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Effect of egg washing and correlation between cuticle and egg penetration by various *Salmonella* strains

Vaibhav C. Gole [a], Juliet R. Roberts [b], Margaret Sexton [c], Damian May [d], Andreas Kiermeier [d], Kapil K. Chousalkar [a,*]

[a] The University of Adelaide, SA, Australia
[b] The University of New England, NSW, Australia
[c] Primary Industries and Regions South Australia, SA, Australia
[d] South Australian Research and Development Institute, SA, Australia

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ABSTRACT

In Australia, Europe and the United States, eggs and egg products are frequently associated with *Salmonella* food poisoning outbreaks. Many of the egg-associated *Salmonella* outbreaks have been due to the products such as mayonnaise, ice-cream and cold desserts which are eaten without cooking following the addition of raw egg. The ability of four *Salmonella* isolates (one each of *S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone) to penetrate washed and unwashed eggs using whole egg and agar egg penetration methods was investigated in the current study. The results of the agar penetration experiment indicated that all the isolates used in the present study have the capacity to penetrate the eggshell. Eggshell penetration by the *S.* Worthington isolate was higher but not significant ($p = 0.06$) in washed eggs compared to unwashed eggs. However, for all other isolates (*S.* Singapore, *S.* Adelaide and *S.* Livingstone), there was no significant difference in penetration of washed and unwashed eggs. Statistical analysis indicated that cuticle score was a significant linear predictor of *Salmonella* eggshell penetration. Whole egg penetration results showed that all of the *Salmonella* isolates used in the present study were capable of surviving on the eggshell surface after 21 days of incubation (at 20 °C) following a high dose of inoculation (10^5 CFU/mL). The combined data of all isolates demonstrated that, the survival rate of *Salmonella* on eggshells (inoculated with 10^5 CFU/mL) was significantly higher ($p = 0.002$) at 20 °C as compared to 37 °C. *S.* Singapore, *S.* Worthington, and *S.* Livingstone were not detected in egg internal contents whereas *S.* Adelaide was detected in one egg's internal contents.

1. Introduction

Salmonellosis, one of the most important foodborne diseases worldwide, is caused by the Gram-negative bacteria of genus *Salmonella*. Infection is characterized by acute onset of fever, abdominal pain, diarrhoea, nausea and sometimes vomiting with incubation period of 6–72 h (World Health Organisation, 2013). In Australia, Europe and the United States, eggs and egg products are frequently associated with *Salmonella* food poisoning outbreaks (The OzFoodNet Working Group, 2012; Braden, 2006; European Food Safety Authority, 2012). Many of the egg-associated *Salmonella* outbreaks have been due to the consumption of contaminated products such as mayonnaise, ice-cream and cold desserts which are prepared or consumed without cooking after addition of raw egg (The OzFoodNet Working Group, 2012). Vertical transmission and horizontal transmission are possible routes by which *Salmonella* can contaminate intact eggs (Messens et al., 2005a). Egg contamination by horizontal transmission occurs when *Salmonella* penetrates the eggshell during or following oviposition, contaminating the internal contents (Miyamoto et al., 1998). For salmonellae other than *S.* Enteritidis, horizontal transmission is the most common route for the contamination of egg internal contents (Humphrey, 1994).

Contact between the eggshell and faeces is difficult to avoid completely. The extent of faecal contamination of the eggshell and the level of *Salmonella* shedding in faeces determines the level of eggshell contamination (Gast and Beard, 1990; De Louvois, 1993). Externally contaminated eggshells pose the risk of egg internal content contamination through horizontal transmission as well as the cross contamination of other food items in the kitchen. Epidemiological investigation from *Salmonella* food poisoning outbreaks in Queensland revealed that the use of dirty and cracked eggs in the restaurants was the major source of bacteria in these outbreaks (Slinko et al., 2009). One way to control such outbreaks is egg washing which reduces the microbial load on the eggshell surface, limiting the chances of contamination of egg internal contents as well as cross contamination of other food items. Egg washing is common practise in the United States, Australia and Japan

* Corresponding author at: School of Animal and Veterinary Science, The University of Adelaide, Roseworthy, 5371, SA, Australia. Tel.: +61 8 8313 1502; fax: +61 8 8303 7356.
E-mail address: kapil.chousalkar@adelaide.edu.au (K.K. Chousalkar).

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(Hutchison et al., 2004). There are, however, some detrimental outcomes associated with egg washing such as damage to the physical barriers of the egg, especially the cuticle (European Food Safety Authority, 2005) which is the first line of defence against bacterial penetration (Board and Halls, 1973). Other factors such as shell porosity and thickness can also affect egg penetration by bacteria (Messens et al., 2005a). However, these findings were not confirmed by the following experiments as it was observed that eggshell penetration by *Salmonella* was independent of shell porosity and thickness (Messens et al., 2005b; De Reu et al., 2006). Increased eggshell translucency has also been associated with greater microbial penetration (Chousalkar et al., 2010). There is, however, a lack of research investigating the relationship between translucency and bacterial penetration.

Although *S.* Typhimurium is the most frequently isolated *Salmonella* serovar from egg products related to food poisoning cases in Australia (The OzFoodNet Working Group, 2012), other *Salmonella* serovars such as *S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone have been frequently isolated from Australian layer farms (Cox et al., 2002; NSW Food Authority, 2012; Gole et al., 2013a). However, egg related food poisoning outbreaks with these serovars have not been reported. In India, *S.* Worthington has been reported as the most frequently isolated non-typhoidal *Salmonella* serovar from human cases (Kumar et al., 2009). In 2004, an outbreak of *S.* Singapore associated with eating sushi was reported in Queensland, Australia however, the source of contamination was not clear (Barralet et al., 2004). In Norway and Sweden, during 2001, an outbreak of *S.* Livingstone, due to contaminated processed fish products, claimed three deaths and 22 hospitalizations (Guerin et al., 2004). The majority of egg penetration studies have, however, investigated the penetration ability of either *S.* Enteritidis or *S.* Typhimurium. To date, little attention has been given to other *Salmonella* isolates such as *S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone.

The objectives of this study were to examine the effect of egg washing on the survival of *Salmonella* isolates on the eggshell surface, to investigate the eggshell penetration ability along with the survival of different *Salmonella* isolates in egg internal content and to study the effect of egg washing and translucency on the penetration of eggshell by various *Salmonella* isolates. The effect of egg washing on cuticle ultrastructure and the relationship between cuticle quality and bacterial penetration were also investigated.

2. Materials and methods

All the *Salmonella* strains (*S.* Singapore 709750, *S.* Adelaide 709043, *S.* Worthington 703775 and *S.* Livingstone 709041) used in this study were isolated from the Australian layer flocks. The strain *S.* Worthington was isolated from egg shell wash by our laboratory whereas the other isolates were obtained from the Australian *Salmonella* Reference Centre (Adelaide, Australia).

2.1. Egg washing

Fresh, visibly clean eggs were collected from hens (40 weeks old) at a HyLine layer farm in South Australia. Before egg washing, all eggs were candled to identify cracks in eggshells. Egg washing processes used in this study involved washing with the aid of a surfactant followed by sanitization and drying. A laboratory based washer with the capacity of 15 eggs at a time (three rows of five rotating rollers) was used for the physical mechanics of the egg washing. Washing was performed using a hydroxide and hypochlorite based solution at the concentration of 0.45% (v/v) equivalent to a pH of ~12 and ~200 ppm hypochlorite in the working solution at 40 °C. Washing was followed by a compatible sanitizer (at a concentration of 0.16% (v/v)) which equated to ~200 ppm hypochlorite in the working solution at 32 °C. Eggs were washed and sanitized for 46 and 22 s, respectively. The pressure of the spray was 3 psi without brushes. Eggs were left on the bench for 15 min to dry and used for further experiments.

2.2. Inoculum preparation

The *Salmonella* isolates used in these experiments (*S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone) were stored at −80 °C in 80% glycerol. Bacteria were recovered from freezing by plating on xylose lysine deoxycholate (XLD) agar (Oxoid, Australia) and incubated overnight at 37 °C. Colonies were selected from XLD agar and resuspended in phosphate buffered saline (PBS) to match the turbidity equivalent with a 0.5 McFarland standard (BioMerieux, Australia). Enumeration of viable bacteria was performed by serial dilution and spread plating on XLD agar and incubation overnight at 37 °C. Following enumeration, a 200 mL inoculum containing 10^3 and 10^5 colony forming units (CFU) per mL was prepared for each isolate. Agar-filled eggs and whole eggs were immersed for 90 s in one of three dilutions: PBS (control), ~10^3, and ~10^5 CFU/mL of *Salmonella*.

2.3. Whole egg penetration experiment

The effects of egg washing on the survival of *Salmonella* isolates on the eggshell surface and penetration across the eggshell, as well as the survival of *Salmonella* isolates in the internal contents of the egg, were investigated using a 'whole egg penetration' approach. Ninety eggs were collected from HyLine Brown hens in early lay and were divided into two groups: washed (n = 30) and unwashed (n = 60). Washed eggs were divided into one control (PBS) and two treatment groups (10^3 and 10^5 CFU/mL) with 10 eggs each. All the washed eggs were incubated at 20 °C after exposure to *Salmonella* or the control PBS treatment. Unwashed eggs were divided into two groups of 30 eggs. Group 1 was further divided into one control and two treatment groups (10^3 and 10^5 CFU/mL) of 10 eggs each. Eggs from group 1 were incubated at 20 °C after exposure to *Salmonella* or the control PBS treatment. Group 2 was also divided into one control and two treatment groups (10^3 and 10^5 CFU/mL) of 10 eggs each. These unwashed eggs were incubated at 37 °C. The reason that only unwashed eggs were incubated at 37 °C is that washed eggs are not used for hatching purposes in Australia. Each egg was dipped into 70% ethanol for 30 s to sterilize the outer shell and allowed to air dry in a biosafety cabinet for 10–15 min. Eggs were then immersed for 90 s in 10^3 CFU/mL or 10^5 CFU/mL of *Salmonella*. After inoculation, eggs were incubated at 20 °C or 37 °C for 21 days.

2.3.1. Isolation of Salmonella from eggshell surface and egg internal contents from whole egg penetration experiment

Eggshell surface samples and egg internal content samples were processed separately by pooling two eggs together. After incubation at 20 °C or 37 °C for 21 days, each pair of eggs was placed in a Whirl-Pak bag (Nasco, USA) containing 20 mL of buffered peptone water (BPW; Oxoid, Australia) and each egg was massaged for 1 min. A 100 μL aliquot of the mixture was spread plated onto XLD plates, incubated overnight at 37 °C and subsequently quantified. The limit of detection for isolation of *Salmonella* from eggshell surface and egg internal contents was 2 CFU/100 μL.

To investigate the penetration and survival of *Salmonella* in the contents of the egg, after the eggshell wash, eggs were dipped in 70% ethanol for 30 s. Eggs were then aseptically opened, emptied into the Whirl-Pak bags and mixed. A 2 mL aliquot of the contents was transferred to 8 mL of BPW and 100 μL of this mixture was plated on XLD agar and incubated overnight at 37 °C. Plates were then observed for *Salmonella* growth. Slopes of suspected *Salmonella* isolates were sent to the Institute of Medical and Veterinary Sciences (IMVS), Adelaide, Australia for confirmation.