides serve as a virulence factor via inhibition of complement-mediated killing, resistance against phagocytosis and bacterial lysis. The Vi antigen is poorly immunogenic and produces low titers of antibody following infection. After that, the serotypes are further subdivided by their resistance to bacteriophages (phage types or lystotypes), antibiotics, sensitivity to or production of bacteriocins (Andino and Hanning, 2015).

The cell diameter of *Salmonella* spp. is about 0.5 to 1.5 microns, and the length around 2 to 5 microns. *Salmonella* spp. are generally motile with peritrichous flagella except for *S.* Galinerum and *S.* Pullorum. It is also facultatively anaerobic bacteria that can survive with or without oxygen. *Salmonella* spp. able to ferment glucose, mannitol, arabinose, maltose, dulcitol and sorbitol to form acid and gas. Lactose, sucrose, salicin or adonitol are not fermented.

Salmonella spp. can survive in foods and other substrates for a long period of time. There are few factors that affect the growth and survival of *Salmonella* spp., such as temperature, pH, water activity and presence of preservatives. *Salmonella* spp. can survive in a wide range of temperature, 5.2 ℃ to 46 ℃ and the optimum temperature is around 35 ℃ to 43 ℃.

Salmonella is the heat sensitive and readily destroyed by pasteurization temperatures. Besides that, the heat resistance of *Salmonella* spp. in food highly depends on the composition of food, pH, and water activity. The heat resistance of *Salmonella* spp. increase as the water activity of food decrease. *Salmonella* spp. can grow in a wide range of pH (3.8 - 9.5). The optimum pH is fall in the range of 7 to 7.5. *Salmonella* spp. able to grow at minimum pH is highly dependent on temperature, presence of salt, nitrate, and type of acid. Apart from that, the water activity (aw) significantly influences the survival and growth of *Salmonella* spp. The optimum water activity 0.99, and the minimum water activity is 0.93. The survival of *Salmonella* spp. increase as the water activity decrease. Hence, *Salmonella* spp. able to survive in a low activity food for a long period of times.

2.4.2 Incidence of *Salmonella* spp. infections in world and Malaysia

Most of the foodborne illness outbreaks are related to the strain of *Salmonella* in the world of country. Humans get an infection with *S*. Enteritidis is more common to occur in poultry and poultry derived products such as egg. The bacteria might present on the gastrointestinal tract of hens. There are approximately 1 million cases of foodborne illness due to *S*. Enteritidis and more than 350 death each year in the United States (Akmar Omar et al., 2018). Salmonellosis is one of the common forms of foodborne illness diseases in the U.S after norovirus. In the United States, surveillance data stated that chicken meat and eggs are the most common food sources contaminated with *S*. Enteritidis. In Canada, there are 50 people who get the infection with *S.* Enteritidis after consumption the contaminated eggs and undercooked eggs. According to the Public Health Agency of Canada (PHAC), the safe handling of raw egg and cook the eggs until yolk and albumen are firm can prevent *Salmonella* infections on human.

Next, the incident of the *Salmonella* outbreak in Australia has risen from 29 outbreaks in 2001 to 116 outbreaks in 2014. A total of 990 outbreaks due to *Salmonella* spp. have been recorded from 1st January 2001 to 31st December 2006 (Ford et al., 2018). Most of the outbreak of salmonellosis in Australia are more prevalent in eggs and egg-based sauce. The number of salmonellosis outbreaks from 2001 to 2006 is shown in Figure 2.2.

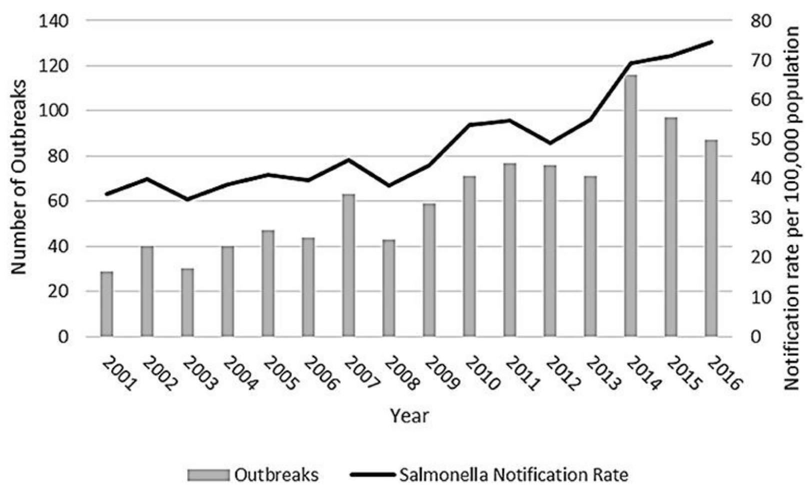

Figure 2.2: Number of salmonellosis outbreak from 2001 to 2006 (Ford et al., 2018)

According to the Ministry of Health (MOH), the incident rate of food poisoning per 100,000 populations has risen from 47.34 in 2015 to 56.62 in 2016 and decreased to 42.25 in 2017. These figures have surpassed the 5-year-median rates for food poisoning, which is 49.79 per 100,000 populations (MOH, 2017). The total episode of food poisoning has been increased by 28.6 %, which is from 409 episodes in 2015 to 526 episodes in 2016.

In contrast, the total episode of food poisoning has been decreased by 28.6 %, which is from 404 episodes in 2015 to 526 episodes in 2016. Table 2.3 had shown the cases of food poisoning in school and premises from 2013 to 2017. Most food poisoning cases occurred in premises if compared to schools (MOH, 2017).

Table 2.3: Cases of food poisoning in school and premises from 2013 to 2017 (MOH, 2017)

Item	Year				
	2013	2014	2015	2016	2017
Total incidents in all premises	495	501	412	524	404
Total incidents in school	130	151	118	167	181

Non-typhoid *Salmonella* can cause many outbreaks and death during its epidemiology. One of the food poisoning incidents happened at the wedding dinner due to the consumption of sweet and spicy chicken meat contaminated with *Salmonella*. The sweet and spicy chicken meat that contaminated with *Salmonella* due to improper preparation of food and caused four people dead. In 2008, *Salmonella* Weltevrede was found in laksa soup that sells at one of the premises located at Kupang, Kedah. The incidence of food poisoning due to insufficient temperature and time to cook the laksa soup and inappropriate storage of unclean noodles. In 2016, there is another case of food poisoning outbreak due to the consumption of contaminated pancakes at one of the residential schools in Perak. The pancake is made of flour, coconut milk, egg and water and the *S.* Enteritidis have been detected in a pancake food sample (Packierisamy et al., 2018). Most of the eggshells or egg content are contaminated with *S.* Enteritidis (Barden, 2006). Based on the Laboratory-Based Surveillance database report, there are few non-typhoid *Salmonella* stereotypes have been identified and recorded in Table 2.4.

Table 2.4: The non-typhoidal *Salmonella* serotypes from 2003 to 2005 (Barden, 2006)

Serotypes	2003	2004	2005
Enteritidis	244	206	155
Weltevreden	200	165	142
Corvallis	1115	117	57
Typhimurium	49	43	37
Stanley	32	11	8
Albany	17	37	5

2.4.3 Types of food involved

The outbreak of Salmonellosis has been associated with a variety of food sources such as poultry and poultry derived products. Most of the *S.* Enteritidis outbreaks are related to consumption of contaminated cooked eggs, egg-based sauce (mayonnaise) and egg-based foods that contain raw and lightly cooked eggs (ice-cream, cake, custard, mousse) (Ford et al., 2018). There are many types of foods had been identified and recorded in Table 2.5.

Table 2.5: Type of foods identified in the outbreak of salmonellosis (Ford et al., 2018)

	Median number of outbreak cases (range)	Outbreaks with statistical or laboratory evidence		Outbreaks with descriptive evidence		Total	
		Number	%	Number	%	Number	%
Raw egg desserts*	10 (2–140)	58/106	55	48/106	45	106/476	22
Egg-based sauce	14 (2–319)	43/79	54	36/79	46	79/476	17
Eggs, other	10 (2–143)	20/47	43	27/47	57	47/476	10
Mixed dishes	10 (2–90)	24/36	67	12/36	33	36/476	8
Poultry	11 (2–391)	20/34	59	14/34	41	34/476	7
Sandwiches, other	10 (3–213)	9/24	38	15/24	63	24/476	5
Desserts, other	13 (4–202)	18/23	78	5/23	22	23/476	5
Vietnamese sandwiches	17 (2–85)	7/17	41	10/17	59	17/476	4
Pork	10 (3–27)	10/15	67	5/15	33	15/476	3
Salads	18 (3–350)	8/10	80	2/10	20	10/476	2
Sushi	9.5 (3–85)	5/8	63	3/8	37	8/476	1
Beef	8.5 (4–15)	2/6	33	4/6	67	6/476	1
Pasta	6 (3–23)	2/6	33	4/6	67	6/476	1
Lamb	6 (3–43)	3/5	60	2/5	40	5/476	1
Fish	3 (3–14)	2/5	40	3/5	60	5/476	1
Sprouts	17 (4–126)	4/5	80	1/5	10	5/476	1
Crustacean/ mollusc	4 (2–23)	2/5	40	3/5	60	5/476	1
Fruits	29 (17–144)	4/4	100	0/4	0	4/476	0.8
Vegetables	10 (5–311)	3/4	75	1/4	25	4/476	0.8
Tahini or helva	24 (3–51)	3/3	100	0/3	0	3/476	0.6
Dips	33 (2–442)	2/3	67	1/3	33	3/476	0.6
Nuts	24 (19–43)	3/3	100	0/3	0	3/476	0.6
Undetermined	18 (2–50)	19/28	68	9/28	32	28/476	6

*Desserts containing raw or lightly cooked eggs (see **Supplementary Information 1** for more information)*

2.4.4 Prevalence of *Salmonella* contamination of eggs

The eggshells can be contaminated with *Salmonella* by either infection of the oviduct or through faecal contamination. *S.* Enteritidis and *S.* Typhimurium are the most common bacterium found in the reproductive system of infected hens. *S.* Enteritidis adhere more tightly to reproductive mucosa in hen if compared to *S.* Typhimurium. *S.* Enteritidis PT4 can be isolated from the reproductive tissue of infected hen in the absence of intestinal colonization (De Buck et al., 2004b).

The outer eggshell contamination can be affected by environmental conditions such as nest box, hatchery truck and egg production area. The chicken manure or faeces promotes the growth and survival of *Salmonella* because it serves as a nutritional reservoir for the growth of *Salmonella*. When the contaminated eggs are stored at 25 °C, the number of microorganisms can increase to 1-2 logs on day one and subsequently increase to 4 - 5 logs on day 3 (Gantois et al., 2009).

Besides, *Salmonella* organisms can survive at low temperature and low relative humidity in the absence of faecal contamination (Messens et al., 2006). *Salmonella* can survive for a long period of time under low temperature due to the slow metabolism induced by unfavorable conditions on the dry eggshell surface. They also examined that high moisture area favors the growth of *Salmonella* on the surface of eggshells and subsequently contaminated the egg contents. The temperature in the storage room increases, the number of *S*. Enteritidis in eggshell and egg contents will increase (Okamura, 2007).

Moreover, the unclean egg-handling equipment and utensil might contaminate the eggs. One of the studies examined that egg-packing plants can contribute to contamination and cross-contamination of eggs (Davies and Breslin, 2003). *Salmonella* is found in a swab of grading tables (30.8 %), floor swab samples (23.2 %), conveyor belts or rollers (23.1 %) and candlers (23.8 %). The presence of undesirable biofilm on the surface of equipment, utensil and plastic can cause contamination of eggshells and egg contents with *Salmonella*. The critical contaminations point in the egg production plants included grading and packaging machine (De Reu et al., 2005).

There are 23 out of 200 egg samples are contaminated with *Salmonella* in Western Australia (WA) (Sodagari et al., 2019). *Salmonella* is isolated from eggshells (9 out of 23 eggs) and egg contents (6 out of 23 eggs). Both eggshells and egg contents are contaminated with *Salmonella* found in another eight eggs. There is a higher prevalence of shell contamination if compared to the egg contents.

In European countries, the prevention method is introduced to minimizing contamination of eggshells, such as vaccination. According to the Food and Agriculture Organization of the United Nations (FAO), vaccination helps to reduce the *Salmonella* contamination on eggshells by 75 % (Wigley et al., 2005). It prevents the *Salmonella* penetrate the eggshells and contaminates the reproductive organ such as the oviduct. However, the penetration of *Salmonella* to egg content still occurs after the vaccination of laying hens (Van Hoorebeke et al., 2009).

2.5 *Escherichia coli*

2.5.1 Characteristics and taxonomy of *Escherichia coli*

Theodor Escherich is the first person who discovers *E. coli* in 1885. He isolates the bacteria from the faeces of newborns, which he originally named as *Bacterium coli commune* (Shulman, Friedmann, & Sims, 2007). *Escherichia* is one of the genera in the large family of Enterobacteriaceae. The genus *Escherichia* consists of five species such as *E. coli*, *E. fergusonni, E. hermanni, E. vulneris* and *E.blattae*. *E. coli* is the most common bacterium and can be further subdivided into biotypes and stereotypes. There are more than 700 different serotypes have been identified.

E. coli is one of the gram-negative, rod-shaped bacterium and non-spore-forming bacteria. It is 1 - 3 × 0.4 - 0.7 µm in size and 0.6 to 0.7 µm in volume and arranged in singly and in pairs. They are generally motile by peritrichous flagella, and some strains may be no motile. *E. coli* have a thin cell wall with one or two layers of peptidoglycan.