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# Fate of mesophilic aerobic bacteria and *Salmonella enterica* on the surface of eggs as affected by chicken feces, storage temperature, and relative humidity

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ABSTRACT

We compared the microbiological quality of chicken eggshells obtained from a traditional wholesale market and a modern supermarket. We also determined the survival and growth characteristics of naturally occurring mesophilic aerobic bacteria (MAB) and artificially inoculated *Salmonella enterica* on eggshells under various environmental conditions (presence of chicken feces, temperature [4, 12, or 25 °C], and relative humidity [RH; 43 or 85%]). The populations of MAB, coliforms, and molds and yeasts on eggshells purchased from a traditional wholesale market were significantly ($P \leq 0.05$) higher than those from a modern supermarket. In the second study, when we stored uninoculated eggs under various storage conditions, the population of MAB on eggshells (4.7–4.9 log CFU/egg) remained constant for 21 days, regardless of storage conditions. However, when eggshells were inoculated with *S. enterica* and stored under the same conditions, populations of the pathogen decreased significantly ($P \leq 0.05$) under all tested conditions. Survival of *S. enterica* increased significantly ($P \leq 0.05$) in the presence of feces, at low temperatures, and at low RH. These observations will be of value when predicting the behavior of microorganisms on eggshells and selecting storage conditions that reduce the populations of *S. enterica* on eggshells during distribution.



## 1. Introduction

*Salmonella* is one of the most common foodborne pathogens (CDC, 2014; HPA, 2012; KMFDS, 2014a) and causes a disease characterized by fever, abdominal pain, diarrhea, nausea, and sometimes vomiting (WHO, 2013). Based on estimates of the number of foodborne illnesses in the United States, nontyphoidal *Salmonella* is the second most common pathogen, causing 11% of foodborne illnesses, following norovirus, and is the leading cause of hospitalization (35%) and death (28%) (Scallan et al., 2011). Outbreaks of *Salmonella* infections are associated with consumption of various foods, including poultry, red meats, pork, vegetables, and fruits that can be contaminated with the pathogen. Eggs are the most common single food associated with outbreaks of salmonellosis (CDC, 2006; EFSA, 2009; Jackson et al., 2013; Moffatt and Musto, 2013). A multi-state outbreak of 1939 cases of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) infection occurred in 11 states of the United States in 2010, and eggshells were suspected as the source of infection (CDC, 2010). Organic shell eggs were recalled by the U.S. Food and Drug Administration due to contamination with *S.* Enteritidis (FDA, 2014). At least six people were infected.

Eggs have high nutritional value and provide high-quality proteins as well as fatty acids, iron, phosphorus, minerals, and vitamins (FAO, 2003; Seuss-Baum, 2007). Per capita consumption of eggs is increasing (FAOSTAT, 2014); thus, outbreaks associated with eggs may also become more common. Generally, there are two possible routes of contamination of eggs with *Salmonella*. One is through colonization of *Salmonella* in the reproductive organs of the hen.

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Colonized *Salmonella* spreads into the yolk, yolk membranes, albumen, shell membrane, and on the surface of eggshells before oviposition (vertical transmission) (Keller et al., 1995; Miyamoto et al., 1997; Shivaprasad et al., 1990). Another possible route is when eggshells are exposed to feces containing *Salmonella* or a contaminated environment, such as a nest or hatchery. *Salmonella* on the eggshell penetrates into the egg contents during or after oviposition (horizontal transmission) (De Reu et al., 2006; Gantois et al., 2009; Miyamoto et al., 1998). When eggshells are contaminated by horizontal transmission, chicken feces and other moist organic materials can protect *Salmonella* against inactivation and promote growth by providing nutrients (Gantois et al., 2009; Howard et al., 2012). The predominant route of egg contamination remains unclear but Wang and Slavik (1998) demonstrated that horizontal transmission appears to play a major role.

Governmental public health regulations for cleaning and storing eggs vary among countries. In the United States, commercially available eggs must be washed using sanitizers meeting certain standards and must be distributed at refrigeration temperature (less than 45 °F [7.2 °C]) (USDA, 2011). In contrast, in the European Union, washing eggs is not permitted and eggs are not refrigerated (European Commission, 2008). In the Republic of Korea, egg washing and conditions for the distribution are not critically regulated by the Korean Ministry of Food and Drug Safety (KMFDS, 2014b), although it is recommended that eggs be distributed at low temperatures (0–15 °C). In reality, eggs washed with water and unwashed eggs are distributed at ambient as well as refrigerated temperatures in Korea. In general, unwashed eggs stored at ambient temperatures (20 ± 5 °C) are sold at traditional wholesale markets and washed eggs stored under refrigeration temperatures (4–10 °C) are sold at modern supermarkets.

Numerous reports have described the survival of *Salmonella* in egg contents (Chen and Thesmar, 2008; Gast et al., 2010; Humphrey and Whitehead, 1993). However, only a few reports have described survival and growth patterns of microorganisms (naturally occurring mesophilic aerobic bacteria [MAB] or *S. enterica*) on the surface of eggshells as affected by storage conditions. One objective of this study was to compare the microbiological quality of the eggshell surfaces of commercial eggs distributed in a traditional wholesale market and a modern supermarket. A second objective was to investigate the survival and growth patterns of naturally occurring MAB and artificially inoculated *S. enterica* on eggshells when eggs were exposed to various environmental conditions (the presence of chicken feces, temperature [4, 12, or 25 °C], and relative humidity [RH; 43 or 85%]).

## 2. Materials and methods

### 2.1. Comparisons of the microbiological quality of commercial eggshell surfaces purchased from a traditional wholesale market and a modern supermarket

To compare the microbiological qualities of chicken eggs as affected by distribution conditions, eggs were purchased from ten stores in a traditional wholesale market or for ten different brands in a modern supermarket in Seoul, Republic of Korea. All eggs were transported to the laboratory under ambient temperatures (20 ± 5 °C) and tested within 3 h. From each package of eggs (15–30 eggs/package), three eggs that were not visibly cracked or contaminated with feces were selected. In total, 30 eggs from each market type were used in this experiment.

To evaluate the microbiological quality of eggshells, eggs were separately placed aseptically in a stomacher bag (BA 6040 standard bags; Seward, West Sussex, UK) containing 40 ml of sterile buffered peptone water (BPW) and gently rubbed by hands for 1 min. After rubbing, the rinsate was serially diluted in 0.1% peptone water. Undiluted rinsate (0.25 ml in quadruplicate and 0.1 ml in duplicate) and diluted rinsate (0.1 ml in duplicate) were surface plated on tryptic soy agar (TSA; BBL/Difco, Sparks, MD, USA), desoxycholate lactose agar (DLA; BBL/Difco), dichloran rose bengal chloramphenicol agar (DRBC; BBL/Difco), and xylose lysine desoxycholate agar (XLD; BBL/Difco) to determine the populations of MAB, coliforms, molds and yeasts (MY), and *Salmonella*, respectively. TSA, DLA, and XLD plates were incubated at 37 °C for 24 h, and DRBC plates were incubated at 25 °C for 5 days. After incubation, typical colonies of coliforms (pink with bile precipitate) formed on DLA were counted, and all colonies formed on TSA and DRBC were counted. The remaining rinsate was enriched by incubating at 37 °C for 24 h. When no presumptive colonies formed on DLA, enriched rinsate was streaked using a loop on DLA and incubated at 37 °C for 24 h. When no colonies presumptive for *Salmonella* were formed on XLD, 0.1 ml of enriched rinsate was added to 10 ml of Rappaport-Vassiliadis R10 broth (RVB; BBL/Difco) and incubated at 42 °C for 24 h. Incubated RVB was streaked using a loop on XLD, incubated at 37 °C for 24 h, and examined for typical *Salmonella* colonies. The detection limits for the direct plating and enrichment were 1.6 log CFU/egg (40 CFU/egg) and 0.0 log CFU/egg (1 CFU/egg), respectively.

### 2.2. Behavior of MAB and S. enterica on eggshells as affected by the presence of feces, storage temperature, and RH

#### 2.2.1. Bacterial strains and preparation of inoculum

Five serovars of *S. enterica* subsp. *enterica* were used: *S.* Enteritidis, *S.* Typhimurium, *S.* Heidelberg, *S.* Hartford, and *S.* Newport. Cryopreserved cells of each serovar were inoculated separately in tryptic soy broth (TSB; BBL/Difco) and activated at 37 °C for 24 h. Cultures were transferred using a loop (ca. 10 μl) into 10 ml of TSB three times at 24-h intervals. Cell suspensions (2 ml each) of the five serovars were combined and centrifuged at 2002 × *g* for 15 min at room temperature (21 ± 2 °C). The supernatant was decanted, and cells in the pellets were resuspended in 10 ml of phosphate buffered saline (PBS; pH 7.2) to give a population of ca. 9 log CFU/ml. This suspension was used as an inoculum for eggshells and chicken feces applied to eggshells.

#### 2.2.2. Preparation of eggs

Chicken eggs were purchased at a traditional wholesale market (Cheongnyang wholesale market, Seoul). The eggs were transported to the laboratory under ambient temperatures (20 ± 5 °C) and tested within 3 h. Eggs not visibly cracked or contaminated with feces were used in the experiments. Chicken feces were collected from the Korea University Farm (Namyangju, Republic of Korea), autoclaved at 121 °C for 15 min, and stored at −21 °C. The feces were thawed at room temperature before use in the experiments.

To determine the patterns of growth and survival of naturally occurring MAB on eggshells during storage, two types of eggs were used: uninoculated eggs without feces and uninoculated eggs on which feces were applied. For the preparation of uninoculated eggs exposed to feces, 0.02 g sterile feces were deposited on the shell of each egg using a sterile spatula. To observe the behavior of *S. enterica* on eggshells during storage, two different types of eggs were prepared: eggs inoculated with *S. enterica* without feces and eggs inoculated with *S. enterica* in feces. For the preparation of eggs inoculated with *S. enterica* without feces, *S. enterica* inoculum (20 μl) was spot-inoculated randomly at 10 locations on the eggshell. For the eggs inoculated with *S. enterica* in feces, 0.02 g feces mixed with 20 μl of inoculum were spotted on the shell of each egg using a sterile spatula. All eggs were dried in a laminar