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PENETRATION OF SPOILAGE AND FOOD POISONING BACTERIA INTO FRESH CHICKEN EGG: A PUBLIC HEALTH CONCERN

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ABSTRACT
Bacterial penetration shortly after inoculation has not been reported extensively. Four strains of bacteria, *Salmonella typhimurium* (ST), *Escherichia coli* (EC), *Pseudomonas earuginosa* (PE) and *Stapylococcus aureus* (SA), in chicken egg components of *Bovan brown* were analyzed by serial dilution and scanning electron microscopy. After exposing the fresh eggs to the bacterial strains at concentrations of 10^6 per ml^{-1}, the egg components (eggshell, shell membrane, albumen and yolk) were examined at 30, 60, 90 and 120 min intervals. Untreated fresh eggs (controls) had no bacteria. Among the 4 strains, PE was the most dominant in the egg components followed by ST, SA and EC. In egg components the four strains generally demonstrated significant increase throughout the experimental period. However, there was a decrease in albumen in SA after 60 min. While in ST, there was a decline in both albumen and yolk after 60 min period. Overall, the porous structure of the eggshell and egg membranes facilitated rapid penetration. The aim of this investigation is to evaluate the rate of penetration in the chicken egg components shortly after inoculation with bacteria. Such studies may be of value in generating awareness of bacterial contamination, especially in hot climates such as Oman.

KEYWORDS: Bacteria penetration, Chicken, Egg components, Food safety

INTRODUCTION

Eggs can undergo rapid microbial deterioration which endangers human health if precautions are not taken during egg collection, preservation and shipping. Many bacterial strains can cause a variety of diseases (Bower and Daeschel, 1999). The ability of food borne pathogens to react and respond to changes in their surrounding is crucial to their survival. Previous investigations have shown that food-borne bacterial pathogens can adapt and survive the harsh environment of different food components (Gast and Beard, 1993; Rowan, 1999). Many bacterial species develop different strategies for survival and multiplying in food components, leading to illnesses, especially food poisoning (Tyler, 1991).

Indar *et al*., (1998) reported outbreaks from food-borne bacteria are on the increase and have become an important public health and economic issue. The consumption of infected eggs is a major factor in incidents of human illness in recent years (Yang *et al*., 2001). Food microbial infection in elderly, young children, pregnant women and immunocompromised individuals is of concern. The common symptoms of infection are diarrhea, vomiting, abdominal cramps, chills, headache and fever (Blumenthal, 1990).

In human, the major outbreak of salmonellosis is associated with eating chicken eggs was reported in Europe and United States for the last 20 years (Gantois *et al*., 2009). To a lesser extent, *Staphylococcus aureus* enterotoxin was also reported in egg products (Yang *et al*., 2001). Members and non-members of Enterobacteriaceae were also reported to cause egg spoilage and contamination. These include *Escherichia coli* and *Pseudomonas* species (Berrang *et al.,* 1999).

Eggs may be infected vertically from the infected ovaries and oviducts prior to oviposition or horizontally if eggs come in contact with contaminated fecal material and oviductal fluid during oviposition (Al-Bahry *et al.,* 2010; Berrang *et al*., 1999). The majority of information on bacterial penetration after inoculation is centered on the examination of the eggshell, membranes, albumen and yolk. There are three mechanisms expressed in bacterial penetration of the egg: adherence to eggshell and penetration through the eggshell pores, survival in albumen, and multiplication and growth in yolk. Models for the penetration of bacteria in eggs has been investigated which are based on simultaneous transport and growth in three egg components, the eggshell, albumen and yolk (Al-Bahry *et al.,* 2009a; Al-Bahry *et al.,* 2011; Messens *et al*., 2005, Grijspeerdt, 2001). The eggs are incubated with bacteria for a period of time since this method was claimed by Messens *et al*., (2005) as the closest to the natural conditions.

Organisms on the eggshell surface are capable of penetrating the pores into the interior of the egg and contaminating the components. Additionally, contaminated fecal material adhered to broken eggshell may infect the egg components (Berrang *et al*., 1999).

The properties of microorganisms differ in the method of penetration. *Salmonella typhimurium*, *E. coli* and *P. aeruginosa* are motile Gram-negative bacteria and can more easily penetrate the egg components. On the other hand, *Staphylococcus aureus* is non-motile Gram positive bacteria and most likely move according to the effective

diffusion of moisture or liquid (Grijspeerdt, 2001). There are several conditions that can stimulate microbial growth. For example, growth response of many microbes is directly proportional to temperature. The optimum growth temperature for the bacteria is between 35-37^{o} C, which favors eggshell invasion (Yang *et al*., 2001). The chicken egg has several physical and chemical defense mechanisms to reduce microbial invasion. The physical barrier includes the eggshell and shell membranes (Tan *et al*., 1992). The chemical defense mechanism is the presence of antimicrobial agents in albumen and yolk such as lysozyme, ovatransferrin and avidin (Board *et al*., 1994). Recently, knowledge of bacterial infections based on electron microscopy examination has been of value in the overall understanding of microbial behavior. The information from previous studies provided important knowledge regarding the behavior of bacteria in eggs (Messens *et al*., 2005). The purpose of this investigation is to analyze penetration rate in chicken egg components for *E. coli* (ATCC 43895), *P. aeruginosa* (ATCC 33354) and *Staphylococcus aureus* (ATCC 6538) and *S. typhimurium* (ATCC 14028). Scanning electron microscopy (SEM) was used to compare penetration of bacterial strains through eggshell and egg membranes. The benefit of such investigation particularly in hot climates such as Oman, will be extremely beneficial in the public health sector, to increase the awareness of microbial contamination.

MATERIALS AND METHODS

Four strains of bacteria were used for this study. These bacteria were *E. coli* (ATCC 43895), *P. aeruginosa* (ATCC 33354), *S. aureus* enterotoxin B producer (ATCC 6538) and *S. typhimurium* (ATCC 14028).

Penetration rate

Rate of penetration in bacteria in egg components was investigated by serial dilution and SEM.

Serial dilution

The eggshell consists of two major layers, the outside shell layer (cuticle) and the inner shell layer (crystalline). The eggshell membrane attached to the crystalline layer is electro-dense surrounding the albumen (Board *et al*., 1994).

A total of 140 experimental and 20 control of the freshly laid *Bovan brown* eggs were examined. All eggs were examined to make sure that they were free of cracks or deformity in the shell. Each experimental egg was disinfected with 70% alcohol and allowed to air dry under aseptic conditions according to the method of Indar 1998. The control eggs were disinfected with 70% alcohol only. The disinfected experimental eggs were dipped in 100 ml of 1x 10^6 of *EC*, *PE, SA* and *ST*. The eggs were dipped for 3 min and were transferred immediately to sterile containers, covered and incubated at 37^{o}C. The control and experimental eggs were then examined at 30, 60, 90 and 120 min intervals. For each interval, 35 experimental and 5 control eggs were examined. Penetration rates of bacteria were analyzed during each interval according to the method of Al-Bahry (2009a). Control and experimental samples of eggshell, egg membranes (1cm^2 each), albumen and yolk (1ml each) were examined. Albumen samples were collected by dipping a sterile syringe after partial removal of the eggshell. Yolk samples were collected after careful separation of albumen from yolk in a sterile Petri-dish. Yolk samples were collected by injecting sterile syringes into the undisturbed yolk to minimize cross contamination. The samples were used for serial dilution method.

Eggshell and egg membranes were taken randomly from all areas of the egg surface. Eggshells were carefully separated from egg membranes. Control and experimental eggs were serially diluted to 10^6 on nutrient agar plates using AutoPlate 4000 (Spiral Biotech, USA). Colony formation units were calculated after 24 hours incubation at 37^{o} C according to Harley and Prescott (2002). Bacterial penetration into the egg components were confirmed serologically as follows: *P. earuginosa,* serotype 06; *S. typhimurium* the O-group (serotype 1, 4, 5, 12) and H-antisera phase 1 (serotype i) and phase 2 (serotype 1,2); *Escherichia coli* O157:H7, and *S. aureus* entrotoxin B producer. The enterotoxin B was detected using staphylococcal enterotoxin (SET) using ELISA immunoassay technique (TECRA®, International Pty Ltd).

Scanning Electron Microscopy (SEM) Studies

The intensity of bacterial penetration and their morphology of each egg component were examined using SEM. Eggshell and egg membranes were aseptically cut into 1 cm^2 using sterile razor blades to the components were placed in carbon discs on SEM-stubs and then coated using the BIO-RAD coating system for 120 s at 1.5 KV. The samples were examined using a JEOL 5600LV SEM at 5 KV (Al-Bahry *et al*., 2009b).

Statistical Analysis

The statistical program of Social Statistics (SPSS) version 15 software package was used to analyze the results using ANOVA and Tukey test for multiple comparison.

RESULTS

Penetration rate of bacteria through the egg components

The bacterial penetration rate for each egg component was compared throughout the four experimental periods. Overall there was a significant increase ($P < 0.05$), when each component was compared to the same three components (Figs 1-4). However, microbial concentration varied among the four strains. *ST* increased in yolk significantly during the first 60 min and then declined at the end of 120 min ($P < 0.05$). In albumen, *ST* reached peak at 90 min but started to decline significantly ($P < 0.05$) at 120 min (Fig. 1).

PE in yolk increased significantly throughout the experimental period. However, in albumen, there was a steady increase during the first 90 min, then declined at the end of the experimental period after 60 min ($P < 0.05$) (Fig. 2).

SA continued to increase in yolk throughout the experimental period, but in albumen a reverse condition occurred ($P < 0.05$), (Fig. 3).

In the yolk and albumen *EC* rose significantly throughout the experimental period ($P < 0.05$), (Fig. 4). The most concentrated strain in the egg components was *PE* followed by *ST*, *SA*, and *EC*.

In the control eggs, the same procedure was followed as the experimental samples. The colony forming units were negligible compared to experimental samples.

Serologically, microbial colonies were not comparable to the experimental eggs.

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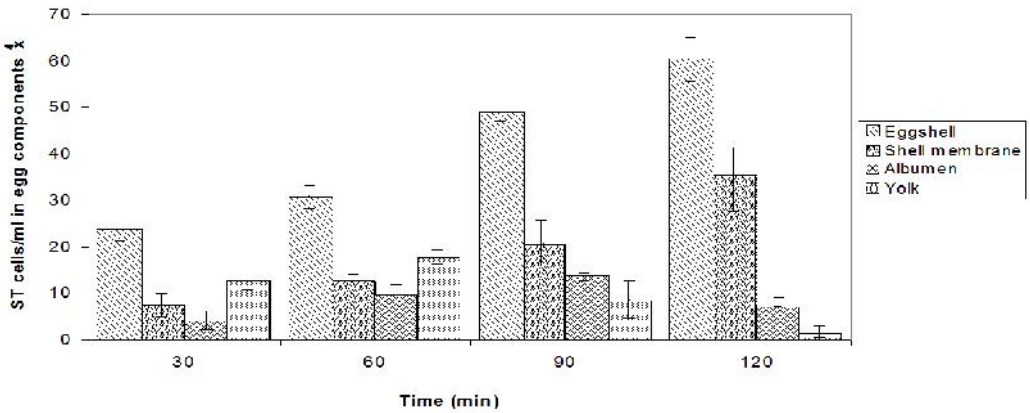

FIGURE 1: Penetration rate of *ST* in egg components during 30-120 min intervals. A significant difference ($P < 0.05$) between the four periods when the penetration rate for each component was compared. Note the significant decrease ($P < 0.05$) in yolk and albumen during the 90 and 120 min periods.

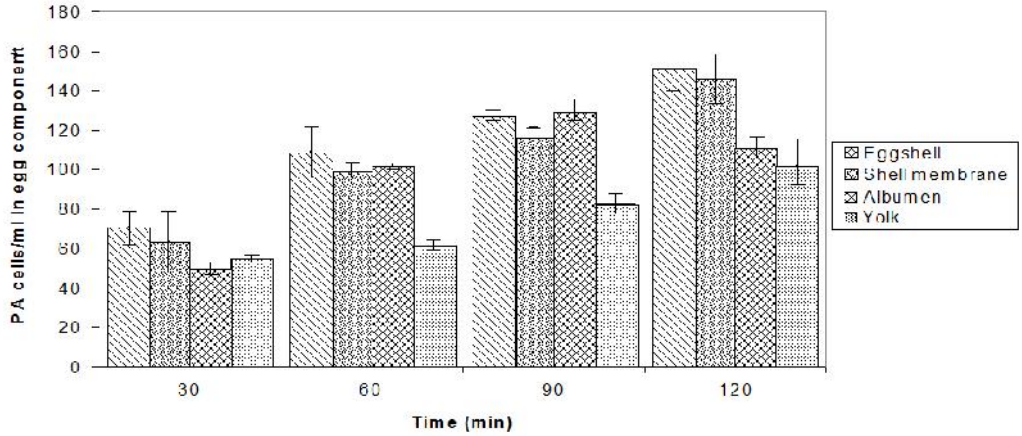

FIGURE 2: Penetration rate of *PE* in egg components during 30-120 min intervals. A significant difference ($P < 0.05$) between the four periods when the penetration rate for each component was compared.

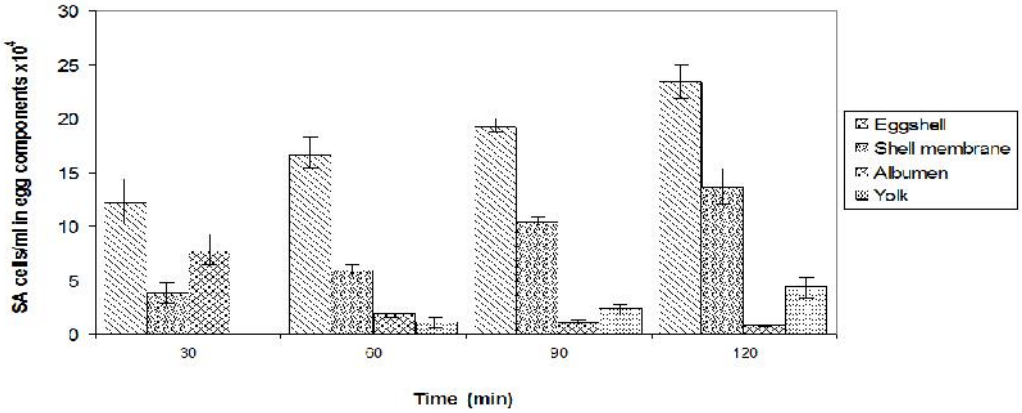

FIGURE 3: Penetration rate of *SA* in egg components during 30-120 min intervals. A significant difference ($P < 0.05$) between the four periods when the penetration rate for each component was compared. Note the significant decrease ($P < 0.05$) of the albumen during the 120 min period.

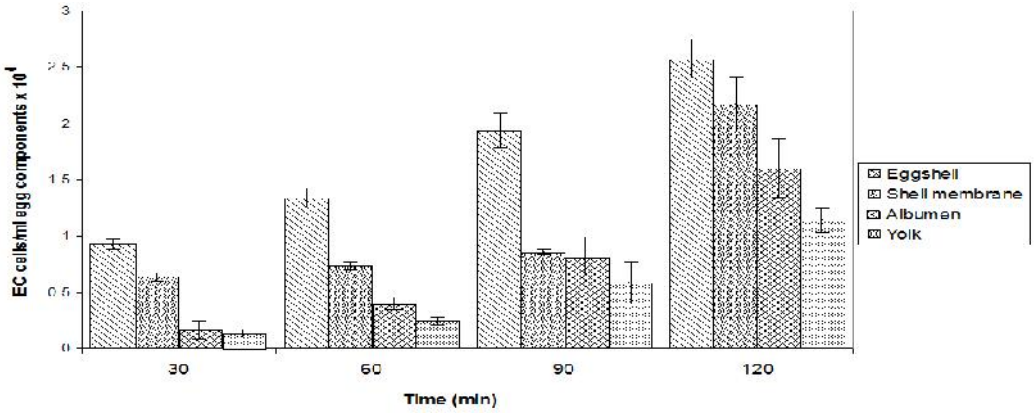

FIGURE 4: Penetration rate of *EC* in egg components during 30-120 min intervals. A significant difference ($P < 0.05$) between the four periods when the penetration rate for each component was compared.

Scanning Electron Microscopy Study (SEM)

Penetration of chicken eggs by bacteria was compared with control eggs (Fig. 5). No bacterial cells were observed in the control eggs components.

When the experimental eggs were examined at 60 min and 120 min, bacteria were found to be capable of penetrating the porous eggshell and eggshell membranes. Intensity of bacteria observed in electron micrographs at 60 min was much lower than at 120 min (Figs 6-9). Entry of bacteria through pores of the eggshells was observed in *ST*, *PE* and *EC*. The outer egg shell layer contained less bacteria than inner shell membranes (Fig. 6-8). However, a layer of *SA* cells on eggshells was obviously covering the eggshell pores (Figs 8a and d). The four bacterial strains appeared predominantly attached to the organic fibers in the inner part of eggshell and shell membranes (Fig. 6-8).

Some of the organic matter was observed in the outer layer of the eggshell formed by *ST* had heavy accumulation after 120 min of incubation (Fig. 6a and e). *ST* cells exhibited two main forms; some were coccobacilli and other were rods (Fig. 6b, c f and g).

PE tends to form bacterial clumps in the inner egg shell and the shell membranes (Fig 7 b, c, d, f, g and h). Bacterial clumping at lower extent in *SA* was also present (Fig 8 d, f and h).

In *EC* no clumping was observed and the bacterial distribution was low (Fig 9 a-h).

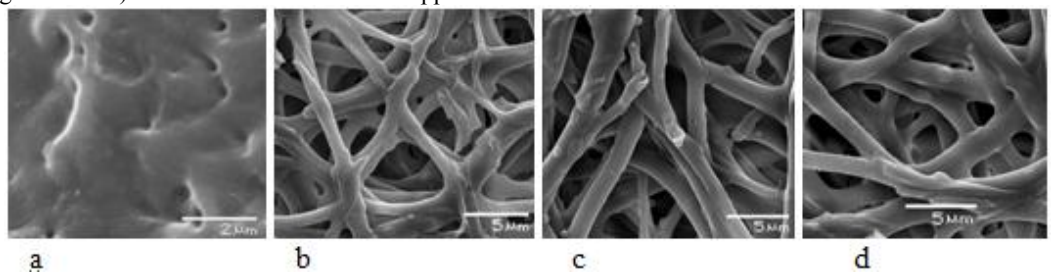

FIGURE 5: Electron micrograph of control chicken egg components. a = outer eggshell, b = inner eggshell, c = outer shell membrane. d = inner shell membrane.

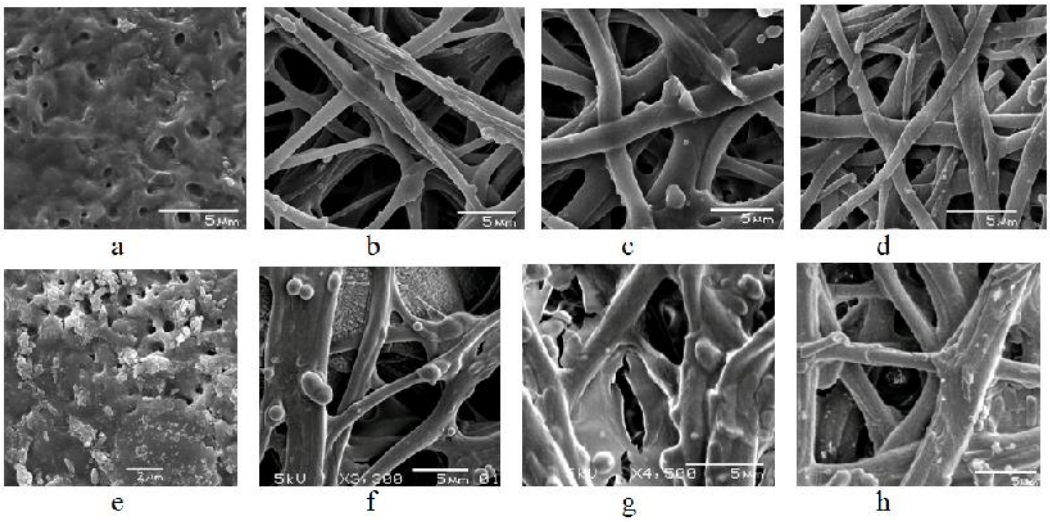

FIGURE 6: Electron micrograph of *Salmonella typhimurium* penetrating chicken egg components at 60 min (a-d) and at 120 min (e-h); a, e = outer eggshell; b, f = inner eggshell; c, g = outer shell membrane; d, h = inner shell membrane.

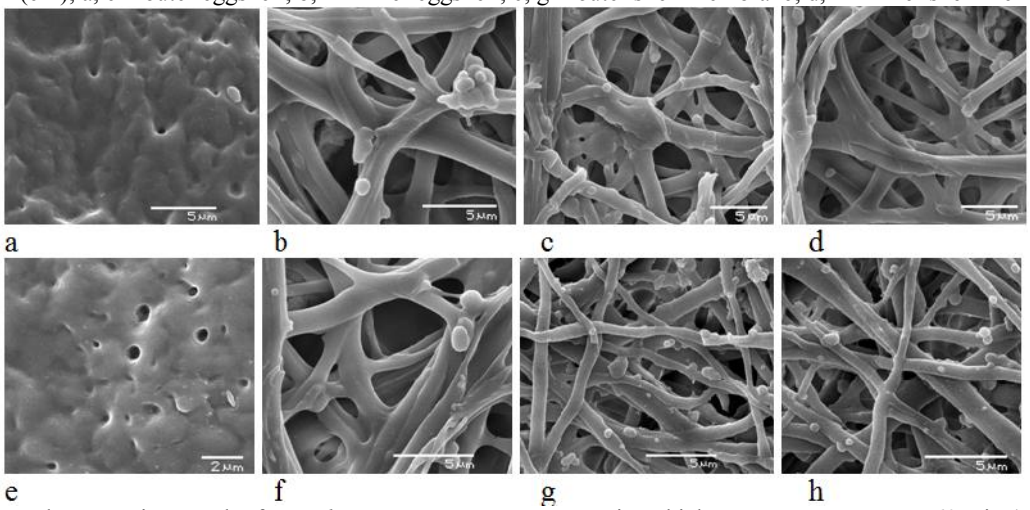

FIGURE 7. Electron micrograph of *Pseudomonas aeruginosa* penetrating chicken egg components at 60 min (a-d) and at 120 min (e-h); a, e = outer eggshell; b, f = inner eggshell; c, g = outer shell membrane; d, h = inner shell membrane.

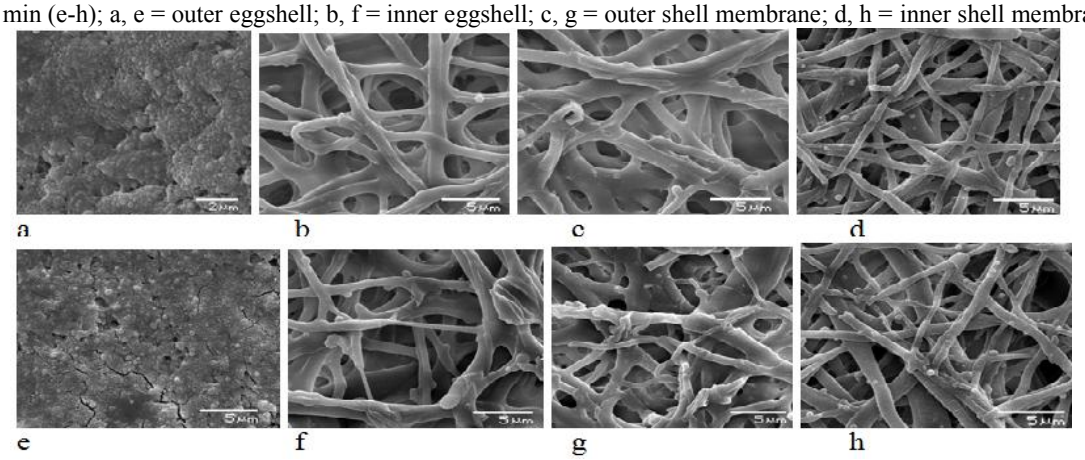

FIGURE 8: Electron micrograph of *Staphylococcus aureus* penetrating chicken egg components at 60 min (a-d) and at 120 min (e-h); a, e = outer eggshell; b, f = inner eggshell; c, g = outer shell membrane; d, h = inner shell membrane.

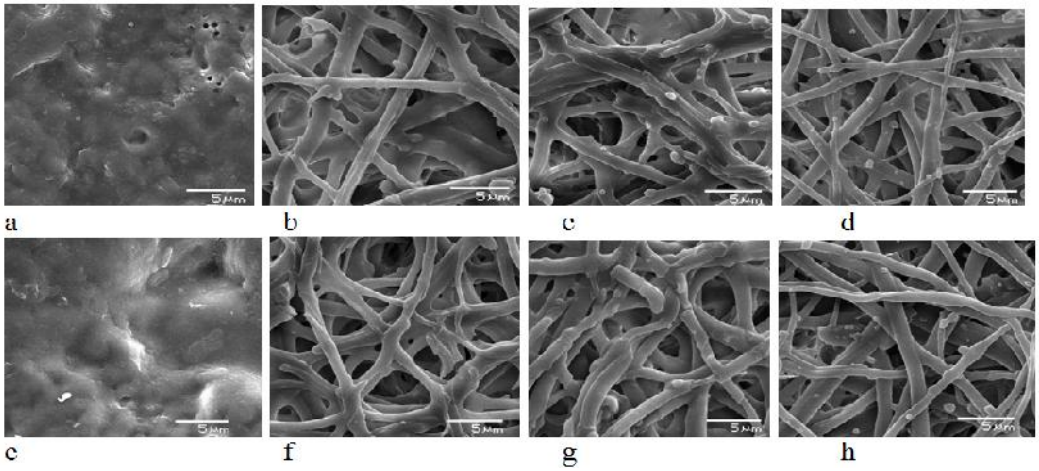

FIGURE 9: Electron micrograph of *Escherichia coli* penetrating chicken egg components at 60 min (a-d) and at 120 min (e-h); a, e = outer eggshell; b, f = inner eggshell; c, g = outer shell membrane; d, h = inner shell membrane.

DISCUSSION

In this investigation, bacteria penetration of egg components was confirmed. Penetration rate of the four strains of bacteria in eggshell and eggshell membranes consistently increased at significant levels throughout the experimental period. Javed *et al.,* (1994) reported that the longer the exposure of eggs to bacteria, the higher the penetration rate, and probably influenced by eggshell porosity. During incubation, a much higher number of

bacteria were found in eggshells and shell membranes than in egg contents (Cason *et al.,* 1993).

All the four strains in this study were isolated at various densities from 30 to 120 minutes. The higher the number of bacteria inoculums used in the culture, the higher the rate of contamination which is in agreement with Miyamoto *et al.,* (1998). Miyamoto *et al.,* (1998) reported that bacterial penetration of the egg components during 2 hours of incubation.

The penetration ability of *ST* in this study was also reported by Javed *et al*., (1994) who tested the extent of *Salmonella* penetration through the eggshell and the shell membrane. They found that within 3-5 minutes of exposure, the bacterial penetration of 1 x 10^6 cfu/ml was accomplished. Other experiments have demonstrated that *S. typhimurium* can penetrate the eggshell, shell membrane and contents within 6 minutes after egg exposure to 10^5 cfu/ml and infect chorioallontoic membranes and yolk sac as well (Cason *et al*., 1993). Berrang *et al*., (1999) proved that all eggshells, shell membrane and contents of the eggs sampled were *Salmonella* positive 30 minutes after inoculation with a bacterial concentration of 10^4 cfu/ml. On the other hand, it was reported that the organism could be detected as early as 2 hr after exposure to infection of 10^3 cfu/ml, indicating that penetration of the organism into the egg is a fairly rapid process. The size of bacterial inoculum is one of the important factors that affect and influence the penetration rate of *Salmonella* spp (Catalon and Knabel 1994). These findings are in agreement with our findings using all four strains.

Results from this investigation revealed that *PE* and *ST* penetrated the eggshell more frequently compared to *EC* and *SA*. Similarly, De Reu *et al*. (2006) compared the penetration of seven selected species with *Pseudomonas* sp and *Salmonella enteritidis* were the most frequent. *ST* and *SA* penetration of chicken eggs indicated that albumen had the least bacteria. This was also confirmed by Al-Bahry *et al*., (2009a). Albumen probably consists of several antimicrobial substances such as lysozymes, ovotransferrin and others (Board *et al.,* 1994). However, in this study, microbial concentration for *PE* and *EC* increased during the experimental period.

Lysozyme play a major role in chemical defense due to the lytic properties. It is a cationic protein that binds to lipopolysaccharide (LPS) of Gram negative bacteria, presumably recognizing the lipid A protein LPS, resulting in the inhibition of several immunostimulatory activities. The structure of LPS, differs from one Gram negative to another. For example, different strains of *EC* may consist of different types of LPS, Liang *et al.,* (1998) who reported the effect of O-antigenic polysaccharide of different strains of *EC* on endotoxin neutralizing activity of lysozyme. Also, *PA* and *EC*, may have the ability to develop certain strategies which inhibit the enzymatic activities and avoids their toxic properties in albumen (Tyler, 1991). In this study, *ST* in yolk decreased with time. Although the yolk has many types of accumulated antibodies, such as immunoglobulin G (IgG), IgA and IgM, considered to an excellent growth medium for bacteria including *EC, PE* and *SA*, (Barrow and Lovell 1991). The yolk is a rich nutrient for optimum growth where some bacteria multiply extensively (Nascimento *et al*., 1992; Board *et al*., 1994).

Although *EC* is a motile bacterium like *PE* and *ST*, its concentration in egg components was the lowest. Probably *EC* has less ability to adhere to eggshell and therefore was found to be the least in egg components. Cason *et al.,* (1993) found that some bacteria have low ability to invade egg contents and are unable to adhere to eggshells and shell membranes.

SA was the only nonmotile strain used in this study. According to the SEM results, *SA* was found abundantly on the outer eggshell. Serial dilution data from this study showed that *SA* was found in higher concentration on eggshell and in other components compared to *EC*. It appears that *SA* uses different strategies to invade the egg components.

In this study, SEM revealed that the bacterial penetration through the eggshell pores of eggshell membranes. It was found that some bacteria had changes in cell morphology within eggshell and shell membrane with some strains dividing to form clumps. An eggshell has numerous pores as portal of entry of bacteria to infect internal egg components (Solomon, 1997).

The results of SEM study concerning the interaction between *ST*, eggshell and shell membrane and their morphology were supported with the results found by Nascimento *et al.,* (1992). Replication of *ST* was observed within egg samples and showed different cell morphology, coccobacilli and rod shaped bacteria. These findings were similar to that of Rowan (1999). In this study microbial concentration of bacteria in yolk varied and may depend on the strategies of bacterial resistance to the activity of anti-microbial proteins and their amounts in the yolk. Chicken eggs have several defense mechanisms that reduce invasion by microorganisms and minimize their influence (Board *et al.,* 1994).

Gantois *et al*., (2009) indicated that a unique combination of genes encoding for improved eggshell protection could be of value to prevent massive bacterial contamination of the egg contents. Handling, storage temperature, and humidity are factors may reduce bacterial infection in eggs, causing contamination and spoilage. In many cases, eggs are kept at storage room temperature (25°C) until they are transported to retail stores where in some places may be stored under non refrigerated conditions (Kim *et al.,* 1989). In hot climates around the world the temperatures in storage warehouses may rich 40^{o}C with high humidity which makes the eggs vulnerable to microbial contamination. Moreover, it was reported that low temperature and humidity are important factors for the survival of certain bacteria on the eggshell (Radkowski, 2002).

Contamination of eggs causes illness and extensive commercial loss. Common microbial contaminants in the chicken oviduct are Enterobacteriaceae such as *EC, Salmonella, Proteus* and *Enterobacter*. The non-members of Enterobacteriaceae from the oviduct include *Pseudomonas, Vibrio* and *Campylobacter* (Berrang *et al.,* 1999).

In conclusion, penetration rate of bacterial strains varied. *PE* and *ST* were the highest followed by *SA* and *EC*. The results of this investigation might be of value to find ways

to reduce microbial contamination of eggs in hot-humid climate such as Oman.

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