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Assessment of *Salmonella enteritidis* Viability in Egg White during Early Incubation Stages by Fluorescent Staining Method

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ABSTRACT

Egg white was considered to play an important role in bacterial-resistance nature of developing embryo especially during early incubation. Previous works were not quite informative in the antimicrobial action of egg white. This study was performed to investigate the survival ability of *Salmonella enteritidis* in egg white during early incubation and to tentatively reveal underline anti-*Salmonella enteritidis* mechanisms of egg white using the combination of flow cytometric analysis and fluorescent strategy. Such properties were also tested at different incubation temperature and pH. The traditional plate count method was also included in this study and the results were in accordance with those of flow cytometric assessment. Following egg white treatment, *Salmonella enteritidis* was discriminated into three subpopulations by flow cytometric assessment: viable, dead and membrane-compromised cells. Furthermore, the results showed that with the extension of incubation time, the population giving green fluorescence increased steadily while red fluorescence emittance didn't change a lot. The incubation temperature and pH were both significant factors in anti-*Salmonella enteritidis* properties of egg white. Besides, the main target for antimicrobial factors in egg white on *Salmonella enteritidis* cells was membrane integrity and the action mechanism of egg white was bacteriostatic rather than bactericidal effect during early incubation.

Key words: *Salmonella enteritidis*, viability, egg white, flow cytometry, incubation

INTRODUCTION

Egg white would provide immune protection to the developing embryo and is considered to be an unfavourable medium for microbial growth (Alabdeh *et al.*, 2011; Stevens, 1996; Tranter and Board, 1984); Lysozyme and ovotransferrin are well-characterized antimicrobials in egg white (Messens *et al.*, 2004). Besides, there are some other biologically active substances with antimicrobial properties in egg white such as cystatin (Jerzy and Pysniak, 2009; Trziszka *et al.*, 2004). Previous researches showed that dramatic changes in egg white would occur during egg incubation, such as pH, viscosity, stability of antimicrobial proteins (Araki *et al.*, 2000; Benton Jr *et al.*, 2001; Tona *et al.*, 2010). These changes in egg white might be important for immune protection to the developing embryo. However, it has been revealed that *Salmonella enteritidis* possessed a strong ability to survive and persist for a long period of time in egg white (Cox *et al.*, 1990; Manijeh *et al.*, 2008; Prakash *et al.*, 2005). Furthermore, researches

showed that eggs inoculated with high numbers of *Salmonella enteritidis* could still hatch (Cason *et al.*, 1994). These findings implicated that *Salmonella enteritidis* have the ability to transmit from generation to generation through eggs which would bring a series of hazards (Gast *et al.*, 2009). To our knowledge, little was known about the viability state and survival mechanism of *Salmonella enteritidis* in egg white during incubation up to now.

Application of multiple stains combined with Flow cytometric (FCM) techniques could provide valuable details about cell physiology and allow analysis of metabolic activity and membrane integrity (Barbesti *et al.*, 2000; Davey and Kell, 1996; Mandal *et al.*, 2011). It also has been proved to be useful for the assessment of viability state of bacteria. The viability state of *Salmonella typhimurium* in dairy products was evaluated by using fluorescent probe Fluorescein Isothiocyanate (FITC) and ethidium bromide (McClelland and Pinder, 1994). In this study, we conducted a rapid assessment of *Salmonella enteritidis* based on FCM detection with a double-staining strategy using carboxyfluorescein diacetate (cFDA) and Propidium Iodide (PI). cFDA is a non-fluorescent dye which would be converted into a fluorescent compound, carboxyfluorescein (cF) by cellular unspecific esterases (Petit *et al.*, 1993). However, the complete outer membrane of gram-negative bacteria has been proved to hinder the entering of viability marker cFDA into the cell (Vaara, 1992). Thus, the positive reaction of cFDA indicates a membrane compromised state of gram-negative bacteria. In contrary, the negative reaction indicates a viable (membrane integrity) state. As to PI, it is a nucleotide-binding probe which was considered not to penetrate and stain cells unless membrane integrity didn't exist (Ananta *et al.*, 2004) and frequently used to indicate death of bacteria. The aim of this study was to illustrate physiological state of *Salmonella enteritidis* cells after exposure to egg white during early incubation by using a rapid assessment based on flow cytometric analysis in combination with double-staining of cFDA and PI.

MATERIALS AND METHODS

Preparation of cell suspension: Three milliliters of original *Salmonella enteritidis*(ATCC 13076) cell suspension in Tryptic Soy Broth (TSB) and one milliliter of sterilized glycerol were mixed and then stored at -80°C until use. It was streaked onto Tryptic Soy Agar (TSA) and then cultured at 37°C for 24 h after thawing at room temperature. As soon as the strain was resuscitated, a loop of SE from TSA was transferred to 100 mL of TSB to grow overnight at 37°C. The harvested bacteria cells were centrifugated at 8000 rpm for 5 min at 4°C. After washed 3 times by 50 mM phosphate buffered saline, pH 7.4 (PBS), the *Salmonella enteritidis* was adjusted to an OD490 of 1.5 (ca. 1.8×10^{10} CFU mL⁻¹). The suspension was then used for inoculating.

Egg white inoculating: Fresh fertilized eggs from Single Comb White Leghorn laid within 24 h were collected from the poultry research centre farm of Huazhong Agricultural University and used in this study. Eggs were incubated at 38°C and 65-75% relative humidity in a forced air incubator for 0, 1, 2, 3 days (Kamanli *et al.*, 2010; Alabi *et al.*, 2012; Seker *et al.*, 2004). Investigations were carried out in daily intervals. At each experimental time, ten eggs were disinfected by immersion in 75% ethanol and under aseptic conditions, they were dried, cracked, and the egg white was collected into a sterile container and thoroughly mixed. Two milliliter of inoculum was injected into 18 mL of albumen samples and then incubated at 38°C for 8 h. To study the effect of incubation temperature on *Salmonella enteritidis* viability in egg white, fresh fertilized egg white inoculated with *Salmonella enteritidis* was incubated for 8 h at 4, 25 and 38°C (King'ori, 2011). The pH of fresh fertilized egg white (pH 9.16) was adjusted to 7.0 and 9.56 by using NaOH

and HCl solution to research in the changes of pH of egg white on *Salmonella enteritidis* viability during early incubation stages (Yesilhk *et al.*, 2011; Wagdy *et al.*, 2011). After double staining with cFDA and PI, all the samples were assessed on a flow cytometer.

Fluorescence labeling: Carboxyfluorescein diacetate (cFDA, Beyotime Institute of Biotechnology, China) and propidium iodide (PI, Beyotime Institute of Biotechnology, China) were used in this study to determine the esterase activity and membrane integrity of *Salmonella enteritidis* cells treated by egg white. Untreated controls and egg white treated samples were filtrated by 8 μm cellulose acetate filter. After that, samples were centrifugated at 8000 rpm for 5 min at 4°C which was followed by washing and resuspension. An appropriate volume of cFDA was added to produce a target concentration of 2 $\mu\text{L mL}^{-1}$ cell suspension. The cells were incubated for 20 min at 37°C and then excessive cFDA was removed by centrifugation before PI staining. The concentration for PI staining was 2 $\mu\text{L mL}^{-1}$ cell suspension. The cells were incubated in ice bath for 10 min before FCM analysis.

Observation with fluorescence microscopy: Stained cells were imaged on Olympus BX51 microscope equipped with a OLYMPUS DP71 CCD (OLYMPUS, Japan). Aliquots of 10 μL of each cell suspension were transferred to a clean glass slide under coverslip. All of the slides were one by one put on the microscopy. CFDA-stained and PI-stained cells were visualized by excitation with blue light (400-440 nm) and UV light (330-385 nm). In addition, *Salmonella enteritidis* controls added glutaraldehyde with a final concentration of 10 g L^{-1} cell suspension were also included in this observation.

Flow cytometric measurement: The stained *Salmonella enteritidis* cells were analyzed with a BD FACSCalibur (America) flow cytometer equipped with a 15 mW, 488 nm air cooled laser. The flow rate in this experiment was set at 600-800 events per second, up to a total sum of 50000 events per sample. The green fluorescence of cF-stained cells was collected in the FL1 channel (525 \pm 20 nm) and red fluorescence of PI-labelled was collected in the FL2 channel (620 \pm 15 nm).

Bacterial plate counts: Enumeration of *Salmonella enteritidis* in egg white was carried out by serial decimal dilution of cell suspensions using 9 mL of sterile PBS. Afterwards, 250 μL portions of the appropriate dilutions of controls and treated samples were added on TSA plates and incubated for 48 h at 37°C.

RESULTS AND DISCUSSION

Basic staining behavior of *Salmonella enteritidis*: The photographs taken with fluorescence microscopy displayed that viable *Salmonella enteritidis* cells unstained or stained with cFDA could hardly emit any fluorescence (Fig. 1a, b). While membrane damage induced by 10 g L^{-1} Glutaraldehyde (GTA) would promote cFDA staining on *Salmonella enteritidis* (Fig. 1c). It was obvious that heat-killed (70°C, 30 min) *Salmonella enteritidis* cells without viability were prone to be labelled by PI and to give a detectable red fluorescence (Fig. 1d). Both green and red fluorescence can be detected in *Salmonella enteritidis* cells after egg albumen treatment following with double staining (Fig. 1e) which indicated that *Salmonella enteritidis* in egg white can be divided into different subpopulations: viable, dead and membrane-compromised.

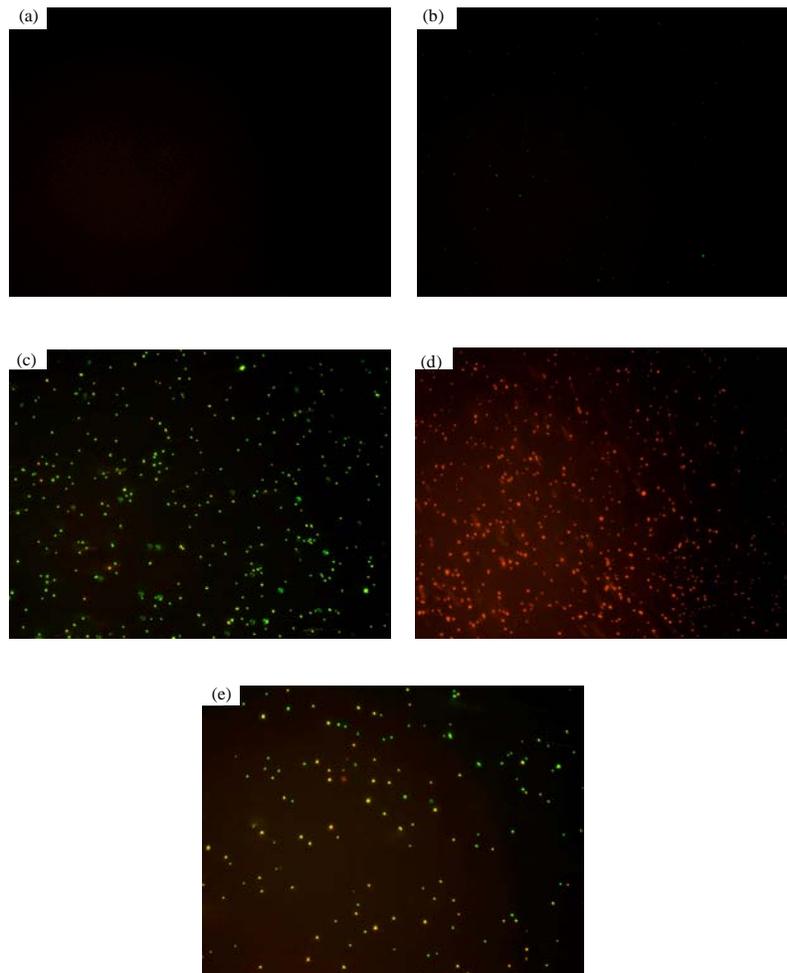


Fig. 1(a-e): Fluorescent microscopy images of viable *Salmonella enteritidis* (a) unstained, (b) stained solely with cFDA, (c) GTA treated *Salmonella enteritidis* stained with cFDA, (d) heat-damaged (70°C, 30 min) *Salmonella enteritidis* stained with PI and (e) egg white treated *Salmonella enteritidis* double-stained with cFDA and PI

To differentiate bacterial population based on their fluorescence properties as well as to assess treatment effect on *Salmonella enteritidis*, the dual-parameter density plot of the green fluorescence (x-axis) and the red fluorescence (y-axis) was used (Ananta *et al.*, 2005). Fluorescence density plots of viable *Salmonella enteritidis* cells and heat-treated cells following staining with cFDA and PI were shown in Fig. 2. The results showed that viable cells that could be stained by cFDA were only 2-3% of total sum (Fig. 2a, in quadrants 2, 4) which was in agreement with the report that cFDA can not permeate into the outer membrane of gram-negative bacteria (Vaara, 1992), 96.3% of *Salmonella enteritidis* cells were stained with PI after heat treatment for 30 min at 70°C (Fig. 2b, in quadrant 1). Double staining treatment of viable *Salmonella enteritidis* showed that most of the cell particles are distributed in quadrant 3 (Fig. 2c).

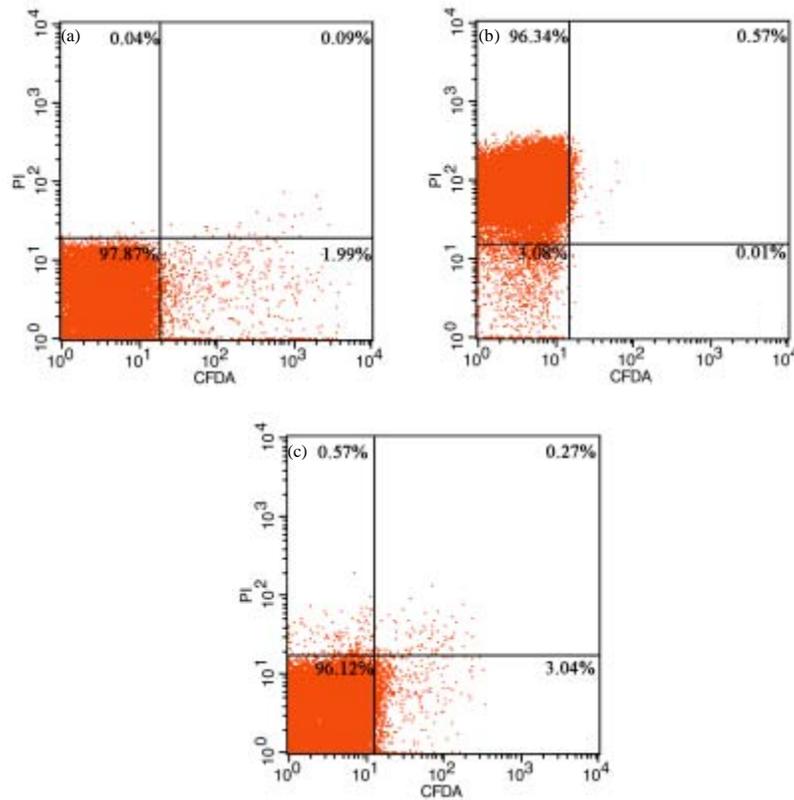


Fig. 2(a-c): (a) Fluorescence density plots of intact *Salmonella enteritidis* cells solely stained with cFDA, (b) heat-treated cells stained with PI and (c) intact *Salmonella enteritidis* cells double stained with cFDA and PI. Heat treatment at 70°C was performed to yield dead, membrane-compromised cells. The figures (in %) in different quadrant are associated with the percentage of the cells in the corresponding quadrant

Changes of viability of *Salmonella enteritidis* in egg white during early incubation:

Figure 3a-d showed fluorescence properties of *Salmonella enteritidis* in egg white collected from the early incubation stages. The population accumulating cF (in quadrant 2 and 4) increased steadily from about 46 to 96% with the extension of incubation time, suggesting that albumen with longer incubation time facilitated penetration of cFDA and its transition into cF by intracellular esterase. It was generally considered that the ability of gram-positive bacteria to accumulate and retain cF was an indicator of metabolic activity because the conversion was dependent on active esterase. However, gram-negative bacteria had a thin cell wall surrounded by an outer membrane as a selective permeability barrier that could limit the passage of certain substances (Kim *et al.*, 2007). Thus, gram-negative bacteria showed a deviating behaviour in their fluorescence property. Only when their special structure of outer membrane was damaged, cFDA could enter into the cells to interact with the esterase and to yield green fluorescence. From this point of view, staining cells of gram-negative bacteria with cFDA would not show green fluorescence unless membrane injury and esterase activity simultaneously exist. Changes of fluorescence property of *Salmonella enteritidis* in egg white indicated that longer incubation time could result in more serious membrane injury.

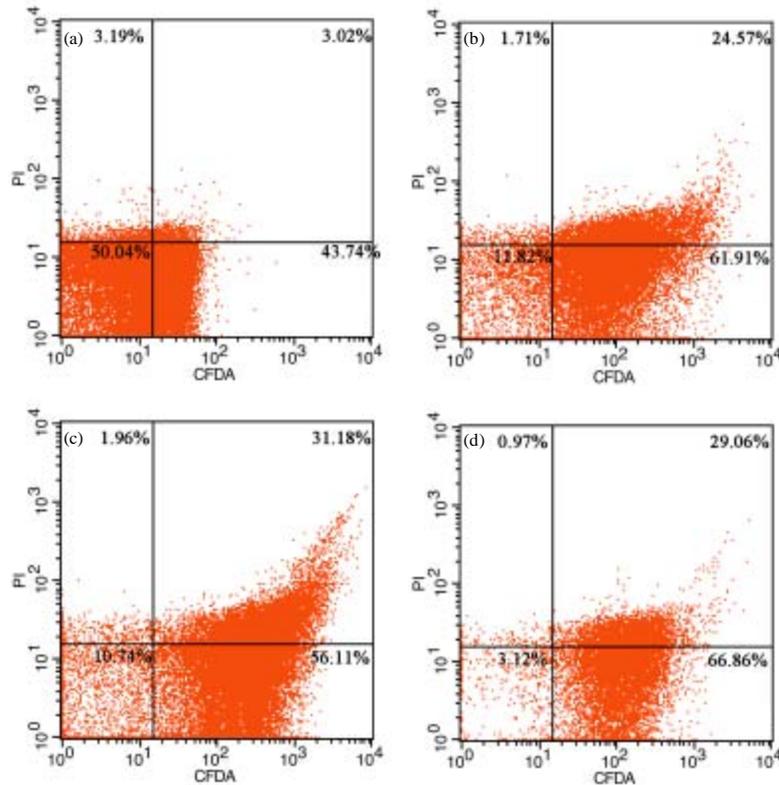


Fig. 3(a-d): Fluorescence density plots of *Salmonella enteritidis* cells treated by egg white collected from fertilized eggs incubated for (a) 0 day, (b) 1 day, (c) 2 day and (d) 3 day. The figures (in %) in different quadrant are associated with the percentage of the cells in the corresponding quadrant

The results of traditional plate count method also showed that anti-*Salmonella enteritidis* activity of egg white was improved as incubation progressed during the early embryonic development (Fig. 6a). Compared the results of flow cytometry with plate counts, it can be inferred that some cells of bacteria with membrane ruptured (in quadrant 4) in egg white could regrow on agar. In another side, the loss of membrane integrity was at least related to the loss of viability of *Salmonella enteritidis*. However, the percentages of *Salmonella enteritidis* cells labelled by PI in quadrant 1 didn't change a lot and all were considerably low (Fig. 3a-d). These findings led to a presumption that the major part of *Salmonella enteritidis* cells treated by egg albumen during the first 3 days of incubation was not dead, indicating the bactericidal activities of egg albumen during early incubation were so limited that they could not reach directly to the death of bacteria cells.

Effect of incubation temperature on *Salmonella enteritidis* viability in egg white: Eggs are incubated at hens' body temperature (38°C), so, we conducted a series of experiments so as to figure out the effect of temperature on the anti-*Salmonella enteritidis* properties of egg white. The viability status of *Salmonella enteritidis* cells treated by egg white at different temperature was

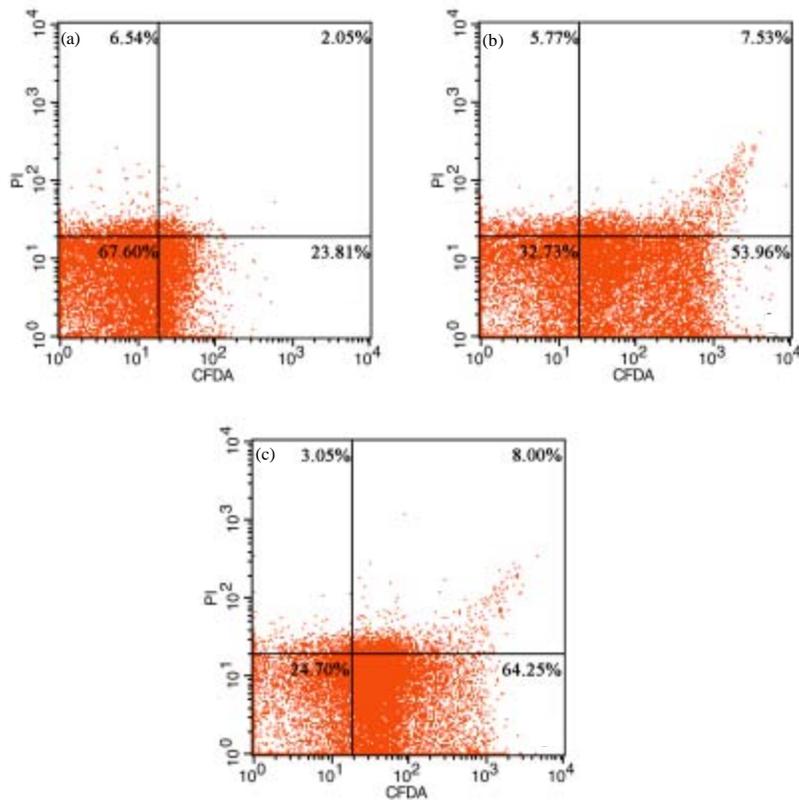


Fig. 4(a-c): Fluorescence density plots of *Salmonella enteritidis* cells inoculated in egg white following incubated at (a) 4°C, (b) 25°C and (c) 38°C for 8 h. The figures (in %) in different quadrant are associated with the percentage of the cells in the corresponding quadrant

depicted in Fig. 4a-c. Percentage of cFDA stained cells was 25.86% at 4°C, 61.49% at 25°C and 72.25% at 38°C of total sum of cells. We selected these three temperatures in this study in order to represent typical refrigeration temperature, room temperature and incubation temperature, respectively. The result suggested that egg white incubated at 38°C (typical incubation temperature) was most able to facilitate the penetration and conversion of cFDA. So, egg white incubated at 38°C possessed the strongest ability to prevent *Salmonella enteritidis* growth. The result was in accordance with that obtained from the traditional plate count method (Fig. 6b). Researches have demonstrated that egg white at high temperature kept higher bacteriostatic properties than at low temperature (Aykes and Taylor, 1956). Avian incubation would provide additional protection through increasing the temperature of the egg white to levels at which its antimicrobial enzymes work optimally (Tranter and Board, 1984) and that exceed the optimum for growth of most microorganisms (Cook *et al.*, 2005). Previous reports have also discovered that there were nuclease activities inside egg albumen which could lacerate both naked and intracellular bacterial DNA (Lu *et al.*, 2003). The nuclease activities were likely to be dependent on temperature, showing higher at 37°C and lower at 4 and 25°C. Based on this study, it was confirmed that the

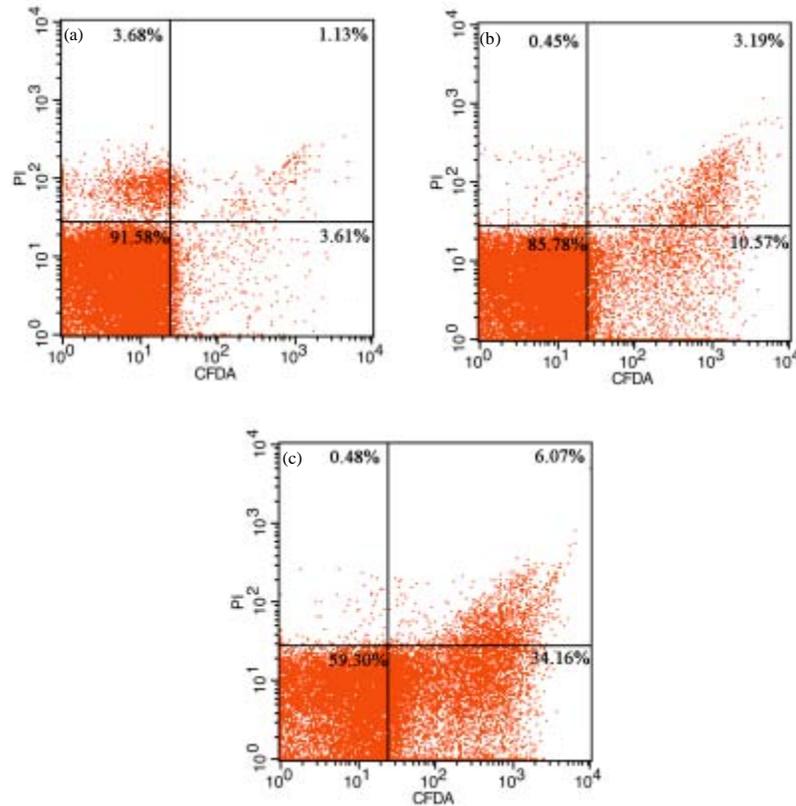


Fig. 5(a-c): Fluorescence density plots of *Salmonella enteritidis* cells inoculated in pH adjusted egg white: (a) 7.0, (b) 9.16 and (c) 9.5. The figures (in %) in different quadrants are associated with the percentage of the cells in the corresponding quadrant

antimicrobial activity of egg white was increased under the incubation conditions. And the anti-*Salmonella enteritidis* mechanism of egg white during early incubation would be cell membrane damage.

Effect of rising of pH during early incubation on *Salmonella enteritidis* viability in egg white: The pH of chicken egg white has been proved to be an important antibacterial defence factor in preventing bacteria growth. During early incubation stages, the albumen pH increased rapidly as a result of sudden mounting up of internal temperature which in turn accelerated CO₂ loss from egg white. In this study, it was evaluated the effect of changes in pH during early incubation on the viability state of *Salmonella enteritidis* in egg white. The pH of fresh egg white used in this study was 9.16. After two days of incubation, the pH of egg white would rise to as high as 9.56 (data not shown). Thus, pH of egg white samples was adjusted by acid and alkali solutions to 7.0, 9.16 and 9.56 to study the effect of pH on *Salmonella enteritidis* viability in egg white during incubation. The result showed that cells in quadrant 2 and 4 were 13.76% at pH 9.16, 40.23% at pH 9.56 and lower than 5% at pH 7.0 (Fig. 5a-c). It can be seen that the anti-*Salmonella enteritidis* activity was increased considerably due to the increase of pH. The survival rate of *Salmonella enteritidis* in response to egg white with different pH confirmed the result of

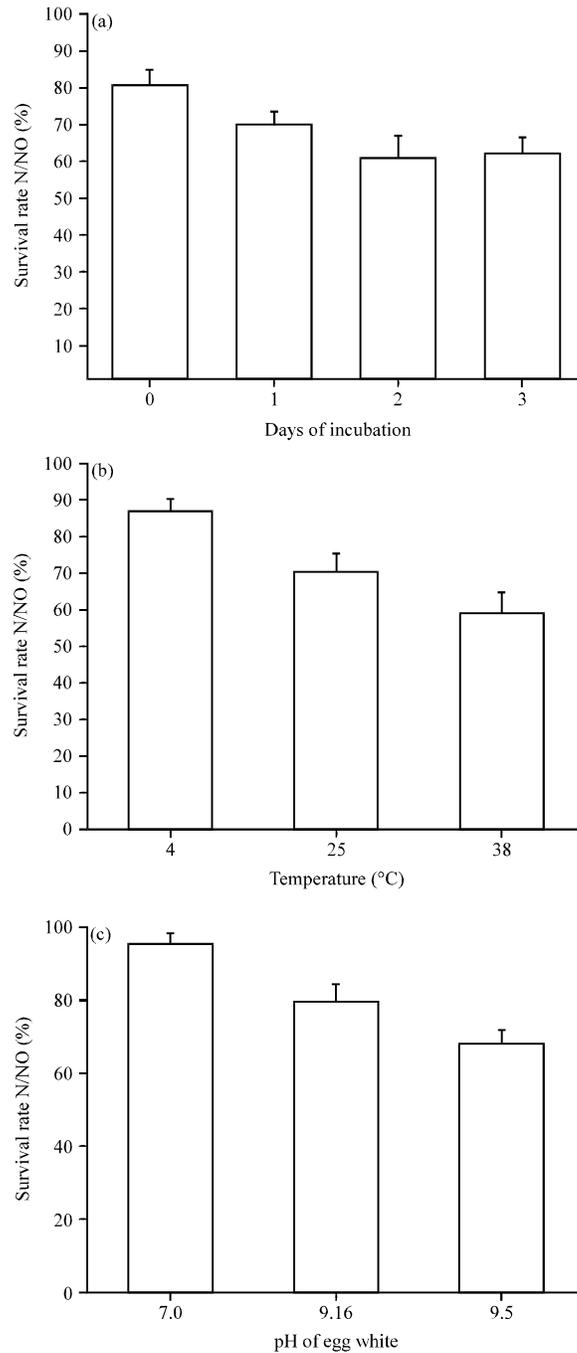


Fig. 6(a-c): Effect of different incubation period (a), incubation temperature (b) and pH during incubation (c), on survival rate of *Salmonella enteritidis* cells in egg white. Data are the mean of three replicates of egg white treatment. Error bars indicate the standard deviations of the mean

assessment by flow cytometry (Fig. 6c). Previous research has shown that egg white was much more toxic to the bacteria at pH 9-10 rather than pH 6-8 (Sharp and Whitaker,

1927). That was probably because high alkalinity of egg white harassed bacterial iron metabolism which prevented bacteria from gaining enough iron for their growth (Tranter and Board, 1984).

CONCLUSION

Based on double-staining strategy by using cFDA and PI, it was found that *Salmonella enteritidis* in egg white could be differentiated into three subpopulations: viable, dead and membrane-compromised cells. As incubation progressed, the viability state of *Salmonella enteritidis* in egg white would change. The main target for antimicrobial factors in egg white was membrane integrity. Moreover, the flow cytometric assessment allowed us to monitor that bacteriostatic properties were much stronger and more obvious than bactericidal activities of egg white during early incubation. It was generally believed that many factors synergistically induce the antimicrobial properties of egg white during early incubation. Based on the data from this study, we could reasonably consider that temperature and pH play a significant role in controlling *Salmonella enteritidis* growth in egg white.

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