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FULL PAPER

Quantitative *Salmonella enterica* serovar Enteritidis risk assessment from consumption of hard-boiled eggs, half-boiled eggs and raw eggs among Malaysians

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Abstract

High occurrences of *Salmonella enterica* serovar Enteritidis outbreak from table eggs have been reported worldwide over the past two decades. Consumptions of hard-boiled and half-boiled eggs are popular among Malaysians. However, there is a lack of study in the risk assessment of salmonellosis associated with different egg consumption patterns. The purpose of this study was to determine the survival rate of *S. enterica* ser. Enteritidis in different methods for cooking eggs (hard-boiled, half-boiled and a minimally cooked egg with hot cocoa drink) using the simulation model of consumers eating habits and the risk associated with different egg consumptions patterns. In this study, *S. enterica* ser. Enteritidis was not detected in the hard-boiled egg samples. However, the survival rate of *S. enterica* ser. Enteritidis in both the half-boiled and the raw egg samples were 3.15 log CFU/mL and 7.01 log CFU/mL, respectively. The Monte Carlo Simulation applying quantitative microbial risk assessments (QMRA) was carried out using 10,000 iterations to access the risk of acquiring salmonellosis by consuming eggs cooked under different heat treatments. The total dosage of *S. enterica* ser. Enteritidis ingested per serving meal in the hard-boiled, half-boiled and minimally cooked eggs were 0.00 CFU/g, 7.526×10^4 CFU/mL and 5.433×10^8 CFU/mL, respectively. The consumptions of half-boiled and minimally cooked eggs were above infectious dosage level (10^2 to 10^4 CFU/mL). The annual risk for the three feature of methods were 0.00, 1.00 and 1.00, respectively. In this study, it was indicated that there was a high probability of acquiring salmonellosis through the consumption of half-boiled and minimally cooked eggs. Thus, the fully cooked eggs should be taken instead of the undercooked eggs to avoid consuming *S. enterica* ser. Enteritidis.

1. Introduction

Salmonellosis is one of the most common but serious foodborne illness globally (Heymann, 2009; Murray, 2016). Since the late 1970s, *Salmonella* spp. especially *Salmonella enterica* serovar Enteritidis has emerged into leading serovar of salmonellosis in many countries while eggs have been recognized to be the principal source of

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the transmission (Poppe *et al*., 1998; Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (WHO), 2002; Lake *et al*., 2004; Elaine *et al*., 2011; Centers of Disease Control and Prevention (CDC), 2018). *S. enterica* ser. Enteritidis has become the main cause of enteric infections towards chickens due to its unusual ability to infect the reproductive organ of hens by colonizing the oviduct tissue without detecting from chicken flocks and make it difficult to control (Poppe *et al*., 1998; Lake *et al*., 2004; Lake *et al.*, 2011). According to the Centers of Disease Control and Prevention (CDC), about 1.2 million cases of *Salmonella* infections associated with eggs were observed each year in the United States with 450 deaths and 23000 hospitalizations (Elaine *et al*., 2011; CDC, 2018). In 2018, the Food and Drug Administration (FDA) announced that more than 200-206 million eggs being recalled from the grocery chains or supermarkets in the United States as the culprit of salmonellosis. The consumption of raw or undercooked eggs has been contributed to 80% of *S. enterica* ser. Enteritidis outbreak worldwide (Lim *et al*., 1991; Adesiyun *et al*., 2007). In the United States, people prefer raw or undercooked eggs when preparing homemade food such as salad dressing, ice creams, or cookies batter (Lim *et al*., 1991; United States Department of Agriculture (USDA), 2006; CDC, 2018). It is suggested to use pasteurized egg when prepared dishes contained undercooked or raw eggs (FDA, 2018). Eggs can become the vehicle of *Salmonella* transmission if they are improperly cooked or stored without refrigeration (Adesiyun *et al*., 2007; Harun *et al*., 2015).

Eggs are frequently consumed in Malaysia regardless of hard-boiled, half-boiled or even eaten in raw eggs. Since eggs are available from breakfast to supper, Malaysians consume 2.8 million eggs daily; 22.2 kg of eggs per capita every year (Astro Awani, 2016; DOSM, 2018). Hard-boiled, half-boiled and raw eggs have become part of Malaysians eating culture. However, it poses a greater risk of *Salmonella* infection to the consumers if the eggs consumed are not fully cooked, especially in undercooked or raw eggs (USDA, 2006; Adesiyun *et al*., 2007).

Salmonellosis associated with egg is not a common foodborne disease in Malaysia, provided with a lack of data talk about the risk assessment regarding egg-salmonellosis to be recorded and identified. Yet, some of the strains have been isolated from the poultry farms and wet markets in Malaysia. However, Malaysians still lack awareness about salmonellosis associated with the consumption of undercooked eggs. The pathogenicity of *S. enterica* ser. Enteritidis poses a microbiological hazard when it comes to the consumption of raw or undercooked eggs (Ong *et al*., 2014; Tan *et al*., 2016). Risk assessment is an important tool to estimate the overall risk for a disease occurrence and its degree of severity (FAO and WHO, 2002; Sani *et al*., 2013; Amoah, 2014). It includes the parameters of risk communication and risk management. It is vital to address the food safety issues transparently. Risk assessment is derived from four important components hazard identification, hazard characterization (dose-response modelling), exposure assessment and risk characterization (FAO and WHO, 2002; Lake *et al*., 2011 Sani *et al*., 2013; Hamouda et al., 2018).

The present study aimed to evaluate the effects of different cooking methods on the survival rate of *S. enterica* ser. Enteritidis in raw eggs. Subsequently, a preliminary qualitative microbial risk assessment (QMRA) was conducted to quantify the risk of acquiring salmonellosis from the consumptions of hard-boiled, half-boiled and minimally cooked eggs.

2. Materials and methods

2.1 Sample preparation and bacterial inoculation

In this study, three dozen Grade A fresh eggs were purchased from the hypermarket in Kampar. Fresh eggs were immediately transported and kept at room temperature (25±5°C). Pure culture of ATCC 13076 *S. enterica* ser. Enteritidis bacterial (0.1 mL) in 10.0 mL of tryptic soy broth (TSB) (Himedia, Dindori, India) was revived at 37°C for 24 hrs in a shaking incubator (INF 29530; Infors HT, Bottmingen, Switzerland) at the rate of 160 RPM.

2.2 Simulation experiments

The simulation study was performed according to the procedures as described by Davis *et al*., (2008), Bryan (2018) and Alfaro (2019), with slight modifications. Only fresh eggs without holes or cracks were chosen, and the eggs shells were not sterilized in order to maintain their original state condition to simulate consumer handling practices. A sterilized pin was used to gently create a tiny hole on the eggshell surfaces before injecting a 0.1 mL of bacterial suspension of *S. enterica* ser. Enteritidis followed by sealing the hole with strong adhesive. After 3 mins, the artificially contaminated eggs were immediately subjected to different heat treatments: 65°C for 7 mins (to resemble condition for cooking a hard-boiled egg), 65°C for 3 mins (to resemble condition for cooking a half-boiled egg), and finally a raw egg was cracked into the hot prepared cocoa drink (65±1°C) (to resemble condition for cooking a minimally cooked egg). For the egg cooking method, different heat treatments were assigned to simulate the cooking process that is usually carried out by Malaysians. In this study, the artificially contaminated eggs were subjected to hard-boiled (cooked with hot water at 65℃ for 12 mins), half-boiled (cooked with hot water at 65℃ for 3 mins) and minimally cooked eggs (cracked eggs inside hot prepared cocoa drink). The simulation studies were performed in triplicate.

2.3 Microbiological analysis

Approximately 10 g of the 'contaminated' hard-boiled egg, 10 mL of the half-boiled and 10 mL of the minimally cooked treated egg samples were mixed with 90.0 mL of 0.85% saline water (NaCl) (ChemSoln, Malaysia) respectively in a sterile stomacher bag. The mixture was then homogenized in BagMixer® 400P stomacher machine (Interscience, France) for 30 s. Serial dilution was performed and each dilution was plated onto three Xylose Lysine Deoxycholate (XLD) agar plates (Oxoid, Basingstoke, England). The XLD agar plates were then incubated at 37℃ for 24 hrs. The presumptive colonies for *S. enterica* ser. Enteritidis (red colonies with a black centre) were identified and recorded as colony-forming units per gram or millilitre (CFU/g or mL).

2.4 Statistical analysis

One-way Analysis of Variance (ANOVA) was performed in order to identify between different cooking methods used to affect the survival rate of *S. enterica* ser. Enteritidis at the significance level of $\alpha = 0.05$. The experimental results were expressed as mean values ± standard deviation.

2.5 Quantitative microbial risk assessment (QMRA)

In addition, the findings from the simulation study were integrated with previous literature data and analyzed using Monte Carlo Simulation @ Risk software (Palisade Corporation, version 7.6). The main objective of Quantitative microbial risk assessment (QMRA) is to determine the risk of acquiring salmonellosis *S. enterica* ser. Enteritidis in the artificially contaminated eggs after subjected to different cooking methods. The risk of acquiring salmonellosis from consumption of hard-boiled, half-boiled and raw eggs was calculated using the risk assessment model. The exposure frequency derived from the Monte Carlo simulation was determined by using @ Risk software with 10,000 iterations to estimate the annual risk of infection.

2.5.1 Hazard identification

S. enterica ser. Enteritidis was chosen as the causative agent in this study because it is the leading cause of egg-salmonellosis among all *Salmonella* strains, while eggs were chosen as the source of contamination due to its high occurrences of *S. enterica* ser. Enteritidis transmission throughout the world (Poppe *et al*., 1998; FAO and WHO, 2002; Lake *et al*., 2004; CDC, 2018).

2.5.2 Exposure assessment

According to the previous studies, Malaysians normally consume at least one egg per day (Norimah *et al*., 2008; Ministry of Health (MOH), 2010; Mohamad-Kasim *et al*., 2018). According to Canada's Food Guide (CFA), the recommended serving size of egg consumption is between 50 to 100 g for each person (CFA, 2019). Based on nutrition fact provided by Alberta (2020), the estimated weight of Grade A eggs consumptions is 53 g per serving for each person. In this experiment, all Grade A eggs used were in 53±0.10 g. Hence, the exposure scenario was inferred to be at 53 g/person/day or 371 g/person/week.

Equation (1) was used to estimate the total amount of *S. enterica* ser. Enteritidis ingested per serving meal of egg (Amoah, 2014).

$$CFU_T = CFU_s/g \text{ or } mL \times W \quad (1)$$

CFU_s/g or CFU_s/mL represent the amount of *S. enterica* ser. Enteritidis, while 'W' represents the weight of egg per serving in g or mL, which is 53 g as described above.

2.5.3 Hazard characterization (dose-response assessment)

The exponential dose-response model, (Equation 2), was utilized in a dose-response relationship manner in order to predict, estimate and quantify the probability and risk of acquiring salmonellosis associated with consumptions of eggs that cooked cooking using different methods. By using this model, the infection probability, P(i) per serving meal was calculated.

$$P(i) = 1 - e^{-r \times N} \quad (2)$$

In this equation, 'N' represents as CFU_T of *S. enterica* ser. Enteritidis, which is obtained from Equation (1), while 'r' represents a parameter of dose-response. In this study, the 'r' used for *S. enterica* ser. Enteritidis was 6.85×10^{-5} (Tromp *et al*., 2010). Monte Carlo Simulation @ Risk software (Palisade Corporation, version 7.6) with 10,000 iterations was used to determine the probability of *S. enterica* ser. Enteritidis infections per day (Amoah, 2014).

2.5.4 Risk characterization

Risk characterization is derived from the integration

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of the estimated populations at risk (Equation 3) and the dose-response model (Equation 2), to calculate the risk estimation for future reference. The estimated number of cases, P(a), reported annually was calculated using Equation 3 as shown:

$$P(a) = 1 - [1 - P(i)]^n \quad (3)$$

'n' in the equation was represented the number of exposure days within the year; in this case, 52 was used due to weekly consumption was accounted for in this study. The mean risks of acquiring *S. enterica* ser. Enteritidis infections, P(i) were derived from @ Risk software (Amoah, 2014).

3. Results

3.1 Simulation process

The microbial counts of *S. enterica* ser. Enteritidis in eggs samples after subjected to different cooking methods are tabulated in Table 1. The survival counts of *S. enterica* ser. Enteritidis in half-boiled and minimally cooked eggs were 3.15 log CFU/mL and 7.01 log CFU/mL, respectively, whereas there was no microbial load detected in a hard-boiled egg. Both half-boiled and minimally cooked eggs achieved a log reduction of 8.23 log CFU/mL and 4.37 log CFU/mL, respectively, compared to the initial microbial loads of 11.38 log CFU/mL, after treated with different cooking methods. Besides, the survival rates of *S. enterica* ser. Enteritidis from minimally cooked eggs were showed significantly higher, compared to half-boiled eggs, followed by hard-boiled eggs ($P < 0.05$).

3.2 Exposure assessment

The total amount of *S. enterica* ser. Enteritidis ingested per serving meal for different consumption patterns are summarized in Table 2. Based on analysis of the Monte Carlo Simulation of 10,000 iterations, the total amount of *S. enterica* ser. Enteritidis ingested per serving of a hard-boiled, half-boiled and minimally cooked egg were 0.00 CFU/g, 7.5261×10^4 CFU/mL, and 5.433×10^8 CFU/mL, respectively. This result has shown that no *S. enterica* ser. Enteritidis was consumed in hard-boiled eggs. Boiling the eggs for 12 mins was sufficient to completely kill *S. enterica* ser. Enteritidis.

As shown in Table 2, the estimated concentrations of *S. enterica* ser. Enteritidis ingested per serving meal, CFU_T were 0.00 CFU/g, 5.68×10^4 CFU/mL and 4.10×10^8 CFU/mL for hard-boiled, half-boiled and minimally cooked eggs, respectively, by taking account into 53 g of eggs per serving meal.

3.3 Hazard characterization (dose-response assessment)

Based on the results generated from @ Risk software (Table 3), the annual risk of acquiring *S. enterica* ser. Enteritidis infection based on weekly consumption of hard-boiled eggs was 0.00. This mediated that none of the individuals was infected with *S. enterica* ser. Enteritidis in any time of year. The annual risk of acquiring *S. enterica* ser. Enteritidis infection through consumption of half-boiled and minimally cooked eggs per week was 1.000. This value was mediated based on a weekly consumption of seven servings of half-boiled and minimally cooked eggs which caused someone to be infected with salmonellosis if the initial microbial level

Table 1. The microbial counts ($\log_{10}$ CFU/g or $\log_{10}$ CFU/mL) and log reduction of *S. enterica* ser. Enteriditis in egg samples after subjected to different methods of cooking.

Cooking methods	Initial microbial load (Log CFU/ mL)	Final loads of microbes (Log CFU/g or mL)		Remaining temperature in eggs (°C)
		Survival	Log reduction	
Hard-boiled	11.38±0.13	ND	11.38±0.13	63.00±0.24
Half-boiled	11.38±0.13	3.15±0.49[a]	8.23±0.37	62.50±0.47
Minimally cooked	11.38±0.13	7.01±0.08[b]	4.37±0.17	62.00±0.82

Values are expressed as mean±SD, n = 3. Values with different superscript within the same column are significantly different ($P<0.05$). ND = Not detected

Table 2. The total amount of *S. enterica* ser. Enteritidis ingested per serving size in three different patterns of egg consumptions

Patterns of consumptions	Final load of *S. enterica ser.* Enteritidis in egg[a] (CFU/g or mL)	Weight of egg (W)	Final amount of microbes ingested per serving (CFU_T)
Hard-boiled	0	53	0
Half-boiled	1.420×10^3	53	7.526×10^4
Minimally cooked	1.025×10^7	53	5.433×10^8

W = The weight of the egg per serving of meal in unit of gram, based on estimation by Norimah *et al.* (2008), MOH (2010), Mohamad-Kasim *et al*. (2018), CFA (2019) and Alberta (2020).
[a]The number of microbes per gram of egg was determined from the survival count tabulated in Table 1.
[T]Total microbes will be ingested per serving meal of egg

Table 3. The probability of acquiring *S. enterica* ser. Enteritidis infection per serving meal of egg and annual risk associated with different patterns of consumption

Patterns of consumption	Total amount of *S. enterica* ser. Enteritidis ingested per serving (CFU_T)[a]	Probability of *S. enterica* ser. Enteritidis infection (per serving meal)	Annual risk of *S. enterica* ser. Enteritidis infection[b]
Hard-boiled	0	0.0000	0.0000
Half-boiled	7.526×10^4	0.9791	1.0000
Minimally Cooked	5.433×10^8	1.0000	1.0000

[a]Mean of three replicates based on the simulation study.
[b]Annual risk of *S. enterica* ser. Enteritidis infection which was based on weekly exposure within the year.

of *S. enterica* ser. Enteritidis in the shell egg was 11.38 log CFU/mL.

4. Discussion

Over the past few years, shell eggs are considered to be safe for consumption in Malaysia. Eggs are an important source of protein in our diet. Nevertheless, *S. enterica* ser. Enteritidis was being reported to be isolated from clean and uninfected table eggs samples (Humphrey, 1994; USDA, 1995; CDC 2018). Thus, we should be aware of the potential harmful hazard occurs when we consume eggs in our daily diet. Adequate cooking time and temperature are the important factors to make sure that the eggs are free of any microorganism.

In this study, *S. enterica* ser. Enteritidis was not detected in the hard-boiled egg samples after subjected to heat treatment of 65℃ for 12 mins. This observation is comparable with the previous studies, whereby the D values required to eliminate 90% of *S. enterica* ser. Enteritidis fall between 54℃ to 60℃ at 4.5 to 6.4 mins (Humphrey *et al*., 1994; Chantarapanont *et al*., 2000; Lake *et al*., 2004; Paula *et al*., 2005; Lake *et al*., 2011).

As for the half-boiled eggs, *S. enterica* ser. Enteritidis has managed to survive at 3.15 log CFU/mL which accounts for a reduction of 8.23 log CFU/mL. These findings were comparable to the study of Paula *et al*. (2005), who had reported that *S. enterica* ser. Enteritidis could reduce approximately 6.67 log CFU/mL when it was subjected to boiling water treatments for 3 mins. In addition, Chantarapanont *et al.* (2000) also had reported that a reduction of 7.30 log CFU/mL of *Salmonella* was observed when the contaminated shell eggs were subjected to boiling water treatment for 3 mins.

As for the minimally cooked eggs, *S. enterica* ser. Enteritidis has managed to recover 7.01 log CFU/mL, which is a 4.37 log CFU/mL reduction of growth. Brackett *et al.* (2001) has found that the artificially contaminated eggshell was able to recover 6.98 to 7.01 log CFU/g of *S. enterica* ser. Enteritidis in hot cocoa. On the other hand, Paula *et al.* (2005) stated that around 1.35 log CFU/mL of *Salmonella* reduction can be achieved if the egg is placed into the boiling water for 1 min. Also, Chantarapanont *et al.* (2000) reported that the treated egg samples were not able to achieve 7.30 log CFU/mL of reduction with less than 3 min of heat treatment. The finding has shown that temperature and time play an important role to control the growth of *S. enterica* ser. Enteritidis.

The survival rate of *S. enterica* ser. Enteritidis for all cooking methods were significantly different from each other ($P < 0.05$) (Table 1). However, the egg is safe to be consumed if there is no pathogenic microorganism found as stated in the zero-tolerance policy according to food safety standards. *Salmonella* is not allowed to be presented in between 25 g to 50 g of foods (FSC, 2004; Greig, 2010; FDA, 2012; Amoah, 2014). In addition, the Food Administration Manual of New Zealand, FAM (1995), and Hong Kong Food Safety Centre, FSC (2014), stated that less than 50 g of foods should be free from *Salmonella* spp. The cooked eggs are considered to be unsafe for consumption even though there is only 1 CFU/mL present in the egg (Duguid and North, 1991). Except for hard-boiled egg, the consumption patterns of half-boiled and minimally cooked eggs were exceeded the minimum standard requirement of *Salmonella* level. The results in this study indicated that the hard-boiled egg was the safest to be taken in our diet.

The infectious dose of *Salmonella* is ranged from 10^2 CFU/mL to 10^4 CFU/mL (FSC, 2004; FDA, 2012; Amoah, 2014). According to Greig (2010), the infectious dose of 10^2 CFU/mL to 10^4 CFU/mL is sufficient to cause a serious *Salmonella* outbreak associated with eggs. Besides, 1 CFU/mL of *S. enterica* ser. Enteritidis is able to multiply from 10^7 CFU/mL to 10^9 CFU/mL in eggs (Duguid and North, 1991). Thus, consumers who prefer half-boiled or minimally cooked eggs should be aware of the risk of getting salmonellosis., especially for these eating cultures are popular among Malaysians, Singaporeans and Hong Kong residents. This is mainly due to the cooking methods for preparing the half-boiled and minimally cooked eggs are not able to completely kill *Salmonella*.

As shown in Table 3, an individual who consumes hard-boiled egg poses the least or even no risk of getting salmonellosis. The probability of being infected is zero. By consuming half-boiled and minimally cooked eggs, the risks of acquiring *S. enterica* ser. Enteritidis infection was 1.000. Findings in this study showed that the annual risk for consumption of half-boiled and minimally cooked eggs are above the tolerable annual risk of 10^{-4} (Amoah, 2014; Hamilton *et al*., 2017). According to Amoah (2014), the annual risk of 10^{-1} (0.100) represents the risk of one person getting the infection out of 10 people. The risk of acquiring salmonellosis is extremely high for those who consume the half-boiled and minimally cooked eggs, especially among the susceptible groups such as young infants and kids, elderly and immunocompromised patients (USDA, 2006; Wida, 2018).

The likelihood of acquiring salmonellosis is very dependent on the initial level of *Salmonella* in eggs, the final concentration of *Salmonella* after subjected to heat treatments and its consumption amount. The hard-boiled egg is proved to be the safest for consumption. Half-boiled and minimally cooked eggs may pose a public health concern since the dosage per serving is adequate to cause *Salmonella* infection. It is advisable to avoid consuming undercooked eggs or minimally cooked eggs as *S. enterica* ser. Enteritidis is easily present in raw or undercooked eggs and these have contributed to 80% of *S. enterica* ser. Enteriditis outbreak worldwide (Lim *et al*., 1991; USDA, 2006; Adesiyun *et al*., 2007; CDC, 2018).

5. Conclusion

In this study, the findings indicated that *S. enterica* ser. Enteritidis have the highest survival rate in half-boiled and minimally cooked eggs as these two cooking methods were not able to completely eliminate *S. enterica* ser. Enteritidis due to the short time of heat exposure. On the other hand, there was no *S. enterica* ser. Enteritidis being detected in hard-boiled eggs. This condition is able to achieve the ‘all kill’ status of *Salmonella* bacteria.

In addition, results also clearly showed that the microbial levels of *S. enterica* ser. Enteritidis present in the half-boiled and minimally cooked eggs were sufficient to contribute to a serious outbreak as the infectious dosage of *Salmonella* is ranged from 10^2 CFU/mL to 10^4 CFU/mL. All in all, the annual risks of acquiring salmonellosis from the consumption of undercooked or minimally cooked egg were exceeded the level of tolerable annual risk of 10^{-4}. Therefore, it will be a high chance of acquiring salmonellosis among the individuals who like to consume half-boiled and minimally cooked eggs. Hence, in order to minimize the chance of obtaining foodborne infections, it is advisable to consume a fully cooked egg.

Conflict of interest

The authors declare no conflict of interest.

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