Evaluation of Lysozyme Product (Inovapure^{TM}) as a Sanitizer for Broiler Hatching Eggs

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ABSTRACT

Hatching egg sanitation is a practice to achieve successful production in the commercial hatchery. Trials were conducted to investigate the effectiveness of EDTA modified lysozyme against *E. coli* penetrating eggshells and its effects on hatching performance and growth performance. In trial one, floor-collected eggs were fumigated with five levels of lysozyme for two exposure times. In trial two, cage-collected eggs were inoculated with *E. coli* then sanitized. In trial one, 2.25% and 3.00% lysozyme solution reduced the penetration of *E. coli* into eggs without affecting hatching performance and growth performance. In trial two, 3.00% lysozyme demonstrated activity against *E. coli* on eggshells, and provided continuous bactericidal action to prevent *E. coli* penetration. The application of lysozyme decreased the presence of *E. coli* in yolk sac of newly hatched chicks. 1.50% lysozyme solution improved hatch weight without affecting growth performance. Lysozyme can be an effective and safe sanitizer for hatching eggs.

LIST OF ABBREVIATIONS USED

Avian pathogenic *Escherichia coli*…….....APEC

Body weight……BW

Buffered peptone water…….BPW

Celsius……..….°C

Colony forming unit……...CFU

Daily body weight gain…….BWG

Eosin methylene blue……EMB

Escherichia coli……...*E. coli*

Ethylenediaminetetraacetic acid……..EDTA

Feed consumption…….FC

Feed conversion ratio…….FCR

Hypochlorous acid……HOCl

Inner shell membrane……..ISM

Nalidixic acid……...NA

N-acetylglucosamine…….NAG

N-acetylmuramic acid……...NAM

Lipopolysaccharides……LPS

Outer shell membrane……...OSM

Relative humidity…….RH

Quaternary ammonium……QA

2,3,5-Triphenyltetrazolium chloride ……..TTC

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CHAPTER 1 INTRODUCTION

The transmission of pathogenic bacteria to eggs is a major concern to the poultry industry. Contaminated eggs could increase the prevalence of these bacteria in live chickens and chicken products, which, when consumed by humans, may cause foodborne illness (Spickler et al., 2011).

Bacterial infection of chickens during production can be the result of bacteria originating from numerous sources. The hatchery may be one of the most important sources. Bacteria can penetrate freshly laid eggs through the shell as the eggs cool down from body temperature at 41°C. Negative pressure generated by the cooling process can pull bacteria from the shell surface through the shell and its membranes (Bruce and Drysdale, 1994). Hatching egg sanitation should be applied as soon as possible after the eggs are laid and collected. The effectiveness of hatchery sanitation and pathogen reduction in day-old chicks is limited if the eggs are already heavily contaminated (Coufal et al., 2003). Bailey et al. (1998) have shown a small percentage of contaminated eggs can spread bacteria from egg to egg within an incubator. With this in mind, it has become common practice to sanitize eggs prior to placement in incubators.

Hatching egg sanitizers should be evaluated on their ability to reduce microbial load and increase hatchability, while demonstrating cost-effectiveness and safety (Shane and Faust, 1996). Formaldehyde was the first commercial sanitizer used in the North American poultry industry. The Occupational Safety and Health Administration (1991) published information on toxicity of formaldehyde for farm workers as well as reporting its poor effectiveness as a disinfectant. Numerous researchers have evaluated different commercial hatching egg sanitizers, including chlorine, hydrogen peroxide and UV light (Coufal et al.,

2003; Fasenko et al., 2009; Spickler et al., 2011). An alternative to formaldehyde, which is less hazardous to humans, economical and efficient, is needed.

Lysozyme is a natural bacteriolytic enzyme commonly isolated from egg white (Fleming, 1922; Salton, 1957). Lysozyme is defined as 1,4-β-N-acetylmuramidase. It cleaves the glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in bacterial peptidoglycan which is an important component of bacterial cell walls (Phillips, 1966). Therefore, it provides protection against bacterial infection. Compared to Gram-positive bacteria, Gram-negative bacteria have an additional outer membrane which consists of lipopolysaccharide and protein. Due to the extra barrier in Gram-negative bacteria, the antibacterial activity of lysozyme is more effective against Gram-positive bacteria than Gram-negative bacteria (Johnson, 1994). Following the addition of substances like EDTA, butyplaraben or tripolyphosphate, the activity of lysozyme was enhanced enough to control growth of the Gram-negative *Escherichia coli* (*E. coli*) (Durance, 1994; Boland et al., 2003).

Several studies have been conducted to evaluate the antimicrobial activity of lysozyme in vegetable, cheese and wine production. Hughey et al. (1989) suggested that applying 100 ppm lysozyme with 5 mM EDTA effectively inhibited the growth of *Listeria monocytogenes* in fresh vegetables and cheese products. According to Makki and Durance (1994), 10 and 50 ppm lysozyme can prevent the growth of Gram-positive bacteria in beer production. However, research regarding the use of lysozyme as an alternative sanitizer for hatching eggs is limited. Evaluation of lysozyme as an alternative to commercial hatching egg sanitizers should include measuring its effectiveness against Gram-negative bacteria such as *E. coli*, which can be problematic for the hatchery industry.

Studies to determine the level of lysozyme that is most effective for preventing contamination of hatching eggs with *E. coli* are needed. An investigation to determine the effectiveness of using lysozyme as a sanitizer for broiler hatching eggs against *E. coli* contamination to improve hatchability, chick quality and growth performance would be useful.

The method used to evaluate the effectiveness of a sanitization procedure should reflect the impact of bacteria that are in position to be problematic to a developing chicken embryo. There are numerous reports that indicate procedures are adequate to reduce the prevalence of microorganisms on the surface of eggshells (Mellor and Banwart, 1965; Gentry and Quarles, 1972; Berrang et al. 1991). However it is important to use a method to determine the amount of bacteria that penetrate the eggshell and contaminate the egg contents. An eggshell penetration assay will be used to evaluate lysozyme application to the surface of hatching eggs.

CHAPTER 2 LITERATURE REVIEW

2.1 Overview of egg formation

The female avian reproductive system consists of two parts: the ovary and the oviduct (Figure 2.1). The ovary is located in the abdominal cavity midway between the neck and the tail of the bird (Bell, 2002). The ovary is responsible for the formation of the yolk, which is a source of food material to sustain development of the embryo. Prior to the hen laying its first egg, there are several thousand small follicles containing ova in the ovary. About 10 to 12 days before the hen lays the first egg, the anterior pituitary gland produces follicle-stimulating hormone to increase the size of the yolk. The yolk (ovum) requires about 10 days to fully develop (Solomon, 1997). A major amount of yolk components are produced in the liver and transported to the developing ovary by the circulatory system. A day or two after the first yolk begins maturing, another follicle starts to develop. Based on that, there are about 10 follicles in the growth phase prior to the first egg being laid. This process is dependent on the light cycle with only one yolk ovulated per day (Bell, 2002).

The mature ova released from the ovary enters the oviduct. After detaching from the ovary, the mature ovum drops into the body cavity and is picked up by the infundibulum. The ovum remains in the infundibulum for about 15 min, then it is forced along the oviduct by multiple contractions (Bell, 2002). The yolk passes from the infundibulum into the magnum where the majority of the albumen (three inner layers) is deposited and the chalaza are formed. This process takes about 2 to 3 h. The amount of albumen produced in the magnum represents half of the volume of albumen present in a fresh laid egg. Additional fluid (outer liquid layer) is added through the formed shell membranes by osmosis in the uterus section. The primary function of chalaza is to anchor the yolk in the center of the

fully formed egg and prevent the yolk being in contact with the external environment (Bell, 2002). The ovum then moves through the magnum and enters the isthmus.

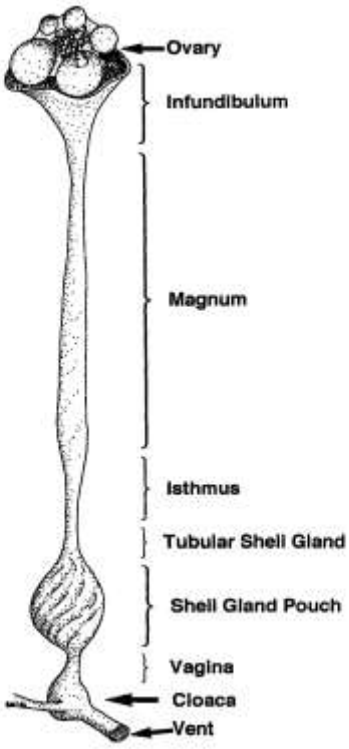

Figure 2.1 The left ovary and oviduct of the laying hen (Roberts, 2004)

Inside the isthmus, the next section of the oviduct, inner shell membrane and outer shell membrane are formed over a 1-hour period (Arias et al., 1993). The outer shell membrane adheres to the shell and is three times thicker than the inner shell membrane (Bell, 2002). Eggs pass from the isthmus to the uterus (shell gland) where the egg spends about 18 h forming the two shell layers. The whole process of eggshell formation can be divided into three stages: the initiation stage (4 h), the rapid calcification stage (12 h) and the terminal stage (2 h). During the first 4 h of the egg entering the uterus, water and salt are added through the shell membrane by the process of osmosis to stretch the shell membranes. This distension separates and exposes the mammillary cones, and is thought to be the stimulus

for the rapid phase of calcification to begin (Bell, 2002). Two main sources of calcium for eggshell calcification are from the digestion of feed and mobilization of calcium from medullary bone of the skeletal system (Bell, 2002).

2.2 Composition of the egg

An egg's physical structure primarily consists of the yolk, albumen, shell membranes and the shell (Romanoff and Romanoff, 1963; Bell, 2002). Figure 2.2 gives a schematic representation of the egg and location of its components.

2.2.1 The cuticle

On the surface of the eggshell, there is an extremely thin protein-rich layer called the cuticle, coating the eggshell. The cuticle consists of two layers: the inner calcified layer and outer non-calcified layer. The inner calcified layer is largely composed of either calcium carbonate crystals or amorphous calcium phosphate (Sparks and Board, 1984). The outer non-calcified layer mostly consists of proteins (Wedral et al., 1974). The cuticle is secreted in the uterus during the last hour of shell formation and deposited onto the surface of the eggshell. The thickness of cuticle varies from 0.005 to 0.036 mm depending on the age and strain of the bird (Romanoff and Romanoff, 1963; Board and Tranter, 1986). The weight of the cuticle is about 0.2% of the entire egg weight (Baker and Balch, 1962). The cuticle is very securely attached to the shell and covers the pores of the shell (Romanoff and Romanoff, 1963). The main functions of cuticle are to waterproof the egg by capping the shell pores and to allow the diffusion of respiratory gases (Board and Scott, 1980).

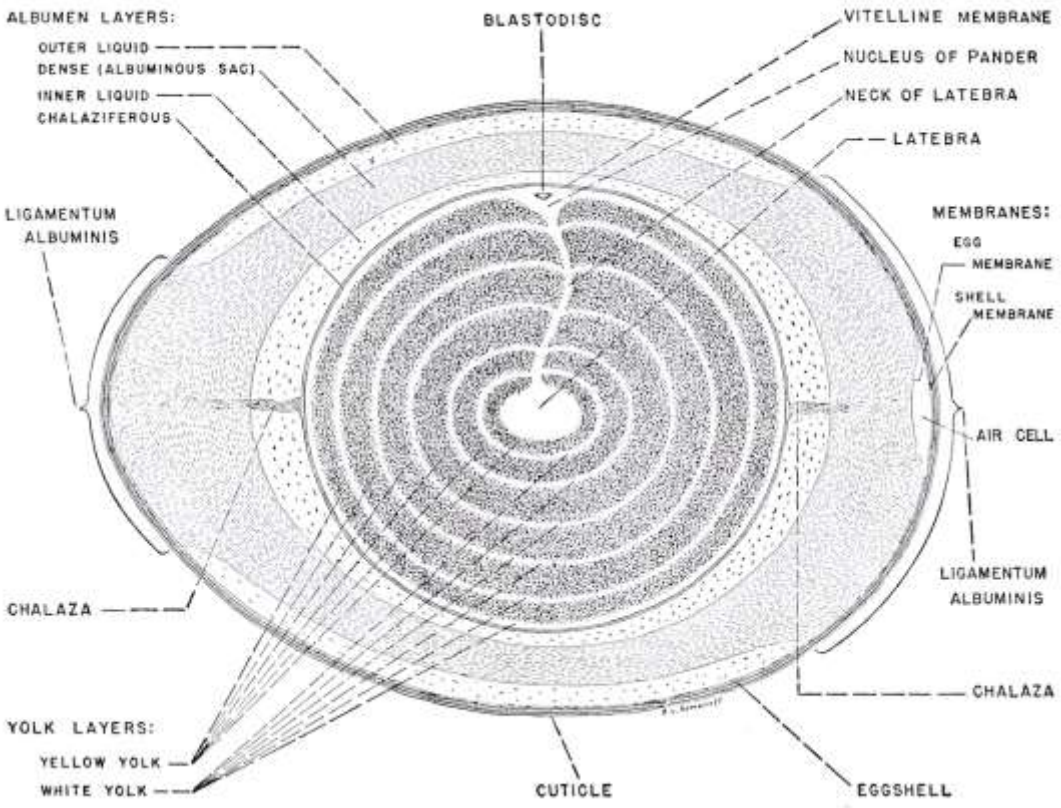

Figure 2.2 Structure of the hen's egg (Romanoff and Romanoff, 1963)

There are a number of proteins present in the cuticle layer that exhibit antimicrobial activity (Hincke et al., 2000). Therefore, the cuticle also acts as a chemical barrier to prevent bacteria from penetrating the shell (Bruce and Drysdale, 1994). At oviposition, the cuticle takes less than 3 min to dry. Sparks and Board (1985) reported that eggshell is easily penetrated by bacteria when the cuticle is still moist. Twelve eggs were collected immediately after oviposition with the cuticle still moist. One side of each eggshell was placed on fresh chicken feces for 15 min. Then the opposite side of egg with the dry cuticle was in contact with feces for the same exposure time. Of the 12 eggs having dry cuticle when placed on feces, only two eggs had contaminated membranes with bacteria. In contrast, all membranes of the eggs, which had been exposed to feces when the cuticle was

wet, were contaminated with bacteria. This suggests that the dry cuticle is an important barrier to prevent bacteria from penetrating the eggshells.

2.2.2 The eggshell

The shell comprises 10 to 12% of the total egg weight (Bell, 2002). The elemental composition of the shell is 94% calcium carbonate, 0.9 % magnesium, 0.9% phosphorus and 1% to 4% protein matrix (Romanoff and Romanoff, 1963; Solomon, 1997). The thickness of the shell ranges between 0.30 and 0.52 mm (Messens et al., 2005). The main function of the shell is to provide mechanical protection for the developing embryo. In addition, the shell is a source of calcium for the developing skeleton of the chick embryo (Romanoff and Romanoff, 1963). The calcified shell consists of three layers that are formed sequentially, starting with the innermost mammillary layer, followed by the palisade layer and the outermost surface vertical crystal layer. The mammillary layer is made of a regular array of cones, the tips of which are embedded in the outer surface of the outer shell membrane. The height of a single mammilla cone corresponds to the thickness of the mammillary layer and each cone is about 0.1 mm in diameter (Rose, 1997). The organic mammillary cores are contained within the mammillary knobs and serve as nucleation sites to attract calcium salts and form the mammillary layer (Solomon, 2010). The structure of the mammillary layer of the shell has an important influence on eggshell strength. Robinson and King (1970) reported that abnormal distribution of the mammillary cones contributed to weaker eggshells.

Superimposed on the top of each cone is a tall column of long calcite crystals. This layer consisting of elongated columns is called the palisade layer, and alternately referred to as the spongy layer (Romanoff and Romanoff, 1963). The palisade layer accounts for the

greatest thickness of the shell and comprises approximately 60% of eggshell (Parsons, 1982). The eggshell consists of two materials: organic matrix and inorganic salts. When the eggshell is decalcified by dilute acid, an intact web of shell matrix protein remains (Romanoff and Romanoff, 1963). The protein matrix fibers pass through the calcite crystals to regulate the process of calcification (Nys et al., 2004). The palisade layer is most closely associated with shell strength. Parsons (1982) found that eggshells with high breaking strength have narrow palisade columns. He noted that the size of palisade column is affected by the mammillary knobs coalesced. Early fusion of the knobs results in narrower palisade columns that may strengthen the shell.

The outer most calcified layer of the shell is the vertical crystal layer. This layer lies directly above the palisade layer and below the cuticle. The thickness of this layer ranges from 3 to 8 μm (Parsons, 1982). The function of the vertical crystal layer has not been determined.

The palisade layer is closely packed together. Occasionally an air space occurs between some columns. This forms a channel though the shell. Numerous funnel-shaped pore canals are found on the surface of an eggshell. The number of pores per egg varies ranging from 7,000 to 17,000 (Tyler, 1953). The pores are distributed unevenly over the surface of eggshell. Romanoff and Romanoff (1963) determined the distribution of the pores in the eggshell. The blunt pole of the egg, where the air cell is located, has the greatest number of pores (126 pores/cm^2). The function of the pores is to provide channels for gas and water exchange between the developing embryo and the external environment (Board and Fuller, 1974). Several researchers suggested that the pores in eggshells provide the entrance of small particles (microorganisms) into the egg and lead to contamination of the egg (Haines and Moran, 1940; Gillespie and Scott, 1950; Lorenz and Starr, 1952), while Messens et al.

(2005) and De Reu et al. (2006) refuted these earlier findings. They did not find a correlation between the number of pores and bacterial eggshell penetration. The fact that some pores do not extend through the entire thickness of the shell (Silyn-Roberts, 1983) and cuticle capping often present on pores prevents microbial penetration (Romanoff and Romanoff, 1963), may contribute to these conflicting opinions.

2.2.3 The shell membrane

The shell membrane consists of inner shell membrane (ISM) and outer shell membrane (OSM). The ISM is in contact with the outer liquid albumen. The outer surface of ISM is firmly attached to the inside of the OSM, except at the blunt end of the egg. In the blunt end of the egg, the space between ISM and OSM creates the air cell. The outer surface of the OSM is firmly embedded in the true shell. On average, the membranes represent about 0.6 % of an egg's weight (Romanoff and Romanoff, 1963). The thickness of membranes varies from 0.050 to 0.092 mm depending on species. In a study of Leghorn eggs, the thickness of ISM was three times thicker than that of OSM (Hays and Sumbardo, 1927). Romanoff and Romanoff (1963) also measured the membranes thickness of Brahma, Leghorn and Bantam hens' eggs. The thickness of ISM was consistently three times thicker than that of OSM. According to electron microscopy, the ISM and OSM are connected by fibers that cross the two membranes (Bellairs and Boyde, 1969). The composition of the shell membrane fibers is still not fully determined. However, the functions of several proteins in the eggshell membranes have been investigated. Type X collagen, a short-chain collagen, was detected in the fibers of eggshell membranes and it contributes to the structural integrity of the eggshell membrane (Wang et al., 2002). Some proteins, such as lysozyme, ovocalyxin-36 and ovotransferrin in the eggshell membrane are known to have

the antimicrobial properties (Hincke et al., 2000; Gautron et al., 2001).

2.2.4 The albumen

The yolk is surrounded and enclosed by the albumen. The albumen represents approximately two-third of the weight of the egg (Romanoff and Romanoff, 1963). The albumen of egg consists of four layers: the outer liquid layer, middle dense layer, inner liquid layer and chalaziferous layer. The proportions of the albumen among the four layers are variable depending on the breed, size of egg and rate of egg production. The middle dense layer albumen represents over 50% of the total relative volume of the albumen in the egg (Table 2.1). Water is the major component (>85%) of albumen (Romanoff and Romanoff, 1963). Albumen is known to play two important roles for embryo development. It provides protection for the embryo from microbial contamination and nutrients (water, protein and vitamin) for the developing embryo (Benton and Brake, 1996).

Table 2.1: Relative volume of each layer of albumen in hen eggs

Albumen layer	Relative volume (%)
Outer liquid layer	23.2
Middle dense layer	57.3
Inner liquid layer	16.8
Chalaziferous layer	2.7

Source: Romanoff and Romanoff (1963).

2.2.5 The yolk

The yolk is one of the most important parts of the egg. It provides the mass of nutrients that support embryonic development. In newly laid eggs, the yolk is firmly held in the center of the albumen. The yolk makes up about 36% of the weight of the egg. The vitelline

membrane surrounding the yolk consists of two layers: the inner layer formed in the ovary and the outer layer deposited in the oviduct (Bellairs et al., 1963). Fromm (1967) reported that the surface of the vitelline membrane is connected to the chalaziferous layer and the strength of the vitelline membrane decreases as the egg ages. The major components of the yolk are proteins and lipids (Table 2.2).

Table 2.2. Composition of the yolk, albumen, eggshell and whole egg

Component	Water (%)	Protein (%)	Lipid (%)	Carbohydrate (%)	Ash (%)
Yolk[1]	46.5-51.0	15.7-16.6	31.8-35.5	0.2-1.0	1.1
Albumen[1]	87.0-89.0	9.7-10.6	0.03	0.4-0.9	0.5-0.6
Eggshell[2]	1.6	6.4	0.03	-	91.1
Whole egg[1]	72.8-75.6	12.8-13.4	10.5-11.8	0.3-1.0	0.8-1.0

Sources: [1]Powrie and Nakai (1986); [2]Zeidler (2002).

2.3 Antimicrobial defense in hen eggs

Bacterial infection of fertilized eggs remains a major concern in the poultry industry, while bacterial contamination of table eggs is a major concern of the public. The egg supplies all the nutrients required for growth of the embryo, but it is also a growth-friendly environment for microorganisms. The combination of the physical organization and the chemical composition of egg provides a natural protection against microbial contamination.

2.3.1 The cuticle

The outer covering layer of the eggshell is the cuticle. The dry cuticle provides both physical and chemical barrier properties to protect the embryo from bacterial invasion. Normally, the pores in the shell are plugged with the cuticle to protect the egg from

microorganisms' entry (Romanoff and Romanoff, 1963). Some hens lay eggs that are partially or completely lacking in cuticle. In this instance, the shell is exposed directly to the environment, and the risk of microbial penetration though the pores increases. The cuticle contains several proteins (lysozyme C, ovotransferrin, ovocalyxin-32 and cystatin) that are known to have antimicrobial activity (Hincke et al., 2000). Bruce and Drysdale (1994) reported that eggs with good quality cuticle significantly decreased the risk of bacterial contamination (26%) compared to eggs with poor quality cuticle (40%). In the egg penetration study of Kim and Slavik (1996), treating eggshells with an acidic solution damaged the microstructure of the cuticle. Increased damage of the cuticle allowed greater rates of bacterial contamination. The deposition of cuticle decreases with flock age and it is one of the factors explaining why eggs produced from older hens are more easily contaminated by bacteria (Sparks and Board, 1984).

2.3.2 The eggshell

The shell plays an important role in preventing microbial contamination of the egg contents. Most eggs are considered sterile at the time of oviposition. However, they are immediately exposed to microorganisms in the environment from contact with sources, such as contaminated nest material. The intact eggshell works as a physical barrier to protect the embryo from infection by bacteria and physical damage. Edema and Atayese (2006) reported that storage of cracked eggs for 4 days increased the load of bacteria, such as *Salmonella*, *Pseudomonads*, *Staphylococcus* and coliforms. In earlier research, Ernst et al. (1998) inoculated eggs with approximately 10^6 cfu *Salmonella enteritidis* per egg for 5 seconds then incubated for 8 days. They found that cracked eggs were more likely to be penetrated by *Salmonella enteritidis* than intact eggs. Up to 77.3% of the eggs with small

line cracks were contaminated versus 2.8% of intact eggs.

Sauter and Peterson (1974) reported that eggs with lower specific gravity or lower eggshell quality are more likely to be penetrated by *Salmonella*. Messens et al. (2005) and De Reu et al. (2006), however, reported that there is not a relationship between *Salmonella* penetration and eggshell characteristics (thickness, eggshell area and the number of pores).

2.3.3 The shell membrane

The shell membranes envelop the whole internal surface of the egg and work as a mechanical filter to microorganisms that penetrate the shell. The OSM is three times thicker than the ISM, while several researchers have indicated that the ISM was a more effective barrier to prevent bacterial penetration (Lifshitz and Baker, 1964; Lifshitz et al., 1964). This paradox might be explained by the research of Simons and Wiertz (1963). They examined shell membranes by using an electron microscope. The OSM has a wider (about 8 x 10 μm) mesh and thicker (up to 3 μm) branched fibers, which comprises the network within the membrane. The ISM has a more compact network formed by smaller (up to 1.5 μm) fibers. The exact role of the shell membranes in the egg's defense is still unknown. Some proteins, detected from the eggshell membrane, are known to have antimicrobial activity. For instance, ovocalyxin-36 is a specific chicken eggshell protein located in the OSM. It may take part in natural defense mechanisms that keep the egg free of microorganisms (Gautron et al., 2001).

2.3.4 The albumen

The albumen possesses both physical and chemical properties to protect the embryo from bacterial invasion. The physical defense is the viscous albumen that works as a physical

barrier to ensure that microorganisms remain localized. But the distribution and thickness of albumen is variable dependent on the breed, breeder age and environment (Roberts, 2004). The chalaza suspension of the yolk in the center of the albumen maintains the yolk at the greatest distance from the shell membrane (Board and Tranter, 1986). Various important compounds with antimicrobial properties in albumen provide the chemical protection against microbial contamination (Table 2.3). Ovotransferrin accounts for 12 % of protein in egg albumen (Zeidler, 2002). It is a member of an iron binding protein group known as transferrins. Ovotransferrin stunts bacterial growth by binding metal ions, a necessary growth factor for pathogens, and forms a protein-metal complex, which is resistant to thermal denaturation and proteolytic attack (Azari and Feeney, 1958). Moreover, the alkaline state of the albumen accentuates the chelating potential of ovotransferrin (Board and Tranter, 1986). Ovomucoid inhibits trypsin. Lineweaver and Murray (1947) found that one molecule of ovomucoid reduced the activity of one molecule of trypsin by 50%.Ovoinhibitor is a proteinase inhibitor that possesses trypsin, α-chymotrypsin inhibitory activity. It also inactivates several bacterial proteases. Ovoflavoprotein chelates riboflavin and avidin binds biotin. Lysozyme represents 3.4% of the protein in egg albumen (Zeidler, 2002). Lysozymes are common enzymes which are mainly obtained from avian egg white and are widespread in many animal tissues and secretions (Fleming, 1922). Lysozyme is capable of hydrolyzing the β(1-4) glycosidic bonds between *N*-acetylneuraminic acid and *N*-acetylglucosamine in bacterial cell wall (Phillips, 1966).

Table 2.3 Biological properties of the main antimicrobial proteins found in egg albumen.

Protein	% of protein in egg albumen[1]	Activity[2]
Ovotransferrin	12	Chelation of metal ions (Fe^{3+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+}, Ni^{2+})
Ovomucoid	11	Inhibition of trypsin
Lysozyme	3.4	Hydrolysis of β(1-4) glycosidic bonds in bacterial cell wall peptidoglycan
Ovoinhibitor	1.4	Inhibition of proteases (trypsin, α-chymotrypsin and fungal proteinase)
Ovoflavoprotein	0.8	Binding of riboflavin, rending it unavailable to bacteria that require it
Avidin	0.05	Binding of biotin, rending it unavailable to bacteria that require it

Sources: [1]Zeidler, 2002; [2]Board and Fuller, 1974.

2.4 Microbiology of the egg

The microbial contamination of eggs increases the incidence of bacterial infection in developing embryos and newly hatched chicks. Contaminated table eggs are one of the major sources for food poisoning. The pathogenic bacteria in hatching eggs may depress hatchability and reduce chick quality (Spickler et al., 2011). It is estimated that more than 90% of eggs are microbiologically sterile at ovipostion (Board, 1966). The shell receives its first load of microorganisms when passing through the cloaca. There is a wide variation in the level of bacterial contamination of eggshells. The population of microorganisms on the surface of eggshells ranges from a few hundred to tens of millions of bacteria per eggshell (Board and Tranter, 1986). Gram-positive bacteria dominate the microflora of the eggshell. In contrast, Gram-negative bacteria are the principal contaminants of rotten eggs

(Board and Tranter 1986), Both *E. coli* and *Salmonella* were among the most common isolates from shells (Musgrove et al., 2006). The potential contamination sources include dust, nesting material and feces.

2.4.1 Biological characteristic of *E. coli*

E. coli are widely distributed in nature, and cause infections in humans and animals. *E. coli* was first described by Theodore Escherich in 1885 (Donnenberg, 2002). Most *E. coli* strains are non-pathogenic, coexisiting in harmony with their host. These strains, in addition to benefiting from the host, may synthesize cofactors and contribute to colonization resistance against pathogenic organisms (Donnenberg, 2002). However, certain serotypes of *E. coli* can cause disease in poultry.

E. coli, a member of the family *Enterobacteriaceae*, is a Gram-negative flagellated rod bacteria (length x width: 2-3 x 0.6 μm). They reside in the digestive tracts of poultry in particularly large number in the lower part of the small intestine and caeca (Ashton, 1990). *E. coli* is a facultative anaerobe and grows at an optimal temperature of 37°C. In the outer membrane of all Gram-negative bacteria, there is an additional outer membrane. This outer membrane consists of lipopolysaccharides, composed of three structural regions (lipid A, core polysaccharide and O antigen) (Raetz and Whitfield, 2002). Due to the extra barrier in Gram-negative bacteria, antibodies are more effective against Gram-positive bacteria than Gram-negative bacteria.

Any localized or systemic infection caused by *E. coli* in birds is called colibacillosis (Ashton, 1990). Avian pathogenic *Escherichia coli* (APEC) possess the virulence factors for colonizing and invading their host (Zhao et al., 2009). The pathogenesis of APEC infections include crossing epithelium and penetration into the mucosa of the respiratory

organs (Dho-Moulin and Fairbrother, 1999). Hatching eggs contaminated with *E. coli* resulted in decreased hatchability, reduced early chick quality and increased embryo mortalities (Spickler et al., 2011). It is estimated that cellulitis caused by APEC results in an economic cost of approximately $ 40 million per year for U. S. poultry industry (Norton, 1997). APEC is classified by several O serogroups (Dziva and Stevens, 2010). The well-recognized virulence factors of APEC include Type 1 and P (Pap/Prs) fimbriae for colonization; IbeA for invasion, iron acquisition systems; TraT and Iss for serum survival and K and O antigens for antiphagocytic activity (Dziva and Stevens, 2010). For poultry, 10% to 15 % of the APEC serotypes present are pathogenic and could cause disease, especially O1, O2, O78 and O157 (Ashton, 1990; Dziva and Stevens, 2010). Most APEC strains are only pathogenic to chickens. However, *E. coli* O157:H7 is able to infect both chicken and humans.

2.4.2 Diseases of the chicken caused by APEC

The transmission of APEC is a serious concern in poultry production, since APEC may cause omphalitis, yolk sac infection, airsacculitis, septicemia, pericarditis, synovitis, osteomyelitis, salpingitis, peritonitis, cellulitis and swollen head syndrome (Ashton, 1990; Lister and Barrow, 2008).

Normally, the defense system of birds would prevent colibacillosis infection. Only a small number of organisms are required for infection via the respiratory tract. Clinical colisepticaemia can be experimentally produced by injection or intratracheal administration of APEC into pathogen-free chickens (Ashton, 1990). Acute disease usually affects young birds between 4 and 12 weeks of age and the first sign is likely to be a drop in feed consumption. This is followed by reducing growth, poorer feed efficiency and

increasing morbidity, mortality and condemnation at processing (Ashton, 1990).

Airsacculitis has been the most common infectious disease caused by *E. coli* for broilers (Ashton, 1990). The *E. coli* organisms enter the upper respiratory tract as the birds breathe and infect the abdominal air sacs. It causes inflammation of the air sac, which fills with a yellowish cheesy material. Birds are condemned when these morbidities are observed. Morbidity can reach over 50% but overall losses are usually less than 5% of the group (Ashton, 1990).

In newly hatched chicks, omphalitis and airsacculitis are the most common diseases causing mortality during the first week after hatch (Ashton, 1990). The role of *E. coli* can be as a primary agent or secondary opportunist (Ashton, 1990). The exposure of *E. coli* may be from the contaminated environment through a poorly healed navel or from fecal contaminated hatching eggs (Ashton, 1990; Lister and Barrow, 2008). Other bacteria, such as *Bacillus cereus*, *Staphylococcus* spp., *Pseudomonas* spp., *Proteus* spp. *and Clostridium* spp., have been isolated from yolk sac infection in birds (Cortes et al., 2004). Nevertheless the most common isolated bacterium is *E. coli* (Lister and Barrow, 2008). It is estimated that yolk sac infection can cause 5% to 10% mortality in the first week after hatch (Ashton, 1990).

2.5 Mechanisms of microbial contamination of eggs

There are two possible routes for eggs to become contaminated with microorganisms. The direct route is through trans-ovarian and oviducal contamination, where eggs are infected during the reproduction process with bacteria in the ovary and oviduct. The other route is the trans-shell contamination route, where eggs are contaminated after laying, due to contact with dirty surfaces (Messens et al., 2005).

2.5.1 Trans-ovarian and oviducal contamination

In the trans-ovarian and oviducal contamination route (vertical transmission), the ovary and oviduct are infected by microorganisms and these microorganisms may be deposited inside the egg via systemic infection (Keller et al., 1995). Miyamoto et al. (1997) reported that trans-ovarian contamination can originate from infection of the ovaries and lower regions of the oviduct. The yolk and albumen are directly contaminated with bacteria during egg formation.

The trans-ovarian contamination route is important for some bacteria. *Salmonella* and, in particular, *Salmonella enteritidis* is the most important potential human pathogen in eggs. The trans-ovarian contamination of *Salmonella* was studied by many researchers (Gast et al., 2004; Arnold et al., 2014). Both naturally and experimentally infected hens have been observed to produce eggs containing microorganisms within the content of eggs. Arnold et al. (2014) found a linear relationship between the prevalence of *Salmonella enteritidis* infected chickens and the rate of contamination of egg contents, while no relationship was been found between the infection prevalence and the rate of eggshell contamination. This suggests that *Salmonella enteritidis* contamination is more likely to take place in the reproductive organs than by eggshell contamination. Gast et al. (2004) evaluated the presence of *Salmonella enteritidis* and *Salmonella heidelberg* in reproductive tissues of infected laying hens and the liquid content of eggs. Chickens were administrated large oral doses of *Salmonella enteritidis* or *Salmonella heidelberg*. Both *Salmonella enteritidis* and *Salmonella heidelberg* were found in ovaries and oviducts at 7 days after inoculation, at frequencies as high as 66.7%. The observed frequencies of internal contamination of eggs ranged from 1.11% for *Salmonella heidelberg* to 7.05% for *Salmonella enteritidis*. This

observation suggested that *Salmonella enteritidis* had the higher ability to contaminate egg contents by trans-ovarian than other serovars.

In some instances, the trans-ovarian route has been considered less important for non-*Salmonella enteritidis* contamination, compared to trans-shell route. When hens orally consume contaminated feed with large numbers of organisms, they produce few contaminated eggs. The ovary and oviduct were surgically removed to examine the presence of microorganisms. All ovaries were found to be contaminated with bacteria; most of the bacteria recovered were determined to be Gram-positive micrococci and lactobacilli, while only 26.3% ova were found to be contaminated with bacteria by using direct plating method (Harry, 1963). This observation suggested that even when the ovaries are contaminated with bacteria, the presence of microorganisms in the content of egg is a very low number. Coliforms and *Pasteurella haemolytica* bacteria were infrequently isolated from oviducts. Schoeni and Doyle (1994) challenged 1-day-old laying hen chicks orally with *E. coli* O157:H7. *E. coli* O157:H7 colonization persisted in cecal tissue longer than 10 to 11 months when chicks were administered 10^8 *E. coli* O157:H7. *E. coli* O157:H7 was isolated from the shell of 14 of 101 (13.9%) eggs but not from the albumen and yolk. Another reason that trans-ovarian contamination prior to laying has been discounted as a major source of infection is that more than 90% of eggs are sterile at ovipostion (Board, 1966). Therefore trans-ovarian contamination has been considered of little importance for bacterial contamination.

2.5.2 Trans-shell contamination

The other route of bacterial contamination is trans-shell contamination (horizontal transmission). For trans-shell contamination, the shell can receive its first load of

microorganisms when passing through the cloaca. Following oviposition, the shell acquires microorganisms from all surfaces with which it makes contact with (Board and Tranter, 1986). The potential sources of contamination include dust, nesting material and feces (Board, 1966). The temperature of a freshly laid egg is generally warmer than ambient air. Bacteria can penetrate eggs through the cuticle-free pores when the eggs cool down. Negative pressure generated by the cooling process can pull the bacteria from the shell surface through the shell and its membranes (Padron, 1990). Stokes et al. (1956) reported that immersion of warm eggs into a cool suspension containing *Salmonella* for 30 min led to penetration of the intact egg by the bacteria. It has been observed that Gram-positive bacteria dominate the microflora on eggshells due to their tolerance of dry condition, whereas Gram-negative bacteria are the principal contaminants of rotten eggs (Board and Tranter, 1986). Trans-shell contamination with non-*Salmonella enteritidis* is probably the most important route. Williams et al. (1968) reported that *Salmonella typhimurium* penetrated the cuticle-free eggshell immediately under ideal moisture and temperature condition. They further noted that there was no relationship between shell thickness and the rate of eggs penetrated by *Salmonella typhimurium* when shell thickness was between 0.24 and 0.42 mm.

2.5.3 Extrinsic factors affecting trans-shell contamination

Several factors can affect trans-shell contamination. A positive relationship has been found between the rate of contamination of egg contents and environmental moisture, storage temperature, condition of cuticle and shell damage. Williams et al. (1968) reported that approximately 76.7% of cracked eggs were penetrated by *Salmonella typhimurium* through the shell after 2 h of incubation, compared to intact eggs at 5.7%.

2.5.3.1 Storage temperature

Microorganisms grow over a very wide range of temperatures. Warm ambient temperature enhances the penetration and multiplication of microorganisms. Bacterial penetration during storage at various temperatures has been studied by many researchers (Stokes et al., 1956; Schoeni et al., 1995; Chousalkar et al., 2010). The rate of *E. coli* penetrating eggshells increases with rising temperature. Sixty percent of eggs were penetrated by *E. coli* when stored at 37°C for 72 h, while 20% penetration was found at 4°C for 72 h (Chousalkar et al., 2010). Stokes et al. (1956) conducted an experiment to evaluate the effects of storage temperature on the rate of eggs contaminated by *Salmonella*. Intact warm eggs were immersed in a chilled suspension containing *Salmonella* for 30 min then incubated at 29°C or 1°C for 29 days. The egg contents were plated to determine total bacterial numbers. After 5 days incubation, the presence of *Salmonella montevideo* and *Salmonella pullorum* in the contents of eggs was over thousands of colonies, while there was no sign of colonization in eggs when stored at 1°C, for as long as 29 days. This suggested that storage of eggs at low temperature could prevent the possible infection with *Salmonella*.

2.5.3.2 Moisture

Eggshells can be penetrated by microorganisms when water, in both liquid and vapor states, is present (Bruce and Drysdale, 1994). Microorganisms can penetrate the eggshell and lead to extensive multiplication in the highly nutritious yolk when in suitable conditions of temperature and moisture. Smith et al. (2000) reported that increasing excreta moisture increased the risk of microbial contamination of intact eggs. Padron (1990) inoculated newly laid eggs by spraying with bacteria or by contacting eggs with contaminated dry nest

litter. The eggshell and membranes of all spraying treated eggs had *Salmonella* present, while only 59% of eggshell and membrane were penetrated by *Salmonella* after placing eggs on *Salmonella* contaminated dry nest box shaving for 10 min. This result suggests that water enhances bacterial penetration though the eggshell.

Refrigeration of eggs is often identified as one of the most critical issues in minimizing the risks associated with bacterial contamination of eggs. However, when eggs are removed from refrigerated storage and placed at room temperature, they may "sweat" due to condensation of moisture from the air (Bruce and Drysdale, 1994). Fromm and Margolf (1958) found that the egg contents were more likely to be contaminated by microorganisms when eggs were permitted to sweat for 3 h. However, Ernst et al. (1998) refuted this early finding. Sweating did not increase the number of eggs contaminated with *Salmonella enteritidis* in their study.

2.5.3.3 Amount of microbial contact on eggshell

In the case of healthy hens, egg contents are generally free from microorganisms when eggs are laid (Mayes and Takeballi, 1983). More frequently, the eggshell becomes contaminated after oviposition due to contact with feces or dust (Board, 1966; Quarles et al., 1970). There is ample evidence that eggs laid into a heavily contaminated environment had higher risk of spoilage than those laid into a clean environment (Bruce and Drysdale, 1994; De Reu et al., 2005). Messens et al. (2005) concluded that higher microbial load on the eggshell increased the risk of microbial penetration. Several studies have been conducted to evaluate the level of eggshell contamination under different housing systems (Smeltzer et al., 1979; Protais et al., 2003; De Reu et al., 2005; Samiullah et al., 2014). Quarles et al. (1970) reported that litter-floor houses had 9 times more bacteria in the air

and 20 to 30 times more aerobic bacteria on the surface of eggshells compared to wire-floor houses. They found that hatchability of eggs produced in wire floor pens was 3% higher than those from litter-floor pens. Ellen et al. (2000) reported that the dust concentration in perchery and aviary systems were 4 to 5 times higher than that in cage system. The total aerobic mesophilic flora count on the surface of eggshells produced in aviary system were at least one log unit higher than that in battery system due to the higher concentration of dust and bacteria in the air (Protais et al., 2003). Smeltzer et al. (1979) found that the eggs collected from an on-floor system had a significantly higher percentage of bacterial penetration (15.3%) than nest-collected eggs (10.5%). The total bacterial and *Enterobacteriaceae* load on the surface of eggshells were significantly higher in a free range system compared with a conventional cage system (Saminullah et al., 2014).

2.6 Methods for enumeration of bacterial penetration

A variety of methods have been conducted to evaluate the population of microorganisms from eggshells and shell membranes. The most commonly used methods involve surface soaks and rinses (Mellor and Banwart, 1965; Gentry and Quarles, 1972), blending eggshells and shell membranes (Brant and Starr, 1962), as well as surface swabbing and blending (Williams and Whittemore, 1967).

Mellor and Banwart (1965) described a very simple soaking method to evaluate the recovery of *Salmonella derby* from the surface of eggshells. The intact egg is first subjected to a positive temperature differential challenge by immersion in a cool bacterial suspension for 15 min. After drying, each egg is placed individually in jars containing broth medium. After 1 hour soaking, the egg is removed and the broth incubated at 37°C. This procedure was further modified by Gentry and Quarles in 1972. They mechanically homogenized the

shell before culturing. An individual freshly laid egg was placed into a sterilized polyethylene bag with 10 mL of sterile phosphate buffered saline. The egg was massaged by hand for 1 min before soaking in the buffer for an additional 5 min. Finally, the total bacterial concentration in buffer was determined by plating (Gentry and Quarles, 1972). Many shell rinse methods are a variation on Gentry and Quarles' method. In order to examine the total bacterial concentration from the inside and outside of the shell, Berrang et al. (1991) described a method in which individual eggs were aseptically cracked, and the empty eggshell was placed into a sterile plastic bag containing buffered peptone water. The eggshell and membranes were gently hand crushed and rubbed through the bag for 30 sec prior to sampling.

Williams and Whittemore (1967) developed a method to test bacterial invasion under simulated fecal contamination conditions. An aluminum tube was securely attached around the surface of an egg with a thin layer of paraffin. The aluminum tube was filled with chicken feces seeded with known bacteria. After inoculation, the egg was emptied and the inside of the eggshell was sampled with a swab. This method is excellent to examine the contamination of specific regions of the shell. However, processing time is also an important consideration in establishing the exact contamination levels; this method may not be adequate for microbial recovery of large numbers of samples. Board and Board (1967) developed a method whereby the entire egg surface can be quickly and easily tested for bacterial penetration. The intact egg was first subjected to a positive temperature differential challenge by immersion in a cool bacterial suspension. After drying, the egg contents were replaced by sterile microbiological growth medium containing 0.1% 2,3,5-triphenyl tetrazolium chloride. The egg was then incubated to allow for bacterial growth.

Where bacterial penetration occurred, bacteria growth in the presence of tetrazolium resulted in reduction to formazon, which is red in color. The agar-filled eggs were candled and visible red colonies on the interior surface of eggshells were recorded.

It is apparent that all methods used to examine the presence of microorganisms did not give the same opportunity for recovery from the eggshell. Penniston and Hedrick (1947) recovered bacteria on washed eggshells by using both a surface rinse method and blending eggshell and membranes. They reported that rinsing and blending methods were equivalent in their ability to recovery bacteria from washed eggs. However, Moats (1980) found that more bacteria were recovered by the blending method than by surface rinsing. That suggests that the microbial load recovery depends on whether bacteria reside on the surface or are embedded within the pores and membranes of the eggshell.

In conclusion, surface rinsing is a simpler, faster method for recovering microbial populations from eggshell surfaces. However, when eggs were washed or previously rinsed, the blending method was more effective at recovering bacteria. Choosing the appropriate method is an important consideration when evaluating the efficiency of a sanitation process.

2.7 Sanitation

Hatching eggs penetrated by pathogenic microorganisms may result in bacterial infection, which could increase the risk of embryo mortality or weaker chicks with poorer subsequent growth performance. Hatching egg sanitation is a common practice to improve success of chicken production. One of the most common routes for eggs to become contaminated with microorganisms is exposure to fecal matter (Hammack et al., 1993). A freshly laid egg is wet, warm and easily penetrated by microorganisms through the eggshell. Hatching eggs

should be collected 3 to 4 times daily to aid in the prevention of contamination with microorganisms (North and Bell, 1990). Jolles and Jolles (1984) stated that although hens have their own immunological systems, the eggs and developing embryos do not develop their own immunoglobulins until approximately 7 days before hatching. Therefore, hatching egg sanitation could provide added protection to the embryo from disease. In addition, hatching egg sanitation works best when it is applied as soon as possible after the eggs are laid and collected. Egg storage is a normal practice after egg collection and sanitation is not always performed prior to storage. The presence of microorganisms on the surface of eggshells could lead to the eggshell being penetrated and the interior of the egg being contaminated especially if storage conditions are suboptimum. Even when there is a small amount of bacteria present on the eggshell surface, it is still a potential hazard that increases the risk of bacterial infection during prolonged storage (Hammack et al., 1993; Gast et al., 2010).

2.7.1 Methods of sanitation of hen eggs

The main objective of egg sanitation is to eliminate bacteria on the eggshell surface and reduce the risk of cross-contamination among hatching eggs during incubation. Table egg washing with application of sanitizer is widely used for eggs consumed by humans in the United States, Australia, Canada and Japan to reduce the microbial load on eggshells. In the European Union, the washing of class A table eggs is not allowed (Hutchison et al., 2004). Brooks (1951) reported that the rate of spoilage for eggs increased when the washing process was carried out under less than optimal conditions. Several application methods are available for hatching egg sanitation. Chemical sanitizers are applied to the shell surface of hatching eggs by spraying, fumigating, washing and dipping (Brake and Sheldon,

1990; Scott and Swetnam, 1993; Fasenko et al., 2009; Spickler et al., 2011). Radiation treatments, ultraviolet (UV) light, pulsed light and X-ray radiation, are physical sanitation methods (Patterson et al., 1990; Wong et al., 1998; Russell, 2003). A novel electrostatic spray-charging system was used to increase the fumigant deposition on eggshell (Russell, 2003). In addition to the choice of sanitation methods, sanitizer concentration and exposure time need to be considered to achieve an effective sanitation process. An example of this is the dipping of chicken eggs in a chlorine dioxide solution reduced hatchability more than 60% with concentrations higher than 100 ppm for more than 5 min. However, embryo viability needs to be considered since prolonged dipping time reduced the hatchability to 11.8% when applying 40 ppm chlorine dioxide solution for 25 min (Patterson et al., 1990).

2.7.2 Sanitizers

Sanitizers, used on hatching eggs, have been evaluated by measuring the reduction of microbial load, hatchability, chick viability, cost-effectiveness and safety to hatchery workers (Shane and Faust, 1996). The properties of sanitizers are highly variable. Choosing the appropriate sanitizer is an important consideration for the sanitation process. Sanitizers may be grouped according to their base active ingredient. The active ingredients of sanitizer products commonly used in broiler breeder farms and hatcheries are chlorine, quaternary ammonium, hydrogen peroxide and formaldehyde.

2.7.2.1 Chlorine and chlorine dioxide

Chlorine is an effective sanitizer widely used in food processing plants to reduce the microbial load on vegetables, fruit and meat products. Elemental chlorine dissolved in water produces hypochlorous acid (HOCl), which has bactericidal action (North and Bell, 1990). Water containing 20 ppm to 250 ppm of chlorine can control spoilage bacteria and

extend poultry carcass shelf-life (Lillard, 1979; McKee et al., 1998; Singh et al., 2002). Shane and Faust (1996) reported that eggshells sprayed with chlorine solution until completely wet demonstrated effective activity against *E. coli* contamination. They determined the residual level of *E. coli* on the surface of eggshell by using surface rinse method and found applying 250 ppm chlorine solution achieved 98.7% *E. coli* reduction on the eggshell surface. Electrolyzed oxidizing water is produced by electrolysis of a weak salt water solution. The acidic solution produced on the positive charged side, contains approximately 50 mg/L free chlorine (pH between 2.5 and 4.5, and 1,150 mV oxidation-reduction potential), has been found to be effective against *E. coli* and *Salmonella enteritidis* on eggshells (Fasenko et al., 2009).

Chlorine dioxide reacting with the proteins in bacterial cell walls, results in damage to the cell and death of the microorganisms (Scott and Swetnam, 1993). Chlorine dioxide is five times as soluble as chlorine in water and has over two times the oxidizing capacity of HOCl (Lillard, 1979). For these reasons, chlorine dioxide could provide equivalent bactericidal activity as chlorine but at much lower levels of use. The bactericidal activities of chlorine-based solutions depend on the application methods. Patterson et al. (1990) conducted a novel method to treat hatching eggs with chlorine dioxide foam for 15 min before placement in an incubator. After 10 days of incubation, eggs were soaked in sterile Ringer's solution for 5 min to evaluate the population of microorganism on the surface of eggshells. Compared to the untreated eggs and formaldehyde fumigation method, hatching eggs subjected to chlorine dioxide foam significantly decreased the amount of *E. coli* on eggshell by 2.6 $\log_{10}$. They further reported that soiled duck eggs treated with 30 ppm chlorine dioxide foam for 15 min had a 10% increase in hatchability, compared to non-

treated eggs. However, chlorine dioxide can react with the protein complex and neutralize the cuticle of eggshells and increase the risk of bacteria penetrating eggshells (Scott and Swetnam, 1993).

2.7.2.2 Quaternary ammonium compounds

Quaternary ammonium has been regarded as an excellent sanitizer against microorganisms on eggshells. Scott and Swetnam (1993) evaluated the effectiveness of 23 sanitizers against microorganisms on eggshells. All quaternary ammonium-based sanitizers (Quat 800, Quam and Super Quam) provided effective activity to reduce the microbial load on eggshells. Brake and Sheldon (1990) found that treatment of hatching eggs with the quaternary ammonium-based disinfectant, Hatching Egg Sanitizer Spray® (HES), at 1.5% or 3.0%, over a period of 30 min of air drying (25°C), reduced total aerobic counts on the eggshell surface by 98.1% and 99.9%, respectively. The hatchability of eggs from a young flock, sprayed with 1.5% and 3.0% HES significantly increased by 6.3% and 6.2%, respectively, with no difference in the water loss during incubation among eggs sprayed with treatments. Quaternary ammonium is used as a disinfectant on floors and walls of hatcheries. However, the use of quaternary ammonium compounds in the poultry industry is banned in some countries. North and Bell (1990) recommended that quaternary ammonium compounds should be withdrawn five days before the birds are marketed.

2.7.2.3 Hydrogen peroxide

Hydrogen peroxide has been used as an antimicrobial agent since the early 1800's. The antimicrobial activity of hydrogen peroxide is due the production of powerful oxidants such as singlet oxygen, superoxide radicals and the hydroxyl radical (Davidson and Branen, 1993). Lillard and Thomson (1983) evaluated the effectiveness of hydrogen peroxide as a

bactericide in carcass chiller water. Water containing 11,000 – 12,000 ppm hydrogen peroxide reduced aerobic organisms and *E. coli* by 94% and 80%, respectively. Hydrogen peroxide has been used as a hatching egg sanitizer as well. Previous research stated that hatching eggs sprayed with 15,000 ppm hydrogen peroxide significantly reduced the total aerobic bacteria count per egg to 3.1 $\log_{10}$, which is significantly lower than the positive control (4.0 $\log_{10}$) (Spickler et al., 2011). Shane and Faust (1996) evaluated the *E. coli* reduction by surface rinsing method and reported that hydrogen peroxide demonstrated great activity against *E. coli* on the surface of eggshells. Disinfectant solution containing 1.5% hydrogen peroxide resulted in a 100% reduction in *E. coli* on eggshells, in contrast to distilled water which only removed 83.2% of *E. coli*.

2.7.2.4 Formaldehyde

Formaldehyde has been a standard disinfectant in poultry industry due to its bactericidal action and ease of application (Funk and Irwin, 1955). Formaldehyde applied by fogging significantly decreased the microbial load on eggshell surface. Williams (1970) reported that thirty-one nest-collected eggs fumigated with the recommended concentration of formaldehyde (1.2 mL of formalin with 0.6 g of potassium permanganate per cubic feet of cabinet space) for 20 min significantly reduced the total microbial load on eggshell by 97.5%. However, the Occupational Safety and Health Administration (1991) published information on toxicity of formaldehyde for farm workers. The potential effects of prolonged exposure to formaldehyde include headaches, nausea, drowsiness, respiratory impairment and kidney injury. Even though formaldehyde gas is effective against bacteria, an alternative sanitizer to formaldehyde, which is less toxic, inexpensive and efficient would be welcomed by the hatchery industry.

2.7.2.5 UV light

UV light naturally occurs in sunlight and is lethal to various types of microorganisms. The antimicrobial activity of UV light has been well documented. Research results indicate that UV light (254±3 nm) was effective against *E. coli* and *Salmonella senftenberg* on the surface of foodstuffs (Huang and Toledo, 1982; Wong et al., 1998).

UV light is used as a sanitation treatment in commercial poultry production based on its advantages including high antimicrobial activity, no residues, does not affect moisture and temperature, is economical and ease of use (Wong et al., 1998). UV light is a safe decontamination agent for eliminating bacteria on the surface of eggshells. Coulfal et al. (2003) treated visibly clean and unwashed eggs with UV light and evaluated the microbial load reduction on the surface of eggshells by using a surface rinsing sampling method. They reported that eggs exposed to UV light at 4 to 14 mW/cm^2 for 4 min significantly decreased the amount of *Salmonella typhimurium* and *E. coli* on the surface of eggshells by 10^4 and 10^5 times, respectively. No difference in hatchability was found between control eggs (84.5%) and eggs treated with UV irradiation (84.9%). Chavez et al. (2002) reported that exposure of unwashed eggs for 30 and 60 sec to UV light at 7.35 mW/ cm^2 significantly decreased the amount of aerobic bacteria on the surface of eggshells. Similarly, Gao et al. (1997) found applying UV light at 9,000 $\mu W/cm^2$ for 15 sec effectively reduced *Salmonella* on the surface of eggshells by 0.5 $\log_{10}$. In addition, Kuo et al. (1997) suggested that egg rotation during UV light exposure significantly improved the level of microbial inactivation on eggshells by increasing the surface area of exposure. They tested the effectiveness of UV light treatment at 4,350 $\mu W/cm^2$ for 20 min and found reduction in the microbial load on eggshell by 2.5 $\log_{10}$ when eggs were rotated at 1 rpm. These findings

indicated that UV light treatment at high intensities for a short exposure time could effectively decreased the risk of bacterial contamination.

2.7.2.6 Propolis

Propolis is a lipophilic resinous material collected by honeybees from living plants. It is used as a remedy in animal medicine for treating infection and a dietary supplement for human based on its antibacterial, antifungal and antiviral properties (De Groot, 2013). Aygun et al. (2012) tested the antimicrobial activity of propolis against *Salmonella* and coliforms on unwashed and visibly clean Japanese quail hatching eggs. They used whole-egg washing technique to determine bacterial load. It was reported that the microbial load on eggshells decreased with an increased propolis concentration. Thirty hatching eggs sprayed with 15 mL 15% propolis had reduced *Salmonella* and coliforms counts of 2.4 $\log_{10}$ and 3.2 $\log_{10}$, respectively. In addition, no significant difference in hatchability was found between spraying 70% ethyl alcohol treatment (97.8%) and the propolis treatment with concentration up to 15% (94.7%).

2.7.3 Lysozyme

Lysozyme is an enzyme discovered by Alexander Fleming in the 1920s. He demonstrated that lysozyme is present in avian egg white and is widespread in many animal tissues and secretions (Fleming, 1922). Lysozyme extracted from egg white is Type C lysozyme, which is a protein consisting of 129 amino acids with ~14,700 molecular weight (Johnson, 1994). Lysozyme extracted from egg white is widely used as a natural food preservative in food processing due to its antimicrobial activity and stability. Lysozyme can be frozen and is stable after drying. No inactivation occurs when lysozyme is dissolved in a phosphate buffer at 63°C for 10 min, while lysozyme is about 50 times more heat sensitive in egg

albumen than in phosphate buffer (Powrie and Nakai, 1986).

2.7.3.1 Antimicrobial activity of lysozyme

Lysozyme is defined as 1,4-β-N-acetylmuramidase. It acts to lyse bacterial cells by inserting a water molecule between N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) in the cell wall, which then degrades the polysaccharide of the bacterial cell wall (Salton, 1957). Lysozyme provides more effective antibacterial activity against Gram-positive bacteria than Gram-negative bacteria (Johnson, 1994).

Common uses of lysozyme include incorporation in vegetable, cheese, wine and pharmaceutical products (Hughey et al., 1989; Makki and Durance, 1997; Tenovuo, 2002). Several researchers have reported that *Bacillus stearothermophilus, Clostridium thermosaccharolyticum* and *Clostridium tyrobutyricum* can be completely inhibited by lysozyme. Lysozyme is moderately effective against *Listeria monocytogenes, Bacillus cereus, Campylobacter jejuni* and *Yersinia enterocolitica*. Hughey et al. (1989) evaluated the effectiveness of lysozyme against *Listeria monocytogenes* in fresh vegetables, pork sausage and cheese products. The result indicated that applying 100 ppm lysozyme with 5 mM EDTA on fresh vegetables effectively reduced *Listeria monocytogenes* by 4 $\log_{10}$. While lysozyme had less antimicrobial activity in animal derived foods, lysozyme with EDTA prevented the growth of *Listeria monocytogenes* in fresh pork sausage for 2 to 3 weeks but did not prevent eventual growth (Hughey et al., 1989). In the wine industry, lysozyme prevented the growth of a wide range of Gram-positive bacteria and increased amine levels (Gerbaux et al., 1997). In beer production, lysozyme at both 10 and 50 ppm prevented the growth of *Lactobacillus brevis* and *Pediococcus damnosus* which cause spoilage (Makki and Durance, 1997). For pharmaceutical application, lysozyme has been

added into oral health care products and human infant formula to improve the immune response of the host without side-effects (Proctor and Cunningham, 1988; Tenovuo, 2002).

2.7.3.2 Application of lysozyme in poultry production

Studies regarding the use of lysozyme as an effective sanitizer for poultry are relatively limited. Several studies have been conducted to evaluate the effectiveness of lysozyme as an alternative feed ingredient to antibiotics in poultry diets to reduce the incidence of intestinal disease. Initially Zhang et al. (2006) conducted an *in vitro* experiment to evaluate the inhibitory effect of lysozyme on *Clostridium perfringens*, which may cause necrotic enteritis in broilers. They found that lysozyme inhibited the growth of *Clostridium perfringens* at 156 ppm and an additional 50 ppm of lysozyme significantly decreased the amount of α-toxin produced. Furthermore, Liu et al. (2010) conducted a cage trial to evaluate the effectiveness of lysozyme as a feed ingredient against *Clostridium perfringens* in the broiler digestive tract. Their results indicated that adding 40 ppm lysozyme into the diet significantly decreased the amount of *Clostridium perfringens* by 1.51 $\log_{10}$ in the ileum of broilers without affecting broiler growth performance. MacIsaac and Anderson (2008) reported that turkeys fed 60 ppm dietary lysozyme had heavier body weights at 55 days of age compared to those fed diets without lysozyme or with antibiotics. Gong (2014) conducted a floor pen trial to evaluate the effect of dietary lysozyme provided during specific critical periods of the growth cycle on controlling broiler digestive tract microbial population. Broilers fed 100 ppm lysozyme during the starter period (5-14 days) reduced the number of *E. coli* in the ileum by 0.74 $\log_{10}$, compared to feeding antibiotics. Limited results have been published from studies focused on the effectiveness of lysozyme as a sanitizer for hatching eggs.

2.8 Knowledge gap

Lysozyme has been widely used as a food preservative and has the potential to be an alternative sanitizer to formaldehyde for hatching eggs. However, recommended concentrations of lysozyme and exposure time in this application have not been determined. The effects of lysozyme as an alternative sanitizer on the development of the embryos, hatching performance and growth performance are not documented. Methods used to determine the microbial load on the surface of eggshells are variable. While methods for evaluating the quality and quantity of microorganisms penetrating eggshells and contaminating eggs are not well documented. The results from these studies may provide specifications for the best combination of lysozyme concentration and exposure time to reduce bacterial contamination and improve the hatching success of broiler hatching eggs.

CHAPTER 3 GROWTH PERFORMANCE OF BROILER CHICKENS FROM FLOOR-COLLECTED HATCHING EGGS FOLLOWING APPLICATION OF LYSOZYME PRODUCT AS A SANITIZER

3.1 Abstract

Lysozyme, an enzyme extracted from egg albumen, has antimicrobial properties. Two experiments were conducted to investigate the effectiveness of application of EDTA modified lysozyme on hatching eggs as a control for bacteria associated with the egg surface. In Experiment 1, an eggshell model was used to determine effective concentration and time of exposure for application of lysozyme to eggs. One hundred eighty emptied and cleaned, physically intact eggshells were filled with nutrient agar. All agar-filled eggs were randomly divided into 12 groups with 3 replicates (5 eggs/rep/trt). Two groups were allocated to a positive control (PC), which was not sprayed with distilled water or lysozyme solution. The remaining 10 groups of eggs were fumigated with 1 of 5 lysozyme levels (0%, 0.75%, 1.50%, 2.25% or 3.00%) for each of 2 exposure times (10 min or 20 min). Fumigated eggs were submerged in a bacterial suspension containing 6.05 x 10^7 cfu/mL nalidixic acid-resistant *E. coli* for 5 min, then incubated at 37 °C for 48 h. Eggshells were candled and visual colonies were counted. In Experiment 2, 900 floor-collected Ross 308 broiler hatching eggs were randomly divided into 12 groups (25 eggs/rep/trt) then fumigated using the same sanitizer treatment combinations as Experiment 1. Hatching and growth performance data were subjected to analysis of variance using the Proc Mixed procedure of SAS. Growth data were analyzed as repeated measures. In Experiment 1, the number of bacteria present was not different for eggs fumigated for 10 min or 20 min within a lysozyme concentration treatment. Fumigation with 2.25% and 3.00% lysozyme solution reduced (P<0.05) *E. coli* to 17 cfu/egg and 13 cfu/egg, respectively, compared to the PC (55 cfu/egg). Chick hatching performance, mortality and growth performance were not affected by lysozyme level or exposure time.
Key words: Lysozyme, egg sanitation, hatching performance, growth performance, *Escherichia coli*

3.2 Introduction

Reducing the presence of microorganisms on the shell of chicken hatching eggs prior to incubation can improve hatching and growth performance. Microorganisms can penetrate the eggshell through the pores or cracks (Berrang et al., 1999) and contaminate the developing embryos. This may result in poor hatchability and chick quality, and have a negative impact on post-hatch growth performance (Williams, 1970; Arhienbuwa et al., 1980). Patterson et al. (1990) found that the hatchability of soiled Peking duck eggs (66.8%)

was lower than that of clean eggs (83.5%). Surface soaking and rinsing are common methods used to evaluate the microbial load on the surface of eggshells (Mellor and Banwart, 1965; Gentry and Quarles, 1972). In order to evaluate the quantity of bacteria penetrating eggshells, Board and Board (1967) inoculated eggs with a bacterial suspension then egg contents were replaced with sterile growth medium. This method is excellent to examine the quantity and location of bacterial penetration. However, cross-contamination while removing the egg contents and filling with growth medium is also a consideration in establishing the contamination level. Commercial sanitation of hatching eggs in the North American poultry industry traditionally involved fumigating with formaldehyde gas or spraying with chlorine solutions (Funk and Irwin, 1955; North and Bell, 1990). However, there are disadvantages to using each of these sanitizers. Formaldehyde poses a threat to worker health and safety (Occupational Safety and Health Administration, 1991). Chlorine solutions can damage the structure of the eggshell cuticle which is a natural barrier to bacteria and increase the risk of bacteria penetrating the eggshell (Scott and Swetnam, 1993).

Lysozyme is a natural enzyme extracted from egg albumen (Fleming, 1922) and provides antimicrobial activity by lysing the bacterial cell wall (Salton, 1957). Lysozyme applied to the surface of hatching eggs is a potential alternative sanitizer to other currently used sanitizers. Vegetable, cheese and wine producers have used lysozyme to effectively reduce the presence of a number of bacteria including *Listeria monocytogenes*, *Bacillus cereus, Campylobacter jejuni* and *Yersinia enterocolitica* (Hughey et al., 1989; Gerbaux et al., 1997; Makki and Durance, 1997). In poultry production, lysozyme has been used as an alternative feed ingredient to dietary antibiotics. Previous studies reported that adding

lysozyme to the diet decreased the amount of *Clostridium perfringens* and *E. coli* found in the ileum of broilers (Liu et al., 2010; Gong, 2014). However, there is no published research on using lysozyme as an alternative sanitizer for hatching eggs.

Surface soaks and rinses are common methods used to evaluate the reduction of microbial load on the surface of eggshells (Mellor and Banwart, 1965; Gentry and Quarles, 1972). However, these methods cannot evaluate the population of bacteria actually penetrating eggshells due to the influence of the eggshell defense mechanisms. A technique, which can determine the quantity of microorganisms penetrating eggshells and observe the site of penetration, is useful to evaluate the effectiveness of sanitizer.

3.3 Objectives

To develop a new technique to evaluate the quantity of *E. coli* penetrating the eggshell and contaminating the egg contents.

To evaluate the application of lysozyme to the surface of eggshells as a procedure to reduce the penetration of *E. coli* into eggs.

To evaluate the impact of surface applied lysozyme on hatching success of naturally soiled broiler hatching eggs and growth performance of broilers.

3.4 Hypotheses

It is hypothesized that the technique of using empty eggshells filled with nutrient agar to measure the ability of *E. coli* to penetrate the shell and evaluate the effectiveness of sanitizing treatments will be acceptable.

Surface application of lysozyme will reduce the penetration of *E. coli* into eggs.

Surface application of lysozyme, as a hatching egg sanitization process, will not have a negative impact on hatching performance and growth performance of broilers.

3.5 Materials and Methods

3.5.1 Eggshell assay experiment (Experiment 1)

3.5.1.1 Development of nalidixic acid-resistant *E. coli*

An *E. coli* isolate was originally collected from the contents of a 35-day-old Ross 308 broiler digestive tract. The isolate was cultured repeatedly with increasing levels of nalidixic acid (NA) (Sigma-Aldrich Co., St. Louis, MO, USA) to develop resistance to NA levels that most native bacteria of the chicken digestive tract would not growth in the presence of. Dilution of digestive tract contents in buffer peptone water (BPW) (Oxoid CM0509, Basingstoke, Hampshire, England) was followed by plating on 3M™ Petrifilm™ *E. coli* count plates (3M, St. Paul, MN, USA) and incubated at 37^{o} C for 48 h. Blue colonies with associated gas bubbles (Figure 3.1) appearing on the Petrifilm™ *E. coli* count plate were transferred to 10 mL BPW containing 20 mg/L NA. After 24 h of incubation, the active *E. coli* culture was diluted and plated on the Petrifilm™ *E. coli* count plates. After incubation, resistant *E. coli* colonies were transferred to 10 mL BPW containing 30 mg/L NA. Resistant *E. coli* were then added to 10 mL BPW and incubated for 48 h to culture the pure 30 mg/L NA-resistant *E. coli*. Well-isolated *E. coli* were stored at -80°C in 20% glycol until further use.

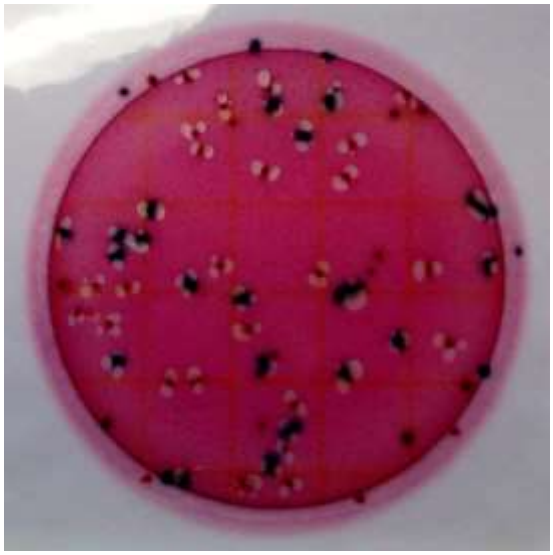

Figure 3.1 Culture of *E. coli* (blue colony with associated gas bubbles) on Petrifilm™ *E. coli*/ coliform count plates (© Xujie Li)

3.5.1.2 Eggshell preparation

One hundred and eighty infertile eggs (58.0 to 60.0 g) were collected from 32-wk-old Lohmann LSL-Lite laying hens housed at the Dalhousie University, Atlantic Poultry Research Centre (APRC). The selected eggs, within a narrow weight range, were considered to have similar surface areas. Eggs were not sprayed or otherwise sanitized before arrival. A small hole with a diameter of about 1 cm was drilled in the large end of each egg by using a DeWalt® cordless drill (DeWalt industrial tool Co., Baltimore, MD, USA). The drill was sanitized using pre-saturated wipes (Kimberly-Clark Professional, Roswell, GA, USA) between eggs. The egg contents were drained and the shell interior was rinsed with distilled water three times. The eggshells were placed in a plastic egg tray with large end down and dried at room temperature. After drying, all eggs were candled to ensure they were free of structural defects. All empty, cleaned and physically intact eggshells were filled with about 55 mL of plate count agar (Oxoid Ltd., Nepean, ON, Canada) containing 30 mg/L NA and 10 mL/L 1% 2,3,5-Triphenyltetrazolium chloride

(TTC) (Sigma-Aldrich Co., St. Louis, MO, USA) solution as a colorimetric indicator. The addition of NA assures that only resistant bacteria are able to grow. After media were solidified, the small hole was sealed with paraffin wax to prevent contamination through the opening.

3.5.1.3 Lysozyme preparation and application

A commercially available Lysozyme product (Inovapure™), naturally extracted from hen egg white, was provided by Neova Technologies Inc. (Abbotsford, BC, Canada). The lysozyme product had an enzymatic activity of 24,000 units/mg (Shugar, 1952). It was commercially prepared as a mixture with ethylenediaminetetraacetic acid (EDTA) at a ratio of 20:80 to enhance the antimicrobial activity against Gram-negative bacteria. All agar-filled eggs were randomly divided into 12 groups (15 eggs/group) with three replicates for each treatment combination of lysozyme level and exposure time. The positive control (PC) consisted of eggs placed in the fumigation room of Dalhousie University Hatchery for 10 or 20 min without fumigant application. The remaining ten groups of eggs were treated with 5 different levels of lysozyme solutions (0.00% 0.75%, 1.50%, 2.25% or 3.00%) for 10 or 20 min. For fumigation application, a Drysan-ss 9 MINI (United AGRI Systems Inc., Abbotsford, BC, Canada) was used to generate the sanitizer to dry and small particles (7 to 10 microns) by ultrasonication. Ten eggs were placed into the fumigation room at the beginning of each fumigation process. After 10 min of fumigation, 5 eggs allocated to the 10 min exposure time were removed. The remaining 5 eggs were fumigated for an additional 10 min. The order of sanitizer application within each replicate was randomized.

3.5.1.4 Egg inoculation and microbiological evaluation

After the fumigation process, all eggs were transported to a biosafety level II lab, to be submerged in a suspension of NA-resistant *E. coli*. The inoculation suspension was prepared by placing a loop of 30 mg/L NA-resistant *E. coli* culture into 800 mL of BPW containing 30 mg/L NA and incubated at 37°C for 12 h to initiate the log growth phase of the NA-resistant *E. coli*. The *E. coli* culture was mixed with 3 L of sterile BPW prior to use. The concentration of the final *E. coli* suspension was 6.05 x 10^7 cfu/mL. This was verified by plating dilutions of the final culture on a 3M™ Petrifilm™ *E. coli* count plate. All agar-filled eggs were submerged in the final NA-resistant *E. coli* suspension for 5 min. Agar-filled eggs were allowed to drip dry and were incubated at 37°C before sampling.

After 48 h of incubation, the eggs were candled to quantify bacterial penetration. Pink colonies, visible with the use of candling, were counted as *E. coli* (Figure 3.2). Any colonies seen to be continuous with the hole were assumed to be contamination during processing and not counted as a penetrating bacteria. Following the sanitation process, all eggs were handled aseptically with new clean rubber gloves for each treatment to prevent cross-contamination among eggs.

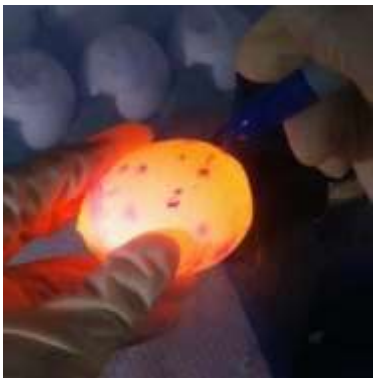

Figure 3.2 Culture of *E. coli* (pink spot) on the interior of an agar-filled eggshell (© Xujie Li)

3.5.2 Hatching trial (Experiment 2-A)

3.5.2.1 Sanitation procedure

Nine hundred floor-collected soiled hatching eggs were obtained from Ross 308 x Ross 308 broiler breeders (42 week of age) from a local hatchery. Eggs were not sprayed or otherwise sanitized before arrival. Before the sanitation process, the eggs with odd-shaped or highly visible contamination were removed and discarded. The remaining eggs were stored at 15°C with 75% relative humidity (RH) for 2 days before sanitation. Each egg was weighed and labeled on the side of the eggshell with a pencil. The average egg weight was 64.6±4.2g. All eggs were randomly divided into 12 groups with three replicates (25 eggs/replicate/treatment) and fumigated with the same sanitizer treatments used in the microbiological experiment described in Chapter 3.5.1.3.

The lysozyme solution preparation and sanitation process followed the methodology described in Chapter 3.5.1.3.

3.5.2.2 Incubation practice

All treated eggs were placed in setting trays with the narrow end down and incubated in

three Chick Master® G90 incubators (Chick Master®, Medina, Ohio, USA) for 18 days of incubation. The setting trays were positioned at a 45 degree angle, and moved 90 degrees every 90 min automatically to prevent the embryo sticking to the shell. Thirty-six eggs were placed in each setting tray with space between eggs to prevent physical contact (Figure 3.3). The incubators were preheated for 48 h to reach the recommended temperature and RH before eggs were set. All the eggs were incubated at a dry bulb temperature of 37.5°C and a RH of 55% for the first 18 days of incubation. The dry bulb temperature and RH of the incubators were recorded twice per day. No abnormal temperature was found during the first 18 days of incubation.

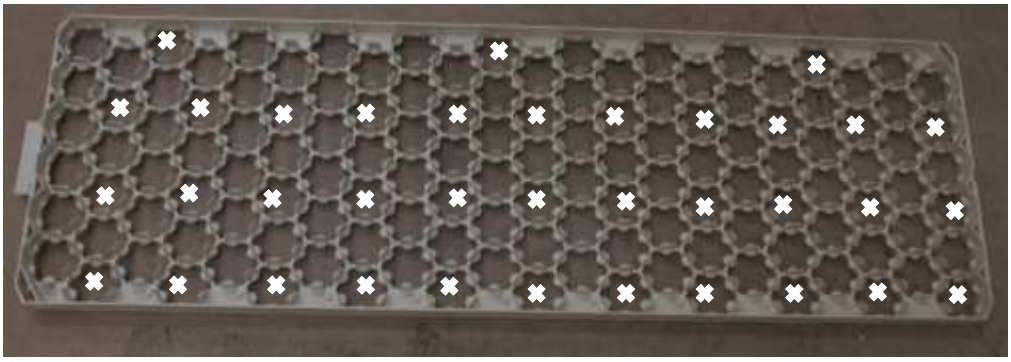

Figure 3.3 Placement positions of hatching eggs on setting tray to avoid egg to egg contact. "X" identified the setting location for each egg (© Xujie Li)

All eggs were candled at day 18 of incubation. The infertile and embryonic mortality eggs were removed and broken open to confirm stage of development. The remaining eggs were weighed individually to calculate moisture loss, and each viable egg was transferred into a plastic pot (width x depth x height: 10 cm x 8 cm x 8 cm) (Figure 3.4) to reduce cross-contamination among eggs during the hatching phase (day 19 to day 21 of incubation). A hole with a diameter of about 1 cm was drilled on each side of the plastic pot to minimize any impact of reduced ventilation. All plastic pots were delivered to the controlled environment room at the Dalhousie University, APRC in Truro, NS. The temperature and

RH of the controlled environment room was set as recommended (Table 3.1). Each egg was lift up straightly to check the pipping site. After the first sign of chick pipping, the hatching performance was monitored every 15 min until the hatch window closed (21 days plus 8 h of incubation). The pipping time (hours from setting), hatch time (hours from setting) and chick hatch weight (g) were recorded to establish chick quality. The spread of hatch, the dispersion around the average incubation duration, was calculated. All unhatched eggs were broken open to determine and record the extent of development.

Figure 3.4 Egg in a plastic pot during the hatching phase (© Xujie Li)

Table 3.1 The temperature and relative humidity of the controlled environment room during the hatch phase (day 18 to day 21 of incubation)

Time of Incubation	Temperature (ºC)	Relative Humidity (%)
Day 18-20	37.5	55%
Day 20	37.5	64%
Day 20.5	37.5	72%
Day 21	37.5	82%
4 hours before hatch window closed	37.5	55%

3.5.3 Broiler production (Experiment 2-B)

3.5.3.1 Post hatching practice

At the end of the hatching phase, all hatched chicks were counted, weighed and feather-sexed. Due to the presence of the rapid-feathering gene, female broilers' convert feathers are shorter than the primary feathers. In male broilers, the convert feathers usually have similar length as primary feathers (Figure 3.4). The chicks were vaccinated with 0.2 mL of Marek's vaccine and separated by sanitation treatments and sex for further processing.

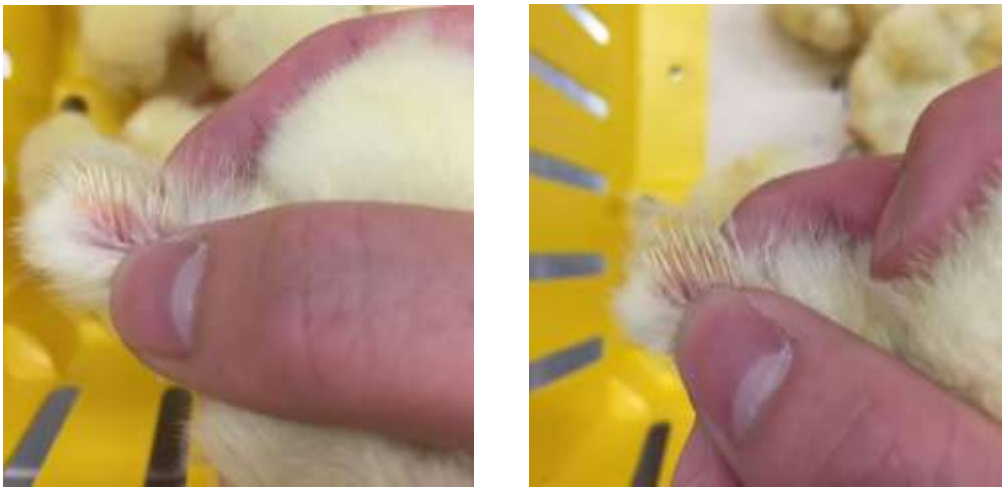

Figure 3.5 Feather sexing of newly hatched broiler chicks. Left: male chick. Right: female chick (© Xujie Li)

3.5.3.2 Bird rearing environment

After hatch, 188 male and 215 female Ross 308 broilers were transferred to a controlled environment room in the APRC and randomly placed into 72 battery cages (width x depth: 60cm x 48 cm), with 6 birds of the same sanitation treatment and sex in each cage. The temperature and lighting of the room were set as 32°C and 20 lux before the chicks arrived. The temperature was reduced by 1°C every 2 days until a temperature of 21°C was reached. The temperature was measured twice daily during health checks throughout the trial. The lighting was reduced by 5 lux every 4 days until 5 lux was reached, then maintained at 5

lux until the end of the trial.

Broiler chicks were managed under the guidance of the Animal Care and Use Committee following the guidelines of the Canadian Council on Animal Care guidelines (CCAC 2009). The diets were formulated to meet or exceed National Research Council (1994) nutrient requirements (Table 3.2). Birds were fed a nutritionally balanced starter, grower and finisher diet from day 0 to 14, day 15 to 24 and day 25 to 34, respectively. Feed in mash form was available from troughs attached at the front of each cage and water from nipple drinkers (2 nipple drinkers per cage) *ad libitum* throughout the trial. Mortalities were removed, as they occurred. The body weight of each dead bird was recorded. All mortalities were examined by a veterinary pathologist. Timing and cause of mortality were determined and recorded.

3.5.3.3 Growth performance measurement

The birds from each cage were weighed as a group on day 0, 7, 14, 24 and 34 using a balance equipped with live weight capability (Mettler PM 34-K Delta Range, Mississauga, ON, Canada). Feed was measured into the troughs as needed and feed remaining in the feeder was weighed on each weigh day and as mortality occurred. Using these data, daily feed consumption (FC), body weight (BW), daily body weight gain (BWG) and feed conversion ratio (FCR) were calculated.

Table 3.2. Ingredient composition and calculated analyses of the starter, grower and finisher diets for broilers (% as fed)

	Starter	Grower	Finisher
Ingredients (%)			
Corn	43.13	51.98	56.90
Soybean meal	38.98	30.98	25.85
Wheat	10.00	10.00	10.00
Tallow-grease blend	3.68	3.80	3.66
MCBS5[1]	0.50	-	-
MCBF5[2]	-	0.50	0.50
Mono-Dicalcium phosphate	0.83	0.47	0.63
Iodized salt	0.43	0.40	0.40
Methionine premix[3]	0.64	0.20	0.53
Lysine 98%	0.06	-	0.13
Limestone	1.67	1.59	1.40
Coban[4]	0.05	0.05	-
Stafac 44[5]	0.03	0.03	-
Calculated Analyses (as fed)			
MEn (kcal/kg)[6]	3050	3150	3200
Protein (%)	23.0	20.0	18.0
Calcium (%)	1.05	0.92	0.85
Nonphosphate phosphorus	0.50	0.40	0.42
Lysine (%)	1.43	1.15	1.09
Methionine (%)	0.69	0.44	0.58
Methionine+cystine (%)	1.07	0.76	0.86
Sodium (%)	0.19	0.18	0.18
Determined analysis			
Crude protein (%)	23.4	22.1	19.0
Total calcium (%)	0.95	0.78	0.81
Total phosphorus (%)	0.63	0.54	0.52

[1]MCBS5, Broiler starter premix (amount per tonne): Vitamin A ($1.00x10^9$ IU kg^{-1}), 1.56 g; Vitamin D3 premix ($3.00x10^7$ IU kg^{-1}), 16 g; Vitamin E ($5x10^5$ IU kg^{-1}), 10 g; Vitamin K (33%), 1.8 g; Riboflavin (80%), 1.9 g; DL Ca-pantothenate (45%), 6 g; Vitamin B12 (1000 mg kg^{-1}), 4.6 g; Niacin (98%), 6 g; Folic acid (3%), 26.6 g; Choline chloride (60%), 267 g; Biotin (400 ppm), 60 g; Pyridoxine (990000 mg kg^{-1}), 1 g; Thiamine (970000 mg kg^{-1}), 0.6 g; Manganous oxide (56%), 23.4 g; Zinc oxide (80%), 20.78 g; Copper sulfate (25%), 20 g; Selenium premix (1000 mg kg^{-1}), 14.85 g; Ethoxyquin (60%), 16.6 g; Ground corn, 401.31 g; Ground limestone, 100 g.

[2] MCBF5, Broiler grower and finisher premix: Vitamin A ($1.00x10^9$ IU kg^{-1}), 1.56 g; Vitamin D3 premix ($3.00x10^7$ IU kg^{-1}), 16 g; Vitamin E ($5x10^5$ IU kg^{-1}), 10 g; Vitamin K (33%), 1.8 g; Riboflavin (80%), 1.9 g; DL Ca-pantothenate (45%), 6 g; Vitamin B12 (1000 mg kg^{-1}), 4.6 g; Niacin (98%), 6 g; Folic acid (3%), 26.6 g; Choline chloride (60%), 267 g; Biotin (400 ppm), 60 g; Pyridoxine (990000 mg kg-1), 1 g; Thiamine (970000 mg kg-1), 0.6 g; Manganous oxide (56%), 23.4 g; Zinc oxide (80%), 20.78 g; Copper sulfate (25%), 20 g; Selenium premix (1000 mg kg-1), 14.85 g; Ethoxyquin (60%), 16.6 g; Ground corn, 401.31 g; Ground limestone, 100 g.

[3]Supplied kg premix-1: DL-Methionine, 0.5kg; wheat middlings, 0.5kg.

[4] Coccidiostat - Coban (active ingredient monensin sodium, 200 g kg-1) Elanco Animal Health, Division Eli Lilly Canada Inc., Guelph, ON, Canada.

[5]Antibiotic - Stafac 44 (active ingredient virginiamycin, 44 g kg-1) Phibro Animal Health Ltd., Regina, SK, Canada.

[6]Nitrogen-corrected apparent metabolizable energy.

3.5.4 Statistical analysis

3.5.4.1 Statistical analysis of eggshell assay

This study was a completely randomized design with 6 x 2 factorial arrangement with lysozyme level and exposure time as factors. Five eggs were used as the experimental unit. The factor lysozyme had six levels: no fumigant application, 0% (distilled water), 0.75%, 1.50%, 2.25% and 3.00% lysozyme. The factor exposure time had two levels: 10 min and 20 min. All bacterial penetration data were subjected to analysis of variance using the Proc Mixed Procedure of the SAS v.9.3 (SAS Inc., Cary, NC) (Littell et al. 1996). If significant effects ($P \leq 0.05$) were found, the Tukey-Kramer test was used to differentiate the means at $\alpha = 0.05$ (Gbur et al., 2012). The statistical model for the microbiological experiment analysis was:

$Y_{ijk}=\mu + \alpha_i+\beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$

Where:

Y_{ijk} was the variable of interest (visible *E. coli* colonies observed by candling);

μ was the overall mean of the response variable (visible *E. coli* colonies observed by using candling);

α_i was the effect of i^{th} lysozyme level (i=1-6);

β_j was the effect of j^{th} sanitation timing (j=1-2);

$\alpha\beta_{ij}$ was the effect of the interaction between lysozyme level and fumigation timing;

ε_{ijk} was the effect of uncontrollable factors.

3.5.4.2 Hatching performance

The experimental design was a randomized complete block design with 6 x 2 factorial arrangement with 6 lysozyme levels (no fumigant application, 0%, 0.75%, 1.50%, 2.25%

and 3.00% lysozyme) and 2 exposure times (10 and 20 min). The incubator was used as blocking factor. There were three blocks involved in experiment. The data for egg weight loss (%), hatchability (%), hatch weight (g) and the spread of hatch were subjected to analysis of variance using the Proc Mixed Procedure of the SAS v.9.3 (SAS Inc., Cary, NC) (Littell et al. 1996). If main effects or interaction effects were found to be significant ($P \leq 0.05$), then Tukey-Kramer test was used to differentiate the means at $\alpha = 0.05$ (Gbur et al., 2012). The statistical model of hatching performance data analysis was:

$$Y_{ijk} = \mu + \theta_i + \alpha_j + \beta_k + \alpha\beta_{jk} + \varepsilon_{ijk}$$

Where:

Y_{ijk} was the variable of interest (egg weight loss, hatchability, hatch weight and TPTH);

μ was the overall mean of the response variable (egg weight loss, hatchability, hatch weight and the spread of hatch);

θ_i was the effect of the blocking factor (i=1-3);

α_j was the effect of j^{th} lysozyme level (j=1-6);

β_k was the effect of k^{th} sanitation timing (k=1-2);

$\alpha\beta_{jk}$ was the effect of the interaction between lysozyme level and fumigation timing;

ε_{ijk} was the random effect of uncontrollable factors.

3.5.4.3 Growth performance

The experiment was a completely randomized design with 6 x 2 x 2 factorial arrangement with 6 lysozyme levels by 2 fumigation timings by gender of birds. Cage was used as the experimental unit, with three replicates for each treatment. Growth performance data including mortality were subjected to analysis of variance using the Proc Mixed Procedure of the SAS v.9.3 (SAS Inc., Cary, NC) (Littell et al. 1996). Growth performance data were

analyzed as repeated measures. In repeated measures analysis, three covariance structures, compound symmetry, toeplitz, and variance components were compared. The covariance structure which provided the smallest corrected Akaike Information Criterion (AICC) and Bayesian Information Criterion (BIC) values, was selected to conduct the ANOVA test. The covariance structure toeplitz was selected. If main effects or interaction effects were found to be significant ($P \leq 0.05$), then Tukey-Kramer test was used to differentiate the means at $\alpha = 0.05$ (Gbur et al., 2012). The statistical model of growth performance data analysis was:

$$Y_{ijklm} = \mu + \alpha_i + \beta_j + \delta_k + \alpha\beta_{ij} + \alpha\delta_{ik} + \beta\delta_{jk} + \alpha\beta\delta_{ijk} + \zeta_l + \alpha\zeta_{il} + \beta\zeta_{jl} + \delta\zeta_{kl} + \alpha\beta\zeta_{ijl} + \alpha\delta\zeta_{ikl} + \beta\delta\zeta_{jkl} + \alpha\beta\delta\zeta_{ijkl} + \varepsilon_{ijklm}$$

Where:

Y_{ijklm} was the variable of interest (FC, BW, BWG and FCR);

μ was the overall mean of the response variable (FC, BW, BWG and FCR);

α_i was the effect of i^{th} lysozyme concentration (i=1-6);

β_j was the effect of j^{th} sanitation timing (j=1-2);

δ_k was the effect of gender (k=1-2);

ζ_l was the effect of period (l=1-4);

$\alpha\beta_{ij}$ was the effect of the two-way interaction between lysozyme concentration and fumigation timing;

$\alpha\delta_{ik}$ was the effect of the two-way interaction between lysozyme concentration and gender;

$\beta\delta_{jk}$ was the effect of the two-way interaction between fumigation timing and gender;

$\alpha\zeta_{il}$ was the effect of the two-way interaction between lysozyme concentration and period;

$\beta\zeta_{jl}$ was the effect of the two-way interaction between fumigation timing and period;

$\delta\zeta_{kl}$ was the effect of the two-way interaction between gender and period;

$\alpha\beta\delta_{ijk}$ was the effect of three-way interaction among lysozyme concentration, fumigation timing and gender;

$\alpha\beta\zeta_{ijl}$ was the effect of three-way interaction among lysozyme concentration, fumigation timing and period;

$\alpha\delta\zeta_{ikl}$ was the effect of three-way interaction among lysozyme concentration, gender and period;

$\beta\delta\zeta_{jkl}$ was the effect of three-way interaction among fumigation timing, gender and period;

$\alpha\beta\delta\zeta_{ijkl}$ was the effect of four-way interaction among lysozyme concentration, fumigation timing, gender and period;

ε_{ijklm} was the random effect of uncontrollable factors.

3.6 Results and Discussions

3.6.1 Eggshell assay experiment (Experiment 1)

The method of replacing egg contents with the growth medium allowed the growth of NA-resistant *E. coli* and was able to evaluate the quantity of resistant *E. coli* penetrating the shell by candling (Figure 3.5). After 48 h incubation, the eggshells were candled and colonies on the interior of the eggshells were counted. The eggshells were also opened aseptically to check the growth of *E. coli*. It was found that the reduced TTC (pink spots) were deposited in both inner and outer shell membranes. It was concluded that NA-resistant *E. coli* had penetrated both the shell and shell membranes, and were thus able to contaminate the content of eggs. The technique described in this study would be useful to determine the quantity of microorganisms penetrating the eggshell. It has an advantage over the method described by Gentry and Quarles (1972). Eggs have their own defense

mechanisms against microorganisms invasion, measuring the microbial load on the surface of eggshells by shell soaking method (Gentry and Quarles, 1972) cannot evaluate the population of bacteria actually penetrating the eggshell or observe the site of penetration. The method described by Board and Board (1967) can quickly and easily test the bacterial penetration and determine the penetration site of the eggshell. The intact eggs were first submerged in the bacterial suspension, then the egg contents were replaced by growth medium. This method may underestimate the number of bacteria penetrating the eggshell. After inoculation, egg content was drained and the interior surface of eggshell was flushed with sterile water. The microorganisms may be killed by the protein in the egg albumen or removed during flushing process.

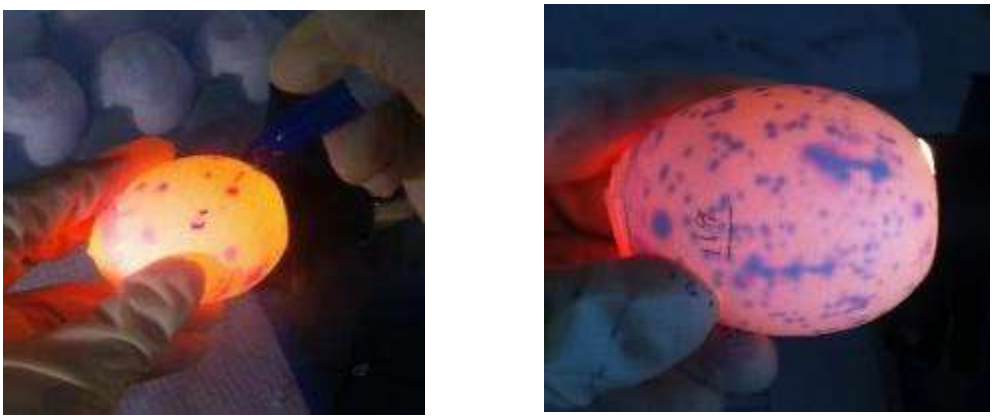

Figure 3.6 *E. coli* colonies grown on the interior surface of the eggshell. (Left: low-level contaminated egg; Right: high-level contaminated egg) (© Xujie Li)

Eggshell penetration results are presented in Table 3.3. All agar-filled eggs were penetrated by NA-resistant *E. coli* after submerging in the bacterial suspension. The application of different levels of lysozyme significantly ($P<0.05$) affected the penetration rate of *E. coli*. *E. coli* colonies growing on the interior surface of eggshells decreased with increasing lysozyme concentration. The lowest and the highest *E. coli* colonies counts found on the interior surface of the eggshells were determined for the 3.00% lysozyme treatment (13

cfu/egg) and no fumigant group (55 cfu/egg), respectively. Fumigating eggs with 3.00% lysozyme reduced *E. coli* penetration by 76%, compared to the no fumigant group. There were no significant differences (P>0.05) in the *E. coli* penetration rate between fumigating eggs with 2.25% and 3.00% lysozyme solutions. Both levels had a significantly lower *E. coli* penetration rate than that of fumigating with 0%, 0.75%, 1.50% lysozyme treatments and the no fumigant groups. In addition, no significant differences (P>0.05) in *E. coli* colony numbers occurred between 10 min and 20 min of fumigation. The highest level of lysozyme demonstrated the most effective activity against *E. coli* penetration.

Table 3.3 Effect of lysozyme concentration and fumigation time on mean *E. coli* counts (cfu egg^{-1}).

Lysozyme concentration	Fumigation time (min)		Concentration mean
	10	20	
	(cfu/egg)	(cfu/egg)	(cfu/egg)
No fumigant	55±11	56±11	55±7[a]
0%	49±11	51±11	50±7[a]
0.75%	30±11	36±11	33±7[a]
1.50%	26±11	43±11	34±7[a]
2.25%	15±11	19±11	17±7[b]
3.00%	11±11	15±11	13±7[b]
ANOVA	P-value		
Lysozyme concentration	**0.0024**		
Timing	0.3680		
Lysozyme concentration x Timing	0.9797		

[a-b]Means ± SE in the lysozyme concentration main effect with no common letters are significantly different according to Tukey-Kramer test (α = 0.05).

A previous study had shown that the total amount of *E. coli* on the surface of the eggshells was significantly reduced by applying chlorine dioxide (Patterson et al., 1990). Shane and Faust (1996) evaluated the effectiveness of chlorine solution on reducing eggshell *E. coli* load. They found spraying eggs with 250 ppm chlorine solution reduced *E. coli* on the surface of the eggshells by 98%. Effectively sanitizing hatching eggs at the breeder farm

can reduce the microbial load on the surface of eggshells and result in increased hatchability and chick quality. Gong (2014) found that adding 100 ppm of the same lysozyme product used in the current study to the diet of broiler chickens reduced the amount of *E. coli* in the ileum by 0.74 $\log_{10}$. The result in the current study clearly showed that the application of EDTA modified lysozyme on the surface of eggshells effectively decreased the total number of *E. coli* penetrating the eggshell and therefore reduced the risk of the egg contents becoming contaminated with *E. coli*.

3.6.2 Hatching trial (Experiment 2-A)

The concentration of lysozyme applied to soiled hatching eggs had no impact (P>0.05) on the percentage of egg weight loss during 18 days of incubation (Table 3.4). The percent of egg weight loss among treatments varied between 11.4% and 13.1%. Egg weight loss is an important parameter for incubation. Peebles et al. (1998) reported that excess moisture loss during the incubation period was disadvantageous for embryonic growth. Measuring egg weight loss during incubation is an indirect method to evaluate the level of damage to the cuticle. Damage to the cuticle increases egg weight loss due to an increase in water loss from the pores without cuticle covering (Peebles et al., 1998). No differences in egg weight loss during incubation among treatments were found in the current study. Therefore, it is suggested that fumigating broiler hatching eggs with lysozyme solution did not damage the shell cuticle. The direct stain method for measuring the deposition of cuticle described by Bain et al. (2013) is recommended for future studies.

Table 3.4 Effect of lysozyme concentration and fumigation time on egg weight loss (%) of hatching eggs during incubation.

Lysozyme concentration	Fumigation time (min)		
	10	20	Concentration mean
No fumigant	12±2.4	12±7.4	12±1.7
0%	11±2.4	12±2.4	12±1.7
0.75%	12±2.4	12±2.4	12±1.7
1.50%	12±2.4	13±2.4	13±1.7
2.25%	12±2.4	11±2.4	12±1.7
3.00%	12±2.4	12±2.4	12±1.7
Timing mean	12±1.0	12±1.0	
ANOVA	P-value		
Lysozyme concentration	0.9989		
Timing	0.9265		
Lysozyme concentration x Timing	0.9984		

No differences (P>0.05) on hatchability were found between eggs fumigated with lysozyme treatments and the no fumigant group (Table 3.5). No differences (P>0.05) in hatchability were observed between 10 min and 20 min exposure times. The results indicated that fumigating broiler hatching eggs with lysozyme solution did not negatively affect the hatchability of soiled broiler hatching eggs. However, the average hatchability was 70.3%, which is much lower than the standard commercial hatchability (87.7%) (Aviagen, 2011). The results suggested that floor-collected hatching eggs are not recommended for incubation, due to the heavy contamination of eggshell which increases the risk of microbial penetration. Messens et al. (2005) concluded that higher microbial load on eggshells increased the risk of microbial penetration. Patterson et al. (1990) reported that the hatchability of soiled Peking duck hatching eggs (66.8%) was significantly lower than that of visibly clean hatching eggs (83.5%). Our study agreed with these previous studies. A possible explanation for no improvement in hatchability with lysozyme treatments is that eggs were originally contaminated with bacteria prior to treating with sanitizer. Hatching egg sanitation should be applied as soon as possible after

the eggs are laid and collected. The effectiveness of hatchery sanitation and pathogen reduction in day-old chicks is limited if the eggs are already heavily contaminated (Coufal et al., 2003).

Table 3.5 Effect of lysozyme concentration and fumigation time on hatchability (%) of contaminated eggs.

Lysozyme concentration	Fumigation time (min)		
	10	20	Concentration mean
No fumigant	72±6.4	62±6.4	67±4.5
0%	85±6.4	72±6.4	79±4.5
0.75%	74±6.4	75±6.4	75±4.5
1.50%	80±6.4	70±6.4	75±4.5
2.25%	69±6.4	59±6.4	64±4.5
3.00%	60±6.4	66±6.4	63±4.5
Timing mean	73±2.6	67±2.6	
ANOVA	P-value		
Lysozyme concentration	0.0994		
Timing	0.1160		
Lysozyme concentration x Timing	0.6467		

No significant reduction in embryonic mortality (P>0.05) was found among treatments at early or middle stages of incubation (Table 3.6). This finding is in agreement with Elibol et al. (2003), who stated that disinfectants did not increase early embryonic mortality. A difference for late embryonic mortality rate among fumigation treatments was identified from the ANOVA (P=0.0206) (Table 3.6), but the Tukey-Kramer test did not differentiate the means (Table 3.7).

Although fumigating hatching eggs with lysozyme did not improve the hatchability in the current study, it was important that the lysozyme solution did not negatively affect hatchability and the development of chicken embryos.

Table 3.6 ANOVA P-value for the rate of early, middle and late embryonic mortality (%) of contaminated eggs fumigated with lysozyme treatments.

ANOVA	Early mortality[1]	Middle mortality[2]	Late mortality[3]
Lysozyme concentration	0.2240	0.7451	**0.0206**
Timing	0.2061	0.8468	0.2871
Lysozyme concentration x Timing	0.0571	0.1112	0.2272

[1]Number of dead embryos between 1 to 7 day of incubation; [2]Number of dead embryo between 8 to 14 day of incubation; [3]Number of dead embryo between 15 day of incubation to external pipping.

Table 3.7 Effect of lysozyme concentration and fumigation time on late embryonic mortality (%) of contaminated eggs.

Lysozyme concentration	Fumigation time (min)		
	10	20	Concentration mean
No fumigant	14±5.3	36±5.3	25±3.8
0%	12±5.3	11±5.3	12±3.8
0.75%	13±5.3	14±5.3	14±3.8
1.50%	12±5.3	15±5.3	14±3.8
2.25%	22±5.3	22±5.3	22±3.8
3.00%	30±5.3	26±5.3	28±3.8
Timing mean	17±2.2	21±2.2	

The fumigation treatments did not impact chick hatch weight (Table 3.8). Chick hatch weight is an indicator of chick quality (Tona et al., 2004). Our study accepted the hypothesis that fumigating hatching eggs with lysozyme did not negatively affect the chick quality.

Table 3.8 Effect of lysozyme concentration and fumigation time on day-old chick body weight (g bird^{-1}) hatched from contaminated eggs.

Lysozyme concentration	Fumigation time (min)		Concentration mean
	10	20	
No fumigant	47±1	46±1	46±1
0%	47±1	48±1	48±1
0.75%	47±1	47±1	47±1
1.50%	46±1	47±1	47±1
2.25%	47±1	46±1	47±1
3.00%	47±1	47±1	47±1
Time mean	47±1	47±1	
ANOVA	P-value		
Lysozyme concentration	0.9222		
Timing	0.8638		
Lysozyme concentration x Timing	0.9572		

Fumigation with lysozyme solutions did not affect (P>0.05) the time from pip to hatch. Total time required from pip to hatch is presented in Table 3.9. The results agreed with Takeshita and McDaniel (1982), who stated that the amount of time from pip to hatch was affected by egg incubation position and pip location. Pip primarily occurred in the large end when eggs were placed with the large end up. The chicks need longer time from pip to hatch when eggs are pipped in the small end, compared to the large end. The earliest hatched chicks stayed longer in the incubator which may cause dehydration.

Table 3.9 Effect of lysozyme concentration and fumigation time on the time (min) from pip to hatch of contaminated eggs.

Lysozyme concentration	Fumigation time (min)		Concentration mean
	10	20	
No fumigant	896±99	917±99	906±70
0%	966±99	868±99	917±70
0.75%	831±99	860±99	846±70
1.50%	864±99	1038±99	951±70
2.25%	985±99	1028±99	1007±70
3.00%	1179±99	843±99	1011±70
Time mean	954±40	925±40	
ANOVA		P-value	
Lysozyme concentration		0.5451	
Timing		0.6294	
Lysozyme concentration x Timing		0.2134	

Hatching began at 480 h of incubation for most groups (Table 3.10). Between 505 h and 510 h of incubation, the eggs fumigated with 0.75% lysozyme had a lower percentage of chickens hatched (11.3%) compared to 3.00% lysozyme treatment (31.4%). There were no significant differences among treatments at other hatching periods (Table 3.11). The information of the spread of hatch is useful to set up the properly condition of incubators for hatching eggs. A narrow hatch window (the time between the first and the last chick hatched) is important for hatchery production. A wider hatch window increases the number of chicks that have the additional holding time in the incubator. Extended post-hatching holding time delays water and food intake, and may cause chicks to become weak and dehydrated (Casteel et al., 1994).

Table 3.10 Effect of lysozyme concentration on spread of hatch for hatched chicks (% of chick hatched).

Lysozyme concentration (%)	Incubation time from setting (hours)					
	480-485	485-490	490-495	495-500	500-505	505-510
0	2.0±1.9	6.9±2.8	14.2±3.7	23.5±4.0	32.3±5.4	20.2±3.6^{abc}
0.75	2.1±1.9	5.8±2.8	20.9±3.7	32.8±4.0	27.3±5.4	11.3±3.6^{c}
1.50	4.4±1.9	2.1±2.8	17.3±3.7	25.7±4.0	32.7±5.4	17.0±3.6^{abc}
2.25	3.5±1.9	10.4±2.8	9.1±3.7	26.9±4.0	22.9±5.4	27.1±3.6^{ab}
3.00	3.0±1.9	2.3±2.8	10.1±3.7	19.3±4.0	33.9±5.4	31.4±3.6^{a}
No fumigant	4.0±1.9	9.2±2.8	7.6±3.7	30.1±4.0	36.4±5.4	12.7±3.6^{bc}

$^{a\text{-}c}$Means ± SE in the lysozyme concentration main effect with no common letters are significantly different according to Tukey-Kramer test ($\alpha = 0.05$).

Table 3.11 ANOVA P-value for the spread of hatch of contaminated eggs fumigated with lysozyme treatments.

	Incubation time from setting (hours)					
	480-485	485-490	490-495	495-500	500-505	505-510
Lysozyme concentration	0.9125	0.2136	0.1180	0.2498	0.5430	**0.0032**
Timing	0.6685	0.7100	0.6505	0.2958	0.1541	0.4406
Lysozyme concentration x Timing	0.1333	0.8002	0.1721	0.5442	0.0757	0.2277

3.6.3 Broiler production (Experiment 2-B)

P-value for effects of fumigating hatching eggs with lysozyme solutions on FC, BW, BWG and FCR are shown in Table 3.12. The concentration of lysozyme solution and exposure time had no effect on FC, BW and BWG of broiler chickens at any age when hatching eggs were applied with lysozyme treatments. FC and BW were affected by gender during different growth periods. BWG was affected by gender and different growth period. The FCR was affected by lysozyme level during different growth periods.

Table 3.12 ANOVA P-value for the effects lysozyme concentration, fumigation time, gender and age and their interactions on broiler chicken growth performance throughout a 34 days production cycle.

Effect	Feed Consumption	Body Weight	Body Weight Gain	Feed Conversion Ratio
Concentration (C)	0.5547	0.3585	0.5041	0.3670
Time (T)	0.9524	0.2236	0.3475	0.3477
C x T	0.4012	0.5081	0.2851	0.7075
Gender (G)	0.5483	0.0081	**0.0010**	0.1200
C x G	0.9970	0.9971	0.9862	0.7895
T x G	0.4293	0.8782	0.3392	0.5739
C x T x G	0.5801	0.5189	0.2352	0.1534
Age (A)	<0.0001	<0.0001	**<0.0001**	<0.0001
C x A	0.5044	0.2791	0.1579	**0.0068**
T x A	0.8041	0.5518	0.4150	0.4992
C x T x A	0.5618	0.6629	0.7527	0.7910
G x A	**0.0215**	**0.0002**	0.0589	0.6498
C x G x A	0.8934	0.9687	0.7051	0.7856
T x G x A	0.5513	0.1062	0.0606	0.4150
C x T x G x A	0.1317	0.5383	0.7931	0.8149

If the $p<0.05$, the effects are significant.

In current study, male broilers were heavier than female broilers at the end of both grower (day 24) and finisher (day 34) periods (Table 3.13). On day 34, the BW of male and female broiler chickens were 2098±10g and 2017±9g, respectively. The recommended

Table 3.13 Effect of lysozyme concentration and fumigation time on body weight (g bird^{-1}) of broiler chickens.

Lysozyme concentration	Time	Day 0		Day 7		Day 14		Day 24		Day 34	
		Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
No fumigant	10	43±1	44±1	125±5	133±5	412±14	407±14	1121±39	1092±39	2033±54	2007±54
	20	44±1	44±1	131±5	129±5	405±14	392±14	1160±39	1069±39	2092±54	1932±54
0%	10	45±1	46±1	137±5	139±5	426±14	431±14	1168±39	1110±39	2045±66	2061±54
	20	46±1	46±1	141±5	139±5	446±14	428±14	1200±39	1122±39	2194±54	2028±54
0.75%	10	46±1	44±1	139±5	135±5	429±14	413±14	1168±39	1123±39	2175±54	2055±54
	20	45±1	44±1	135±5	139±5	397±14	420±14	1129±39	1129±39	2059±54	2056±54
1.50%	10	45±1	44±1	138±5	134±5	442±14	412±14	1162±39	1115±39	2149±54	2045±54
	20	44±1	44±1	123±5	132±5	409±14	403±14	1063±39	1068±39	2093±66	1984±54
2.25%	10	46±1	44±1	135±5	131±5	436±17	413±14	1180±47	1129±39	2084±66	2091±54
	20	47±1	44±1	129±5	123±5	415±17	393±14	1097±47	1051±39	2093±66	1954±66
3.00%	10	45±1	45±1	128±5	129±5	413±14	394±14	1121±39	1058±39	2098±54	1967±54
	20	45±1	45±1	126±5	130±5	392±14	394±14	1051±39	1090±39	2076±66	2038±54
Gender x Age		45±10^{g}	45±9^{g}	132±10^{f}	132±9^{f}	419±10^{e}	408±9^{e}	1135±10^{c}	1096±9^{d}	2098±10^{a}	2017±9^{b}

$^{a\text{-}g}$Means ± SE in the same group: gender x age effects with no common letters are significantly different according to Tukey-Kramer test ($\alpha = 0.05$).

BW for male and female Ross 308 broiler at 34 days are 2179g and 1922g, respectively (Aviagen, 2014). The results suggested that the application of lysozyme solution on the surface of hatching eggs did not affect the final BW. Research describing the effect of applying lysozyme to hatching eggs on growth performance of broiler chickens is limited. Previous studies showed that the application of disinfectant on hatching eggs did not affect the growth performance of broiler chickens. Fasenko et al. (2009) found that spraying hatching eggs with sanitizer solution did not affect BW at the end of a 39 day growth period. Copur et al. (2010) applied oregano oil disinfectant and formaldehyde on the surface of broiler hatching eggs. The effects of essential oil and formaldehyde treatments on BW and BWG were not significant. In the current study, average daily body weight gain was only affected by gender and different growth periods. The average daily body weight gain of male broiler chickens was higher than that of female broilers (Table 3.14).

The application of lysozyme on the surface of the eggshell had no effect on FC during the 34 day growth period (Table 3.15). Gender affected daily FC during the grower period (day 15 to 24). Daily FC of male broiler chickens (107g $bird^{-1}$ day^{-1}) was higher than that of female broiler chickens (102g $bird^{-1}$ day^{-1}). The FCR was affected by different growth periods (Table 3.16). The FCR during the first week was poorer than that during the second week. The possible explanation is that feed provided inside the cage during the first week had elevated levels of wastage that could not be measured. The lysozyme level and exposure time had no effect on FCR during day 8-14, 15-24 and 25-34 (P>0.05) (Table 3.16). During day 0 to 7, FCR differences were detected by ANOVA (P= 0.0271), but the Tukey-Kramer test did not

Table 3.14 Effect of lysozyme concentration and fumigation time on body weight gain (g bird^{-1} day^{-1}) of broiler chickens.

Lysozyme concentration	Time	Day 0 - 7		Day 8 - 14		Day 15 - 24		Day 25 - 34	
		Male	Female	Male	Female	Male	Female	Male	Female
No fumigant	10	12±1	13±1	41±2	39±2	71±3	68±3	91±4	91±4
	20	13±1	12±1	39±2	38±2	76±3	67±3	93±4	86±4
0%	10	13±1	13±1	41±2	42±2	74±3	68±3	86±5	95±4
	20	13±1	13±1	55±2	41±2	75±3	69±3	99±4	91±4
0.75%	10	13±1	13±1	42±2	40±2	74±3	71±3	101±4	93±4
	20	13±1	14±1	38±2	40±2	74±3	71±3	93±4	93±4
1.50%	10	14±1	13±1	44±2	40±2	72±3	70±3	99±4	93±4
	20	11±1	12±1	41±2	39±2	65±3	67±3	99±5	92±4
2.25%	10	13±1	13±1	43±2	41±2	75±4	72±3	91±5	96±4
	20	12±1	11±1	41±2	38±2	68±4	66±3	100±5	89±5
3.00%	10	12±1	12±1	41±2	38±2	71±3	67±3	98±4	91±4
	20	12±1	12±1	38±2	38±2	66±3	70±3	106±5	95±4
Age		13±1[d]		40±1[c]		70±1[b]		94±1[a]	
Gender		Male		Female					
		55±0.4[x]		53±0.4[y]					

[a-d]Means ± SE in the age effect with no common letters are significantly different according to Tukey-Kramer test (α = 0.05)
[x-y]Means ± SE in the gender effect with no common letters are significantly different according to Tukey-Kramer test (α = 0.05)

Table 3.15 Effect of lysozyme concentration and fumigation time on feed consumption (g bird^{-1} day^{-1}) of broiler chickens.

Lysozyme concentration	Time	Day 0 - 7		Day 8 - 14		Day 15 - 24		Day 25 - 34	
		Male	Female	Male	Female	Male	Female	Male	Female
No fumigant	10	18±1	17±1	52±2	50±2	111±4	103±4	154±10	148±10
	20	16±1	17±1	51±2	50±2	100±4	103±4	141±10	151±10
0%	10	18±1	18±1	50±2	53±2	105±4	101±4	125±10	155±10
	20	19±1	18±1	57±2	51±2	115±4	104±4	163±10	156±10
0.75%	10	16±1	19±1	54±2	51±2	107±4	105±4	148±10	145±10
	20	20±1	19±1	52±2	55±2	109±4	101±4	151±10	144±10
1.50%	10	19±1	18±1	52±2	52±2	108±4	104±4	150±10	139±10
	20	23±1	19±1	48±2	50±2	108±4	99±4	132±10	147±10
2.25%	10	17±1	19±1	53±2	52±2	109±4	108±4	137±10	149±10
	20	19±1	17±1	52±2	49±2	101±4	99±4	139±10	127±10
3.00%	10	19±1	18±1	51±2	49±2	107±4	98±4	140±10	141±10
	20	17±1	17±1	49±2	50±2	109±4	101±4	131±10	139±10
Gender x Age		18±2^{e}	18±2^{e}	52±2^{d}	51±2^{d}	107±2^{b}	102±2^{c}	145±2^{a}	143±2^{a}

$^{a-e}$Means ± SE in the same group: gender x age effects with no common letters are significantly different according to Tukey-Kramer test ($\alpha = 0.05$).

Table 3.16 Effect of lysozyme concentration and fumigation on feed conversion ratio of broiler chickens.

Lysozyme concentration	Time	Day 0 - 7	Day 8 - 14	Day 15-24	Day 25-34
No fumigant	10	1.44±0.04	1 .02	1.54±0.02	1.70±0.08
	20	1.37±0.04	1.31±0.02	1.47±0.02	1.70±0.07
0%	10	1.36±0.04	1.24±0.02	1.51±0.02	1.62±0.08
	20	1.37±0.04	1.28±0.02	1.52±0.02	1.69±0.07
0.75%	10	1.34±0.04	1.26±0.03	1.50±0.02	1.52±0.07
	20	1.38±0.05	1.31±0.03	1.47±0.02	1.59±0.07
1.50%	10	1.40±0.04	1.26±0.02	1.50±0.02	1.50±0.07
	20	1.47±0.05	1.24±0.02	1.48±0.02	1.60±0.08
2.25%	10	1.42±0.05	1.26±0.03	1.49±0.02	1.54±0.08
	20	1.54±0.05	1.27±0.03	1.49±0.02	1.50±0.08
3.00%	10	1.48±0.05	1.27±0.02	1.50±0.02	1.56±0.08
	20	1.47±0.04	1.31±0.02	1.48±0.02	1.50±0.08
ANOVA		P-value			
Lysozyme concentration		**0.0271***	0.3605	0.8479	0.0970
Timing		0.2716	0.0528	0.1715	0.6362
Lysozyme concentration x Timing		0.3118	0.5697	0.7328	0.8590

*Tukey-Kramer option did not differentiate among these means.

differentiate means among lysozyme levels. The results of this study agree with the findings of Copur et al. (2010), who reported that no significant differences with respect to feed conversion ratio following essential oil and formaldehyde treatments.

No significant differences in mortality were observed among lysozyme levels and exposure time during the 34 days of growth period (Table 3.17). Total mortality was 12.0%, with most of these occurring in the finisher period (day 25 to 34). The majority of mortalities were caused by ascites. Low environmental temperature during post-hatch holding period may be a potential explanation for ascites. Ascites is also known as pulmonary hypertension syndrome (Ladmakhi et al., 1997). The factors that induce ascites include poor ventilation, low environment temperature and oxygen concentration, and pre-existing respiratory pathology (Shlosberg et al., 1992). Shlosberg et al. (1992) reported that lower than optimum temperature is the main cause for ascites.

Table 3.17 Effect of lysozyme concentration and fumigation time on mortality (%) of broiler chickens throughout a 34 days production cycle.

Lysozyme concentration	Fumigation time (min)		
	10	20	Concentration mean
No fumigant	6±5.3	17±5.3	11±3.8
0%	8±5.3	14±5.3	11±3.8
0.75%	14±5.3	8±5.3	11±3.8
1.50%	11±5.3	11±5.3	11±3.8
2.25%	11±5.9	18±5.9	14±4.2
3.00%	14±5.3	13±5.3	13±3.8
Time mean	11±2.2	11±2.2	

3.7 Conclusion

The current study demonstrated a new technique for measuring *E. coli* penetration though the eggshell was useful for evaluation of the effectiveness of sanitizers for reducing

eggshell bacterial contamination. Lysozyme was used to effectively to reduce *E. coli* penetration for eggs when applied at a level higher than 2.25%. Lysozyme did not negatively affect the hatching performance of floor-collected eggs or growth performance of broiler chickens.

Commercial hatching egg sanitizer was not included in the present study. Both commercial sanitizer and lysozyme should be tested to determine whether commercial sanitizer can be replaced by lysozyme for reducing bacterial infection of hatching eggs. Floor collected hatching eggs were covered with fecal material, which increased the risk of bacterial infection. These soiled eggs are not recommended for incubation process, even with the application of a sanitizer. Also the microbial loads on the surface of floor-collected eggs were variable. Visibly clean hatching eggs inoculated with a known bacterial strain and concentration should be considered in future studies.

CHAPTER 4 APPLICATION OF LYSOZYME AS A SANITIZER BEFORE AND AFTER INOCULATION OF E. COLI ON EGGS INCUBATED FOR HATCHING BROILER CHICKENS

4.1 Abstract

Two microbiological experiments were conducted to evaluate the effectiveness of EDTA modified lysozyme against *E. coli* on eggshells. An animal experiment was conducted to evaluate lysozyme applied to the surface of hatching eggs on hatching and growth performance of broiler chicks. In the first microbiological experiment, sixty agar-filled eggs were inoculated with 5.3 x 10^7 cfu/mL NA-resistant *E. coli* suspension for 1 min, then fumigated with distilled water (negative control), 1.50% or 3.0% lysozyme solutions or a quaternary ammonium product at 0.125% (positive control) for 10 min. In the second microbiological experiment, another sixty agar-filled eggs were fumigated with the same sanitizer treatments first, and then inoculated with the *E. coli* suspension for 1 min. All eggs were candled to detect *E. coli* growth on the interior surface of eggshells after incubation for 48 h at 37°C. In the animal experiment, a total of two thousand eighty hatching eggs were collected from a Ross 308 commercial breeder flock. All eggs were submerged in a nutrient broth containing 5.7 x 10^6 cfu/mL NA-resistant *E. coli* for 1 min. After drip drying, eggs were randomly divided into four fumigation treatment groups with four replicates. Fumigation treatments were as the first microbiological experiment. The eggs were incubated in 8 incubators (2 incubators per treatment) and the broiler chicks were grown to 33 days of age. Hatching and growth performance data were subjected to analysis of variance using the Proc Mixed procedure of SAS. Growth data were analyzed as repeated measures. In microbiological experiments, inoculated eggs fumigated with 3.00% lysozyme and 0.125% quaternary ammonium reduced (P<0.05) the total amount of *E. coli* to 11 cfu/egg and 10 cfu/egg, respectively. When eggs were treated with sanitizers prior to inoculation, 3.00% lysozyme demonstrated (P<0.05) continuous bactericidal action to prevent *E. coli* penetration. No significant differences were found in hatchability (P=0.058) among treatments (distilled water 89.8%; 1.50% lysozyme 93.3%; 3.0% lysozyme 89.7%; 0.125% quaternary ammonium 89.7%). Hatch weight (P<0.05) was significantly increased by applying 1.5% lysozyme solution to the eggs before incubation, with no effect on the ratio of yolk sac weight to yolk-free body weight (P>0.05) at hatch. Application of sanitizers decreased (P<0.05) the presence of NA-resistant *E. coli* in yolk sac of newly hatched chicks. Daily feed consumption, average body weight and feed conversion ratio were not affected (P>0.05) by treatments. However, average daily body weight gain was significantly reduced (P<0.05) in the chicks that hatched from eggs fumigated with 0.125% quaternary ammonium. Treatments did not influence chick mortality post-hatch. Overall 3.00% lysozyme demonstrated acceptable activity against *E. coli* on eggshell, and provided continuous bactericidal action to prevent *E. coli* penetration. 1.50% lysozyme solution improved hatch weight without negatively affecting growth performance.

Key words: Lysozyme, hatching performance, growth performance, *Escherichia coli*

4.2 Introduction

Numerous microorganisms can infect an egg before and after the egg is laid. The application of hatching egg sanitizer can decrease risk of bacterial contamination. The effectiveness of hatching egg sanitation is limited if the eggs are already heavily contaminated (Coufal et al., 2003). In an *in vitro* egg contamination model, the penetration of microorganisms through the cuticle, shell and shell membranes has been observed. Eggs inoculated with a marked bacterial strain and known concentration may be an effective way to evaluate bacterial penetration. Bacterial penetration may cause a rapid multiplication of bacteria in yolk material (Gast et al., 2006). Yolk sac infection is responsible for a significant amount of chicks mortality during the first week of the posthatch (Rai et al., 2005). Several studies have reported that surface application of lysozyme effectively reduced the population of *Listeria monocytogenes* in fresh vegetables, meat and wine products (Hughey et al., 1989; Gerbaux et al., 1997; Mangalassary et al., 2008). In addition, Mangalassary et al. (2008) reported that the pre-surface application of a nisin-lysozyme treatment was effective at preventing the growth of *Listeria monocytogenes* in turkey bologna for up to three weeks of storage. However, there are no published studies focused on determining the effect of applying lysozyme on the surface of eggshells prior to contamination with *E. coli*.

4.3 Objectives

To evaluate the effectiveness of lysozyme product against *E. coli* on contaminated eggshells.

To evaluate the anti-microbial activity of lysozyme product applied to eggshells prior to

contamination with *E. coli*.

To evaluate the effect of lysozyme on hatching and growth performance of broiler chicks when applied to the surface of hatching eggs under commercial production condition.

4.4 Hypotheses

Lysozyme will demonstrate acceptable activity against *E. coli* on eggshell, and provide continuous bactericidal action to prevent *E. coli* penetration.

Surface application of lysozyme will not negatively affect hatching performance of artificially inoculated hatching eggs and growth performance of broiler chickens.

4.5 Materials and Methods

4.5.1 Eggshell assay experiment

4.5.1.1 Bacterial isolate

The pure 30 mg/L NA-resistant *E. coli* culture isolation followed the protocols described in Chapter 3.5.1.1.

4.5.1.2 Eggshell preparation

One hundred and twenty infertile eggs weighing (58.0 to 60.0 g) were collected from 36-wk-old Lohmann LSL-Lite laying hens housed at the Dalhousie University, APRC. The formula for detection agar and the protocols for preparing agar-filled eggs were previously described in Chapter 3.5.1.2.

4.5.1.3 Sanitizer preparation and application

Quaternary ammonium (QA), a common disinfectant, used in food-processing application, was obtained from Sani Marc Group (Victoriaville, QC, Canada). Quaternary ammonium

bears a positive charge, which attaches to the negative charge of the microorganism and can damage the bacterial cell wall and result in cell death. The lysozyme preparation was as described in Chapter 3.5.1.3. One hundred and twenty agar-filled eggs were randomly divided into 2 groups (60 eggs/group). One group of eggs (post-treated group) were submerged in a nutrient broth containing 5.3 x 10^7 cfu/mL 30 mg/L NA-resistant *E. coli* culture for 1 min. Eggs were further divided into 4 groups with three replicates (5 eggs/replicate/treatment) then fumigated with distilled water (negative control), 1.5% or 3.0% lysozyme solutions or 0.125% QA (positive control) for 10 min. The other sixty agar-filled eggs (pre-treated) were fumigated using the same sanitizer treatments for 10 min. After 30 min air drying, all 60 eggs were submerged in the 5.3 x 10^7 cfu/mL NA-resistant *E. coli* suspension for 1 min. The fumigation application followed the protocols described in Chapter 3.5.1.3.

4.5.1.4 Microbiological sampling procedure

After incubation at 37°C for 48 h, eggs were candled and pink colonies were counted to evaluate bacterial penetration. Clean rubber gloves were required whenever the eggs were handled among treatments.

4.5.2 Hatching trial

4.5.2.1 Egg allocation and sanitation process

A total of 2080 cage-collected hatching eggs were collected from a 63-wk-old Ross 308 commercial breeder flock. Eggs were not sprayed or otherwise sanitized before arrival. Before the sanitation process, the odd-shaped eggs were removed and discarded. The remaining eggs were stored at 15°C and 75% relative humidity (RH) for 2 days before

sanitation. A subset of 160 eggs weighing within a narrow weight range (61.0 to 62.0 g) was identified, and labeled. All 2080 hatching eggs were submerged in a nutrient broth containing 5.7 x 10^6 cfu/mL *E. coli* for 1 min. After drip drying, eggs were randomly divided into four treatment groups with four replicates (Figure 4.1). Treatments included fumigation with distilled water (negative control), 1.5% or 3.0% lysozyme solutions and 0.125% quaternary ammonium (positive control) for 10 min.

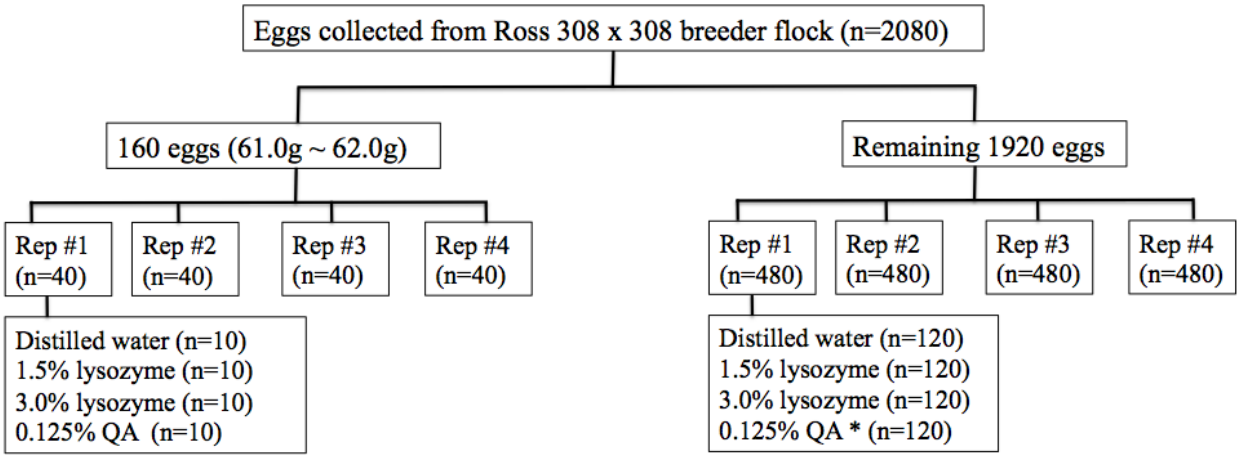

Figure 4.1 Flowchart showing the allocation of eggs to different treatment groups.

4.5.2.2 Incubation practice

All treated eggs were placed in the setting trays with the narrow end down and incubated in eight Chick Master® G90 incubators (Chick Master®, Medina, Ohio). During the first 18 days of incubation, the temperature was set at 37.5ºC and at 55% RH. On day 18 of incubation, the subset of eggs was placed into individual labeled plastic pots to evaluate chick quality individually. The remaining eggs were removed from the setting racks and transferred into hatching trays. Treated eggs followed the standard procedure of the

hatchery until the hatch window closed. The incubation condition schedule is listed in Table 4.1. After the hatch window closed, hatch weight and hatchability were recorded. The chicks hatched from the 160 subset eggs were euthanized by cervical dislocation and weighed. From these chicks, the yolk sacs were dissected, weighed and packaged using aseptic techniques for further microbiological testing.

Table 4.1 The temperature and relative humidity of the incubator during the incubation period.

Time of Incubation	Temperature (^{o}C)	Relative Humidity (%)
Day 1-20	37.5	55%
Day 20	37.5	64%
Day 20.5	37.5	72%
Day 21	37.5	82%
4 hours before hatch window closed	37.5	55%

4.5.3 Broiler production

4.5.3.1 Post hatching process

The process of gender identification and vaccination process followed the protocols described in Chapter 3.5.2.2.1.

4.5.3.2 Animal rearing environment

After 21.5 days of incubation, 640 male and 640 female day-old broilers (Ross 308 x Ross 308) were transported to APRC and randomly distributed into 32 floor pens, with 40 birds of same sanitizer treatment and sex in each pen. Each pen (2.13 m x 1.40 m) was prepared with wood shaving litter at a depth of 4 cm. The stocking density of 40 birds per pen was 0.07 m^2 $bird^{-1}$. Temperature and lighting program of the room were set (Table 4.2). The temperature was measured twice daily during scheduled health-checks throughout the trial.

Table 4.2: Temperature and lighting schedules for broiler chickens housed at Atlantic poultry research center during a 33 days production cycle.

Days post hatch	Temperature (^{o}C)	Light Hours	Light Intesity (lux)
0-1	32	24	20
2-3	31	23	20
4	30	23	20
5	30	16	15
6	29	16	15
7-8	29	16	10
9-10	28	16	5
11-12	27	16	5
13-15	26	16	5
16-17	25	16	5
18-19	24	16	5
20-22	23	16	5
23-26	22	16	5
27	21	16	5
28-31	21	17	5
32-33	21	18	5

All the procedure were carried out in accordance with the Canadian Council on Animal Care guidelines (CCAC 2009). The diets were formulated to meet or exceed National Research Council (1994) nutrient requirements (Table 4.3). The nutritionally balanced starter diet in crumble form was supplied from day 0 to 14. The grower and finisher diets in pellet form were supplied from day 15 to 25 and day 26 to 33, respectively. Starter diet was provided on cardboard box lids (width x depth x height: 53.3 cm x 43.2 cm x 5.1cm) during the first 7 days after placement. After day 7 of placement, feed was provided from tube feeders. Water was provided from three nipple drinkers per pen.

Table 4.3 Ingredient composition and calculated analyses of the starter, grower and finisher diets for broilers (% as fed).

	Starter	Grower	Finisher
Ingredients (%)			
Corn	43.58	43.72	51.00
Soybean meal	38.68	36.72	29.25
Wheat	10.00	10.00	10.00
Tallow-grease blend	3.97	5.37	5.10
MCBS5[1]	0.50	-	-
MCBF5[2]	-	0.50	0.50
Mono-Dicalcium phosphate	1.76	1.55	1.45
Iodized salt	0.45	0.42	0.42
Methionine premix[3]	0.60	0.50	0.49
Lysine 98%	-	-	0.01
Limestone	1.45	1.20	1.22
Amprolium[4]	0.01	0.01	-
BMD[5]	-	-	-
PEL-STIK[6]	-	-	0.50
Calculated Analyses (as fed)			
MEn (kcal/kg)[6]	3025	3150	3200
Protein (%)	23.0	21.0	18.0
Calcium (%)	1.05	0.90	0.85
Nonphosphate phosphorus (%)	0.50	0.45	0.42
Lysine (%)	1.30	1.17	0.97
Methionine+cystine (%)	0.94	0.84	0.76
Sodium (%)	0.19	0.18	0.18
Determined analysis			
Crude protein (%)	24.5	22.0	18.8
Total calcium (%)	0.97	0.96	0.778
Total phosphorus (%)	0.74	0.73	0.645

[1] MCBS5, Broiler starter premix (amount per tonne): Vitamin A ($1.00x10^9$ IU kg^{-1}), 1.56 g; Vitamin D3 premix ($3.00x10^7$ IU kg^{-1}), 16 g; Vitamin E ($5x10^5$ IU kg^{-1}), 10 g; Vitamin K (33%), 1.8 g; Riboflavin (80%), 1.9 g; DL Ca-pantothenate (45%), 6 g; Vitamin B12 (1000 mg kg^{-1}), 4.6 g; Niacin (98%), 6 g; Folic acid (3%), 26.6 g; Choline chloride (60%), 267 g; Biotin (400 ppm), 60 g; Pyridoxine (990000 mg kg^{-1}), 1 g; Thiamine (970000 mg kg^{-1}), 0.6 g; Manganous oxide (56%), 23.4 g; Zinc oxide (80%), 20.78 g; Copper sulfate (25%), 20 g; Selenium premix (1000 mg kg^{-1}), 14.85 g; Ethoxyquin (60%), 16.6 g; Ground corn, 401.31 g; Ground limestone, 100 g.

[2] MCBF5, Broiler grower and finisher premix: Vitamin A ($1.00x10^9$ IU kg^{-1}), 1.56 g; Vitamin D3 premix ($3.00x10^7$ IU kg^{-1}), 16 g; Vitamin E ($5x10^5$ IU kg^{-1}), 10 g; Vitamin K (33%), 1.8 g; Riboflavin (80%), 1.9 g; DL Ca-pantothenate (45%), 6 g; Vitamin B12 (1000 mg kg^{-1}), 4.6 g; Niacin (98%), 6 g; Folic acid (3%), 26.6 g; Choline chloride (60%), 267 g; Biotin (400 ppm), 60 g; Pyridoxine (990000 mg kg^{-1}), 1 g; Thiamine (970000 mg kg^{-1}), 0.6 g; Manganous oxide (56%), 23.4 g; Zinc oxide (80%), 20.78 g; Copper sulfate (25%), 20 g; Selenium premix (1000 mg kg^{-1}), 14.85 g; Ethoxyquin (60%), 16.6 g; Ground corn, 401.31 g; Ground limestone, 100 g.

[3] Supplied kg premix-1: DL-Methionine, 0.5kg; wheat middlings, 0.5kg.

[4] Amprolium -- AMPROL® 25% FEED MIX Huvepharma AD, Bio Agri Mix LP, Mitchell, ON, Canada (amprolium 25% w/w)

[5] BMD – Bacitracin Methlyene Disalicylate, Alpharma, Inc., Fort Lee, NJ, USA (providing 4.4 mg tonne-1 mixed feed)

[6] PEL – STIK -- Pellet Binder – Tembec Inc., Montreal, QC, Canada.

4.5.3.3 Data collection

Microbiological sampling

On hatch day, the chicks hatched from the 160 eggs subset were euthanized by cervical dislocation for collecting yolk sac samples. The chick weight, yolk sac weight and yolk sac free body weight were measured using a top pan balance (Thermo Fisher Scientific Inc. Waltham, MA, USA). The yolk sac sample was collected from each chick using aseptic techniques and placed in sterile plastic bags. All samples were transported to a biosafety level 2 lab, Faculty of Agriculture, Dalhousie University and stored at -80^{o}C freezer until analysis of presence of NA-resistant *E. coli*.

The eosin methylene blue (EMB) agar (BD Ltd., Mississauga, ON, Canada) containing 30 mg/L NA was poured into sterile petri plates and left to solidify. The yolk sac sample was placed in a filtered stomacher bag (Mix 2, AES Laboratories, Bruz, France), weighed and diluted 1:10 with BPW. Each yolk sac sample culture (10^{-1}) was further diluted to obtain 10^{-2} and 10^{-3} in BPW. One mL of each diluted culture was individually spread on the surface of the solidified agar plates and plated on Petrifilm™ *E. coli* count plates. All samples were inoculated in duplicate for each dilution. All plates were incubated at 37^{o}C. After 24 h, blue-black colonies with a green metallic sheen on EMB agar were enumerated as *E. coli*.

Growth performance measurement

Birds were mass weighed per pen on days 0, 7, 14, 25 and 33. The feed remaining in the feeders was weighed on each weigh day and as mortality occurred. Mortality was recorded and the dead birds were sent to the veterinary pathologist for necropsy (Animal Health Laboratory, Truro, Canada). Performance was determined by measuring FC, BW, BWG

and FCR.

4.5.4 Statistical analysis

4.5.4.1 Eggshell assay experiment

Each experiment was a completely randomized design. Five eggs were used as the experimental unit with three replicates for each treatment. The factor sanitizer had four levels: distilled water, 1.50% and 3.00% lysozyme and 0.125% QA. All bacterial penetration data were subjected to analysis of variance using the Proc Mixed Procedure of the SAS v.9.3 (SAS Inc., Cary, NC) (Littell et al. 1996). If significant effects ($P \leq 0.05$) were found, the Tukey-Kramer test was used to differentiate the means at $\alpha = 0.05$ (Gbur et al., 2012). The statistical model for microbiological experiment analysis was:

$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$

Where:

Y_{ij} was the variable of interest (*E. coli* colonies);

μ was the overall mean of the response variable (*E. coli* colonies);

α_i was the effect of i^{th} treatment level (i=1-4);

ε_{ij} was the random effect of uncontrollable factors.

4.5.4.2 Hatching trial

Hatching trial was a completely randomized design. One hundred and thirty eggs were used as the experimental unit with four replicates for each treatment. The factor sanitizer had four levels: distilled water, 1.50% and 3.00% lysozyme and 0.125% QA. Mean differences of the positive presence of 30 mg/L NA-resistant *E. coli* in yolk sac samples were separated by the Chi-square test pairwise comparison using MINITAB software. The data of

hatchability, hatch weight, yolk sac weight, yolk sac free body weight and yolk sac absorption were subjected to analysis of variance using the Proc Mixed Procedure of the SAS v.9.3 (SAS Inc., Cary, NC) (Littell et al. 1996). If significant effects ($P \leq 0.05$) were found, the Tukey-Kramer test was used to differentiate the means at $\alpha = 0.05$ (Gbur et al., 2012). The statistical model of hatching performance data analysis was:

$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$

Where:

Y_{ij} was the variable of interest (hatchability, hatch weight, yolk sac weight, yolk sac free body weight and yolk sac absorption);

μ was the overall mean of the response variable (hatchability, hatch weight, yolk sac weight, yolk sac free body weight and yolk sac absorption);

α_i was the effect of i^{th} lysozyme level (i=1-4);

ε_{ij} was the random effect of uncontrollable factors.

4.5.4.3 Broiler production

The experiment was a randomized complete block design with 4 x 2 factorial arrangement with 4 sanitizers (distilled water, 1.50% and 3.00% lysozyme and 0.125% QA) and 2 gender of birds. Room was the blocking factor. Pen was used as the experiment unit with four replicates for each treatment. All growth performance data were subjected to analysis of variance using the Proc Mixed Procedure of the SAS v.9.3 (SAS Inc., Cary, NC) (Littell et al. 1996). Growth performance data were analyzed as repeated measures. In repeated measures analysis, three covariance structures, compound symmetry, toeplitz, and variance components were compared. The covariance structure which provided the smallest

corrected Akaike Information Criterion (AICC) and Bayesian Information Criterion (BIC) numbers, was selected to conduct the ANOVA test. The covariance structure toeplitz was selected. If main effects or interaction effects were found to be significant ($P \leq 0.05$), then Tukey-Kramer test was used to differentiate the means at $\alpha = 0.05$ (Gbur et al., 2012). The statistical model of growth performance data analysis was:

$$Y_{ijkl} = \mu + \theta_i + \alpha_j + \beta_k + \alpha\beta_{jk} + \zeta_l + \alpha\zeta_{jl} + \beta\zeta_{kl} + \alpha\beta\zeta_{jkl} + \varepsilon_{ijkl}$$

Where:

Y_{ijkl} was the variable of interest (FC, BW, BWG and FCR);

μ was the overall mean of the response variable (FC, BW, BWG and FCR);

θ_i was the effect of blocking factor (i=1-2);

α_j was the effect of j^{th} sanitizer (j=1-4);

β_k was the effect of k^{th} gender of bird (k=1-2);

ζ_l was the effect of l^{th} period (l=1-4);

$\alpha\beta_{jk}$ was the effect of the two-way interaction between sanitizer and gender;

$\alpha\zeta_{jl}$ was the effect of the two-way interaction between sanitizer and period;

$\beta\zeta_{kl}$ was the effect of the two-way interaction between gender and period;

$\alpha\beta\zeta_{jkl}$ was the effect of the three-way interaction among sanitizer, gender and period;

ε_{ijkl} was the random effect of uncontrollable factors.

4.6 Results and Discussion

4.6.1 Eggshell assay experiment

For inoculated eggs, the NA-resistant *E. coli* population grown on the interior surface of

eggshells was significantly reduced by the application of sanitizers (Table 4.4). Compared to the eggs fumigated with distilled water (20 cfu/egg), the contaminated eggs treated with 3.00% lysozyme or 0.125% quaternary ammonium treatments had significantly reduced population of *E. coli* (11 cfu/egg and 10 cfu/egg, respectively). These results support the hypothesis that fumigating contaminated eggs with lysozyme treatment would reduce the risk of *E. coli* penetration. Cox et al. (2007) found that the application of a quaternary ammonium sanitizer reduced the population of *Salmonella* on eggshells by 95%. The results support our findings of a decrease risk for bacterial penetration when eggshells were sanitized with quaternary ammonium. Brake and Sheldon (1990) determined that quaternary ammonium effectively eliminated the total aerobic bacteria count on freshly laid eggshells by 99%, while no significant differences for coliform counts were found between eggshells sprayed with water and quaternary ammonium treatments due to the variable and small amount of coliforms present. The current study indicates that fumigating with quaternary ammonium and lysozyme treatments on the surface of eggshell would reduce *E. coli* load on the eggshell.

Table 4.4 Effect of applying sanitizer treatments after contamination (post-treated) on mean *E. coli* counts (cfu egg^{-1}).

Sanitizer	Colony
Distilled water	20 ± 2^{a}
1.50% Lysozyme	13 ± 2^{ab}
3.00% Lysozyme	11 ± 2^{b}
0.125% QA*	10 ± 2^{b}
ANOVA	P-value
Sanitizer	**0.0005**

[a-b]Means ± SE in the sanitizer main effect with no common letters are significantly different according to Tukey-Kramer test ($\alpha = 0.05$).
*Quaternary ammonium.

When eggs were sanitized prior to inoculation, 3.00% lysozyme reduced the number of *E. coli* colonies penetrating the shell (10 cfu/egg) compared the distilled water treatment (22 cfu/egg) (Table 4.5). The result indicates that lysozyme provided continuous protection that prevented *E. coli* from penetrating eggshells. A previous study showed that the pre-surface application of a nisin-lysozyme treatment effectively prevented the growth of *Listeria monocytogenes* in turkey bologna for up to three weeks of storage (Mangalassary et al., 2008). The above result supports our findings that applying lysozyme on the surface of eggshell reduces the total amount of *E. coli* on the eggshells and provides continuous bactericidal action to prevent *E. coli* penetration.

Table 4.5 Effect of applying sanitizer treatments prior to contamination (pre-treated) on mean *E. coli* counts (cfu egg^{-1}).

Sanitizer	Colony
Distilled water	22 ± 2^{a}
1.50% Lysozyme	14 ± 2^{ab}
3.00% Lysozyme	10 ± 2^{b}
0.125% QA*	16 ± 2^{ab}
ANOVA	P-value
Sanitizers	**0.0321**

[a-b]Means ± SE in the sanitizer main effect with no common letters are significantly different according to Tukey-Kramer test ($\alpha = 0.05$).
* Quaternary ammonium.

4.6.2 Hatching trial

The difference of egg weight loss during 18 days incubation among sanitizer treatments was identified from ANOVA ($P<0.05$), but Tukey-Kramer test did not differentiate the means among treatments (Table 4.6). The rates of egg weight loss varied between 8.54% and 10.15% among all treatments. The eggs treated with 1.50% lysozyme had the lowest rate of egg weight loss. Egg weight loss is an important parameter for assessing incubation condition and eggshell porosity (Scott and Swetnam, 1993). Brake and Sheldon (1990) reported that the application of quaternary ammonium on the surface of eggshell did not affect the moisture loss during incubation. However, Scott et al. (1993) evaluated the effect of quaternary ammonium-based sanitizers on moisture loss during incubation. The hatching eggs treated with sanitizer solutions containing quaternary ammonium had lower moisture loss than that applied with formaldehyde treatment. The possible explanation for differences on moisture loss is that the sanitizer application methods affect the deposition of cuticle.

Table 4.6 Egg weight loss (%) of artificial inoculated eggs during 18 days incubation.

Sanitizer	Egg weight loss (%)
Distilled water	10.0±0.39
1.50% Lysozyme	8.5±0.39
3.00% Lysozyme	10.1±0.39
0.125% QA*	10.2±0.39
ANOVA	P-value
Sanitizer	**0.0395****

* Quaternary ammonium.
**Tukey-Kramer option did not differentiate among these means.

The hatchability of eggs fumigated with sanitizers is shown in Table 4.7. No differences in hatchability of fertile eggs were found among sanitizer treatments, indicating that fumigating eggs with lysozyme or quaternary ammonium solution did not negatively affect the hatchability of broiler hatching eggs. The hatchability for artificially inoculated hatching eggs treated with distilled water, 1.50% lysozyme, 3.00% lysozyme and 0.125% quaternary ammonium solutions were 89.75%, 93.32%, 89.67% and 89.72%, respectively. These results agreed with Scott et al. (1993), who found no significant differences between using quaternary ammonium and formaldehyde as disinfectants for broiler hatchability. In addition, Brake and Sheldon (1990) reported that the application of quaternary ammonium increased the hatchability of fertile eggs from a 32-week-old flock but did not have the same effect when used on eggs from an older flock. The improved hatchability in the eggs from the younger flock could be due to the decreased deposition of cuticle as the flock ages (Sparks and Board, 1984). For the hatching eggs with poor quality cuticle, the embryo may be contaminated with bacteria before applying sanitizer.

Table 4.7 Hatchability (%) of eggs inoculated with *E. coli* and sanitized.

Sanitizer	Hatchability (%)
Distilled water	89.75±0.99
1.50% Lysozyme	93.32±0.99
3.00% Lysozyme	89.67±0.99
0.125% QA*	89.72±0.99
ANOVA	P-value
Sanitizer	0.0582

* Quaternary ammonium.

Chick hatch weight is often used as an indicator of chick quality. For chicks hatched in this study, the chicks from eggs fumigated with lysozyme and quaternary ammonium treatments had significantly higher body weight compared with the chicks that hatched from the control group (Table 4.8).

Table 4.8 Chicks hatch weight (g $bird^{-1}$) of chicks from eggs fumigated after being inoculated with *E. coli*.

Sanitizer	Hatch weight
Distilled water	48 ± 0.3^{b}
1.50% Lysozyme	50 ± 0.3^{a}
3.00% Lysozyme	49 ± 0.3^{a}
0.125% QA*	49 ± 0.3^{a}
ANOVA	P-value
Sanitizer	**0.0038**

[a-b]Means ± SE in the sanitizer main effect with no common letters are significantly different according to Tukey-Kramer test ($\alpha = 0.05$).
* Quaternary ammonium.

There were no significant differences for yolk sac weight (Table 4.9) and yolk sac weight as a percentage of yolk-free body weight (Table 4.10). In this experiment, the yolk sac samples could only be collected if the egg resulted in a chick. For this reason, the numbers of yolk sac sample varied between treatments. For samples collected from eggs fumigated

with distilled water, 1.50% lysozyme, 3.00% lysozyme and 0.125% quaternary ammonium treatments, there were 35, 32, 32 and 29 chicks, respectively. The weight of the residual yolk sac is an indicator of energy utilization by the embryo during development. A reduced yolk sac may indicate the embryo development is more mature (Deeming, 2005). Our results indicated that applying lysozyme treatments did not have a negative effect on the energy utilization by the embryo during development.

Table 4.9 Yolk sac residue (g) of chicks from eggs fumigated after being inoculation with *E. coli*.

Sanitizer	Yolk sac residue
Distilled water	6.3±0.20
1.50% Lysozyme	6.3±0.20
3.00% Lysozyme	6.2±0.20
0.125% QA*	6.7±0.20
ANOVA	P-value
Sanitizers	0.3734

* Quaternary ammonium.

Table 4.10 Effect of sanitizers' fumigation on the ratio (g/g*100) between yolk sac weight and yolk-free body weight of contaminated eggs.

Sanitizer	Ratio (yolk sac : yolk-free body weight)
Distilled water	15.1±0.53
1.50% Lysozyme	14.5±0.53
3.00% Lysozyme	14.3±0.53
0.125% QA*	15.6±0.53
ANOVA	P-value
Sanitizers	0.3068

* Quaternary ammonium.

When the trans-ovarian route of contamination of a chicken egg occurs, the bacteria are deposited in the yolk from the infected ovary or other parts of the reproductive tract. In this

case, penetration of bacteria into egg contents may occur during the incubation period. The results of NA-resistant *E. coli* present in the yolk sac sample of newly hatched chicks are shown in Table 4.11. Fourteen out of 35 yolk sac samples from the eggs treated with distilled water treatment were observed to contain NA-resistant *E. coli*. The yolk sac samples from day-old chicks hatched from eggs fumigated with 1.50% lysozyme, 3.00% lysozyme and 0.125% quaternary ammonium were all lower than that amount and not different from each other. In the current study, the application of sanitizers decreased the risk of penetration of *E. coli* into hatching eggs where contamination of the developing embryos during incubation can occur. Kizerwetter-Swida and Binek (2008) measured the bacterial counts in liver and yolk sac of chicks hatched from the eggs without sanitation. *E. coli* was present in 14 out of 25 yolk sac samples with an average number 1.16×10^6 cfu/g. The penetration of pathogenic bacteria to the yolk leads to infection of the embryo. The APEC is one of the most common bacteria isolated from yolk sac of infected chicks (Dho-Moulin and Fairbrother, 1999). The infection of the yolk sac increases the mortality of young chicks during the first week of the post-hatching period (Rai et al., 2005). Fumigating hatching eggs with lysozyme greatly reduced this problem and improved chick quality. In future studies, evaluating bacterial counts in yolk sac and other organs during growth period is recommended.

Table 4.11 Effect of sanitizer fumigation on the NA-resistant *E. coli* positive in yolk sac samples.

Sanitizer	NA-resistant *E. coli* positive sample/total sample
Distilled water	14/35[a]
1.50% Lysozyme	0/32[b]
3.00% Lysozyme	1/32[b]
0.125% QA*	0/29[b]

[a-b]Means in the sanitizer main effect with no common letters are significantly different according to Chi-square test pairwise comparison ($\alpha = 0.05$).
* Quaternary ammonium.

4.6.3 Broiler production

On day 25 post hatch, BW of male chicks was heavier than that of female chicks (Table 4.12). There were no differences in final body weight at day 33 among sanitizer treatments. On day 33, the BW of male and female broiler chickens were 2491±7g and 2171±7g, respectively. The expected BW for male and female Ross 308 broilers at 33 days are 2075g and 1838g, respectively (Aviagen, 2014). The application of lysozyme and quaternary ammonium on the surface of eggshell did not affect the final BW. Limited studies have evaluated the effect of sanitizer on broiler production parameters. Results of the current study agreed with Fasenko et al. (2009), who reported no significant differences on BW at the end of a 39 days growth period when hatching eggs were treated with electrolyzed oxidizing water. Likewise, Copur et al. (2010) found that that the use of oregano oil or formaldehyde as hatching egg sanitizers did not affect broiler BW and BWG during the production period.

Table 4.12. Effect of sanitizers applied to *E. coli* inoculated hatching eggs on body weight (g bird^{-1}) of broiler chickens.

Sanitizer	Day 0		Day 7		Day 14		Day 25		Day 33		Treatment mean
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	
Distilled water	47±1	47±1	193±2	183±2	533±5	486±5	1546±20	4326±20	2537±23	2171±23	907±6
1.5% Lysozyme	48±1	48±1	189±2	186±2	517±5	495±5	1520±20	1363±20	2510±23	2181±23	906±6
3.0% Lysozyme	47±1	46±1	186±2	189±2	527±5	492±5	1524±20	1356±20	2483±23	2182±23	903±6
0.125% QA*	47±1	47±1	182±2	180±2	507±5	487±5	1505±20	1343±20	2434±23	2149±23	888±6
Gender x Age	47±7^{h}	47±7^{h}	187±7^{g}	184±7^{g}	521±7^{e}	490±7^{f}	1524±7^{c}	1347±7^{d}	2491±7^{a}	2171±7^{b}	

ANOVA	P-value
Room	0.1175
Sanitizer	0.2516
Gender	<0.0001
Sanitizer x Gender	0.3525
Age	<0.0001
Sanitizer x Age	0.1046
Gender x Age	**<0.0001**
Sanitizer x Gender x Age	0.7051

$^{a-h}$Means ± SE in the same group: Gender x Age effects with no common letters are significantly different according to Tukey-Kramer test ($\alpha = 0.05$).
* Quaternary ammonium.

BWG for the chicks hatched from the eggs fumigated with 0.125% quaternary ammonium was significantly ($P<0.05$) lower than those fumigated with distilled water, 1.50% lysozyme and 3.00% lysozyme (Table 4.13). During the 33 day growth period, the 0.125% quaternary ammonium treated chicks gained 64±0.4g per day, while the other treatment chicks gained 66±0.4g per day. Hatching eggs treated with lysozyme as sanitizer did not negatively impact the BWG of broilers during the entire production period. There was no significant difference in BWG between male and female chicks during the first week. After day 7, the BWG of male chicks was higher than that of female chicks. The differences of BWG between male and female chicks during the grower and finisher periods agreed with the breeder company expectations (Aviagen, 2014). Male chicks are expected to grower faster during grower and finisher periods.

Gender affected FC during the grower and finisher period. Male chicks had higher daily FC than females (Table 4.14). During the grower period, male chicks consumed 132±1g feed /day and female consumed 114±1g feed /day. During the finisher period, male chicks consumed 204±1g feed /day and female consumed 178±1g feed /day. The FC of male chicks and female chicks were the same during the first 14 days (Table 4.14). The FCR was affected ($P>0.05$) by gender through the experiment (Table 4.15). Male chicks had better FCR than female chicks through the 33 days production period. During the finisher period, the chicks expressed a poor FCR than during other periods. These results support the finding of Copur et al. (2010), who concluded that the use of formaldehyde and oregano oil as hatching egg sanitizer did not affect broiler viability, BW and FCR during production period.

Table 4.13 Effect of sanitizers applied to *E. coli* inoculated hatching eggs on body weight gain (g bird^{-1}day^{-1}) of broiler chickens.

Sanitizer	Day 0-7		Day 8-14		Day 15-25		Day 26-33		Treatment mean
	Male	Female	Male	Female	Male	Female	Male	Female	
Distilled water	21±0.2	20±0.2	49±0.7	43±0.7	92±1.6	77±1.6	124±2.1	106±2.1	66±0.4^{x}
1.5% Lysozyme	20±0.2	20±0.2	47±0.7	44±0.7	91±1.6	79±1.6	124±2.1	103±2.1	66±0.4^{x}
3.0% Lysozyme	20±0.2	20±0.2	49±0.7	43±0.7	91±1.6	79±1.6	120±2.1	104±2.1	66±0.4^{x}
0.125% QA*	19±0.2	19±0.2	46±0.7	44±0.7	91±1.6	78±1.6	116±2.1	101±2.1	64±0.4^{y}
Gender x Age	20±0.7^{g}	20±0.7^{g}	48±0.7^{e}	44±0.7^{f}	91±0.7^{c}	78±0.7^{d}	121±0.7^{a}	103±0.7^{b}	

ANOVA	P-value
Room	0.0325
Sanitizer	**0.0137**
Gender	<0.0001
Sanitizer x Gender	0.2753
Age	<0.0001
Sanitizer x Age	0.1060
Gender x Age	**<0.0001**
Sanitizer x Gender x Age	0.6432

$^{a-g}$Means ± SE in the same group: Gender x Age effects with no common letters are significantly different according to Tukey-Kramer test (α = 0.05).

x-y Means ± SE in the sanitizer effect with no common letters are significantly different according to Tukey-Kramer test (α = 0.05).

* Quaternary ammonium.

Table 4.14 Effect of sanitizers applied to *E. coli* inoculated hatching eggs on feed consumption (g bird^{-1}day^{-1}) of broiler chickens.

Sanitizer	Day 0-7		Day 8-14		Day 15-25		Day 26-33		Treatment mean
	Male	Female	Male	Female	Male	Female	Male	Female	
Distilled water	25±1	24±1	62±1	58±1	132±4	112±4	206±2	182±2	100±1
1.5% Lysozyme	24±1	24±1	61±1	60±1	132±4	114±4	208±2	179±2	100±1
3.0% Lysozyme	24±1	25±1	65±1	58±1	133±4	115±4	203±2	176±2	100±1
0.125% QA*	24±1	23±1	60±1	58±1	132±4	113±4	199±2	178±2	98±1
Gender x Age	24±1^{g}	24±1^{g}	62±1^{e}	58±1^{f}	132±1^{c}	114±1^{d}	204±1^{a}	178±1^{b}	

ANOVA	P-value
Room	0.1203
Sanitizer	0.5182
Gender	<0.0001
Sanitizer x Gender	0.8779
Age	<0.0001
Sanitizer x Age	0.3396
Gender x Age	**<0.0001**
Sanitizer x Gender x Age	0.5589

$^{a-g}$Means ± SE in the same group: Gender x Age effects with no common letters are significantly different according to Tukey-Kramer test ($\alpha = 0.05$).

* Quaternary ammonium.

Table 4.15 Effect of sanitizers applied to *E. coli* inoculated hatching eggs on feed conversion ratio of broiler chickens.

Sanitizer	Day 0-7		Day 8-14		Day 15-25		Day 26-33		Treatment mean
	Male	Female	Male	Female	Male	Female	Male	Female	
Distilled water	1.20±0.04	1.25±0.04	1.29±0.02	1.34±0.02	1.43±0.05	1.46±0.05	1.66±0.03	1.72±0.03	1.42±0.01
1.5% Lysozyme	1.19±0.04	1.23±0.04	1.31±0.02	1.36±0.02	1.45±0.05	1.45±0.05	1.68±0.03	1.74±0.03	1.43±0.01
3.0% Lysozyme	1.22±0.04	1.25±0.04	1.32±0.02	1.33±0.02	1.47±0.05	1.47±0.05	1.69±0.03	1.71±0.03	1.43±0.01
0.125% QA*	1.22±0.04	1.20±0.04	1.30±0.02	1.32±0.02	1.46±0.05	1.45±0.05	1.72±0.03	1.77±0.03	1.43±0.01
Age mean	1.22±0.01[d]		1.32±0.01[c]		1.45±0.01[b]		1.71±0.01[a]		
Gender	Male	Female							
	1.41±0.01[y]	1.44±0.01[x]							

Room	0.9396
Sanitizer	0.8386
Gender	**0.0311**
Sanitizer x Gender	0.6768
Age	**<0.0001**
Sanitizer x Age	0.9066
Gender x Age	0.7389
Sanitizer x Gender x Age	0.9991

[a-d]Means ± SE in the age effect with no common letters are significantly different according to Tukey-Kramer test (α = 0.05).
[x-y]Means ± SE in the gender effect with no common letters are significantly different according to Tukey-Kramer test (α = 0.05).
*Quaternary ammonium.

No significant differences in mortality were observed among sanitizer treatments during the 33-day production period (Table 4.16). Total mortality calculated through the study was 2.5%.

Table 4.16 Effect of sanitizers' fumigation on mortality (%) of chicks hatched from contaminated eggs.

Sanitizer	0-33 day		
	Male	Female	Treatment mean
Distilled water	5.0±1.0	1.3±1.0	3.1±0.7
1.50% Lysozyme	3.1±1.0	1.3±1.0	2.2±0.7
3.00% Lysozyme	3.1±1.0	1.3±1.0	2.2±0.7
0.125% QA*	4.4±1.0	0.6±1.0	2.5±0.7

* Quaternary Ammonium.

Cause of mortality was mainly leg deformities and heart disease. Diseases were not related to bacterial infection. The mortality rate of chicks hatched from artificially inoculated eggs was much lower than that of chicks hatched from floor-collected eggs (Chapter 3). Floor-collected eggs with high bacterial contamination increases the risk of bacteria penetrating eggshells and contaminating egg contents before sanitation process. Hatching egg sanitation should be applied as soon as possible after the eggs are laid and collected.

4.7 Conclusion

The results of the current study demonstrated that both 0.125% quaternary ammonium and 3.00% lysozyme solution provided acceptable activity against *E. coli* on eggshell. In addition, 3.00% lysozyme provided a continuous bactericidal action to prevent *E. coli* penetration. The application of lysozyme solution to inoculated hatching eggs nearly completely eliminated *E. coli* from the yolk sac of newly hatched chicks and increased their hatch weight. Lysozyme did not negatively affect the growth performance of broiler

chickens during a 33-day production period.

The lack of differences in broiler production parameters between the control broiler chickens (distilled water) and the chicks hatched out from quaternary ammonium treatment indicated that *E. coli* isolated from broiler digestive tract content was not enough of a challenge to restrict growth or affect the health of these birds.

Based on the results of this study, lysozyme may provide an effective, safe, and less toxic means for sanitation of hatching eggs.

CHAPTER 5 PROJECT CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The new technique for measuring bacterial penetration through eggshells effectively determined the location and quantity of *E. coli* penetrating eggshells. EDTA modified lysozyme is a reasonable sanitizer for hatching eggs due to demonstrated equivalent antimicrobial activity against *E. coli* as quaternary ammonium solution for contaminated eggs. Use of 3.00% lysozyme provided a continuous bactericidal action to prevent *E. coli* from penetrating during storage and incubation periods.

With no difference in egg weight loss during incubation, there is indirect evidence that the application of lysozyme did not negatively affect the structural integrity of the cuticle on the surface of eggshells. Fumigating hatching eggs with up to 3.0% lysozyme treatments did not negatively affect hatching performance of contaminated broiler hatching eggs and the number of yolk sacs from newly hatched chicks containing NA-resistant *E. coli* was significantly lower, compared to eggs fumigated with distilled water. Reduction in the presence of *E. coli* in yolk sac may contribute to a reduction in broiler mortality during the first week of production by reducing the bacteria present in the young broilers.

5.2 Recommendations

In future research, inoculation of hatching eggs with pathogenic bacteria is recommended to provide challenge to developing embryos and to evaluate the hatching performance of broiler hatching eggs and growth performance of broilers.

These findings warrant larger field studies in a commercial environment to more accurately determine the effects of using lysozyme as a hatching egg sanitizer for not only reducing

the risk of bacterial penetration but also improving hatching performance of broiler hatching eggs and growth performance of broiler chickens.

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