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Investigating the Efficacy of Natural Antimicrobial Molecules in Reducing Egg-borne Transmission of Salmonella enterica serovar Enteritidis in Layer Hens

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Investigating the Efficacy of Natural Antimicrobial Molecules in Reducing Egg-borne Transmission of *Salmonella enterica* serovar Enteritidis in Layer Hens

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Salmonella Enteritidis (SE) is a major foodborne pathogen worldwide. Eggs constitute the most common food product associated with SE infections in humans. Chickens serve as the natural host of SE, with the cecum being the principal site of colonization. In layer hens, SE colonizes the intestine and migrates to various organs, including the oviduct, leading to egg yolk and shell contamination. Despite several control measures adopted for reducing SE by pre-harvest and post-harvest approaches, *Salmonella* is widespread in poultry. In this Ph. D. dissertation, the efficacy of two natural molecules, *trans*-cinnamaldehyde (TC), a phytophenol, and caprylic acid (CA), a medium chain fatty acid, were investigated for reducing egg-borne transmission of SE in layer hens. Additionally, cell culture and gene expression studies were performed to elucidate the mechanisms behind the antimicrobial action of TC and three other phytophenols, namely eugenol (EUG), thymol (THY) and carvacrol (CR). Moreover, the efficacy of TC, CR and EUG as an antimicrobial wash on table eggs, and the efficacy of TC and EUG as fumigation agents for reducing SE on embryonated eggs was investigated. Results revealed that in-feed supplementation of TC and CA reduced SE in the yolk and on the shell of eggs, and in the cecum, liver and oviduct of chickens ($P < 0.01$). Feeding of TC and CA did not adversely affect the body weight, feed intake, and egg production in chickens or the consumer

acceptability of eggs ($P > 0.05$). Follow up studies revealed that TC, EUG, THY and CR reduced SE attachment and invasion of cultured chicken oviduct epithelial cells ($P < 0.01$). Real-Time quantitative PCR results indicated that the expression of major SE genes specific for colonization in the chicken oviduct were down-regulated by TC, EUG, THY and CR ($P < 0.05$). As an antimicrobial wash, TC, CR and EUG rapidly decreased SE on table eggs ($P < 0.01$). Moreover, TC and EUG reduced SE on the shell and embryo of embryonated eggs when used as fumigating agents in a hatching incubator. These results indicate the potential use of TC, EUG, THY, CR and CA for controlling egg-borne transmission of SE.

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Investigating the Efficacy of Natural Antimicrobial Molecules in Reducing Egg-borne Transmission of *Salmonella enterica* serovar Enteritidis in Layer Hens

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TABLE OF CONTENTS

Approval page……..i

Acknowledgements…….ii

Table of contents……iv

List of figures……...viii

List of tables……..……....xi

List of abbreviations……..xii

Chapter I: Introduction…….**1**

Chapter II: Review of Literature…….**4**

1. Epidemiology of Salmonella Enteritidis *and egg-related sources of infection*……....5

2. Routes of egg-borne transmission of Salmonella Enteritidis *in layers*.....8

3. Mechanisms of egg contamination by Salmonella Enteritidis.....10

3.1. Outer shell contamination.....10

3.2. Colonization of hen's reproductive system.....11

3.2.1. Genes specific for oviduct colonization.....15

4. Chickens as a host of Salmonella. 17

5. Salmonellosis in humans.....19

6. Significance of pre-harvest intervention strategy for controlling SE in eggs.....20

7. Significance of post-harvest intervention strategy for controlling SE in eggs 22

8. Plant derived polyphenols….....24

8.1. Trans-cinnamaldehyde.....24

8.2. Carvacrol, Thymol and Eugenol.....26

9. Medium chain fatty acids.....28

9.1. Caprylic acid.....29

References.....32

Chapter III: Effect of Plant Derived Antimicrobials on *Salmonella* Enteritidis Adhesion to and Invasion of Primary Chicken Oviduct Epithelial Cells *in vitro* and Virulence Gene Expression

Abstract……..66

1. Introduction……....67

2. Materials and Methods…….…….…….…….…….…….....69

2.1. Bacterial strains and culture conditions.....69

2.2. PDAs and SIC determination.....70

2.3. Isolation of chicken oviduct epithelial cells…….……..…….....70

2.4. SE adhesion and invasion assay.....71

2.5. Macrophage cultivation and SE survival assay.....72

2.6. RNA isolation and RT-qPCR…….....73

2.7. Statistical analysis…….....74

3. Results and discussion.....74

References.....80

Chapter IV: In-feed supplementation of *trans*-cinnamaldehyde reduces egg-borne transmission of *Salmonella* Enteritidis in layer chickens

Abstract.....102

1. Introduction.....103

2. Materials and Methods.....105

2.1. Bacterial strains and dosing.....105

2.2. Experimental birds and housing.....106

2.3. Determination of SE on egg surface and egg contents.....107

2.4. Determination of SE in internal organs.....108

2.5 Sensory evaluation.....109

2.6. Statistical analysis.....109

3. Results.....110

4. Discussion.....111

References.....113

Chapter V: Reducing egg-borne transmission of *Salmonella* Enteritidis in layer chickens by in-feed supplementation of caprylic acid

Abstract.....126

1. Introduction……..128

2. Materials and Methods…….…….....130

2.1. Bacterial strains and dosing …….....130

2.2. Experimental birds and housing…….....131

2.3. Experimental design…….…….....131

2.4. SE determination on egg surface and egg contents:.....132

2.5. SE determination in internal organs: ……..…….....133

2.6. Sensory evaluation……..……...….....133

2.7. Statistical analysis ……..……...….....134

3. Results……..……..…….....134

3.1. Effect of caprylic acid on SE contamination of eggshell and yolk…….....134

3.2. Effect of caprylic acid on SE colonization of internal organs and cloaca……..….....135

3.3. Effect of CA on sensory characteristics of eggs…….....……..136

4. Discussion……..…….…….……....136

References……..……..……139

Chapter VI: Rapid inactivation of *Salmonella* Enteritidis on shell eggs by washing with plant-derived antimicrobials

Abstract……..……..……149

1. Introduction……..151

2. Materials and Methods……..……..…….……153

3. Results and Discussion.....155

References.....160

Chapter VII: Reducing *Salmonella enterica* serovar Enteritidis on embryonated eggs by fumigation with *trans*-cinnamaldehyde and eugenol

Abstract……..……..……174

1. Introduction……..176

2. Materials and Methods……..……..…….……178

2.1. Bacterial strains and culture conditions…….…….…….....….……178

2.2. Preparation and inoculation of eggs…….…….....….……...179

2.3. Fumigation treatment with TC and EUG and determination of SE ……..…..…….179

2.4. Statistical analysis…….…….…….…….…….…...……..180

3. Results and Discussion.....181

References.....185

Chapter VIII: Summary.....196

List of Figures

	Title	Page
	Chapter III	
FIG. 1	Adhesion to and invasion of 10 strains of *Salmonella* Enteritidis (SE) on chicken oviduct epithelial cells (COEC).	96
FIG. 2a	Effect of *trans*-cinnamaldehyde (TC), carvacrol (CR) thymol (THY) and eugenol (EUG) on SE adhesion to primary COEC.	97
FIG. 2b	Effect of *trans*-cinnamaldehyde (TC), carvacrol (CR) thymol (THY) and eugenol (EUG) on SE invasion of primary COEC.	98
FIG. 3a	Effect of *trans*-cinnamaldehyde (TC), carvacrol (CR) thymol (THY) and eugenol (EUG) on survival of SE 21 in HTC (Chicken macrophages).	99
FIG. 3b	Effect of *trans*-cinnamaldehyde (TC), carvacrol (CR) thymol (THY) and eugenol (EUG) on SE 28 survival of HTC (Chicken macrophages).	100
	Chapter IV	
FIG. 1	Effect of TC on SE contamination of eggs in 40-week old birds for 7 weeks post inoculation (a) on egg shell, (b) egg yolk (c) cumulative effect of TC treatment for 7 weeks on shell, (d) cumulative effect of TC treatment for 7 weeks on yolk.	122 123
FIG. 2	Effect of TC on SE contamination of eggs in 25-week old birds for 7 weeks post inoculation (a) on egg shell, (b) egg yolk (c) cumulative effect of TC treatment for 7 weeks on shell, (d) cumulative effect of TC treatment for 7 weeks on yolk.	

FIG. 3 Effect of TC on SE in internal organs (liver, cecum, oviduct) of (a) 40-week-old layer hens, P<0.001. (b) 25-week-old layer hens, P < 0.001. 124

Chapter V

FIG. 1 Effect of 0.7% and 1% CA on SE contamination of eggs in 40 week old birds for 7 weeks post inoculation (a) on egg shell and (b) egg yolk. 143 144

FIG. 2 Cumulative effect of 0.7% and 1% CA treatment for 7 weeks on egg shell, in egg yolk, with N = 5061 and P < 0.001. 145

FIG. 3 Effect of CA in (a) internal organs (liver, cecum, oviduct) P<0.001 (b) cumulative effect of CA on SE in cloacal swabs, P<0.001. 146 147

Chapter VI

FIG. 1a Effect of CR at 0.25, 0.5, and 0.75% on SE inoculated on shelled eggs at 32^{o}C. 166

FIG. 1b Effect of EUG at 0.25, 0.5, and 0.75% on SE inoculated on shelled eggs at 32^{o}C. 167

FIG. 1c Effect of TC at 0.25, 0.5, and 0.75% on SE inoculated on shelled eggs at 32^{o}C. 168

FIG. 2a Effect of CR at 0.25, 0.5, and 0.75% on SE inoculated on shelled eggs at 42^{o}C. 169

FIG. 2b Effect of EUG at 0.25, 0.5, and 0.75% on SE inoculated on shelled eggs at 42^{o}C. 170

FIG. 2c Effect of TC at 0.25, 0.5, and 0.75% on SE at inoculated on shelled eggs 42^{o}C. 171

FIG. 3 Effect of TC, CR, EUG at 0.5% on SE inoculated on shelled eggs at 32^{o} C in the presence of 3% organic matter. 172

Chapter VII

FIG. 1 Efficacy of fumigation treatment with TC and EUG in reducing SE on eggshell of embryonated eggs. 193

FIG. 2 Efficacy of fumigation treatment with TC and EUG in reducing SE in embryo of eggs. 194

List of Tables

Chapter III

TABLE 1.	List of *S.* Enteritidis strains screened	90
TABLE 2.	List of primers used for RT-qPCR to validate the primary COEC line.	91
TABLE 3.	List of primers used for RT-qPCR of SE genes and their function	92
TABLE 4.	Expression of SE genes critical for virulence and oviduct colonization in the presence of TC, CR, THY and EUG.	94

List of Abbreviations

CA	Caprylic acid
CDC	Centers for Disease Control and Prevention
COEC	Chicken oviduct epithelial cells
CR	Carvacrol
EG	Eugenol
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GRAS	Generally recognized as safe
HACCP	Hazard analysis and critical control points
PDA	Plant-derived antimicrobial
RT-qPCR	Real-time quantitative polymerase chain reaction
SAS	Statistical analysis software
SIC	Sub-inhibitory concentration
SE	*Salmonella* Enteritidis
TC	*Trans*-cinnamaldehyde
TSA	Tryptic soy agar
TSB	Tryptic soy broth
THY	Thymol
USDA	United States Department of Agriculture
USDA FSIS	United States Department of Agriculture Food Safety and Inspection Service
WHO	World Health Organization

Chapter I

Introduction

Eggs constitute an important part of the American diet, and their annual per capita consumption has steadily increased during the last decade (USDA-ERS, 2007). Approximately 90 billion eggs are produced and 67.5 billion shell eggs being consumed annually in the USA (USDA, 2012). Thus, the microbiological safety of eggs is a major concern to the government, poultry industry and consumers, due to its potential impact on public health and the economy. *Salmonella enterica* serovar Enteritidis (SE) is one of the most common bacterial agents causing enteric disease in humans, largely due to the consumption of contaminated eggs (Latimer et al., 2002; Bialka et al., 2004; Namata et al., 2008; Thomas et al., 2009). Humans contract SE infection *via* the consumption of contaminated, raw or undercooked eggs. Several epidemiological studies have revealed an association between human salmonellosis and consumption of eggs (Guard-Petter, 2001; Braden, 2006).

Cecum is the primary site of SE colonization in chickens (Allen-Vercoe and Woodward, 1999; Stern, 2008), with cecal carriage of the pathogen leading to contamination of ovaries by transovarian route (Keller et al., 1995). Contamination of egg contents (yolk, albumen and eggshell membranes) by SE can occur before oviposition (Miyamoto et al., 1997; Okamura et al., 2001), where *Salmonella* colonized in the reproductive organs invades and multiplies in the granulosa cells of the preovulatory follicles (Thiagarajan et al., 1994; 1996). Trans-shell contamination of eggs with SE may also occur from various sources such as workers, pets, rodents, contaminated feed, litter, and water (Jones et al., 1995; Latimer et al., 2002). The survival of SE on the outer shell surface of eggs is supported by the presence of chicken manure and other moist organic materials following oviposition (Gantois et al., 2009).

Moreover, SE contaminated fertile eggs in a hatchery environment can increase the potential of *inovo* transmission to the embryo from the shell, followed by chick contamination (Magwood, 1964), thus underscoring the microbiological safety of hatching eggs.

The overall objective of this dissertation was to investigate the antimicrobial potential of several natural antimicrobials, including trans-cinnamaldehyde (TC), carvacrol (CR), thymol (THY) and eugenol (EUG) and caprylic acid (CA) for controlling egg-borne transmission of SE in layer chickens. The specific objectives were:

1. To investigate the effect of TC, CR, THY and EUG on SE attachment and invasion of chicken oviduct epithelial cells, and survival in chicken macrophages *in vitro*.
2. To determine the effect of TC as a feed additive in reducing egg-borne transmission of SE in layer chickens.
3. To determine the effect of CA as a feed additive in reducing egg-borne transmission of SE in layer chickens.
4. To determine the efficacy of TC, CR, and EUG in rapidly reducing SE on table eggs as a post-harvest antimicrobial wash treatment.
5. To determine the efficacy of TC and EUG in reducing SE on embryonated eggs as a fumigation treatment.

Chapter II

Review of Literature

Eggs are an inexpensive, healthy and vital part of the American diet. However, the microbiological safety of eggs is a concern, since they constitute the primary source of *Salmonella enterica* serovar Enteritidis (SE), the most common infectious agent causing disease in humans (Guard-Petter, 2001; Latimer et al., 2002; Bialka et al., 2004; Namata et al., 2008; Thomas et al., 2009; De Vylder et al., 2009). *S*. Enteritidis is a Gram-negative, non-spore forming, and motile bacillus belonging to the *Enterobacteriaceae* family. Similar to other bacteria of the *Enterobacteriaceae* family, SE is a facultative anaerobe, which grows at temperatures between 8^{o}C to 45^{o}C and a pH range of 4 to 8. Like most of the non-typhoidal Salmonellae, SE is predominantly regarded as zoonotic pathogen. *S*. Enteritidis demonstrates a wide host range, including chickens, turkeys, swine, cattle, and other domestic and wild animals and birds. In chickens, SE is recovered from both poultry meat and eggs, with eggs being the major source of food-borne infections in humans. Historically, Gartner first isolated an organism causing "meat poisoning" in Germany in 1888, and named it *Bacillus enteritidis*. However, it was designated as *Salmonella enteritidis* in early 20th century, and since the mid 1980's, this serovar has received significant attention worldwide due to its association with food-borne illnesses involving a variety of food vehicles.

1. Epidemiology of SE and egg-related sources of infection

Numerous surveys conducted in various parts of the world have indicated that SE is the most common serotype of *Salmonella* isolated from poultry products (Machado and Bernardo, 1990; Plummer et al., 1995; Uyttendaele et al., 1999; Antunes et al., 2003). It was reported that 80% of the known-source SE outbreaks during 1985 to 1999 in the United States were egg- associated (Patrick et al., 2004). A report by the USDA-FSIS

estimated the number of SE-contaminated eggs in the U.S. to be 2.2 million per year (Ebel and Schlosser, 2001). Among the many SE outbreaks with a confirmed food vehicle during the period from 1985 to 2003, 79% were egg-based or contained egg ingredients (Braden, 2006). The US Centers for Disease Control and Prevention (CDC) reported 677 outbreaks of egg-borne SE with 23,366 illnesses, 1,988 hospitalizations, and 33 deaths in the United States during the period from 1990 to 2001 (Anonymous, 2003). Another study reported an estimated 700,000 cases of egg-borne salmonellosis in the U.S., which accounted for ~ 47% of total food-borne salmonellosis, costing more than $ 1 billion annually (Frenzen et al., 1999). Yet another study published by the USDA's Food Safety Inspection Service (FSIS) estimated that consumption of SE–contaminated shell eggs caused 182,060 illnesses in the U.S. in 2000 (Schroeder et al., 2005). Likewise, several epidemiological studies in US during the last 20 years highlighted the association between human salmonellosis and egg consumption (Anonymous, 1988; 1990; 1996; Angulo and Swerdlow, 1999; Braden, 2006; Guard-Petter, 2001). Similarly, the European Surveillance System reported that 62.5% of the 165,023 cases of salmonellosis in the European Union in 2006 were due to SE contamination of egg and egg products (EFSA, 2007). This is further underscored by a recent report from the UK, which implicated eggs as the primary source of SE, causing 247 cases of salmonellosis in humans (Anonymous, 2014). These aforementioned studies clearly indicate that contaminated eggs are a major source of human salmonellosis and potentially constitute a major public health hazard.

S. Enteritidis is the most frequently isolated *Salmonella* from layer flocks (Baird-Parker, 1990; Braden, 2006; Gast et al., 2005; EFSA 2007). The primary colonization site of SE in chicken is the ceca (Allen-Vercoe and Woodward, 1999; Filho et al., 2000;

Stern, 2008), with cecal carriage of SE leading to horizontal transmission of the infection, contamination of eggshell with feces, and probably retro-contamination of ovaries (Keller et al., 1995; Gantois et al., 2009). Egg contamination with SE results by penetration through the eggshell from contaminated feces during or after oviposition (De Reu et al., 2006; Gast and Beard 1990; Messens et al., 2005, 2006). Trans-shell route of egg contamination with SE can also occur from other sources such as farmers, pets and rodents (Latimer et al., 2002). Following oviposition, *Salmonella* survival and/or growth on the outer shell surface of eggs is supported by the presence of chicken manure and other moist organic materials (Gantois et al., 2009). In a study conducted by De Reu et al., 2006, when intact eggs were dipped in a bacterial suspension, egg contents were most frequently contaminated by SE (33%) when compared to other bacteria, thereby suggesting that although shells can be penetrated by various bacterial species, SE possesses mechanisms to better survive and/or grow in the internal egg contents. Similarly, in an *in vivo* trial, numerous *Salmonella* serotypes, such as Enteritidis, Typhimurium and Hadar, were isolated from eggshells, but strikingly only Enteritidis was isolated from egg contents (Humphrey et al., 1991).

In light of the mounting evidence linking human salmonellosis with shell eggs, the USDA- FSIS, in partnership with the FDA, issued a "farm-to-table" risk assessment of SE in eggs in 1996, which served as the basis for the Federal and State Egg Safety Action Plan (Braden, 2006). In addition, the U.S. President's Council on Food Safety in 1999 identified egg safety as a major public health problem warranting immediate federal, inter-agency action. The council published a report with the objective of reducing, and ultimately eliminating eggs as a source of human SE illnesses

(Anonymous, 2000). In July 2009, the FDA announced that eggs constitute the primary source of SE infections, and issued a final rule that requires shell egg producers to implement strict measures to prevent SE from contaminating eggs on the farm and further growth during storage and transportation. Additionally, egg producers are required to maintain records concerning compliance with the rule and to register with the FDA (FDA, 2009).

2. Routes of egg-borne transmission of *Salmonella* Enteritidis in layers

Besides the cecal carriage of SE which leads to increased fecal load and contamination of the eggshell (horizontal transmission), contamination of egg contents (yolk, albumen, and eggshell membranes) with SE can occur by direct transmission of the pathogen from infected hens' ovaries and/or oviducts via transovarian route (vertical transmission) before oviposition (Borland, 1975; Miyamoto et al., 1997; Okamura et al., 2001; Timoney et al., 1989; Shivaprasad et al., 1990). Gantois and others (2009) reported that SE has the ability to persist in the reproductive organs of hens, despite the immune response mounted by the birds. Several researchers believe that internal contamination of eggs with SE is the outcome of its colonization in the hen's reproductive organs (Keller et al., 1995; Methner et al., 1995; Gast and Holt, 2000). Many studies have established that systemic colonization of birds with *Salmonella* results in the pathogen spreading to the reproductive organs (Baskerville 1992; Leach et al., 1999; Vazquez- Torres et al., 1999). The uptake of *Salmonella* by hen's macrophages after bacterial invasion of intestinal cells is believed to disseminate the pathogen within the host, including the reproductive organs (Miyamoto et al., 1997; Okamura et al., 2001; Gast et al., 2007; Gantois et al., 2008). In addition to systemic spread, SE colonization of hen's

reproductive tract can result from an ascending infection from the cloaca (Reiber et al., 1995; Miyamoto et al., 1997) or a descending infection from the ovary (Keller et al., 1995). *S*. Enteritidis can colonize many sites in the hen's reproductive tract, including the ovary, infundibulum, magnum, isthmus and vagina, resulting in the contamination of the egg (Gantois et al., 2009). Another potential source of SE contamination is generations of infected primary breeding flocks that are maintained for establishing, continuing or improving parent lines and hatching chicks. Thus hatchery sanitation is essential to ensure chick quality as the poultry hatch environment can be contaminated with a variety of bacteria, especially Salmonellae (Lock et al., 1992, Bruce et al., 1994, Cox et al., 1999). Breeding flocks may serve as a crucial source for vertical transmission of SE. A small number of hens in the primary flock infected with SE can potentially transmit the infection to the successive generations asymptomatically (Poppe, 1999). The introduction or persistence of infection as a result of inadequate breeding and multiplier stock management (Laszlo et al., 1985), negligent hatchery management, infected personnel handling vaccination and other standard procedures, including shipment are also implicated in the transmission of infection (Poppe, 1999). Furthermore, contaminated feed, litter, water, rodents, and insects can also play a role in the spread of SE (Bhatia et al., 1979; Sterski et al., 1981). Additionally, the movement of colonized birds, contaminated equipment, egg flats, feed trucks, and service personnel in the grow-out houses also facilitate flock-to-flock transmission. Other sources of SE transmission include airborne droplets, dust particles, and fomite-contact based transmission (Poppe, 1999). Once contamination of egg shell takes place, *Salmonella* can penetrate the shell and membranes of hatching eggs, and contaminate the developing embryo (Berrang et al.,

1997, Cox et al., 2000). Moreover, transovarian transmission of SE leads to contamination of newly formed fertile eggs, thereby adversely affecting the hatchability and infecting hatching chicks (De Buck et al., 2004).

3. Mechanisms of egg contamination by *Salmonella* Enteritidis:

3.1. Outer shell contamination:

As described earlier, eggs can be contaminated due to intestinal carriage of SE or by infection of the hen's oviduct (Miyamoto et al., 1997; Okamura et al., 2001). Once the egg is laid, contaminated environment in the surroundings of the eggs, including litter, nest box, hatchery environment or hatchery truck can result in outer shell contamination (Gantois et al., 2009). In addition, chicken manure, fecal and organic matter is reported to facilitate SE growth and survival on eggshell (Schoeni et al., 1995). Following deposition on the shell, SE contaminate the inside by surpassing not only the physical barriers (Ruiz and Lunam, 2002), but also the chemical barriers present in the form of various antibacterial proteins in albumen (Hincke et al., 2000; Gautron et al., 2001). The physical barriers on egg shell such as the cuticle, crystalline eggshell and the shell membranes (inner, outer and limiting membrane), along with antimicrobial proteins such as lysozyme, ovotransferrin and ovocalyxin-36 protect the yolk and inner environment of eggs (Ruiz and Lunam, 2002, Hincke et al., 2000; Gautron et al., 2001, Gantois et al., 2009). In comparison to other serotypes of *Salmonella*, which are limited to eggshell, SE is reported to be the only serotype consistently isolated from eggshell and egg contents (De Reu et al., 2006).

Due to the potential of SE penetration occurring before examining and washing the eggs, quick removal of any fecal contamination on the shell is essential. Despite

implementation of intensive control measures in the United States, such as removing eggs with cracks, and washing and disinfecting eggs, egg contamination with SE is still persistent (Braden, 2006). The eggshell is most susceptible to penetration by the pathogen immediately after the egg is laid (Sparks and Board, 1985; Padron, 1995; Miyamoto et al., 1998a; Gantois et al., 2009). For a brief period after oviposition, the cuticle is not completely mature allowing the pores to be open. Additionally, when the egg is exposed to an environment cooler than the chicken body temperature (42^{o}C), SE migrates more easily due to the negative pressure (Board, 1966; Bruce and Drysdale, 1994). Moreover, dehydration occurs on the cuticle of older eggs, leading to its shrinkage and exposing the pores to bacterial penetration (Mayes and Takeballi, 1983, Gantois et al., 2009). Studies have reported that cuticle deposition is vital for the prevention of SE penetration; its absence leads to frequent bacterial contamination of egg's inside (De Reu et al., 2006; Messens et al., 2007). However, apart from shell penetration, there are other factors that can contribute to SE infection, including the bacterial ability to colonize the hen reproductive tract and subsequent survival and multiplication inside eggs.

3.2. Colonization of hen's reproductive system:

Multiple studies suggest that egg contamination with SE most commonly occurs during the formation of the egg in the reproductive organs rather than by eggshell penetration (Lister, 1988, Keller et al., 1995; Methner et al., 1995; Gast and Holt, 2000). Even in the absence of intestinal colonization, Lister (1988) demonstrated SE isolation from the reproductive tissue of infected birds. Despite the innate and adaptive immune response of hens, SE persists in the reproductive tissues of naturally and experimentally infected hens, indicating the potential of the pathogen to escape the host defense

mechanisms. Therefore deposition of SE inside eggs is potentially a consequence of reproductive tissue colonization in infected laying hens (Keller et al., 1995; Methner et al., 1995; Gast and Holt, 2000, Gantois et al., 2009).

The hen's reproductive system can be anatomically subdivided into the ovary, the oviduct (infundibulum, magnum, isthmus, uterus) and vagina. Briefly, the infundibulum is responsible for capturing the ovulatory follicles, the magnum for production of albumen, the isthmus for depositing the eggshell membranes, the uterus for eggshell formation and the vagina for oviposition (Gantois et al., 2009). Depending on the site of colonization, SE can infect albumen, the eggshell membranes or the eggshell itself (magnum, isthmus and uterus, respectively). Numerous studies have reported both yolk and albumen contamination, but suggested that the albumen is most frequently contaminated, highlighting the oviduct tissue as the major colonization site (Gast and Beard, 1990; Humphrey et al., 1991; Keller et al., 1995; Miyamoto et al., 1997; De Buck et al., 2004a). In contrast, a few studies found the yolk to be primarily contaminated by SE, suggesting the ovary as the main colonization site of the pathogen (Bichler et al., 1996; Gast and Holt, 2000). However, it has been generally reported that SE can be isolated from all sites in the hen's reproductive tract; thereby suggesting that contamination of any part of the egg is possible.

Colonization of SE in the reproductive organs has been attributed to the systemic spread of *Salmonella* from the intestine (Vazquez-Torres et al., 1999). *S*. Enteritidis invades the intestinal epithelial cells, followed by immune cell infiltration especially the macrophages, thereby resulting in its uptake by these cells. Several studies have reported the ability of SE to survive and replicate in chicken's macrophages, thereby leading to

reproductive organ colonization (Keller et al., 1995; Miyamoto et al., 1997; Okamura et al., 2001; Gast et al., 2007; Gantois et al., 2008). Other parts of the hen's reproductive organ, including the ovary have been reported to support a higher frequency of colonization when compared with the frequency of recovery from the oviduct (De Buck et al., 2004; Gast et al., 2007). Hence, there is an increased possibility of SE interacting with the cellular components of preovulatory follicles. It has also been reported that SE attaches to the developing and mature follicular granulosa cells (Thiagarajan et al., 1994), thereby resulting in increased bacterial number in the membranes of the preovulatory follicles than in the yolk itself. This suggests that during transovarian transmission, SE could potentially remain attached to the egg vitelline membranes. Previous studies have also confirmed such association of yolk contamination with vitelline membrane contamination (Gast and Beard, 1990; Gast and Holt, 2000). In addition, *in vitro* attachment of SE to granulosa cells has been reported as a potential mechanism of infection aided by the type 1 fimbriae causing subsequent invasion and multiplication in granulosa cells (Thiagarajan et al., 1996). Howard and co-workers (2005) studied the penetration of SE in immature follicles and suggested the practical implications leading to contamination of eggs, since the maturation of these follicles could result in continuous transovarian infection of egg and contents throughout the hen's reproductive cycle. However, this observation is questionable since not all white follicles mature and SE growth in the nutrient-rich follicles would cause follicle degeneration (Kinde et al., 2000, Gantois et al., 2009).

Although many studies have reported the vitelline membrane as one of the most common sites of SE contamination (Bichler et al., 1996; Gast and Holt, 2000; Gantois et

al., 2009), other research highlights albumen as the principal site of egg contamination (Shivaprasad et al., 1990; Humphrey et al., 1991; Keller et al., 1995), which is representative of SE colonization of oviduct tissues. Some researchers also advocate the colonization of the reproductive tract as a result of a potential ascending infection from the cloaca (Reiber et al., 1995; Miyamoto et al., 1997), a descending infection from the ovary (Keller et al., 1995) and/or a systemic spread of *Salmonella* (Gantois et al., 2009).

Several researchers have elucidated the role of vagina in the production of SE-contaminated eggs (Keller et al., 1995; Reiber et al., 1995; Miyamoto et al., 1999; Okamura et al., 2001; Mizumoto et al., 2005). Many studies have indicated a high egg contamination rate post-intravaginal infection, marking a high risk of eggshell contamination as the egg passes via vagina colonized by SE (Miyamoto et al., 1997, 1998b; Okamura et al., 2001). In addition, a comparative study with six different *Salmonella* serotypes showed that SE was the major serotype recovered from the vagina when compared to strains belonging to other serotypes after intravaginal inoculation (Miyamoto et al., 1998). This is suggestive of SE expressing an increased ability to attach to the vaginal epithelium. Mizumoto et al. (2005) elaborated that the invasiveness of SE to the vaginal epithelium was dependent on the lipopolysaccharide (LPS) type O9, which is one of the major virulent factors of the pathogen.

Predominantly, the isthmus part of the oviduct is considered as the main colonization site of SE that results in contaminated eggshell membranes (Bichler et al., 1996; De Buck et al., 2004; Miyamoto et al., 1997; Okamura et al., 2001). Moreover, many studies suggest that SE most frequently migrates into eggs through the upper oviduct (Gast and Beard, 1990; Hoop and Pospischil, 1993; Humphrey and Whitehead,

1993; Schoeni et al., 1995). In addition, SE has been detected by immuno-histochemical staining in association with secretory cells of the upper and lower magnum of the oviduct, thereby proving the hypothesis that the pathogen may contaminate forming eggs through the albumen (Hoop and Pospischil, 1993; Keller et al., 1995; De Buck et al., 2004, Gantois et al., 2009).

3.2.1. Genes specific for oviduct colonization:

Many studies have characterized the genes responsible for oviduct colonization and invasion of SE in the hen's reproductive system (Gantois et al., 2008; De Buck et al., 2004). Most recently, to obtain information about the oviduct colonization mechanisms of SE, Raspoet and coworkers (2014) performed a genome-wide microarray-based transposon library screening. This study demonstrated that mutants harboring transposon insertions in genes important for oviduct colonization are less persistent in oviduct cells, and hence following oviduct-cell passage, decrease in numbers compared to mutants in which genes that are not essential for oviduct colonization are inactivated. The critical genes that were identified *in vitro* and *in vivo* included those aiding in SE colonization and invasion of chicken reproductive tract such as those regulating *Salmonella* motility, namely *flgG* (Gantois 2008), *fimD*, and *prot6E* (De buck et al., 2004); adherence and invasion, *sopB*, and *invH* (Li et al., 2009); type three secretion system (TTSS), *sipA, sipB, pipB, ssaV,* and *orf245*; cell membrane and cell wall integrity, *hflK, lrp, ompR,* and *tatA*; (Gantois et al., 2008) exo/endonuclease activity, *xthA* and *mrr1/SEN4287* (Li et al., 2009) and those involved in metabolism such as *rfbH*, *rpoS*, and *ssrA* (Gantois et al., 2009, Shah et al., 2012, Bohez et al., 2008). Among these genes, *ssaV* and *pipB* (Li et al., 2009), although are integral to *Salmonella* TTSS, have also been reported to play a major

role in macrophage survival of SE in the host cells. In addition, *ssrA* has been associated with *Salmonella* survival in macrophages (Li et al., 2009). Other genes reported to play a role in *Salmonella* survival in macrophages are *sodC*, *spvB* and *mgtC* (Retamal et al., 2009). The gene *spvB* ribosylates actin of the macrophages and destabilizes the cytoskeleton (Lesnick et al., 2001, Otto et al., 2000). Yet another virulence gene, *invH,* is an outer membrane lipoprotein responsible for *Salmonella* adhesion and invasion of the host cell, which in turn is facilitated by *sopB* that allows the uptake of the pathogen into the host system (Li et al., 2009). On the other hand, *orf245* and *prot6E* are specific to oviduct colonization of SE. Other important genes include *pipB, sipA* and *sipB* which aid in *Salmonella* invasion and translocation of proteins through the TTSS (Li et al., 2009). It has also been established that *Salmonella* pathogenicity island-2 (SPI-2) is essential to cause a systemic infection (Arpaia et al., 2001). One such study using a deletion mutant in the regulator of SPI-2 (*ssrA*) showed that after intravenous infection of laying hens, the SE with the *ssrA* mutant were significantly lower in the oviducts and the ovaries as compared with the wild-type strain, thus implying the role of *ssrA* in SE spread or colonization of the reproductive tract tissue (Bohez et al., 2008).

The uptake of a pathogen by macrophages has been suggested as a critical process that aids the host to defend against an invading bacterium and elicit a specific immune response. However, the ability of a pathogen to survive in the hostile environment within the macrophage offers it protection from the immune system and helps in its dissemination (Townsend et al., 2007). Li et al. (2009) reported that *sodC* is a critical gene for *Salmonella* survival in macrophages (Li et al., 2009). Upon exposure to reactive oxygen species (ROS), SE present in polymorphonuclear cells and macrophages up-

regulates the expression of *sod,* thereby producing the enzyme superoxide dismutase and neutralizing ROS (Erturk, 1999).

4. Chickens as a host of *Salmonella*:

SE is reported to be the most genetically homogenous serotype among all *Salmonellae* (Porwollik et al., 2005; Swaminathan et al., 2001). Despite limited genomic diversity, the clinical isolates of the serotype vary in their potential to form biofilms, production of LPS, and survival within egg albumen (Clavijo et al., 2006; Jain and Chen, 2007). In chickens, SE isolates possess variations in their virulence potential in causing mortality or colonizing the intestinal tract, leading to systemic spread and subsequent reproductive tract colonization (Gast and Benson, 1995; 1996). Numerous on-farm investigations have revealed that once chickens are exposed to the pathogen, the entire flock is at risk of SE colonization (Foley et al., 2008; Berrang et al., 2009). This is partly due to the ability of *Salmonella* to rapidly proliferate in the gastrointestinal tract followed by spread to the liver, spleen and oviduct of layers (Poppe, 2000).

Multiple investigations have reported that SE is invasive in both young and adult chickens (Hinton et al., 1990; Shah et al., 2011). Young chickens can develop systemic disease with varying rates of mortality (Duchet-Suchaux et al., 1995; Velge et al., 2005). The affected chicks may show signs of anorexia, depression, ruffled feathers, huddling together in groups, drowsiness, dehydration, white diarrhea, stained and pasted vents, and stunted growth (McIllroy et al., 1989). Large doses of SE cause clinical salmonellosis with high mortality, whereas infection with low doses results in clinically healthy carrier birds (Gast and Benson, 1995; Desmidt et al., 1997; Van Immerseel, 2004). Likewise, layers once colonized with the pathogen may remain as asymptomatic carriers, shedding

the pathogen to the environment continuously or intermittently (Velge et al., 2005; Golden et al., 2008). Moreover, due to the transovarian transmission of SE causing contamination of newly formed fertile eggs, the pathogen adversely affects hatching and can infect hatching chicks as well (De Buck et al., 2004). Thus *Salmonella* contamination of eggs is suggested as a potential bacterial strategy facilitating environmental dissemination and eventual human infection (Shah et al., 2011).

In order to establish a successful microhabitat in chickens, SE has to overcome a hostile environment. The bacterium needs to adjust to differences in temperature, osmolality, oxidation-reduction potentials, iron concentrations, acidity, organic and inorganic nutrient environments, antimicrobial substances, host immune response, peristalsis, mucus and numerous other microenvironments (Slauch et al., 1997). The ability of SE to colonize the oviduct aided by other virulent mechanisms such as motility, adhesion to host cells and invasion of epithelial cells are critical for its persistence within the host. In general, most of the virulent *Salmonellae,* including SE employ mechanisms such as resistance to lytic action of complement (D'Aoust, 1991), increased expression of siderophores (that accumulate iron for growth), virulence plasmids (Slauch et al., 1997), cytotoxins, diarrhoegenic enterotoxins (D'Aoust, 1991), and antimicrobial resistance (Travers and Barza, 2002). Moreover, SE has to compete with a larger number of endogenous bacteria consisting of obligate anaerobes, or adopt strategies to find new ecological niches to avoid encounter with the normal flora (Dhawi et al., 2011). S. *Enteritisis* possesses an inclination to colonize close to the mucosa, where nutrients and oxygen are freely available, thus eliminating the possibility of obligate anaerobe colonization (Poulsen et al., 1995). In addition, they have to utilize available electron

receptors (Gennis and Stewart, 1996) and incorporate different patterns of nutrient metabolism (Pullinger et al., 2008). Furthermore, *Salmonella* is reported to utilize an unusual source of carbon such as gluconate during the colonization process in the host (Chang et al., 2004; Fabich et al., 2008). The systemic spread of SE demands the production of immune-resistant virulent mechanisms, especially against avian defensins (β–defensin), followed by counteracting the antimicrobial components in the oviduct (Ebers et al., 2009). *S*. Enteritidis needs to express oviduct specific genes to withstand the growth restricting factors such as lysozyme, ovotransferrin, and other proteinase-inhibiting enzymes. Thus, SE persistence in layers is related to modulation of several genetic loci, especially those involved in nutrient metabolism, virulence, stress tolerance, and oviduct colonization (Gantois et al., 2009).

5. Salmonellosis in humans

Salmonellosis in humans is characterized by fever, headache, abdominal pain, vomiting and diarrhea, and is often self-limiting (Anonymous, 2013). The incubation period of the disease typically ranges from 12-72 hours, with the illness lasting for 2-7 days. Patients usually recover within a week without any antibiotic treatment except in cases of severe diarrhea, where intravenous fluid therapy is warranted. However, severe illness caused by antibiotic resistant strains of SE may result in extended treatment period (Lee et al., 1994). The susceptible populations such as infants, children, the elderly and immune-compromised are prone to severe and invasive disease, characterized by bacteremia and occasionally death (FAO, 2002). In addition, a small percentage of healthy individuals can become predisposed to chronic reactive arthritis, osteoarthritis, appendicitis, meningitis, and peritonitis (Bell, 2002; FAO, 2002). Also, *Salmonella* has

been implicated as a triggering organism for reactive arthritis and Reiter's syndrome in humans. Furthermore, SE is reported to cause extra-intestinal infections, especially urinary tract pathologies (Ghosh and Vogt, 2006; Gordon et al., 2008; Katsenos et al., 2008; Kobayashi et al., 2009; Mutlu et al., 2009).

6. Significance of pre-harvest intervention strategy for controlling SE in eggs:

The cecal carriage of SE results in its dissemination in cloacal contents and feces, thereby leading to contamination of eggshells and trans-shell infection of eggs. Intestinal colonization of layers with SE also results in its systemic spread to reproductive organs, thus contributing to direct transmission of the bacterium to eggs from infected ovaries and/or oviducts by transovarian route. In addition, contaminated feed, litter, water, rodents, and insects can also play a role in the spread of SE to layers on a farm. This is especially important since the infection of chickens after oral ingestion of SE from environmental sources (contaminated feed, fluff, dust) resulting in systemic spread and subsequent colonization of ovary and oviduct has been reported (Davies and Breslin, 2004). Moreover, the presence of SE in eggs is difficult to detect until bacterial populations reach high levels (> 10 CFU). Due to the multitude of SE sources, implementation of strict biosecurity measures at the farm/production sites is critical to reducing the pathogen spread. Therefore, decreasing the level of SE in the gastrointestinal tracts of birds can reduce the contamination of eggs by both horizontal and vertical transmission routes. Minimizing *Salmonella* prevalence in flocks has been reported to result in a direct reduction in human health risk (Altekruse et al., 1993). Control measures implemented at the flock level can reduce human salmonellosis from egg consumption and thus should be a primary focus of control at farm level (Namata et al., 2008). This is

a key strategic approach for delivering a microbiologically safer egg and decreasing disease outbreaks in consumers.

A variety of approaches, including feeding chicks with competitive exclusion bacteria (Blankenship et al., 1993; Stern et al., 2001), probiotic bacteria (Pascual et al., 1999; Tellez et al., 2001), bacteriophages (Carrillo et al., 2005), caprylic acid (Kollanoor Johny et al., 2009), mannanoligosaccharides (Spring et al., 2000; Fernandez et al., 2002), fructooligosaccharides (Bailey et al., 1991), chicory fructans (Yusrizal and Chen, 2003), organic acids such as acetic, propionic, and formic acids (Hinton and Linton, 1988; Thompson and Hinton, 1997; Al-Tarazi and Alshawabkeh, 2003; Heres et al., 2004), tannic acid (Kubena et al., 2001), and antibiotics and other antimicrobials (Chadfield and Hinton, 2003) have been investigated with limited success for reducing *Salmonella* persistence in chickens. The widespread use of antibiotics at therapeutic and subtherapeutic levels has contributed to the emergence of multi-drug resistant strains of pathogenic bacteria, creating a major public health concern worldwide (Shea, 2003; Bywater, 2005). Likewise, SE strains that are resistant to a variety of antibiotics have been reported by numerous investigators (Chadfield and Hinton, 2003; Daly et al., 2005; Dias de Oliveira et al., 2005; Erdem et al., 2005). Additionally, vaccination approaches to control SE in chickens have been undertaken (Barrow, 1997; Dueger et al., 2001; Khan et al., 2003), however, there is no fully effective commercial vaccine currently available to prevent SE colonization of chickens. This is partly due to the commensal relationship of SE with chickens, thereby resulting in an inadequate immune response in vaccinated birds (Bailey, 1993).

7. Significance of post-harvest intervention strategies for controlling SE in eggs:

The exterior surface of eggs becomes contaminated with SE from infected hens's feces, and a variety of environmental sources, including contaminated feed, litter, water and nest (Jones et al., 1995; Latimer et al., 2002; Humphrey, 1994). In addition, egg-shell contamination can occur at egg processing facilities from transfer belts and packaging materials (Mayes and Takeballi, 1983). It was reported that ~ 7.8% of egg shells were contaminated with *Salmonella* before washing, but only 1.1% of the washed egg shells contained the pathogen (Humphrey, 1994). Hence, the cleanliness of egg shell and disinfection of egg surface are a cornerstones in preventing/reducing *Salmonella* contamination on eggs (Kuo et al., 1997; Park et al., 2005). Reducing or eliminating SE on shell eggs would potentially result in microbiologically safer egg products.

Controlling SE contamination of hatching eggs and day-old chicks or poults from infected breeding flocks is becoming increasingly relevant in the operation of hatcheries (Samberg and Meroz, 1995). Hatchery sanitation is essential to ensure chick quality as the poultry hatch environment can be contaminated with a variety of bacteria, especially Salmonellae (Lock et al., 1992, Bruce et al., 1994, Cox et al., 1999). Due to on-farm contamination, around 0.6% of eggs laid by infected breeding flocks have been reported to be infected internally by SE (Humphrey et al., 1991, Davies and Wray, 1994). Laying/breeding flocks have been considered as a critical link between systemic infection of SE in birds and human food poisoning incidents due to contaminated eggs (Altekruse et al., 1993, Davies and Wray, 1994). Additionally, the presence of salmonellae in/on fertile eggs has been identified as a vital step in *Salmonella* contamination of hatching chicks. Since the invading bacteria do not decompose the egg, the infected chick hatching from contaminated eggs could potentially serve as an extensive bacterial reservoir in

commercial hatcheries (Maclaury, and Moran, 1959, Cox et al., 2000).

Cleaning and sanitation of shell eggs by washing is a common practice mandatory for plants operating under Federal Grading Service. Currently, egg washers use a variety of detergents and sanitizers in order to reduce the microbial load on the eggshell surface (USDA, 2008; Zeidler, 2002). Washing eggs under optimum conditions could potentially reduce the total bacterial load by 2 to 3 log CFU/egg (Zeidler, 2002). Additionally, for hatching egg sanitation, fumigation with formaldehyde, potassium permanganate or hydrogen peroxide has been performed against SE and *Staphylococcus aureus*. In selecting a suitable disinfectant to clean egg shells, a number of factors such as the antimicrobial effectiveness of the agent to eliminate target bacterium from egg shell, safety, and cost should be considered (Scott and Sweetnam, 1993). The chemicals used to wash eggs are considered potential food additives, and hence are regulated by the FDA. An ideal egg wash antimicrobial should be effective in reducing large populations of the target pathogen in a rapid time-frame, even in the presence of organic matter. Further, it should be safe to workers and the environment, cost effective (Scott and Sweetnam, 1993), and should be easily incorporated in a Fazard Analysis Critical Control Point program (HACCP) plan.

A variety of disinfectants in egg wash water, including hydrogen peroxide (Padron, 1995), chlorine and iodine-based sanitizer (Knape et al., 1999), ozone (Koidis et al., 2000), quaternary ammonium and sodium carbonate (Wang and Slavik, 1998), zinc sulfate and formaldehyde fumigation (Beirer and Barnett, 1962), and electrolyzed oxidizing water (Russel, 2003) have been investigated with varying degrees of success to reduce or eliminate pathogens on table eggs and hatching eggs. However, many of the

aforementioned chemicals have been shown to possess limited antimicrobial effect, especially in the presence of organic matter, and many did not render eggs pathogen-free (Frank and wright, 1956; Moats, 1978; Wang and Slavik, 1998).

In recent years, there has been a renewed interest in the use of natural antimicrobial substances for pre-harvest and post-harvest control of pathogens due to concerns regarding the safety of synthetic compounds (Cleveland et al., 2001; Salamci et al., 2007). The various naturally occurring molecules investigated in this Ph.D. dissertation for controlling egg-borne transmission of SE in layers are discussed below.

8. Plant-derived polyphenols

Plant essential oils are a group of natural molecules that have been historically used as dietary constituents (Wollenberger, 1988), especially to preserve foods and enhance food flavor. The antimicrobial properties of several plant essential oils have been well characterized (Burt 2004; Holley and Patel, 2005), and many active components in these oils have been identified. The plant-derived polyphenols investigated for reducing egg-borne transmission of SE in this Ph.D. dissertation were *trans*-cinnamaldehyde (TC), carvacrol (CR), eugenol (EUG), and thymol (THY).

8.1 *Trans*-cinnamaldehyde:

Trans-cinnamaldehyde (TC) is a major component of the bark extract of cinnamon. It is classified as Generally Recognized as Safe (GRAS), and approved for use in foods by the FDA. The U. S. Flavoring Extract Manufacturers' Association reported based on chronic studies that TC possesses a wide margin of safety between conservative estimates of intake and no observed adverse effect levels (Adams et al., 2004). The report also revealed absence of genotoxic and mutagenic effects due to TC. Additionally,

Michiels et al. (2008) reported that oral supplementation of TC in piglets at 13.0 mg/kg BW did not induce any toxic effects. Moreover, published data from our laboratory indicated that the TC at 0.5% and 1.5% did not produce any cytotoxic effect on human epithelial cell lines and urinary tract cells in *vitro,* respectively (Amalaradjou et al., 2009; 2010). Nutley et al. (1990) fed groups of four male Fischer 344 rats with a single oral dose of 2.5 mmol cinnamaldehyde that resulted in 16% recovery of the compound in excreta, with 0.7% retained in the carcass. In another similar study in rats, Sapienza et al. (1993) reported that a single oral dose of 500 mg/kg of radioactive cinnamaldehyde resulted in 3.2% recovery in excreta after 24 hours. Radioactivity of the compound was seen primarily in gastrointestinal tract, kidney, liver and fat. In addition, they reported that after a 7-day supplementation of cinnamaldehyde 4.5% recovery was observed in feces, indicating that continuous supplementation might result in higher recovery why.

Trans-cinnamaldehyde possesses antimicrobial activity towards a wide range of foodborne pathogens, including Gram-positive and Gram-negative bacteria (Bowles and Miller, 1993; Bowles et al., 1995; Friedman et al., 2002). The antibacterial effect of TC against *Clostridium botulinum* (Bowles and Miller, 1993), *Clostridium difficile* (Mooyottu et al., 2014), *Staphylococcus aureus* (Bowles et al., 1995, Huang et al., 2014), *Vibrio spp*., (Brackman et al., 2008) and *Escherichia coli* 0157:H7 has been previously documented. Trans-cinnamaldehyde was found to be inhibitory on various pathogenic bacteria *in vitro* without exerting any harmful effect on the natural microflora. The compound was found to reduce the *Salmonella* Typhimurium DTI04, *E. coli* 0157: H7 and other coliforms with little inhibition towards *Lactobacilli* and *Bifidobacteria in vitro* (Si et al., 2006). In addition, TC was effective in inhibiting biofilm formation and

inactivating mature biofilms of *Cronobacter sakazakii* (Amalaradjou and Venkitanarayanan, 2011) , enteropathogenic *Escherichia coli* (de Oliveira et al., 2012) and uropathogenic *E. coli* (Amalaradjou et al., 2010). Additionally, our laboratory reported that feed-supplemented TC was effective in reducing cecal colonization of SE in broilers (Kollanoor-Johny et al., 2012). Furthermore, Hernandez et al (2004) observed that feeding plant extracts containing TC to broilers improved their digestibility.

8.2 Carvacrol, thymol and eugenol:

Carvacrol (CR) is a principal ingredient in oregano oil obtained from *Origanum vulgare*, a common herb found in Europe and the Mediterranean. The essential oil obtained from *O. vulgare* has been found effective against bacterial and fungal infections of the gastrointestinal and genitourinary tract (Adam et al., 1998; Blumenthal et al., 2000; Chun et al., 2005). Oregano oil was also reported to possess significant antibacterial activity against *Helicobacter pylori* (Chun et al., 2005), *Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae, Enterobacter cloacae* (Hersch-Martinez et al., 2005), and *Streptococcus mutans* (Botelho et al., 2007*)*. In addition, the antimicrobial activity of carvacrol has been extensively reviewed by Nostro et al., in 2012 depicting its effect against a wide range of pathogens including *Enterobacter sakazakii, Haemophilus influenza* and methicillin-resistant *Staphylococcus aureus* (Hersch-Martinez et al., 2005).

Similar to CR, thymol (THY) is another ingredient in oregano. Thymol was reported to reduce *S.* Typhimurium and *Listeria monocytogenes* in fish patty (Ilhak and Guran et al., 2014). In addition, THY has been shown to be antibacterial against oral pathogens, including *Candida albicans* (Knezevic et al., 2012) and *Streptococcus mutans*

(Botelho et al., 2007). Recently, Jentsch et al. (2014) observed that THY in combination with a commonly employed mouthwash was effective against a variety of plaque causing pathogens. Likewise, Si et al., (2006) reported that THY was inhibitory on various pathogenic bacteria such as *S.* Typhimurium DTI 04 and *Escherichia coli* 0157: H7 in pigs without exerting any harmful effect on the natural intestinal microflora, especially *Lactobacilli* and *Bifidobacteria*.

Eugenol (EUG) is yet another natural molecule present as an active ingredient in the oil from cloves (*Eugenia caryophillis*) (Ali et al., 2005). The antibacterial activity of clove oil and EUG has been documented by many researchers (Stecchini et al., 1993; Menon and Garg, 2001; Suhr and Nielsen, 2003; Ali et al., 2005). EUG has been reported to exert antibacterial activity against other pathogens including *Salmonella* Typhi (Devi et al., 2010) and *Proteus mirabilis* (Devi et al., 2013). Additionally, EUG has been studied as antimicrobial packaging film or coating to counteract *Aeromonas hydrophila* and *Enterococcus faecalis* (Sanla-Ead et al., 2012). Michiels et al. (2008) observed that supplementation of eugenol at 12.5 mg/kg BW in piglets did not result in any toxic effects.

A critical property of essential oils or their components is their hydrophobicity, which helps them to target the lipid containing bacterial cell membrane (Sikkema et al., 1994; Smith-Palmer et al., 2004). This makes these membranes more permeable leading to leakage of ions and other cell contents (Carson et al., 2002; Ultee et al., 2002). Besides the effect on cell membrane, TC is also believed to kill bacteria by inhibiting energy generation and glucose uptake (Gill and Holley, 2004). Another mechanism by which TC and EUG kill microorganism is by their inhibitory effect on key enzymes such as amino

acid decarboxylases (Wendakoon and Sakaguchi, 1995). Since plant derived compounds contain a number of different chemical groups in their structure, their antimicrobial activity is attributed to more than one specific mechanism (Carson et al., 2002; Burt, 2004). Therefore unlike to antibiotics, it is hypothesized that the potential for bacteria to develope resistance to plant antimicrobials is negligible (Smith-Palmer et al., 2004; Ohno et al., 2003; Domadia et al., 2007).

9. Medium chain fatty acids (MCFAs):

The primary medium chain fatty acids (MCFAs) are caproic (C6), caprylic (C8), capric (C10), and lauric (C12) acids (Bach and Babayan, 1982; Van Immerseel et al., 2006). These molecules are present in the natural extracts of coconut oil, palm kernel oil, and in bovine, caprine and human milk (Sprong et al., 2001; Jensen, 2002). Several MCFAs have been used in a variety of nutritional applications due to their distinctive digestive and metabolic properties. Their smaller molecular size and high ionization capacity at physiological pH, enables better solubility in aqueous biological fluids than long chain fatty acids (Odle, 1997). Moreover, medium chain triglycerides have been traditionally used in the treatment of gastroenteritis, pancreatic and biliary insufficiency, diabetes, and in total parenteral nutrition and preterm infant formulas (Odle, 1997). In addition, MCFAs or their triglycerides are commonly used in foods, cosmetics, and drugs (Traul et al., 2000). Although there is a wealth of information on the antimicrobial effect of free fatty acids and their esters in various laboratory media (Oh and Marshall, 1992; Wang and Johnson, 1992; Petschow et al., 1996; Bergsson et al., 1999) studies validating their efficacy in controlling pathogens in animals are relatively limited.

9.1 Caprylic acid:

Caprylic acid (octanoic acid, CA) is a natural, eight-carbon MCFA predominant in breast milk, bovine milk (Jensen, 2002), and coconut oil (Sprong et al., 2001). It is a GRAS-status, food-grade compound approved by the FDA (GRAS, CFR 184.1025). Previously, Vasudevan et al. (2005) observed that CA was effective in rapidly reducing SE in chicken cecal contents *in vitro.* Similarly, CA was antimicrobial against *Escherichia coli* O157:H7 in bovine rumen fluid, killing the pathogen by > 5.0 log in 24 hours (Annamalai et al., 2004). Another study by Van Immerseel et al. (2004) reported that MCFAs, particularly caproic acid supplemented to chicks (at 3 g/kg of feed) resulted in significantly reducing SE colonization in the ceca and internal organs of the birds. Previous research conducted in our laboratory and by our collaborators indicated that CA supplementation through feed reduced *Campylobacter jejuni* and SE carriage in broiler chickens (Solis de los Santos et al., 2008, 2009; Kollanoor Johny et al., 2009). In a study that involved day-old chicks CA, Solis de los Santos et al. (2008) demonstrated that feed-supplemented with 0.7% CA consistently reduced *C. jejuni,* compared to control birds not receiving the fatty acid. Similarly, Kollanoor Johny et al. (2009) observed that prophylactic CA supplementation to day-old chicks at 0.7 and 1% in the feed significantly reduced SE colonization, without adversely affecting body weight, feed intake, cecal endogenous flora in birds. Follow up research in our laboratory revealed that CA reduced SE invasion of avian intestinal epithelial cells by down-regulating critical colonization-associated genes in the pathogen (Kollanoor Johny et al., 2012b).

Although the mechanisms behind the antimicrobial effect of MCFAs are unclear, several hypotheses have been suggested. It is believed that the diffusion of fatty acids into bacterial cells in the undissociated form and subsequent dissociation in the

protoplasm lead to intracellular acidification and death of bacteria (Sun et al., 1998). Another theory is suggestive of fatty acid penetration and incorporation into the bacterial plasma membrane, thereby deleteriously affecting bacterial cell membrane permeability (Bergsson et al., 2001). Tsuchido et al. (1985) reported that fatty acids mediate cell lysis by inducing autolytic enzymes. Another potential mechanism may involve MCFA-mediated inhibition of bacterial virulence genes that aid in pathogen colonization in the host.

In summary, SE is a major food-borne pathogen in the U.S., with eggs being the primary source of infection. Contamination of eggs with SE occurs by transmission of the pathogen from layers' infected ovaries and oviducts by transovarian route, or by penetration through eggshell from contaminated feces. Decreasing *Salmonella* colonization in layers can potentially reduce contamination of eggs, and in turn human salmonellosis. Moreover, effective wash treatments that rapidly reduce SE populations on eggs decrease the risk of trans-shell penetration of the pathogen during storage. Additionally, reducing SE on fertile eggs used for hatching is critical for controlling *inovo* transmission and subsequent chick contamination by SE.

Based on the published literature and preliminary research, the hypothesis of this Ph.D. dissertation is that TC and CA are effective as feed additives in controlling SE colonization and egg-borne transmission in layer hens. Further, TC, CR, or EUG are effective in reducing SE on table and/or embryonated eggs. The specific objectives are to investigate

1. The effect of TC, CR, TH and EUG on SE attachment and invasion of chicken oviduct epithelial cells, and survival in chicken macrophages *in vitro*.

2. The efficacy of TC as a feed additive in reducing egg-borne transmission of SE in layer chickens.
3. The efficacy of CA as a feed additive in reducing egg-borne transmission of SE in layer chickens.
4. The efficacy of TC, CR, and EUG in rapidly reducing SE on eggs when used as an antimicrobial wash treatment.
5. The efficacy of TC and EUG in reducing SE on fertile eggs as a fumigation treatment.

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Chapter III

Effect of plant-derived antimicrobials on *Salmonella* Enteritidis adhesion to and invasion of primary chicken oviduct epithelial cells *in vitro* and virulence gene expression

Abstract

Salmonella Enteritidis (SE) is a major foodborne pathogen in the United States, largely transmitted to humans by consumption of contaminated eggs. The pathogen colonizes chicken intestinal tract, and migrates to reproductive organs via systemic route, or invades the oviduct epithelial cells via ascending infection from the cloaca. Since adhesion to- and invasion of chicken oviduct epithelial cells (COEC) are critical steps in SE colonization of chicken reproductive tract and subsequent yolk contamination, reducing these virulence attributes could potentially decrease egg-borne transmission of the pathogen. Oviduct epithelium collected from laying hens was cultured, and COEC isolated. Ten different isolates of SE were examined for their adhesive and invasive abilities on COEC. The efficacy of sub-inhibitory concentrations (concentrations below MIC, not inhibiting SE growth) of four plant derived antimicrobials (PDAs), namely *trans*-cinnamaldehyde, carvacrol, thymol and eugenol in reducing SE adhesion to and invasion of COEC, and its survival in chicken macrophages was investigated. In addition, the effect of PDAs on major SE genes critical for oviduct colonization and macrophage survival was determined using real-time quantitative PCR (RT-qPCR). All PDAs significantly reduced SE adhesion to and invasion of COEC ($P<0.05$). The PDAs, except thymol consistently decreased SE survival in macrophages ($P<0.05$). RT-qPCR results revealed down-regulation of critical genes involved in SE colonization of chicken oviduct ($P<0.05$). The PDAs could potentially be used as feed additives to attenuate SE virulence and decrease its colonization in chicken reproductive tract.

1. Introduction

Eggs constitute a vital part of the American diet with an annual per capita consumption of approximately 250 eggs (1). Approximately 90 billion eggs are produced and 67.5 billion shell eggs being consumed annually in the U.S (1). Thus, the microbiological safety of eggs is a major concern from public health and economic perspectives. *Salmonella enterica* serovar Enteritidis (SE) is one of the most common bacterial agents causing enteric disease in humans, largely due to the consumption of contaminated eggs (2, 3, 4, 5, 6). Humans contract SE infection via consumption of contaminated, raw or undercooked eggs. In light of increasing evidence linking human salmonellosis with consumption of eggs, the Food and Drug Administration (FDA) in 2009 (7) announced that eggs constitute the primary source of SE infections to humans, and issued a final rule that requires egg producers to implement measures to prevent the pathogen from contaminating eggs on the farm and further growth during storage and transportation (7).

The primary colonization site of SE in chicken is the cecum (8, 9), with cecal carriage of the pathogen leading to contamination of ovaries by transovarian route (10). Additionally, uptake of *Salmonella* by hen's macrophages after bacterial invasion of intestinal cells aids in its dissemination within the host, including the reproductive organs (11, 12, 13, 14). Contamination of egg contents (yolk, albumen, and eggshell membranes) by SE can occur before oviposition (11, 12), where *Salmonella* originating from infected reproductive organs invades and multiplies in the granulosa cells of the preovulatory follicles in the reproductive tract (15, 16). Since cecal colonization of layers with SE results in the systemic spread of the pathogen to reproductive organs by transovarian

route, decreasing the pathogen prevalence in flocks has been reported to result in a direct reduction in human health risk (17). Control measures implemented at the flock level could reduce human salmonellosis from egg consumption, and was thus suggested as a primary focus of control at farm level (4). Therefore, innovative on-farm strategies for preventing colonization of birds with SE are critical to prevent the pathogen contamination of eggs. Apart from reducing the colonization of SE in chicken cecum, a viable approach could be one that potentially reduces the bacterial virulence mechanisms, thereby reducing their colonization in the reproductive tract of birds, where eggs are formed (18, 10, 19).

Historically, plants have served as a source of information for the development of novel drugs against human and animal diseases. Plants produce a large number of compounds, most of which as a defense mechanism against predation by pathogenic microorganisms and insects. Several plant compounds form dietary constituents as well as active components in a number of herbal and traditional medicines (20). In recent years, the use of natural compounds has gained attention due to increasing concerns over the safety of synthetic chemicals (21, 22) and emerging antibiotic resistance in bacteria (22). The antimicrobial properties of several plant-derived essential oils have been previously reported, and a variety of ingredients in these oils have been identified (23, 24 25). Among the various plant-derived antimicrobials (PDAs), *trans*-cinnamaldehyde (TC) is a major ingredient in cinnamon (*Cinnamomum zeylandicum*). Carvacrol (CR) and thymol (THY) are extracted from oregano oil, which is obtained from *Origanum glandulosum*, whereas eugenol (EUG) is a component of clove oil (*Eugenia caryophillis).* All these aforementioned PDAs are reported to be effective against several Gram-

negative and Gram-positive bacteria (26, 27, 28), and are classified as generally recognized as safe (GRAS) by the FDA (29, 30, 31, 32). Our laboratory previously reported that the aforementioned molecules were effective in killing SE *in vitro* (33, 34) and in broiler chickens (35). Additionally, research conducted in our laboratory revealed that sub-inhibitory concentrations (SIC, concentration not inhibiting bacterial growth) of TC suppressed the attaching and invading abilities of uropathogenic *Escherichia coli* on human urinary tract epithelial cells, by down-regulating the genes critical for host tissue colonization (36). The current study was undertaken to investigate the efficacy of SICs of TC, CR, THY and EUG in reducing the attachment to and invasion of primary chicken oviduct epithelial cells (COEC) by SE *in vitro*. Moreover, the effect of TC, CR, THY and EUG on various virulence genes critical for SE colonization in the chicken oviduct was studied.

2. Materials And Methods:

2.1. Bacterial strains and culture conditions

Ten strains of SE (Table 1) were screened to determine their adhesive and invasive properties on COEC. Since we did not observe any strain variability in their adhesive and invasive abilities, three strains of SE*,* namely SE 28 (oviduct isolate), SE 21 (intestinal isolate), and SE 457 (egg yolk isolate) were selected for further investigations. All bacteriological media were purchased from Difco (Becton Dickinson, Sparks, MD). Each strain of SE was cultured separately in 10 ml of sterile tryptic soy broth (TSB) in 50 ml screw-cap tubes, and incubated at 37°C for 18 h. Following incubation; the cultures were sedimented by centrifugation (3600 × g for 15 min) at 4°C. The resultant pellet was washed twice, resuspended in 10 ml of sterilized phosphate buffered saline (PBS, pH 7.2)

and serial, ten-fold dilutions were plated on duplicate tryptic soy agar (TSA) and xylose lysine deoxycholate agar (XLD) agar plates, followed by incubation at 37°C for 24 h (33, 37).

2.2. PDAs and SIC determination

Trans-cinnamaldehyde, CR, EUG and THY (99% purity) were purchased from Sigma-Aldrich Inc (St.Loius, MO). The SICs of each plant compound were determined as described previously (34, 35, 36). Briefly, sterile 24-well polystyrene tissue culture plates (Costar, Corning Incorporated, Corning, NY) containing 1 ml of TSB were inoculated separately with ~6.0 log CFU of SE, followed by the addition of 1 to 10 µl of TC, CR, THY or EUG in increments of 0.5 µl. The plates were incubated at 37°C for 24 h, and bacterial growth was determined by plating on TSA and XLD plates. The highest two concentrations of each plant compound below its respective minimum inhibitory concentration that did not inhibit bacterial growth after 24 h of incubation were selected as its SICs for this study. Duplicate samples for each plant compound were included and the experiment was repeated three times.

2.3. Isolation of chicken oviduct epithelial cells

Primary COEC were isolated as described previously (38), with slight modifications. The oviduct tissues of 25–28 week old, *Salmonella*-free layer hens (single comb, white leghorn) were obtained from the University of Connecticut poultry farm. The isthmal epithelium of the oviduct was removed, flushed thoroughly with HBSS (Sigma-Aldrich) containing 200 U/ml penicillin (Sigma-Aldrich) and 200 mg/ml streptomycin (Sigma-Aldrich). The cells were then treated with 20 ml of HBSS containing 1 mg/ml collagenase (Sigma-Aldrich) for 30 min at 37°C. After collagenase

treatment, the supernatant was discarded and trypsinization of tissue fragments was done using 0.25% trypsin and 3 mM EDTA in 20 ml of HBSS for 10 min at 37°C. The cell suspension was added with 10% heat-inactivated fetal bovine serum (HI-FBS; Gibco, Invitrogen) to stop the activity of trypsin. The cell suspension was then passed through a cell strainer (100 μm, Fisher Scientific) in order to remove any undigested tissue. The epithelial cells were centrifuged at 50 × *g* for 5 min to separate cell aggregates from erythrocytes, platelets, and other immune cells. The supernatant obtained after centrifugation was discarded, and the pellet containing epithelial cells was resuspended in minimal essential medium (MEM, Invitrogen, Grand Island, NY) supplemented with 10% HI-FBS, 2% heat-inactivated chicken serum (HICS; Gibco, Invitrogen), insulin (0.12 U/ml, Sigma-Aldrich), and estradiol (50 nM, Sigma-Aldrich). The COEC were incubated in a tissue culture flask for 2 h at 39°C in 5% CO_2 to allow fibroblast attachment. Following incubation, the epithelial cells were collected by gentle pipetting, followed by centrifugation at 125 × *g* for 10 min. The pelleted epithelial cells were resuspended in whole medium and allowed to grow until a monolayer was formed. After four successive passages, the cells were seeded onto 24-well cell culture plates (~ 2 X 10^5 cells per well), and grown at 37°C under 5% CO_2 for 24-36 h. The identity of COEC was confirmed by determining the constitutive expression of avian β- defensin (AvβD) genes (Table 2) by reverse transcriptase quantitative PCR (RT-qPCR) (38).

2.4. SE adhesion and invasion assay

The adhesive and invasive abilities of ten *S*. Enteritidis isolates (Table 1) on COEC were investigated (39). The COEC were seeded on a 24-well tissue culture plates at ~ 10^5 cells per well, and inoculated with ~ 6.0 log CFU of each SE (MOI 10). The

inoculated COEC cells were incubated at 37°C in a humidified, 5% CO_2 incubator. The infected monolayer was incubated for 1 h to facilitate SE attachment, followed by washing to remove unattached bacteria. The cells were then lysed with 0.1% Triton X-100 (Invitrogen). The number of viable adherent SE was determined by serial dilution and plating on TSA and XLD plates. For the invasion assay, the monolayers were incubated for 1 h following SE infection, rinsed three times in minimal media and incubated for another 2 h in whole media-10% FBS containing gentamicin (100 μg/ml; Sigma-Aldrich) to kill the extracellular bacteria. The wells were then washed with PBS three times, one ml of PBS containing 0.1% Triton X (Invitrogen) was added, followed by incubation at 37°C for 15 min to lyse the cells and release the invaded SE. The cell lysates were serially diluted, plated on TSA/XLD plates and incubated at 37°C for 24 h. The assays were run in duplicates and replicated three times.

The effect of TC, CR, THY and EUG on *Salmonella* adhesion to and invasion of COEC was determined as above, except that the bacteria were grown to midlog phase without (control) and with the respective SICs of each plant compound before inoculating onto COEC. The numbers of adherent/invaded SE in control samples were taken as 100 percent and the populations of bacteria in the treatments were expressed as a percentage relative to that of the control (36).

2.5. Macrophage cultivation and SE survival assay

Chicken macrophages (HTC, chicken monocyte cell line; [40]) were cultivated in RPMI 1640 with 10% FBS. The cells were activated and plated as described previously (41), with slight modifications. Twenty four hours prior to infection, the cells were seeded on to 24-well tissue culture plates and incubated at 37°C under 5% CO_2 to form a

monolayer. Each SE isolate grown to midlog phase in the presence or absence of SICs of TC, CR, THY, or EUG was centrifuged (3600 X g), and resuspended in RPMI media with 10% FBS. About 10^5 macrophages were separately infected with 6.0 log CFU/ml of each SE isolate at an MOI of 10, and incubated at 37°C for 45 min under 5% CO_2. After incubation, the macrophages were resuspended in whole media supplemented with 100 µg of gentamicin/ml and incubated at 37°C for 2 h under 5% CO_2. The macrophages were then washed twice and maintained in whole media supplemented with 10 µg of gentamicin/ml for 24, 48 and 72 h. The cells were washed every 24 h and the medium replaced. Macrophages were washed twice, lysed with 0.5% Triton X, serially diluted, and plated on TSA and XLD agar plates to determine the surviving population of SE at the aforementioned time intervals. All assays were performed in duplicates at least three times.

2.6. RNA isolation and RT-qPCR

To determine the basal level expression of avian β- defensin (AvβD) genes, RT-qPCR was performed (38) using total RNA extracted from COEC, and primers specific for the AvβD genes (Table 3). Specific amplification of AvβD genes, including AvβD -4, AvβD -5, AvβD -9, AvβD -10, AvβD -11, and AvβD -12 was achieved with primers specific for each gene, and β-actin gene serving as the endogenous control. In addition, the effect of TC, CAR, THY and EUG on the expression of SE virulence genes was investigated using RT-qPCR. Each SE strain was grown separately with and without the respective SICs of PDAs in TSB at 37°C to mid-log phase, and total RNA was extracted using RNeasy RNA isolation kit (Qiagen, Valencia, CA). Complementary DNA (cDNA) was synthesized using the Superscript II Reverse transcriptase kit (Invitrogen, Carlsbad,

CA), and was used as the template for RT-qPCR. The primers for each gene (Table 4) were designed from published GenBank, SE sequences using Primer Express® software (Applied Biosystems, Foster city, CA). Relative gene expression was determined according to comparative critical threshold (Ct) method using a 7500 Fast Real-Time PCR system (Applied Biosystems). Data were normalized to the endogenous control (16S rRNA), and the level of candidate gene expression between treated and control samples was determined.

2.7. Statistical Analysis

Data from the adhesion, invasion, and macrophage survival assays were analyzed separately. Completely randomized design (CRD) with factorial treatment structure was followed for all the trials with factors including four plant molecules (TC, CR, EUG, THY), two concentrations and three bacterial strains (21, 28, 457). Each experiment was replicated six times (n=6). Data were analyzed using the mixed procedure of SAS (version 9.3, SAS institute, Inc., Cary, NC) for all assays, and with the repeated measures statement used for the macrophage survival assay (measurements taken at 24, 48 and 72 h of incubation). Least-squares means were generated for significant F tests ($P < 0.001$) and separated using least significant differences. For gene expression assays, differences between independent treatments were analyzed using two-tailed t tests, and a P value of < 0.001 was considered significantly different.

3. Results and Discussion

Salmonella Enteritidis is a major cause of food-borne disease worldwide, with the consumption of contaminated egg as the common source of human infection. In chickens, there are two possible ways by which the pathogen contaminates the eggs; by directly

contaminating the outer surface of the eggs while transiting the cloaca, or by reaching the ovarian tissue via circulatory route and contaminating the yolk before the egg is laid. *Salmonellae* have been found on the mucosal surface and within the epithelial cells lining the oviduct in naturally and experimentally infected hens (42, 43). Since the attachment and invasion of SE in chicken oviduct cells are essential steps in its colonization of ovarian tissue and contamination of egg yolk (10, 13, 44), a potential strategy for controlling trans ovarian transmission of SE to eggs is to reduce pathogen colonization in the ovarian tissue, thereby decreasing its entry into the eggs. Therefore, this study investigated the efficacy of PDAs in reducing SE adhesion to and invasion of COEC.

Although all parts of chicken reproductive tract are prone to SE colonization, the isthmus is likely a critical site in terms of persistent reproductive tract colonization and egg membrane contamination by the pathogen (45, 46). Since no established chicken oviduct epithelial cell lines are available commercially, we isolated primary COEC from the isthmus of chicken oviduct. The primary COEC model has been reported as a useful tool in studying the early interactions between SE and chicken oviduct epithelium (38, 44, 47). Several studies have suggested that the epithelial cells (along with the lymphocytes and macrophages associated with the epithelial cells) of laying hens express a number of beta-defensins (AvβDs), which are antimicrobial peptides that play significant roles in the innate immune systems of the chickens (48, 49, 50). Hence, in order to validate the COEC isolated in the current study, the presence of six AvβD genes was detected using RT-qPCR (38, 51, 52). Our results indicated that the oviduct epithelial cells derived from laying hens constitutively expressed all AvβD genes (data not shown),

thereby confirming that the cell line used in the study was indeed of chicken oviduct origin (38).

The results on the adhesion to and invasion of 10 isolates on the COEC are presented in Fig. 1. There was no significant difference (P>0.05) between the adhesion and invasion capabilities of the isolates on the primary cell line; ~ 5.0 log and 4.0 log CFU of SE attached and invaded the COEC, respectively. However, 2 strains SE-180 and SE-90 had a significantly lower invasion than the other strains. The mean attachment and invasion efficiencies of the 10 SE isolates on COEC were 85% and 65%, respectively. This is in accordance with a previous study, wherein a similar adhesion and invasion efficiency of SE on COEC was observed (53). From the 10 isolates, three strains, namely SE 21, SE 28 and SE 457 were chosen for the subsequent experiments. Since no significant difference was observed between the three strains studied (P > 0.05), the results corresponding to SE 28 are presented in the manuscript.

The two SICs of the PDAs that did not inhibit SE growth as compared to control were selected, which included 0.565 and 0.750 mM for TC, 0.500 and 0.650 mM for CR and THY, and 1.2 and 1.8 mM for EUG. The average initial SE population in the control and treated samples was ~ 6.0 log CFU/ml. Following incubation at 37°C for 24 h, ~ 8.0 log CFU/ml of SE was recovered from all wells, irrespective of control and compound treatment (data not shown). This confirmed that the concentrations used in the assay were not inhibitory on the bacterium.

All four PDAs significantly reduced SE adhesion and invasion of COEC (P<0.05) (Fig. 2a, 2b). In general, the PDAs decreased SE adhesion and invasion of COEC by 40% and 50%, respectively. It was also observed that all PDAs at their higher tested SIC were

more inhibitory on SE adhesion and invasion of COEC compared to the lower SIC (P<0.05).

Since SICs of antimicrobials, including antibiotics can modulate bacterial physio-chemical functions, including that of genes, they are used for studying the effect of antimicrobials on bacterial gene expression and virulence (54, 55). Moreover, since the SICs do not inhibit bacterial growth or reduce their populations, the reduction in SE adhesion and invasion of COEC may be due to the effect of PDAs in modulating the expression of genes associated with virulence in the bacterium. To ascertain this, we determined the effect of PDAs on transcription of 20 published genes critical for colonization of chicken reproductive tract by RT-qPCR (Table 4). The RT-qPCR results indicated that all four PDAs significantly down-regulated (P<0.05) several oviduct-specific colonization genes in SE (Table 3). The down-regulated genes included those critical for regulating *Salmonella* motility, namely *flgG* (14), *fimD* (42, 56), and *prot6E* (19); adherence and invasion - *sopB* (53), and *invH* (57); type three secretion system (TTSS) genes - *sipA, sipB, pipB, ssaV*, and *orf245* (53); cell membrane and cell wall integrity - *hflK, lrp, ompR*, and *tatA*, (14); exo/endonuclease activity – *xthA* (58) and *mrr1/SEN4287* (19) and those involved in metabolism such as *rfbH* (59), *rpoS,* (60), *ssrA* (61), and *sodC* (62). The functions of specific genes are provided in Table 3. Although all four PDAs significantly down-regulated the expression of the aforementioned virulence genes, EUG followed by THY, CR and TC were effective in descending order in decreasing the expression of most of the tested genes (Table 4). Conversely, TC was more effective than the other PDAs in down-regulating *ompR* and *tatA*. Similarly, TC was found to be more effective in decreasing the expression of *rfBH, sodC, ssaV, and*

XthA compared to EUG. These results collectively suggest that these PDAs may be acting through different mechanisms, and genome-wide studies are needed to fully understand the mechanisms by which TC, CR, THY and EUG attenuate virulence in SE.

Macrophage uptake of a pathogen is an innate mechanism that helps a host to defend against an invading bacterium. However, the ability of a pathogen to survive the hostile environment within the macrophage offers it protection from the immune system and helps in its dissemination (41). *S*. Enteritidis has the ability to persist in chicken macrophages, enabling its spread to the circulatory system and various internal organs, including the reproductive system (53). The results from the macrophage survival assay revealed that the PDAs significantly decreased the survival of SE in chicken macrophages, although at different levels (Fig 3a and 3b). For example, except THY, all PDAs decreased SE survival in macrophages by ~ 30 to 50% at 24 and 48 h, and 40 to 80% by 72 h of incubation compared to controls ($P< 0.05$). Since SE 457 failed to survive in macrophages even in the absence of PDAs (control), no data are available for inclusion in the manuscript. RT-qPCR results supported the findings from macrophage survival assay, where the PDAs significantly down-regulated *sod*, a critical gene for *Salmonella* survival in macrophages. Pathogens present in polymorphonuclear cells and macrophages are exposed to reactive oxygen species (ROS) that function to kill bacteria. As a defense mechanism, bacteria up-regulate the expression *sod*, producing the enzyme, superoxide dismutase to neutralize ROS (63). Thus, the down-regulating effect of PDAs on *sod* expression in SE could be attributed to its reduced survival within macrophages.

In conclusion, we found that four PDAs, TC, CR, THY, and EUG significantly reduced SE colonization of cultured chicken oviduct epithelial cells by down-regulating

the transcription of critical virulence genes in the bacterium. In addition, the PDAs decreased the survival of SE in chicken macrophages.

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Table 1: List of *S.* Enteritidis strains screened

Strains of SE	Source
SE-21	Chicken intestine isolate (Connecticut Veterinary Diagnostic Medical Laboratory)
SE-28	Chicken oviduct isolate (Connecticut Veterinary Diagnostic Medical Laboratory)
SE-12	Chicken liver isolate (Connecticut Veterinary Diagnostic Medical Laboratory)
SE-31	Chicken gut isolate (Connecticut Veterinary Diagnostic Medical Laboratory)
SE-457	Chicken egg yolk isolate (University of Pennsylvania)
SE-1294	Human egg outbreak (New York Department of Health)
SE-565	Food outbreak (Lunch – II)
SE-61697	Human isolate (University of Pennsylvania)
SE-180	Human isolate (New York Department of Health)

Table 2: List of primers used for RT-qPCR to validate the primary COEC line*

Gene	Sequence
β-Actin-F	5'-TGCGTGACATCAAGGAGAAG-3'
β-Actin-R	5'-GACCATCAGGGAGTTCATAGC-3'
AvBD-4-F	5'-CATCTCAGTGTCGTTTCTCTGC-3'
AvBD-4-R	5'-CGCGATATCCACATTGCATG-3'
AvBD-5-F	5'-CTGCCAGCAAGAAAGGAACCTG-3'
AvBD-5-R	5'-GTAATCCTCGAGCAAGGGACA-3'
AvBD-9-F	5'-GCAAAGGCTATTCCACAGCAG-3'
AvBD-9-R	5'-GGAGCACGGCATGCAACAA-3'
AvBD-10-F	5'-TGGGGCACGCAGTCCACAAC-3'
AvBD-10-R	5'-CATGCCCCAGCACGGCAGAA-3'
AvBD-11-F	5'-ACTGCATCCGTTCCAAAGTCTG-3'
AvBD-11-R	5'-GTCCCAGCTGTTCTTCCAG-3'

*Ebers et al. (2009) (38)

Table 3: List of primers used for RT-qPCR of SE genes and their function:

Accession number	Gene	Gene function	Sequence (5'-3')
NC_011294.1	*fimDF*	Outer membrane usher protein FimD	5'CGCGGCGAAAGTTATTTCAA 3'
	fimDR		5'CCACGGACGCGGTATCC 3'
NC_011294.1	*flgGF*	Flagellar basal body rod protein	5'GCGCCGGACGATTGC 3'
	flgGR		5'CCGGGCTGGAAAGCATT 3'
NC_011294.1	*hflKF*	FtsH protease regulator	5'AGCGCGGCGTTGTGA 3'
	hflKR		5'TCAGACCTGGCTCTACCAGATG 3'
NC_011294.1	*invHF*	Cell adherence /invasion protein	5' CCCTTCCTCCGTGAGCAAA 3'
	invHR		5'TGGCCAGTTGCTCTTTCTGA 3'
NC_011294.1	*lrpF*	Leucine-responsive transcriptional regulator	5'TTAATGCCGCCGTGCAA 3'
	lrpR		5'GCCGGAAACCAAATGACACT 3'
NC_011294.1	*mrr1F*	Pseudo/ restriction endonuclease gene	5'CCATCGCTTCCAGCAACTG 3'
	mrr1R		5'TCTCTACCATGAACCCGTACAAATT 3'
NC_011294.1	*ompRF*	Osmolarity response regulator	5'TGTGCCGGATCTTCTTCCA 3'
	ompRR		5'CTCCATCGACGTCCAGATCTC 3'
NC_011294.1	*orf24F*	Pathogenicity island protein	5'CAGGGTAATATCGATGTGGACTACA 3'
	orf245R		5'GCGGTATGTGGAAAACGAGTTT 3'
NC_011294.1	*pipBF*	Pathogenicity island protein	5'GCTCCTGTTAATGATTTCGCTAAAG 3'
	pipBR		5'GCTCAGACTTAACTGACACCAAACTAA 3'
NC_011294.1	*prot6F*	Fimbrial biosynthesis	5'GAACGTTTGGCTGCCTATGG 3'
	prot6ER		5'CGCAGTGACTGGCATCAAGA 3'
NC_011294.1	*rfbHF*	DehydrataseRfbH	5'ACGGTCGGTATTTGTCAACTCA 3'
	rfbHR		5'TCGCCAACCGTATTTTGCTAA 3'
NC_011294.1	*rpoSF*	RNA polymerase sigma factor RpoS	5'TTTTTCATCGGCCAGGATGT 3'

	rpoSR		5’CGCTGGGCGGTGATTC 3’
NC_011294.1	*sipAF* *sipAR*	Pathogenicity island 1 effector protein	5’CAGGGAACGGTGTGGAGGTA 3’ 5’AGACGTTTTTGGGTGTGATACGT 3’
NC_011294.1	*sipBF* *sipBR*	Pathogenicity island 1 effector protein	5’GCCACTGCTGAATCTGATCCA 3’ 5’CGAGGCGCTTGCTGATTT 3’
NC_011294.1	*sodCF* *sodCR*	Superoxide dismutase	5’CACATGGATCATGAGCGCTTT 3’ 5’CTGCGCCGCGTCTGA3’
NC_011294.1	*sopBF* *sopBR*	Cell invasion protein	5’GCGTCAATTTCATGGGCTAAC 3’ 5’GGCGGCGAACCCTATAAACT 3’
NC_011294.1	*ssaVF* *ssaVR*	Secretion system apparatus protein SsaV	5’GCGCGATACGGACATATTCTG 3’ 5’TGGGCGCCACGTGAA3’
NC_011294.1	*ssrAF* *ssrAR*	Sensor Kinase	5’CGAGTATGGCTGGATCAAAACA 3’ 5’TGTACGTATTTTTTGCGGGATGT 3’
NC_011294.1	*tatAF* *tatAR*	Twin arginine translocase protein A	5’AGTATTTGGCAGTTGTTGATTGTTG 3’ 5’ACCGATGGAACCGAGTTTTTT 3’
NC_011294.1	*xthAF* *xthAR*	Exonuclease III	5’CGCCCGTCCCCATCA 3’ 5’CACATCGGGCTGGTGTTTT 3’
NC_011294.1	*16S f* *16S r*	SENr010, 16S ribosomal RNA	5’CCAGGGCTACACACGTGCTA 3’ 5’TCTCGCGAGGTCGCTTCT 3’
NC_011294.1	*mgtCF* *mgtCR*	Mg (2+) transport ATPase protein C	5’CGAACCTCGCTTTCATCTTCTT 3’ 5’CCGCCGAGGGAGAAAAAC 3’
NC_019120.1	*spvBF* *spvBR*	Actin ADP ribosyltransferase 2C toxin SpvB	5’ TGGGTGGGCAACAGCAA 3’ 5’ GCAGGATGCCGTTACTGTCA 3’

Table 4: Expression of SE genes critical for virulence and oviduct colonization in the presence of TC, CR, THY and EUG:

GENE	TC 0.56 mM	TC 0.75 mM	CR 0.50 mM	CR 0.65 mM	THY 0.50 mM	THY 0.65 mM	EUG 1.2 mM	EUG 1.8 mM
fimD	-1.10	-1.90	-1.88	-1.95	-3.04	-3.67	-6.11	-6.87
flgG	-1.10	-1.26	-1.81	-2.19	-1.13	-1.24	-3.55	-3.83
hflK	-0.65	-0.76	-1.40	-2.44	-0.30	-1.90	-3.18	-3.32
invH	-3.29	-3.76	-16.39	-17.78	-28.55	-29.76	-18.89	-19.81
lrpF	-3.98	-4.48	-10.05	-13.15	-41.66	-47.61	-1.08	-1.14
mrr1	-3.01	-3.61	-4.98	-6.22	-8.20	-10.88	-23.95	-24.13
ompR	-10.21	-11.76	-3.10	-3.66	-5.75	-6.13	-0.15	-0.18
orf245	-4.80	-5.73	-21.39	-25.37	-60.32	-64.81	-73.29	-76.92
pipB	-3.73	-4.01	-15.34	-19.16	-65.48	-66.90	-54.38	-54.59
prot6E	-3.11	-3.32	-1.99	-3.25	-5.99	-8.65	-7.11	-7.92
rfbH	-6.63	-8.00	-10.41	-11.11	-12.09	-16.66	-1.17	-1.20

rpoS	-3.45	-3.33	-4.20	-6.89	-3.33	-5.23	-1.33	-1.48
sipA	-6.50	-6.50	-29.66	-35.24	-78.59	-88.86	-76.30	-77.14
sipB	-3.46	-5.87	-7.12	-8.33	-14.50	-17.48	-50.29	-53.42
sodC	-1.67	-3.81	-4.14	-4.20	-8.67	-9.25	-0.22	-0.27
sopB	-4.21	-4.56	-22.49	-24.10	-43.22	-46.60	-48.58	-49.25
ssaV	-2.19	-2.36	-5.05	-6.93	-0.10	-0.10	-1.07	-1.27
ssrA	-0.66	-1.85	-5.97	-7.04	-3.29	-4.11	0.09	-0.11
tatA	-1.39	-1.42	-0.61	-1.74	-0.78	-2.08	-0.94	-1.15
xthA	-9.79	-12.82	-5.02	-5.74	-8.01	-8.26	-0.19	-0.27

***Fold change in gene expression, relative to control (0 mM PDA)**

Fig. 1: Adhesion to and invasion of 10 strains of SE on COEC. Ten strains of SE were screened to determine their adhesive and invasive properties on COEC. The COEC were seeded on a 24-well tissue culture plates at ~ 10^5 cells per well, and inoculated with ~ 6.0 log CFU of each SE (MOI 10). The infected monolayer was incubated for 1 h following which the cells were lysed and the number of viable adherent SE was determined. For the invasion assay, the monolayers incubated for 1 h following SE infection, were rinsed and incubated for another 2 h in whole media-10% FBS containing gentamicin (100 μg/ml) following which the cells were lysed and SE was enumerated. Grey bars indicate SE adhesion on COEC, whereas black bars indicate invasion. Strains were not significantly different from each other (P>0.05) except for invasion of SE 180 and SE 90 (P<0.05).

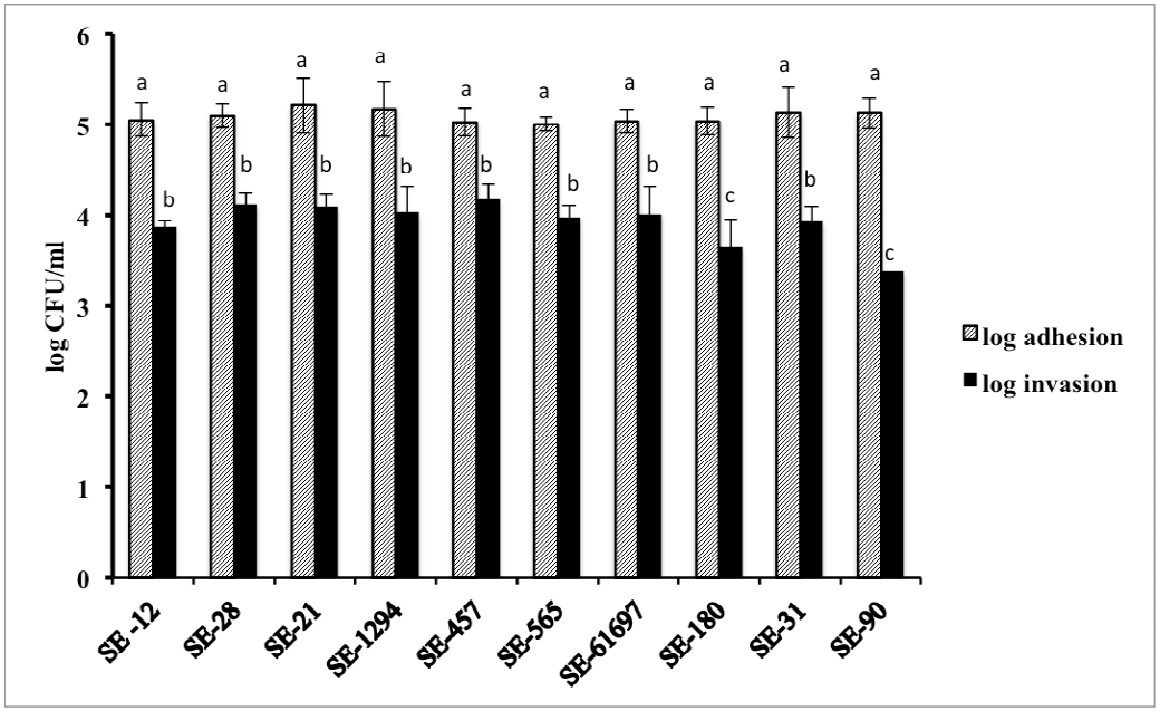


Fig. 2a: Effect of *trans*-cinnamaldehyde (TC), carvacrol (CR) thymol (THY) and eugenol (EG) on SE adhesion to primary COEC. 10^5 COEC were inoculated with 6.0 log CFU of each SE (MOI 10). After incubating the infected monolayer for 1 hour, the cells were lysed and viable SE adhered were enumerated. For invasion, following incubation for 1 hour, the infected cells were rinsed and incubated for another 2 h in whole media supplemented with 10% FBS containing gentamicin (100 μg/ml). The cells were lysed and invading SE was determined. Since there was no significant difference between three strains studied, results are shown for SE 28 (P <0.05). Treatments for each compound differed significantly from the control (open column) at P<0.05.

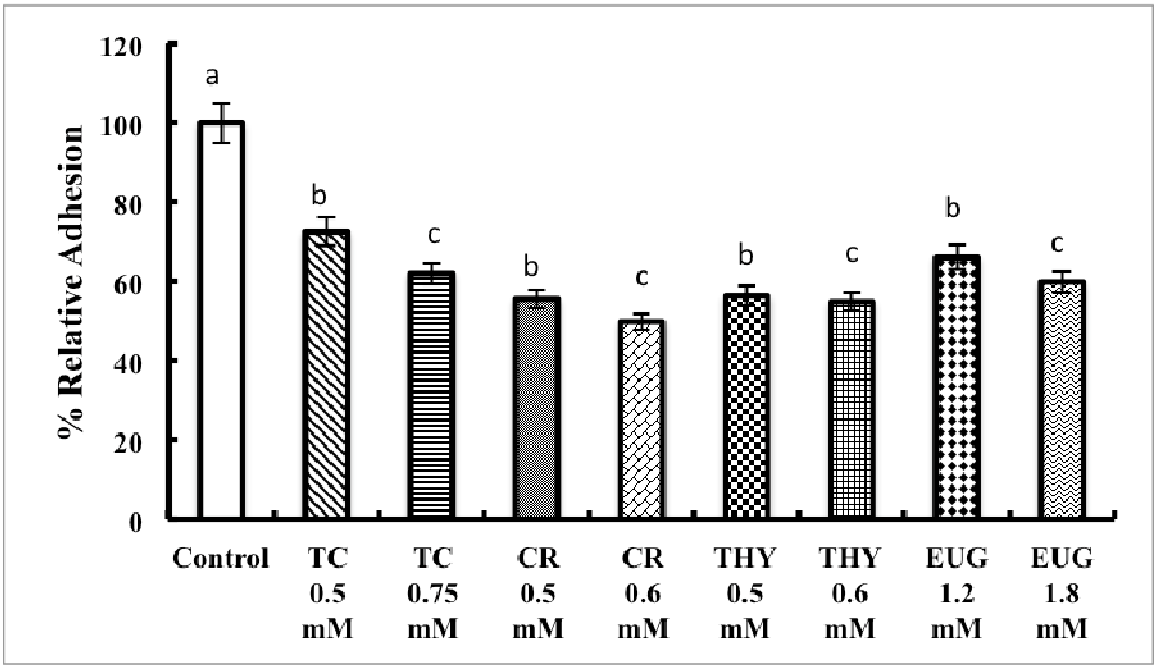


Fig. 2b: Effect of *trans*-cinnamaldehyde (TC), Carvacrol (CR) Thymol (THY) and Eugenol (EG) on SE invasion of primary COEC line. The COEC were seeded on a 24-well tissue culture plates at ~ 10^5 cells per well, and inoculated with ~ 6.0 log CFU of each SE (MOI 10). The infected monolayer was incubated for 1 h. The cells were lysed and the number of viable adherent SE was enumerated. For the invasion assay, the monolayers incubated for 1 h following SE infection, were rinsed and incubated for another 2 h in whole media-10% FBS containing gentamicin (100 μg/ml). Following the incubation, the cells were lysed and invading SE was enumerated. Since there was no significant difference between three strains studied, results are shown for SE 28 (P<0.05). Treatments for each compound differed significantly from the control (open column) at P<0.05.

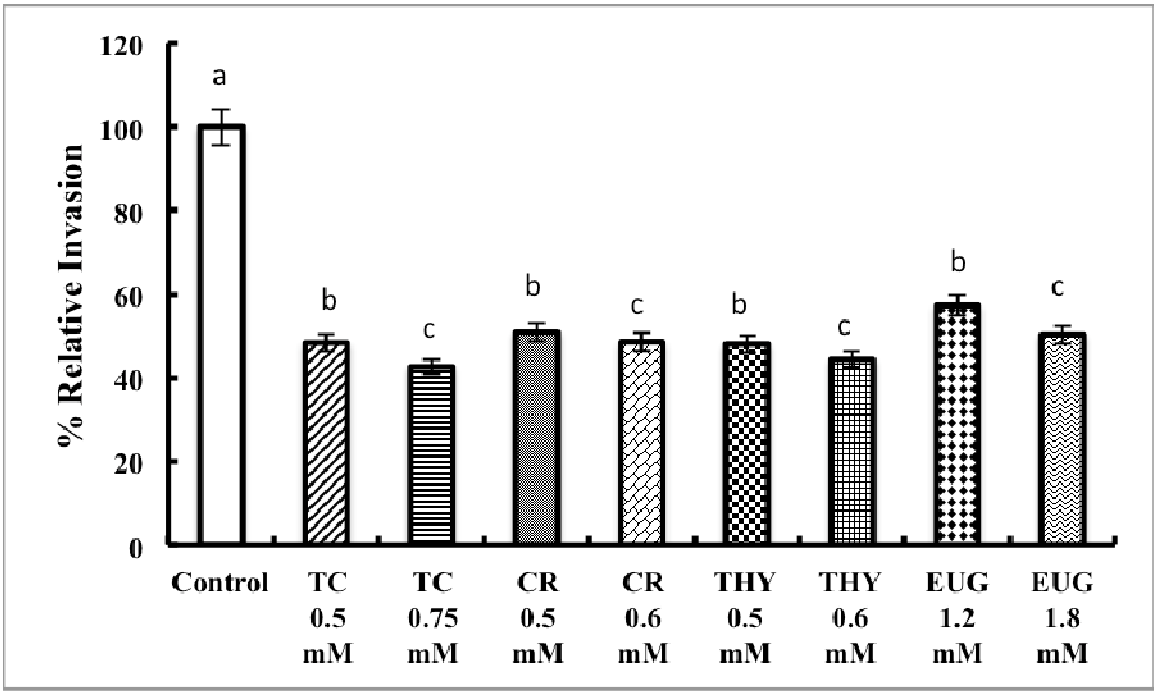


Figure 3a: Effect of *trans*-cinnamaldehyde (TC), carvacrol (CR) thymol (THY) and eugenol (EG) on survival of SE 21 in chicken macrophages. About 10^5 macrophages were infected with 6.0 log CFU/ml of SE, and incubated at 37°C for 45 min under 5% CO_2. The macrophages were then washed twice and maintained in whole media supplemented with 10 µg of gentamicin/ml for 24, 48 and 72 h. At their respective time points, the cells were lysed and the surviving SE was enumerated on XLD and TSA. All treatments except for THY differed significantly from the control (black column) at $P<0.05$.

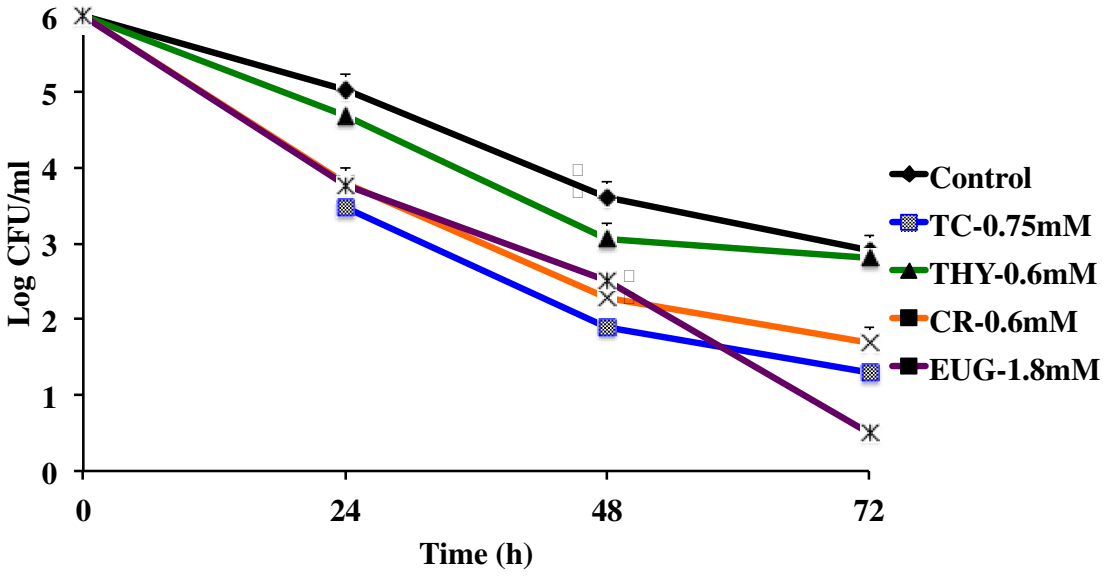
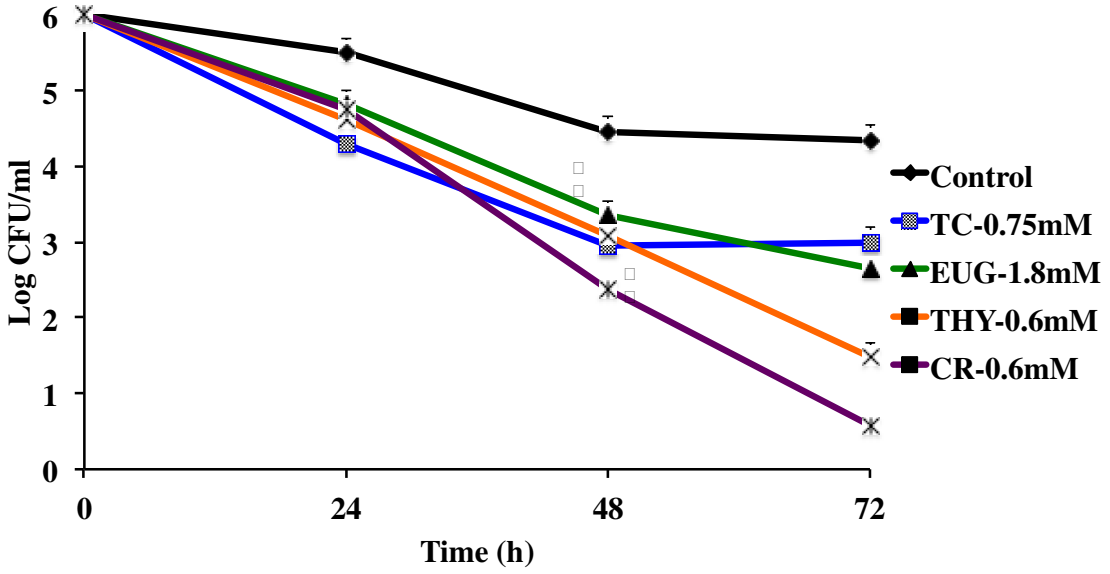


Figure 3b: Effect of *trans*-cinnamaldehyde (TC), carvacrol (CR) thymol (THY) and eugenol (EG) on SE 28 survival of HTC (Chicken macrophages). 10^5 macrophages were infected with 6.0 log CFU/ml of SE, and incubated for 45 min, following which they were washed and maintained in whole media supplemented with 10 µg of gentamicin/ml for 24, 48 and 72 h. The cells were lysed at each time point and surviving SE was determined by plating on XLD and TSA. Treatments for each compound differed significantly from the control (black column) at $P < 0.05$.



Chapter IV

In-feed supplementation of *trans*-cinnamaldehyde reduces egg-borne transmission of *Salmonella* Enteritidis in layer chickens

Abstract

Salmonella Enteritidis (SE) is a major foodborne pathogen in the United States, causing gastroenteritis in humans, primarily through consumption of contaminated eggs. Chickens are the reservoir host of SE. In layer hens, SE colonizes the intestine and migrates to various organs including the oviduct, leading to egg contamination. This study investigated the efficacy of in-feed supplementation of *trans*-cinnamaldehyde (TC), a GRAS-status, plant compound obtained from cinnamon, in reducing SE cecal colonization and systemic spread in layers. The consumer acceptability of eggs was also determined by triangle test.

Supplementation of TC in feed for 66 days at 1 or 1.5% (vol/wt) to 40-week or 25-week-old layer chickens decreased SE on eggshell and in the yolk ($P<0.001$). Additionally, SE persistence in the cecum, liver and oviduct of TC-supplemented birds was decreased compared to control ($P<0.001$). No significant differences in feed intake, body weight or egg production in birds, and consumer acceptability of eggs was observed ($P>0.05$). The results suggest that TC could potentially be used as a feed additive to reduce egg-borne transmission of SE.

1. Introduction

Salmonella enterica serovar Enteritidis (SE) is one of the major foodborne pathogens in the United States responsible for causing enteric illnesses in humans (1). Eggs are the primary source of SE infection to humans (1,2). Chickens act as asymptomatic carriers for SE resulting in their environmental dissemination and potential infection to humans. Humans contract SE infection *via* consumption of contaminated, raw or undercooked eggs, and several epidemiological studies have confirmed this association between human salmonellosis and egg consumption (4, 5).

Despite the implementation of various pre- and post-harvest control measures, SE remains a major cause of egg-borne disease outbreaks in the US (1). Recently, the United States Centers for Disease Control and Prevention (CDC) reported that food-borne salmonellosis did not decrease significantly in the last decade, highlighting the need for renewed efforts and alternative approaches for controlling *Salmonella* (6). Moreover, in light of increasing evidence linking human salmonellosis with consumption of eggs, the Food and Drug Administration (FDA) in 2009 announced that eggs constitute the primary source of SE infections to humans, and issued a final rule that requires egg producers to implement measures to prevent the pathogen from contaminating eggs on the farm and further growth during storage and transportation (7).

Cecum is the primary site of SE colonization in chickens (8, 9), with cecal carriage of the pathogen leading to contamination of ovaries by transovarian route (10). Additionally, the uptake of *Salmonella* by hen's macrophages following bacterial invasion of intestinal cells aids in its dissemination within the host, including the reproductive organs (11-14). Contamination of egg contents (yolk, albumen and eggshell

membranes) by SE can occur before oviposition (11, 12), where *Salmonella* colonized in reproductive organs invades and multiplies in the granulosa cells of the pre-ovulatory follicles in the reproductive tract (15, 16). Since SE colonization in the ceca of layers results in the transovarian spread of the pathogen to reproductive organs, decreasing pathogen prevalence in flocks has been reported to result in a direct reduction in human health risk (17). Control measures implemented at the flock level could reduce human salmonellosis from egg consumption, and thus suggested as a primary focus of control at farm level (18). Therefore, innovative on-farm strategies for preventing SE colonization of birds are critical to prevent pathogen contamination of eggs. Besides reducing the cecal colonization of SE in chicken cecum, a viable approach could also be one that potentially reduces bacterial virulence, thereby preventing its colonization in the reproductive tract and eventual trans-ovarian transmission to eggs (10, 19, 20).

Various approaches for reducing SE colonization in poultry have been investigated with varying degrees of success. These include feeding chickens with competitive exclusion bacteria (21, 22), bacteriophages (23), organic acids (24, 25), oligosaccharides (26, 27) antibiotics (28), and vaccination of birds (29). Due to the limited efficacy of aforementioned approaches along with concerns over toxicity of synthetic chemicals and the development of multi-drug resistance in bacteria, there is a growing interest in exploring the potential of natural antimicrobials for controlling pathogens (30, 31).

Since ancient times, plants have played a critical role in human health and well-being. Plant extracts have been widely used in herbal medicine, both prophylactically to prevent infections, and therapeutically for the treatment of various ailments and diseases

(32). The antimicrobial activity of several plant-derived compounds has been previously reported (33, 34), and a wide array of active components has been identified (35). A majority of these compounds are secondary metabolites, and are produced by plants in response to microbial infection or animal predation (36, 37). Among the various plant compounds, *trans*-cinnamaldehyde (TC), a major ingredient in cinnamon (*Cinnamomum zeylandicum*) has been reported to exhibit antibacterial properties against both Gram-negative and Gram-positive bacteria (33). It is a GRAS (generally regarded as safe)-status chemical approved for addition in foods by the United States FDA (TC – 21CFR182.60). Previously, our laboratory observed that TC was effective in reducing SE in chicken cecal contents *in vitro*, and in various internal organs in broilers (38). In addition, TC was found to inhibit biofilm formation in *Cronobacter sakazakii* (39) and uropathogenic *Escherichia coli* (40) by down-regulating critical genes involved in biofilm synthesis.

The objective of this study was to investigate the efficacy of feed-supplemented TC in reducing SE colonization, systemic spread and contamination of eggs in layer chickens. Moreover, the effect of TC supplementation in birds on consumer acceptability of eggs was studied.

2. Materials and Methods

2.1 Bacterial strains and dosing

A four-strain mixture of SE isolated from chickens (obtained from the Connecticut Veterinary Diagnostic Medical Laboratory, University of Connecticut) was used to inoculate the birds. The isolates were SE-12 (chicken liver, phage type 14b), SE-

21 (chicken intestine, phage type 8), SE-28 (chicken ovary, phage type 13a), and SE-31 (chicken gut, phage type 13a). Each strain was pre-induced for resistance to 50 µg/ml of nalidixic acid (NA; Sigma Aldrich, St. Louis, MO, USA) for selective enumeration (38). One hundred microliters of each NA-resistant strain was cultured separately in 10 ml tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) overnight, transferred to flasks containing 100 ml TSB supplemented with 50 µg/ml of NA, and incubated overnight at 37^{o}C with shaking (100 rpm). Equal volume of each SE culture was combined and centrifuged at 3,600 x g for 15 min at 4^{o}C. The pellet was washed and resuspended in 100 ml of phosphate buffered saline (PBS, pH 7.0), and used as the inoculum (~10^{10} CFU/ml). The bacterial count in the individual cultures and the four-strain cocktail was confirmed by plating 0.1 ml portions of appropriate dilutions on xylose lysine desoxycholate agar (XLD; Difco) plates containing NA (XLD-NA), and incubating the plates at 37^{o}C for 24 h.

2.2. Experimental Birds and Housing

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut. Twenty five and 40-week-old *Salmonella*-free layer hens (single comb, white leghorn) were procured from University of Connecticut poultry farm, and allocated to floor pens with *ad libitum* non-medicated feed, *Salmonella*-free water, age appropriate ambient temperatures, and bedding at the Isolation Facility of University of Connecticut.

Two separate experiments with TC were conducted, wherein 40-week old (experiment 1) and 25-week old layers (experiment 2) were randomly allocated to 6 treatments (20 birds/treatment group). The treatments included a negative control (no SE

challenge and no supplemental TC), low dose compound control (no SE challenge but, 1.0 % supplemental TC vol/wt), high dose compound control (no SE challenge 1.5% supplemental TC vol/wt), a positive control (SE challenge but, no supplemental TC), a low dose treatment (SE challenge and 1% supplemental TC), and a high dose treatment (SE challenge and 1.5% supplemental TC). On day 0, two birds from each experimental group were randomly selected and sacrificed to confirm that the birds were initially devoid of any *Salmonella*. *Trans*-cinnamaldehyde was supplemented in the feed for 66 days starting on day 0. Appropriate amount of TC was added in feed and mixed thoroughly to obtain the concentrations of 1 and 1.5% in the feed. On day 10, birds in the positive control, low dose and high dose treatments were challenged with SE (10 $\log_{10}$ CFU/bird) by crop gavage. After 3 days of SE challenge (day 13), three birds from each treatment group were sacrificed to determine pathogen colonization in the ceca, liver and oviduct. After 7 days of challenge (day 17), eggs were collected daily from each treatment group and tested for the presence or absence of SE. Birds were re-inoculated on day 35 followed by egg collection and testing until 66 days. In addition, cloacal swabs from all birds were analyzed weekly until 66 days for the presence or absence of *Salmonella*. At the end of 66 days, the birds from all treatment groups were euthanized by CO_2 asphyxiation, and cecum, oviduct and liver samples from birds were collected for SE detection.

2.3. Determination of SE on egg surface and egg contents

After 7 days of SE challenge, eggs from each treatment group were collected daily and checked for the presence or absence of the pathogen until 66 days of experiment. The SE presence on egg-shell surface and in egg contents was determined

according to the method of Miyamoto et al. (11). Each egg was rinsed separately in a sterile stomacher bag containing 50 ml of selenite cysteine broth supplemented with NA (50 μg/ml) for 2 min. After washing, the egg was removed and the broth was incubated at 37°C for 48 h, followed by streaking on XLD + NA plates to detect SE presence on eggshell. The bacterial colonies were confirmed as *Salmonella* using the *Salmonella* rapid detection kit (Microgen Bioproducts Ltd. Camberley, UK).

The eggs that were washed in selenite cysteine broth above were disinfected by wiping with 70% ethanol, dried, cracked open aseptically, and the shell and egg contents were collected into separate, stomacher bags containing 50 ml of selenite cysteine broth containing NA. The bags with the eggs contents or shell were homogenized for 1 min in a stomacher, and incubated at 37°C for 24-48 h to detect *Salmonella* present inside the egg. The bacterial colonies were confirmed as SE as described previously.

2.4. Determination of SE in internal organs

SE populations in oviduct, liver and cecum were determined as described previously (38). The organ samples and their contents from each bird were weighed and homogenized. Each homogenate was serially diluted (1:10) in PBS, and appropriate dilutions were plated on XLD-NA plates for bacterial enumeration. Representative colonies from XLD-NA plates were confirmed as *Salmonella* using the *Salmonella* rapid detection kit (Microgen Bioproducts Ltd.). When colonies were not detected by direct plating, samples were tested for surviving *Salmonella* by enrichment in 100 ml selenite cysteine broth (SCB; Oxoid) for 48 h at 37°C (38), followed by streaking on XLD-NA plates. In addition, cecal endogenous bacteria were enumerated by plating appropriate

dilutions of the ceca samples on duplicate thioglycollate agar plates (TGA; Difco), followed by incubation at 37^{o}C under 5% CO_2 for 24 h.

2.5. Sensory Evaluation of eggs

The sensory evaluation of eggs collected from TC-treated and control birds was conducted at the Sensory Laboratory, Department of Poultry Science, Auburn University, Alabama. Eggs were collected from control and TC-treated groups of birds once a week for three weeks, and were tested using the triangle test (44) to assess whether consumers can detect a difference between the eggs from TC supplemented and control birds. The sensory testing was done with 36 panelists (students, staff, faculty, local townsmen) per experiment, and the experiment was repeated thrice over a period of three weeks. The panelists were randomly served with three coded scrambled egg samples for tasting and detection of organoleptic differences in a sensory booth under white light. The effect of residual taste in the mouth was minimized by using a water-based mouth rinse between each sampling.

2.6. Statistical analysis

The number of SE colonies in the organs was logarithmically transformed (log_{10} CFU/g) before analysis to achieve homogeneity of variance. These data were analyzed using the PROC-GLM procedure of the statistical analysis software (version 9.2, SAS Institute Inc. Cary, NC). Differences among the means were detected at $P<0.001$ using Fisher's Least Significance Difference (LSD) test. For cell culture and RT-qPCR assays, the results are provided as mean values with their standard errors. Differences between two independent treatments were analyzed using two-tailed t test and $P<0.001$ was considered statistically significant. For the sensory study, analysis of results was done for

a probability level of 5% using a table of "Minimum Numbers of Correct Judgments" (44).

3. Results

The dietary supplementation of TC at 1 or 1.5% did not significantly alter ($P > 0.05$) the body weight or egg production in birds compared to controls in experiment 1 and experiment 2. In both experiments, TC supplementation (1 and 1.5%) decreased SE on shell and in the yolk ($P<0.001$). In experiment 1, a total of 2195 eggs from the inoculated birds were evaluated over a period of 7 weeks for the presence of *Salmonella* on the shell and in yolk. As observed in Figures 1a and 1b, TC at 1% and 1.5% consistently decreased *Salmonella* both on the shell (1a) and in yolk (1b) from week 1 to week 7 of supplementation ($P<0.001$). The cumulative data on the prevalence of *Salmonella* from 2195 eggs over the 7-week period revealed that dietary supplementation of TC at 1.5% decreased SE presence to 16% on the shell and 4% in yolk, when compared to control birds which yielded 60% SE-positive eggs (Figure 1c) on shell and 40% in yolk (Figure 1d).

In the experiment with 25-week old birds, a total of 2350 eggs from inoculated birds were assayed for *Salmonella* presence in eggs. As observed in experiment 1, TC supplementation at both tested concentrations decreased SE contamination eggshell and yolk ($P<0.001$) compared to untreated control birds (Figures 2a and 2b). In-feed supplementation of TC at 1.5 and 1% levels reduced SE contamination of eggshell and yolk to 15% and 2%, and to 28% and 4%, respectively as compared to control birds, which produced 63% positive eggs on shell and ~ 39% in yolk (Figure 2c and 2d).

Similar to the results observed in eggs, TC supplementation reduced SE colonization of cecum, liver and oviduct in both 40-week and 25-week old birds ($P<0.001$). In 40-week old layers, 60% of cecal samples, 20% of liver samples and 30% of oviduct samples from control birds tested positive for SE (Figure 3a). However, TC supplementation decreased SE in all the aforementioned organs, with the pathogen recovered from only 35% of the cecum and 10% of liver and oviduct samples from birds. Similar results were also observed in the experiment with 25-week old birds (Figure 3b). In addition, the cecal endogenous bacterial counts did not differ ($P>0.05$) among birds from the various treatment groups (data not shown).

When the eggs were subjected to sensory analysis by triangle test, only 43 of the 108 panelists were able to detect the eggs from TC-treated birds, and the remaining 65 of them failed to identify the treatments from controls, thus resulting in a 0.005 confidence that the panelists were not able to detect a difference between the eggs from TC-supplemented and untreated birds.

4. Discussion

Despite substantial progress achieved in food safety through pathogen reduction programs, SE remains as one of the most common foodborne pathogens transmitted to humans through the consumption of contaminated eggs. Since chickens serve as the reservoir of SE, innovative on-farm strategies for reducing pathogen colonization in birds are critical to control human infections. An antimicrobial treatment that can be applied through feed represents the most practical and economically viable method for controlling SE in chickens. In addition, a natural and safe feed additive will be better accepted by producers, including organic farmers without concerns for toxicity.

S. Enteritidis primarily colonizes the chicken cecum (47, 48), from where it spreads to the spleen and liver by lymphatic or circulatory routes, serving as a repertoire for subsequent colonization and spread (47, 48). In addition, SE colonizes the reproductive organs in layers, thereby contaminating the yolk. The results from the chicken trials indicated that in-feed administration of TC significantly reduced SE colonization in layer chickens, and egg-borne transmission of the bacterium. TC supplementation to birds not only decreased SE on eggshell and in the yolk, but also reduced the pathogen populations in the cecum, liver and oviduct compared to control birds ($P<0.001$).

In summary, TC supplementation to chickens reduced SE contamination of egg yolk and shell without adversely affecting egg production or consumer acceptability of eggs from treated birds. We conclude that TC could potentially be used as an antimicrobial feed additive to reduce egg-borne transmission of SE in combination with standard hygienic practices used in the farm. This manuscript reports the first study demonstrating the effectiveness of a feed-supplemented natural antimicrobial compound in reducing the transovarian route of transmission of SE in layer chickens.

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Figure 1: Effect of TC on SE contamination of eggs in 40-week old birds for 7 weeks post inoculation (a) on egg shell, (b) egg yolk (c) cumulative effect of TC treatment for 7 weeks on shell, (d) cumulative effect of TC treatment for 7 weeks on yolk, N = 2195, P<0.001. Negative and compound controls were not included in the statistical analysis since SE was not recovered from those treatments.

Figure 1 (a)

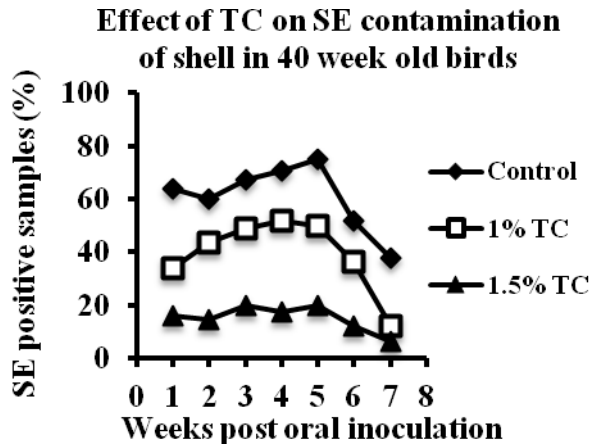


Figure 1 (b)

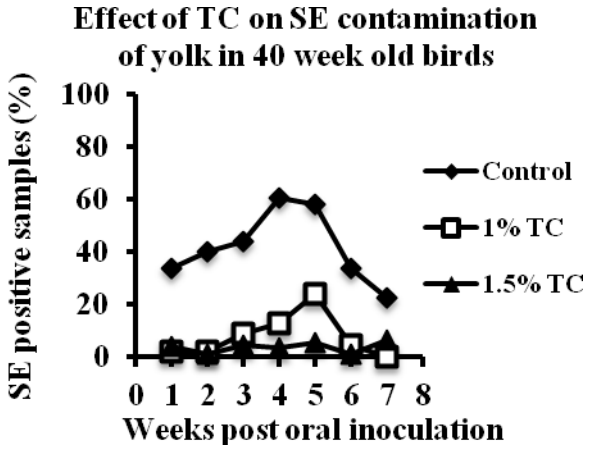


Figure 1 (c)

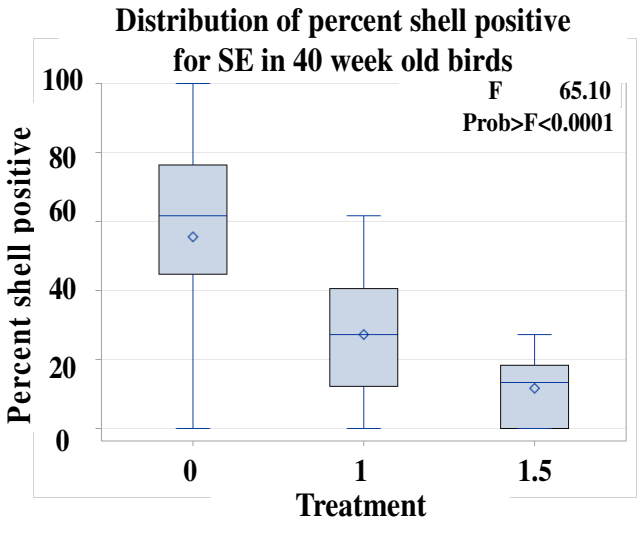


Figure 1 (d)

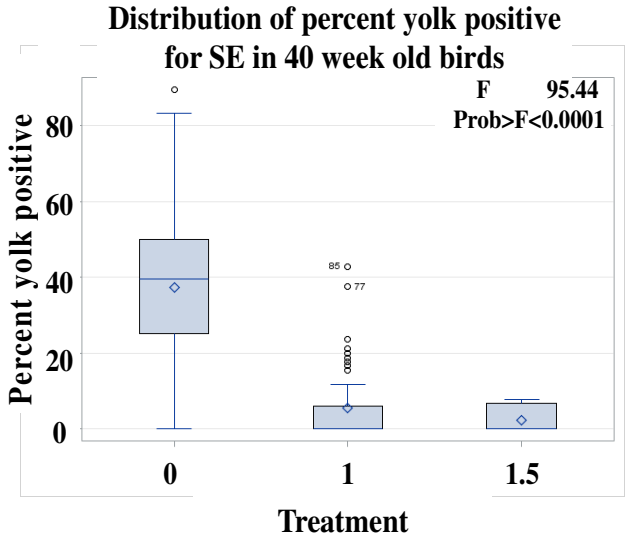


Figure 2: Effect of TC on SE contamination of eggs in 25-week old birds for 7 weeks post inoculation (a) on egg shell, (b) egg yolk (c) cumulative effect of TC treatment for 7 weeks on shell, (d) cumulative effect of TC treatment for 7 weeks on yolk, N = 2350, P<0.001. Negative and compound controls were not included in the statistical analysis since SE was not recovered from those treatments.

Figure 2 (a)

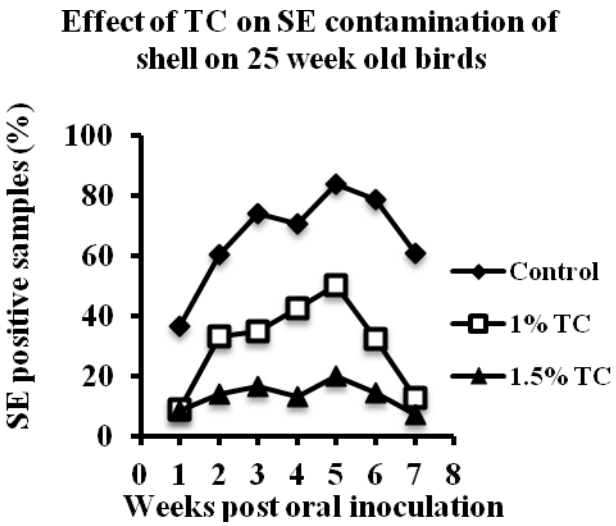


Figure 2 (b)

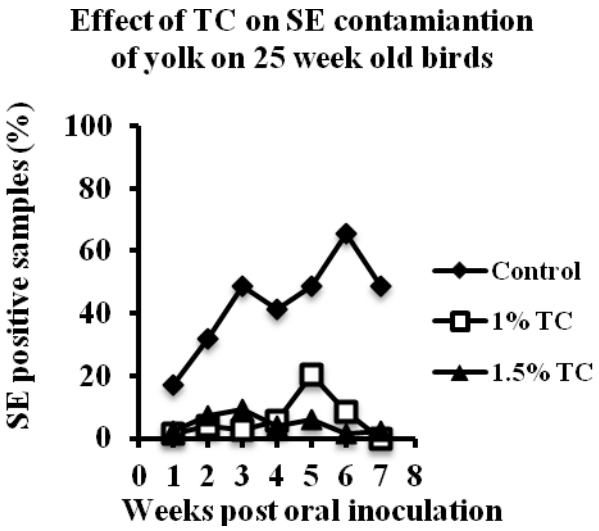


Figure 2 (c)

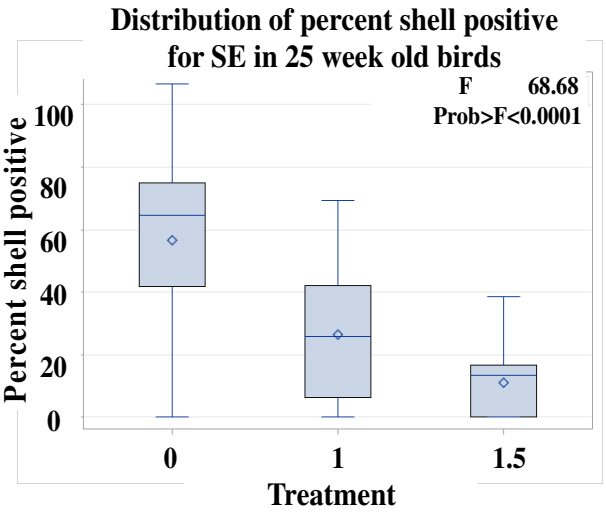


Figure 2 (d)

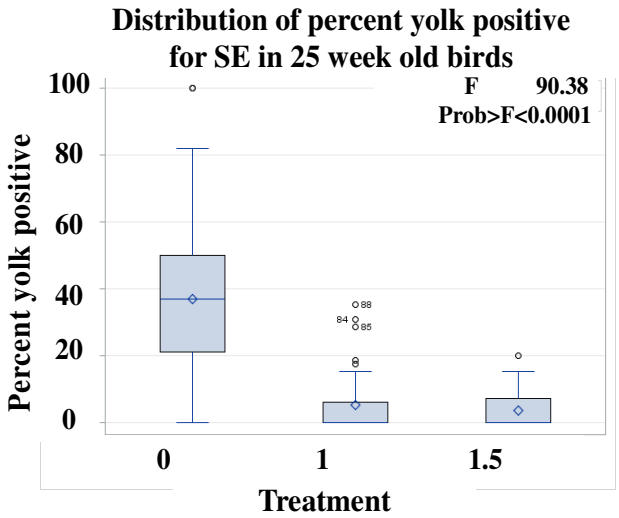


Figure 3: Effect of TC on SE in internal organs (liver, cecum, oviduct) of (a) 40-week-old layer hens, P<0.001. (b) 25-week-old layer hens, P < 0.001. In the 40-week-old layer hens, values with different letters (a b c, a' b' c', a" b" c") differ significantly within the organ between treatments (P<0.001).

Figure 3 (a)

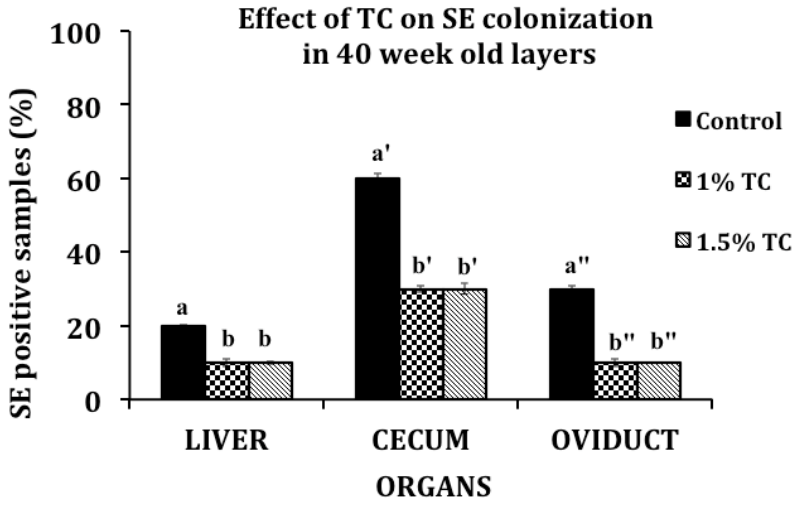
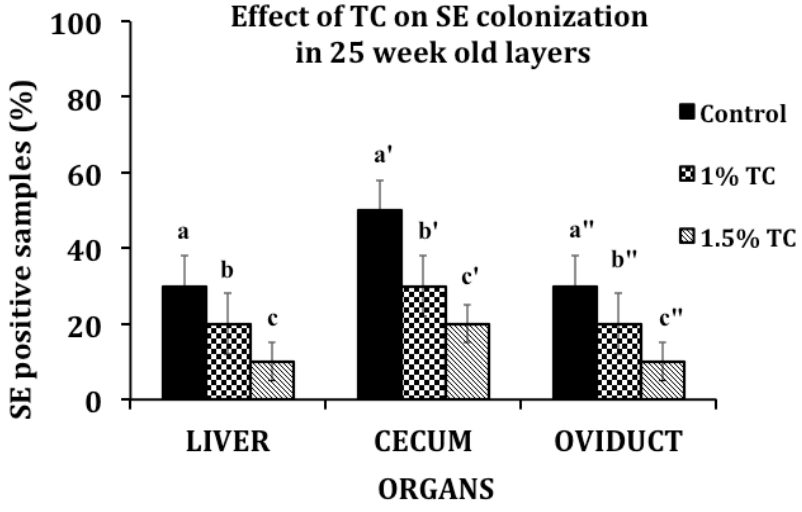


Figure 3 (b)



Chapter V

Reducing egg-borne transmission of *Salmonella* Enteritidis in layer chickens by in-feed supplementation of caprylic acid

Abstract

Salmonella Enteritidis (SE) is a major foodborne pathogen in the United States, responsible for causing gastro-intestinal infections in humans, predominantly due to the consumption of contaminated eggs. In layer hens, SE colonizes the intestine and migrates to various organs, including the oviduct, thereby leading to egg yolk and shell contamination. This study investigated the efficacy of a medium chain fatty acid, caprylic acid (CA), in reducing SE colonization and egg contamination in layers. White leghorn birds (40 week old; N=120/experiment) were randomly assigned to six treatments (n=20/treatment): negative control (-ve SE, -ve CA), compound controls (-ve SE, +ve 0.7% or 1% CA), positive control (+ve SE, -ve CA), low (+ve SE, +ve 0.7% CA) and high dose treatment (+ve SE, +ve 1% CA). Caprylic acid was supplemented in the feed for 66 days, starting on day 0. On days 10 and day 35, birds in the positive controls, low and high dose treatments were challenged with 10^{10} CFU/ml of SE by crop gavage. After 7 days post inoculation, eggs were collected daily and tested for SE on the shell and in the yolk separately. The birds from each treatment were sacrificed on day 66 of the experiment to determine pathogen colonization in the ceca, liver and oviduct. The consumer acceptability of eggs was also determined by triangle test. The experiment was repeated twice.

In-feed supplementation of CA (0.7%, 1%) to birds consistently decreased SE on eggshell and in the yolk ($P<0.001$). Additionally, a reduction in SE populations in the cecum, liver and oviduct was observed in treated birds compared to control ($P<0.001$). No significant difference in feed intake, body weight or sensory properties of eggs was

observed ($P>0.05$). The results suggest that CA could potentially be used as a feed additive to reduce egg-borne transmission of SE.

1. Introduction

Salmonella enterica serovar Enteritidis (SE) is a major cause of food-borne illnesses worldwide (CDC, 2010). The bacterium is responsible for causing gastroenteritis in humans with contaminated eggs as the primary source of infection (CDC, 2010, Mead et al., 1999). Approximately 90 billion eggs are produced and 67.5 billion shell eggs are consumed annually in the USA (USDA, 2012), thereby emphasizing the significance of the microbiological safety of eggs. Chickens are asymptomatic carriers of SE resulting in pathogen dissemination to the environment and potential infection to humans. In addition, the most common food associated with SE infection in humans is contaminated, raw or undercooked egg, and several epidemiological studies have confirmed this association between human salmonellosis and consumption of eggs (Guard-Petter, 2001, Braden, 2006).

Despite adopting various pre- and post-harvest control measures, SE remains a major cause of egg-borne disease outbreaks in the US (CDC, 2010). The United States Centers for Disease Control and Prevention (CDC) recently reported that food-borne salmonellosis did not decrease significantly in the last decade, underscoring the need for novel approaches to control *Salmonella* (CDC, 2012). Due to the increasing evidence linking human salmonellosis with consumption of eggs, the Food and Drug Administration (FDA) in 2009 issued a final rule requiring egg producers to implement measures to prevent SE from contaminating eggs on the farm and further growth during storage and transportation (FDA, 2009).

The cecum is the primary site of SE colonization in chickens (Allen-Vercoe and Woodward, 1999, Stern, 2008), and cecal carriage of the pathogen leads to contamination

of ovaries by transovarian route (Gantois et al., 2009). Moreover, contamination of egg contents (yolk, albumen and eggshell membranes) by SE can occur prior to oviposition (Miyamoto et al., 1997, Okamura et al., 2001), where *Salmonella* colonized in reproductive organs invades and multiplies in the granulosa cells of the pre-ovulatory follicles in the reproductive tract (Thiagarajan et al., 1994, 1996). Since SE colonization of the ceca results in the transovarian spread of the bacterium and subsequent egg-borne transmission, interventions implemented at the flock level to decrease the pathogen prevalence in birds represent a viable strategy for reducing human salmonellosis from egg consumption (Altekruse et al., 1993; Namata et al., 2008; Gantois et al., 2009, Keller et al., 1995, Clavijo et al., 2006).

Although several approaches, including feeding chickens with competitive exclusion bacteria (Mead et al., 1996; Stern et al., 2001; Mead, 2002), bacteriophages (Carrillo et al., 2005), organic acids (Byrd et al., 2001; Heres et al., 2004), oligosaccharides (Schoeni and Wong, 1994, Spring et al., 2000; Fernandez et al., 2002) antibiotics (Chadfield and Hinton, 2003) and vaccination of birds (Deuger et al., 2001; Inoue et al., 2008) have been investigated for reducing SE colonization in chickens, limited efficacy of the aforementioned approaches along with concerns for the development of multi-drug resistance in *Salmonella* have triggered research investigating the potential of natural antimicrobials to control pathogens (Abee et al., 1995, Salamci et al., 2007). Lipids and their esters have been studied extensively in the past for their microbicidal activity (Bergsson et al., 1998, 1999, Nair et al., 2005). Free fatty acids, especially medium-chain fatty acids (MCFAs) are bactericidal against Gram-positive and Gram-negative bacteria (Dierick et al., 2004; Nakai and Siebert 2002). Caprylic acid

(CA, octanoic acid) is a natural, 8-carbon MCFA present in coconut oil, breast milk, and bovine milk (Jensen 2002; Sprong et al., 2001). It is classified as generally recognized as safe (GRAS) by the US FDA (CFR 184.1025). Our previous research indicated that supplementation of CA through feed reduced *Campylobacter jejuni* and SE carriage in broiler chickens (Solis de los Santos et al., 2008, 2009; Kollanoor Johny et al., 2012a). Prior research from our laboratory also revealed that CA reduced SE invasion of avian intestinal epithelial cells by down-regulating critical colonization-associated genes in the pathogen (Kollanoor Johny et al., 2012a). The present study was undertaken to investigate the prophylactic efficacy of CA as a feed supplement in reducing SE colonization and egg-borne transmission in layer chickens. Moreover, the effect of CA supplementation on consumer acceptability of eggs was studied.

2. Materials and Methods

2.1 Bacterial strains and dosing

A four-strain mixture of SE strains isolated from chickens (obtained from the Connecticut Veterinary Diagnostic Medical Laboratory, University of Connecticut) was used to inoculate the birds. The SE isolates were SE-12 (chicken liver, phage type 14b), SE-21 (chicken intestine, phage type 8), SE-28 (chicken ovary, phage type 13a), and SE-31 (chicken gut, phage type 13a). Each strain was pre-induced for resistance to 50 μg/ml of nalidixic acid (NA; Sigma Aldrich, St. Louis, MO, USA) for selective enumeration (Kollanoor-Johny et al., 2012b). One hundred microliters of each NA-resistant strain was cultured separately in 10 ml tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) overnight, transferred to flasks containing 100 ml TSB supplemented with 50 μg/ml of NA, and incubated overnight at 37^{o}C with shaking (100 rpm). Equal volumes of

the SE cultures were combined and centrifuged at 3,600 x g for 15 min at 4°C. The pellet was washed and resuspended in 100 ml of phosphate buffered saline (PBS, pH 7.0), and used as the inoculum (~10^{10} CFU/ml). The bacterial count in the individual cultures and the four-strain cocktail was confirmed by plating 0.1 ml portions of appropriate dilutions on xylose lysine desoxycholate agar (XLD; Difco) plates containing NA (XLD-NA), and incubating the plates at 37°C for 24 h.

2.2 Experimental Birds and housing

All experiments with chickens were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut. Forty-week-old *Salmonella*-free layer hens (single comb, white leghorn) were procured from University of Connecticut poultry farm, and allocated to floor pens with *ad libitum* non-medicated feed, *Salmonella*-free water, age appropriate ambient temperatures, and bedding at the Isolation Facility of University of Connecticut.

2.3 Experimental design

Two experiments with CA were conducted, wherein 40-week old hens were randomly allocated to 6 treatments. Caprylic acid from Sigma Aldrich (99% purity, SAFC grade) was used for all the experiments. Briefly, the treatments included a negative control (no SE challenge and no supplemental CA), low dose compound control (no SE challenge but, 0.7 % supplemental CA vol/wt), high dose compound control (no SE challenge 1% supplemental CA vol/wt) a positive control (SE challenge but, no supplemental CA), a low dose treatment (SE challenge and 0.7% supplemental CA), and a high dose treatment (SE challenge and 1% supplemental CA). On day 0, two birds from each experimental group were randomly selected and sacrificed to confirm that the birds

were initially devoid of any *Salmonella*, and CA was supplemented in the feed for 66 days starting from day 0. Appropriate amount of CA was added in feed and mixed thoroughly to obtain concentrations of 0.7 and 1.0% in the feed. On day 10, birds in the positive control, low dose and high dose treatments were challenged with the four-strain mixture of SE (10 $\log_{10}$ CFU/bird) by crop gavage. After 3 days of SE challenge (day 13), three birds from each treatment group were sacrificed to determine pathogen colonization in the ceca, liver and oviduct. After 7 days of challenge (day 17), eggs were collected daily from each treatment group and tested for the presence or absence of SE until 66 days. In order to simulate a re-infection occurring in layer flock, the birds were re-inoculated orally with SE (10 $\log_{10}$ CFU/bird) on day 35. Additionally, cloacal swabs from all birds were analyzed weekly until 66 days for the presence or absence of SE. At the end of 66 days, the birds from all treatment groups were euthanized via CO_2 asphyxiation, and cecum, oviduct and liver samples from birds were collected in 10 ml of sterile PBS for SE detection.

2.4 SE determination on egg surface and egg contents

The presence of SE on eggshell surface and in egg contents was determined according to the method of Miyamoto et al. (1997). After 7 days of SE challenge, eggs from birds were collected daily and checked for the presence or absence of the pathogen until 66 days of infection. Each egg was rinsed separately in a sterile stomacher bag containing 50 ml of selenite cysteine broth supplemented with NA (50 μg/ml) for 2 min. After washing, the egg was removed and the broth from stomacher bag was incubated at 37^{o}C for 48 h, followed by streaking on XLD + NA plates to detect SE on eggshell. The bacterial colonies were confirmed as *Salmonella* using the *Salmonella* rapid detection kit

(Microgen Bioproducts Ltd. Camberley, UK). The eggs that were washed in selenite cysteine broth were disinfected by wiping with 70% ethanol, dried, cracked open aseptically, and the shell and egg contents were collected into separate, stomacher bags containing 50 ml of selenite cysteine broth containing NA. The bags with the eggs contents or shell were homogenized for 1 min in a stomacher, and incubated at 37^{o}C for 24-48 h to detect *Salmonella* present inside the egg. The bacterial colonies were confirmed as SE as described previously.

2.5 SE determination in internal organs

The presence of SE in the oviduct, liver and cecum were determined as described previously (Kollanoor-Johny et al., 2012b). The organ samples and their contents from each bird were weighed and homogenized. Each homogenate was serially diluted (1:10) in PBS, and appropriate dilutions were plated on XLD-NA plates for bacterial enumeration. Representative colonies from XLD-NA plates were confirmed as *Salmonella* using the *Salmonella* rapid detection kit (Microgen Bioproducts Ltd. Camberley, UK). When colonies were not detected by direct plating, samples were tested for surviving *Salmonella* by enrichment in 100 ml selenite cysteine broth (SCB; Oxoid) for 48 h at 37^{o}C (Kollanoor-Johny et al., 2012), followed by streaking on XLD-NA plates. In addition, cecal endogenous bacteria were enumerated by plating appropriate dilutions of the ceca samples on duplicate thioglycollate agar plates (TGA; Difco), followed by incubation at 39^{o}C under 5% CO_2 for 24 h.

2.6 Sensory evaluation of eggs

The sensory evaluation of eggs was conducted at the Sensory Laboratory, Department of Poultry Science, Auburn University, Alabama. Eggs were collected from

control and CA-treated birds once a week for three weeks, and were tested using the triangle test (Roessler et al., 1978) to assess whether consumers can detect a difference between the eggs from CA treatments and control birds. Sensory testing was done with 36 panelists (students, staff, faculty, local townsmen) per experiment and the experiment was repeated thrice over a period of three weeks. The panelists were randomly served with three, coded scrambled egg samples for tasting and detection of organoleptic differences in a sensory booth under white light. The effect of residual taste in the mouth was minimized by using a water-based mouth rinse between each sampling.

2.7 Statistical analysis

The number of SE colonies in the organs was logarithmically transformed ($\log_{10}$ CFU/g) before analysis to achieve homogeneity of variance. These data were analyzed using the PROC-GLM procedure of the statistical analysis software (version 9.2, SAS Institute Inc. Cary, NC). Differences among the means were detected at $P < 0.001$ using Fisher's Least Significance Difference (LSD) test. For the sensory study, analysis of results was done for a probability level of 5% using a table of "Minimum Numbers of Correct Judgments" (Roessler et al., 1978).

3. Results

3.1 Effect of caprylic acid on SE contamination of eggshell and yolk

In-feed supplementation of CA at 0.7% or 1% did not significantly change ($P > 0.05$) the body weight or the egg production in birds compared to controls (data not shown). However, CA supplementation (0.7% and 1%) decreased SE on shell and in the yolk ($P < 0.001$). A total of 2543 eggs from the first experiment, and 2518 eggs from the second experiment, were tested over a period of 7 weeks for the presence of *Salmonella*

on the shell and in yolk. The average weekly results (N= 5061) from both experiments are depicted in Figures 1a and 1b, wherein CA at 0.7% and 1% consistently decreased *Salmonella* both on the shell (1a) and in yolk (1b) from week 1 to week 7 of supplementation ($P < 0.001$). A significant difference between the controls and treated groups was observed from week 1, wherein control birds yielded ~ 72% of SE contaminated shell, while birds fed with CA had only 60% (0.7% CA) and 42% (1% CA) of their shells positive for SE (Fig 1a). A similar decrease in SE was observed in treated birds during week 2 and week 3 (Fig 1a). Moreover, despite re-inoculation of SE at the end of 3rd week, CA at both concentrations effectively reduced the pathogen on the shell and in the yolk throughout the rest of the experiment until week 7 (Fig 1a and 1b). Analysis of the cumulative data on SE prevalence from 5061 eggs over the 7-week period revealed that dietary supplementation of CA at 1.0% decreased SE presence to ~14% on the shell and ~10% in yolk, when compared to controls which yielded ~60% positive samples on shell and ~ 43% in yolk (Figure 2).

3.2 Effect of caprylic acid on SE colonization of internal organs and cloaca

As observed in egg, CA supplementation significantly reduced SE colonization of the cecum, liver and oviduct. At the end of 66 days, 70% of the cecal samples and liver samples from control birds tested positive for SE (Figure 3a). Moreover, 40% of the oviduct samples from control birds were positive for the pathogen (Figure 3a). However, feeding 1% CA-supplemented diet decreased SE in all the aforementioned organs, with the pathogen recovered from only 25% of the cecum and 20% of liver and oviduct samples from birds ($P<0.001$). In addition, the cecal endogenous bacterial counts did not differ ($P > 0.05$) among birds from the various treatment groups (data not shown).

The presence of SE in cloaca was determined by cloacal swabs collected weekly for 7 weeks in the inoculated group. The results showed that birds fed with CA consistently yielded reduced number of SE positive samples compared to control birds ($P<0.001$). The cumulative data at the end of the experiment revealed that 75% of the cloacal swabs from control birds were positive for SE (Figure 3b), whereas 0.7% and 1% CA supplemented birds yielded only 40% and 35% positive samples, respectively ($P<0.001$).

3.3 Effect of CA on sensory characteristics of eggs

When the eggs were subjected to consumer acceptance by triangle test, only 43 of the 111 panelists were able to detect the eggs from CA-treated birds, and the remaining 68 of them failed to identify the treatments from controls, thus resulting in a 0.005 confidence that the panelists were not able to detect a difference between the eggs from CA-supplemented and untreated birds.

4. Discussion

The cecal colonization of layers with SE results in the systemic spread of the pathogen to reproductive organs, thus contributing to direct transmission of the bacterium to eggs from infected ovaries and/or oviducts by transovarian route. In addition, the pathogen can reach the spleen and liver by lymphatic or circulatory routes (Thiagarajan et al., 1994, 1996). Therefore, in the current study, we determined the efficacy of in-feed supplementation of CA in chickens in reducing SE in the aforementioned organs. Additionally, due to the fecal and systemic transmission of SE to the egg, CA's effect on decreasing the bacterium in feces, eggshell and yolk was investigated.

The results from this study indicated that in-feed administration of CA significantly reduced SE colonization in layer chickens. Specifically, both concentrations

of CA consistently decreased SE on eggshell and yolk from week 1 to 7, with the higher concentration of 1% CA being more effective ($P<0.001$). Since re-infection with *Salmonella* can occur in a flock due to its persistence on a variety of sources in the farm environment (Hassan et al., 1991, Wilson, 2002), the birds were re-inoculated with the bacterium at the end of 3 weeks. However, despite the re-inoculation, CA was effective in decreasing the pathogen prevalence on egg shell and yolk compared to control birds (Fig. 1a and 1b). Moreover, CA supplementation to birds reduced SE populations in the internal organs such as the cecum, liver and oviduct (Fig. 3a), and in feces (Fig. 3b) ($P<0.001$).

The mechanism behind CA-mediated SE reduction in chickens could be multi-fold. Fatty acids, including CA can diffuse into bacterial cells in their undissociated form and subsequently dissociate in the protoplasm leading to acidification (Sun et al., 1998). Another potential bactericidal mechanism of CA involves inducing alterations in bacterial membrane permeability, thereby resulting cell death (Bergsson et al., 1998, 2001). Additionally, the inhibitory effect of medium chain fatty acids on virulence associated genes in SE has been suggested as a mode of antibacterial action. For example, Van Immerseel et al. (2004) observed that medium chain fatty acids suppressed the expression of *hilA,* a key gene regulator involved in *Salmonella* invasion, thereby resulting in decreased *Salmonella* colonization in chicks. Previous research from our laboratory also revealed that CA reduced SE invasion of avian intestinal epithelial cells by down-regulating critical colonization-associated genes in the pathogen (Kollanoor Johny et al., 2012a).

In conclusion, prophylactic supplementation of CA to chickens reduced SE contamination of egg yolk and shell without adversely affecting body weight, egg production or consumer acceptability of eggs. These results suggest that CA could potentially be used as an antimicrobial feed additive to control egg-borne transmission of SE when coupled with other standard biosecurity measures in the farm. Our future experiments will validate the findings of this study in commercial poultry farms.

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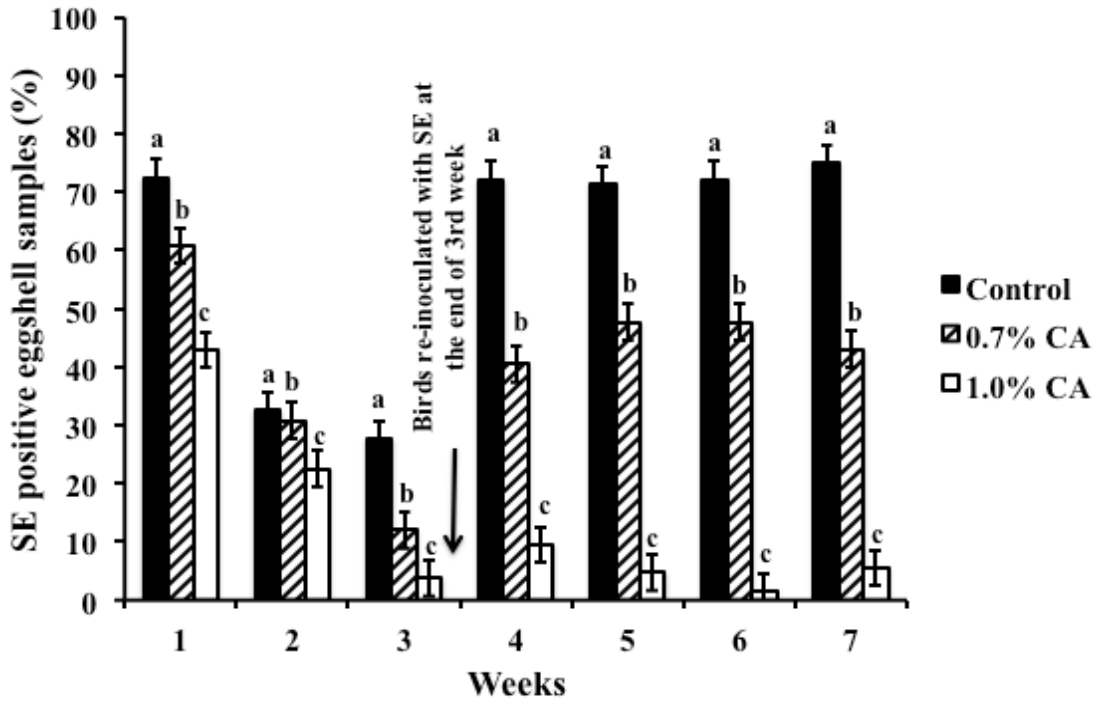
Figure 1: Effect of 0.7% and 1% CA on SE contamination of eggs in 40 week old birds for 7 weeks post inoculation (a) on egg shell and (b) egg yolk. Values with different letters (a, b, c) differ significantly within the week between treatments. Error bars represent ± standard error.

Figure 1a:



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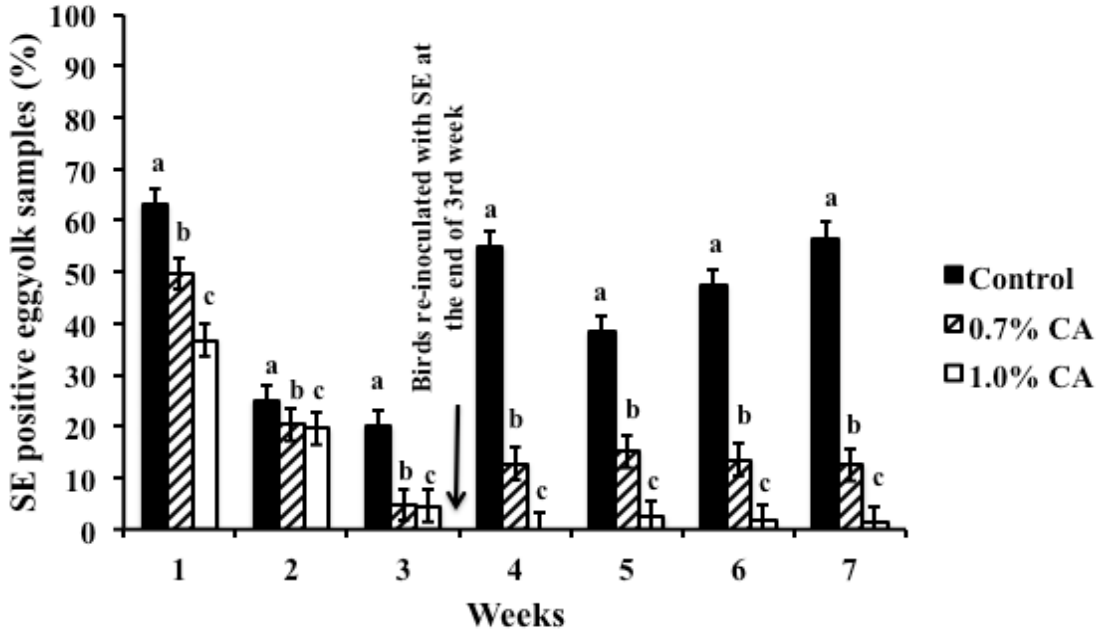

SE positive eggyolk samples (%)
100
90
80
70
60
50
40
30
20
10
0
Birds re-inoculated with SE at the end of 3rd week
a
b
c
1
2
3
4
5
6
7
Weeks
Control
0.7% CA
1.0% CA

Figure 2: Cumulative effect of 0.7% and 1% CA treatment for 7 weeks on egg shell and in egg yolk, with N = 5061 and P < 0.001. Bar graphs represent significant difference between treatment and control ± standard error.

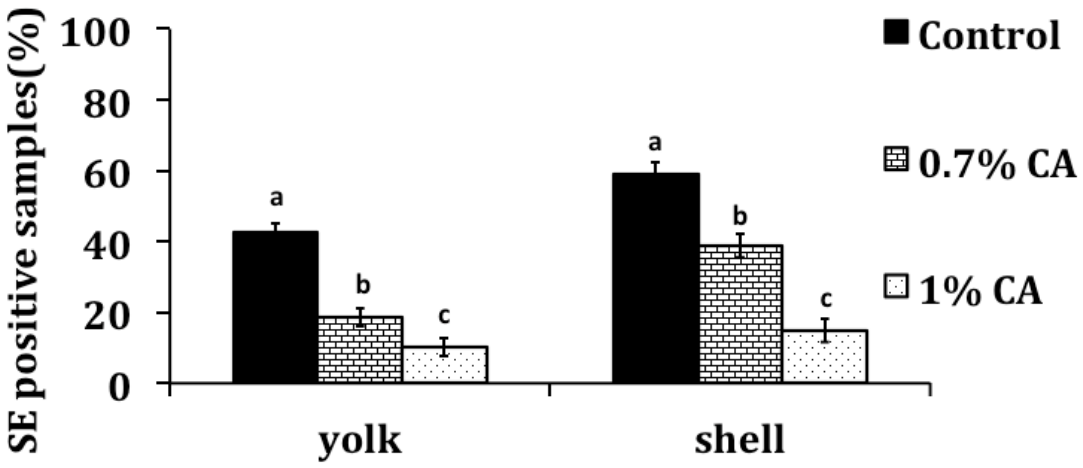


Figure 3: Effect of CA in (a) internal organs (liver, cecum, oviduct) P<0.001, values with different letters (a, b, c) differ significantly within the organ between treatments, and (b) cumulative effect of CA on SE in cloacal swabs, P<0.001, values with different letters (a, b, c) differ significantly between treatments. Error bars represent ± standard error.

Figure 3a:

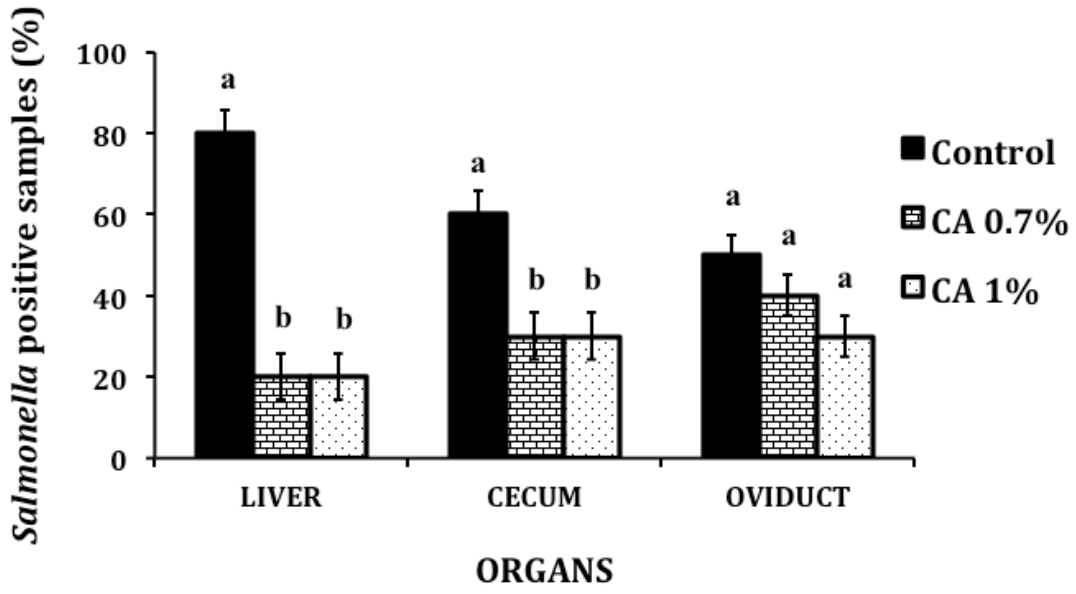
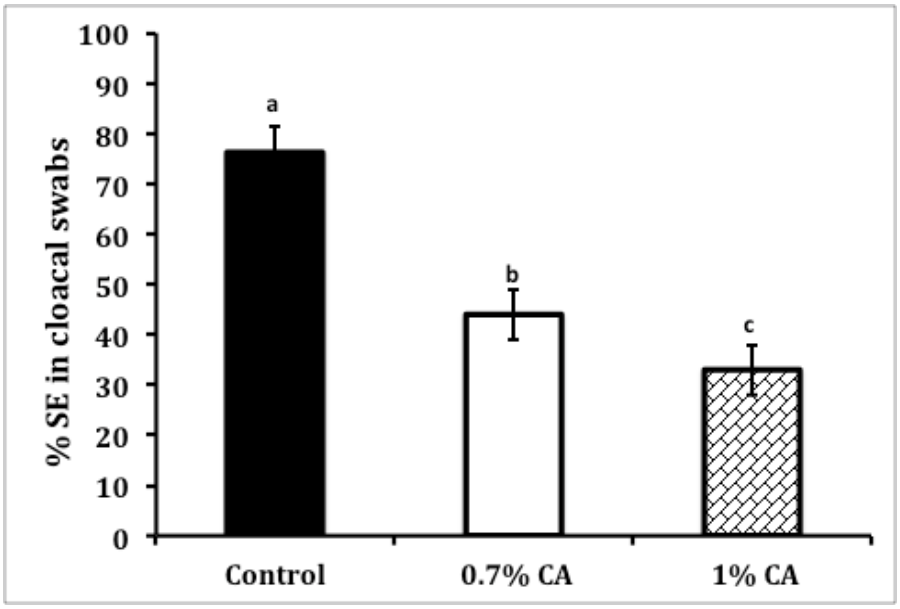


Figure 3b:


100
90
80
70
60
50
40
30
20
10
0
% SE in cloacal swabs
a
b
c
Control
0.7% CA
1% CA

Chapter VI

Rapid inactivation of *Salmonella* Enteritidis on shell eggs by washing with plant-derived antimicrobials

Abstract

Salmonella Enteritidis (SE) is a major foodborne pathogen transmitted to humans by consumption of contaminated eggs. The external surface of eggs becomes contaminated with SE from various sources on farms, the main sources being hens' droppings and contaminated litter. Therefore, effective egg surface disinfection is critical to reduce pathogens on eggs and potentially control egg-borne disease outbreaks. This study determined the efficacy of GRAS status, plant-derived antimicrobials (PDAs), namely *trans*-cinnamaldehyde (TC), carvacrol (CR), and eugenol (EUG) as an antimicrobial wash for rapidly killing SE on shell eggs in the presence or absence of chicken droppings. White-shelled eggs inoculated with a 5-strain mixture of nalidixic acid (NA) resistant SE (8.0 log CFU/mL) were washed in sterile deionized water containing each PDA (0.0, 0.25, 0.5 or 0.75%) or chlorine (200 ppm) at 32 or 42^{o}C for 30sec, 3 min, or 5 min. Approximately 6.0 log CFU of SE was recovered from inoculated and unwashed eggs. The wash water control and chlorine control decreased SE on eggs by only 2.0 log CFU/egg even after washing for 5 min. The PDAs were highly effective in killing SE on eggs compared to controls ($P < 0.05$). All treatments containing CR and EUG reduced SE to undetectable levels as rapidly as within 30 sec of washing, whereas TC (0.75%) completely inactivated SE on eggs washed at 42^{o}C for 30 sec ($P < 0.05$). No SE was detected in any PDA or chlorine wash solution; however, substantial pathogen populations (~ 4.0 log CFU/ml) survived in the antibacterial-free control wash water ($P < 0.05$). Carvacrol and EUG were also able to eliminate SE on eggs to undetectable levels in the presence of 3% chicken droppings at 32^{o}C ($P < 0.05$). This study demonstrates that PDAs could effectively be used as a wash treatment to reduce SE on shell eggs. Sensory

and quality studies of PDA-washed eggs are warranted before recommending their use to the poultry industry.

1. Introduction

Eggs constitute a vital part of the American diet with an annual per capita consumption of approximately 250 eggs (USDA, 2012). Due to the universal acceptance of eggs as an economical and nutritious food source, and considering the public health significance, the microbiological safety of this product is critical (Howard et al., 2012). The primary source of bacterial contamination in eggs is *Salmonella enterica* serovar Enteritidis (SE), which is the most common serotype of *Salmonella* (Braden, 2006), transmitted to humans largely due to the consumption of infected eggs (Mead et al., 1999). It is also the most frequently isolated *Salmonella* from chickens, especially layer flocks (Baird-Parker, 1990; Gast et al., 2005; EFSA 2007). The primary colonization site of SE in chickens is the ceca (Allen-Vercoe and Woodward, 1999; Stern, 2008), with cecal carriage of the pathogen leading to transmission of the organism via contaminated eggs from infected ovaries, or contaminated eggshell with feces (Keller et al., 1995; Gantois et al., 2009). In the former case, contamination of egg contents (yolk, albumen, and eggshell membranes) by SE occurs before oviposition (Miyamoto et al., 1997, Okamura et al., 2001,), where *Salmonella* originating from infected ovaries invades and multiplies in the preovulatory follicles of the reproductive tract (Thiagarajan et al., 1994, 1996). In the latter, SE contamination could also result from penetration of the bacteria through the eggshell from contact with feces infected with the pathogen during or after oviposition (Barrow et al., 1990; Gast and Beard 1990; Messens et al., 2006). Trans-shell contamination of eggs with SE, may occur through environmental sources such as farmers, pets, rodents, contaminated feed, litter, and water (Jones et al., 1995; Latimer et al., 2002). Following oviposition, SE survival on the outer shell surface of eggs is

supported by the presence of chicken manure and other moist organic materials (Gantois et al., 2009). Once the egg is subjected to processing, eggshell contamination can occur at the processing facilities from transfer belts and packaging materials as well (Mayes and Takeballi, 1983). In view of multiple sources of egg contamination, the cleanliness and disinfection of the eggshell is pivotal in controlling SE contamination on eggs (Kuo et al., 1997; Park et al., 2005). Therefore, reducing or eliminating SE population on shell eggs could potentially result in microbiologically safer egg products.

For reducing the microbiological load on shell eggs, including *Salmonella,* a variety of disinfectants for egg washing have been investigated with varying degrees of success. The commonly employed antimicrobials include chlorine and iodine-based sanitizers (Knape et al., 1999), hydrogen peroxide (Padron, 1995), ozone (Koidis et al., 2000), quaternary ammonium compounds (Wang and Slavik, 1998)), and electrolyzed oxidizing water (Russel, 2003). However, many of the aforementioned chemicals have been shown to possess limited antimicrobial effect, especially in the presence of organic matter, and do not render eggs pathogen-free (Moats, 1978; Wang and Slavik, 1998).

The use of natural antimicrobial molecules for inactivating pathogenic microorganisms has received renewed attention due to toxicity concerns of synthetic chemicals (Salamci et al., 2007, Isman 2000). Historically, plants have served as sources of novel drugs, contributing to human health and wellbeing. Plants are capable of synthesizing a large number of molecules, many of which are phenolic compounds or their derivatives (Geissman, 1963). *Trans*-cinnamaldehyde (TC) is an aldehyde present as a major component of bark extract of cinnamon (*Cinnamomum zeylandicum*). Carvacrol (CR) is an antimicrobial ingredient in oregano oil obtained from *Origanum glandulosum*.

Eugenol (EUG) is an active ingredient in the oil obtained from cloves (*Eugenia caryophillis*). The aforementioned molecules are classified by the United States Food and Drug Administration as GRAS (generally regarded as safe) (Adams et al., 2004, 2005; Knowles et al., 2005). Previous research conducted in our laboratory has shown that various plant-derived antimicrobials (PDAs), including TC, CR and EUG, were effective in inactivating SE and *Campylobacter jejuni* in chicken cecal contents *in vitro* (Kollanoor Johny et al., 2010c). We also previously reported that the PDAs increased the sensitivity of *S*. Typhimurium DT104 to several antibiotics (Kollanoor Johny et al., 2010a), and two of the PDAs, namely TC and EUG, significantly reduced *S.* Enteritidis populations in the cecum of young and market-age broiler chickens (Kollanoor Johny et al., 2012b,d). The objective of the present study was to investigate the efficacy of TC, CR, and EUG as wash treatments for reducing SE on eggshell surface.

2. Materials and Methods

Five isolates of SE pre-induced for resistance to 50 µg/ml of nalidixic acid (NA**;** catalogue no. N4382, Sigma-Aldrich, St. Louis, MO) were used for the study. The strains included SE12 (chicken liver, phage type 14b), SE 22 (chicken intestine, phage type 8), SE 28 (chicken ovary, phage type 13a), SE31 (chicken gut, phage type 13a) and SE 90 (human, phage type 8). Each strain was cultured separately in 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) containing 50 µg/ml NA, and incubated at 37°C for 24 h. After three passages, equal volumes of the cultures were combined and sedimented by centrifugation (3600 g for 15 min at 4 °C). The pellet was washed twice, re-suspended in phosphate buffered saline (PBS; pH 7.0), and used as the inoculum. The bacterial count in the individual cultures and the 5-strain mixture was confirmed by

plating 0.1 ml proportions of appropriate dilutions on tryptic soy agar (TSA+NA, Difco) containing 50 µg/ml NA and xylose lysine desoxycholate agar (XLD+NA; Difco) containing 50 µg/ml NA, and incubating the plates at 37°C for 24 h (Kollanoor Johny et al. 2010b,d).

Freshly laid eggs from single-comb White Leghorn layer chickens obtained from the University of Connecticut poultry farm were washed in sterile deionized water at room temperature (23^{o}C) to remove visible dirt, if any, and kept for drying under a laminar flow hood for 15 min. A batch of eight eggs was dipped completely in 500 ml of sterile PBS inoculated with ~8.0 log CFU/ml of a 5-strain mixture of SE in the presence or absence of 3% fresh layer droppings (Stringfellow et al., 2009). An immersion time of 30 min was given for each batch, and the eggs were dried under a hood for 1 h at 23^{o}C (Cason et al., 1993; Russel, 2003) before applying the treatments.

Each egg was placed in a separate Whirlpak containing 100 ml of sterile deionized water containing 0.0, 0.25, 0.5 or 0.75% of TC, CR, or EUG, and washed in a shaker water bath at 32 or 42^{o}C for 1, 3 or 5 min. The deionized water used was previously tempered for 5 min in the shaker water bath prior to treatments and was monitored using a thermocouple. Water with 200 ppm chlorine was included as chlorine control. After treatment, each egg was transferred to a sterile stomacher bag containing 30 ml of neutralizing broth (Fisher) and was gently rubbed by hand for 1 min (Park et al., 2005). SE was enumerated by plating the neutralizing broth directly or after 10-fold serial dilutions on XLD+NA and TSA+NA plates. The plates were incubated at 37^{o}C for 48 h before counting the colonies. Ten ml aliquots of the neutralizing broth were added to 100 ml of selenite cysteine broth (SCB; Difco) and enriched at 37^{o}C for 48 h. The culture was

streaked on Brilliant Green Agar (BGA; Oxoid) plates and incubated at 37°C for 48 h. Representative bacterial colonies from the BGA plates were confirmed as SE using the *Salmonella* rapid detection kit (Microgen Bioproducts Ltd, Camberley, UK). In addition, we also tested the egg wash solution (deionized water with/without PDAs) after each washing period for the presence of SE.

Five eggs per treatment at every sampling point for each temperature were included in all three replicated experiments. Each experiment was a completely randomized design (CRD) with a 3 X 6 X 3 X 2 factorial treatment structure. The factors included 3 compounds (CR, EUG, TC), 6 treatments (baseline, chlorine, 0 %, 0.25 %, 0.5 %, and 0.75 % of respective compounds), 3 sampling times (30 s, 3 min, and 5 min), and 2 temperatures (32 and 42°C). The experiment was repeated in the presence of 3% organic matter utilizing a CRD with 3 X 4 X 3 factorial treatment structure. The factors included 3 compounds, 4 treatments (baseline, chlorine, 0 % and 0.5 % of respective compounds) and 3 sampling times (30 s, 3 min, and 5 min). All experiments were replicated three times. Data were analyzed using PROC-MIXED procedure of the Statistical Analysis Software (SAS Institute, Inc., Cary, N.C.). Differences among means were detected at $P<0.05$ using the Fisher's least significant difference test with appropriate corrections for multiple comparisons.

3. Results and Discussion:

Cleaning and sanitation of shell eggs by washing is a common practice mandatory for retail shell eggs from plants operating under voluntary USDA grade standards (USDA, 2008) and also required by individual state laws in the US. Zeidler et al. (2002) reported that washing eggs under optimum conditions could potentially reduce the total

bacterial load by 2 to 3 log CFU/egg. An ideal egg wash antimicrobial should be effective in reducing large populations of the target pathogen in a rapid time frame, even in the presence of organic matter. Further, it should be safe to workers and the environment, cost effective (Scott and Sweetnam, 1993), and be easily incorporated into a HACCP plan.

Chlorine and chlorine-containing compounds are the most commonly used antimicrobial agents for egg washing (Cao et al., 2008). However, chlorine is minimally effective in reducing pathogen loads on the egg surface, and does not render the egg pathogen-free (Wang et al., 2010). Moreover, chlorine can combine with organic matter releasing trihalomethanes and other organochlorine compounds, which are potentially carcinogenic. Therefore, it is critical to develop safe and effective antimicrobial interventions to wash eggs in order to reduce or eliminate SE on the eggshell surface.

In the current study, we investigated the efficacy of three PDAs namely, CR, TC, and EUG added to wash solutions for reducing SE on shell eggs in the presence and absence of organic matter. Since selective media can inhibit the recovery of bacteria stressed by exposure to antimicrobials, we used a non-selective medium (TSA) for enumerating SE from treated eggs, although a selective medium (XLD) was also used. However, we did not find any significant differences ($P < 0.05$) between the SE counts recovered on the selective and non-selective media (data not shown). Therefore, *Salmonella* counts from the XLD plates were used for statistical analysis and discussion.

Based on the USDA recommendation on the minimum temperature for the water used for egg washing (USDA, 2008), the efficacy of PDAs as an antimicrobial wash for killing *S*. Enteritidis on shell eggs was investigated at 32^{o}C. A higher temperature of 42^{o}C

[above the reported average internal temperature of eggs ranging from 31.1 to 35.6°C (Curtis et al., year) was also chosen to determine if an increase in the wash water temperature could enhance the efficacy of PDAs. The effect of various egg washing treatments at 32°C on SE counts in the absence of organic matter is depicted in Figures 1-3. The average SE count recovered from unwashed eggs after inoculation (baseline) was ~6.4 log CFU/ml. Washing of eggs in water or water containing chlorine (200 ppm) decreased SE counts by ~ 2.0 log CFU/ml. However, washing of eggs in water containing CR (0.5 and 0.75%) decreased SE counts to undetectable levels by 30 sec, and the eggs consistently tested negative for the pathogen (by plating and enrichment) throughout the subsequent sampling points (3 and 5 min) (Fig. 1a). Although 0.25% CR reduced SE to undetectable levels by plating at all time points, the eggs tested positive for the pathogen on enrichment at 30 sec. Similarly at 32°C, EUG (0.5 and 0.75%) completely inactivated SE (negative by enrichment) at all sampling time points, whereas 0.25% EUG reduced SE populations by ~ 5.0 log CFU/ml at 5 min of washing ($P<0.05$) (Fig. 1b). On the other hand, TC was least effective among the three PDAS, and its highest tested concentration of 0.75% decreased SE counts on eggs by ~ 5.0 log CFU/ml at the end of 5 min (Fig. 1c).

At 42°C, all concentrations of CR and EUG reduced SE counts on eggs to undetectable levels as quickly as 30 sec of washing ($P<0.05$) (Fig 2a and 2b). In the case of TC, the highest concentration (0.75%) completely inactivated SE on eggs at 30 sec (negative on enrichment), whereas 0.25% and 0.5% TC brought about the same magnitude of reduction in SE populations at 5 min of washing ($P<0.05$) (Fig 2c).

The presence of organic matter could potentially reduce the efficacy of antimicrobials used in egg wash. For example, Knape et al. (2002) observed that although

distilled deionized water and chlorine (200 ppm) decreased SE populations on eggs as compared to dry egg controls, the efficacy of egg sanitizers was affected by the level of total dissolved compounds in the wash water. Therefore, we investigated the efficacy of the aforementioned PDAs in the presence of poultry droppings, which is one of the common contaminants on eggshell surfaces. We examined the efficacy of 0.5% of each PDA on SE at 32^{o}C in the presence of chicken droppings. We observed that CR decreased SE to undetectable levels as rapidly as 30 seconds (enrichment negative), whereas EUG completely inactivated the pathogen at 3 min (Fig 2a and Fig 3). However, TC could reduce SE counts on eggs by 5.0 log CFU/ml only after 5 min (P<0.05). Washing of eggs in water or water containing chlorine brought about similar reductions in SE counts as observed in the absence of chicken droppings (Fig 3). Since similar results were observed with the use of PDA's with or without organic matter at 32 ^{o}C, at a higher temperature of 42^{o}C there could be similar or enhanced effect.

It was found that ~ 4.0 log CFU/ml of SE survived in the antimicrobial-free deionized wash water after treating the eggs, whereas no bacteria were recovered from the water containing chlorine or the PDAs. The recovery of viable SE in the water after washing eggs is of concern due to potential cross-contamination or recontamination of sequential batches of eggs, if the same solution is used for washing. In addition, the disposal of wash water needs to be addressed to prevent potential environmental contamination.

We found significant difference between the two temperatures, i.e. 32 and 42 ^{o}C, especially in EUG and TC's efficacy in reducing SE on eggs ($P < 0.05$). For example, EUG at 0.25% decreased SE counts by > 5.0 log CFU/ml only after 5 min at 32^{o}C,

whereas same concentration of the compound completely inactivated the pathogen after 30 sec of washing at 42^{o}C (Fig. 1b and 2b). Similarly, none of the tested concentrations of TC reduced SE counts by more than 5.0 log CFU/ml at 32^{o}C (Fig. 1c), while all three TC concentrations decreased the pathogen to undetectable levels after 5 min of washing at 42^{o}C (Fig. 2c). The antimicrobial activity of lipid-soluble PDAs is attributed to their hydrophobicity and deleterious effects on bacterial cell membrane (Sikkema et al. 1994; Cox et al., 2006). The heat-induced damage of bacterial plasma membrane potentiates the effect of PDAs, thereby resulting in an enhanced bactericidal effect with increase in temperature. A similar finding was reported by Shibasaki and Kato (1978), who observed that heating makes the bacterial plasma membrane more fluid, thereby increasing the antimicrobial activity of lipid-soluble small molecules. To conclude, results of this study indicate that the PDAs, especially CR and EUG were effective (P<0.05) in rapidly reducing SE on shell eggs when compared to washing in untreated or chlorine treated water. Although washing of eggs with the PDA's revealed no visible difference in shell color or consistency compared to control eggs, sensory and quality analyses of PDA-treated eggs are required before recommending their use.

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Figure 1a: Effect of CR at 0.25, 0.5, and 0.75% on SE inoculated on shelled eggs at 32^{o}C. A control (0%) and chlorine (200 ppm) under similar experimental conditions were also tested. Five eggs per treatment per sampling point (30 s, 3 min and 5 min) were included, and the experiment was repeated three times. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n=15).

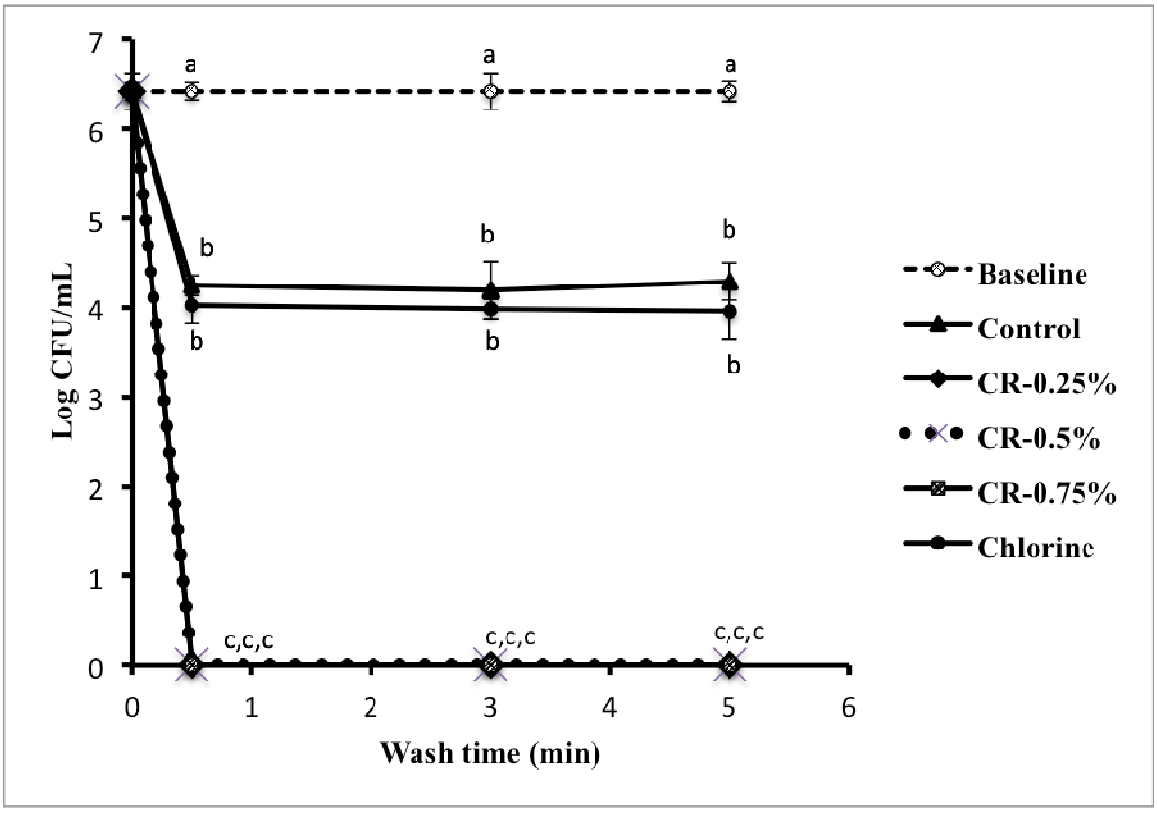


Figure 1b: Effect of EUG at 0.25, 0.5, and 0.75% on SE inoculated on shelled eggs at 32^{o}C. A control (0%) and chlorine (200 ppm) at similar experimental conditions were also tested. Five eggs per treatment per sampling point (30s, 3min and 5min) were included, and the experiment was repeated three times. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n=15).

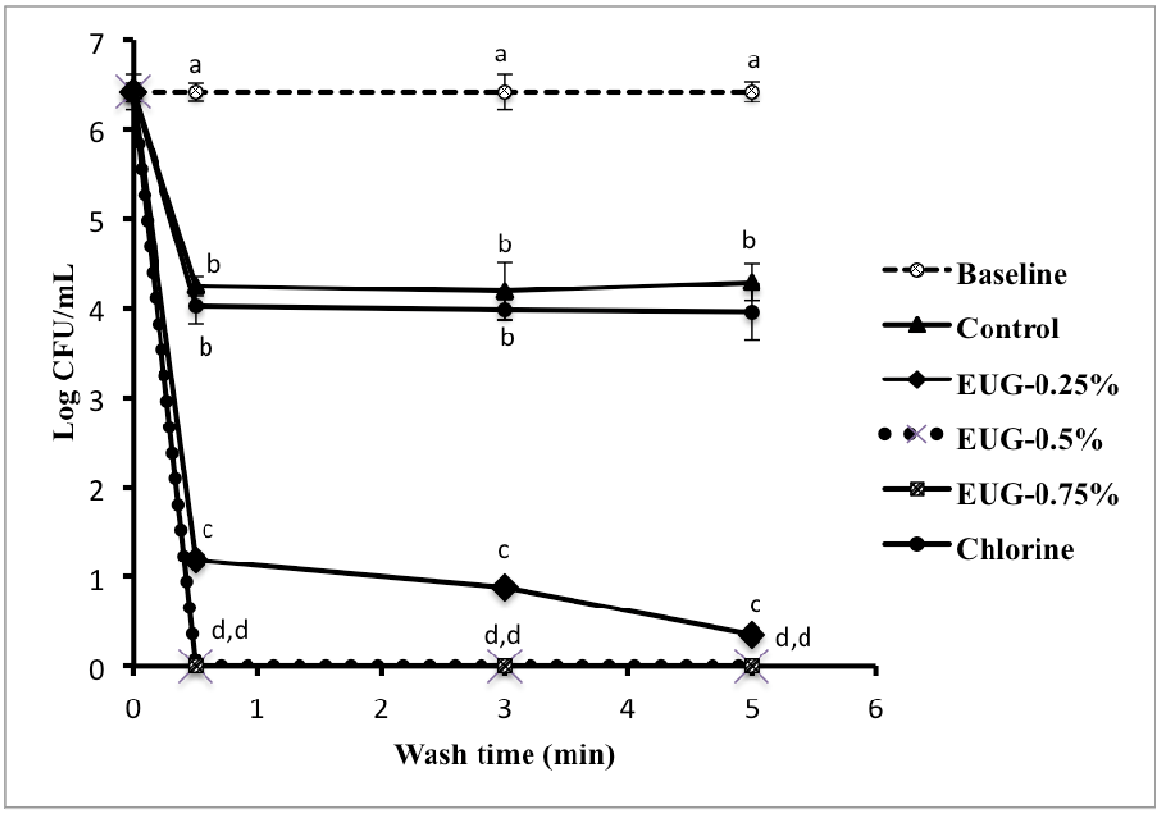


Figure 1c: Effect of TC at 0.25, 0.5, and 0.75% on SE inoculated on shelled eggs at 32^{o}C. A control (0%) and chlorine (200 ppm) under similar experimental conditions were also tested. Five eggs per treatment per sampling point (30s, 3min and 5min) were included, and the experiment was repeated three times. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n=15).

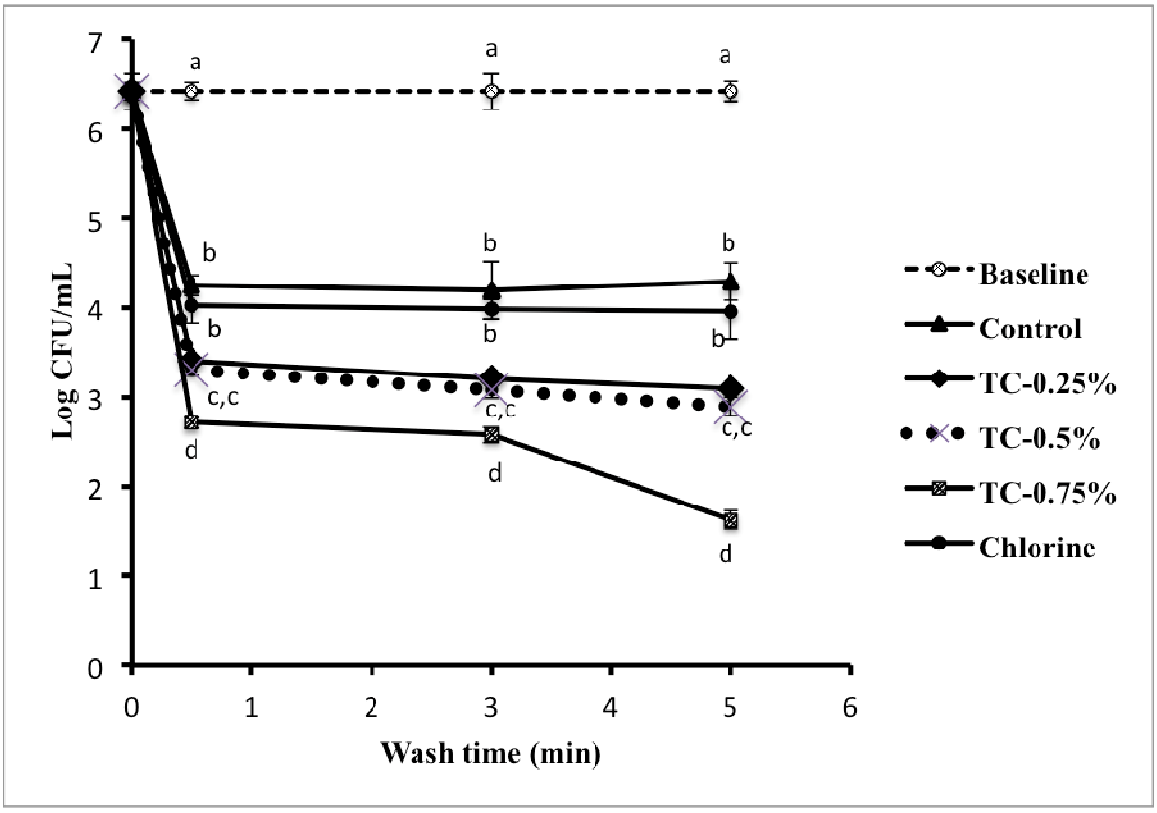


Figure 2a: Effect of CR at 0.25, 0.5, and 0.75% on SE inoculated on shelled eggs at 42°C. A control (0%) and chlorine (200 ppm) under similar experimental conditions were also tested. Five eggs per treatment per sampling point (30s, 3min and 5min) were included, and the experiment was repeated three times. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n=15).

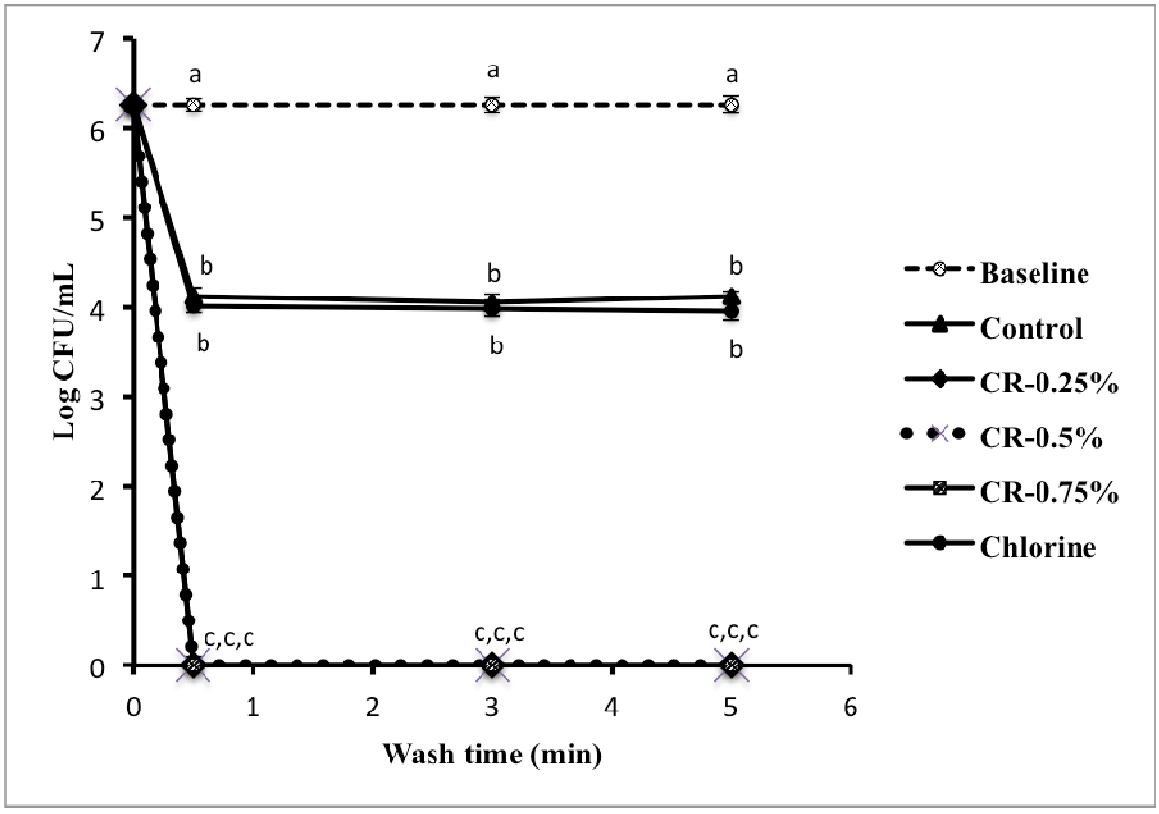


Figure 2b: Effect of EUG at 0.25, 0.5, and 0.75% on SE inoculated on shelled eggs at 42°C. A control (0%) and chlorine (200 ppm) under similar experimental conditions were also tested. Five eggs per treatment per sampling point (30s, 3min and 5min) were included, and the experiment was repeated three times. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n=15).

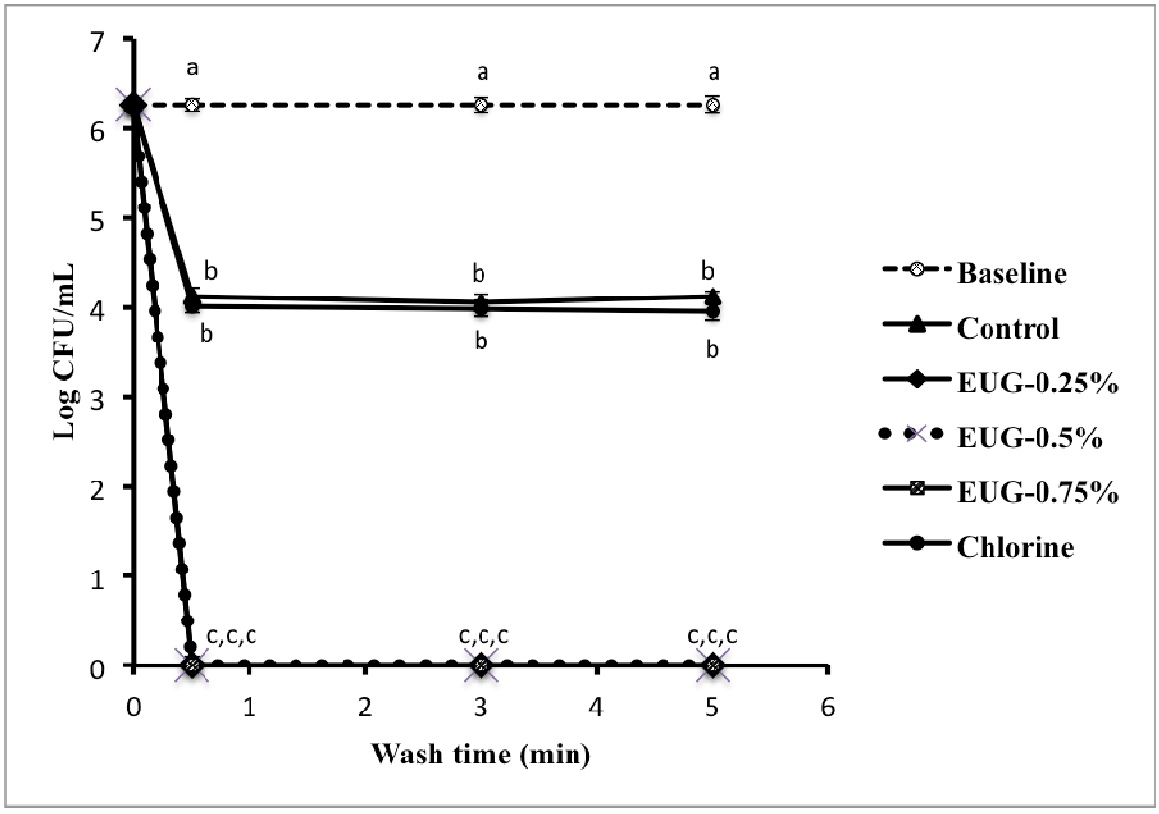


Figure 2c: Effect of TC at 0.25, 0.5, and 0.75% on SE at inoculated on shelled eggs 42^{o}C. A control (0%) and chlorine (200 ppm) under similar experimental conditions were also tested. Five eggs per treatment per sampling point (30s, 3min and 5min) were included, and the experiment was repeated three times. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n=15).

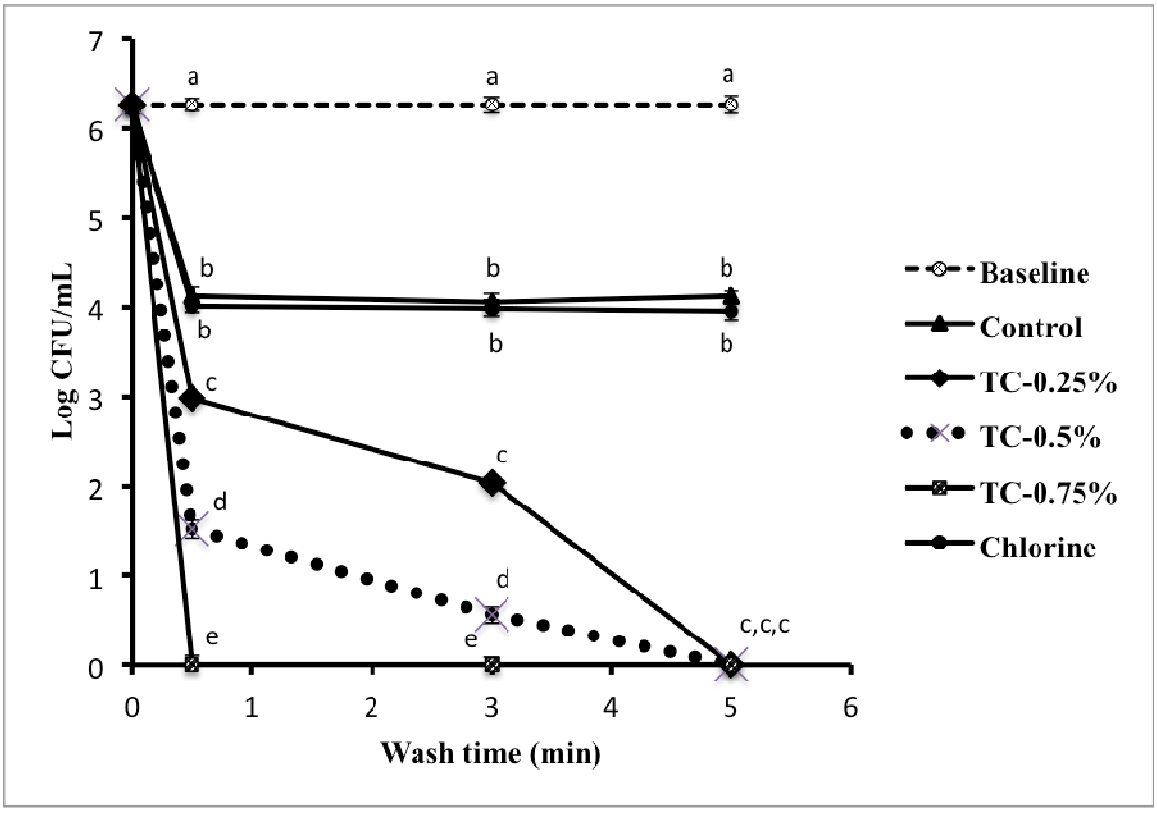
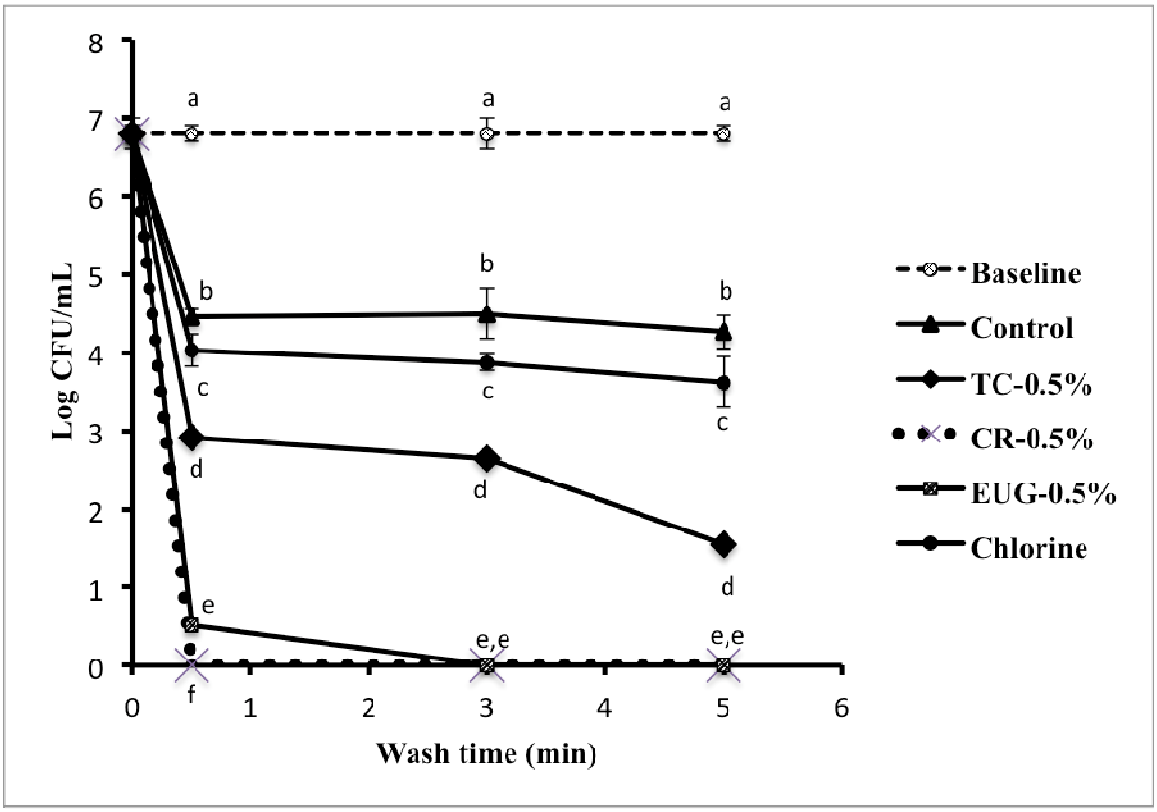


Figure 3: Effect of TC, CR, EUG at 0.5% on SE inoculated on shelled eggs at 32° C in the presence of 3% organic matter. A control (0%) and chlorine (200 ppm) under similar experimental conditions were also tested. Three eggs per treatment per sampling point (30s, 3min and 5min) were included, and the experiment was repeated twice. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n=6).



Chapter VII

Reducing *Salmonella enterica* serovar Enteritidis on embryonated eggs by fumigation with *trans*-cinnamaldehyde and eugenol

Abstract

Salmonella Enteritidis (SE) is a major foodborne pathogen in the United States, with eggs being the most common food product associated with SE infections in humans. The presence of *Salmonella* in fertile hatching eggs has been identified as a vital link to the contamination of hatching chicks, thus underscoring the significance of effective embryonated egg sanitation for protecting public health and decreasing economic losses to the poultry industry. This study investigated the efficacy of two GRAS (generally regarded as safe)-status, plant-derived antimicrobials (PDAs), namely *trans*-cinnamaldehyde (TC) and eugenol (EUG) applied as a fumigation treatment in reducing SE on embryonated eggs. Day-old embryonated eggs were spot inoculated with a 4-strain mixture of SE (~ 6.5 log CFU) and subjected to fumigation with the aforementioned PDAs (0 or 1% concentration) for 20 min in a hatching incubator, and SE on the shell and embryo was enumerated on days 1, 3, 6, 9, 13, 16 and 18. On day 13, the eggs were re-inoculated, followed by fumigation treatment for 20 min. Since the PDAs were dissolved in ethanol (final concentration 0.04%), eggs fumigated with ethanol were included as a control.

Approximately 6.0 log CFU/ml of SE were recovered from the shell of untreated, inoculated eggs on days 1 and 13. The fumigation of embryonated eggs with the PDAs was more effective in reducing SE on the shell and embryo compared to controls ($P<0.05$). On day 18, the eggs fumigated with ethanol were SE positive on the shell, whereas no pathogen was detected on eggs subjected to fumigation with TC and EUG. Similarly, although the embryos of eggs subjected to fumigation with ethanol yielded 1 log CFU/ml of SE on day 18, the embryos of TC and EUG treated eggs were devoid of

the pathogen. Although this study showed that TC and EUG could potentially be used as a fumigation treatment for reducing SE on embryonated eggs, follow up studies ascertaining the quality traits of eggs, including the hatchability are necessary.

1. INTRODUCTION

Hatchery sanitation is essential to ensure chick quality as the poultry hatch environment can be contaminated with a variety of bacteria, especially Salmonellae (Lock et al., 1992, Bruce et al., 1994, Cox et al., 1999). *Salmonella enterica* serovar Enteritidis (SE), the most common serotype contaminating eggs (Braden, 2006) is transmitted to humans largely due to the consumption of infected eggs (Mead et al., 1999). It is also the most frequently isolated *Salmonella* from chickens, especially layer flocks (Baird-Parker, 1990; Gast et al., 2005). Moreover, the presence of salmonellae in/on fertile eggs has been identified as a vital step in *Salmonella* contamination of hatching chicks. The primary colonization site of SE in chickens is the ceca (Allen-Vercoe and Woodward, 1999; Stern, 2008), with cecal carriage of the pathogen leading to transmission of the organism via contaminated eggs from infected ovaries, or contaminated eggshell with feces (Keller et al., 1995; Gantois et al., 2009). In the former case, contamination of egg contents (yolk, albumen, and eggshell membranes) by SE occurs before oviposition (Miyamoto et al., 1997, Okamura et al., 2001), where *Salmonella* originating from infected ovaries invades and multiplies in the pre-ovulatory follicles of the reproductive tract (Thiagarajan et al., 1994, 1996). In the latter, SE contamination results from bacterial penetration through the eggshell from contact with feces infected with the pathogen during or after oviposition (Barrow and Lovell, 1991; Gast and Beard 1990; Messens et al., 2006). Trans-shell contamination of eggs with SE may occur through environmental sources such as farmers, pets, rodents, contaminated feed, litter, and water (Jones et al., 1995; Latimer et al., 2002). In the case of fertile eggs, eggshell contamination can occur from hatching cabinets, grow-out facilities, and

personnel handling vaccination and other standard procedures, including shipment (Poppe, 1999, Mayes and Takeballi, 1983). Since the invading bacteria do not decompose the egg, the infected chick hatching from contaminated eggs could potentially serve as an extensive bacterial reservoir in commercial hatcheries (Maclaury, and Moran, 1959; Cox et al., 2000). Additionally, since chicks are more susceptible to infection by SE, effective egg surface disinfection in the hatching environment is critical for ensuring chick quality and microbiological safety. This is especially significant in light of the annual hatching egg production reaching more than a billion in the United States (USDA, 2014).

Although formaldehyde fumigation of hatching eggs for disinfection was routinely employed by the poultry industry, adverse health effects associated with the use of formaldehyde triggered the search for alternate egg sanitizers in hatcheries (Cox et al., 1999). These include a variety of disinfectants such as chlorine and iodine-based sanitizers (Knape et al., 1999), chlorine dioxide (Patterson et al., 1990), hydrogen peroxide (Padron, 1995), ozone (Koidis et al., 2000), quaternary ammonium compounds (Wang and Slavik, 1998), sodium hydroxide (Olsen and Mcnally, 1947), and electrolyzed oxidizing water (Russel, 2003). However, many of the aforementioned chemicals were shown to possess limited efficacy, and did not render eggs pathogen-free (Moats, 1978; Wang and Slavik, 1998).

The use of natural antimicrobial molecules for inactivating pathogenic microorganisms has received renewed attention due to toxicity concerns of synthetic chemicals (Salamci et al., 2007, Isman 2000). Plants are capable of synthesizing a large number of molecules, many of which are phenolic compounds or their derivatives (Geissman, 1963). *Trans*-cinnamaldehyde (TC) is an aldehyde present as a major

component of bark extract of cinnamon (*Cinnamomum zeylandicum*). Eugenol (EUG) is an active ingredient in the oil obtained from cloves (*Eugenia caryophillis*). The aforementioned molecules are classified by the United States Food and Drug Administration as GRAS (generally regarded as safe) (Adams et al., 2004, 2005; Knowles et al., 2005). Previous research conducted in our laboratory has shown that several plant-derived antimicrobials (PDAs), including TC and EUG, were effective in inactivating SE and *Campylobacter jejuni* in chicken cecal contents *in vitro* (Kollanoor Johny et al., 2010), and significantly reducing SE colonization in young and market-age broiler chickens (Kollanoor Johny et al., 2012). The PDAs were also found to be effective as an antimicrobial wash treatment for rapidly decreasing SE on table eggs (Upadhyaya et al., 2013). The objective of the present study was to investigate the efficacy of TC and EUG as fumigating agents to reduce SE on embryonated eggs incubated under hatching environment.

2. MATERIALS AND METHODS:

2.1 Bacterial strains and culture conditions:

Four isolates of SE pre-induced for resistance to 50 µg/ml of nalidixic acid (NA**;** catalogue no. N4382, Sigma-Aldrich, St. Louis, MO) were used for the study. The strains included SE12 (chicken liver, phage type 14b), SE 22 (chicken intestine, phage type 8), SE 28 (chicken ovary, phage type 13a) and SE31 (chicken gut, phage type 13a). Each strain was cultured separately in 10 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) containing 50 µg/ml NA, and incubated at 37°C for 24 h. After three passages, equal volumes of the cultures were combined and sedimented by centrifugation (3600 g for 15 min at 4°C). The pellet was washed twice, re-suspended in

phosphate buffered saline (PBS; pH 7.0), and used as the inoculum. The bacterial population in the individual cultures and the 4-strain mixture was confirmed by plating 0.1 ml proportions of appropriate dilutions on tryptic soy agar (TSA+NA, Difco) containing 50 µg/ml NA and xylose lysine desoxycholate agar (XLD+NA; Difco) containing 50 µg/ml NA, and incubating the plates at 37°C for 24 h (Kollanoor Johny et al. 2010).

2.2 Preparation and inoculation of eggs

Freshly laid fertile eggs from single-comb White Leghorn layer chickens were obtained from the University of Connecticut poultry farm. Eggs were transported to the laboratory one day prior to treatment, and incubated by placing in a thermostat incubator (2362N hova-bator, GQF Manufacturing Company Inc., GA) with automatic egg turner (1611 egg turner with 6 universal racks, GQF Manufacturing Company Inc.) for 10-12 h at 37.8^{o}C and 55% RH. The eggs were then inoculated on day 1 by spot inoculation (Jin et al., 2013) with ~ 6.5 log CFU/ml of the 4-strain SE mixture, and re-inoculated on day 13 to simulate hatching egg re-contamination from equipment (Poppe, 1999). The eggs were dried in the hova-bator for 30 min at 37.8^{o}C before applying the treatments.

2.3 Fumigation treatment with TC and EUG and determination of SE:

Following inoculation and drying, the eggs were subjected to fumigation with 1% TC and 1% EUG (TC and EUG dissolved in 0.04% ethanol, vol/vol) for 20 minutes (Garip et al., 2011) on day 1 and day 13 of incubation using a commercial humidifier (Bell and Howell, IL). The treatment concentration of 1% TC and EG was selected based on preliminary experiments, wherein a range of concentrations was tested for their efficacy in reducing SE on eggs. The lowest concentration of each PDA that reduced

more than 3 log CFU/ml of SE on egg shell with one application was (1% TC and EUG) used for further study. A non-treated control (baseline), distilled water control and ethanol (0.04%) control were also included. After treatment, four eggs per group were transferred to a sterile stomacher bag containing 30 ml of neutralizing broth (Fisher), and gently rubbed by hand for 1 min (Park et al., 2005). The surviving SE on eggs was enumerated by plating the neutralizing broth directly or after 10-fold serial dilutions on XLD+NA and TSA+NA plates. The plates were incubated at 37^{o}C for 48 h before counting the colonies. One ml aliquots of the neutralizing broth were also added to 50 ml of selenite cysteine broth (SCB; Difco) and enriched at 37^{o}C for 48 h. The culture was streaked on XLD+NA plates and incubated at 37^{o}C for 48 h. Representative bacterial colonies from the XLD+NA plates were confirmed as SE using the *Salmonella* rapid detection kit (Microgen Bioproducts Ltd, Camberley, UK).

On days 1, 3, 6, 9, 13, 16, and 18, the eggs washed in neutralizing broth were disinfected by wiping with 70% ethanol, dried, cracked open aseptically, and egg contents/embryo were collected into separate, stomacher bags containing 30 ml of neutralizing broth. The bags with the eggs contents or embryo were homogenized for 1 min in a stomacher, and incubated at 37^{o}C for 24-48 h to detect *Salmonella* present inside the egg. The bacterial colonies were confirmed as SE as described previously.

2.4 Statistical Analysis:

Four eggs per treatment at every sampling point rature were included in the replicated experiments (n=8). Each experiment was a completely randomized design (CRD) in a 2 X 4 X 7 factorial treatment structure, with 2 compounds (EUG, TC), 4 treatments (baseline, ethanol control, 0%, and 1% of respective compounds) and 7 time

points (day 1, 3, 6, 9, 13, 16, and 18). The data were analyzed using PROC-MIXED procedure of the Statistical Analysis Software (SAS Institute, Inc., Cary, N.C.). Differences among means were detected at $P<0.05$ using the Fisher's least significant difference test with appropriate corrections for multiple comparisons.

3. RESULTS AND DISCUSSION:

Laying and breeding flocks are considered as a critical link between systemic SE infection in birds and human foodborne outbreaks due to contaminated eggs (Altekruse et al., 1993). In natural infections, around 0.6% of eggs laid by infected breeding flocks are reported to be internally contaminated with SE (Humphrey et al., 1991). In addition, contaminated environment in the surroundings of the eggs, including litter, nest box, hatchery environment or hatchery truck can result in bacterial contamination of egg shell (Gantois et al., 2009). *Salmonella* can penetrate the shell and membranes of hatching eggs, and contaminate the developing embryo (Lock et al, 1992; Cox et al., 1999; Bruce and Drysdale, 1994). Moreover, transovarian transmission of SE leads to contamination of newly formed fertile eggs, thereby adversely affecting the hatachability and/or infecting hatching chicks (De Buck et al., 2004). Since *Salmonella* have the ability to persist for long periods of time in commercial hatcheries (Cox et al., 2000, Berrang et al., 1997), controlling SE contamination of hatching eggs and day-old chicks from infected breeding flocks is crucial to successful hatchery operations (Samberg and Meroz, 1995).

In the current study, we investigated the efficacy of two PDAs, namely TC and EUG as fumigation agents in reducing SE on embryonated eggs (shell and embryo). Since the PDAs were dissolved in ethanol before use, we included eggs fumigated with ethanol as a control, in addition to water control. However, no differences in SE counts

were observed between ethanol and water controls ($P > 0.05$); the results from ethanol control are compared with TC and EUG treatments in the manuscript. Since *Salmonella* contamination can occur when the eggs are set, and re-infection potentially occurs during incubation from contaminated incubator (Hassan et al., 1991, Wilson, 2002), the eggshells were inoculated with SE on day 1 followed by re-inoculation on day 13. The eggs were subjected fumigation with 1% TC or EUG was for 20 minutes to simulate a commercial hatchery setting of formaldehyde fumigation (Garip & Dere, 2011). The inactivation of on SE on the shell of embryonated eggs during 18 days of incubation is depicted in Figure 1. The average SE count recovered from untreated eggs after first inoculation on day 1 (baseline) was ~ 6.0 log CFU/ml. Eggs fumigated with ethanol control had an average SE count of ~ 5.2 log CFU/ml on the shell on day 1. There were no significant differences between SE counts on eggshells subjected to water and ethanol fumigation at any of the time points (data not shown) ($P > 0.05$). However, fumigation of eggs with 1% TC reduced the pathogen on eggshell to undetectable levels on day 1, and these eggs consistently tested negative for the pathogen on the shell (by plating and enrichment) throughout the subsequent sampling points (day 3, 6 and 9) until re-inoculation (Figure 1). Although ethanol fumigation was unable to significantly reduce SE when compared to TC on day 1, it reduced the pathogen count on day 3 to 1 log CFU/ml, and these eggs tested negative for SE (by enrichment) on days 6 and 9 similar to TC treatment ($P > 0.05$). Similarly, EUG at 1% decreased SE on eggshells by ~3.5 log CFU/ml on day 1. On days 6 and 9, there was no significant difference between SE counts recovered from eggs fumigated with water, ethanol, TC or EUG ($P > 0.05$) (Fig.1).

After re-inoculation on day 13, the eggs were re-fumigated for 20 minutes. The shell of control eggs yielded a SE count of 5.5 log CFU/ml (Fig. 1) when compared to TC, which reduced the pathogen population to 1.8 log CFU/ml (Fig.1). Similarly, fumigation with EUG decreased SE counts on eggshells to 2.5 log CFU/ml on day 13 (Fig. 1). The eggs fumigated with ethanol were SE positive on shell until day 18, however, no pathogen was detected on eggs subjected to TC and EUG treatment (negative by plating and enrichment).

The transmission of SE from eggshell to embryo due to bacterial penetration of the shell membranes is one of the major causes of contamination in hatching chicks and subsequent SE persistence in the flock (Cox et al., 2000). The effect of 1% TC or EUG fumigation on SE counts in the embryos of fertile eggs until 18 days of incubation is shown in Fig. 2. Fumigation with ethanol yielded ~ 2 log CFU/ml counts of SE on day 1 and 1 log CFU/ml on day 3 in the chick embryo. However, TC and EUG decreased SE in the embryo to 1 log CFU/ml on day 1, and to undetectable levels (negative by enrichment) at all the subsequent time points until re-inoculation ($P < 0.05$). After re-inoculation and fumigation on day 13, the embryos of eggs subjected to ethanol, EUG and TC fumigations yielded a SE count of ~ 3.5 log, 1.5 log and 1 log CFU/ml, respectively. On day 18, the embryos of eggs subjected to ethanol fumigation yielded 1 log CFU/ml of SE, but TC and EUG decreased the pathogen to undetectable levels (negative by plating and enrichment) (Fig. 2). Fumigation of eggs with TC was generally more effective than EUG in decreasing SE counts both on eggshell (day 1, 3, 13 and 16) and embryo (day 13 and 16).

Although PDAs have been investigated as an antimicrobial wash treatment for

reducing SE on table eggs (Upadhyaya et al., 2013), this manuscript reports the first study evaluating their efficacy as fumigation agents against SE on embryonated eggs. The fumigation of eggs with TC or EUG produced no visible differences in shell color or consistency compared to control eggs, however, follow up studies on the quality characteristics of eggs, including hatchability are necessary before recommending their use.

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Figure 1: Efficacy of fumigation treatment with TC and EUG in reducing SE on eggshell of embryonated eggs. Effect of TC and EUG at 1% on SE spot inoculated on shell of embryonated eggs from day 1 to day 18 of incubation. An ethanol control (0%) under similar experimental conditions was also tested. Four eggs per treatment per sampling point (day 1, 3, 6, 9, 13, 16 and 18) were included, and the experiment was repeated two times. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n=8).

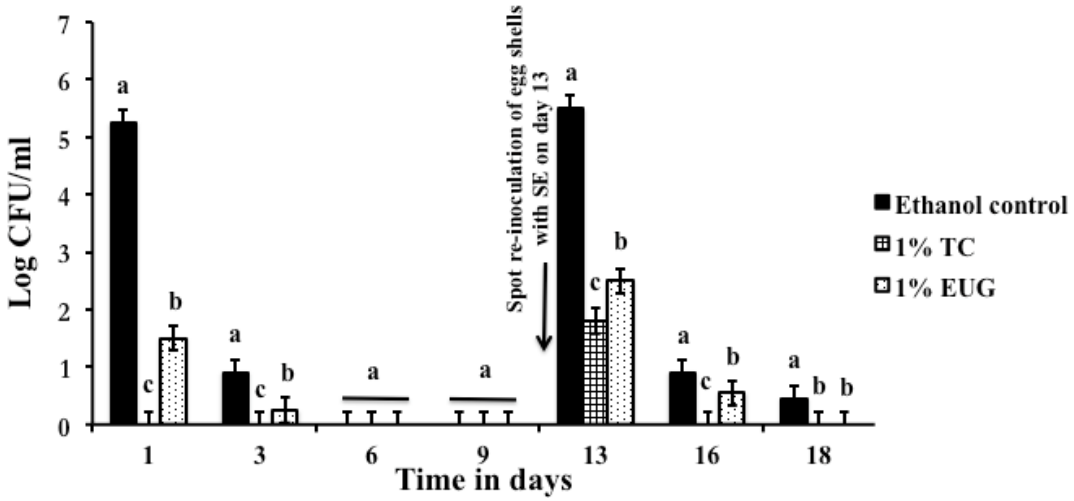
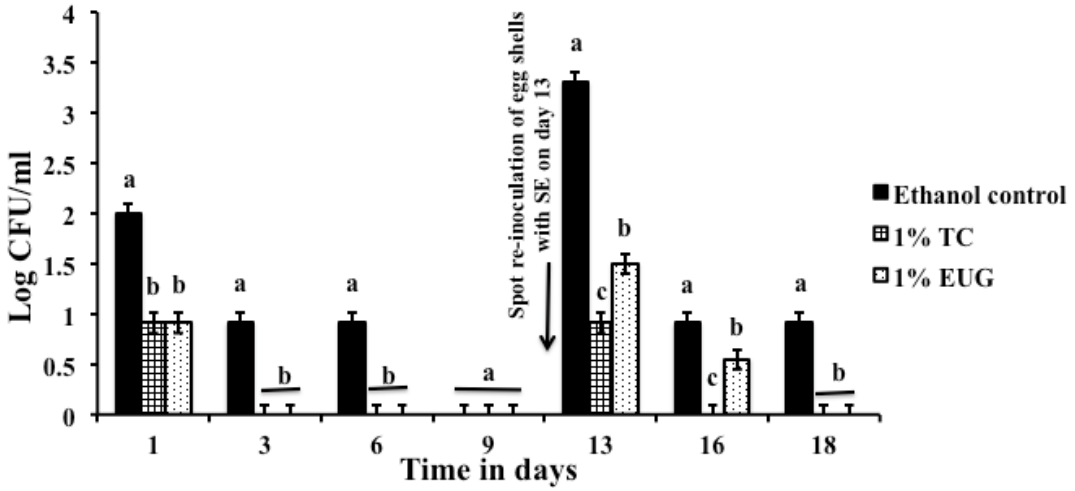


Figure 2: Efficacy of fumigation treatment with TC and EUG in reducing SE in embryo of eggs. Effect of TC and EUG at 1% on SE spot inoculated in embryo of eggs from day 1 to day 18 of incubation. An ethanol control (0%) under similar experimental conditions was also tested. Four eggs per treatment per sampling point (day 1, 3, 6, 9, 13, 16 and 18) were included, and the experiment was repeated two times. The differences between the means were compared at a significance level of 5%. Error bars represent SEM (n=8).



Chapter VIII

Summary

Salmonella Enteritidis (SE) is a major foodborne pathogen in the United States, causing gastroenteritis in humans, primarily through the consumption of contaminated eggs. Chickens are the reservoir host of SE, where the bacterium colonizes the intestine and migrates to various organs, including the oviduct, thereby leading to egg contamination. Despite substantial progress achieved in food safety through pathogen reduction programs, SE remains as one of the most common foodborne pathogens transmitted to humans through the consumption of eggs. Therefore, innovative on-farm strategies for reducing SE colonization and egg-borne transmission in layers are critical to control human infections of this pathogen. An antimicrobial treatment that can be applied through feed represents the most practical and economically viable method for adoption by the farms. In addition, a natural and safe antimicrobial will be better accepted by producers, including organic farmers without concerns for toxicity.

This study investigated the efficacy of several natural antimicrobials, including caprylic acid (CA), a medium chain fatty acid, and four plant-derived antimicrobials (PDAs), namely *trans*-cinnamaldehyde (TC), carvacrol (CR), thymol (THY), and eugenol (EUG) in reducing eggborne transmission of SE. The first objective of this dissertation investigated the efficacy of sub-inhibitory concentrations of four plant-derived antimicrobials (PDAs), namely TC, CR, THY and EUG in reducing SE adhesion to and invasion of chicken oviduct epihelial cells (COEC), and survival in chicken macrophages. In addition, the effect of PDAs on SE genes critical for oviduct colonization and macrophage survival was determined using real-time quantitative PCR (RT-qPCR). All the four PDAs significantly reduced SE adhesion to and invasion of COEC ($P<0.001$). The PDAs, except thymol consistently decreased SE survival in

macrophages (P<0.001). RT-qPCR results revealed that the PDAs down-regulated the expression of SE genes involved in its colonization and macrophage survival (P<0.001).

Reducing SE populations in the chicken cecum would lead to a decreased fecal excretion of the pathogen, which in turn could reduce contamination of eggshell and yolk. In addition, decreasing the cecal load of SE leads to reduced systemic dissemination of the bacterium, including its colonization of the reproductive tract and subsequent egg-borne transmission. Therefore, the second and third objectives of this dissertation investigated the efficacy of TC and CA as feed supplements in reducing SE cecal colonization, systemic spread and egg-borne transmission in layers. In the second objective, TC supplementation in feed for 66 days at 1 or 1.5% (vol/wt) to 40-week or 25-week-old layer chickens decreased SE on eggshell and in the yolk (P<0.001). Additionally, SE persistence in the cecum, liver and oviduct of TC-supplemented birds was decreased compared to control (P<0.001). The results suggest that TC could potentially be used as a feed additive to reduce egg-borne transmission of SE. Similarly in the third objective, in-feed supplementation of CA (0.7%, 1%) to birds consistently decreased SE on eggshell and in the yolk (P<0.001). Additionally, a reduction in SE populations in the cecum, liver and oviduct was observed in treated birds compared to control (P<0.001). No significant differences in feed intake, body weight or egg production in birds, and consumer acceptability of eggs was observed (P>0.05), thereby suggesting that TC and CA could potentially be used as feed additives to reduce egg-borne transmission of SE.

The external surface of eggs becomes contaminated with SE from various sources on farms, especially hens' droppings and contaminated litter. Therefore, effective

egg surface disinfection is critical to reduce SE on eggs and potentially control egg-borne disease outbreaks. Cleaning and sanitation of shell eggs by washing is a common practice mandatory for retail shell eggs from plants operating under voluntary USDA grade standards. Therefore, the fourth objective of this dissertation was to determine the efficacy of TC, CR, and EUG as an antimicrobial wash for rapidly killing SE on shell eggs in the presence or absence of chicken droppings.

White-shelled eggs inoculated with a 5-strain mixture of nalidixic acid (NA)-resistant SE (8.0 log CFU/mL) were washed in sterile deionized water containing each PDA (0.0, 0.25, 0.5 or 0.75%) or chlorine (200 ppm) at 32 or 42^{o}C for 30 sec, 3 min, or 5 min. The wash water control and chlorine control decreased SE on eggs by only 2.0 log CFU/egg even after washing for 5 min. However, all treatments containing CR and EUG reduced SE to undetectable levels as rapidly as within 30 sec of washing, whereas TC (0.75%) completely inactivated SE on eggs washed at 42^{o}C for 30 sec ($P < 0.05$). Carvacrol and EUG were also able to eliminate SE on eggs to undetectable levels in the presence of 3% chicken droppings at 32^{o}C ($P < 0.05$). This study demonstrated that the PDAs could effectively be used as a wash treatment to reduce SE on shell eggs.

The presence of *Salmonella* in fertile hatching eggs has been identified as a vital link to the contamination of hatching chicks, thus underscoring the significance of effective embryonated egg sanitation for protecting public health and decreasing economic losses to the poultry industry. Additionally, since chicks are more susceptible to infection by SE, effective egg surface disinfection in the hatching environment is critical for ensuring chick quality and microbiological safety. Therefore, the fifth and final objective of this dissertation investigated the efficacy of TC and EUG applied as a

fumigation treatment in reducing SE on embryonated eggs. Day-old embryonated eggs were spot inoculated with a 4-strain mixture of SE (~ 6.5 log CFU) and subjected to fumigation with the aforementioned PDAs (0 or 1% concentration) for 20 min in a hatching incubator, and SE on the shell and embryo was enumerated on days 1, 3, 6, 9, 13, 16 and 18. On day 13, the eggs were re-inoculated, followed by fumigation treatment for 20 min. Since the PDAs were dissolved in ethanol (final concentration 0.04%), eggs fumigated with ethanol were included as a control. The fumigation of embryonated eggs with the PDAs was found to be more effective in reducing SE on the shell and embryo compared to controls ($P<0.05$). On day 18, the eggs fumigated with ethanol were SE positive on the shell, whereas no pathogen was detected on eggs subjected to fumigation with TC and EUG. Similarly, although the embryos of eggs subjected to fumigation with ethanol yielded 1 log CFU/ml of SE on day 18, the embryos of TC and EUG treated eggs were devoid of the pathogen. These results suggest that TC and EUG could potentially be used as a fumigation treatment for reducing SE on embryonated eggs; however, quality traits of eggs, including the hatchability need to be determined.

To conclude, the results of this Ph.D. dissertation indicate the potential of TC, CR, EUG, THY and CA for controlling egg-borne transmission of SE.