

Quality and Control of *Staphylococcus aureus* and *Clostridium perfringens* in Salted Egg Production

Rosarin Wongvilairat

Department of Microbiology and Parasitology, Faculty of Medical Science,
Naresuan University, Phitsanulok, 65000 Thailand
Corresponding author. E-mail: Rosarinw@nu.ac.th

ABSTRACT

Traditional salted egg production often pertains some microbial contaminations in the food causing food hazard which is a major concern in food biotechnology industry. Brining in lime juice are introduced to control these hazard in order to guarantee the safety and reserve sensory acceptance of the product. The aim of this study was to assess the microbiological quality of salted eggs based on Thai Industrial Standard Institute (TISI) and to assess the possibility of lime juice to control the hazard and their sensory quality. It was found that procedure used in making salted eggs nowadays has been passed the microbiological quality standard in term of mesophilic aerobic bacteria and *Salmonella* spp. ; however has not been passed the microbiological quality standard in terms of hazard related to *Staphylococcus aureus* and *Clostridium perfringens*. Lime juice was added into brining solution to observe its effectiveness in inhibiting the growth of *S. aureus* and *C. perfringens* in the production of salted eggs. Our data showed that 1.35% (v/v) lime juice and pH 4.59 in the brining process could significantly eliminate *S. aureus* and *C. perfringens* in salted eggs. Moreover, this condition could maintain the safety quality but could not retain the sensory acceptance. Our findings could lead to development of a standard procedure in salted egg production industries to improve quality of the food product.

Keywords: salted egg, quality, lime juice, control, safety.

INTRODUCTION

Salted egg is a traditional preserving egg which is characterized by salty, sticky and redness. It has been one of the most popular foods consuming in Thailand for many centuries. Process in making salted egg is varied depend upon locations and there is no standardized procedure. In general, the process is to immerge eggs in a high-salted concentration for a period of time. Most salted eggs contain considerable levels of the antimicrobial NaCl (18-20%) that could prevent the survival and growth of pathogens (Stadelmen and Cotterill, 1977). However, based on the fact that several strains of bacteria have developed greatly in their salt toleration, and gained remarkable ability to survive in different environmental stress conditions make it very difficult for food processing to exclude them from the products (Andersson *et al.*, 1995).

It is well known that eggs and egg products revealed the presense of considerable levels of food poisoning bacteria such as *Salmonella* spp., *S. aureus*, *C. perfringens* and *Enterobacteriaceae* (Stadelmen and Cotterill, 1977). *S. aureus* is responsible for one of the most common type of food poisoning. The main symptoms are vomiting (3-12 times) and diarrhea (Miwa *et al.*, 2001). The

incubation period is between 1.5 to 4 hr with the median time of 2.7 hr (Miwa *et al.*, 2001). *C. perfringens* is a serious foodborn pathogen and is a major concern in the food industry (Todd *et al.*, 1997). Symptoms caused by *C. perfringens* in human are gastroenteritis, including acute abdominal pain and diarrhea. These symptoms usually appear 8-16 hr after ingestion of the vegetative form of the organism and can last 24-48 hr (Duncan *et al.*, 1972). *Salmonella* spp. is also a serious foodborn pathogen and the main symptom is grouped to two types as enteritis and systemic. The enteritis type usually appears as mild fever, nausea, vomiting, abdominal pain and diarrhea between 6 and 48 hr. The systemic type causes septicaemia (Adams and Moss, 1995).

Salmonella spp., *S. aureus* and *C. perfringens* are the potential bacteria caused outbreaks of food borned illness associated with consumption of eggs and egg products (Stadelmen and Cotterill, 1997). It was stated in the previous research that locally commercial salted eggs were detected mesophilic aerobic bacteria, *S. aureus* and *C. perfringens* but not *Salmonella* spp. (Rosarin, 2002) (unpublished). This result is in correspondence with other researchers who found that the probability of detection of *Salmonella* spp. in salted egg is extremely low (about 0.05 according to 10% (w/w) NaCl and with water activity below 0.94, preventing the growth of *Salmonella* spp.) (Henry *et al.*, 1979). Therefore, *S. aureus* and *C. perfringens* are considered to be potential hazard which are a major concern for this product. Benzoic acid and organic acids, mainly ascorbic, citric, lactic, malic, propionic, succinic and tartaric acids, which are naturally found in a variety of fruits, have been used by several researchers as antimicrobial agents in food processing (Sengun and Karapinar, 2004). Therefore, lime juice could be considered to be used as a possible food preservative to reduce or eliminate *S. aureus* and *C. perfringens* contamination in salted egg production.

To develop a better understanding of the microbiological problems associated with salted egg production, it becomes extremely necessary to study the microbiological quality and to examine procedure in order to justify method to diminish their contamination in order to control *S. aureus* and *C. perfringens* associated with the salted egg production. This is to ensure the safety of salted eggs being produced, and possibly standardized procedures for further application.

MATERIALS AND METHODS

The microbiological quality of salted egg

Sample collection

Five locally commercial salted eggs were collected by random from producer A located in Phitsanulok. All samples were packed in aseptic polyethylene bags and kept at 4°C in the laboratory until they were analysed. The acceptable criteria for the microbiological testings are according to the ICMSF as followed: n=5, c=0, m=0 for *Salmonella* spp.; n=5, c=0, m=0 for *S. aureus* and n=5, c=0, m=0 for *C. perfringens* where n is the number of unit samples, c is the number of sample that have a count more than m, and m is the critical limit count of microorganisms (ICMSF, 1986).

Sample preparation

For microbial enumeration and detection, salted egg was processed after shell disinfection. The egg shell was wiped with a sterile cotton wool swab moistened with sterilized water, then wiped with a cotton ball soaked with 70% ethanol, and finally it was sterilized by a quick passing over an opened flame. This procedure was performed to avoid contamination from germs colonizing the egg shells. After disinfection, the eggs were cracked with a sterilized surgical knife and their contents were dropped into a sterilized glass container. The salted eggs were cut using a clean sterilized knife to yield an appropriate weight.

For microbial enumeration, decimal dilutions were made in sterile peptone saline solution (0.1% peptone, 0.85 %NaCl). Appropriate dilutions for *Salmonella* spp., *S. aureus* and *C. perfringens* detection were performed according to The Compendium of Methods Evaluation Division of Canada (The Compendium of Methods Evaluation Division, 2005).

Microbiological analysis:

Total aerobic plate bacteria

Samples (10g) were homogenized in 90 ml sterile peptone saline using a stomacher. Further decimal dilutions were made with the same diluent. Duplicate counting plates were prepared using appropriate dilutions. For pour-plating, 1ml of the dilution was mixed with molten standard plate count agar (PCA). For spread-plating, 0.1 ml of the dilution was spread on the surface of a dried agar plate. After incubation at 35°C for 24-48 hr, the colonies appearing on the selected plates were counted and calculated as colony forming unit (cfu) per gram fresh weight of each sample (ICMSF, 1986).

Salmonella spp. Detection

Twenty-five grams of each salted egg sample were homogenized in 225ml of buffered peptone water, and incubated at 35°C for 24 hr. One milliliter was transferred to 9 ml of tetrathionate broth (TT) and to 9 ml of selenite cystine broth. The broths were incubated for 24 hr at 35°C and 43°C, respectively. After incubation, broths were streaked on xylose lysine deoxycholate (XLD) agar and hektoen enteric agar (HEA), and incubated at 35°C for 24 hr. Typical *Salmonella*-like colonies were inoculated in a triple sugar iron agar (TSI) and lysine iron agar (LIA). The agar plates were incubated at 35°C for 24 hr (The Compendium of Methods Evaluation Division, 2005).

S. aureus detection

Twenty-five grams of each salted egg sample were homogenized in 225 ml of buffered peptone water. One milliliter was transferred to 9 ml of peptone water. Aliquots of 10⁻¹ and 10⁻² dilutions (1 ml and 0.1ml) were poured and spread on manitol salted egg yolk agar (MSEY) respectively. After incubation at 30°C for 24-48 hr, typical *S. aureus*-like colonies were confirmed and identified by the test of coagulase, DNase test and acetylmethyl carbinol production test (The Compendium of Methods Evaluation Division, 2005).

C. perfringens detection

Twenty-five grams of each salted egg sample were homogenized in 225 ml of buffered peptone water. One milliliter of the homogenate was transferred to 9 ml of cooked meat medium overlaid with 5 ml of paraffin oil, after incubated at 35°C for 24 hr under the anaerobic condition. 1 ml of 10⁻¹ dilution was pour and 0.1 ml aliquot of cooked meat medium were spread on tryptose sulfite cycloserine containing egg yolk respectively and incubated at 35°C for 24 hr under anaerobic condition. *C. perfringens*-like colonies (black colony) were identified and confirmed by fermentation of lactose, gelatin liquefaction, motility and nitrate reduction (The Compendium of Methods Evaluation Division, 2005).

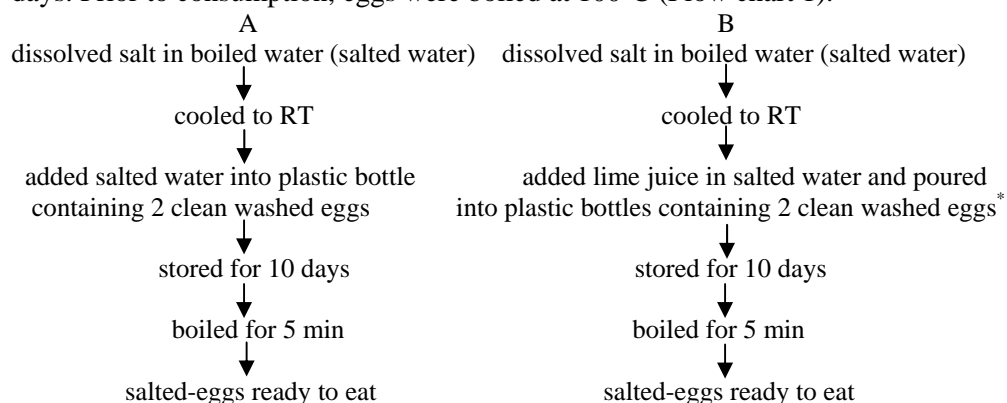
Physicochemical analysis

For pH determination, 10 g of sample were dissolved with 10 ml of distilled water, then the pH was measured using a pH meter (ORION 420A Scincetech Ltd.) (AOAC, 1995). Water activity was determined using NOVASINA, Aw-center200 (AOAC, 1995). %Salinity was determined using a salinity refractometer (ATAGO S/Mill). Acidity of lime juice was determine by titration with sodium hydroxide (0.1N) and expressed as % citric acid (Rangana, 1997).

S. aureus and *C. perfringens* in salted egg production

Salted egg production

The experiments were divided into two groups. Each group was performed in five replicates. In the control, the salted eggs were prepared by traditional- salted egg process. The treatment group prepared salted eggs by adding lime juice in salted water. The procedure to prepare salted water to make salted eggs was as followed: 50 g of NaCl were dissolved in 300 ml of water (14.86%NaCl), brought it to boil, and used as brining solution for the control group. The brining solution for the treated group was prepared as for the control except that 0.14 ml of lime juice 1.35% (v/v) citric acid equivalent was added. Brining solutions for the appropriate group were added into plastic bottles containing 2 eggs, then kept at room temperature for ten days. Prior to consumption, eggs were boiled at 100°C (Flow chart 1).



Flow chart 1 Preparation of salted eggs

A: salted eggs brined in salted water (control group)

B: salted eggs brined in salted water containing limejuice (treated group)

*control point, lime juice (1.35 % (v/v), citric acid equivalent)

Sample preparation

Five replicates of salted eggs brined in salt water (control group) and five replicates of salted egg brined in salted water containing lime juice (treated group) were taken from the final state of preparation and subsequently tested for *S. aureus* and *C. perfringens* detection, pH and a_w determination described previously.

Sensory evaluation

A portion of salted eggs from control and treatment groups was presented to the panelists at ambient temperature. The panelists (n=10) evaluated the salted eggs for their tastes (sour and salinity) , egg yolk colour and overall acceptability on a 9-point hedonic scale (9=like extremely, 8=like extreme, 7=like medium, 6= like little, 5= neither like nor dislike, 4=dislike little, 3=dislike medium, 2=dislike extreme, 1=dislike extremely). Sensory scores of sality taste, egg yolk colour, overall acceptability above 5.0 were assumed to be acceptable and sensory score of sour taste less than 5 was assumed to be acceptable. (Meilgaard *et al.*, 1998).

Statistical analysis

S. aureus and *C. perfringens* in salted egg production

The effect of lime juice in the brining solution was compared between the control group (n=5) and the treated group (n=5). Relation of the lime juice addition and un-detection of bacteria were evaluated by using Fisher exact test ($p<0.05$).

Sensory evaluation

pH and a_w difference of the salted eggs between control (n=5) and treated groups (n=5) were evaluated by using non-parametric statistics with 2-independent parameter. Significance was established at p-value less than 0.05.

Sensory difference between control and treated group were evaluated by panelists (n=10) using non-parametric models procedures with 2-dependent parameter. Significance was established at p -value less than 0.05.

RESULTS AND DISCUSSION

The results of microbiological quality of the salted eggs are presented in the Table 1.

Table 1 Microbiological quality of locally commercial salted eggs in the microbiological experiment

No. of sample	The detection			Counts (cfu/g)
	<i>Salmonella</i> spp. in 25 g sample	<i>S. aureus</i> in 0.1 g sample	<i>C. perfringens</i> in 0.1 g sample	Mesophilic aerobic bacteria
1	-	+	-	2.80 x 10 ³
2	-	+	+	3.60 x 10 ³
3	-	+	+	1.84 x 10 ²
4	-	+	+	2.60 x 10 ³
5	-	+	+	1.96 x 10 ²

+ : detected
 - : not detected

The Thai Industrial Standard Institute (TISI, 2003) stated that the microbiological standard of salted egg were that total aerobic plate count found is less than 10^4 cfu/g, and that there is no presence of *Salmonella* spp., *S. aureus* and *C. perfringens* in 25g, 0.1g, and 0.1 g of samples, respectively.

The two-class plan was used for the detection of *Salmonella* spp., *S. aureus* and *C. perfringens* due to the fact that these bacteria are moderated, direct, potentially extensive spread and there is no complete exclusion of these microorganisms from salted eggs even the most stringent criteria. Therefore, the absences of *Salmonella* spp., *S. aureus*, and *C. perfringens* in 25, 0.1g and 0.1g of samples, respectively, are considered acceptable (ICMSF, 1986; Henrik *et al.*, 1995).

The results showed that *Salmonella* spp. was not detected in all 5 samples, but *S. aureus* were detected in every sample. Four out of 5 samples were detected *C. perfringens*. Every sample had total aerobic count less than 10^4 cfu/g. According to the TISI standard, these salted eggs prepared using normal process were failed to pass the quality control.

The presence of these bacteria suggested that *S. aureus* and *C. perfringens* could enter the eggs through transovarian transmission, or probably during the procedure in salted egg production (Srikaro and Hourigan, 2001).

Moreover, at pH 6.70 ± 0.09 and A_w 0.921 ± 0.04 of the salted eggs as shown in Table 2, the conditions were suitable for the growth of *S. aureus* and *C. perfringens*. These bacteria could multiply to large number of vegetative cells (greater than 10^6 cfu/g) which once ingested could cause illness (Novak and Juneja, 2002; Oranussietal, 2003). As it appeared that the contamination was in the eggs and was taking place during processing, it is possible to control such contamination in egg farms or in salted egg manufacturing to lower levels of *S. aureus* and *C. perfringens* by adherence to good manufacturing practice (GMP) (Henrik *et al.*, 1996). However, this may not be practical in Thailand. Therefore, a simpler method needs to be developed to inhibit the growth of *S. aureus* and *C. perfringens* during the production salted eggs. Therefore, lime juice was introduced into the brining solution, and the results are presented in the Table 3.

The results of the physicochemical analyses of salted eggs (pH, a_w , %lime juice as citric acid equivalent) are presented in Table 2.

Our results showed that locally commercial salted eggs have pH around 6.70 ± 0.09 and a_w about 0.921 ± 0.04 which could be categorized as high moisture and neutral pH. Dehydration by salting can reduce the a_w values, however, such condition of a_w is still suitable for the growth of microorganisms (Banwart, 1989). Lime juice (0.14 ml) in 300 ml salted solution was assessed total titrable acidity by 0.1 N sodium hydroxide and calculated to be equivalent to 1.35% (v/v) citric acid.

Lime juice-added salted solution in treated brining solution could significantly reduced pH of the solution to 4.59 ± 0.24 in comparison to pH 8.18 ± 0.01 of the control ($p < 0.05$). The average of % NaCl of the control was 14.86 ± 0.02 , and that of the treated was 15.12 ± 0.46 , which is not significantly difference ($p > 0.05$) using non-parametric, 2-independent groups.

Table 2 The physical and chemical properties of salted egg between local and laboratory making

Properties	Salted eggs ^A	Salted eggs in the control group ^B	Salted eggs in the treated group ^C	Brining solution of the control group ^D	Brining solution of the treated group ^E
pH	6.70±0.09	6.66±0.12 ^a	5.36±0.15 ^b	8.18±0.01 ^a	4.59±0.24 ^b
a _w	0.921±0.04	0.925±0.05 ^a	0.923±0.02 ^a	-	-
% NaCl	-	-	-	14.86±0.02 ^a	15.12±0.46 ^a
% (v/v)	1.35	-	-	-	-
Lime juice as citric acid equivalent					

A : The means of pH& a_w from five samples collected from a locally commercial producer

B : The means of pH& a_w from five independent replicates of the control group

C : The means of pH& a_w from five independent replicates of the treated group

D : The means of pH, %NaCl from five independent replicates of brining solution in the control group

E : The means of pH, %NaCl from five independent replicates of brining solution in the treated group

Means in the same row with different letters are significantly different ($p < 0.05$).

Salted eggs brined in lime juice and salt had significantly lower pH (5.36±0.15) than salted eggs brined in salt alone (6.66±0.12) using non-parametric, 2-independent groups ($p < 0.05$), while the a_w values were not significantly different (0.923±0.02 and 0.925±0.05, respectively) using non-parametric, 2-independent groups ($p > 0.05$).

The results of effect of lime juice on the detection levels of *S. aureus* and *C. perfringens*

Table 3 Effect of lime juice on the detection levels of *S. aureus* and *C. perfringens*

Process step	Detection									
	<i>S. aureus</i>					<i>C. perfringens</i>				
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
control (salted solution)	+	+	+	+	+	-	+	+	+	+
treated (1.35%(v/v) citric acid equivalent in salted solution)	-	-	-	-	-	-	-	-	-	-

+ : detected

- : not - detected

All 5 salted eggs in the lime juice-treated brining solution showed no detectable level of *S. aureus*, whereas those in the control had detectable level of *S. aureus*. For the *C. perfringens* test, results showed that none of the eggs in the lime juice-treated brining solution had *C. perfringens*, but 4 out of 5 samples were positive for the control. These data implied that lime juice could significantly reduce the growth or survival rates of *S. aureus* and *C. perfringens* based on Fisher exact test ($p < 0.05$).

The results showed that lime juice (1.35 % (v/v) of citric acid equivalent) exhibited antimicrobial effect on *S. aureus* and *C. perfringens*. The acidic pH (4.59 ± 0.24) of the brining solution could cause membrane disruption, inhibited essential metabolic reactions and the accumulation of toxic anion resulted in the loss of their cell viability (Brul and Coote, 1999; Derrickson-Tharrington *et al.*, 2001; Sengun and Karapinar, 2004).

The drop of pH value of the brining solution to 4.59 ± 0.24 , when lime juice was added, was still within the minimal pH range (4.0) for the growth of *S. aureus* (Adams and Moss, 1995), however, less than the minimal pH range (5.0) for the growth of *C. perfringens* (Novak and Junija, 2002). *S. aureus* was highly sensitive to pH (Lanciotti *et al.*, 2001 and Sengun and Karapinar, 2004). The growth of *C. perfringens* can be inhibited by the decreasing pH (Kamra and Srivastawa, 1991). However, further quantitative antibacterial activity of lime juice should be investigated.

It should be emphasized that the brining process in making salted eggs should add lime juice (1.35% (v/v) citric acid equivalent) and measure the pH to 4.59 in order to eliminate *S. aureus* and *C. perfringens* in salted eggs.

Sensory properties

Sensory property test scores of salted eggs from brining solution containing only salt, and brining solution with salt and lime juice (1.35%(v/v) citric acid equivalent) are shown in Table 4.

Table 4 Comparison of sensory property scores of salted eggs from a brining solution containing only salt, versus the lime juice-added brining salted solution

sensory property *	salted egg type	
	brined in salt	brined in lime juice and salt
sour	3.40 ^a	4.30 ^b
salinity	6.10 ^a	5.20 ^b
egg yolk colour	5.10 ^a	4.10 ^b
overall acceptability	7.90 ^a	6.90 ^b

* : Hedonic scale, 9= like extremely, 8= like extreme, 7= like medium, 6= like little, 5 = neither like nor dislike, 4= dislike little, 3= dislike medium, 2= dislike extream, 1= dislike extremely. Values are the means of ten replicates (n=10). The means in the same row with different letter are significantly different ($p<0.05$).

The 9-point hedonic scale was used to evaluate the sensory quality of salted eggs in this study. The sensory scores for sour taste, salinity, egg yolk colour and overall acceptability between salted eggs brined in salt and salt with lime juice were significantly different that the p-value less than 0.05, using non-parametric, 2-dependent groups. However, the salinity and the overall acceptance of salted eggs brined in salt and lime juice were scored higher than 5.0, which are considered acceptable. The sensory scores for sour taste in salted egg brined in salt with lime juice were less than 5.0, considered acceptable, meaning that the product is not too sour. The sensory scores for egg yolk colour of salted egg brined in salt with lime juice were less than 5, which was not acceptable. To overcome the problem, the concentration of lime juice may need to be quantitating in order to achieve colour acceptance, and yet pass the safety tests. This result will benefit producers to produce acceptable salted eggs with high degree of safety with respect to food-borne pathogenic microorganisms.

CONCLUSIONS

The microbiological quality of salted eggs was assessed in the present survey. The results showed that normally processed salted eggs did not pass a microbiological quality standard. To a great extent, the safety quality of salted eggs can be credited to the use of 1.35%(v/v) lime juice with pH 4.59 in the brining process , in order to eliminate *S. aureus* and *C. perfringens* contaminations and also to maintain the safety quality. Concentration of lime juice used may need adjustment in order to achieve better scoring in all aspects in sensory test.

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