

**EFFECTS OF ZINC ON *SALMONELLA* IN THE LAYER HOUSE
ENVIRONMENTS AND LAYING HENS, AND THE ABILITY
OF ZINC TO INDUCE MOLT IN LAYING HENS**

A Dissertation

by

SHINYOUNG PARK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2003

Major Subject: Food Science and Technology

**EFFECTS OF ZINC ON *SALMONELLA* IN THE LAYER HOUSE
ENVIRONMENTS AND LAYING HENS, AND THE ABILITY
OF ZINC TO INDUCE MOLT IN LAYING HENS**

A Dissertation

by

SHINYOUNG PARK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved as to style and content by:

Steven C. Ricke
(Co-Chair of Committee)

Sarah G. Birkhold
(Co-Chair of Committee)

Leon F. Kubena
(Member)

Jimmy T. Keeton
(Member)

Alan R. Sams
(Head of Department)

Rhonda K. Miller
(Chair of Food Science and
Technology Faculty)

December 2003

Major Subject: Food Science and Technology

ABSTRACT

Effects of Zinc on *Salmonella* in the Layer House Environments and Laying Hens,
and the Ability of Zinc to Induce Molt in Laying Hens. (December 2003)

Shinyoung Park, B.S., Korean Samyook University;

M.S., Mississippi State University

Co-Chairs of Advisory Committee: Dr. Sarah G. Birkhold
Dr. Steven C. Ricke

There is increasing interest in developing methods to detect and identify *Salmonella*, to eliminate or reduce the risk of contamination of shell eggs, and to retain the economic advantages of induced molting without increasing the risk of *Salmonella enteritidis* infection. *S. enteritidis* and *S. typhimurium* are the most common serotypes among 2449 known serotypes of the *Salmonella*, and are the causes of most egg-related foodborne salmonellosis in humans in the U. S. These two species are also responsible for environmental contamination and the incidence of infections.

Therefore, this study was conducted in three phases consisting of ten *in vivo* and *in vitro* experiments. This study investigated the effects of zinc on *Salmonella* growth and survivability in poultry environments, and its ability to induce a molt in single comb white leghorn hens. In part, the antibacterial properties of zinc may reduce environmental contamination in a poultry house by interrupting airborne routes. The first phase involved detecting airborne bacteria by aerosol sampling methods, and then screening any *Salmonella* mutant (s) that survived desiccation by transposon footprinting. The second phase examined, *in vitro*, the addition of zinc on the growth

kinetics of *Salmonella* under aerobic or anaerobic conditions, the effects of combining zinc and acidic conditions on the growth kinetics of *Salmonella in vitro* under aerobic or anaerobic conditions, and the effects of zinc amended feed on the survival of a dry inoculum of *Salmonella*. The third phase investigated the ability of zinc propionate, as an alternative salt form of zinc, to induce molt in laying hens, the influence of zinc acetate and zinc propionate on gastrointestinal tract fermentation, and susceptibility of laying hens to *S. enteritidis* during an induced molt, and the comparison of digestive microbial crop and cecal communities among molted hens fed by either zinc acetate or zinc propionate amended molting diets with hens undergoing feed withdrawal or full fed nonmolted hens using molecular-based denaturing gradient gel electrophoresis.

DEDICATION

My doctor of philosophy and this dissertation are dedicated with my greatest gratitude and deepest love to my parents, ChongKi Park, and ChungKum Lee for their endless love and sacrifice.

ACKNOWLEDGEMENTS

Thanks to be God Almighty in whom all things are possible.

My sincerest appreciation is expressed to Dr. Steven C. Ricke, my co-advisor, for his academic advice. With patience and attention, he led me through this project and all of my graduate studies. I have studied and worked with him since 1998, and I appreciate his great interest in scientific research and his diligence in his work. He is the model for my academic life.

My sincerest appreciation is expressed to Dr. Sarah G. Birkhold, my major co-advisor for her special guidance and invariable encouragement. With consideration and affection, she spiritually and substantially helped me to get through this project and this dissertation.

My sincere appreciation is also extended to Dr. Leon F. Kubena and Dr. Jimmy T. Keeton for their service as committee members.

My special appreciation is expressed to Mr. Bill and Dale Hyatt who work at the Poultry Research Center and are my senior friends. They helped me when I had difficulties catching birds during the initial time of field research, and in many other ways. I cannot forget their big hearts.

My special appreciation is also expressed to my ever-present lab companions: I. B. Zabala Diaz, X Li, K. L. Medvedev, J. Nutt, C. Froelich, Z. Howard, Aswari and Vesela.

My special appreciation is extended to Pilgrim's Pride, Inc. for their financial support and to the Department of Poultry Science for providing a wonderful academic environment.

I would greatly like to thank my Pastor Chong Kim and my new friends from the Korean Mission Church of Texas A&M. Just like a parent, Pastor Chong Kim, always remained alongside me and supported me with his invaluable prayers for my successful studies. My friends from the church have been a safe haven for me where I have always been able to find comfort when I was troubled and needed peace in the midst of my problems.

I would like to thank all of my friends for their friendship and support.

Finally, I cordially express my profound gratitude and admiration to my beloved parents, my brother and two sisters for their extraordinary love and sacrifice, encouragement, and sustaining support. I am especially indebted to my father and mother for all the times they stood behind me, for all my special dreams they helped make come true, for the never-ending love I have found in them, and for daily praying for my needs. Lastly, I am keenly aware of everything I am; and my world is a better place because of their support and sacrifices.

TABLE OF CONTENTS

	Page
ABSTRACT.....	iii
DEDICATION.....	v
ACKNOWLEDGEMENTS.....	vi
TABLE OF CONTENTS.....	viii
LIST OF FIGURES.....	xii
LIST OF TABLES.....	xv
CHAPTER I INTRODUCTION.....	1
CHAPTER II REVIEW OF LITERATURE.....	3
<i>Salmonella</i> and Human Salmonellosis.....	3
Molting.....	4
Vertical Contamination.....	8
Horizontal Contamination.....	9
Desiccation.....	17
Detection and Identification Methods.....	19
Alternative Methods to Induced Molting.....	23
Zinc.....	27
Hormones and Ovulation.....	38
Summary and Objectives of Research.....	39
CHAPTER III OPTIMIZATION AND COMPARISON OF BACTERIAL LOAD AND SAMPLING TIME FOR BIOAEROSOL DETECTION SYSTEMS IN A POULTRY LAYER HOUSE.....	42
Overview.....	42
Introduction.....	43
Materials and Methods.....	45
Results and Discussion.....	47
Conclusions.....	63

	Page
CHAPTER IV APPLICATION OF A TRANSPOSON FOOTPRINTING TECHNIQUE FOR RAPID IDENTIFICATION OF <i>SALMONELLA</i> <i>TYPHIMURIUM</i> TN5 MUTANTS REQUIRED FOR SURVIVAL UNDER DESICCATION STRESS CONDITIONS.....	64
Overview.....	64
Introduction.....	65
Materials and Methods.....	66
Results and Discussion.....	70
CHAPTER V <i>IN VITRO</i> COMPARISON OF ANAEROBIC AND AEROBIC GROWTH RESPONSE OF <i>SALMONELLA TYPHIMURIUM</i> TO ZINC ADDITION.....	77
Overview.....	77
Introduction.....	78
Materials and Methods.....	79
Results and Discussion.....	82
CHAPTER VI THE COMBINATION OF ZINC COMPOUNDS AND ACIDIC PH LIMITS AEROBIC GROWTH OF A <i>SALMONELLA</i> <i>TYPHIMURIUM</i> POULTRY ISOLATE MARKER STRAIN IN RICH AND MINIMAL MEDIA.....	91
Overview.....	91
Introduction.....	91
Materials and Methods.....	93
Results and Discussion.....	95
CHAPTER VII INFLUENCE OF OXIDATION-REDUCTION MEDIA REDUCTANTS ON <i>SALMONELLA TYPHIMURIUM</i> GROWTH KINETICS REPOSE IN AN ANAEROBIC ATMOSPHERE AFTER INITIAL PH ADJUSTMENT AND ZINC COMPOUND ADDITION.....	103
Overview.....	103
Introduction.....	104
Materials and Methods.....	106
Results and Discussion.....	108
CHAPTER VIII SURVIVAL OF <i>SALMONELLA TYPHIMURIUM</i> POULTRY	

	Page
MARKER STRAIN ADDED AS A DRY INOCULUM TO ZINC AND SODIUM ORGANIC ACID AMENDED FEED.....	118
Overview.....	118
Introduction.....	119
Materials and Methods.....	120
Results and Discussion.....	124
 CHAPTER IX EFFECTS OF HIGH ZINC DIETS USING ZINC PROPIONATE ON MOLT INDUCTION, ORGANS, POSTMOLT EGG PRODUCTION AND QUALITY IN LAYING HENS.....	 134
Overview.....	134
Introduction.....	135
Materials and Methods.....	136
Results and Discussion.....	141
 CHAPTER X COMPARISON OF ZINC ACETATE AND PROPIONATE ADDITION ON GASTROINTESTINAL TRACT FERMENTATION AND SUSCEPTIBILITY OF LAYING HENS TO <i>SALMONELLA ENTERITIDIS</i> DURING FORCED MOLT.....	 164
Overview.....	164
Introduction.....	165
Materials and Methods.....	168
Results and Discussion.....	173
Conclusions.....	191
 CHAPTER XI DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) AS A RAPID METHOD FOR ASSESSING GASTROINTESTINAL TRACT MICROFLORA RESPONSES IN LAYING HENS FED SIMILAR ZINC MOLT INDUCTION DIETS.....	 193
Overview.....	193
Introduction.....	194
Materials and Methods.....	196
Results and Discussion.....	199
 CHAPTER XII EFFECTS OF STORAGE CONDITIONS ON BONE BREAKING STRENGTH AND BONE ASH IN LAYING HENS AT	

	Page
DIFFERENT STAGES IN PRODUCTION CYCLES.....	206
Overview.....	206
Introduction.....	207
Materials and Methods.....	208
Results and Discussion.....	210
CHAPTER XIII CONCLUSION.....	215
REFERENCES.....	217
APPENDIX.....	280
VITA.....	283

LIST OF FIGURES

FIGURE		Page
3-1	Day 1 axial fan accumulation with impinger at each time point.....	48
3-2	Day 2 axial fan accumulation with impinger at each time point.....	49
3-3	Filter and impinger, direct comparison.....	52
3-4	Comparison of axial fan glass filter and circulating fan.....	55
3-5	Comparison of axial fan puralator and glass filters.....	56
3-6	Comparison of axial fan puralator filter and circulating fan.....	57
4-1	Experimental approach for desiccation and recovery of <i>S. typhimurium</i> Tn5 mutant pools.....	68
4-2	Survival of <i>S. typhimurium</i> Tn5 mutants during initial desiccation....	71
4-3	Footprints of different <i>S. typhimurium</i> Tn5 mutant pools on day zero.....	73
4-4	Comparison of transposon footprints of <i>S. typhimurium</i> Tn5 mutant pools 1 and 5 from different days.....	75
5-1	Aerobic growth rate response of <i>Salmonella typhimurium</i> poultry isolate in the presence of Zn compounds in TSB medium.....	83
5-2	Aerobic growth rate response of <i>Salmonella typhimurium</i> poultry isolate in the presence of Zn compounds in M9 medium.....	85
5-3	Anaerobic growth rate response of <i>Salmonella typhimurium</i> poultry isolate in the presence of Zn acetate in M9 medium.....	87
5-4	Anaerobic growth rate response of <i>Salmonella typhimurium</i> poultry isolate in the presence of Zn sulfate in M9 medium.....	89
6-1	Aerobic growth response of <i>S. typhimurium</i> poultry isolate to the presence of Zn acetate in TSB medium.....	96
6-2	Aerobic growth response of <i>S. typhimurium</i> poultry isolate to the presence of Zn sulfate in TSB medium.....	98

FIGURE	Page
6-3	100
Aerobic growth response of <i>S. typhimurium</i> poultry isolate to the presence of Zn acetate in M9 medium.....	
6-4	101
Aerobic growth response of <i>S. typhimurium</i> poultry isolate to the presence of Zn sulfate in M9 medium.....	
7-1	110
Anaerobic growth response of <i>S. typhimurium</i> poultry isolate in the presence of Zn acetate in different pH levels of TSB medium without and with reductants.....	
7-2	111
Anaerobic growth response of <i>S. typhimurium</i> poultry isolate in the presence of Zn sulfate in different pH levels of TSB medium without and with reductants.....	
7-3	113
Anaerobic growth response of <i>S. typhimurium</i> poultry isolate in the presence of Zn acetate in different pH levels of M9 medium without and with reductants.....	
7-4	115
Anaerobic growth response of <i>S. typhimurium</i> poultry isolate in the presence of Zn sulfate in different pH levels of M9 medium without and with reductants.....	
8-1	126
Moisture contents of poultry layer ration, Zn acetate (1% zinc) amended feed, and Zn propionate (1% zinc) amended feed over 9 days of storage at room temperature.....	
8-2	129
Survival of <i>Salmonella typhimurium</i> populations in poultry layer ration, Zn acetate (1% zinc) amended feed, and Zn propionate (1% zinc) amended feed over 9 days of storage at room temperature..	
8-3	131
Survival of <i>Salmonella typhimurium</i> populations in poultry layer ration, 1% Na acetate amended feed, and 1% Na propionate amended feed over 9 days of storage at room temperature.....	
9-1	156
Percent hen-day egg production by four treatments on a weekly basis both during the pre-molt and during the post-molt.....	
9-2	157
Percent average egg production after induced molting by four treatments (weeks 3 to 12).....	

FIGURE	Page	
11-1	Denaturing gradient gel electrophoresis of ceca or crop bacterial 16S amplicon patterns from leghorn hens on nonmolted control (C), molted feed withdrawal (Mo), zinc acetate (Za), and zinc propionate (Zp) in trial 1.....	203
11-2	Denaturing gradient gel electrophoresis of ceca or crop bacterial 16S amplicon patterns from leghorn hens on nonmolted control (C), molted feed withdrawal (Mo), zinc acetate (Za), and zinc propionate (Zp) in trial 2.....	204
11-3	Denaturing gradient gel electrophoresis of ceca or crop bacterial 16S amplicon patterns from leghorn hens on nonmolted control (C), molted feed withdrawal (Mo), zinc acetate (Za), and zinc propionate (Zp) in trial 3.....	205
12-1	Effect of storage conditions on breaking strength (breaking force divided by bone weight expressed as kilograms per gram) of tibia obtained from hens of different ages.....	211
12-2	Effect of storage conditions on percentage bone ash (tibia ash weights divided by tibia dry weight and multiplying by 100) of tibia obtained from hens of different ages.....	212

LIST OF TABLES

TABLE		Page
2-1	Common airborne microorganisms in pig or poultry houses.....	12
2-2	Common airborne pathogens of pigs and poultry.....	13
3-1	Direct comparison of impinger and axial fan filters.....	51
3-2	Comparison of four different samplers.....	54
3-3	Comparison of four samplers.....	58
3-4	Enumeration of total viable counts from each sampling before and after adjusting calibrated airflow rate.....	60
3-5	Standard deviations of samplers.....	62
8-1	Composition of the soybean meal-based diet.....	122
8-2	Decline of <i>Salmonella typhimurium</i> populations in different poultry feed over 9 days of storage at room temperature.....	128
9-1	Composition of TAMU layer ration.....	138
9-2	Effect of molting and nonmolting diets on the crop pH, feed intake, body weight loss and ovary weight of Single Comb White Leghorn (SCWL) hens.....	142
9-3	Effect of molting and nonmolting diets on the relative organ weight of Single Comb White Leghorn (SCWL) hens.....	147
9-4	Effect of molting and nonmolting diets on the bone breaking strength (kg/g) and bone ash (%) of Single Comb White Leghorn (SCWL) hens.....	151
9-5	Effect of molting and nonmolting diets on the concentrations of zinc in kidney and liver of Single Comb White Leghorn (SCWL) hens.....	153
9-6	Effect of molting and nonmolting diets on the internal egg quality of Single Comb White Leghorn (SCWL) hens during the postmolt.....	159
9-7	Effect of molting and nonmolting diets on the external egg quality of	

TABLE	Page
Single Comb White Leghorn (SCWL) hens during the postmolt.....	161
10-1 Effects of nonmolting and molting diets on feed intake, body weight lost, and ovary weight of hens.....	174
10-2 Effects of nonmolting and molting diets on the crop lactic acid concentration and pH of hens.....	178
10-3 Effects of nonmolting and molting diets on the cecal volatile fatty acid and lactic acid concentrations.....	182
10-4 Effects of nonmolting and molting diets on <i>S. enteritidis</i> crop colonization of hens.....	185
10-5 Effects of nonmolting and molting diets on <i>S. enteritidis</i> cecal colonization of hens.....	187
10-6 Effects of nonmolting and molting diets on <i>S. enteritidis</i> colonization of the liver, spleen, and ovary of hens.....	189
11-1 Oligonucleotide primer sequences.....	198

CHAPTER I

INTRODUCTION

Since 1970, the reported case of nontyphoidal salmonellosis have continued to increase in the United States (Tietjen and Fung, 1995; CDC 1999). Nontyphoidal salmonellosis is the leading cause of human illness, resulting in an approximately 1.4 million cases (Mead *et al.*, 1999). Annually, 16,430 people are hospitalized and 600 people die due to the nontyphoidal salmonellosis in the U. S. (Mead *et al.*, 1999).

Symptoms of non-typhoidal salmonellosis include non-bloody diarrhea, abdominal pain, fever, nausea, and vomiting, which normally appear 8 to 72 h after initial contact with infectious agent (Tietjen and Fung, 1995). Non-typhoidal salmonellosis also can lead to more severe symptoms such as septicemia, arthritis, meningitis, and pneumonia, but is usually limited to gastroenteritis that does not require antibiotics (Cohen *et al.*, 1987). The very young, elderly, and immunocomprised are especially at risk of death (Tauxe, 1991). The direct and indirect medical costs to society due to salmonellosis could exceed 4 billion dollars in the U. S. (Todd, 1989).

Most outbreaks of human salmonellosis are caused by contaminated food products such as egg and egg products (Hedberg *et al.*, 1993; Tietjen and Fung, 1995; Mead *et al.*, 1999), poultry, other meat products (Mishu *et al.*, 1994; Tietjen and Fung, 1995), and dairy products (El-Gazzar and Marth, 1975; Tietjen and Fung, 1995).

This dissertation follows the style and format of Poultry Science.

Among the 2449 known serotypes of the *Salmonella* (Brenner *et al.*, 2000), the most common serotypes causing human salmonellosis are *Salmonella typhimurium* and *S. enteritidis* (Hedberg *et al.*, 1993; Baumler *et al.*, 2000). *S. enteritidis* is introduced and transmitted to uninfected birds through many different routes. The uninfected birds can be contaminated via direct contact with infected birds, vertical transmission, and horizontal transmission in an integrated poultry layer house.

The molting process is a means of increasing productivity in older laying flocks. It is commonly used in an estimated 75 to 80% of all commercial U. S. egg layer operations (Bell, 2001). In 2001, 75 million (27%) of the nation's 276 million egg layers either were actively in or had completed an induced molt in the U. S. (USDA, 2000). The poultry layer industry needs to consider alternative molting procedures that do not require feed withdrawal, but allow layer house managers to retain the economic advantages of obtaining a second laying cycle via molting without increasing the risk of *S. enteritidis*. High concentrations of zinc have been shown to induce molt in laying hens. However, the effects of zinc on the growth of *Salmonella* in the laying house environment and under conditions similar to the hen's gastrointestinal tract have not been completely elucidated.

CHAPTER II

REVIEW OF LITERATURE

Salmonella and Human Salmonellosis

Salmonella are facultative, gram-negative, and non-sporing rods in the family Enterobacteriaceae (Grimont *et al.*, 2000). *Salmonella* serovars can be divided into three groups based on their epidemiological characteristics (Lax *et al.*, 1995). Group I correspond to the infection of humans only. These include *S. typhi*, *S. paratyphi* A, *S. paratyphi* C and the agents of typhoid and paratyphoid fevers; group I causes the most severe of all human diseases. Group II correspond to the host-adapted serovars; some of which are human pathogens and may be contracted from foods. These include *S. galinarum* (poultry), *S. dublin* (cattle), *S. abortus-equi* (horses), *S. abortus-ovis* (sheep), and *S. choleraesuis* (swine). Group III correspond to unadapted serovars, with no host preference. These include most foodborne serovars and pathogens for humans and other animals. *Salmonella* species and serovars can cause several diseases such as gastroenteritis and systemic disease in different hosts.

Salmonella enterica serovar Enteritidis (*S. enteritidis*) and Typhimurium (*S. typhimurium*) are of public health significance due to their complex epidemiology involving extensive fecal excretion with associated environmental contamination and the existence of many different infections (Barrow, 1993). *Salmonella enteritidis* and *typhimurium* are reported to be as the most common pathogens of human salmonellosis (Baumler *et al.*, 2000). Approximately 60% of U. S. human salmonellosis in 1995 was caused by *S. enteritidis* (24.7%) and *S. typhimurium* (23.5%) (Rabsch *et al.*, 2001). The

largest outbreak of human salmonellosis occurred in Illinois was caused by *S. typhimurim* in milk (Lecos, 1986). In the last 10 years, the infection of greatest concern in the U. S. has been caused by *S. enteritidis* (Angulo and Swerdlow, 1998). In 1989, there were more than 49 outbreaks caused by *S. enteritidis* in U. S. and Puerto Rico (Blumenthal, 1990). In 1993, a large outbreak in Florida was caused by *S. enteritidis* in ice cream produced from milk that was transported in tanker trucks that had previously hauled unpasteurized liquid eggs (Anonymous, 1994). Therefore, it is note that *S. enteritidis* and *typhimurium* are important causes of foodborne salmonellosis in humans in the U. S.

Molting

Natural and Induced Molting

Annually, many wild birds naturally experience a body weight of loss up to 40%, accompanied by feather loss and regression of the reproductive system (Brake and Thaxton, 1979; Mrosovsky and Sherry, 1980). This is caused by self- induced decreased feed intake and activity during the late summer and early fall when the day length naturally begins to shorten (North and Bell, 1990). These physiological changes cause the cessation of reproduction. Therefore, wild birds take a self-induced rest to rejuvenate body tissues and build up energy stores. Based on these characteristics, the practice of induced molting by the U. S. poultry industry by light manipulation and reduced daily feed intake closely mimics what occurs naturally. The combination of feed removal and light reduction from 16 h of darkness to 8h of light for 10 to 14 days is

the most widely used practice for inducing molting in the U. S. commercial egg layer industry (Bell, 1987; Holt, 1993). Induced molting allows an egg producer to achieve a second productive laying cycle (North and Bell, 1990; Holt, 1999). Light stimulation results in the release of the follicle-stimulating hormone (FSH) from the pituitary, which is followed by an increase in the growth of the ovarian follicles. Once reaching maturity, the ovum is released by leutenizing hormone (LH).

A general increase in reproductive performance results from the rejuvenation effect. This rejuvenation may be associated with an increased tissue sensitivity or efficiency and reorganization of metabolic processes (Brake and Thaxton, 1979). In addition, the loss of adipose tissue may be associated with the overall increase in performance (Brake and Thaxton, 1979). However, both the regression and redevelopment of organs and tissues are related to the increased reproductive performance postmolt. The decrease in body weight of hens by feed withdrawal is directly related to decreased muscle, adipose tissue, liver, and the involution of reproductive organs (Brake and Thaxton, 1979; Berry and Brake, 1985). Approximately 25% of the body weight loss is connected to the decrease in liver weight and involution of the reproductive organs (Brake and Thaxton, 1979). The loss of primary flight feathers is also involved in postmolt reproductive performance (Andrews *et al.*, 1987a, 1987b; Herremans *et al.*, 1988). The loss of primary feathers is clearly due to the loss of estrogenic influence on the feather papilla (Péczy, 1992). Therefore, molt occurs during a nadir in estrogen production.

Lee (1982) reported that molted birds have more egg production, better feed efficiency, better shell quality, and less mortality than unmolted birds. A greater primary feather loss is associated with greater egg production (Lee, 1982). One of the main reasons for increased postmolt egg production is decreased postmolt production of shell-less eggs (Roland and Brake, 1982). Hens that lay shell-less or poorly shelled eggs show increased shell gland lipid (Roland *et al.*, 1977). This lipid is largely confined to the calcium secreting glandular epithelium (Baker *et al.*, 1983) and remains during the feed withdrawal periods until more than 25% of bird's initial weight is lost (Brake, 1992).

Current Issues on Induced Molting

The practice of feed withdrawal is an efficient method to induce a molt because it is easy management friendly, economically advantageous and results in satisfactory postmolt performance for the commercial layer industry (Brake, 1993). However, a long period of feed withdrawal is more controversial. Poultry scientists, commercial poultry laymen and others are increasing concerned over issues related to animal welfare during the induced molting, even though birds experiencing a natural molt reject feed for a prolonged period (Mrosovsky and Sherry, 1980). Of concern is whether it is detrimental to initiate molt before the bird is physiologically ready (Ruszler, 1998). In addition, birds molted by feed withdrawal may be more susceptible to *Salmonella enteritidis* infection because induced molting by feed withdrawal depresses the cellular immune response (Holt, 1992; Holt and Porter, 1992a, 1992b ; Holt, 1993; Holt *et al.*, 1994; Holt *et al.*, 1995). The increased susceptibility to *S. enteritidis* infections in molted birds has

been related to a rapid and severe inflammatory response in ceca, which was not generally seen in nonmolted infected birds (Isobe and Lillehoj, 1992; Porter and Holt, 1993; Macri *et al.*, 1997). During the molting periods, *S. enteritidis* is readily transmitted to uninfected birds from infected birds in adjacent cages (Holt and Porter, 1992b), and can be transmitted to uninfected birds in cages some distance way from infected bird, suggesting that airborne transmission of the organism may be important (Holt *et al.*, 1998).

S. enteritidis colonizes the gastrointestinal tract in birds prior to dissemination to multiple organs (Gast and Beard, 1993; Gast, 1994). The cecum is the main site for colonization by *S. enteritidis*, followed by the colon and ileum (Fanelli *et al.*, 1971; Turnbull and Snoeyenbos, 1974; Holt *et al.*, 1995). Colonization of the ovary may cause the production of *S. enteritidis* contaminated eggs, which in turn could lead to human salmonellosis if infected eggs are mishandled or undercooked. Therefore, the large amount of *S. enteritidis* that could be shed into the house environment during molting, may pose a greater problem for the producer and for future flocks that would occupy that house.

Effects of Induced Molting on the Microenvironment of Crop and Ceca

Induced molting through feed withdrawal alters the microenvironment of crop and ceca which are the main sites of *Salmonella* colonization in the chicken intestine (Brownell *et al.*, 1970; Soerjadi *et al.*, 1981; Impey and Mead, 1989). Carrier *et al.* (1997) reported that induced molting had no apparent effect on pH or on the oxidation-reduction potential of the ceca, but induced molting decreased the concentrations of

acetic, propionic, and total volatile fatty acids (VFA) of the ceca. Corrier *et al.* (1997) suggested that increased susceptibility of molted hens to *S. enteritidis* colonization may be related to decreased fermentation and production of VFA-producing bacteria present in the ceca, and addition of lactose in the drinking water during the water may enhance resistance to *S. enteritidis* colonization. VFA in the cecal contents in chicken are fermentation products of normal indigenous anaerobic bacteria (Barnes *et al.*, 1979, 1980) and the concentrations of VFA may represent the degree of fermentation activity of bacteria in the ceca (Barnes *et al.*, 1980).

Durant *et al.* (1999) reported that induced molting increased crop pH, and decreased the *Lactobacilli* populations and the concentrations of lactic, acetic, propionic, butyric, and VFA of the crop. They also reported that increased *S. enteritidis* colonization in the crop was due to the high pH and lowered concentrations of lactate and total VFA. *Lactobacilli* that are the predominant microflora in the crop, play an important role in maintaining a low pH that prevented coliform establishment in the crop (Fuller and Brooker, 1974) and in preventing the growth of *E. coli in vitro* (Fuller, 1977). The native intestinal microflora functions to inhibit *Salmonella* colonization of the ceca (Nurmi and Rantala, 1973; Barnes *et al.*, 1980; Nisbet *et al.*, 1994; Corrier *et al.*, 1995) and crop (Barnes *et al.*, 1980) in chickens.

Vertical Contamination

Vertical contamination is transovarian transmission of microorganisms from hens to progeny or table eggs (Guthrie, 1992). This transmission rapidly increases the

incidence of disease in poultry flocks and permits cross-contamination due to contamination of the egg passage via the chicken (Guthrie, 1992).

S. enteritidis may be carried in the large intestines of adult laying hens and shed in their feces, which may lead to contamination of the egg shell surface by *S. enteritidis* (Coyle *et al.*, 1988). Additionally, *S. enteritidis* oral inoculation to adult laying hens can infect the reproductive tracts following localization and colonization of the large intestine, that is, by transovarian transmission although there are no noticeable clinical signs in the hens (Timoney *et al.*, 1989; Shivaprasad *et al.*, 1990). Ovarian infections of *S. enteritidis* cause both the laying of contaminated eggs and infected chicks hatched from the contaminated hatching eggs. These infected chicks grow up to become pullets and subsequently lay contaminated eggs (Hopper and Mawer, 1988; Lister, 1988).

Horizontal Contamination

Airborne Transmission of the Microorganisms

The primary mechanism of horizontal contamination of microorganism to uninfected birds is via direct contact with infected birds (Gast and Beard, 1990a, 1990b; Holt and Porter, 1992a, 1992b ; Holt and Porter 1993; Holt, 1995) or via contaminated drinking water (Nakamura *et al.*, 1994). However, airborne transmission could not be ruled out as an important factor because high bird density in a commercial poultry house may increase the disease spread by airborne microorganisms and accentuate airborne contamination (Sauter *et al.*, 1981). Therefore, these problems require the development of information regarding airborne bacteria and fungi present in poultry house.

Airborne contamination is a complex combination of the following four fractions: non-viable fraction, viable fraction, particulate fraction, and gaseous fraction (Hartung, 1994a). The non-viable particulate fraction, dust, is mainly associated with particles of feed (80 to 90%), litter (55 to 68%), animal surfaces (2 to 12 %), feces (2 to 8%), manure, and dander (Hartung, 1994a). They can become airborne by animal movement and air flow (Hartung, 1994a).

Gases are widely produced directly by animals and from their feces. Ammonia, hydrogen sulfide and methane are produced from the microbial degradation of manure (Jones *et al.*, 1984). Carbon dioxide is produced from both animal and microbial degradation, as well as combustion of fuel for heating the animal house (Jones *et al.*, 1984). Carbon monoxide can also be produced from incomplete fuel combustion (Jones *et al.*, 1984). Nitrogen oxides are by-products of fuel combustion (Jones *et al.*, 1984). The chemical analysis of dust from pig and poultry houses is a complex mixture, which mainly yields ammonia, proteins, proteolytic enzymes, and endotoxins (Donham *et al.*, 1986a). Endotoxins are fragments of gram-negative bacterial walls, lipopolysaccharides, which cause allergic and immunological reactions in humans (Hartung, 1994a). Therefore, dust is a hazardous airborne agent that has allergenic agents, infectious microorganisms and toxic gases.

The viable fraction or bioaerosol contains bacteria, fungi, and viruses (Clark *et al.*, 1983). Airborne microorganisms in swine houses mainly contain gram positive bacteria with up to 40% *Streptococci* and 17% *Micrococci* (Donham *et al.*, 1986b). Gram negative coliform bacteria and fungi in poultry and swine house are detected in

less than 1% and 13-45% of these houses, respectively (Clark *et al.*, 1983). Common airborne microorganisms found in pig or poultry houses are listed in Table 2-1. All airborne pathogens detected from both pig and poultry are listed in Table 2-2.

The overall populations and identification of microorganisms, and their pathogenic or toxin producing potential should be considered. Although many of the microbes are harmless and are required for decomposition of waste organic matter, some microorganisms are pathogenic for either man, birds or both. Petersen *et al.* (1978) analyzed the total aerobic microflora found in a poultry house and found the total to be 8% *Staphylococcus aureus* and 4-5% *Escherchia coli*. *Staphylococcus aureus* was isolated from the skin and nasal passages of poultry (Derviese *et al.*, 1975). The transmission and survival of bioaerosol organisms is affected by environmental parameters such as air currents, temperature, and relative humidity. According to Lighthart and Mohr (1987), the faster the upwind speed, the more the sample concentration resembles the source concentrations. In general, airborne microorganisms can be inactivated quickly by high temperature (Sattar and Ijaz, 1987). The response of airborne bacteria to atmospheric humidity is species dependent. For example, during the aerosolization, *E. coli* K-12 was shown to survive better at low humidities than at high humidities (Cox, 1966). Bacteria are more susceptible to harsh environmental stress than fungal spores and enteric viruses during transport through the air (Knudsen and Spurr, 1987).

Airborne transmission of *Salmonella* spp. within the rearing environment may have an important role in the cross infection of birds and the maintenance of the

TABLE 2-1. Common airborne microorganisms in pig¹ or poultry² houses

	Species	Pig	Poultry
Bacteria	<i>Staphylococcus aureus</i>	x	x
	<i>Escherichia coli</i>	x	x
	<i>Streptococcus</i>	x	x
	<i>Aerococcus</i>	x	
	<i>Micrococcus</i>	x	x
	<i>Proteus</i>		x
	<i>Bacillus</i>	x	x
	<i>Aerobic spores</i>	x	x
	<i>Enterobacter</i>	x	
	<i>Pseudomonas</i>	x	x
	<i>Aerobic spores</i>	x	x
	<i>Anaerobic spores</i>	x	
	Fungi	<i>Penicillium</i>	
<i>Aspergillus</i>			x
<i>Scopulariopsis</i>		x	x
<i>Cladosporium</i>		x	x
<i>Acremonium</i>			x
<i>Mucor</i>		x	x
<i>Alternaria</i>			x
<i>Trichothecium</i>			
	<i>Monocillium</i>		x

¹Pig house source: Crook *et al.* (1991); Hartung (1994a)

²Poultry house source: Sauter *et al.* (1981); Hartung (1994a)

TABLE 2-2. Common airborne pathogens of pigs and poultry

Airborne pathogens	Species
Bacteria	<i>Bordetella bronchiseptica</i>
	<i>Brucella suis</i>
	<i>Cornebacterium equi</i>
	<i>Erysipelothrix rthusiopathiae</i>
	<i>Escherichia coli</i>
	<i>Haemophilus gallinarus</i>
	<i>Haemophilus parasuis</i>
	<i>Haemophilus pleuropneumoniae</i>
	<i>Listeria monocytogenes</i>
	<i>Leptospira Pomona</i>
	<i>Mycobacterium arium</i>
	<i>Mycobacterium tuberculosis</i>
	<i>Mycoplasma gallisepticum</i>
	<i>Mycoplasma hyorhinus</i>
	<i>Mycoplasma suis pneumoniae</i>
	<i>Mycoplasma nultocida</i>
	<i>Mycoplasma pseudotuberculosis</i>
	<i>Salmonella pullorum</i>
	<i>Salmonella typhimurium</i>
	<i>Straphylococcus aureus</i>
<i>Streptococcus suis type II</i>	
Fungi	<i>Aspergilluse flavus</i>
	<i>Aspergilluse fumigatus,</i>
	<i>Aspergilluse nidulans</i>
	<i>Aspergilluse niger</i>
	<i>Coccidioides immitis</i>
	<i>Crptococcus neoformans</i>
	<i>Histoplasma farcinorum</i>
	<i>Rhinosporidium seeberi</i>
Viruses	African swine fever
	Avian encephalomyelitis
	Avian leucosis
	Foot-and-mouth disease
	Fowl plague
	Hog cholera
	Inclusion body rhinitis
	Infectious bronchitis of fowls
	Infectious laryngotracheitis of fowls
	Infectious nephrosis of fowls
	Infectious porcine encephalomyelitis
	Marek's disease
	Newcastle disease
	Ornithosis
	Porcine enterovirus
Swine influenza	
Transmissible gastroenteritis of swine	

Source: Wathes, 1995.

pathogens within poultry flocks. *S. enteritidis* PT4 has been shown to infect poultry (Baskerville *et al.*, 1992) and *S. typhimurium* (Wathes *et al.*, 1988) to infect calves when carried experimentally by aerosol. Airborne contamination by *S. enteritidis* PT4 and *S. typhimurium* more generally has been experimentally demonstrated to lead to the cross-infection of birds sharing the same rearing environment (Lever and Williams, 1996; Holt *et al.*, 1998; Leach *et al.*, 1999). The airborne coliform bacteria and *E. coli* detected in dust samples may be involved in coli-septicemia of chickens in poultry house (Harry 1964; Carlson and Whenham, 1968). *Salmonella* detected in eggshell fragments in adjacent hatching trays was in the mixed hatching tray containing chicks from *Salmonella* inoculated eggs (Cason *et al.*, 1994). Laying hens exposed to aerosol containing *S. enteritidis* develop a systematic infection and excrete the organism in the fecal materials (Baskerville *et al.*, 1992). Airborne transmission of *S. enteritidis* can be spread 3-4 times more rapidly in molted laying hens than unmolted laying hens (Holt *et al.*, 1998). Fan driven air in the hatching cabinet may move *Salmonellae* from contaminated eggs to uncontaminated eggs (Cason *et al.*, 1994; Berrang *et al.*, 1995). Therefore, the airborne transmission of microorganisms is regarded as a potential and favorable microenvironment route that favors infection (Harry, 1964; Wathes, 1995).

Eggborne Source

An important source of human *Salmonella* infection is contaminated eggs or egg products (St. Louis *et al.*, 1988; Hedberg *et al.*, 1993); this makes it a unique threat to food safety. Although the on-farm environment of the chicken is a rich source of a number of *Salmonella* serotypes (Caldwell *et al.*, 1995; Byrd *et al.*, 1999), *S.*

enteritidis is a primary pathogen that causes frequent human illness associated with egg contamination. Although laying hens may harbor *S. enteritidis*, they show no clinical signs of infection, and the eggs they produce appear normal (Garber *et al.*, 2003). The rate of egg contamination is between 1 and 11 contaminated eggs per 100,000 eggs laid (USDA-Food Safety and Inspection Service, 1998). Egg contamination by *S. enteritidis* has been a continuing international public health problem for more than a decade (Angulo and Swerdlow, 1998; CDC, 1999). Foodborne outbreaks of *S. enteritidis* infection in the U. S. for which vehicle could be identified were most often associated with Grade-A shell eggs (St. Louis *et al.*, 1988; Hedberg *et al.*, 1993; Mishu *et al.*, 1994).

Egg contamination results from penetration through the eggshell by *S. enteritidis* contained in feces after the egg is laid, horizontal transmission (Gast and Beard, 1990a, 1990b; Barrow and Lovell, 1991; Humphrey *et al.*, 1991) or by direct contamination of reproductive organs with *S. enteritidis* before the egg is laid, vertical transmission (Timoney *et al.*, 1989; Shivaprasad *et al.*, 1990). The incidence of *S. enteritidis*-positive eggs increases threefold within the first 10 weeks after hens are subjected to forced molting (USDA-Animal and Plant Health Inspection Service, 1994). *S. gallinarum* and *S. pullorum* are strict host-adapted serovars that can infect the ovary and cause transovarian transmission into the egg (Snoeyenbos *et al.*, 1969). *S. heidelberg*, *S. kentucky*, *S. saintpaul*, *S. thompson*, *S. typhimurium*, and *S. hadar* are vague-adapted serovars that have been isolated from the reproductive organs of hens and from egg contents (Snoeyenbos *et al.*, 1967; Snoeyenbos *et al.*, 1969; Humphrey *et al.*, 1989; Barnhart *et al.*, 1993; Waltman and Horne, 1993).

Feedborne Source

Feed is an important source of *Salmonella* contamination in poultry (Morris *et al.*, 1969; Dougherty, 1976; MacKenzie and Bains, 1976; Williams, 1981; Cox *et al.*, 1983; Veldman *et al.*, 1995). Feed serves as a source of horizontal transmission once it has been contaminated by feathers, feces, or airborne *Salmonella* (Hoover *et al.*, 1997).

Veldman *et al.* (1995) reported that *Salmonella* contamination rates for mash layer-breeder feeds (21.4%) were higher than those of pellet layer-breeder feeds (1.4%). Jones *et al.* (1991) also reported that *Salmonella* contamination rates for mash type feeds (35%) were higher than those of pellet type feeds (6.3%) at the feed mills. Therefore, pelleting poultry feed is a valuable method for reducing the incidence of contamination with *Salmonella* (Veldman *et al.*, 1995). However, layer-breeder feeds are usually not pelleted; this prevents dirty egg shells and maintains a low level of feed intake by birds (Vanderwal, 1979; Veldman *et al.*, 1995). Mash layer-breeder feeds with acid can reduce the vertical transmission of *Salmonella* (Vanderwal, 1979; Humphrey and Lanning, 1988). Fish meal, meat and bone meal, tapioca, and maize grits used as feed components for layer-breeder feeds influence the incidence of *Salmonella* contamination with 31, 4, 2, and 27%, respectively, for feeds formulated with each ingredient (Veldman *et al.*, 1995).

Other Sources in Laying House Environment

Numerous potential sources of *Salmonella* exist in the laying house environment (Potter, 1987; Elliott, 1990; Jones *et al.*, 1995). Jones *et al.* (1995) reported that *Salmonella* was isolated from egg belts (72.7%), egg collectors (63.6%), ventilation fans

(100%), and flush water (100%) in the commercial laying house environment. Murase *et al.* (2001) suggest that *Salmonella* spp can be spread via egg belts from one house to others. Garber *et al.* (2003) recently reported that overall 7.1% of layer houses and 3.7% of mice were culture positive for *S. enteritidis* from a total of 200 layer houses and 129 house mice, respectively, in 15 states throughout the U. S. Mice may both amplify and spread *S. enteritidis* in layer house (Guard-Petter *et al.*, 1997; Hassan and Curtiss, 1997). According to Henzler and Opitz (1992), up to 10^5 colony-forming units of *Salmonella* may be present in a single mouse fecal pellet. Laying flocks with high levels of manure contamination are more likely to produce contaminated eggs and thus, pose the greatest risk to human health (Henzler *et al.*, 1998). Thirteen and 38% of *S. enteritidis* was detected on manure in the layer houses in 1995 and in 1992, respectively, by the Pennsylvania Egg Quality Assurance Program; under the right conditions, this *Salmonella* could be spread within the laying flock (White *et al.*, 1997).

Desiccation

Desiccation is the most fundamental stressor that is experienced by all airborne bacteria in the indoor environment and is caused by evaporation of water from bacteria carrying droplets, which can result in a loss of cellular water (Cox, 1989). Desiccation can stress or injure airborne microorganisms (Benbough, 1967; Cox and Baldwin, 1967; Stersky and Hedrick, 1972; Cox, 1989). However, when microorganism survive in a desiccation condition indoors, the microorganisms can be disseminated and transferred

to different locations by the airborne route (Goodlow and Leonard, 1961; Anderson *et al.*, 1968).

The rate of desiccation generally increases with increasing temperature; bacteria carrying droplets undergo desiccation regardless of environmental relative humidity (RH). However, high RH (>80%) or low RH (<20%) are both deleterious to bacterial survival (Webb, 1959). According to Benbough (1967), air stress at low humidities is attributed to the toxic effects of oxygen present, but at high RH values mechanisms lethal to airborne bacteria must be independent of the presence of oxygen. The toxic effects of oxygen may be due to free radical formation in the cell, because it has been found that metabolic inhibitors and free radical scavengers protect aerosolized bacteria (Benbough, 1967). There also may be irreversible changes in protein structure at high RH due to the weaker exchanges of bonded water with atmospheric water vapor (Benbough, 1967). More strongly held water would be involved at lower RH, so that the chances of lethal strong bond exchanges deaths occurring are greatly increased (Webb, 1959). Water loss leads to structural changes that result in membrane destabilization. The structures of proteins including enzymes, DNA, and RNA may also be affected (Marthi, 1994). Therefore, damage to surface structures can affect permeability and transport of substances into and out of a cell. Air stressed bacteria tend to lose K^+ , Na^+ , and other ions by leakage through damaged membranes (Benbough, 1967; Anderson *et al.*, 1968). Stersky and Hedrick (1972) reported that air stressed *S. newbrunswick* and *E. coli* are unable to grow on selective media containing bile salts such as sodium deoxycholate and sodium taurocholate. Water loss also causes the reduction of water

activity (a_w) and thus impair cellular functions. Cell damage of air stressed bacteria can be repaired in the presence of Mg^{2+} , Fe^{3+} , and Zn^{2+} (Hambleton, 1971). These ions help stabilize the cell envelope by providing strength and by preventing lysis (Hambleton, 1971).

Salmonella do not multiply in a poultry house with a_w below 0.84 (Hayes *et al.*, 2000). Hayes *et al.* (2000) noted that a low a_w environment (<0.84) functions as a physical barrier to the establishment or continuation of *Salmonella* contamination in a poultry house. In contrast, a high a_w environment may provide a high risk of continuing *Salmonella* contamination of poultry, provided the organism is introduced into the favorable environmental condition in a poultry house (Hayes *et al.*, 2000). Desiccation can reduce the number of *Salmonella* in poultry associated environments such as manure and litter (Riemann *et al.*, 1998). However, *Salmonella* spp. may be able to survive and adapt to more severe desiccation conditions. Juven *et al.* (1984) reported that the survival of *Salmonella* was greater at a_w of 0.43 than at 0.75. *Salmonella* spp. may survive for greater than 120 days in spray-dried milk and greater than 6 months in chalk (Beckers *et al.*, 1985; Hoffmans and Fung, 1993). When surviving in a desiccated state, *Salmonella* spp. could be transmitted or could infect new flocks via dust, feed, litter, feather, or dust particles.

Detection and Identification Methods

Bioaerosol Detection Methods

Efficient collection of microorganisms from the air is required to monitor

airborne microorganisms. An appropriate method for analysis of air samples is also necessary. The variety and complexity of bioaerosols complicate monitoring and exposure assessment research (Alvarez *et al.*, 1995).

Conventional techniques of direct culturing of litter and drag-swab sampling are mainly used to evaluate the contamination of poultry houses, and to detect *Salmonella* in poultry houses. *S. infantis*, *S. bredeney*, *S. havana*, *S. johannesburg*, *S. montevideo*, and *S. drypool* were isolated in the culture of 6-week-old floor litter at the poultry farm (Bhatia *et al.*, 1980). In broiler flocks, *S. typhimurium* and *S. havana* contamination was detected in 9 out of 13 sheds by drag swab and in 7 out of 13 sheds by litter culture (Kingston, 1981). Caldwell *et al.* (1994) found that *Salmonella* could be more detected by drag swab testing in occupied poultry houses than in the vacant poultry houses. The presence of chickens may play a major role in the amplification and dissemination of *Salmonella* to a greater area of the poultry house (Caldwell *et al.*, 1994). Additionally, feed contamination or early neonatal infection of chicks at the hatchery may also be important (Caldwell *et al.*, 1994). Hayes *et al.* (2000) compared drag swab and litter sampling methods for detection of *Salmonella* spp. in 86 commercial poultry houses. Drag-swab testing detected *Salmonella* spp more often (51.2%) than did litter culture (25.6%). The contamination with *Salmonella* spp. from the farms sampled was detected in 48 (55.8%) of 86 houses when combining the results of the two testing methods (Hayes *et al.*, 2000).

However, these conventional techniques have some difficulties in ease of sampling and sampling size due to the physical collection (Kingston, 1981; Caldwell *et*

al., 1994). When airborne microorganisms are conventionally detected by total count and culture techniques, the microorganisms that are not culturable under the specific growth conditions used in the laboratory remain undetected (Byrd *et al.*, 1991). The microorganisms may be exposed to the stress of aerosolization and sampling which may result in a loss of culturability (Walter *et al.*, 1990; Buttner and Stetzenbach, 1991). In addition, culture-based techniques can take several days to weeks to detect and identify a specific airborne microorganism. Therefore, polymerase chain reaction (PCR) amplification is used as an alternative, rapid, and accurate technique to detect and identify microorganisms in environmental samples. PCR amplification has specificity, sensitivity, and reduced processing time in aerobiological monitoring; this technique can effectively detect even small numbers of targeted microorganism (Alvarez *et al.*, 1995). The PCR method is very sensitive to interference by the complex organic load in either litter or drag-swab samples (Cohen *et al.*, 1994; Pillai *et al.*, 1994; Pillai and Ricke, 1995, Maciorowski *et al.*, 1998; Kwon *et al.*, 1999; Peña *et al.*, 1999). Therefore, airborne sampling, bioaerosol can be used as an alternative to solve the sample size labor and cost problems (Kwon *et al.*, 2000). Because this technique is an environmental sampling method, airborne sampling could provide representative monitoring of an entire poultry house. Cason *et al.* (1994) and Berrang *et al.* (1995) reported that *Salmonella* was transmitted from contaminated eggs to uncontaminated eggs during hatching and this transmission may be through fan-driven air. This airborne sampling technique shows less interference when combined to PCR amplification (Pillai *et al.*, 1996). Kwon *et al.* (1999) reported that when PCR was conducted by the spike samples of *S. typhimurium*,

PCR amplification of *Salmonella*-specific DNA was more readily detected in air filter samples than in litter samples.

Transposon Footprinting

Transposon footprinting is a novel and efficient genetic approach for identification of bacterial genes which are required for survival in diverse harsh conditions (Kwon *et al.*, 2002). This method could be applied to a stressful nongrowth environmental condition typically encountered by *Salmonella* spp. (Park *et al.*, 2002a). Kwon and Ricke (2000) devised an efficient PCR-based method for specific amplification of transposon-flanking sequences. This method requires the sequence information of only transposon-specific sequences. It consists of two simple steps of ligation and amplification and does not exhibit nonspecific background amplification. It can amplify multiple independent insertions either within a mutant or in a pool of multiple mutants. This method, termed transposon footprinting, simultaneously amplifies the transposon -flanking sequences in a complex pool of the transposon mutants. Because the length of the amplified DNA fragment is unique for each distinct transposon mutant, the polymerase chain reaction (PCR) products can be separated on an agarose gel to generate a transposon footprint with each band in the footprint representing the corresponding transposon mutant. The missing DNA band(s) in this footprints can easily be visualized on the agarose gel and the respective mutants identified.

Transposons are defined as DNA elements that can move or transpose from one place in the DNA to a different place with the action of transposase enzymes (Snyder

and Champness, 1997). Because they also have an insertion element at each end, transposons can readily move from place to place carrying their genes with them. The transposon itself usually encodes its own transposases, so that it carries with it the ability to move each time it moves. For this reason, transposons have been called “the jumping gene” (McClintock, 1950). In all transposition events, the transposase enzyme cuts the donor DNA at the ends of the transposon and then inserts the transposon into the target DNA. When transposons are experimentally inserted into the genome of bacteria, target gene(s) results in a loss of function and downstream gene(s), sequences that lie in the 5' direction on the coding strand of a DNA region, in an operon, a region on DNA encompassing genes that are transcribed from the same promoter, could be affected by the inserted transposon (Tsolis and Heffron, 1994). An altered phenotype mutant resulted from the constitutive expression, a gene expressed constantly, of downstream gene(s) can be isolated if the transposon has a constitutive promoter, a region on DNA to which RNA polymerase binds to initiate transcription (Lee *et al.*, 1992). For the identification of the sequence which flanks the transposon, gene(s) affected by the inserted transposon should be characterized when a transposon mutant with the altered phenotype of interest is isolated.

Alternative Methods to Induced Molting

Several Alternative Methods

Alternative methods of inducing a molt may ameliorate the situation based on animal welfare concerns and *S. enteritidis* infection caused by feed withdrawal.

Supplementing laying hens' rations with an ingredient that restricts feed intake may be a reliable alternative for reducing body weight and induced molting. A self-restricting diet is accomplished by adding drugs or chemicals to a regular diet, or by feeding a diet deficient in one or several essential nutrients, so that the laying hens would voluntarily restrict their feed intake even though they are provided free access to the diet (Lee *et al.*, 1971). Low-calcium (Nevalainen, 1969; Blair and Gilber, 1973; Gilbert and Blair, 1975; Hurwitz *et al.*, 1975; Campos and Baiao, 1979; Gilbert *et al.*, 1981; Mather *et al.*, 1982; Said and Sullivan, 1984), low-sodium (Hughes and Whitehead, 1974; Nesbeth *et al.*, 1974; Whitehead and Shannon 1974; Begin and Johnson, 1976; Dilworth and Day, 1976; Nesbeth *et al.*, 1976a, 1976b; Monsi and Enos, 1977; Whitehead and Sharp, 1976; Herbert and Cerniglia, 1978; Hughes and Whitehead, 1979; Ross and Herrick, 1981; Bird and Sunde, 1982; Ingram *et al.*, 1982; Mather *et al.*, 1982), and high- zinc diet (Scott and Creger, 1976; Herbert and Cerniglia, 1978; Robertson and Francis, 1979; Shippee *et al.*, 1979; McCormick and Cunningham, 1987) have been evaluated. A low-calcium diet with less than 0.2-0.3% Ca reduced the rate of egg production to less than 5% within 10 to 14 days and in some cases, there was complete cessation of egg laying within 21 days. A low-sodium diet less than 40 ppm Na reduced the rate of egg production to less than 5% within 14 to 21 days and in some cases resulted in a complete cessation of egg laying within 4 weeks. A high-zinc diet has received more attention in molting practice since the adjust addition of a trace component is much easier to execute in molting practice than to procedure a well balanced low calcium and sodium diet (Ruszler, 1998). A diet with 10,000 to 25,000 ppm Zn added as zinc oxide or zinc

acetate resulted in cessation of egg production within 5 to 7 days (Shippee *et al.*, 1979). Vermaut *et al.* (1997) recently reported that a 12% jojoba supplementation diet was effective as another alternative molting method. Jojoba meal, which is a by-product after the extraction of oil from jojoba seeds, contains approximately 30% crude protein, and supplementing feed with defatted jojoba meal inhibits feed intake in chickens (Ngou Ngoupayou *et al.*, 1982; Arnouts *et al.*, 1993; Vermaut, 1997). Rolon *et al.* (1993) found that a low energy, low density, and low calcium diet will cause an egg pause in laying hens when compared to feed withdrawal. Holt *et al.* (1994) also reported that laying hens on the molt diet shed less *S. enteritidis*, showed less susceptibility to an *S. enteritidis* infection and less intestinal inflammation as compared to fasted hens, although the percentage of *S. enteritidis* positive birds did not differ between molted groups. Recently, Seo *et al.* (2001) found that feeding wheat middings, by-product of wheat flour that is low in fiber but high in energy, resulted in cessation of egg production within 7 days, with no increased risk for *S. enteritidis*.

Zinc Rich Diets

In the U. S. commercial egg layer industry, feed withdrawal for a period of several days is commonly practiced to induce molt. This practice is easy to arrange and allows the commercial layer industry to save money without providing feed to hens (Brake, 1993). However, alternative methods have been investigated because public awareness of molt induction by the use of feed withdrawal has increased over the years (Anonymus, 1999; Ruzsler, 1998; Keshavarz and Quimby, 2002; Bar *et al.*, 2003) and the stress associated with feed withdrawal results in increased susceptibility to

Salmonella enteritidis infection (Holt and Porter, 1992a, 1992b; Holt, 1993; Holt *et al.*, 1994; Holt *et al.*, 1995). High concentrations of zinc added to a poultry layer ration have been evaluated as an alternative method to induce molt. Scott and Creger (1976), Creger and Scott (1977), and Roberson and Francis (1979) reported that zinc at 20,000 ppm added to the diet was effective to induce molt and generally gave results comparable, if not significantly better, than those obtained with feed removal. Scott and Creger (1976) and Creger and Scott (1977) reported that the 20,000 ppm (2%) of zinc as zinc oxide stopped a egg production completely within 5 days and resulted in significant improvements of production in the periods of postmolt as compared with that observed immediately premolt. Shippee *et al.* (1979) found that the addition of 10,000 ppm (1%) zinc as either zinc oxide or zinc acetate to the layer ration for 14 days caused egg production to decline from 60 to 0% in 6 days. Berry and Brake (1985) observed that hens fed high concentrations of zinc ceased ovulating a day sooner than hens molted by feed withdrawal. According to McCormick and Cunningham (1987), there were no differences in the reproductive systems between hens molted by feed withdrawal and hens molted by high dietary zinc. Also they indicated that the effectiveness of zinc at high concentrations might be due to a depression of feed intake. However, Breeding *et al.* (1992) reported that the moderate concentrations of zinc ($\leq 2,800$ ppm) were sufficiently by effective to be suppressive on hen reproduction systems.

Zinc

Zinc Biochemistry

Zinc is ubiquitous in all living organisms and acts both structurally and catalytically in metalloenzymes (O'Dell, 1992). Zinc metalloenzymes are recognized in all six enzyme types that are oxidoreductase (catalyzing oxidoreductions between two substrates), transferase (catalyzing transfer of a group other than hydrogen), hydrolase (catalyzing hydrolysis of esters, ether, peptide, glycosyl, acid anhydride, C-C, C-halide, or P-N bonds), lyase (catalyzing removal of groups from substrates by mechanisms other than hydrolysis, leaving double bonds), isomerase (catalyzing interconversion of optical, geometric, or positional isomers), and ligase (catalyzing the linking together of two components coupled to the breaking of a pyrophosphate bond in ATP or similar compound) (Vallee and Auld, 1990). These well-characterized zinc metalloenzymes are discussed below.

Carbonic anhydrase plays a role in the transport of carbon dioxide from tissues to lungs (Keilin and Mann, 1940). CO_2 and H_2O are bound simultaneously to the catalytic zinc atom in the carbonic anhydrase ($\text{H}_2\text{O} + \text{CO}_2 \rightarrow \text{H}^+ + \text{HCO}_3^-$) (Chlebowski and Coleman, 1976). Superoxide dismutase plays an important role in protecting cells and tissue from damage by superoxide radical ($2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$) (Bannister *et al.*, 1971; McCord *et al.*, 1971). Alcohol dehydrogenases catalyze the oxidation of ethanol, vitamin A, alcohol, and steroids using NAD as a cofactor, and the reduction of aldehydes and ketones in the presence of NADH ($\text{CH}_3\text{CH}_2\text{OH} + \text{NAD} \rightarrow \text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+$) (Vallee and Hoch, 1955; VonWartburg *et al.*, 1964). Each subunit of this enzyme

contains two zinc atoms and binds one molecule of NAD(H). One zinc atom is essential for the catalytic activity and the other atom is involved in stabilizing the polymeric structure (Drum *et al.*, 1967). The catalytic zinc atom is liganded in a tetrahedral geometry to two cysteinyl sulfurs, the imidazole group of histidine, and a water molecule (Riordan and Vallee, 1976). DNA and RNA polymerases are nucleotidyl transferases that catalyze the replication and transcription of the cellular genome. Liberman *et al.* (1963), and Fujioka and Liberman (1964) observed that DNA polymerase and the synthesis of DNA are inhibited by the presence of EDTA. Duncan and Hurley (1978), and Prasad (1982) also observed that DNA and RNA polymerase are decreased in cell cultures when zinc chelators are added. These observations indicate that inadequate zinc results in damaged cellular functions caused by decreased DNA or RNA synthesis. Carboxypeptidase A catalyzes the hydrolysis of aromatic amino acids such as phenylalanine, tyrosine or tryptophan, or the blanched aliphatic amino acids (Neurath, 1960). Carboxypeptidase B catalyzes hydrolysis of the basic amino acids, lysine, arginine and ornithine from the carboxyl-terminal peptide bonds in polypeptides (Folk, 1971).

Zinc Cellular Immunity

Inadequate intracellular concentrations of zinc cause abnormal development of T lymphocyte in humans (Dardenne and Bach, 1993) and cause lower weights of spleen and thymus mice (mice fed 5 ppm) when compared to control (mice fed with 100 ppm) (Beach *et al.*, 1982). Involution of the thymus and small spleen weights are characteristics of zinc deficiency, and appear to be primarily due to an absence of white

cells (Vruwink *et al.*, 1993). In addition, zinc deprived mice demonstrated significant growth retardation as reflected by lower body weight (Beach *et al.*, 1982). Inadequate intracellular concentration of zinc also causes damage to the lymphocyte function that is the ability of T and B cell proliferation (Cunninghna-Rundles and Cunnungham-Rundles, 1988). Prasad (1993) hypothesized that DNA synthesis may be dependent on zinc. According to Prasad (1993), the activity of deoxythymidine kinase as a zinc-dependent enzyme is greatly reduced when the concentrations of zinc are deficient. This reduction is accompanied by decreased protein and collagen synthesis in rats (Prasad, 1993). Impaired DNA synthesis results from zinc deficiency both in mammalian culture (Lieberman *et al.*, 1963) and in developing rat embryos (Duncan and Hurley, 1978). These results may suggest that inadequate zinc results in impaired cellular functions because of a decrease in DNA synthesis.

Dietary Zn may also influence growth and infectivity of bacterial pathogens in animals. Zn deficiency in animals is associated with increased infections with microorganisms and causes gram-negative sepsis in rats by increased bacterial populations present in liver, lungs, and kidneys (Srinivas *et al.*, 1989). Chickens have hypozincemia when infected with the Newcastle disease virus (Squibb *et al.*, 1971) or *E. coli* endotoxin (Butler *et al.*, 1973b). Zinc concentrations in the liver are increased by *E. coli* endotoxin infection (Klasing, 1984). Temporal and quantitative changes in zinc concentrations in immune tissues may be important in the response to infection because the host uses zinc as a cofactor for enzymes involved in defense against pathogens (Pekarek *et al.*, 1972; Klasing, 1984). Increased liver weight by infection could be due

to interleukin (IL-1) stimulation of acute phase reactant protein synthesis such as metallothionein and ceruloplasmin (Pekarek *et al.*, 1972; Klasing, 1984). Zn concentrations in serum and plasma are initially depressed when birds are infected with *Salmonella gallinarum* (Hill, 1989), *Escherichia coli* (Tufft *et al.*, 1988), or *E coli* endotoxin (Butler and Curtis, 1973). Sobocinski *et al.* (1977) reported that the effectiveness of zinc inhibition of bacterial growth results from changing the active transport system and impeding the initial phase of bacterial mating. They also propose that zinc treatments such as zinc chloride enhanced survival incidence in rats infected with *Francisella tularensis* or *S. pneumoniae*. This can be explained by zinc's affection to defense mechanism such as leukocytosis, phagocytosis, and cell mediated immunity as well as by zinc's direct inhibition of bacterial proliferation.

Bacterial Zinc Transport

Bacteria can keep zinc homeostasis because bacteria have specific energy dependent uptake and efflux transport systems for zinc. However, the mechanism of bacterial zinc transport has not been completely investigated. At low concentrations of zinc, ATP-binding cassette (ABC) transporters as binding proteins are usually induced from periplasmic membranes in gram-negative bacteria and allow zinc to enter the cytoplasm (Nies, 1999; Hantke, 2001). This uptake system uses ATP hydrolysis as the energy source (Nies, 1999; Hantke, 2001). Patzer and Hantke (1998) reported that the gene *znuA* (zinc uptake) encodes a periplasmic binding protein, *znuB* encodes an integral membrane protein, and *znuC* encodes the ATPase component of the transporter in *E. coli*. Specific transporters at medium concentrations of zinc have not been completely

identified but, pit (phosphate inorganic transport) may act as a co-transporter to help meet the zinc demands of the cell (Patzner and Hantke, 1998). Gram-positive bacteria, which do not possess an outer membrane, have many binding sites for divalent cations in the teichoic acids, the phosphates bound to the thick peptidoglycan layer. This uptake system may act cation exchangers on the surface of gram-positive bacteria (Hantke, 2001).

At high concentrations, zinc disrupts the homeostatic balance of the cells, thereby exerting toxicity (Nies, 1999; Hantke, 2001). Zinc efflux system plays a critical role in protection the cells from zinc poisoning. There are three different types of zinc efflux systems in bacteria: CDF (cation diffusion facilitators) type exporters; RND (resistance, nodulation, cell division) type exporters, and P-type ATP ases (Nies, 1999; Hantke, 2001). RND type exporters are found in *Ralstonia metallidurans* (Rensing *et al.*, 1997). The gene *czcA* (cobalt/zinc/cadmium) encodes cation/proton antiporter for Cd^{2+} , Co^{2+} and Zn^{2+} , and *czcB/C* encodes *czcB/C* couple *czcA* to the outer membrane to allow extrusion of Zn^{2+} in *R. metallidurans*. CDF type exporters are also found in *R. metallidurans* (Anton *et al.*, 1999) and *Staphylococcus aureus* (Xiong and Jayaswal, 1998). The gene *czcD* and *zntA* encode cation diffusion facilitator in *R. metallidurans* and *S. aureus*, respectively. P-type ATPases, named for the phosphorylated aspartate enzyme intermediate, have a main type of cation-transporting membrane protein found in eukaryotes and bacteria (Hantke, 2001). P-type ATPases specifically transport cations (Zn^{2+} , Cd^{2+} , and Pb^{2+}) into or out of a cell, intracellular compartment through the cellular membrane (Lutsenko and Kaplan, 1995; Gatti *et al.*, 2000). The common characteristics

include the utilization of energy derived from hydrolysis of the pyrophosphate bond of ATP for transport of cations across the cell membrane and energy state catalytic cycle involving a phosphorylated acyl intermediate (Lutsenko and Kaplan, 1995; Gatti *et al.*, 2000). ATP-dependent phosphorylation at a site on the cytoplasmic side of the protein is coupled with binding and occlusion of one or more intracellular cations (Lutsenko and Kaplan, 1995; Gatti *et al.*, 2000). This generates a high energy intermediate and induces conformational changes causing translocation of the cation across the membrane (Agranoff and Krishna, 1998). Guffanti *et al.* (2002) recently reported that the CzcD protein of *Bacillus subtilis*, a CDF protein, uses an antiporter mechanism, catalyzing active efflux of Zn^{2+} in exchange for K^+ and H^+ .

In bacteria, the capacity for K^+ uptake found in antiporters catalyses the efflux of toxic monovalent or divalent cation substrates from the cytoplasm (Guffanti *et al.*, 2002). This capacity, in CzcD protein may enhance its effectiveness in conferring resistance to the toxic metals (Guffanti *et al.*, 2002). Paulsen *et al.* (1997) and Gotoh *et al.* (1999) reported that RND proteins have two different subproteins: the MFP (membrane fusion proteins), which is maybe localized in the periplasmic space and the OMF (outer membrane factor) proteins, which facilitate passage of substrates into external medium. RND proteins are regarded as proton-motive force dependent transporters because no ATP-binding cassette has been identified in their sequences and because the reduced accumulation of substrates in the cells was restored by the addition of inhibitors of the cytoplasmic membrane proton gradient (Nies, 1995; Nikaido, 1996; Ocaktan *et al.*, 1997).

Intestinal Zinc Transport

Intestinal zinc absorption is a carrier mediated step of facilitated diffusion, that is not an energy dependent process, but this exact uptake mechanism have not been investigated (Song and Adham, 1979; Menard and Cousins, 1983). According to Davis (1980) and Smith and Cousins (1980) studies, there are two steps of zinc uptake as a function of the luminal zinc concentration in rats. A fast mucosal uptake step across the brush border membrane was followed by a slower step that may involve transport of zinc across the basolateral membrane. The fast step of uptake, with increasing luminal zinc concentrations, may represent saturability of binding sites on the brush border membrane. The membranes at high concentrations may become leaky and allow zinc to enter the cell and bind nonspecifically to cell proteins and other ligands. The chemical form of transported zinc is not unknown. Uptake at the membrane surface via an unidentified receptor on the brush border surface may require binding of free ions or prior binding to specific compounds.

Intestinal zinc absorption is affected by dietary levels of zinc, the presence of other minerals in the intestinal lumen, availability of zinc chelating agents in the diet, and the synthesis of zinc carrier molecule(s) in the mucosal cells of small intestine (Song, 1987). Zinc uptake by high molecular weight proteins in the intestinal mucosa is an active process requiring ATP (Menard and Cousins, 1983). A low molecular weight zinc-binding ligand (10^5 LMW-ZBL) as a zinc transport carrier in the diet may stimulate zinc absorption (Hurley *et al.*, 1979; Song and Adham, 1979) although the role of LMW-ZBL in stimulating zinc absorption is not well understood. A LMW-ZBL not

only increases the bioavailability of zinc but also participates in carrying zinc across the mucosal cells of small intestine (Hurley *et al.*, 1979). Chelation by LMW-ZBL is required for zinc to absorption through small intestine. Since free zinc ion forms an insoluble zinc hydroxide at neutral pH and the zinc transport rate is not dependent on the intraluminal zinc concentrations, zinc is transported across epithelial cells through a zinc carrier molecule (Song and Adham, 1979). Therefore, ZBL plays a key role in regulating the intestinal zinc transport mechanism. Vohra *et al.* (1965) reported that ethylenediaminetetraacetic acid (EDTA) has a high affinity for zinc and it has been demonstrated to enhance zinc absorption in animals (Forbes, 1961; Vohra and Kratzer, 1966; Sahagian *et al.*, 1967). Suso and Edwards (1968) also reported that EDTA chelates zinc and enhances zinc absorption in chicks. Other potential zinc transport carriers include citrate (Hurley *et al.*, 1979), picolinic acid (Evans and Johnson, 1980), histidine (Wapnir *et al.*, 1983) and a metallothionein (Cousins *et al.*, 1978).

Zinc in Poultry Nutrition

Zinc is a trace element that is necessary for normal growth, maintenance, bone development, feathering, enzyme structure and function, and appetite regulation for all avian species (Batal *et al.*, 2001). Zinc at 0.012 to 0.018% on a total weight basis is commonly added as a supplement to all formulated poultry diets (Lesson and Summers, 1997; Batal *et al.*, 2001). Currently there are two inorganic feed-grade zinc sources commercially used by the poultry feed industry (Wedekind and Baker, 1990; Batal *et al.*, 2001): zinc oxide (ZnO: 72% Zn) and zinc sulfate monohydrate (ZnSO₄·H₂O: 36% Zn). Of the supplemental zinc fed, 80 to 90% is ZnO, which is less bioavailable for poultry

than reagent-grade or feed-grade Zn sulfate (Wedekind and Baker, 1990; Sandoval *et al.*, 1997; Edwards and Baker, 2000). However, the sulfate (acid salt) is highly water soluble, allowing reactive metal ions to promote free radical formation, which can facilitate reactions that lead to the breakdown of vitamins and ultimately to the degradation of fats and oils, decreasing the nutrient value of the diet (Batal *et al.*, 2001). Oxide is less reactive, but also less bioavailable (Batal *et al.*, 2001).

Dietary zinc is relatively nontoxic to animals and humans; both exhibit considerable tolerance to high intakes of zinc (Fosmire, 1990). However, high levels of zinc in the diet result in reduced growth rates in chicks (Roberson and Schaible, 1960) and laying hens (Dewar *et al.*, 1983), high mortality in chicks (Blalock and Hill, 1988), and reduced feed intake and egg production in laying hens (Hermayer *et al.*, 1977). Zinc toxicity is responsive to supplemental copper, and both iron and zinc interfere with copper and iron metabolism (Cox and Harris, 1960; Rama and Blanas, 1981). Stahl *et al.* (1989) reported that 0.2% zinc fed to chicks caused reduced tissue iron and copper concentrations. Although zinc interferes with iron metabolism in chicks, iron-deficient chicks are more susceptible to the effects of zinc toxicity than are iron-adequate chicks (Blalock and Hill, 1988). This is because iron may induce the synthesis of metallothionein in the liver (McCormick, 1984). Metallothionein is a nonspecific metal-buffering ligand to sequester or displace zinc from normal sites (Richards and Cousins, 1975).

Zinc in Poultry Immunity

The effects of zinc supplementation on the poultry immune system and infectious

disease resistance have not been thoroughly studied although zinc nutrition is associated with the immune system and infectious disease resistance. However, Zinc-methionine, (Zn-Met) and cellular poultry immunity have been studied. Zn-Met is more bioavailable than Zn-Sul (sulfate) and Zn-O when fed to chicks in corn-soybean diets (Kidd *et al.*, 1996). The Zn-Met organic amino complex contains zinc sulfate (ZnSO_4) and DL-methionine (Met) with the chemical ratio of Met to Zn to SO_4 is 1:1:1 (Zinpro, 1993).

Dietary Zn-Met supplementation (80 mg/kg for old, 40 mg/kg for young broilers) in the broiler diet improves immunity in the progeny of old (Kidd *et al.*, 1992) and young broiler breeders (Kidd *et al.*, 1993). Kidd *et al.* (1992) and Kidd *et al.* (1993) note that the non-specific cellular immunity of the progeny can be enhanced due to Zn-Met supplementation. Kidd *et al.* (1992) also reported that antibody responses to *S. pullorum* were not different between Zn-Met and Zn-O supplementation. Zn-Met supplementation enhances *in vitro* macrophage phagocytosis of *S. enteritidis* in young turkeys (Kidd *et al.*, 1994). Dietary Zn-Met supplementation in the turkey diet increases activity of macrophage phagocytosis in young turkeys (Ferket and Qureshi, 1992). Dietary Zn-Met supplementation (40 or 80 mg/kg) in the layer diet improves survival of *E. coli* challenged in old laying hens (Flinchum *et al.*, 1989).

Mechanism of Dietary Zinc to Induced Molting

The mechanism of an induction molt by the use of dietary zinc is not completely understood, but it was studied by Scott and Creger (1976), Shippee *et al.*, (1979), Berry and Brake (1985), and McCormick and Cunningham (1987). Most studies reported that dietary zinc at high concentrations (10,000-20,000 ppm) induce follicular atresia and

ceasation of laying egg by interfering with ovulation in adult chicken because this cation (Zn^{2+}) reduces feed intake to 10-15% from the normal level. However, dietary zinc at moderate concentrations (2,800 ppm) in the absence of calcium supplementary diet has a direct suppressive effect on the reproductive organs because calcium is required for the initiation and stimulation of gonadotropin-releasing hormone stimulated luteinizing (LH) release (Luck and Scanes, 1980). Additionally, zinc-calcium antagonism exists. Dietary zinc at high concentrations can reduce calcium utilization and dietary calcium is the first limiting mineral for ovulation during the induced molt (Garlich and Parkhurst, 1982). Hens treated with high concentrations of dietary zinc have low plasma progesterone and the sensitivity of progesterone to LH is reduced as compared to fasted hens or hens treated with a low-calcium diet (Verheyen *et al.*, 1987). Calcium plays an important role in gonadotropin secretion and reproduction in avian ovarian cells (Simkiss, 1961; Taylor, 1965). Zinc causes calcium to fall below a critical level essential for gonadotropin production and release (Berry and Brake, 1985). Breeding *et al.* (1992) found that the effectiveness of zinc is not related to feed consumption and body weight difference, and zinc inhibits production of luteinizing hormone (LH)-stimulated progesterone and formation of cyclic adenosine monophosphate (cAMP) in granulosa cells of the hen's ovary. This inhibition is not caused by a toxic effect of zinc on granulosa cells of the hen ovary. The LH-stimulated progesterone in granulosa cells is dependent on the formation of cAMP and the mobilization of extracellular and intracellular calcium. Extracellular calcium is necessary for gonadotrophin-stimulated cAMP formation (Asem *et al.* 1987).

Hormones and Ovulation

A main function for lutenizing hormone (LH) is to induce ovulation. Premature ovulation is stimulated following injection of LH (Imai, 1973). Other functions of LH are associated with steroidogenesis and reduction of plasminogen activator activity (Shahabi *et al.*, 1975; Etches and Cunningham, 1976). LH peaks 4-6 hr before the hen ovulates (Johnson and van Tienhoven, 1980). Some studies reported that LH has an additional peak 14-11 hr prior to ovulation (Etches and Cheng, 1981) although the significance of this second peak was not determined. Shahabi *et al.* (1975) reported that mammalian LH *in vivo* increases plasma concentrations of progesterone, estrogen, and testosterone production of the hen's ovary. A main function for follicle-stimulating hormone (FSH) is closely related to granulosa cell differentiation and the induction of steroidogenesis in prehierarchal follicle granulosa cells (Etches, 1990). FSH stimulates both cAMP formation and progesterone secretion by the granulosa of intermediate stage follicles. A rise in the plasma concentrations of follicle-stimulating hormone (FSH) is determined 15 hr prior to ovulation in the domestic hen (Scanes *et al.*, 1977). Progesterone can induce both a preovulatory surge of LH and premature ovulation. The highest plasma concentrations of progesterone occurs 6-4 hr prior to ovulation and coincide with the preovulatory LH peak (Etches, 1990). Although the main function of androgens is unclear, high circulating concentrations of testosterone are necessary to stimulate LH secretion and induce ovulation. Moreover, ovulation can occur in the absence of any preovulatory increase in plasma testosterone (Johnson and van Tienhoven, 1984). Like testosterone, estrogens are not directly related to the

induction of LH secretion or ovulation (Etches, 1987). Moreover, ovulation can occur in the absence of a provulatory increase in plasma estrogens (Etches, 1987). However, estrogens are associated with reproduction, including the regulation of calcium metabolism for shell formation (Etches, 1987), induction of progesterone receptor in the ovary and reproductive parts (Pageaux *et al.*, 1983). Corticosterone controls the timing of the preovulatory LH surge (Wilson and Cunningham, 1980), but there is no ovulation-related increase in circulating corticosterone. In many avian species, circulating prolactin increases at the onset of egg laying. Rozenboim *et al.* (1993) reported that prolactin can reduce hypothalamic GnRH levels and inhibit LH secretion.

Circulating plasma concentrations of prolactin, growth hormone, LH, and ovarian steroids are lower in molting laying hens and in unmolting laying hens (Hoshino *et al.*, 1988). During the molting periods, plasma concentrations of prolactin are less than half those determined in laying turkey (Proudman and Opel, 1981). On the other hand, plasma concentrations of corticosterone, testosterone, and triiodothyroxine (T3), but not thyroxine (T4) increase during the molting. Thyroid hormones are elevated in blood plasma during the loss and regeneration of feathers (Brake *et al.*, 1979; Lien and Siopes, 1989).

Summary and Objectives of Research

Salmonella spp can be introduced to the laying hens and ultimately table eggs via many routes. These sources include feed, the layer house environments such as litter and water, horizontal infection from laying hen to laying hen via wild rodents, insects,

domestic animals, humans, and air currents and via vertical infection from a hen to her eggs. When *Salmonella* spp are spread and survive in any harsh environmental conditions such as desiccation of a laying house, it is imperative that detection and identification of the organism should be performed by rapid, efficient, and economical methods. A management strategy should also be developed that eliminates or reduces the risk of *Salmonella* contamination in a layer house, while inducing molt in laying hens. Zinc supplemented diets (0.012 to 0.018% zinc) have been used to provide zinc as a nutrient and higher concentrations (1 to 2.5% zinc) have been used to induce molt in laying hens. It is not known whether zinc addition would inhibit *Salmonella* growth in the bird's gastrointestinal tract or whether *Salmonella* survives in zinc amended feed during the storage. Zinc may prevent and/or interrupt the spread of *Salmonella* in the layer house environment by reducing the bioaerosol fraction of the environment, and colonization of *Salmonella* in bird's gastrointestinal tract.

Therefore, the objectives of the first phase of research were to compare the different bioaerosol detection methods for the collection of total airborne bacteria in a poultry layer house and to investigate a novel transposon footprinting method for rapid identification of a *Salmonella* gene required for survival during desiccation in a poultry layer house. The objectives of the second phase of research were to investigate the effects of zinc addition on the growth kinetics of *Salmonella in vitro* under both aerobic and anaerobic atmospheric conditions without or with acidic pH media, and to investigate the effect of zinc amended feed on the survival of a dry inoculum of *Salmonella*. The growth of *Salmonella in vitro* in zinc amended media and the survival

of *Salmonella* on zinc amended feeds were used to support the concept that zinc containing molt diets may be effective for limiting *in vivo Salmonella* colonization in the gastrointestinal tract of laying hens. Additionally, the use of zinc amended feeds may function as a potential barrier to interrupt the horizontal transmission of *Salmonella* through feces during a period of molting. The objectives of the third phase of research were to investigate the effects of zinc propionate, as an alternative salt form of zinc, on the induced molt of laying hens, and to compare zinc acetate and zinc propionate addition on gastrointestinal tract fermentation and susceptibility of laying hens to *Salmonella enteritidis* during molting.

CHAPTER III

**OPTIMIZATION AND COMPARISON OF BACTERIAL LOAD
AND SAMPLING TIME FOR BIOAEROSOL DETECTION
SYSTEMS IN A POULTRY LAYER HOUSE**

Overview

In order to develop an effective bacterial aerosol sampling system for commercial poultry housing, total bacterial populations were monitored using different air sampling systems in a poultry layer house. To find the optimum sampling time for the detection of airborne bacteria, axial fan Puralator filter (a home air conditioning filter), circulating fan filter, and all-glass impinger samples were compared over 2 days. Accumulated samples were collected continuously over 36 hours and non-accumulated samples were collected concurrently during four distinct time periods within this 36 hour collection period. The air sampling results also were compared to conventional drag-swab samples from the same poultry house environment. Total bacterial colony forming units recovered from impingers and circulating fan filters were typically less variable. The axial fan samples by comparison had higher variability. Overall aerosol bacterial counts were higher when collected by the impactors before adjusting for airflow rate. Higher yields of total bacterial colony forming units were recovered from impingers after being recalculated for airflow rate. The further development of aerosol sampling systems may allow for continuous monitoring of microbial populations in a poultry layer house.

Introduction

Today's large scale poultry production has led to increased bird densities within poultry houses (Sauter *et al.*, 1981; Charles *et al.*, 1994). In the UK, it was estimated that during the 1950's only about 8% of all flocks were in battery cages, in the 1990's the estimate was closer to 85% (Charles *et al.*, 1994). The large animal densities within confined spaces have increased the health problems related to aerosol pollutants (Sauter *et al.*, 1981; Clark *et al.*, 1983; Hartung, 1994a; Hartung, 1994b), which include gases and particulate matter. Non-viable particulates originate from feces, litter, feed, dust and dander; whereas viable particulates or bioaerosols include bacteria, fungi, and viruses. These pollutants become airborne through animal excretion, respiration and subsequent movement of birds in combination with high airflow (Jones *et al.*, 1984; Cox and Wathes, 1995; Wathes, 1995). The bioaerosols in the confined poultry growout house can cause respiratory problems because of infection as well as general respiratory stress due to constant contact with non-pathogenic bacteria, allergens and particulates (Hartung, 1994a; Hartung, 1994b; Wathes 1994). Due to the health effects of pollutants, the resulting stressed birds could lead to increased incidence of disease and subsequent losses in production (Donham, 1991; Hartung, 1994a; Hartung, 1994b).

Most of the work on bioaerosols has been centered on both animal and worker health from inhalation of airborne bacteria, viruses and subsequent concerns for determining exposure limits of airborne particulates (Jensen *et al.*, 1992; Wathes, 1995; Wathes, 1998). Within the poultry environment, bioaerosol contents have been identified (Madelin and Wathes, 1989; Hartung, 1994a; Hartung, 1994b; Wathes, 1998).

For the detection of pathogens within the poultry house, studies involving drag swab, litter collection, and sampling of boot covers have been conducted and standard protocols have been suggested (Kingston, 1981; Mallinson *et al.*, 1989; Caldwell *et al.*, 1994; Caldwell *et al.*, 1998). However, these methods are all limited by the difficulty of sampling and multiple numbers of independent samples required (Kinston, 1981; Caldwell *et al.*, 1994). These methods can also pose detection interference problems due to high organic loads when very sensitive detection methods i.e. PCR are employed (Kwon *et al.*, 1999). Bioaerosol collection represents another method in which to monitor bacterial populations and more importantly pathogens in the poultry environment. *E. coli* and *Salmonella* have been detected in the airborne environment and dust around naturally and experimentally infected birds (Harry, 1964; Carlson and Whenham, 1968; Arp *et al.*, 1978; Sauter *et al.*, 1981; Baskerville *et al.*, 1992; Holt *et al.*, 1998). Even when the numbers of *Salmonella* and pathogenic *E. coli* are not high enough to cause infection via aerosol transmission, their presence in aerosols could create more favorable microenvironments that favors infection (Harry, 1964; Wathes, 1995). Thus aerosol sampling could be a potential diagnostic tool for the poultry house as a whole.

Several different bacterial aerosol collection devices have been developed. These collection devices fall into two basic categories: impinger and impactor. Impingement is collection by drawing a vacuum across a liquid surface such that the aerosol is collected into the liquid media. Impaction can be further divided by the type of surface in which the aerosol impacts; solid media or filters. The aerosol is drawn

across a medium or through a filter using fans or vacuum (Jensen *et al.*, 1994).

Although, each device or method has its own advantages and limitations for use, each aerosol sampling environment must be evaluated on a case by case basis to determine the optimal collection device for that environment (Cox and Wathes, 1995). Most commercial devices tend to be expensive and present logistical problems for routine use in a poultry house. In addition, different devices have the potential of collecting different bacterial populations based on collection efficiencies as well as the bacteria's ability to withstand desiccation (Hartung, 1994a; Hartung, 1994b; Jensen *et al.*, 1994).

If bacterial monitoring of poultry houses by the aerosol route is to become feasible and routine, it is assumed that the collection device must be relatively portable, and inexpensive. In order to achieve this eventual goal, we compared different air sampling systems to conventional air sampling systems for the collection of total airborne bacteria in a poultry house and determined the optimal sampling time in the poultry house for these devices.

Materials and Methods

Experimental Design

Environmental sampling was performed at the Texas A&M University Poultry Research Center, poultry layer house, which had approximately 600 hens of varying breeds and age. House dimensions were 150 x 40 ft in size, with all side windows open and a system fan inside the house.

Time Frame Analysis of Airborne Samplers

Long term accumulation of aerosols on filter samples: Axial fan Puralator 2000 filter samples [1] were accumulated over a 36 hour total time frame with 2 samples periodically removed 6 times during the 36 hour time period. Microbial counts [2] recovered from the two samples were averaged for each time frame. Airborne microorganisms were collected along with the Axial fan samples using an air impinger [3] concurrently during the 6 sampling times with the experiment repeated in its entirety. Reported values were calculated for all collection time counts except 0 hour where airflow rate was considered to be 0. These trials were performed during the month of July, 1999.

Short term accumulation on filter samples with direct comparison to impinger:

Sampling of airborne microorganisms at select time intervals was performed concurrently four times a day over two time trials with axial fan puralator filter sampling and air impinger sampling methods. The first trial and the second trial were performed in October, 1998 and July, 1999 respectively.

Compasrison of Airborne Sampers

For comparison of the four different methods [1, 3, 4, 5] samples were collected concurrently three times a day over two day periods in April, 1999. Drag swab samples were also taken [6]. Data analysis is described [7].

Results and Discussion

Long Term Accumulation on Filter Samples

To find the optimal time for sampling on an axial fan puralator filter (AFPF) with particular consideration to our poultry facilities, aerosol accumulation onto filters continued for 36 hours with samples removed intermittently. Figure 3-1 and Figure 3-2 represent two days of sampling, combining data from fan filters accumulation and superimposed with impingers at each time point. Total aerobic bacterial counts were sampled from the filters at each time point. For microbial populations recovered from an AFPF, the total bacterial counts on the filters did not continue to increase throughout the full 36 hrs. On the first repetition of sampling, the highest bacterial populations recovered occurred at 24 hours ($4.52 \log_{10}$ CFU/m³ or 3.22×10^7 CFU/20 ml) with considerable fluctuation during the collection period. On the second repetition of sampling, the highest bacterial populations recovered occurred at 16 hours ($4.09 \log_{10}$ CFU/m³ or 2.55×10^7 CFU/20 ml). Maximum load or potential clogging of the filters appeared to have occurred within 2 hours. Maximum saturation for recovery of viable counts most likely was maintained during the longer time frame due to bacterial dehydration and bacterial accumulation being in a continual state of flux.

At each time of sampling using the AFPF or air impinger (AI) the aerosol samples could be collected for only 20 minutes, as opposed to collection from several hours to 36 hours on the AFPF. Impinger sampling could be conducted for a period of only 20 minutes before liquid evaporation in the impinger became a factor. This limited

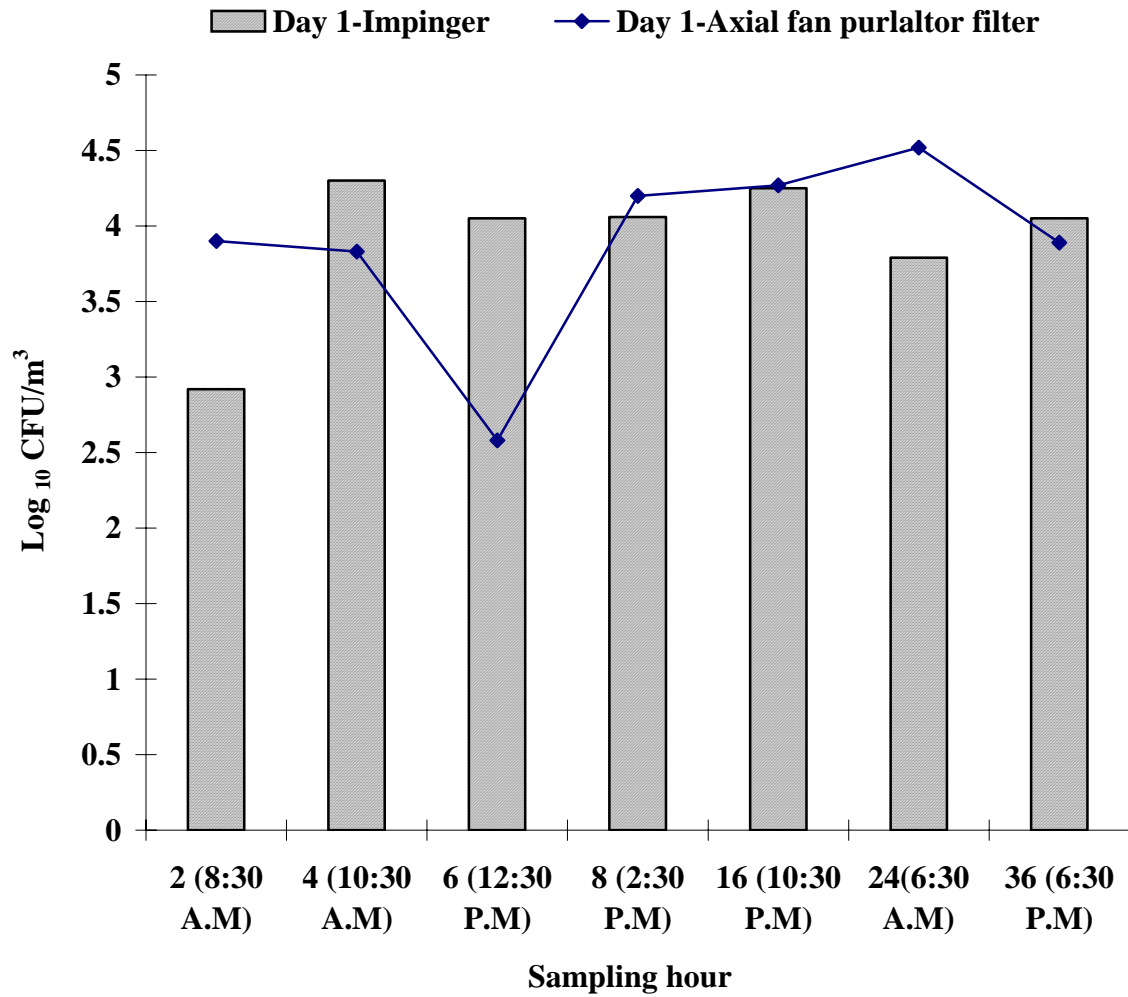


FIGURE 3-1. Day 1 axial fan accumulation with impinger at each time point

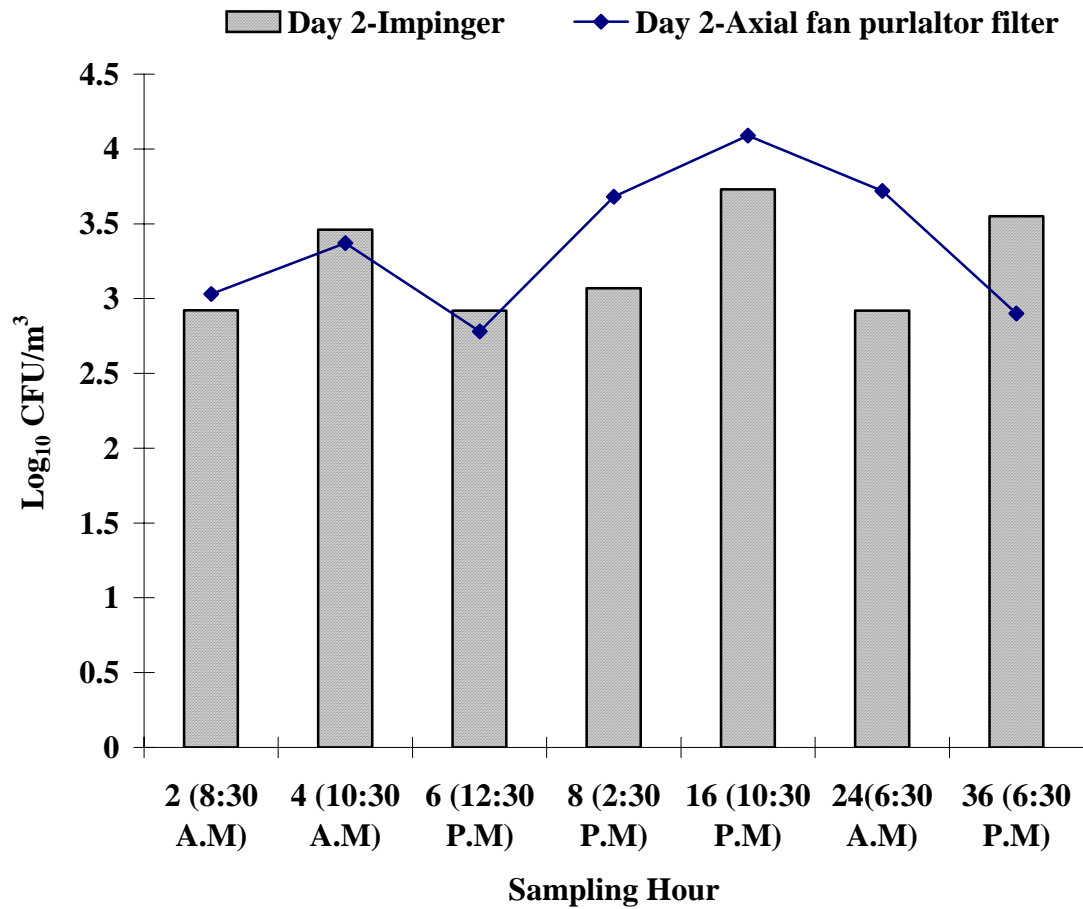


FIGURE 3-2. Day 2 axial fan accumulation with impinger at each time point

time span for impinger aerosol collection is consistent with previous studies where Madelin and Wathes (1989) utilized a 10 min. impingement time and Terziera *et al.* (1996) a 15 min. time period. On the first repetition of sampling, the highest bacterial populations recovered occurred at 4 hours ($4.30 \log_{10}$ CFU/m³ or 4.90×10^3 CFU/20 ml) and 16 hours ($4.25 \log_{10}$ CFU/m³ or 4.40×10^3 CFU/20 ml). On the second repetition of sampling, the highest bacterial populations recovered occurred at 16 hours ($3.73 \log_{10}$ CFU/m³ or 1.30×10^3 CFU/20 ml). The highest total bacterial plate counts occurred at 10:30 am and 10:30 pm. The higher bacterial populations detected in aerosols during the morning hours could be due to increased bird activity during feeding, and egg collection generating more aerosols, but would not explain the 10:30 pm activity unless the hens were overly active in response to the presence of personnel and samplers during off hours [8].

Short Term Accumulation on Filter Samples with Direct Comparison to Impinger

The second experiment was designed to more closely compare filter samples to the impinger samples. Based on the previous experiment in which an extended period of time did not increase total viable bacterial yield, a sampling time of 30 minutes was chosen. Total aerobic bacterial counts are shown in Table 3-1. Figure 3-3 represents the comparison between impinger samples and filter (AFPF) samples at each time and day. Any pair with a set of impinger or filter samples not represented is excluded from the graph. The linear regression between AFPF and Impinger samplers with a 90% mean prediction interval of the paired data shows an R^2 of 0.13. The filter and the impinger time did not increase total viable bacterial yield, a sampling time of 30 minutes was

TABLE 3-1. Direct comparison of impinger and axial fan filters

Day	Time	Sampler	CFU/TAFR ¹	Log CFU/m ³
1	1	Filter	2.68	3.43
1	1	Impinger	0.83	2.92
1	2	Filter	6.78	3.82
1	2	Impinger	5.42	3.73
1	3	Filter	23.42	4.33
1	3	Impinger	14.17	4.15
1	4	Filter	19.73	4.30
1	4	Impinger	5.83	3.77
2	1	Filter	5.93	3.77
2	1	Impinger	0.83	2.92
2	2	Filter	0.35	2.54
2	2	Impinger	4.58	3.66
2	3	Filter	Below detection ²	N/A
2	3	Impinger	2.08	3.32
2	4	Filter	0.32	2.50
2	4	Impinger	2.08	3.32

¹TAFR is Total Air Flow Rate

²Detection Limit= 200 CFU/20211(total air flow rate) = 0.009

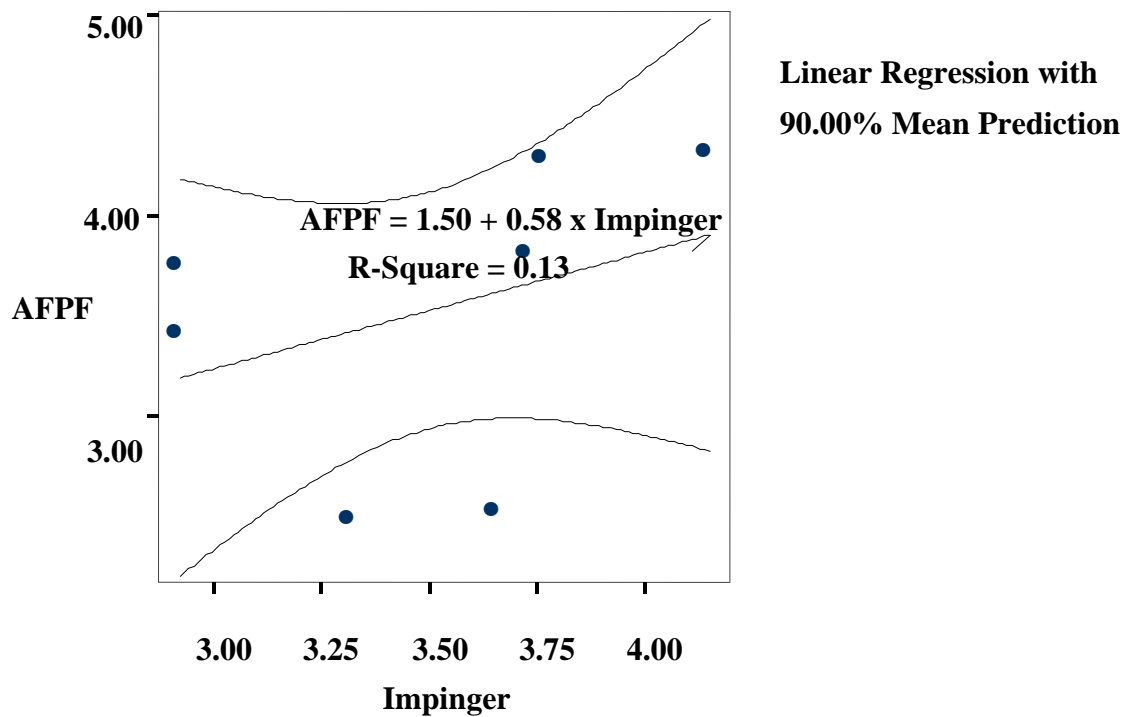


FIGURE 3-3. Filter and impinger, direct comparison. Axial Fan Puralator Filter (AFPF) and Impinger Sampler are compared by their bacterial concentration Log_{10} CFU/m^3 . The straight line represents the linear regression and the curved lines are the 90% confidence limit

chosen. Total aerobic bacterial counts are shown in Table 3-1. Figure 3-3 represents the comparison between impinger samples and filter (AFPF) samples at each time and day. Any pair with a set of impinger or filter samples not represented is excluded from the graph. The linear regression between AFPF and Impinger samplers with a 90% mean prediction interval of the paired data shows an R^2 of 0.13. The filter and the impinger samples do not show good correlation (Pearson's correlation coefficient of 0.34, significance = 0.44), and therefore, may not be recovering similar bacterial counts from the poultry environment.

Comparison of Aerosol Samplers

In an attempt to find a suitable sampler, we compared impinger aerosol sampling with bacterial counts from drag swabs of the poultry house floor along with two different filter types on a household box fan. We also used glass filters with a circulating fan that was capable of generating a more even and measurable airflow across a filter. Table 3-2 represents the data from each sampling type at each time and day. All axial fan filters (puralator and glass) are averages of three filter samples removed from the fans at each time and day, and all impinger, circulating fan and drag swabs values are single samples. On the first day relative humidity and temperature during sampling was 79 °F, and 78% RH, the second day was 74 °F, and 89% RH, during April, 1999. Dehydration and temperature increase will lead to increased bacterial death in aerosols and high humidity will lead to an increase in bacterial sedimentation rates and therefore less airborne bacteria to collect (Stark *et al.*, 1998; Yongyi and Lighthart, 1998).

The linear regression graphs for pairs of air samplers are shown in Figures 3-4 to

TABLE 3-2. Comparison of four different samplers

Day	Sample Time	Treatment	CFU/TAFR	Log CFU/m ³
1	1	Glass Filter	66.40*	4.46
1	1	Puralator Filter	112.42*	5.02
1	1	Impinger	N/A	N/A
1	1	Circulating Fan	64.47	4.81
1	2	Glass Filter	13.43*	3.81
1	2	Puralator Filter	18.81*	4.26
1	2	Impinger	17.50	4.24
1	2	Circulating Fan	18.73	4.27
1	3	Glass Filter	3.21*	3.41
1	3	Puralator Filter	50.14*	4.66
1	3	Impinger	10.42	4.02
1	3	Circulating Fan	16.07	4.21
2	1	Glass Filter	0.91*	2.87
2	1	Puralator Filter	0.29*	2.45
2	1	Impinger	10.42	4.02
2	1	Circulating Fan	8.65	3.94
2	2	Glass Filter	9.34*	3.53
2	2	Puralator Filter	0.43*	2.62
2	2	Impinger	28.75	4.46
2	2	Circulating Fan	6.40	3.81
2	3	Glass Filter	9.71*	3.36
2	3	Puralator Filter	1.67*	3.20
2	3	Impinger	7.03	3.85
2	3	Circulating Fan	2.71	3.43

*Represents an average of three filters taken from the axial fans at the same time

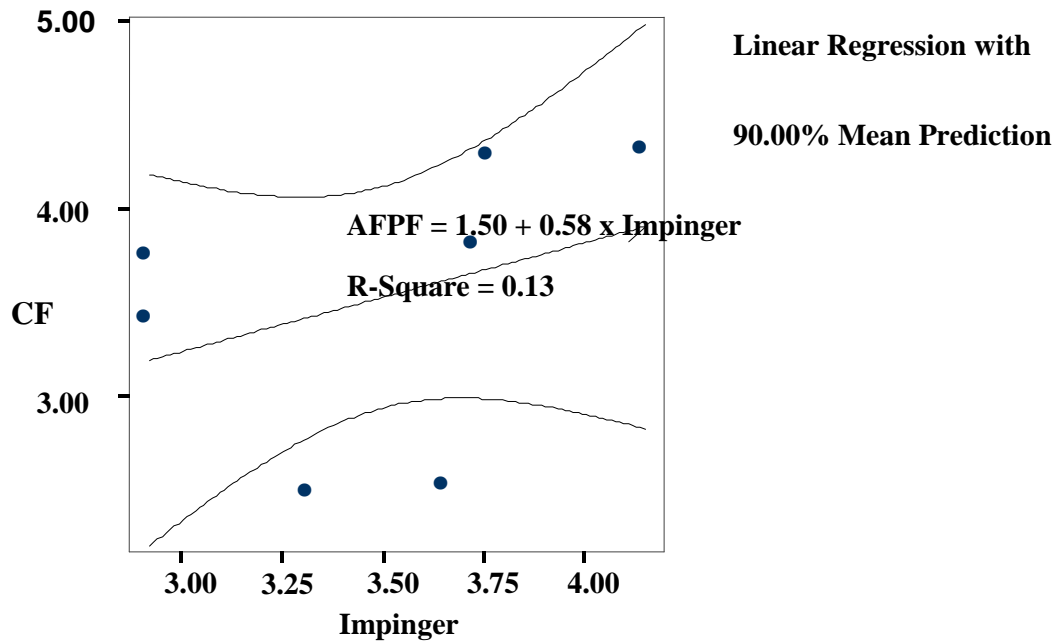


FIGURE 3-4. Comparison of axial fan glass filter and circulating fan. Axial Fan Puralator Filter (AFPF) and Circulating Fan (CF) are compared by their bacterial concentration $\text{Log}_{10} \text{CFU}/\text{m}^3$. The straight line represents the linear regression and the curved lines are the 90% confidence limit

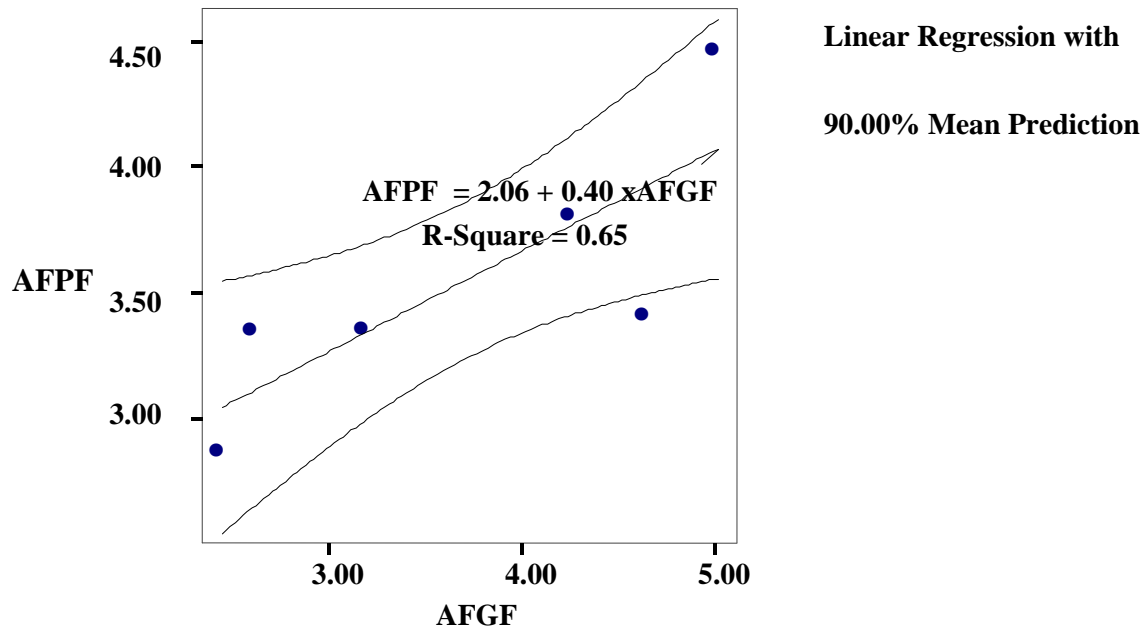


FIGURE 3-5. Comparison of axial fan puralator and glass filters. Axial Fan Puralator Filter (AFPF) and Axial Fan Glass Filter (AFGF) are compared by their bacterial concentration $\text{Log}_{10} \text{CFU}/\text{m}^3$. The straight line represents the linear regression and the curved lines are the 90% confidence limit

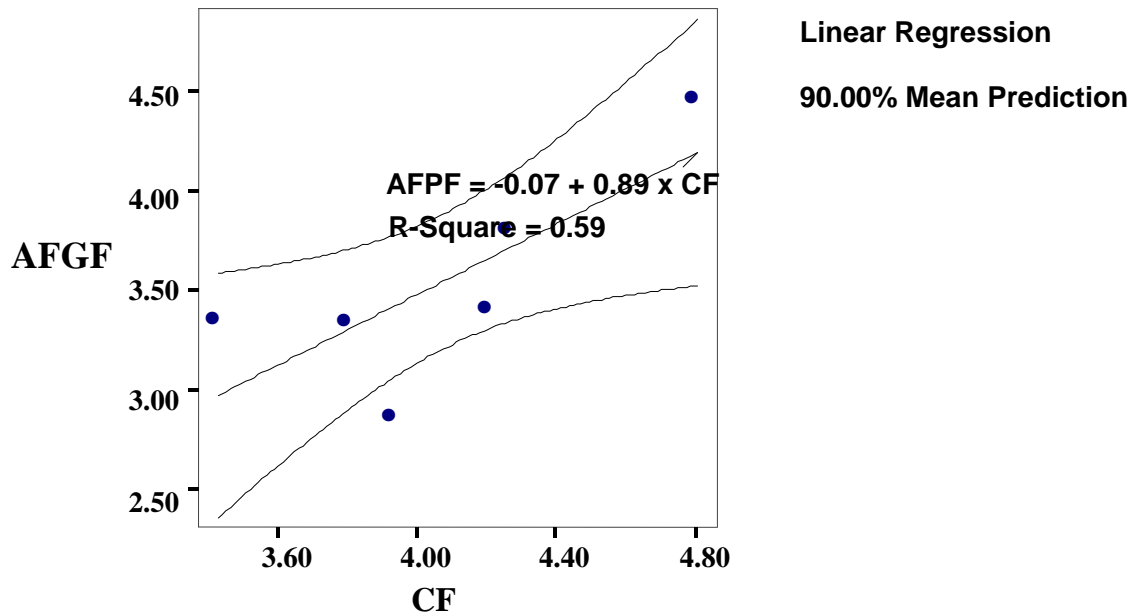


FIGURE 3-6. Comparison of axial fan puralator filter and circulating fan. Circulating Fan (CF) and Axial Fan Glass Filter (AFGF) are compared by their bacterial concentration $\text{Log}_{10} \text{CFU}/\text{m}^3$. The straight line represents the linear regression and the curved lines are the 90% confidence limit

TABLE 3-3. Comparison of four samplers

		Correlations			
		AFPF	AFGF	IMPINGER	CF
AFPF	Pearson Correlation	1.000	0.806	0.300	0.766
	Sig. (2 tailed)	.	0.053	0.623	0.076
	N	6	6	5	6
AFGF	Pearson Correlation	0.806	1.000	-0.148	0.776
	Sig. (2 tailed)	0.053	.	0.813	0.070
	N	6	6	5	6
IMPINGER	Pearson Correlation	0.300	-0.148	1.000	0.332
	Sig. (2 tailed)	0.623	0.813	.	0.586
	N	5	5	5	5
CF	Pearson Correlation	0.766	0.776	0.332	1.000
	Sig. (2 tailed)	0.076	0.070	0.586	.
	N	6	6	5	6

3-6. The R^2 of axial fan glass filter and circulating fan was 0.60, circulating and axial fan puralator filter was 0.65, and axial fan puralator filter and circulating fan was 0.59. All other comparisons which included the impinger exhibited very low R^2 values. The correlations between each sampler are shown in Table 3-3. Only the fans with puralator or glass fiber filters showed somewhat related ($P < 0.1$) correlations. This is not surprising since the samplers with the greatest correlation utilize the same type of fan apparatus with the only difference being the type of filter used for collection. Therefore, one would expect to achieve similar results for total bacteria counts.

Each sampler device has its advantages and disadvantages. The impinger can only be used for short time frames due to liquid evaporation. This short time frame may necessitate determining peak time periods for sampling to maximize effects during peak animal activity, weather conditions, and other environmental conditions which generate high levels of aerosols (Wathes, 1995). The liquid matrix in the impinger does allow for collection of a greater number of viable bacteria during collection by avoiding dehydration stress but less total bacteria overall are recovered because of the limited time period for collection (Table 3-4). When attempting to collect bacterial counts in extremely low numbers in an aerosol with a high bacterial population background i.e. detection of pathogens, using nonviable detection methods such as polymerase chain reaction, immunoassays or fluorescent antibody techniques may be required to increase the probability of detecting specific bacteria and avoiding a potential false negative result. Davies *et al.* (1997) demonstrated that serum positive birds appeared long before salmonellae could be detected by enrichments.

TABLE 3-4. Enumeration of total viable counts from each sampling before and after adjusting calibrated airflow rate

	AFPF	AFGF	CF	Impinger	Drag awab
CFU/20ml ¹	1.83 x 10 ⁴ – 1.34 x 10 ⁶	5.80 x 10 ³ – 2.27 x 10 ⁶	5.30 x 10 ⁴ – 1.26 x 10 ⁶	1.70 x 10 ³ – 6.90 x 10 ³	9.5 x 10 ⁵ – 7.3 x 10 ⁷
AFL (Lit/min) ²	673.7	673.7	1303	12	
ST (Minute) ³	30	30	15	20	
TAFR (Liter) ⁴	20211	20211	19545	240	
CFU/TAFR (Liter/min)	0.905- 66.30	0.287- 112.32	2.71- 64.47	7.03- 28.75	
CFU/TAFR (m ³) ⁵	905- 66300	287- 112320	2710- 64470	7030- 28750	
Log ₁₀ (CFU/m ³)	2.96-4.82	2.46-5.05	3.43-4.81	3.85-4.46	

¹CFU/(Colony Forming Units)/20ml= counts (in 1 ml) x 20

²AFL (Air Flow Rate)

³ST (Sampling Time)

⁴TAFR (Total Air Flow Rate)

⁵CFU/TAFR (m³)=CFU/TAFR (L) x 1000

Impactors have the potential to collect more bacteria per unit time but development of dehydration problems may decrease the survival of bacteria collected. Collection efficiency of aerosols is also influenced by the source of the aerosol, either from a liquid or fecal source or from an already dehydrated source (Byrd *et al.*, 1997). Impactors do have the advantage of remaining in place for longer periods of time and thus could bypass problems related to sample timing in weather condition changes and animal and/or management activity differences. Time restraints are more dependent on the eventual clogging the filters by excess particulate accumulation. Donham *et al.* (1991) successfully used a 3 to 4 hr. sampling time on filters and despite their drawbacks, filtration sampling remains popular and less expensive (Thorne *et al.*, 1992; Watehes, 1995).

Enumeration of total viable counts from each sampling before and after adjustment for a calibrated airflow rate are listed in Table 3-4. Bacterial counts were highest in the drag swab method and least in the impinger before adjusting for airflow. The samplers and the drag swab bacterial counts could not be directly compared because the units of measurement were considered different. To better understand the possibility of samplers acquiring more representative data with fewer replications needed, the standard deviation of the average of total bacterial counts was used to compare the samplers (Table 3-5). If the standard deviation was high, it would indicate that the sampler was less consistent in bacterial counts than a sampler with a lower standard deviation. With this comparison, the axial fan samplers tended to yield total bacterial counts with the greatest deviation from their mean values with the impinger and the

TABLE 3-5. Standard deviations of samplers

Descriptive Statistics					
	N	Minimum	Maximum	Mean	Std. Deviation
AFPF	6	2.87	4.47	3.5450	0.5426
AFGF	6	2.45	5.02	3.7033	1.0925
IMPINGER	5	3.85	4.46	4.1180	0.2361
CF	6	3.43	4.81	4.0783	0,4692
DRAG SWAB	6	5.98	7.87	7.0650	0.6393
VALID N(LISTWISE)	5				

circulating fan sampler tended to deviate the least. The differences in variation among

the samplers is not surprising since the airflow across the impinger and the circulating fan sampler is designed to maintain very consistent air intake through the given sample area. The household box fans, because of the fan assembly and motor tend to generate an uneven airflow across the filters. However, the axial household box fan should be sufficient for routine measurement of fluctuating airborne bacterial populations and represents an economical apparatus for continuous collection and monitoring.

Conclusions

Household box fans (axial fans) with irregular airflow across filters can be used for detection of bacteria but detection based on this approach can not be used to accurately correlate with airflow in the poultry house and so therefore should only be used as a positive/negative detection and not for quantitation. Dehydration and bacterial death associated with the use of filters as a collection system might interfere with detection of bacteria if using plating methods, but could most likely be used with indirect and more sensitive molecular and immunological detection methods. The airflow collection devices that utilize equipment for exact measurements of airflow to correlate with bacterial collection may be required for quantitation and regulatory reasons but the less expensive axial fan may provide an in house means of routine monitoring of bacteria and other aerosolized particles.

CHAPTER IV

**APPLICATION OF A TRANSPOSON FOOTPRINTING
TECHNIQUE FOR RAPID IDENTIFICATION OF *SALMONELLA*
TYPHIMURIUM TN5 MUTANTS REQUIRED FOR SURVIVAL
UNDER DESICCATION STRESS CONDITIONS***

Overview

Salmonella spp. are one of the foodborn pathogens that can be isolated in the environments of poultry houses and desiccation is a potential stress condition that can influence the survival of *Salmonella* spp. in this environment. In order to investigate the desiccation survival mechanism of *Salmonella* spp. the genome of *S. typhimurium* ATCC 14028 was screened for the genes potentially required for survival during desiccation using a novel method based on Tn5 mutagenesis previously developed in our laboratory. This method, termed transposon footprinting, simultaneously amplifies the Tn5-flanking sequences in a complex pool of the Tn5 mutants. As the length of the amplified DNA fragment should be unique for each distinct Tn5 mutant, the polymerase chain reaction (PCR) products separated on an agarose gel generate transposon footprints with each band in the footprint representing the corresponding Tn5 mutant. By comparing the transposon footprints from the

* Reprinted with permission from: Application of a transposon footprinting technique for rapid identification of *Salmonella typhimurium* Tn5 mutants required for survival under desiccation stress conditions, by Park *et al.*, 2002, J. Rapid Meth. Autom. Microbiol. Vol. 10, pp. 197-206. Copyright 2002 by Food & Nutrition Press, Inc.

pools of *S. typhimurium* Tn5 mutants before and after exposure to desiccation, Tn5 mutants that were not recovered after the selection were rapidly identified that would be easily isolated for further genetic analysis.

Introduction

Poultry is regarded as the most primary source of food associated outbreaks of human salmonellosis. Because the transmission of *Salmonella* spp. has been suggested to be cyclic between human and poultry hosts and the environment (Williams, 1981). Poultry contaminated with *Salmonella* spp. may occur at virtually all phases of poultry production, and the control of infection at early stages of production is not only important (Pillai *et al.*, 1994) and but the most cost effective strategy. The primary sources of *Salmonella* contamination include contaminated feed, litter and water, vertical transmission from parent to progeny through the hatchery, and horizontal transmission via wild rodents, insects, domestic animals and humans (Mead, 1993) and *Salmonella* spp. can survive for as long as 16 months in poultry feed (Williams and Benson, 1978).

Salmonella do not multiply in a poultry house with an Aw below 0.84 (Hayes *et al.*, 2000). Desiccation can reduce the number of *Salmonella* in poultry associated environments such as manure and litter (Riemann *et al.*, 1998). However, *Salmonella* spp. may be able to survive and adapt to more severe desiccation conditions. Juven *et al.* (1984) reported that the survival of *Salmonella* was greater at an Aw of 0.43 than at 0.75. *Salmonella* spp. may survive for greater than 120 days in spray-dried milk and greater than 6 months in chalk (Beckers *et al.*, 1985; Hoffmans and Fung, 1993). When

surviving in a desiccated state, *Salmonella* spp. could be transmitted or could infect new flocks via dust, feed, litter, feather, or dust particles.

Kwon and Ricke (2000) devised an efficient PCR-based method for specific amplification of transposon-flanking sequences. This method requires the sequence information of only transposon-specific sequences, consists of two simple steps of ligation and amplification and does not exhibit nonspecific background amplification. It can amplify multiple independent insertions present either within a mutant or in a pool of multiple mutants. This method, termed transposon footprinting, simultaneously amplifies the Tn5-flanking sequences in a complex pool of the Tn5 mutants. As the length of the amplified DNA fragment should be unique for each distinct Tn5 mutant, the polymerase chain reaction (PCR) products can be separated on an agarose gel to generate a transposon footprint with each band in the footprint representing the corresponding Tn5 mutant. Our overall goal in this study was to determine whether this rapid footprinting method could be applied to a stressful non-growth environmental condition typically encountered by *Salmonella* spp.

Materials and Methods

Generation of *S. typhimurium* Mutant by Mini-Tn5 Mutagenesis

Escherichia coli SM 10 λ *pir* transformed with the suicide plasmid pUT/km was used to generate mini-Tn5 mutants of *S. typhimurium* ATCC 14028 that had been selected for resistance to nalidixic acid as described by Herrero *et al.* (1990). The donor cells, *E. coli* SM 10 λ *pir* carrying the plasmid pUT with kanamycin resistance as a

selectable marker were mixed with recipient cells, *S. typhimurium* with a nalidixic acid resistance mutation in the chromosome. After incubation, the cells were plated on Luria-Bertani (LB; Difco, Laboratories, Detroit, MI) medium containing kanamycin (Km-75 $\mu\text{g}/\text{mL}$) and nalidixic acid (NA-25 $\mu\text{g}/\text{mL}$). The resulting colonies on the plate were considered the recipient cells with transposon inserted into chromosome. The total number of *S. typhimurium* ATCC 14028 Tn5 mutants generated in this study consisted of 8 pools containing 96 mutants (12 mutants/pool).

Pools of Tn5 Mutants Exposed to Desiccation Condition

The steps for desiccation and recovery of Tn5 mutant pools are illustrated in Figure 4-1. Briefly, a 100 μL sample from each pool were aseptically spotted into sterile petri dishes and the petri dishes were to allow to dry in a vacuum container for 10 min to remove residual moisture. The petri dishes were stored in a 37 C incubator and were removed for recovering cells on day 0, 2, and 4 of storage.

Preparation of Cell Recovery

The culture (referred to as input pool, 100 μL) on day 0 of storage was transferred into a sterile 1.5 mL microcentrifuge tube and the tube was centrifuged (Labnet, Woodbridge, NJ) for 3 min with 1,300 rpm. The desiccated mutant cells (100 μL) on day 2, and 4 of storage was resuspended in 1 mL phosphate buffered saline (PBS, pH 7.2) and shaken on a orbit shaker (Lab-Line Instruments, Inc., Melrose Park, IL) for 10 min at 2 rpm. The resuspension of mutant cells was serially diluted and inoculated in LB (Km-75 $\mu\text{g}/\text{mL}$, NA-25 $\mu\text{g}/\text{mL}$) plates. After overnight incubation at 37 C, the

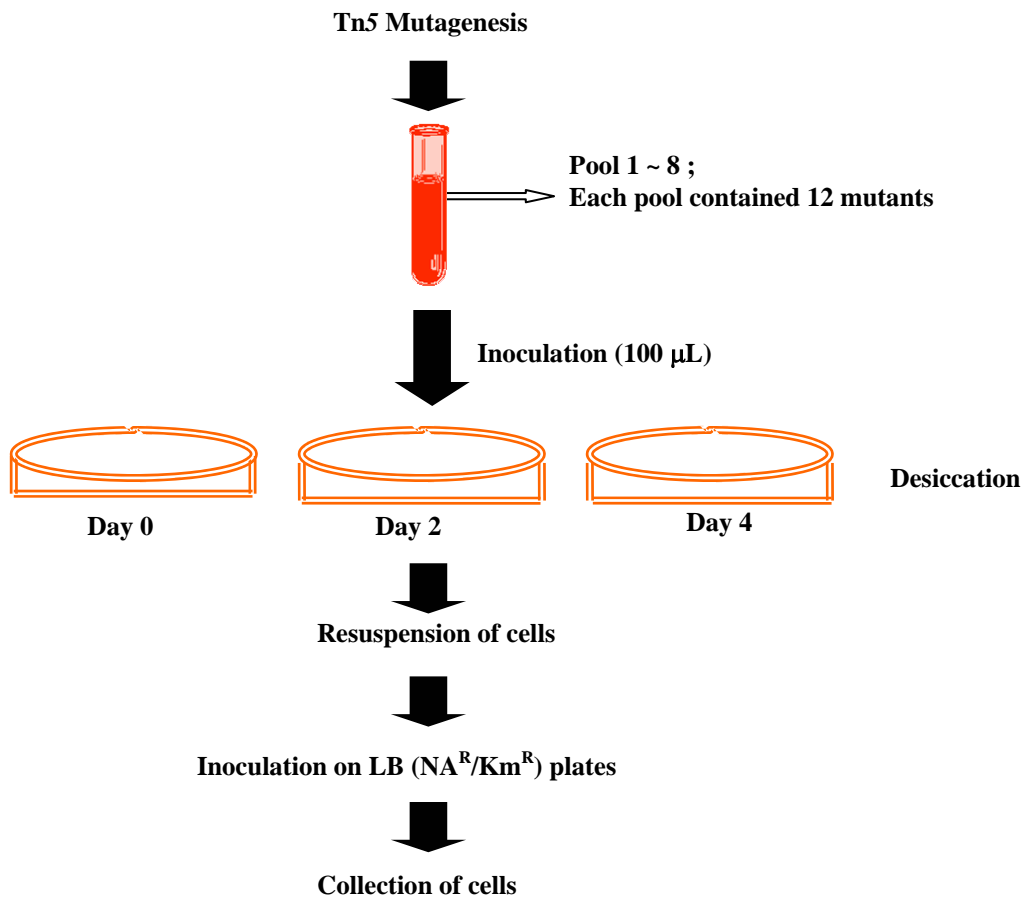


FIGURE 4-1. Experimental approach for desiccation and recovery of *S. typhimurium* Tn5 mutant pools

grown up mutant colonies (over 30 colonies) were collected into 2 mL of PBS (pH 7.2). This mixture in PBS was considered the outpool pool.

Preparation of Template DNA for PCR

The oligonucleotide sequences used in this study were used by the protocol described previously by Kwon and Ricke (2000). A 9 μL of linker 2 (350 ng/ μL) was first phosphorylated at the 5' end using T4 polynucleotide kinase (PNK). After heat denaturation of PNK at 65 C for 20 min, 9 μL of linker 1 (350 ng/ μL) was added to a final volume of 29 μL . The mixture of linkers 1 and 2 were heated to 95 C for 2 min and cooled slowly to room temperature to allow annealing. Bacterial genomic DNA was isolated from mini-Tn5 mutants of *S. typhimurium* by the method of Pitcher *et al.* (1989) and completely digested with *Nla*III (New England BioLabs, Beverly, MA). Approximately, 40 ng of the digested DNA was ligated to 1 μg of the Y linker with 1 μL of T4 DNA ligase (1 units/ μL ; Invitrogen, Life Technologies, Carlsbad, CA) in a final volume of 20 μL . After overnight incubation at room temperature, the reaction mixture was diluted with double distilled water to a final volume of 200 μL and heated at 65 C for 10 min to denature T4 DNA ligase. Subsamples (2 μL) were used as templates in the PCR amplification.

PCR Amplification and Gel Electrophoresis

All PCR reactions were performed using GeneAmp® PCR system 2400 (PE Applied Biosystems, Foster City, CA) along with a primer specific to mini-Tn5 (Tn5 primer) and a primer specific to the Y linker (Y linker primer). The Tn5 primer was designed to specifically anneal to the I end of the mini-Tn5 (Auerswald *et al.*, 1981;

Herrero *et al.*, 1990). The reaction containing 5 μ L 10X PCR buffer (166 mM $(\text{NH}_4)_2\text{SO}_4$, 670 mM Tris (pH 8.8), 67 mM MgCl_2 , 100 mM β -mercaptoethanol), 350 ng of each primer, 3 μ L dNTPs mix (25 mM for each dNTP), 3 μ L DMSO, and 2 μ L template DNA in a 49 μ L reaction mixture was incubated at 95 C for 2 min. As a next step, 1 μ L Tag DNA polymerase (5 units/ μ L; PE Applied Biosystems) was added during a hot-start incubation at 80 C to prevent non-specific priming. The target sequences were amplified through 30 cycles of 95 $^\circ\text{C}$ for 30 sec, 58 $^\circ\text{C}$ for 1 min, and 70 $^\circ\text{C}$ for 1 min, followed by a final cycle of 70 $^\circ\text{C}$ for 5 min. The PCR products were analyzed on a 1.5% agarose gel and stained with ethidium bromide.

Results and Discussion

Although desiccation can reduce populations of *Salmonella* spp. in associated environments such as poultry manure and litter (Riemann *et al.*, 1998), *Salmonella* can survive at much lower A_w levels in other environments. Our overall goal in this work was to use drying over a short time period to simulate desiccation shock to determine if our novel transposon footprinting method could be applied to a typical non-growth condition. Typical survival responses of *S. typhimurium* Tn5 mutants from pool 1 and pool 2 during initial desiccation are shown in Figure 4-2. On day 2 of storage, the populations of *S. typhimurium* Tn5 mutants from pool 1 and pool 2 were reduced 53.50 and 51.46%, respectively. However, as exposure time continued to day 4 of storage at 37 C, the reduction of *S. typhimurium* Tn5 mutants from pool 1 and pool were

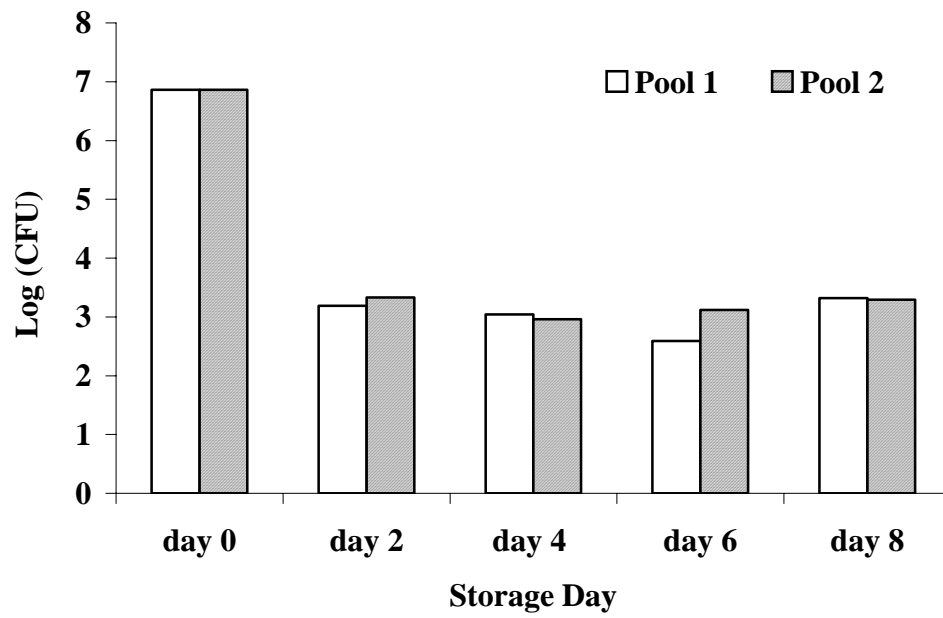


FIGURE 4-2. Survival of *S. typhimurium* Tn5 mutants during initial desiccation

less marked as shown Figure 4-2. By day 8, reduction of the population of *S. typhimurium* Tn5 mutants from both pool 1 and pool 2 was fairly stable with minimal decreases compared to that of *S. typhimurium* Tn5 mutants on day 4 of storage. Based on this, we selected pools from *S. typhimurium* Tn5 mutants on days 0, 2, and 4 of storage for mutant analysis because this is the time frame where most of the population reduction occurred.

The bacterial genomic DNA of the eight pools of inoculum (input pools), each containing twelve Tn5 mutants, and output pools recovered from the desiccation condition with each input pool were used in this study. The genomic DNA was digested completely with *Nla*III, ligated to, and used as templates in PCR amplification with a Y linker primer and a Tn5 primer to amplify the transposon-flanking sequences. The amplified DNA fragments within a pool on day 0 of storage were separated by a 1.5% agarose gel electrophoresis to generate a unique transposon footprint for each pool shown in Figure 4-3. Based on the gel results shown in Figure 4-3, the number of DNA bands in the transposon footprints were less than 8 (Lanes 2, 3, 4, 5, 6, and 7) for all of the pools (Pools 2, 3, 4, 6, 7, and 8) on day 0 of storage even though each pool contained 12 mutants initially. This might be because the transposon-flanking sequences were potentially too long to be amplified in the PCR amplification condition used in this study. Kwon *et al.* (2001) suggested that if this is true for some of the mutants, it should be possible to amplify the transposon-flanking sequence either by using a 4-bp recognition restriction enzyme other than *Nla*III to prepare template for PCR or by using a protocol for long PCR (Cheng *et al.*, 1994).

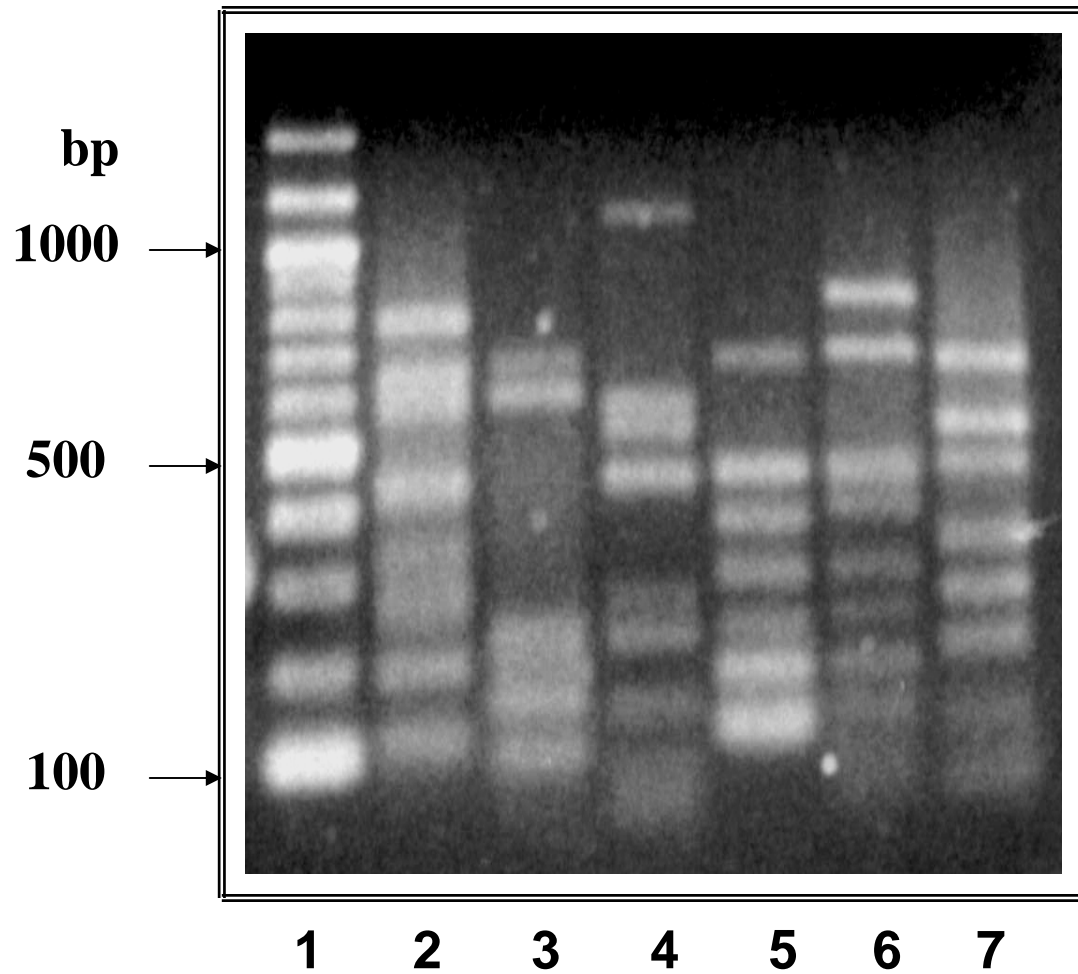


FIGURE 4-3. Footprints of different *S. typhimurium* Tn5 mutant pools on day zero. Lane 1. 100 bp ladder, Lane 2. Pool 8, Lane 3. Pool 7, Lane 4. Pool 6, Lane 5. Pool 4, Lane 6. Pool 3, Lane 7. Pool 2

Mini-Tn5 flanking sequences in pool 5 were not distinguished among time intervals during the desiccation condition as shown in Figure 4-4. In general, pool 5 could be considered representative of the sets of generated mutants which were not distinguishable from day 0 or mutant pools from other time intervals during desiccation. The number of DNA bands in the transposon footprints in pool 5 were less than 8 on all storage of days (Lane 2, 3, and 4) even though the pool contained 12 mutants initially. The number of DNA bands in the transposon footprints of pool 5 were observed to yield the same number of DNA bands as the number of DNA bands in the transposon footprints of other *S. typhimurium* Tn5 mutant pools on day 0 of storage. Therefore, no unique mutants appeared and the genes reflected by this banding pattern would not be considered involved in desiccation stress. Mini-Tn5 flanking sequences in pool 1 exhibited a different pattern of bands (Lane 5) on day 4 of storage on the desiccation condition as shown in Figure 4-4. A mutant (as indicated by an arrow) was not recovered from the day 4 of storage in pool 1 based on the gel results shown in Figure 4-4. The result may indicate that the potential mutant has a survival defect incurred by desiccation.

Based on the results of the present study, this transposon footprinting method can be applied to find *S. typhimurium* potential gene(s) required for survival from short term desiccation. Recently it was reported that there are 4,950 kilobases within the entire genome of *S. typhimurium* (McClelland *et al.*, 2001). In this study, 1 potential mutant was detected out of 96 mutants based on the transposon footprints patterns. Therefore, if calculated for the entire genome (assuming 1 kilobase-sized genes), over 50 potential

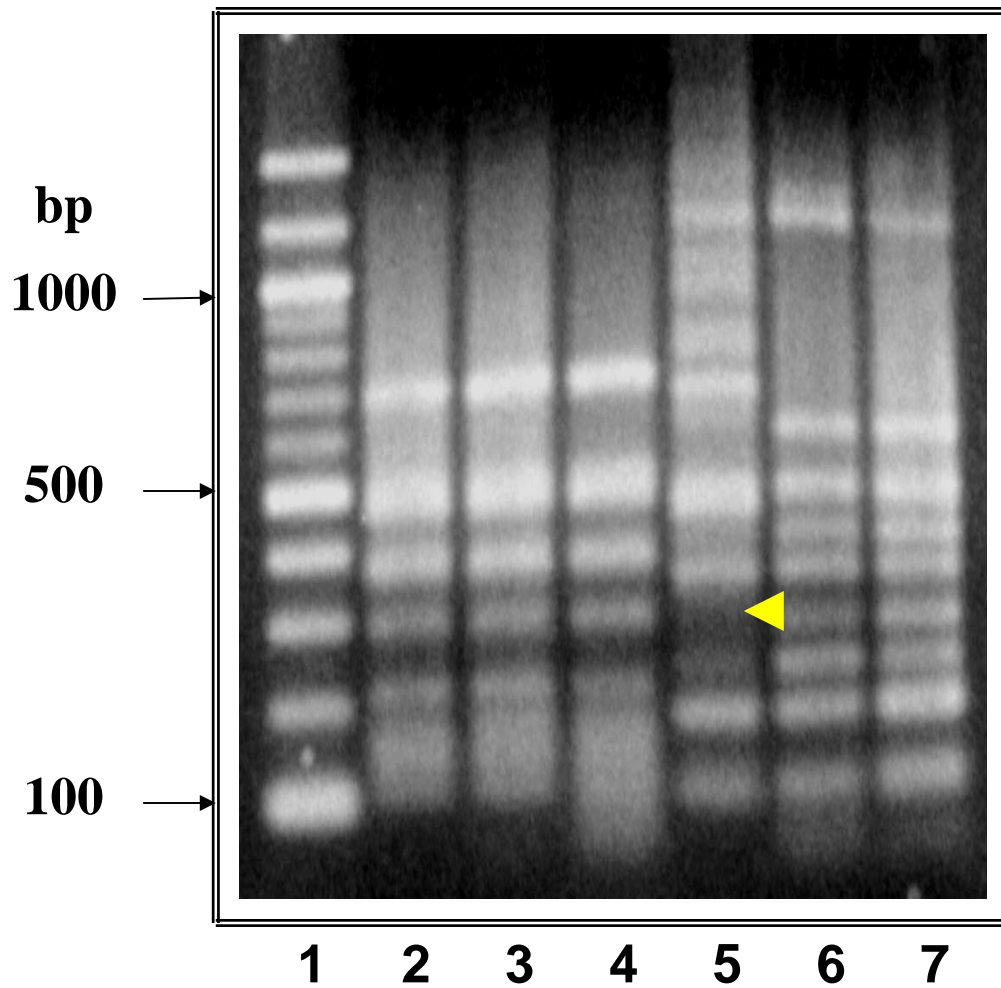


FIGURE 4-4. Comparison of transposon footprints of *S. typhimurium* Tn5 mutant pools 1 and 5 from different days. Lane 1. 100 bp ladder, Lane 2. Pool 5 on day 4 of storage, Lane 3. Pool 5 on day 2 of storage, Lane 4. Pool 5 on day 0 of storage, Lane 5. Pool 1 on day 4 of storage, Lane 6. Pool 1 on day 2 of storage, Lane 7. Pool 1 on day 0 of storage

genes may be associated either directly or indirectly with desiccation stress in *Salmoella*. In conclusion, the successful application of transposon footprinting method should lead to more rapid screening of mutants with potential genes associated with desiccation as well as other nongrowth stressors. This in turn may lead to a better understanding of genetic regulation of *Salmonella* response to these stressors in environments such as poultry houses.

CHAPTER V

***IN VITRO* COMPARISON OF ANAEROBIC AND AEROBIC
GROWTH RESPONSE OF *SALMONELLA TYPHIMURIUM*
TO ZINC ADDITION***

Overview

Zinc supplemented diets have been used to provide zinc as a nutrient and higher concentrations have been used to induce molt in laying hens. It is not known if the zinc in these diets would inhibit *Salmonella* spp growth. This study examines the effects of zinc compounds on the growth of *S. typhimurium* poultry isolate under aerobic and anaerobic conditions. The aerobic growth response of *S. typhimurium* poultry isolate was determined either in tryptic soy broth (TSB) or minimal (M9) broth containing five different concentrations (0.67, 2.01, 3.35, 4.69, and 6.03% [wt/vol]) of either Zn acetate [$\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$] or Zn sulfate [$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$] while anaerobic growth response was determined in M9 broth with or without reductants (L-cysteine hydrochloride [$\text{C}_3\text{H}_7\text{NO}_2\text{S} \cdot \text{HCl}$], sodium sulfide [$\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$]). Aerobic growth rates were more ($P < 0.05$) inhibited by Zn acetate than by Zn sulfate in TSB medium. The Zn source and concentration also significantly ($P < 0.05$) decreased aerobic growth response of *S. typhimurium* poultry isolate in M9 medium. The growth rates of *S. typhimurium* poultry isolate under anaerobic growth conditions were less responsive to Zn salts but were

* Reprinted with permission from: *In vitro* comparison of anaerobic and aerobic growth response of *Salmonella typhimurium* to zinc addition, by Park *et al.*, 2002, J. Food Safety. Vol 22, pp. 219-229. Copyright 2002 by Food & Nutrition Press, Inc.

generally less ($P < 0.05$) in the presence of reductant than in the absence of reductants at each concentration of Zn compound. The results of this study provide evidence that Zn may inhibit *S. typhimurium* under *in vitro* aerobic and anaerobic atmospheric conditions and *S. typhimurium* grow less optimally under anaerobic growth conditions.

Introduction

Zinc is a required trace mineral and is commercially added in poultry feeds either as Zn sulfate or Zn oxide at 0.012 to 0.018% on a total weight basis (Batal *et al.*, 2001; Leeson and Summers, 1997). Dietary Zn may also influence growth and infectivity of bacterial pathogens in animals. Zn deficiency in animals is associated with increased infections with microorganisms and causes gram-negative sepsis in rats by increased bacterial populations present in liver, lungs, and kidneys (Srinivas *et al.*, 1989). Zn concentrations in serum and plasma can be decreased by infection of *Salmonella gallinarum* (Hill, 1989), *Escherichia coli* (Tufft *et al.*, 1988), or *E coli* endotoxin (Butler and Curtis, 1973). Zn-methionine supplementation diets in mature chicken have also been demonstrated to increase primary antibody response to *S. pullorum* (Kidd *et al.*, 1992) and macrophage phagocytosis of *S. enteritidis* (Kidd *et al.*, 1994).

Zinc may also possess antibacterial properties of *S. enteritidis* in food processing and food animals as well. Zn chloride is bactericidal to *S. typhimurium* and effective in inhibiting attachment to poultry skin under aerobic conditions (Nayak *et al.*, 2001). Likewise, feeding moderate and high Zn molting diets has been shown to decrease *S. enteritidis* colonization in laying hens compared to feed deprived hens (Ricke *et al.*,

2001; Kubena *et al.*, 2001). This may indicate that Zn is bactericidal in the gastrointestinal tract as well, since *Salmonella* can come in contact with the gastrointestinal tract via an oral route and colonize the intestine or invade the epithelial cells of the small intestine (Finlay, 1994). However, the microbiological conditions in avian and mammalian gastrointestinal tracts are generally anaerobic in adult animals (Hungate, 1966; Barnes *et al.*, 1979) and it is not known if Zn compounds would be inhibitory under anaerobic and aerobic conditions. This is important for food packaging as well since microaerophilic and anaerobic growth conditions are often encountered by *Salmonella* during food production or processing systems. Packaging of food products with plastic films may limit oxygen permeability and thus increase the level of CO₂ due to the enzymatic activity of the tissue, and the growth of microorganisms (Farkas, 2001). Likewise, vacuum packaging limit aerobic spoilage organisms but favor growth of obligate and facultative anaerobic pathogens under these storage conditions (Cutter, 2002). The present study was conducted to examine the effects of zinc compounds on the growth of a *S. typhimurium* poultry isolate under aerobic and anaerobic conditions.

Materials and Methods

Bacterial Strain

A chicken isolate of *Salmonella typhimurium* resistance to novobiocin (NO) and nalidixic acid (NA) was used in the study (Ziprin *et al.*, 1990). Tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) was used for maintenance and growth of the bacterial strain. For bacterial culture maintenance, NO (25µg/ml) and NA (25µg/ml) were added

for the *S. typhimurium* poultry isolate (NO/NA) strain.

Preparation of Aerobic Culture Media

Tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) and a minimal salts M9 medium (0.5g of NaCl, 6.0 g of Na₂HPO₄, 3.0 g of KH₂PO₄, 1.0 g of NH₄Cl, 1.0 ml of 1 M MgSO₄·7H₂O, 10.0 ml of 0.01 M CaCl₂, and 10.0 ml of 20% Glucose; Miller, 1972; Sambrook *et al.*, 1989) were used in this study. The media were dispensed into 16 X 150 mm test tubes (5mL/tube), and autoclaved. Filter sterile solutions (1X) of, Zn acetate [Zn(C₂H₃O₂)₂·2H₂O] or Zn sulfate [ZnSO₄·7H₂O] were added to TSB or M9 media at five different concentrations (0.67, 2.01, 3.35, 4.69, and 6.03% [wt/vol]).

Preparation of Anaerobic Culture Media

For the preparation of anaerobic M9 medium, the aerobically prepared M9 medium before autoclaving as described for aerobic medium was gassed with 80% N₂ and 20% CO₂ to adjust to a neutral pH of approximately pH 7.0 using NaOH and HCl. A reducing agent, 0.05% [wt/vol] of L-cysteine hydrochloride [C₃H₇NO₂S·HCl] (Kim *et al.*, 1996) was added to M9 medium. The M9 medium was dispensed into 16X100 mm test tubes (5mL/tube), sealed with butyl rubber stopper and aluminum sealer, and autoclaved for 20 min. Zn acetate or Zn sulfate at five different concentrations (0.67, 2.01, 3.35, 4.69, and 6.03% [wt/vol]) were added with a sterile 1 cc syringe, as an appropriate amount of Zn acetate [Zn(C₂H₃O₂)₂·2H₂O] or Zn sulfate [ZnSO₄·7H₂O] stock solution (1X) to the anaerobic M9 medium. Final anaerobic preparation was done using 2.4% [wt/vol] of sodium sulfide [Na₂S·9H₂O] as the reducing agent (LeVan *et al.*, 1998; Kim *et al.*, 1996) in a gassing manifold described in

the following section. A 200 μL solution of sodium sulfide [$\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$] were added with a sterile 1 cc syringe to anaerobic M9 medium (5 mL/tube).

Gassing Manifold

A gassing manifold was originally described by Balch and Wolfe (1976) with some modification as described by Kim *et al.* (1996). The gassing manifold was created to add a substrate anaerobically at elevated pressures of approximately 2 or 3 atm to stoppered culture tubes. The high pressure gas tank (80% CO_2 , and 20% N_2) was connected to the manifold by oxygen impermeable tubing. The manifold was composed of a two-way valve connected to a gas tank and a vacuum pump joined together with a pipeline. At the tip of each pipeline was a glass syringe needle (21 gauge) and pressure in the manifold was controlled by a two-stage regulator on the gas tank.

Growth Rate Measurement

Overnight fresh cultures (20 μL and 50 μL) were used for inoculation in aerobic and anaerobic TSB and M9 medium, respectively. Growth of *S. typhimurium* in individual tubes at 37 °C was measured as optical density (A_{600}) on a spectrophotometer (Milton Roy Co., Rochester, NY). Batch culture growth rate was determined by the linear regression of the change in the natural logarithm of density A_{600} during exponential growth as described by Ricke and Schaefer (1991).

Statistical Analysis

Growth rates (h^{-1}) under aerobic growth conditions presented in Figure 5-1 and Figure 5-2, represent the average data generated from two (Figure 5-1) or three independent trials (Figure 5-2) using three tubes per trial per treatment. Growth rates (h^{-1})

¹) under anaerobic growth conditions presented in Figure 5-3 and Figure 5-4, represent the average data generated from two runs using two tubes per run per treatment. Growth rates (h^{-1}) were estimated using natural logarithm of A_{600} versus time by linear regression analysis in the linear portion of the plot (Ricke and Schaefer, 1991). The values were analyzed by least squares mean separations of the ANOVA procedure in SAS statistical analysis software program, version 6.11 (SAS Institute Inc., Cary, NC). All statistical analyses were considered significantly different at the $P < 0.05$ level.

Results and Discussion

Aerobic Growth Response of *S. typhimurium* in TSB Medium

The growth rates of *S. typhimurium* grown in TSB medium in the presence of five different concentrations of either Zn acetate or Zn sulfate are shown in Figure 5-1. TSB medium was chosen for this initial study to compare the influence of Zn compounds under optimal growth conditions of an aerobic atmosphere and nutrient rich medium. Zn acetate and Zn sulfate were chosen because Zn acetate at 10,000-25,000 ppm of Zn has been used as a hen's molting diet (Shippee *et al.*, 1979), while Zn sulfate at 0.012 to 0.018% is commercially used as a zinc supplement poultry diet (Leeson and Summers, 1997), and at 1,400 - 4,200 ppm Zn has been used as molting diet (Breeding *et al.*, 1992).

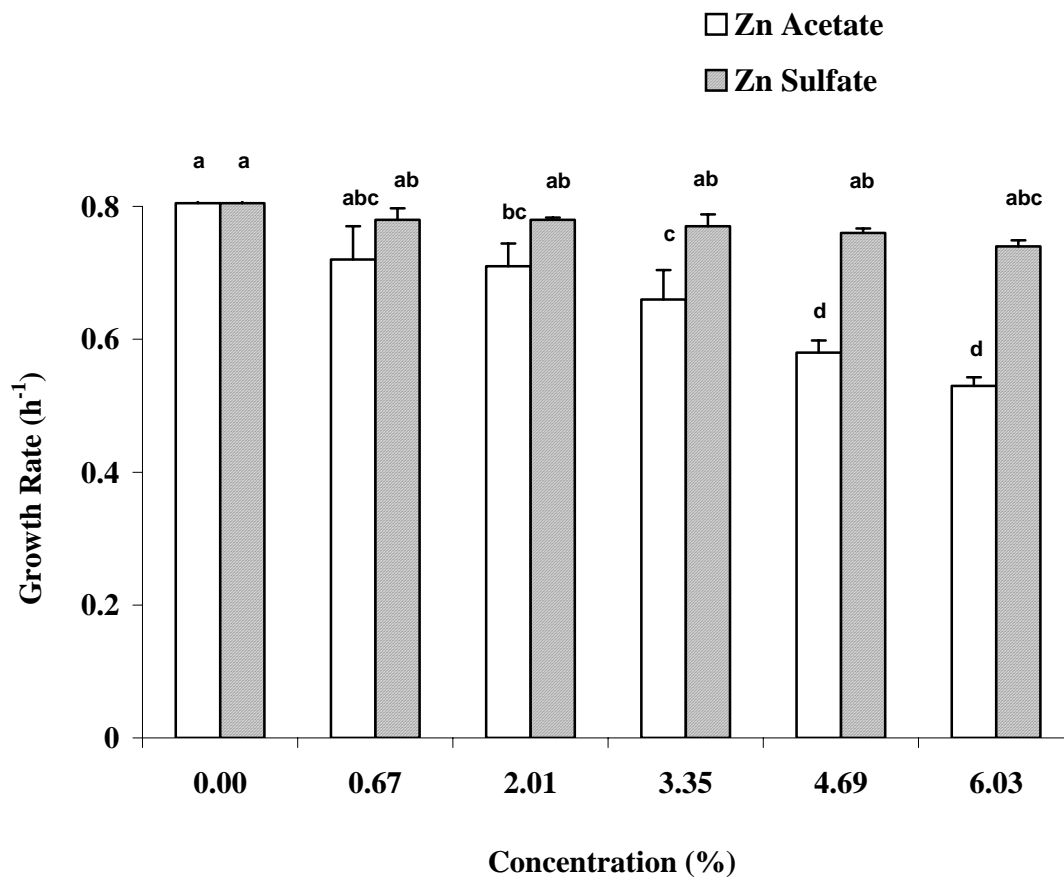


FIGURE 5-1. Aerobic growth rate response of *Salmonella typhimurium* poultry isolate in the presence of Zn compounds in TSB medium. The data present means of two runs with standard errors (three tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$)

The interaction of Zn compounds and concentrations of Zn compounds significantly ($P < 0.05$) affected the growth rate of *S. typhimurium* poultry isolate in TSB medium.

When overall main effects of Zn compounds or concentrations of Zn compounds in TSB medium were compared, growth rates of the *S. typhimurium* poultry isolate were decreased ($P < 0.05$) by stepwise increase (0.67 to 6.03%) of Zn acetate. Growth rates of the *S. typhimurium* were not inhibited ($P > 0.05$) even in the presence of highest concentration (6.03%) of Zn sulfate in TSB medium. Growth rates of the *S. typhimurium* were more reduced ($P < 0.05$) by Zn acetate than by Zn sulfate at concentrations of 3.35, 4.69, and 6.03%. No significant differences were observed between the zinc sources at 0.67 and 2.01%, *S. typhimurium* poultry isolate in Zn acetate and in Zn sulfate were 0.67 and 0.77 (h^{-1}), respectively.

Aerobic Growth Response of *S. typhimurium* in M9 Medium

The respective growth rates of *S. typhimurium* in minimal M9 (glucose, salts, and ammonia) media of five different concentrations of either Zn acetate or Zn sulfate are shown in Figure 5-2. The interaction of Zn compounds and concentrations of Zn compounds significantly ($P < 0.05$) affected the growth rate of *S. typhimurium* in M9 medium. The growth rates of *S. typhimurium* in M9 media were more inhibited ($P < 0.05$) in the presence of Zn acetate than in that of Zn sulfate at 0.67%. Although the effects of medium for growth rates were not statistically compared, growth rates appeared to be generally less in M9 medium than in TSB medium for all concentrations of Zn compounds. The overall growth rates of *S. typhimurium* in TSB medium and M9

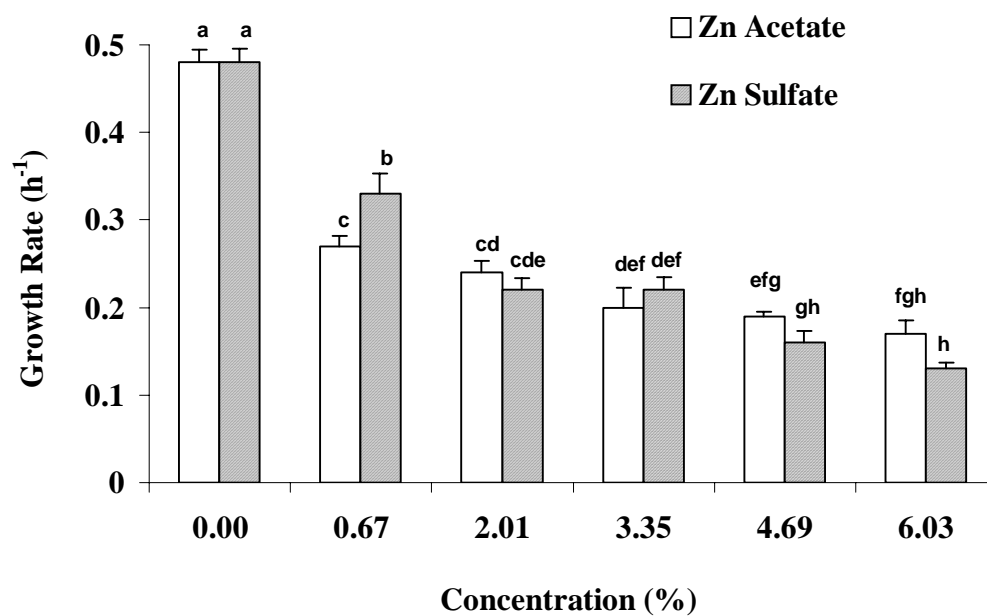


FIGURE 5-2. Aerobic growth rate response of *Salmonella typhimurium* poultry isoate in the presence of Zn compounds in M9 medium. The data present means of three runs with standard errors (three tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$)

medium were 0.67 (Zn acetate) or 0.77 (h^{-1})(Zn sulfate), and 0.26 (Zn acetate) or 0.25 (h^{-1})(Zn sulfate), respectively. Because M9 medium is a minimal nutrient medium, the growth of *S. typhimurium* poultry isolate was expected to be more limited in a M9 than in a TSB medium.

Anaerobic Growth Response of *S. typhimurium*

Microaerophilic and anaerobic growth conditions could be encountered by *Salmonella* during food production or processing systems as well as packaging of certain food products with plastic films that limit oxygen permeability (Farkas, 2001; Cutter, 2002). In addition, the microecological conditions in avian and mammalian gastrointestinal tracts are generally anaerobic in adult animals (Hungate, 1966; Barnes *et al.*, 1979). In order, to determine the physiological response of *S. typhimurium* when exposed to an anaerobic atmosphere under *in vitro* conditions, growth rate comparisons were made with anaerobically prepared media.

The growth rates of *S. typhimurium* poultry isolate in M9 medium in the presence of five different concentrations of Zn acetate are shown in Figure 5-3. When effects of Zn acetate were statistically analyzed, the interaction of Zn acetate concentration and presence or absence of reductants significantly ($P < 0.05$) affected the growth rate of *S. typhimurium* under anaerobic conditions. The growth rates of *S. typhimurium* poultry isolate were 2 to 3 fold ($P < 0.05$) more inhibited in the presence of reductants than in the absence of reductants at each concentration of Zn acetate. When growth rate responses to individual Zn acetate concentrations were compared in the presence or absence of reductants, the majority were not significantly different.

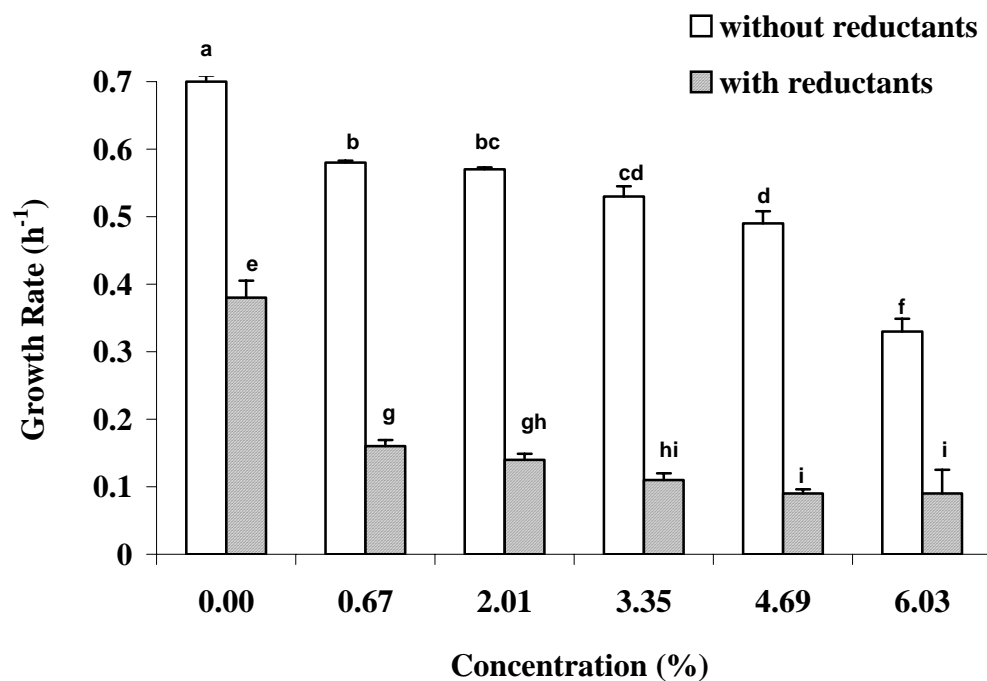


FIGURE 5-3. Anaerobic growth rate response of *Salmonella typhimurium* poultry isoate in the presence of Zn acetate in M9 medium. The data present means of two runs with standard errors (two tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$)

The growth rates of *S. typhimurium* poultry isolate in M9 medium in the presence of five different concentrations of Zn sulfate are shown in Figure 5-4. When effects of Zn sulfate were statistically analyzed, the interaction of concentrations of Zn sulfate and presence or absence of reductants did not significantly ($P > 0.05$) affect the growth rate of *S. typhimurium* under anaerobic conditions. However, the overall main effects of either increasing Zn sulfate concentration or addition of reductants significantly decreased ($P < 0.05$) *S. typhimurium* poultry isolate growth rates. The overall growth rates of the *S. typhimurium* poultry isolate in the presence of reductants and in the absence of reductants were 0.26 and 0.54 (h^{-1}), respectively, and the overall growth rates of the *S. typhimurium* poultry isolate in Zn sulfate ranged from 0.49 (at 0.67%) to 0.25 (h^{-1})(at 6.03%). In general, a similar pattern was observed with the growth of *S. typhimurium* poultry isolate response to Zn acetate in the presence of or absence of reductants as was observed with Zn sulfate.

The decreased growth rates for *S. typhimurium* may indicate that the use of both hydrogen sulfide and cysteine as reducing agents to reduce oxidation-reduction potential may either be somewhat inhibitory to *S. typhimurium* growth or the resulting anaerobic conditions decrease bacterial cell metabolism and growth due to less energy availability from anaerobic energy metabolism. According to Carlsson *et al.* (1979), the oxidation of cysteine can be formed in the presence of hydrogen peroxide under anaerobic conditions, *Peptostreptococcus anaerobius* strain was rapidly killed by 20 μM hydrogen peroxide in anaerobic solution. The effects of Zn compounds for growth rates under anaerobic condition appeared to be less pronounced with Zn acetate addition than with

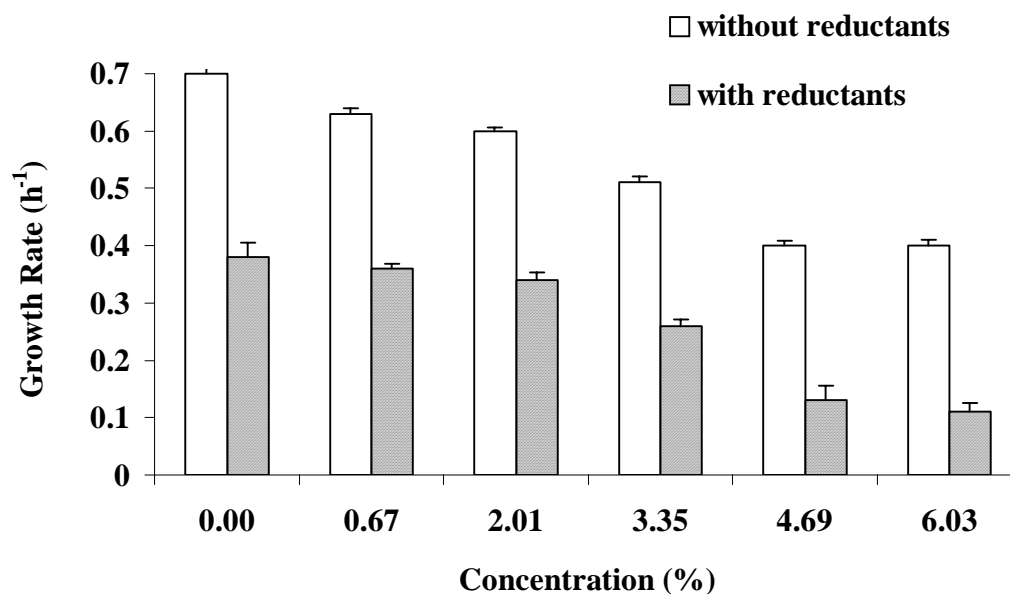


FIGURE 5-4. Anaerobic growth rate response of *Salmonella typhimurium* poultry isoate in the presence of Zn sulfate in M9 medium. The data present means of two runs with standard errors (two tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$)

the Zn sulfate in the presence of reductants at all concentrations. The anaerobic growth response to Zn compounds were somewhat similar to the growth response of *S. typhimurium* to Zn compounds under aerobic conditions but less differentiation was detectable among individual Zn concentrations.

The results in this study provide additional evidence that Zn may inhibit *S. typhimurium* under *in vitro* aerobic or anaerobic atmospheric conditions and that *S. typhimurium* grows less optimally under anaerobic growth conditions. In addition, this *in vitro* inhibition may support the concept of zinc containing molt diets being effective for limiting *in vivo* *Salmonella* colonization in the gastrointestinal tract of laying hens (Ricke *et al.*, 2001; Kubena *et al.*, 2001). The effectiveness of zinc under anaerobic conditions observed in this study may also suggest a potential extension of the application of zinc compounds to poultry skin (Nayak *et al.*, 2001) to limiting *Salmonella* surface contamination and multiplication on carcasses packaged under vacuum as well. However, *in vivo* studies must be conducted to assess the influence of the environmental matrix on the effectiveness of these compounds.

CHAPTER VI

**THE COMBINATION OF ZINC COMPOUNDS AND ACIDIC PH
LIMITS AEROBIC GROWTH OF A *SALMONELLA*
TYPHIMURIUM POULTRY ISOLATE MARKER
STRAIN IN RICH AND MINIMAL MEDIA ***

Overview

The objective of the present study was to examine the combined effects of zinc compounds with different acidic pH levels on the aerobic growth of a *S. typhimurium* poultry isolate under either rich or minimal media. When overall main effects of pH levels of medium or concentrations of Zn compounds were compared, growth rates of the *S. typhimurium* poultry isolate were significantly ($P < 0.05$) decreased by stepwise increase of pH levels of medium (pH 4, 5, 6, and 7) or concentrations (0.67, 3.35, and 6.03%) of Zn compounds (Zn acetate and Zn sulfate). In general growth rates of *S. typhimurium* poultry isolate appeared to be more reduced by Zn acetate than by Zn sulfate and more reduced in minimal media compared to rich media.

Introduction

Zinc is one of the most abundant elements within the group of metals referred to

* Reprinted with permission from: The combination of zinc compounds and acidic pH limits aerobic growth of a *Salmonella typhimurium* poultry isolate marker strain in rich and minimal media, by Park *et al.*, 2003, J. Environ. Sci. Health. Part B. Accepted.

as transition metals (Outten and O'Halloran, 2001). Zinc is a common micronutrient in human milk, infant formula as well as enriched dairy and fruit products (De Jong *et al.*, 2000; Dorea, 2000; Rodriguez *et al.*, 2000). In poultry production zinc is an important trace element that is necessary for avian physiological processes such as growth, bone development, feathering, enzyme structure, and cellular and immunological functions (Duncan and Hurley, 1978; Dardenne and Bach, 1993; Batal *et al.*, 2001). Dietary Zn may also influence growth and of foodborne pathogens in environments associated with poultry production. Park *et al.* (2002b) demonstrated that various zinc compounds would inhibit *in vitro* aerobic and anaerobic growth of a poultry *Salmonella typhimurium* isolate. *In vivo* studies involving feeding, moderate and high Zn containing diets to induce molting in laying hens have also been shown to decrease *S. enteritidis* colonization in laying hens compared to feed deprived hens (Kubena *et al.*, 2001; Ricke *et al.*, 2001). However, zinc resistance has also been shown to occur in multiple metal resistant *Pseudomonas* strains (Choudhury and Srivastava, 2001). It has been reported that increasing Zn chloride concentrations are effective in inhibiting growth of a wide range of yeasts but inter- and intra-generic differences in zinc tolerance were also observed (Batič *et al.*, 1996).

Zinc can be added in a variety of forms including salts in combination with organic and inorganic acids. Park *et al.* (2002b) observed more growth inhibition of *S. typhimurium* with zinc acetate than zinc sulfate. However, the initial pH of most media amended with the these zinc compounds was close to neutral. *Salmonella* grows at pH 4.5 to 9.5 with an optimum pH for growth of 6.5 to 7.5 (Chung and Goepfert, 1970). It

is known that a lowered pH enhances the antibacterial activity of short chain fatty acids by increasing the concentration of the undissociated form which can penetrate across the bacterial cell membrane (Cherrington *et al.*, 1991). Therefore, pH level may be one of the important parameters to control or inhibit in growth of *Salmonella* in the presence of zinc. Since *S. typhimurium* can come in contact with foods containing high levels of acids a potential synergism may exist for zinc in the presence of a lower pH. The objective of the present study was to examine the combined effects of zinc compounds with different acidic pH levels on the aerobic growth of a *S. typhimurium* poultry isolate under either rich or minimal media.

Materials and Methods

Bacterial Strain

A poultry isolate of *Salmonella typhimurium* previously selected for resistance to novobiocin (NO) and nalidixic acid (NA) was used in the study (Ziprin *et al.*, 1990). Tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) was used for maintenance and growth of the bacterial strains. For bacterial culture maintenance, NO (25µg/ml) and NA (25µg/ml) were added for the *S. typhimurium* poultry isolate (NO/NA) strain.

Preparation of Culture Media with Different pH Levels

Tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) and a minimal salts M9 medium (0.5g of NaCl, 6.0 g of Na₂HPO₄, 3.0 g of KH₂PO₄, 1.0 g of NH₄Cl, 1.0 ml of 1 M MgSO₄·7H₂O, 10.0 ml of 0.01 M CaCl₂, and 10.0 ml of 20% Glucose; Miller, 1972;

Sambrook *et al.*, 1989) were used in this study. The TSB or M9 medium was adjusted to pH 4, 5, 6, and 7 based on measurements with a pH meter (Corning 240; Corning Inc., Corning, NY) and dispensed into 16X150 mm test tubes (5mL/tube), followed by autoclaving for 20 min. For the preparation of Zn compounds in aerobic TSB or M9 media, Zn acetate [$\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 2\text{H}_2\text{O}$] or Zn sulfate [$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$] at three different concentrations (0.67, 3.35, and 6.03% [wt/vol]) was added, as an appropriate amount of filter-sterilized Zn acetate or Zn sulfate stock solution (1X) to the aerobic TSB or M9 media.

Growth Rate Measurement

Overnight fresh cultures (20 μl) were used for inoculation in TSB and M9 medium, respectively. Growth of *S. typhimurium* in individual tubes at 37 °C was measured as optical density (A_{600}) on a spectrophotometer (Milton Roy Co., Rochester, NY). Batch culture growth rate was determined by the linear regression of the change in the natural logarithm of density A_{600} during exponential growth as described by Ricke and Schaefer (1991).

Statistical Analysis

Growth rates (hr^{-1}) were estimated using natural logarithm of A_{600} versus time by linear regression analysis in the linear portion of the plot (Ricke and Schaefer, 1991). The values were analyzed by least squares mean separations of the ANOVA procedure in SAS statistical analysis software program, version 6.11 (SAS Institute Inc., Cary, NC). All statistical analyses were considered significantly different at the $P < 0.05$ level.

Results and Discussion

To determine the growth response of *S. typhimurium* in the presence of various concentrations of zinc and levels of pH growth rate was determined. TSB medium chosen for this initial study to compare the influence of Zn compounds under optimal growth conditions of an aerobic atmosphere and nutrient rich medium. Zn acetate and Zn sulfate were chosen as discussed previously because 1,400-4,200 ppm Zn has been used as molting diets for laying hens (Shippee *et al.*, 1979; Breeding *et al.*, 1992) and 0.012 to 0.018% Zn sulfate is commercially used as a Zn dietary supplement in poultry (Leeson and Summers, 1997).

The growth rates of *S. typhimurium* grown in the four different pH levels of TSB medium in the presence of three different concentrations of Zn acetate are shown in Figure 6-1. The interaction of pH levels and concentrations of Zn acetate significantly ($P < 0.05$) affected the growth rate of *S. typhimurium* poultry isolate in TSB medium. Although the growth rates of *S. typhimurium* were not significantly ($P > 0.05$) different between 0.67, 3.35, and 6.03% of Zn acetate in pH 4 of TSB, the growth rates of *S. typhimurium* were more significantly ($P < 0.05$) inhibited in three concentrations of pH 4 than in those of pH 5, 6, or pH 7 of TSB. When growth rate responses to individual Zn acetate concentrations were examined for each different pH levels of TSB, at pH 6 and 7 growth rate was significantly less ($P < 0.05$) for 6.03% Zn acetate compared to the lower concentrations of Zn acetate. At pH 5, growth rate was significantly less for 6.03% Zn acetate but not 3.35. The overall main effects of either decreasing pH level or increasing Zn acetate concentration significantly decreased ($P < 0.05$) *S. typhimurium* poultry

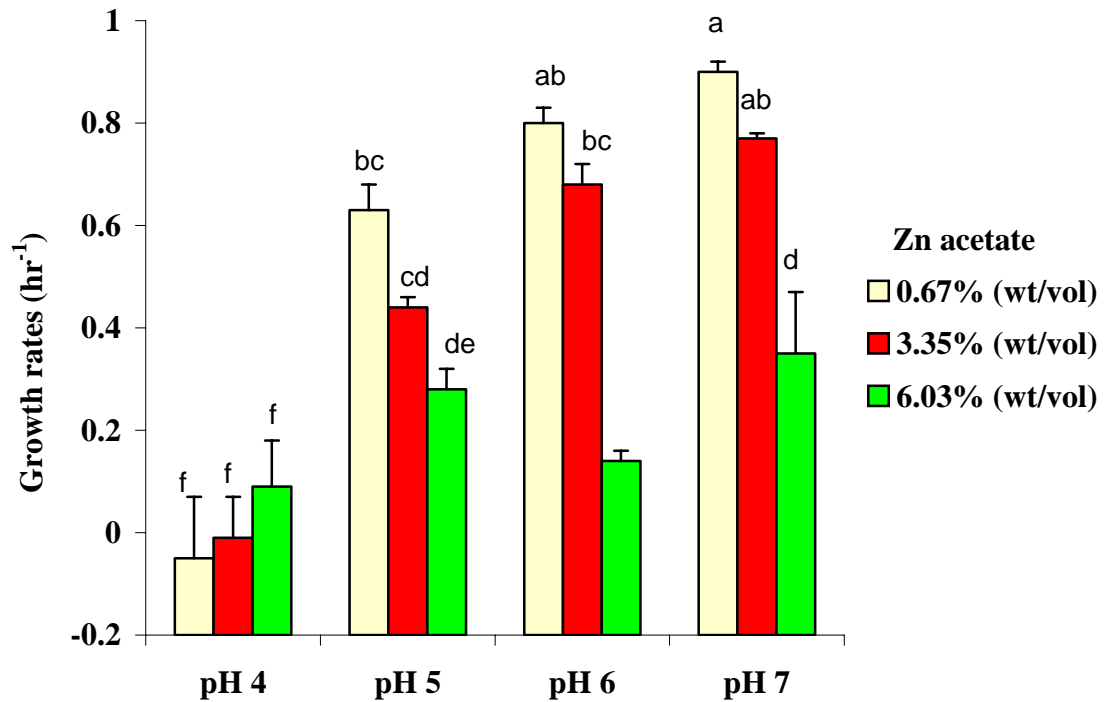


FIGURE 6-1. Aerobic growth response of *S. typhimurium* poultry isolate to the presence of Zn acetate in TSB medium. The data present means of two runs with standard errors (two tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$). Control (with no zinc added): pH 4 ($0.05 \pm 0.02 \text{ hr}^{-1}$), pH 5 ($0.63 \pm 0.06 \text{ hr}^{-1}$), pH 6 ($0.69 \pm 0.06 \text{ hr}^{-1}$), pH 7 ($0.91 \pm 0.04 \text{ hr}^{-1}$)

isolate. When overall main effects of pH levels or concentrations of Zn acetate in TSB medium were compared, growth rates of the *S. typhimurium* were decreased ($P < 0.05$) by stepwise decrease (pH 4-7) of pH levels or increase (0.67-6.03%) of Zn acetate.

The growth rates of *S. typhimurium* grown in the four different pH levels of TSB medium in the presence of three different concentrations of Zn sulfate are shown in Figure 6-2. The interaction of pH levels and concentrations of Zn sulfate did not significantly ($P > 0.05$) affect the growth rate of *S. typhimurium* poultry isolate in TSB medium. However, the overall main effects of either decreasing pH level or increasing Zn sulfate concentration significantly decreased ($P < 0.05$) *S. typhimurium* poultry isolate growth rates. The overall growth rates of the *S. typhimurium* in the pH 4, 5, 6, and 7 of TSB medium were 0.06, 0.60, 0.65, and 0.83 (h^{-1}), respectively, and the overall growth rates in Zn sulfate at 0.67, 3.35, and 6.03% were 0.57, 0.54, and 0.49 (h^{-1}), respectively. Although the effects of Zn compounds for growth rates in TSB medium were not statistically compared, growth rates appeared to be approximately 2 fold more inhibited in the presence of Zn acetate than in Zn sulfate at 6.03% at each pH level. To determine aerobic growth response of *S. typhimurium* poultry isolate to Zn compounds at different pH levels under less optimal growth conditions, the respective growth rates of *S. typhimurium* poultry isolate were determined in minimal M9 (glucose, salts, and ammonia) medium of three different concentrations of either Zn acetate are shown in Figure 6-3. The interaction of pH levels and concentrations of Zn acetate did not significantly ($P > 0.05$) affect the growth rate of *S. typhimurium* poultry isolate in M9 medium. However, the overall main effects of either decreasing pH level or

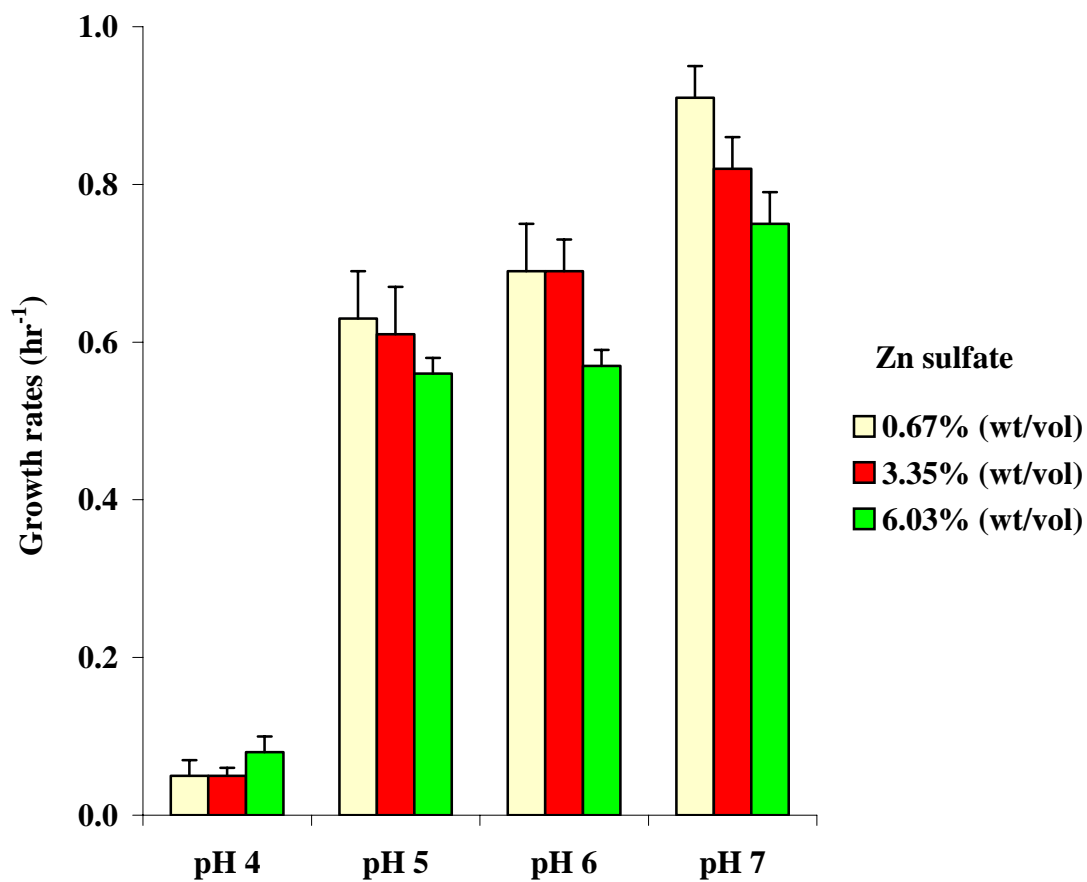


FIGURE 6-2. Aerobic growth response of *S. typhimurium* poultry isolate to the presence of Zn sulfate in TSB medium. The data present means of two runs with standard errors (two tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$). Control (with no zinc added): pH 4 ($0.05 \pm 0.02 \text{ hr}^{-1}$), pH 5 ($0.63 \pm 0.06 \text{ hr}^{-1}$), pH 6 ($0.69 \pm 0.06 \text{ hr}^{-1}$), pH 7 ($0.91 \pm 0.04 \text{ hr}^{-1}$)

increasing Zn acetate concentration significantly decreased ($P < 0.05$) *S. typhimurium* poultry isolate growth rates. The overall growth rates of the *S. typhimurium* in the pH 4, 5, 6, and 7 of M9 medium were 0.05, 0.06, 0.06, and 0.07 (h^{-1}), respectively, and the overall growth rates in Zn acetate at 0.67, 3.35, and 6.03% were 0.08, 0.05, and 0.04 (h^{-1}), respectively.

The growth rates of *S. typhimurium* grown in the four different pH levels of M9 medium in the presence of three different concentrations of Zn sulfate are shown in Figure 6-4. The interaction of pH levels and concentrations of Zn sulfate did not significantly ($P > 0.05$) affect the growth rate of *S. typhimurium* poultry isolate in M9 medium. However, the overall main effects of either decreasing pH level or increasing Zn acetate concentration significantly decreased ($P < 0.05$) *S. typhimurium* poultry isolate growth rates. The overall growth rates of the *S. typhimurium* in the pH 4, 5, 6, and 7 of M9 medium were 0.07, 0.09, 0.09, and 0.10 (h^{-1}), respectively, and the overall growth rates in Zn acetate at 0.67, 3.35, and 6.03% were 0.11, 0.08, and 0.07 (h^{-1}), respectively. Although the effects of Zn compounds for growth rates in M9 medium were not statistically compared, growth rates appeared to be less inhibited in the presence of Zn acetate than in Zn sulfate at all three concentrations at each pH level.

Although the effects of medium for growth rates at different pH levels were not statistically compared, growth rates appeared to be greatly less in M9 medium

than in TSB medium for all three concentrations of Zn compounds at each pH level except pH 4. Because M9 medium is a minimal nutrient medium, the growth of *S. typhimurium* poultry isolate was expected to be more limited in a M9 than in TSB

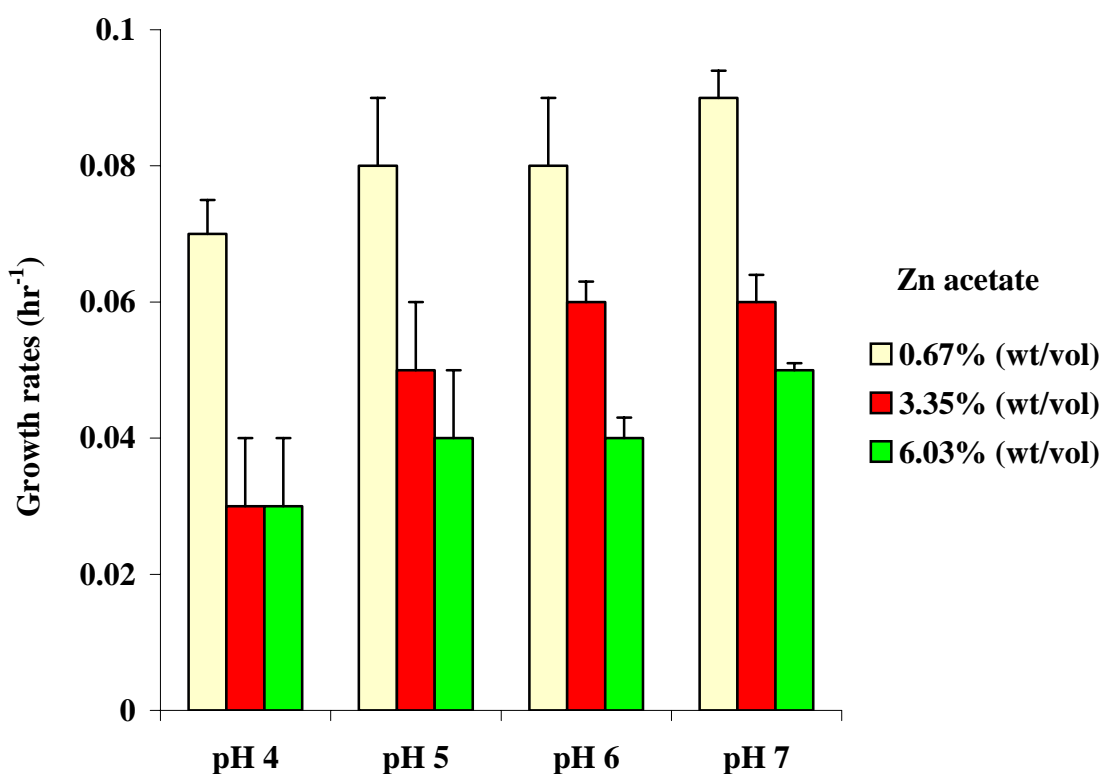


FIGURE 6-3. Aerobic growth response of *S. typhimurium* poultry isolate to the presence of Zn acetate in M9 medium. The data present means of two runs with standard errors (two tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$). Control (with no zinc added): pH 4 ($0.07 \pm 0.008 \text{ hr}^{-1}$), pH 5 ($0.11 \pm 0.005 \text{ hr}^{-1}$), pH 6 ($0.61 \pm 0.0006 \text{ hr}^{-1}$), pH 7 ($0.18 \pm 0.006 \text{ hr}^{-1}$)

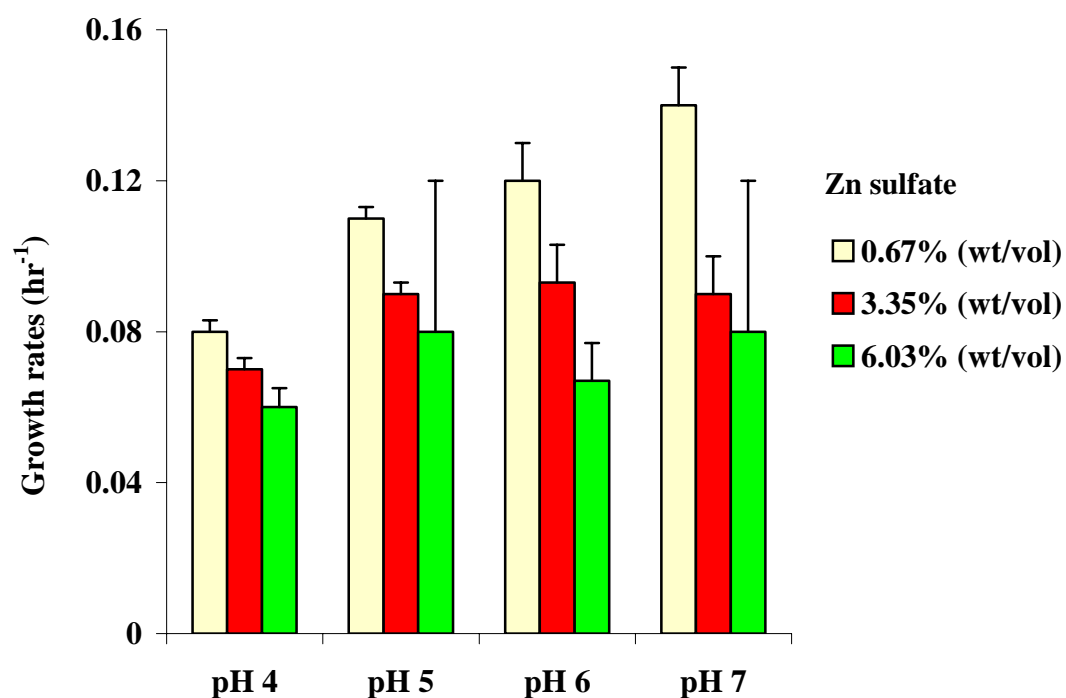


FIGURE 6-4. Aerobic growth response of *S. typhimurium* poultry isolate to the presence of Zn sulfate in M9 medium. The data present means of two runs with standard errors (two tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$). Control (with no zinc added): pH 4 ($0.07 \pm 0.008 \text{ hr}^{-1}$), pH 5 ($0.11 \pm 0.005 \text{ hr}^{-1}$), pH 6 ($0.61 \pm 0.0006 \text{ hr}^{-1}$), pH 7 ($0.18 \pm 0.006 \text{ hr}^{-1}$)

medium.

The results in this study provide evidence that Zn greatly inhibits *S. typhimurium* growth aerobic growth in acidic rich or minimal media. In addition, the effectiveness of zinc at lower acidic medium observed in this study may also suggest a potential extension of the application of the combination of zinc compounds and acidic condition to poultry skin (Nayak *et al.*, 2001) and other food matrices for to limiting *Salmonella* surface contamination. However, application of these compounds directly on surfaces must be conducted to assess the effectiveness of these compounds in the presence of organic compounds and other components associated with these matrices.

CHAPTER VII

INFLUENCE OF OXIDATION-REDUCTION MEDIA

REDUCTANTS ON *SALMONELLA TYPHIMURIUM* GROWTH

KINETICS RESPONSE IN AN ANAEROBIC ATMOSPHERE

AFTER INTIAL PH ADJUSTMENT AND

ZINC COMPOUND ADDITION

Overview

The objective of this study was to examine the interaction between acidic pH, addition of reductants and zinc compounds (Zn acetate and Zn sulfate) on the anaerobic growth of a *S. typhimurium* poultry isolate in rich or minimal media. The anaerobic growth of a *S. typhimurium* poultry isolate in TSB medium was significantly inhibited by either acidic pH levels (pH 4: 0.18, pH 5: 0.20, pH 6: 0.40, pH 7: 0.57 h⁻¹) or higher concentrations of Zn acetate (0.67%: 0.43, 3.35%: 0.33, and 6.03%: 0.26 h⁻¹). *S. typhimurium* anaerobic growth in M9 minimal medium was significantly inhibited by either acidic pH level or higher concentrations of Zn acetate or Zn sulfate. Most anaerobic growth rates of the *S. typhimurium* poultry isolate in acidic and higher concentrations of Zn acetate or Zn sulfate were less than 0.10 h⁻¹. The overall anaerobic growth rates of *S. typhiurium* were more inhibited in the presence (0.089 h⁻¹) of reductants than in the absence (0.102 h⁻¹) of reductants in M9 medium. The results in

this study suggest that a potential combination of Zn compounds and acidic pH reduces growth of foodborne *Salmonella* under anaerobic conditions.

Introduction

Given the public concerns associated with increased antibiotic resistance in pathogens that make treatment of clinical disease more difficult (Glynn *et al.*, 1998; Koutsolioutsou *et al.*, 2001), alternative antimicrobial compounds such as organic acids have been recently considered once again as possible alternatives for antibiotics (Ricke, 2003). Organic acids can effectively reduce *E. coli*, *Salmonella*, and *Campylobacter* (Russell and Diez-Gonzalez, 1998; Dibner and Buttin, 2002). Organic acids have commonly been used a food and animal feed additives because organic acids possess antibacterial properties that are pH dependent in these matrices (Khan and Katamay, 1969; Cherrington *et al.*, 1991). *Salmonella* grows at pH 4.5 to 9.5 with an optimum pH for growth of 6.5 to 7.5 (Chung and Goepfert, 1970) and an increased pH may be associated with increased *Salmonella* survivability and growth. Therefore, pH level may be one of the important parameters to control or inhibit in growth of *Salmonella* during food processing and preservation.

As food processing involves more implementation of packaging materials that limit oxygen transfer and modify the atmosphere surrounding the food, there is a need to understand how these changes influence effectiveness of antimicrobial compounds. Microaerophilic and anaerobic growth conditions are potentially encountered by foodborne *Salmonella* during food production or processing systems as well as

packaging of food products with plastic films that limit oxygen permeability (Farkas, 2001; Cutter, 2002). Anaerobic growth conditions in vacuum-packaged foods may be suitable for the growth of anaerobic pathogens such as *Clostridium botulinum* (Brody, 1989). In addition, the restraint of aerobic spoilage organism in vacuum-packaged foods may create favorable conditions for the growth of pathogenic aerobic bacteria such as *Listeria monocytogens*, *Yersinia enterocolitica*, *Aeromonas hydrophila*, and enterotoxigenic *Escherichia coli* (Brody, 1989). Therefore, vacuum packaging may selectively favor the growth of obligate and facultative anaerobic pathogens on fresh foods (Brody, 1989).

Nayak *et al.* (2001) noted that electropositive charge of zinc ions may interfere with *Salmonella* attachment to poultry skin by selectively binding to the electronegative charges on the cell membrane and zinc chloride may also interfere with *Salmonella* attachment by competing with specific receptor sites on the skin surface. The current study represents an examination of zinc ions to potentially serve as antimicrobial compounds in anaerobic environments likely encountered by foodborne *Salmonella* during food production. Park *et al.* (2002b) recently reported that Zn acetate or Zn sulfate addition would inhibit *in vitro* aerobic and anaerobic growth of a *Salmonella typhimurium* when initial pH was neutral. When initial pH was decreased in a second study, *S. typhimurium* aerobic growth was more reduced by Zn acetate than by Zn sulfate and more reduced in acidic minimal media compared to acidic rich media (Park *et al.*, 2003). The objective of the present study was to examine the influence of zinc compounds and acidic pH when *S. typhimurium* was grown under a strict anaerobic

atmosphere and comparing growth response in the presence or absence of reducing compounds added to the media.

Materials and Methods

Bacterial Strain

A poultry isolate of *Salmonella typhimurium* previously selected for resistance to novobiocin (NO) and nalidixic acid (NA) was used in the study (Ziprin *et al.*, 1990). Tryptic soy broth (TSB; Difco Laboratories, Detroit, MI) was used for maintenance and growth of the bacterial strains. For bacterial culture maintenance, NO (25µg/ml) and NA (25µg/ml) were added for the *S. typhimurium* poultry isolate (NO/NA) strain.

Preparation of Anaerobic Culture Media with Different pH Levels

After initial gassing and preparation of anaerobic medium as previously described (Park *et al.*, 2002), respective media was adjusted to an initial of either pH 4, 5, 6, and 7 with 2M hydrochloride (HCl) solution. A reducing agent, 0.05% [wt/vol] of L-cysteine hydrochloride [$C_3H_7NO_2S \cdot HCl$] (Kim *et al.*, 1996) was added to TSB or M9 medium. The TSB or M9 medium was dispensed into 16x100 mm test tubes (5ml/tube), sealed with butyl rubber stoppers and aluminum seals, and autoclaved for 20 min. Zn acetate or Zn sulfate at three different concentrations (0.67, 3.35, and 6.03% [wt/vol]) were added with a sterile 1 cc syringe, as an appropriate amount of Zn acetate [$Zn(C_2H_3O_2)_2 \cdot 2H_2O$] or Zn sulfate [$ZnSO_4 \cdot 7H_2O$] stock solution (1X) to the anaerobic TSB or M9 medium. Final anaerobiosis was achieved by adding a 2.4% [wt/vol]

solution (200 μ l) of sodium sulfide [$\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$] as an additional reducing agent (Levan *et al.*, 1998; Kim *et al.*, 1996) in a gassing manifold described briefly in the following section. A 200 μ l solution of sodium sulfide [$\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$] were added with a sterile 1 cc syringe to anaerobic TSB or M9 medium (5 ml/tube).

Gassing Manifold

A gassing manifold (Balch and Wolfe, 1976) modified by Kim *et al.* (1996) was used for all growth studies and described previously by Park *et al.* (2002b). A gas tank (80% CO_2 , and 20% N_2) was connected to the manifold by oxygen impermeable tubing. The manifold consisted of a two-way valve connected to a gas tank and a vacuum pump. Glass syringe needles (21 gauge) were located at the end of each line and pressure in the manifold was controlled by a two-stage regulator on the gas tank.

Growth Rate Measurement

Overnight fresh cultures (50 μ l) were used for inoculation in anaerobic TSB and M9 medium, respectively. Growth of *S. typhimurium* in individual tubes at 37 °C was measured as optical density (A_{600}) on a spectrophotometer (Milton Roy Co., Rochester, NY). Batch culture growth rate was determined by the linear regression of the change in the natural logarithm of density A_{600} during exponential growth as described by Ricke and Schaefer (1991).

Experimental Design and Statistical Analysis

Growth rates (h^{-1}) were estimated using natural logarithm of A_{600} versus time by linear regression analysis in the linear portion of the plot (Ricke and Schaefer, 1991). The values were analyzed by least squares mean separations of the ANOVA procedure

in SAS statistical analysis software program, version 6.11 (SAS Institute Inc., Cary, NC). All statistical analyses were considered significantly different at the $P < 0.05$ level.

Results and Discussion

Anaerobic Growth Response of *S. typhimurium* at Different pH Levels in Medium

One of the important environmental conditions that *Salmonella* are expected to meet during its passage through food production and gastrointestinal tracts of food animals is acidic pH conditions. The microecological conditions in avian and mammalian gastrointestinal tracts are generally anaerobic and acidic pH occurs in some segments of the gastrointestinal tract in most food animals (Hungate, 1966; Barnes *et al.*, 1979; Watkins and Miller, 1983; Dibner and Buttin, 2002). Likewise, anaerobic atmospheric environments can occur during food packaging when plastic films that limit oxygen penetration are employed (Farkas, 2001; Cutter, 2002). Therefore, to simulate the physiological response of *S. typhimurium* poultry isolate when exposed to an anaerobic atmosphere, growth rate comparisons were made under anaerobic conditions with different pH levels. To determine anaerobic growth response of *S. typhimurium* poultry isolate to the combination of different pH levels of TSB medium without and with reductants, the anaerobic growth rates of *S. typhimurium* poultry isolate amended with three different concentrations of Zn acetate are shown in Figure 7-1. Three way interactions of pH levels of media, concentrations of Zn acetate, and absence or presence of reductants or any of two way interactions of pH levels of media and concentrations of Zn acetate, pH levels of media and absence or presence of reductions, or concentrations

of Zn acetate and absence or presence did not significantly ($P > 0.05$) affect the anaerobic growth rate of *S. typhimurium* poultry isolate. Overall main effect of absence or presence of reductants did not significantly affect ($P > 0.05$) *S. typhimurium* poultry isolate growth rates. However, overall main effects of either lower pH levels or increasing concentrations of Zn acetate significantly decreased ($P < 0.05$) *S. typhimurium* poultry isolate growth rates. The overall growth rates of *S. typhimurium* poultry isolate in the pH 4, 5, 6, and 7 levels of TSB were 0.18, 0.20, 0.40, and 0.57 (h^{-1}), respectively, and the overall growth rates of *S. typhimurium* poultry isolate in 0.67, 3.35, and 6.02% of Zn acetate were 0.43, 0.33, and 0.26 (h^{-1}), respectively.

To determine anaerobic growth response of *S. typhimurium* poultry isolate to the combination of different pH levels of TSB medium and without and with reductants, the anaerobic growth rates of *S. typhimurium* poultry isolate in different pH levels of TSB medium in the presence of three different concentrations of Zn sulfate are shown in Figure 7- 2. Three way interactions of pH levels of media, concentrations of Zn sulfate, and absence or presence of reductants or any of two way interactions of pH levels of media and concentrations of Zn sulfate, pH levels of media and absence of presence of reductions, or concentrations of Zn sulfate and absence of presence did not significantly ($P > 0.05$) affect the growth rate of *S. typhimurium* poultry isolate under anaerobic conditions. However, overall main effects of lower pH levels decreased ($P < 0.05$) *S. typhimurium* poultry isolate growth rates. The overall growth rates of *S. typhimurium*

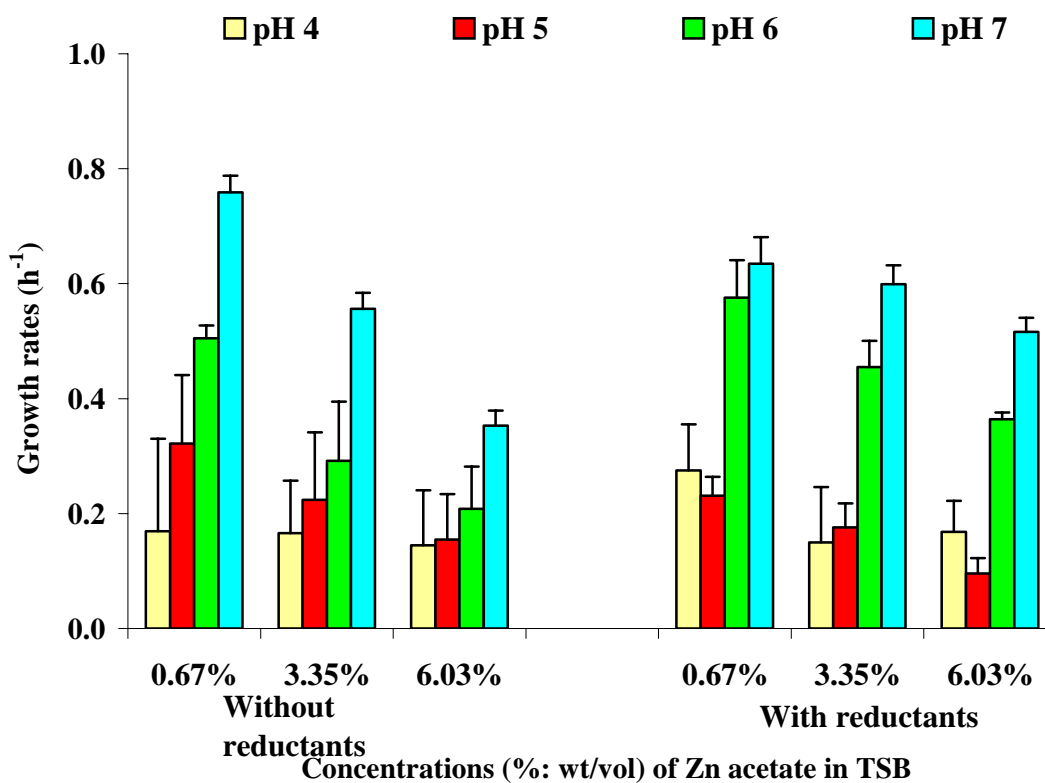


FIGURE 7-1. Anaerobic growth response of *S. typhimurium* poultry isolate in the presence of Zn acetate in different pH levels of TSB medium without and with reductants. The data present means of two runs with standard errors (two tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$). Control-without reductants is the same as Fig. 7-2 (with no zinc added): pH 4 ($0.20 \pm 0.06 \text{ h}^{-1}$), pH 5 ($0.44 \pm 0.01 \text{ h}^{-1}$), pH 6 ($0.68 \pm 0.01 \text{ h}^{-1}$), pH 7 ($0.95 \pm 0.12 \text{ h}^{-1}$). Control of with reductants is the same as Fig. 7-2 (with no zinc added): pH 4 ($0.29 \pm 0.05 \text{ h}^{-1}$), pH 5 ($0.49 \pm 0.06 \text{ h}^{-1}$), pH 6 ($0.62 \pm 0.02 \text{ h}^{-1}$), pH 7 ($0.79 \pm 0.08 \text{ h}^{-1}$)

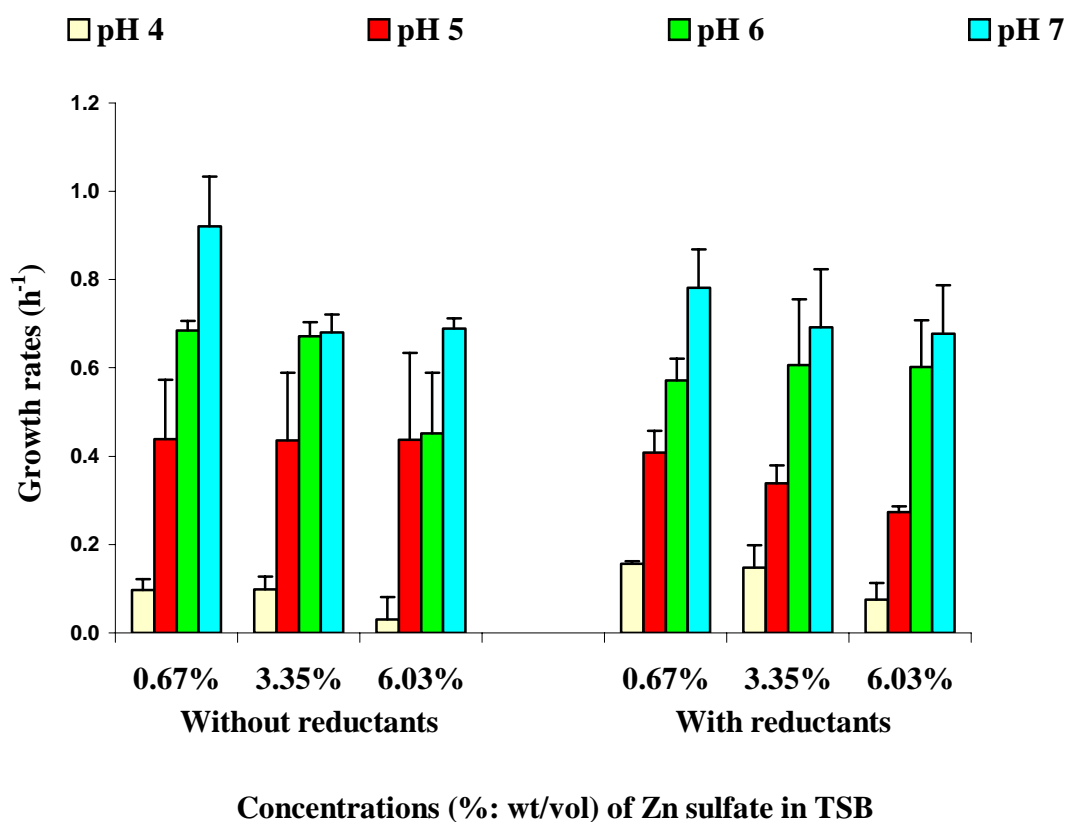


FIGURE 7-2. Anaerobic growth response of *S. typhimurium* poultry isolate in the presence of zinc sulfate in different pH levels of TSB medium without and with reductants. The data present means of two runs with standard errors (two tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$). Control-without reductants is the same as Fig. 7-1 (with no zinc added): pH 4 (0.20 ± 0.06 h⁻¹), pH 5 (0.44 ± 0.01 h⁻¹), pH 6 (0.68 ± 0.01 h⁻¹), pH 7 (0.95 ± 0.12 h⁻¹). Control of with reductants is the same as Fig. 7-1 (with no zinc added): pH 4 (0.29 ± 0.05 h⁻¹), pH 5 (0.49 ± 0.06 h⁻¹), pH 6 (0.62 ± 0.02 h⁻¹), pH 7 (0.79 ± 0.08 h⁻¹)

poultry isolate in the pH 4, 5, 6, and 7 levels of TSB were 0.10, 0.39, 0.60, and 0.74 (h^{-1}), respectively.

When anaerobic growth rate response to the pH levels of TSB medium (no Zn compound added) were compared in the presence or absence of reductants, the growth rates of *S. typhimurium* poultry isolate were inhibited in the presence of reductants than in the absence of reductants at only pH 6 or pH 7 of TSB medium. However, overall main effects of the presence or absence of reductants did not significantly ($P > 0.05$) affect the growth rate of *S. typhimurium* poultry isolate under anaerobic conditions.

Anaerobic Growth Response of *S. typhimurium* at Different pH Levels in Minimal Medium

To determine anaerobic growth response of *S. typhimurium* poultry isolate to the combination of different pH levels of M9 medium without and with reductants, the growth rates of *S. typhimurium* poultry isolate in different pH levels of M9 medium in the presence of three different concentrations of Zn acetate are shown in Figure 7- 3. The three way interactions of pH levels of media, concentrations of Zn acetate, and absence or presence of reductants did not significantly ($P > 0.05$) affect the anaerobic growth rate of *S. typhimurium* poultry. However, two way interactions of pH levels of media and absence of presence significantly ($P < 0.05$) affected the anaerobic growth rates of *S. typhimurium* poultry isolate. Overall main effects of either lower pH levels or increasing concentrations of Zn acetate significantly decreased ($P < 0.05$) *S. typhimurium* poultry isolate growth rates. The overall growth rates of *S. typhimurium* poultry isolate in the pH 4, 5, 6, and 7 levels of M9 were 0.04, 0.05, 0.07, and 0.09 (h^{-1}),

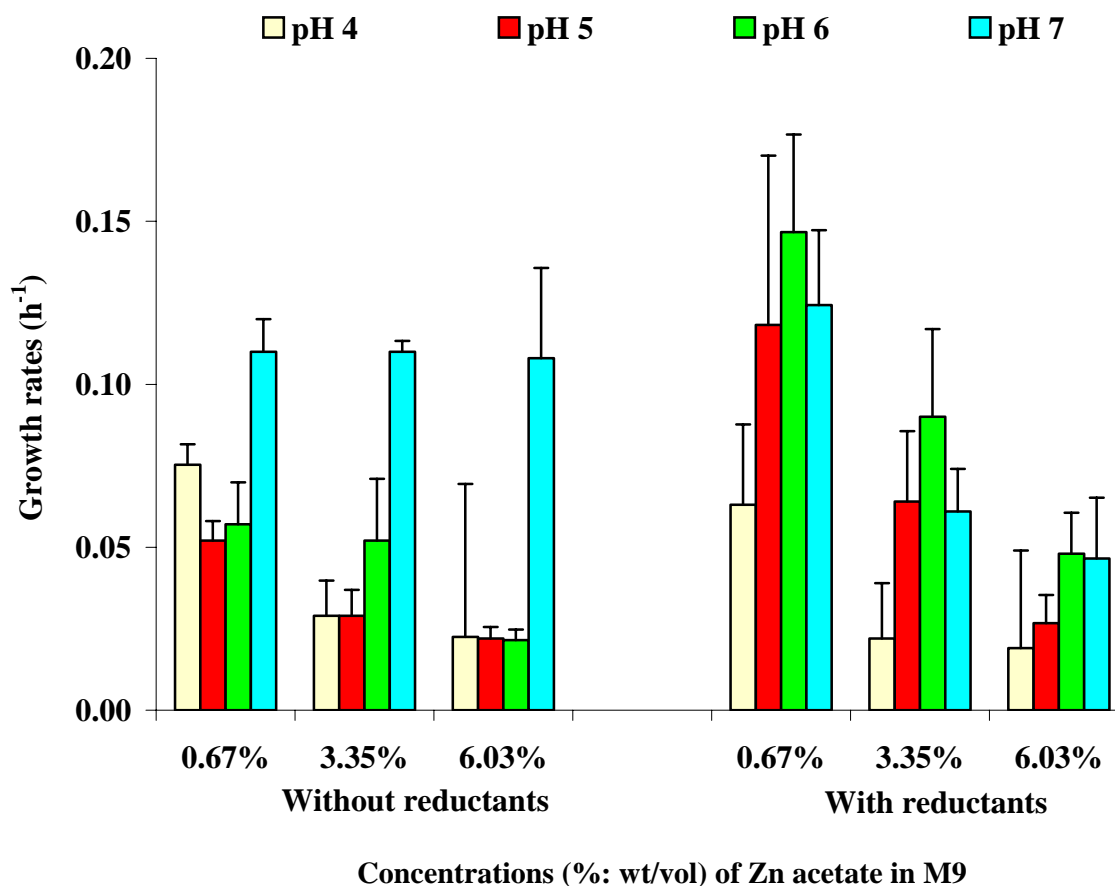


FIGURE 7-3. Anaerobic growth response of *S. typhimurium* poultry isolate in the presence of zinc acetate in different pH levels of M9 medium without and with reductants. The data present means of two runs with standard errors (two tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$). Control-without reductants is the same as Fig. 7-4 (with no zinc added): pH 4 ($0.08 \pm 0.01 \text{ h}^{-1}$), pH 5 ($0.20 \pm 0.02 \text{ h}^{-1}$), pH 6 ($0.36 \pm 0.01 \text{ h}^{-1}$), pH 7 ($0.54 \pm 0.01 \text{ h}^{-1}$). Control-with reductants is the same as Fig. 7-4 (with no zinc added): pH 4 ($0.10 \pm 0.03 \text{ h}^{-1}$), pH 5 ($0.19 \pm 0.02 \text{ h}^{-1}$), pH 6 ($0.21 \pm 0.02 \text{ h}^{-1}$), pH 7 ($0.29 \pm 0.02 \text{ h}^{-1}$)

respectively, and the overall growth rates of *S. typhimurium* poultry isolate in 0.67, 3.35, and 6.02% of Zn acetate were 0.09, 0.06, and 0.04 (h^{-1}), respectively.

To determine anaerobic growth response of *S. typhimurium* poultry isolate to the combination of different pH levels of M9 medium without and with reductants, the anaerobic growth rates of *S. typhimurium* poultry isolate in different pH levels of M9 medium in the presence of three different concentrations of Zn sulfate are shown in Figure 7- 4. The three way interactions of pH levels of media, concentrations of Zn acetate, and absence or presence of reductants did not significantly ($P > 0.05$) affect the anaerobic growth rate of *S. typhimurium* poultry. However, two way interactions of pH levels of media and concentrations of Zn sulfate or absence or presence of reductants and concentrations of Zn sulfate significantly ($P < 0.05$) altered of *S. typhimurium* anaerobic growth rates. Overall main effects of either lower pH levels or increasing concentrations of Zn sulfate significantly decreased ($P < 0.05$) *S. typhimurium* poultry isolate growth rates. The overall growth rates of *S. typhimurium* poultry isolate in the pH 4, 5, 6, and 7 levels of M9 were 0.05, 0.08, 0.08, and 0.10 (h^{-1}), respectively, and the overall growth rates of *S. typhimurium* poultry isolate in 0.67, 3.35, and 6.02% of Zn acetate were 0.11, 0.08, and 0.05 (h^{-1}), respectively. When anaerobic growth rate response to the pH levels of M9 medium (no Zn compound added) were compared in the presence or absence of reductants, the growth rates of *S. typhimurium* poultry isolate were inhibited in the presence of reductants than in the absence of reductants at only pH 5, pH 6 or pH 7 of M9 medium. However, overall main effect of the presence or

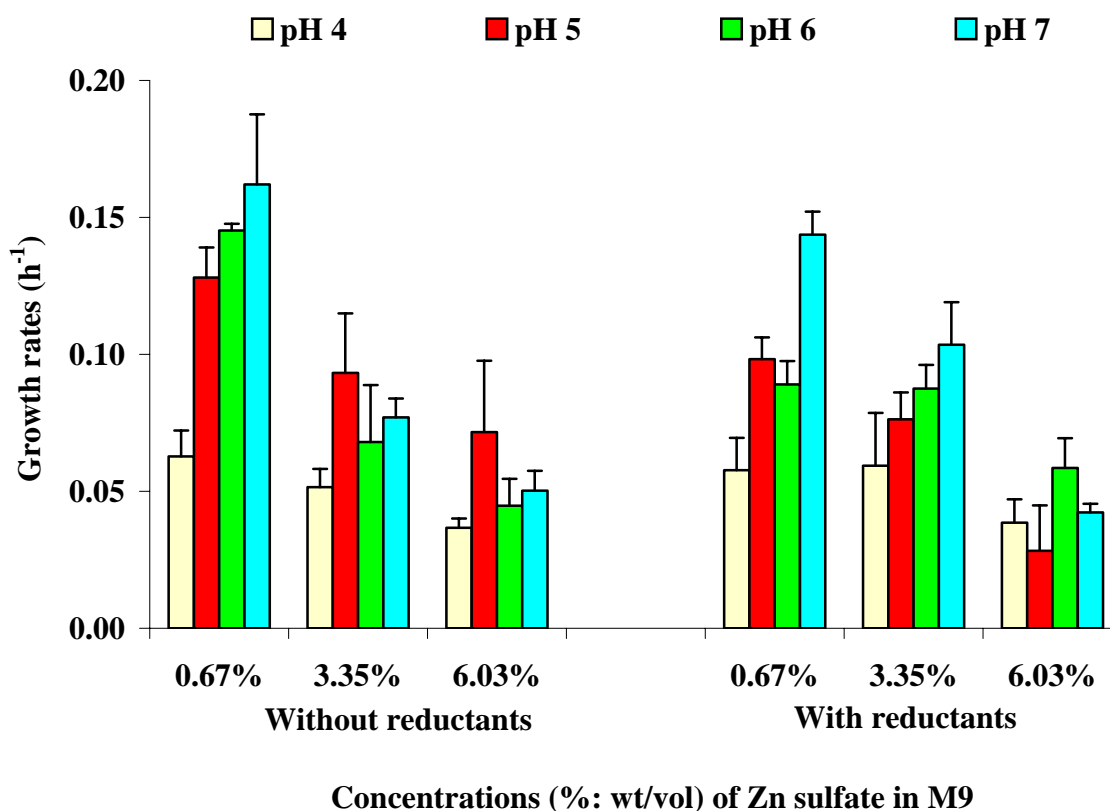


FIGURE 7-4. Anaerobic growth response of *Salmonella typhimurium* poultry isolate in the presence of zinc sulfate in different pH levels of M9 medium without and with reductants. The data present means of two runs with standard errors (two tubes/run/treatment). Means with no common subscript differ significantly ($P < 0.05$). Control-without reductants is the same as Fig. 7-3 (with no zinc added): pH 4 ($0.08 \pm 0.01 \text{ h}^{-1}$), pH 5 ($0.20 \pm 0.02 \text{ h}^{-1}$), pH 6 ($0.36 \pm 0.01 \text{ h}^{-1}$), pH 7 ($0.54 \pm 0.01 \text{ h}^{-1}$). Control-with reductants is the same as Fig. 7-3 (with no zinc added): pH 4 ($0.10 \pm 0.03 \text{ h}^{-1}$), pH 5 ($0.19 \pm 0.02 \text{ h}^{-1}$), pH 6 ($0.21 \pm 0.02 \text{ h}^{-1}$), pH 7 ($0.29 \pm 0.02 \text{ h}^{-1}$)

absence of reductants did not significantly ($P > 0.05$) affect the growth rate of *S. typhimurium* poultry isolate under anaerobic conditions.

Although we did not statistically compare the anaerobic growth rates of *S. typhiurium* between Zn acetate and Zn sulfate, or between TSB medium and M9 medium in this study, the anaerobic growth rates of *S. typhiurium* were generally lower in Zn acetate or M9 medium than sulfate or TSB medium. Additionally, presence or absence of reductants in TSB medium did not affect the anaerobic growth rates of *S. typhiurium*. However, the overall anaerobic growth rates of *S. typhiurium* were 2 fold more inhibited in the presence (0.089 h^{-1}) of reductants than in the absence (0.102 h^{-1}) of reductants in M9 medium. These observations were similar to Park *et al.* (2002b) who also reported that growth rates of *S. typhimurium* under anaerobic growth conditions and neutral initial pH were generally lower in the presence of reductants than in the absence of reductants in M9 medium at each concentrations of Zn acetate or Zn sulfate. Some of this reduction in growth may possibly be attributed to the generation of toxic compounds. When oxidation of cysteine occurred in the presence of hydrogen peroxide under anaerobic conditions, *Peptostreptococcus anaerobius* was rapidly killed by $20 \mu\text{M}$ hydrogen peroxide in anaerobic solution (Carlsson *et al.*, 1979). *S. typhimurium* growth rates under anaerobic atmospheric conditions and neutral initial pH also appeared to be more reduced by Zn acetate than by Zn sulfate and more reduced in minimal media compared to rich media (Park *et al.*, 2002b). In addition, the combination of Zn compounds and acidic pH inhibited *S. typhimurium* growth more than did the single variable of Zn or acidic pH under *in vitro* aerobic atmospheric incubation conditions

(Park *et al.*, 2003). However, in the current study, three way interactions of pH, addition of reductants, and zinc compounds (Zn acetate and Zn sulfate) or the single effect of addition of reductants did not significantly influence *S. typhimurium* anaerobic growth in acidic media.

In previous work, Zn compounds have been shown to inhibit *S. typhiurium* under *in vitro* aerobic or anaerobic atmospheric conditions when initial pH was close to a neutral pH of 7 (Park *et al.*, 2002). The results in the current study suggest that combining an initial acidic media pH and zinc ion amendment may enhance antimicrobial effectiveness of zinc compounds and allow the use of lesser concentrations of zinc. Therefore, potential incorporation of Zn compounds and acidic amendment combinations to food production or processing systems may be helpful towards reducing anaerobic growth and/or survival of *Salmonella* and therefore limiting the dissemination of foodborne *Salmonella* in food production.

CHAPTER VIII

SURVIVAL OF A *SALMONELLA TYPHIMURIUM* POULTRY MARKER STRAIN ADDED AS A DRY INOCULUM TO ZINC AND SODIUM ORGANIC ACID AMENDED FEED

Overview

The effects of different organic acid compounds on the survival of a *Salmonella typhimurium* marker strain added to poultry feed were determined. Organic acids were added as 1% Zn (w/w) or Na salts (w/w) to poultry layer ration and stored at room temperature ($21\pm 1^{\circ}\text{C}$) for 9 days. Reduction of *S. typhimurium* populations from 0 to day 9 days of storage was not substantially different between poultry layer ration (96.76%) and Zn acetate (99.72%) or Zn propionate (99.60%) amended feed. However, over 90% reduction of *S. typhimurium* populations occurred in feed containing either Zn acetate or Zn propionate by day 3 while poultry layer ration populations reached 90% reduction by day 5. *S. typhimurium* populations after 9 days were nearly 40% more reduced in Na propionate amended feed than Na acetate amended feed. The results of this study indicated that compared to Zn acetate, Zn propionate at 1 % of Zn may have more potential to reduce survival of *S. typhimurium* in a poultry diet during storage.

Introduction

Annually, 4 million cases of *Salmonella* associated gastroenteritis were estimated to occur in the United States (Tauxe, 1991). In addition, one billion dollars is spent each year on foodborne illness caused by *Salmonella* infections (Stavric and D'Aoust, 1993). Poultry contaminated with *Salmonella* spp. are regarded as a primary source of food-associated outbreaks of human salmonellosis. Contaminated feed is considered one of the important vectors for the transmission of *Salmonella* to animals (Jones *et al.*, 1982). *Salmonella* has been isolated in poultry feed, animal feed or feed ingredients (Williams, 1981; Cox *et al.*, 1983; Stuart, 1984; Veldman *et al.*, 1995).

Zn is a required trace element and is commercially supplemented in poultry feeds either as Zn sulfate or Zn oxide at 0.012 to 0.018% on a total weight basis (Leeson and Summers, 1997; Batal *et al.*, 2001). Zn is also known to exhibit antibacterial properties in food processing and in food animal production. Zn chloride is bactericidal to *S. typhimurium* and is effective in inhibiting attachment to poultry skin under aerobic conditions (Nayak *et al.*, 2001). Zn acetate or Zn sulfate at high concentrations greatly inhibit *S. typhimurium* under *in vitro* aerobic and anaerobic atmospheric conditions (Park *et al.*, 2002). Likewise, feeding moderate and high Zn molting diets has been shown to decrease *S. enteritidis* colonization in laying hens compared to feed deprived hens (Kubena *et al.*, 2001; Ricke *et al.*, 2001). This may indicate that Zn is bactericidal in the gastrointestinal tract as well, since *Salmonella* can come in contact with the gastrointestinal tract via an oral route and colonize the intestine or invade the epithelial cells of the small intestine (Finlay, 1994).

Zn supplemented diets at high concentrations may also indirectly decrease *Salmonella* infections by limiting *Salmonella* growth in the feed. Consequently, Zn anion combinations which display synergistic efficacy could dictate the most effective means for providing Zn as a supplement. Organic acids have commonly been used a food and animal feed additives and are known to exhibit antibacterial properties in these matrices (Khan and Katamay, 1969; Cherrington *et al.*, 1991). Acetic acid addition has been reported to reduce the number of *Enterobacteriaceae* and *Salmonella* in feed (Vanderwal, 1979). Propionic acid and its salts have been used in poultry feed as effective mold inhibitors (Stewart *et al.*, 1977; Paster, 1979; Dixon and Hamilton, 1981). It has also been demonstrated that high concentrations of propionic acid can decrease *Salmonella spp.* in poultry feed (Vanderwal, 1979; Hume *et al.*, 1993; Matlho *et al.*, 1997; Ha *et al.*, 1998a). The present study was conducted to examine the effects of Zn compound as Zn acetate or Zn propionate added at 1% concentration of Zn to poultry layer ration, on survival of *S. typhimurium* inoculated in a dry form to simulate storage conditions for commercial poultry feeds.

Materials and Methods

Microorganism and Preparation of Inoculation Methods

A primary poultry isolate of *Salmonella typhimurium* previously selected for resistance to novobiocin (NO) and nalidixic acid (NA) was used as a marker strain for recovery studies (Ziprin *et al.*, 1990). Tryptic soy broth (TSB, Difco Laboratories, Detroit, MI) was used for maintenance and growth of the bacterial strain. For bacterial

culture maintenance, NO (25 µg/ml) and NA (25 µg/ml) were added for the *S. typhimurium* poultry isolate (NO/NA) strain. Cells grown for 18 to 24 h at 37 °C on TSB were then applied to blocks of chalk for preparing dry inoculum (Hoffmans and Fung, 1993). Approximately, 1 g of charged chalk [10^8 *S. typhimurium* colony forming units (CFU) g⁻¹] was aseptically added to 100 g of each poultry mash. Inoculated feed was placed in a sterile stomacher bag (Stomacher 400, Seward Medical, London, UK) to give an initial Salmonella concentration of 10^6 CFU g⁻¹ mash. The method for mixing was based on the approach of Ha et al. (1998a). Briefly, the bags were closed by twisting and the contents were vigorously shaken manually for 1 min (120 times at a 45 °angle).

Mash Composition and Treatments

Soybean meal-based poultry mash containing 200 g crude proteins kg⁻¹ diet was formulated. Composition of the mash is presented in Table 8-1. Zn acetate [Zn(C₂H₃O₂)₂·2H₂O] (Sigma Chemical Co., St. Louis, MO) or Zn propionate [Zn(C₃H₅O₂)₂] (Kemin Americas, Inc., Des Moines, IA) at 1% concentration of Zn was added to poultry layer mash. For non-Zn salts additive trials, 1% sodium propionate [C₃H₅O₂Na](Sigma Chemical Co., St. Louis, MO) or 1% sodium acetate [C₂H₃O₂Na·3H₂O] was added to poultry mash. Organic acids were not added to control mash of poultry layer ration, and the controls were inoculated with *S. typhimurium* by the same methods and enumerated on the same days as Zn amended mash/salts of Zn

TABLE 8-1. Composition of the soybean meal-based diet

Ingredients	Amount (g kg ⁻¹ mash) ^a
Corn, yellow	567.18
Soybean meal	316.33
Vegetable oil	76.82
Mono-Ca phosphate	16.86
Calcium carbonate	15.62
Methionine 98%	1.69
Vitamine premix ^b	2.50
Salt (NaCl)	2.50
Trace mineral premix ^c	0.5
Total	1000.00

^a For diet formulations, crude fat concentrations were fixed at 100 g kg⁻¹

^b Provides mg kg⁻¹ of diet unless otherwise noted: vitamin A, 8818 IU; vitamin D, 2205 IU; vitamin E, 5.86 IU; vitamin K, 2.2; thiamine, 1.1; riboflavin, 4.4; niacin, 22; pantothenic acid; choline, 500; vitamin B12, 0.013; biotin, 0.055

^c Trace mineral premix, Nutrius Premix Division, Bioproducts Inc., Cleveland, OH 44141. Provides mg kg⁻¹ of diet unless otherwise noted: Mn, 68.2; Zn, 55; Cu, 4.4; I, 1.1g; Se, 0.1

amended mash for comparison. Before adding the chemicals to poultry layer mash, the poultry layer mash was sterilized in an autoclave for 15 min at 121 °C to eliminate indigenous feed fungi and bacteria.

Feed Storage Study

Triplicate samples (1 g) for Zn amended mash or duplicate samples for salts of Zn amended mash transferred into sterile petri dishes were stored at room temperature (21 ± 1 °C). These samples were analyzed initially (day 0) and after 1, 3, 5, 7, and 9 days of storage. Moisture contents of Zn amended mash were determined by drying 2 g samples of feed on aluminum pans at 105 °C for 20 to 24 h until constant weights were attained.

Microbiological Analysis

The samples in petri dishes were directly poured into sterile disposable centrifuge tubes and were vortexed for 30 s with 9 ml of sterile phosphate buffer (0.31 mM of KH_2PO_4 and 0.22 mM of NaOH adjusted to pH 7.2). Serial dilution of the samples were made with sterile phosphate buffer by standard methods (Swanson *et al.*, 1992). *S. typhimurium* NO-NA were plated on brilliant green agar (BGA, Difco Laboratories, Detroit, MI) plates containing 25 μg NO-NA ml^{-1} (Nisbet *et al.*, 1993). The plates were incubated at 37 °C for 48 h. The number of *S. typhimurium* NO-NA were determined by colony enumeration and results were expressed as \log_{10} CFU g^{-1} .

Statistical Analysis

Colony enumerations expressed as logarithmic functions were analyzed by least

squares mean separations which were accomplished by the Pdiff option of the GLM procedure in the SAS statistical analysis software program, version 6.04 (SAS, 1988). Model and parameter adequacy were considered significant at $P < 0.05$ level unless otherwise noted. Each mean was the average of three (Figure 8-1) or two samples (Figure 8-2), and means were considered significantly different at $P < 0.05$.

For the calculations of the slope of *Salmonella* population, a linear regression was performed for each triplicate or replicate versus time, and correlation coefficients (R^2) were calculated. The resulting slopes were analyzed for differences by regression analysis with the equation $y = \beta_0 + \beta_1 x_1 + \epsilon$, where x_1 = a class variable representing the matrix in which *Salmonella* spp. survived on the different poultry mash. Least squares mean slope differences were analyzed by SAS as described above and considered significant at $P < 0.05$.

Results and Discussion

An evaluation of the effects of two different Zn compounds on survival of *S. typhimurium* in sterile poultry layer ration was performed in this study. We used a total of 9 days of storage to recover *S. typhimurium* in sterile poultry layer ration because 9 days were typically used in previous *Salmonella* infection studies as the period of an induction molt in laying hens by the use of either feed withdrawal or high concentrations of dietary Zn feed (1% Zn) added to layer ration (Corrier *et al.*, 1997; Durant *et al.*, 1999; Kubena *et al.*, 2001). Because Duncan and Adams (1972) noted that different feed compositions may affect survivability of bacteria through differing water activities,

S. typhimurium was added as in a dry chalk carrier form to poultry feeds in this study. The dry inoculation method to avoid potential osmotic shock or a change in the water activity (a_w) in the feed was based on Hoffmans and Fung (1993), and Ha *et al.* (1998a). Interaction of storage days and feed types significantly ($P < 0.05$) influenced the moisture content in feeds (Figure 8-1). Although the days of storage did not ($P > 0.05$) significantly influence moisture contents in feed, the Zn propionate and Zn acetate amended feed contained more ($P < 0.05$) moisture (%) than layer ration. The moisture contents of Zn propionate amended feed, Zn acetate amended feed, and layer ration were 10.91, 10.87, and 10.30%, respectively. This result may indicate that Zn propionate or Zn acetate in a layer ration are hygroscopic and could absorb more water than feeds where these salts were not added. The chemicals in the form of either powder of Zn acetate or Zn propionate particles may influence moisture contents in feed. Choi and Chan (2002), and Peng *et al.* (2001) reported that water soluble organic acids such as succinic acid, malonic acid, citric acid, and glutaric acid and glycerol can easily absorb water from the atmosphere. These water soluble organic compounds possess hygroscopic properties of atmospheric aerosols. The effect of organic compounds on the hygroscopic properties of atmospheric aerosols is dependent on relative humidity (RH) (Choi and Chan, 2002). At low RH (<30%), a significant amount of the inorganics become crystallized, but the mixtures may contain water due to the presence of nondeliquescent organic acids (Seinfeld, 1989; Choi and Chan, 2002). At moderate RH,

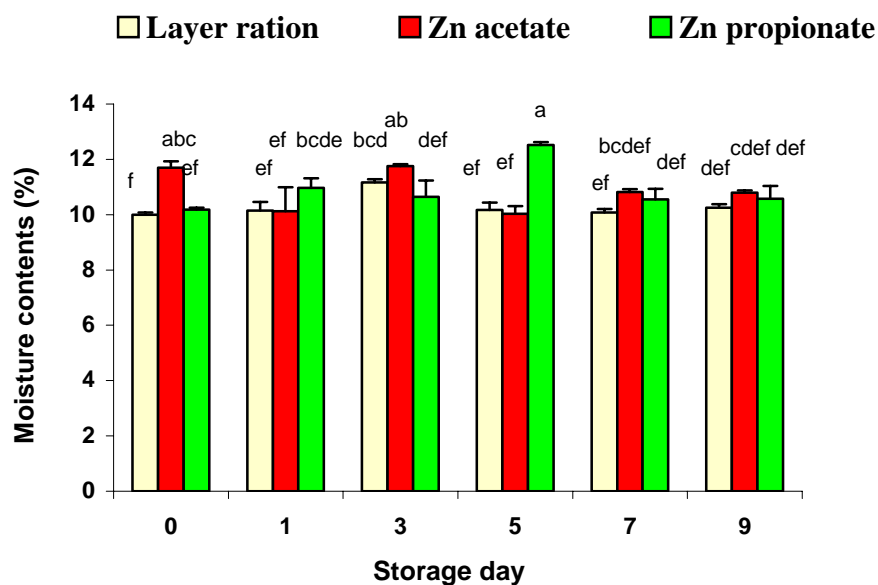


FIGURE 8-1. Moisture contents of poultry layer ration, Zn acetate (1% zinc) amended feed, and Zn propionate (1% zinc) amended feed over 9 days of storage at room temperature. The data represent means with standard errors (three samples/days/treatment). Means with no common superscript letters differ significantly ($P < 0.05$)

the effect is complex due to the change of both deliquescence RH and crystallization RH (Choi and Chan, 2002). At high RH (>80%), aerosol particles become completely deliquesced (Choi and Chan, 2002).

The feed types (Zn amended mash) did not ($P > 0.05$) significantly influence the slope estimations of the *Salmonella* decay curves (Table 8-2). The values of linear slope on Zn acetate amended feed and Zn propionate feed were -0.21 and -0.30, respectively. These decay values were approximately 100-fold times more than the slopes (-0.0079 to -0.0111) calculated for survival over 56 days in poultry mash containing soybean meal at different concentrations (Ha *et al.*, 1998b).

The recovery of *S. typhimurium* was not ($P > 0.05$) significantly affected by the interaction of days of storage and feed types (Figure 8-2). However, storage days or feed types as single independent variables significantly ($P < 0.05$) influenced survivability of *S. typhimurium*. *S. typhimurium* populations were significantly ($P < 0.05$) decreased by stepwise increases in days of storage. The overall average counts of *S. typhimurium* on Zn propionate amended feed over 9 days of storage were lower than on Zn acetate amended feed or layer ration and were 2.67, 3.06, and 3.44 \log_{10} (CFU/g), respectively although the initial *S. typhimurium* counts on feeds were similar (4.11 from Zn propionate amended feed, 4.28 from Zn acetate feed, and 4.32 \log_{10} (CFU/g) from layer ration). Over 90% reduction of *S. typhimurium* population occurred in both Zn propionate and Zn acetate amended feed by day 3 and layer ration by day 5 of storage. More than 99% reduction occurred in Zn amended layer ration in the form of either Zn acetate (2.55 \log_{10} (CFU/g), 99.72%) or Zn propionate (2.40 \log_{10} (CFU/g), 99.60%) by

TABLE 8-2. Decline of *Salmonella typhimurium* populations in different poultry feed over 9 days of storage at room temperature

Feeds	<i>Salmonella</i> decay slope ¹ , (R ²)
Zn amended mash	
Layer ration	-0.257±0.131 (0.300)
Zn acetate ²	-0.210±0.117 (0.289)
Zn propionate ³	-0.301±0.132 (0.341)
Na amended mash	
Layer ration	-0.160±0.152 (0.155)
Na acetate ⁴	-0.134±0.134 (0.143)
Na propionate ⁵	-0.111±0.105 (0.157)

¹Calculated as the change of *Salmonella typhimurium* populations (log₁₀ colony forming units g⁻¹) in poultry mash over time (days)

²Zn acetate = a feed containing 10,000 mg/kg zinc as zinc acetate

³Zn propionate = a feed containing 10,000 mg/kg zinc as zinc propionate

⁴Na acetate = a feed containing 10,000 mg/kg sodium acetate

⁵Na propionate = a feed containing 10,000 mg/kg sodium propionate

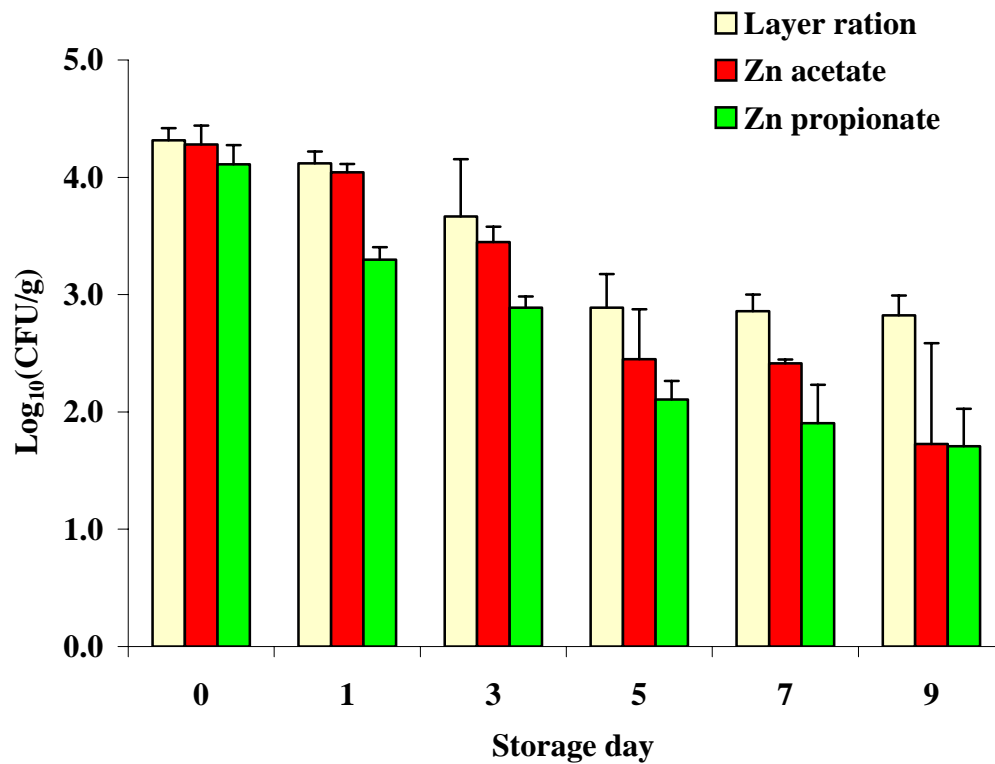


FIGURE 8-2. Survival of *Salmonella typhimurium* populations in poultry layer ration, Zn acetate (1% zinc) amended feed, and Zn propionate (1% zinc) amended feed over 9 days of storage at room temperature. The data represent means with standard errors (three samples/days/treatment)

day 9 of storage when compared to layer ration ($1.49 \log_{10}(\text{CFU/g})$, 96.76%). The reductions seen in the current study for *Salmonella* under dry conditions are in agreement with previous *in vitro* liquid culture studies and *in vivo* infection studies. Park *et al.* (2002) recently reported that Zn may inhibit *S. typhimurium* under *in vitro* aerobic or anaerobic atmospheric conditions. Ricke *et al.* (2001) and Kubena *et al.* (2001) demonstrated that Zn molting diets could decrease *S. enteritidis* colonization in laying hens compared to feed deprived hens. Although the effects of Zn on the growth or colonization of *Salmonella* was not determined in the current study, the results provide evidence that Zn may limit *S. typhimurium* survival in poultry feed as well.

In a second series of studies, we added 1% Na acetate and Na propionate to layer mash to compare the effects of salts form of Zn compounds on the survivability of *S. typhimurium*. The feed types (Na amended mash) did not ($P > 0.05$) significantly alter the slope of *Salmonella* curves when compared to the nonamended layer ration (Table 8-2). The values of linear slope on Na acetate amended feed and Na propionate feed were -0.13 and -0.11, respectively. As noted for the zinc studies, these values were approximately 100-fold more than the previous work with poultry mash containing soybean meal (Ha *et al.*, 1998b). However, in the previous work survival was monitored over a much longer period of time (56 days).

The recovery of *S. typhimurium* was not ($P > 0.05$) significantly affected by the interaction of days of storage and feed types (Figure 8-3). However, days of storage or feed types when statistically analyzed as single independent variables significantly ($P < 0.05$) influenced survivability of *S. typhimurium*. *S. typhimurium* population was

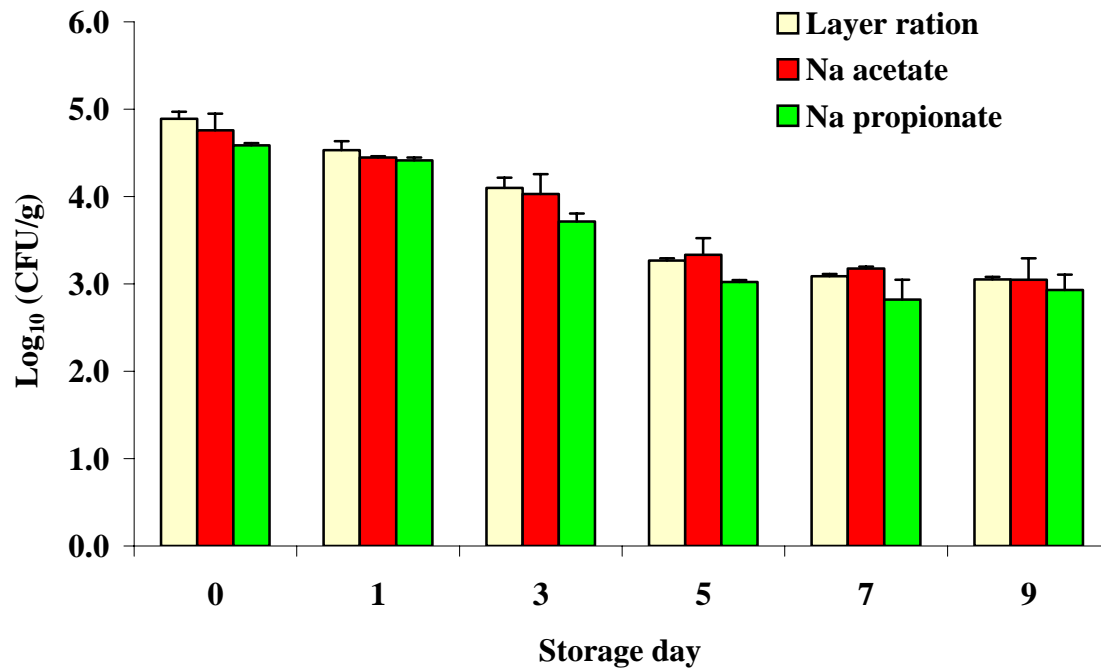


FIGURE 8-3. Survival of *Salmonella typhimurium* populations in poultry layer ration, 1% Na acetate amended feed, and 1% Na propionate amended feed over 9 days of storage at room temperature. The data represent means with standard errors (three samples/days/treatment)

significantly ($P < 0.05$) decreased by stepwise increase in days of storage. The average counts of *S. typhimurium* on Na propionate amended feed were significantly ($P < 0.05$) lower than by Na acetate amended feed or by layer ration. Overall average counts of *S. typhimurium* were reduced 39.74% more by Na propionate than by Na acetate amended feed. Reduction in *S. typhimurium* counts by layer ration, Na acetate or Na propionate amended feed from day 0 to day 9 of storage was approximately $1.84 \log_{10}(\text{CFU/g})$ (98.55%), 1.71 (98.05), and 1.66 (97.81), respectively. Over 97% reduction in *S. typhimurium* counts occurred in both Na acetate and Na propionate amended feed by day 5 of storage. These observations concur with previous reports in related studies. Kwon *et al.* (1998) and Kwon and Ricke (1999) reported that *S. typhimurium* growth in rich media was gradually decreased as the concentrations of propionic acid or Na propionate increased. Ha *et al.* (1998a) suggested that higher concentrations of buffered propionic acid may have the potential for reducing fungal and *Salmonella* spp. contamination in poultry mash.

In conclusion, *Salmonella* population was over 92% more reduced by Zn amended feed (1% Zn) in the form of either Zn acetate or Zn propionate compared to the populations by layer ration. This may be due to the combination of antibacterial effects of Zn and organic acid in the form of either acetate or propionate. In this study, the counts of *S. typhimurium* were approximately 56% more reduced by Zn propionate amended feed than by Zn acetate amended feed. This study indicated that compared to Zn acetate, Zn propionate at 1% of Zn may have a greater ($P < 0.05$) potential to inhibit survival of *S. typhimurim* in the molting feed. Also, this study suggested that high

dietary Zn compounds may function as a potential barrier to interrupt the transmission of *Salmonella* since the spread of *Salmonella* spp. has been suggested to be cyclic between human and poultry hosts and the environment including poultry feed (Williams, 1981). Use of these amended poultry feeds may be particularly important during times when increased horizontal spread of *Salmonella* transmission in poultry houses is known to occur such as during molting to induce a second egg laying cycle (Nakamura *et al.*, 1994; Holt, 1995; Holt *et al.*, 1998).

CHAPTER IX

**EFFECTS OF HIGH ZINC DIETS USING ZINC PROPIONATE ON
MOLT INDUCTION, ORGANS, AND POSTMOLT EGG
PRODUCTION AND QUALITY IN LAYING HENS***

Overview

This study was conducted to determine the ability of an alternative salt form of 1% zinc, Zn propionate, to induce molt in 66 wk-old hens. The hens were randomly assigned to 4 treatment groups of 27 or 28 birds each, either a) molted conventionally by feed withdrawal b) 1% zinc as Zn acetate c) 1% zinc as Zn propionate or d) non-molted control for 9 days. Feed intake was significantly ($P < 0.05$) depressed in both Zn acetate and Zn propionate hens when compared to non-molted control hens during the 9 days. Ovary weight of hens undergoing feed withdrawal, Zn acetate, or Zn propionate were not ($P > 0.05$) significantly different from each other, but all were significantly ($P < 0.05$) lighter than the ovary weight of non-molted control hens. Zinc concentrations in the kidney and liver were significantly ($P < 0.05$) increased in both Zn acetate and Zn propionate molted hens when compared to either non-molted control fed hens or feed withdrawal molted hens. Bone ash were significantly ($P < 0.05$) increased in both Zn acetate and Zn propionate molted hens or non-molted control hens as compared to feed withdrawal molted hens. Over the entire 3-month postmolt period, there were no

* Reprinted with permission from: Effects of high zinc diets using zinc propionate on molt induction, organs, and postmolt egg production and quality in laying hens, by Park *et al.*, 2003, Poultry Sci. Accepted.

significant differences in interior egg qualities but egg weights from Zn propionate fed hens was significantly ($P < 0.05$) heavier than that from feed withdrawal treatment hens. The data of the current study demonstrated that feeding a Zn propionate (1% zinc) supplemented diet can induce molt.

Introduction

In nature, all adult avian species undergo annually bird molting to renew their feathers. This results in body weight losses up to 40% of their mass (Mrosovsky and Sherry, 1980), and a pause in oviposition due to regression of the reproductive tract (Brake and Thaxton, 1979). After completion of the molting process, the reproductive tracts of old birds more closely resemble those of young birds (Brake and Thaxton, 1979).

In the U. S. commercial layer industry, older hens can be artificially induced to molt before the end of a first laying cycle, rested, and enter into a second egg laying cycle (North and Bell, 2002). The most commonly practiced method of molt induction is by the withdrawal of feed for a period of several days. This is an efficient method to induce a molt because it is easy management friendly, economically advantageous and results in satisfactory postmolt performance for the commercial layer industry (Brake, 1993). However, increased public awareness of the animal stress associated with feed withdrawal has led researches to investigate alternative molting processes. Additionally, the stress results in increased susceptibility to *Salmonella enteritidis* infection (Holt and

Porter, 1992; Holt, 1993; Holt *et al.*, 1995); this may lead to increased risk of food borne illness to consumers of these products.

Molting induction by feeding hens a diet containing high levels, 10,000 to 20,000 ppm of added zinc as Zn oxide or Zn acetate results in egg production cessation within 5 days (Scott and Creger, 1976; Creger and Scott, 1977). Berry and Brake (1985) reported that the high zinc fed hens stopped ovulating up to a full day sooner than did fasted hens. In several studies, it has also been reported that the effectiveness of zinc to induce follicular atresia and halt egg laying is probably caused by this cation's ability to depress feed intake (Scott and Creger, 1976; Shippee *et al.*, 1979; Berry and Brake 1985; McCormick and Cunningham, 1987).

Propionic acid at higher concentrations may reduce chicks' feed intake by decreasing palatability (Ryś and Koreleski, 1974; Cave, 1982, 1984). Previously reported methods of dietary zinc for an induction molt were restricted in Zn acetate and Zn oxide. Therefore, the objectives of this study were to investigate as to whether Zn propionate, an alternative salt form of zinc, would induce molt, effect organ systems, egg quality and egg production as the hens entered the second cycle of laying in production.

Materials and Methods

General

One hundred forty two Single Comb White Leghorn (SCWL) hens, 66 wk of age, were obtained from a commercial laying facility. Hens were housed one per cage (30 x 35 cm). The hens were maintained under an artificial lighting program of hours of light

(L) and dark (D) of 16L: 8D and provided *ad libitum* access to a complete layer ration and water via nipple drinkers to prevent cross-contamination of water and feed for a period of 8 wks prior to molting. The individual feeders were stainless steel. During the 8 wk acclimation period, egg production was monitored to insure that all hens were healthy and in active production.

Molting Procedure

After acclimation, one hundred ten hens were moved into a nearby house and housed two per cage for the molting procedure. Hens were assigned to one of the following four treatment groups: 27 hens to Zn acetate (1% of Zn added to layer ration) diet; 27 hens to Zn propionate (1% of Zn added to layer ration) diet; 28 hens fed layer ration without additives to serve as the non-molted control; or 28 hens to feed withdrawal (negative control). All hens were allowed *ad libitum* access to water and their respective diets. Hens were placed on an artificial lighting program of 8L: 16D for 1 wk prior to molting procedure. Treatments were randomly assigned to cages throughout the house to ensure there was no variability in egg production or reproductive tract regression due to light stimulation.

During the molt procedure, hen weights were monitored at day 1, 3, 5, 7, and 9. In accordance with Texas A&M University Lab Animal Care Committee (ULACC) animal use protocols, any hen reaching 25% weight loss prior to the end of the trial (day 9) were removed from their respective diet and immediately placed on full feed. After molting, the remaining hens were placed on fed layer ration (Table 9-1) *ad libitum*, and the light program changed to an artificial lighting program of 16L: 18D to

TABLE 9-1. Composition of TAMU layer ration^a

Ingredients	Amount (g kg ⁻¹ mash) ^b
Corn, yellow	567.18
Soybean meal	316.33
Vegetable oil	76.82
Mono-Ca phosphate	16.86
Calcium carbonate	15.62
Methionine 98%	1.69
Vitamine premix ^c	2.50
Salt (NaCl)	2.50
Trace mineral premix ^d	0.5
Total	1000.00

^aProvides mg kg⁻¹ of diet unless otherwise noted: Mn, 68.2; Zn, 55; Cu, 4.4; I, 1.1g; Se, 0.1.

^bFor diet formulations, crude fat concentrations were fixed at 100 g kg⁻¹.

^cProvides mg kg⁻¹ of diet unless otherwise noted: vitamin A, 8818 IU; vitamin D, 2205 IU; vitamin E, 5.86 IU; vitamin K, 2.2; thiamine, 1.1; riboflavin, 4.4; niacin, 22; pantothenic acid; choline, 500; vitamin B12, 0.013; biotin, 0.055.

^dTrace mineral premix, Nutrius Premix Division, Bioproducts Inc., Cleveland, OH 44141.

stimulate egg production. Daily egg production was monitored for 12 wks after the molting diets were replaced with full feed.

Collection of Organs and Determination of Crop pH

At the end of the molt procedure, 55 hens were euthanized with CO₂ gas according to the approved ULACC protocol, and the crop, ovary, kidney, liver, spleen, heart, proventriculus, gizzard, pancreas, and the entire intestine excised aseptically and weighed. Relative organ weights (grams per 100 g of body weight) were calculated (Edrington *et al.*, 1997) and presented as a percentage. The pH of crop was determined by the insertion of sterile glass pH electrode (Model 05669-20; Cole Palmer Model, Niles, IL) through an incision in the crop wall, ensuring that the electrode remained in contact with the crop mucosal surface as described by Ramirez *et al.* (1997).

Bone Shear Strength and Bone Ash

The right or left tibia of each of hens was randomly chosen from a group of hens, excised from the fresh carcass, and defleshed. The tibias were individually sealed in 4oz plastic Whirl-Pak bag (Nasco, Fort Atkinson, WI) to minimize moisture loss, and stored at 4 °C. The tibias were dried at 105 °C for 24 h, cooled to room temperature in a desiccator, and weighed to the nearest hundredth gram. Tibia shear strengths (breaking force divided by bone weight expressed as kilograms per gram) (Shafer *et al.*, 2001) were conducted using an Model 1011 Instron (Instron Corp., Canton, MA) with 50-kg-load cell at 50-kg-load range with a crosshead speed of 50 mm/min with tibia supported on a 3.35-cm span. Tibia ash weights were determined by ashing in tared ceramic crucibles for 24 h at 615 °C (Shafer *et al.*, 2001). Percentage tibia ash was calculated by

dividing tibia ash weights by tibia dry weight and multiplying by 100 (AL-Batshan *et al.*, 1994).

Zinc Concentration in Kidney and Liver

Approximately 0.2 g (frozen) of each liver and kidney sample was dried at 75 °C for three days and subsequently predigested in nitric acid for three days. The predigested sample was further digested for two hours at 100 °C using an Inovative Microwave (CEM Corporation, Matthews, NC) (Davis, 1998; Dougherty, 2002). Atomic absorption spectrophotometry (Perkin-Elmer 4000 Atomic Absorption, Norwalk, CT) was used to determine zinc concentrations of the liver and kidney samples.

Egg Production and Quality Parameters

Egg production and quality were monitored and compared after molting. Egg weight (g) was measured using a balance and recorded to the nearest hundredth of a gram. Egg circumference (cm) was measured using a tape measure and recorded to the nearest tenth of a centimeter. Egg length (cm) and width (cm) were measured using a caliper and recorded to the nearest centimeter. Albumen height (mm) was measured using a micrometer and recorded to the nearest tenth of a millimeter. Shell thickness was evaluated using NaCl solutions whose specific gravity ranged from 1.065 to 1.110% in increments of 0.005%. This method using concentrations of NaCl solutions was based on an approach described by Kenshavarz and Quimby (2002) with minimal modifications. Shell strength (kg) was also measured using an Model 1011 Instron (Instron Corp., Canton, MA) with 50-kg-load cell at a 10-kg-load range with a crosshead speed of 50 mm/min. Yolk color was expressed as Hunter color, 'L', 'a', and 'b'

(Hunter and Harold, 1987) using a colorimeter (Model CR-200 Minolta, Minolta Corp., Ramsey, NJ). Each yolk pool was poured into a clean 60 x 15 mm glass Petri dish. A glass lid was placed flush against the yolk surface to prevent air pockets. The tip of the Chroma Meter measuring head was placed flat against the surface of the Petri dish and yolk reflective color was determined from the average of three consecutive pulses from the optical chamber of the Chroma Meter (Herber-McNeil and Van Elswyk, 1998).

Daily egg production was monitored for 8 wks before molting, and 12 wks after molting.

Statistical Analysis

Data were analyzed using a one-way ANOVA to analyze the differences among treatment groups (Feed withdrawal, Zn acetate, Zn propionate, and Layer ration) using SAS® (SAS Institute, 1985, Cary, NC) General Linear Models procedures. Differences among treatment groups, when significant, were compared using Duncan's multiple range test. Level of significance used in all results was ($P < 0.05$).

Results and Discussion

Crop pH

Hens fed 1% zinc in the form of either Zn acetate (5.98) or Zn propionate (5.68) chemical added layer ration did not exhibit significantly ($P > 0.05$) different crop pH levels when compared to hens that had undergone feed withdrawal (5.67). Nonmolted hens fed layer ration (4.89) had a significantly ($P < 0.05$) lower crop pH than all of molted hen treatments (Table 9-2). Humphrey *et al.* (1993) reported that when chickens are undergoing malnutrition or starvation, the pH of crop can increase due to decreased

TABLE 9-2. Effect of molting and nonmolting diets on the crop pH, feed intake, body weight loss and ovary weight of Single Comb White Leghorn (SCWL) hens

Diet Treatment	Crop pH	Feed Intake (g/day)	Body Weight Lost (%)	Ovary Weight (g)
Feedwithdrawal ¹	5.67 ± 0.08 ^a	N/A ⁵	25.12± 1.51 ^c	6.13± 0.73 ^b
Zn acetate ²	5.98 ± 0.09 ^a	22.61± 3.27 ^b	15.52± 1.44 ^b	7.43± 0.65 ^b
Zn propionate ³	5.68 ± 0.15 ^a	26.59± 4.34 ^b	15.66± 1.43 ^b	6.34± 0.92 ^b
Layer ration ⁴	4.89 ± 0.08 ^b	76.29± 6.36 ^a	1.15± 0.81 ^a	31.04±5.01 ^a

^{a-c}Means within a column with no common superscripts differ significantly ($P < 0.05$)

¹Feed withdrawal = hens that were undergoing feed withdrawal for 9 days

²Zn acetate = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

³Zn propionate = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

⁴Layer ration =(nonmolted control) hens that received TAMU layer ration for 9 days

⁵N/A = not applicable

Lactobacillus fermentation within the crop. Feed withdrawal for 9 days resulted in a decrease in lactic acid in the crop, accompanied by an increase in crop pH (Durant *et al.*, 1999). During the present study, the effects of dietary molting treatments on the population of the *Lactobacilli* and lactic acid concentration in the crop were not determined. However, the results of the present study may indicate that the increases in crop pH by dietary molting treatment either feed withdrawal, Zn acetate, or Zn propionate feeding may reduce either the normal resident *Lactobacilli* population or lactic acid concentration in the crop. Zn acetate, or Zn propionate feeding may be inhibitory to *Lactobacilli* population due to the effect of zinc on microorganism growth. Dietary zinc may influence growth and infectivity of bacterial pathogens in animal. Park *et al.* (2002) recently reported that zinc may inhibit *S. typhimurium* under *in vitro* aerobic or anaerobic atmospheric conditions. Earlier studies have shown that zinc molting diets have been shown to decrease *S. enteritidis* colonization in laying hens compared to feed deprived hens (Kubena *et al.*, 2001; Ricke *et al.*, 2001). Reduced feed intake may be the main factor causing the decrease in the *Lactobacilli* population (Humphrey *et al.*, 1993; Corrier *et al.*, 1999; Durant *et al.*, 1999), thereby allowing the pH to rise in hens that are deprived of feed.

Feed Intake

Hens fed the non-molted control diet had significantly ($P < 0.05$) greater feed intake (76.29 g/bird/day) than did hens fed 1% zinc in the form of either Zn acetate or Zn propionate. However, there was no significant ($P > 0.05$) difference in feed intake between hens fed Zn acetate (22.61 g/bird/day) or hens fed Zn propionate (26.59

g/bird/day) (Table 9-2). There were approximately 65 and 70% reduction of feed intake in Zn propionate fed hens and Zn acetate fed hens, respectively, when compared to the feed intake of hens fed non-molted control layer ration.

Similar feed intake reduction was seen by Shippee and coworkers (1979) who reported that 1% zinc as Zn oxide or Zn acetate resulted in the average daily feed intake of 22 g/bird and 16 g/bird, respectively, during the second week of the forced resting period when hens reached 0 % egg production. The reduced feed intake could be due to appetite depression (Brink *et al.*, 1950) or low palatability of high levels of zinc (Fox, 1989). It has also been reported that the reduced feed intake could be due to the ability of zinc cation (Zn^{2+}) to induce follicular atresia and halt egg laying (Scott and Creger, 1976; Shippee *et al.*, 1979; Berry and Brake, 1985; McCormick and Cunningham, 1987; Johnson and Brake 1992). Therefore, it is likely that the efficiency of high dietary zinc treatments as a method to induce molt in this study was directly related to the suppression of feed intake.

Body Weight Loss

Hens undergoing feed withdrawal (25.12%) had significantly more ($P < 0.05$) body weight loss than hens fed 1% zinc in the form of either Zn acetate or Zn propionate. However, there was no significant ($P > 0.05$) difference in body weight loss between hens fed Zn acetate (15.52%) and hens fed Zn propionate (15.60%). Non-molted hens had the least body weight loss (1.15%) compared to other treatments of molted hens (Table 9-2).

The extent of body weight loss by Zn acetate or Zn propionate feeding was similar to values previous study by McCormick and Cunningham (1987) who reported that body weight loss during the 4 days for fasted and zinc fed hens was 16.4% and 15.2%, respectively. The body weight loss is a major factor contributing to the induced molting because body weight loss has influence on the successful results of an induced molting procedure (Brake and Thaxton, 1979; Brake *et al.*, 1981; Brake and McDaniel, 1981; Baker *et al.*, 1983). Baker and coworkers (1983) suggest that the extreme body weight loss during the induced molt is directly related to a hen's postmolt performance. Approximately 25% body weight loss in hens while subjected to feed withdrawal has been directly associated with decreased muscle weight, decreased liver weight, decreased use of adipose tissue, involution of the reproductive tissue, and greater reproductive regression (Brake and Thaxton, 1979; Berry and Brake, 1985).

Ovarian Weight

The regression of the ovary is the most important factor for induced molt because the loss of reproductive weight may be linked to the overall rejuvenation process (Brake and Thaxton, 1979). Therefore, ovarian weight was measured in the current study as an indication of molting.

Hens fed 1% zinc in the form of either Zn acetate (7.43 g) or Zn propionate (6.34 g) did not have significantly ($P > 0.05$) different ovarian weights when compared to hens that were undergoing feed withdrawal (6.13 g). Nonmolted hens fed the layer ration had a significantly ($P < 0.05$) higher ovarian weights (31.04 g) when compared to all other molted hen dietary treatments (Table 9-2).

Berry and Brake (1985) reported that the ovarian weights of hens fed 2% zinc as Zn oxide for 8 days were 9.00 g. McCormick and Cunningham (1984) reported that high zinc fed hens can cause reproductive involution. Although we did not monitor or measure for reproductive involution in the current study, hens treated both by high dietary zinc as Zn acetate or Zn propionate may have experienced reproductive involution as indicated by substantial ovarian regression and reduced ovarian weight. The results may have an important indication that the major effects of high dietary zinc on the reproductive organs may be somewhat linked general physiological state of high zinc fed hens (Berry and Brake, 1985).

Organ Weight

The proventriculus, gizzard, and intestines of chickens fed high doses of zinc appear normal and there are generally no histological changes (Dewar *et al.*, 1983). According to Fosmire (1990), zinc is relatively nontoxic to animals and human; both exhibit considerable tolerance to high intakes of zinc. However, 1-2% zinc in the diet resulted in anemia in rat (Duncan *et al.*, 1953), reduced growth rates in chicks (Roberson and Schaible, 1960), and high mortality in chicks (Blalock and Hill, 1988). Therefore, the relative organ weights were measured in the current study to investigate whether feeding 1% zinc feeding may influence the hens' overall health condition based on each organ when compared to those of nonmolted hens or molted hens by feed withdrawal. There were no significant ($P > 0.05$) differences in relative weights of the heart, pancreas, intestine, or kidney between all molted hens and non-molted hens (Table 9-3). Hens fed Zn propionate had significantly ($P > 0.05$) higher relative liver weight (2.11%)

TABLE 9-3. Effect of molting and nonmolting diets on the relative organ weight¹ of Single Comb White Leghorn (SCWL) hens

Diet Treatment	Liver (g)	Spleen (g)	Heart (g)	Proventriculus (g)
Feed withdrawal ²	1.74±0.09 ^b	0.10±0.006 ^b	0.47±0.03	0.42±0.01 ^b
Zn acetate ³	1.90±0.10 ^{ab}	0.12±0.009 ^{ab}	0.41±0.02	0.49±0.02 ^a
Zn propionate ⁴	2.11±0.09 ^a	0.13±0.007 ^a	0.41±0.02	0.44±0.02 ^b
Layer ration ⁵	1.97±0.07 ^{ab}	0.10±0.005 ^b	0.39±0.03	0.42±0.01 ^b

Diet Treatment	Gizzard (g)	Pancrease (g)	Intestine (g)	Kidney (g)
Feed withdrawal ²	1.76±0.01 ^{ab}	0.16±0.02	3.28±0.26	0.21±0.02
Zn acetate ³	1.66±0.08 ^b	0.19±0.02	3.70±0.15	0.28±0.04
Zn propionate ⁴	1.84±0.05 ^a	0.17±0.01	3.92±0.20	0.29±0.03
Layer ration ⁵	1.42±0.03 ^c	0.19±0.02	3.35±0.29	0.27±0.02

^{a-c}Means within a column with no common superscripts differ significantly ($P < 0.05$)

¹Relative organ weight = organ weight/100g of body weight

²Feed withdrawal = hens that were undergoing feed withdrawal for 9 days

³Zn acetate = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

⁴Zn propionate = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

⁵Layer ration = (nonmolted control) hens that received TAMU layer ration for 9 days

than hens on feed withdrawal (1.74%). Brake and Thaxton (1979) and Berry and Brake (1985) noted that involution of the reproductive organs and the decrease in liver weight resulted in approximately 25% of the total body weight reduction. Berry and Brake (1985) noted that the decrease in liver weight resulted from the removal of hepatic energy stores as glycogen and lipids that were metabolized in the liver. The liver is the target organ of yolk phospholipoprotein synthesis, which is dependent on ovarian steroids, primarily estrogen (Sturkie, 1976). McCormick and Cunningham (1984) noted that glycogen in the liver may not have been depleted because zinc interferes with insulin secretion. They also noted that the possible mechanism of zinc detoxification in the liver, including a high rate of bile production and synthesis of large quantities of the zinc storage protein metallothionein, may have contributed to the maintenance of liver weight. Hens fed Zn propionate (0.13%) also exhibited significantly ($P > 0.05$) higher relative spleen weights than hens undergoing feed withdrawal (0.10 g) or non-molted control hens (0.10%). Hens fed Zn acetate (0.49%) had significantly ($P < 0.05$) higher relative proventriculus weight than hens fed Zn propionate (0.44%), hens undergoing feed withdrawal (0.42%), or non-molted hens (0.42%). However, hens fed Zn propionate (1.84%) had significantly ($P < 0.05$) higher relative gizzard weight than fed Zn acetate (1.66%) or non-molted hens (1.42%). Brake and Thaxton (1979) found that no consistent trends over trials were observed in the relative weight of the spleen from birds going through a forced molt.

No consistent trends were exhibited in the relative weights of organs in the current study. Compared to the feed withdrawal hens or non-molted control diet fed

hens, zinc fed hens obtained statistically higher, lower or the same value of relative organ weights and were dependent on organ type. Therefore, we could not definitively conclude that zinc feeding caused a harmful or toxic effect on these organs or overall health condition of the hens.

Crop pH

Hens fed 1% zinc in the form of either Zn acetate (5.98) or Zn propionate (5.68) chemical added layer ration did not exhibit significantly ($P > 0.05$) different crop pH levels when compared to hens that had undergone feed withdrawal (5.67). Non-molted control hens (4.89) had a significantly ($P < 0.05$) lower crop pH than molted hen treatments (Table 9-2).

Humphrey and coworkers (1993) reported that when chickens are undergoing malnutrition or starvation, the pH of crop can increase due to decreased *Lactobacillus* fermentation within the crop. Feed withdrawal for 9 days resulted in a decrease in lactic acid in the crop, accompanied by an increase in crop pH (Durant *et al.*, 1999). During the present study, the effects of dietary molting treatments on *Lactobacilli* populations and lactic acid concentrations in the crop were not determined. However, the results of the present study indicate that the increases in crop pH by dietary molting treatment for either feed withdrawal, Zn acetate, or Zn propionate regimens may reduce either the normal resident *Lactobacilli* population or lactic acid concentration in the crop. Zn acetate, or Zn propionate feeding may be inhibitory to *Lactobacilli* population due to the effect of zinc on microorganism growth. Dietary zinc may influence growth and infectivity of bacterial pathogens in animal. Park *et al.* (2002b) recently reported that

zinc compounds would inhibit *in vitro* aerobic or anaerobic growth of *S. typhimurium*. Kubena *et al.* (2001) and Ricke *et al.* (2001) observed that high zinc containing molting diets decreased *S. enteritidis* colonization in laying hens when compared to hens undergoing feed withdrawal. Reduced feed intake may be the main factor causing the decrease in the *Lactobacilli* population (Humphrey *et al.*, 1993; Corrier *et al.*, 1999; Durant *et al.*, 1999), thereby allowing the pH to rise in hens that are deprived of feed.

Bone Breaking Strength and Bone Ash

Although there were no significant ($P > 0.05$) differences in tibia breaking strength between all molted hens and nonmolted hens, tibia ash (%) of hens on feed withdrawal was significantly ($P < 0.05$) less than those of the other molted hens and non-molted hens (Table 9-4). The tibia ash (%) of feed withdrawal hens, Zn acetate fed hens, Zn propionate fed hens, and non-molted hens were 39.98, 45.32, 46.01, and 45.32%, respectively.

Bone-breaking force (Crenshaw *et al.*, 1981; Merkley, 1981; Ruff and Hughes, 1985), bone ash (Garlich *et al.*, 1982), and bone ash concentration (Garlich *et al.*, 1982; Cheng and Coon, 1990) have been used as indicators of bone status in the mineral nutrition of poultry and swine. In the current study, feed withdrawal hens had the lowest tibia ash (%) among treatment groups. This result may indicate that hens undergoing feed withdrawal have smaller amounts of mineral in tibia. This may be due to a rapid depletion of calcium reserves in fasting laying hens (Berry and Brake, 1985).

TABLE 9-4. Effect of molting and nonmolting diets on the bone breaking strength (kg/g)¹ and bone ash (%)² of Single Comb White Leghorn (SCWL) hens

Diet Treatment	Breaking strength (kg/g)	Bone ash (%)
Feed withdrawal ³	1.56±0.13	39.98±1.62 ^b
Zn acetate ⁴	2.34±0.26	46.78±1.79 ^a
Zn propionate ⁵	1.82±0.19	46.01±1.08 ^a
Layer ration ⁶	2.17±0.24	45.32±0.94 ^a

^{a-c}Means within a column with no common superscripts differ significantly ($P < 0.05$)

¹Bone breaking strength (kg/g) = tibia breaking force/tibia weight

²Bone ash (%) = (tibia ash weight/ tibia dry weight) x 100

³Feed withdrawal = hens that were undergoing feed withdrawal for 9 days

⁴Zn acetate = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

⁵Zn propionate = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

⁶Layer ration = (nonmolted control) hens that received TAMU layer ration for 9 days

Medullary bone is the storage site for the calcium necessary for egg shell calcification (Mueller *et al.*, 1964). Dietary calcium depletion of laying hens can cause a decrease in the amount in bone and an increase in the osteoid (Bloom *et al.*, 1958; Zambonin Zallone and Teti, 1981). The excess zinc consumed by the hens may have interacted with both blood and cellular calcium. Zinc may have interacted with calcium absorption or metabolism, probably through interference with metal containing enzymes (Underwood, 1977).

Zinc Concentrations in Kidney and Liver

The accumulation of zinc in various tissues is dependent on tissue type and zinc feeding (McCormick and Cunningham, 1987). Therefore, zinc concentrations in the kidney and liver in the current study were determined to investigate whether zinc feeding in the form of either Zn acetate or Zn propionate would affect or increase zinc concentration in these tissues.

Hens fed 1% zinc in the form of either Zn acetate (361.59 ppm) or Zn propionate (486.04 ppm) added to the layer ration had significantly ($P > 0.05$) higher zinc concentrations in kidney when compared to hens that were undergoing feed withdrawal (170.58 ppm) or non-molted hens (77.26 ppm) (Table 9-5). Hens fed 1% zinc in the form of either Zn acetate (1546.14 ppm) or Zn propionate (1661.59 ppm) diets had significantly ($P > 0.05$) higher zinc concentrations in the liver when compared to feed withdrawal hens (552.13 ppm) or non-molted hens (127.98 ppm) (Table 9-5). There was a significant increase in zinc concentrations in renal tissue of hens fed high zinc (1%) with approximately a 3.7 and 5.3 fold increase in the renal zinc concentrations from Zn

TABLE 9-5. Effect of molting and nonmolting diets on the concentrations of zinc in kidney and liver of Single Comb White Leghorn (SCWL) hens

Diet Treatment	Kidney (ppm)	Liver (ppm)
Feed withdrawal ¹	170.58±44.8 ^b	552.13±86.10 ^b
Zn acetate ²	361.59±80.34 ^a	1546.14±230.63 ^a
Zn propionate ³	486.04±35.00 ^a	1661.59±321.19 ^a
Layer ration ⁴	77.26±12.96 ^b	127.98±56.32 ^b

^{a-b}Means within a column with no common superscripts differ significantly ($P < 0.05$)

¹Feed withdrawal = hens that were undergoing feed withdrawal for 9 days

²Zn acetate = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

³Zn propionate = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

⁴Layer ration = (nonmolted control) hens that received TAMU layer ration for 9 days

acetate fed hens and Zn propionate fed hens, respectively, compared to that of feed withdrawal hens. Relative to kidney, the accumulation of zinc in liver was considerably higher in all molted hens and non-molted hens. There were approximately a 11 and 12 fold increase in the hepatic zinc concentrations from Zn acetate fed hens and Zn propionate fed hens, respectively, compared to that of feed withdrawal hens.

According to McCormick and Cunningham (1984), there was a 4-fold, 10-fold, and 27-fold increase in the concentration of zinc in the kidney, liver, and pancreas, respectively, after feeding either 1% zinc or 2% zinc as Zn oxide for 4 days. McCormick and Cunningham (1984), and McCormick and Cunningham (1987) also noted approximately 30% of the increase in zinc accumulation in kidney and liver was due to reduction of feed intake. Although urinary zinc excretion was not measured in the current study, we could speculate that urinary zinc concentrations may be increased during the molting process. The increase in urinary zinc excretion during starvation is considered to originate in skeletal muscle, which is being catabolized during starvation to provide a source of precursors for gluconeogenesis, amino acids for continuing protein synthetic activity by other tissues and substrate for oxidation by the muscle (Fell *et al.*, 1973). Elia *et al.* (1984) suggest that kidney, liver, and pancreas tissues have a “buffering” action with respect to the zinc released during the reduction of lean body mass, thus preventing excessive losses of zinc. Zinc concentrations in the liver and kidney may have accumulated due to the zinc (1%) feeding in the current study.

Egg Production

Percent hen-day egg production by four treatments on a weekly basis both during the pre-molt and during the post-molt is shown in Figure 9-1. As expected, non-molted control hens had a significantly ($P < 0.05$) higher level of egg production than hens from all other treatments in the first and second week after molting periods. However, there were no significant ($P > 0.05$) differences in overall egg production between molted hens and non-molted control hens in relation to body weight loss from week 3 to the end of 12 weeks after the molting periods (Figure 9- 2). This may be because age of the hens is an important factor in the overall response to induced molting (McCormick and Cunningham, 1987). Egg production in feed withdrawal hens, Zn acetate, and Zn propionate fed hens, and non-molted control hens from week 3 to the end of 12 weeks after molting was 66.38, 77.43, 70.31, and 71.04%, respectively. All molted hens reached more than 50% egg production from the third week to the end of trial. All molted hens reached more than 50% egg production from the third week to the end of trial. Zn propionate fed hens had 0% egg production on the first week postmolt, whereas Zn acetate fed hens had 2.04% egg production on the same week postmolt.

From the second week postmolt, all molted groups of hens initiated egg production. The egg production by hens fed 1% zinc in either Zn acetate or Zn propionate and hens undergoing feed withdrawal stopped by the fourth day and fifth day, respectively (data not shown). Although hens fed 1% zinc in the form of either Zn acetate or Zn propionate molted a day earlier than hens undergoing feed withdrawal in the current study, overall egg production was not significantly ($P > 0.05$) different for all

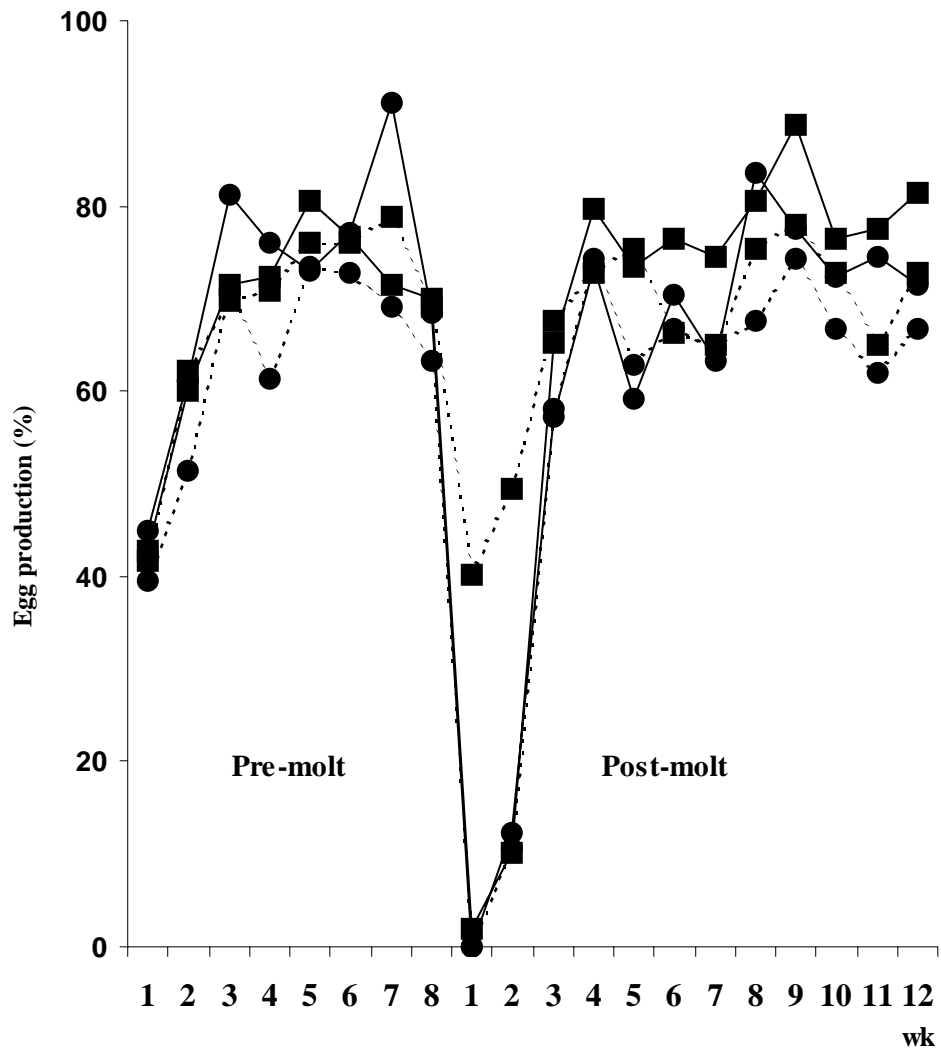


FIGURE 9-1. Percent hen-day egg production by four treatments on a weekly basis both during the pre-molt and during the post-molt. Solid line with ■ represents Zn acetate (1% zinc) treatment, solid line with ● represents Zn propionate (1% zinc) treatment, dashed line with ■ represents non-molted treatment, and dashed line with ● represents feed withdrawal treatment

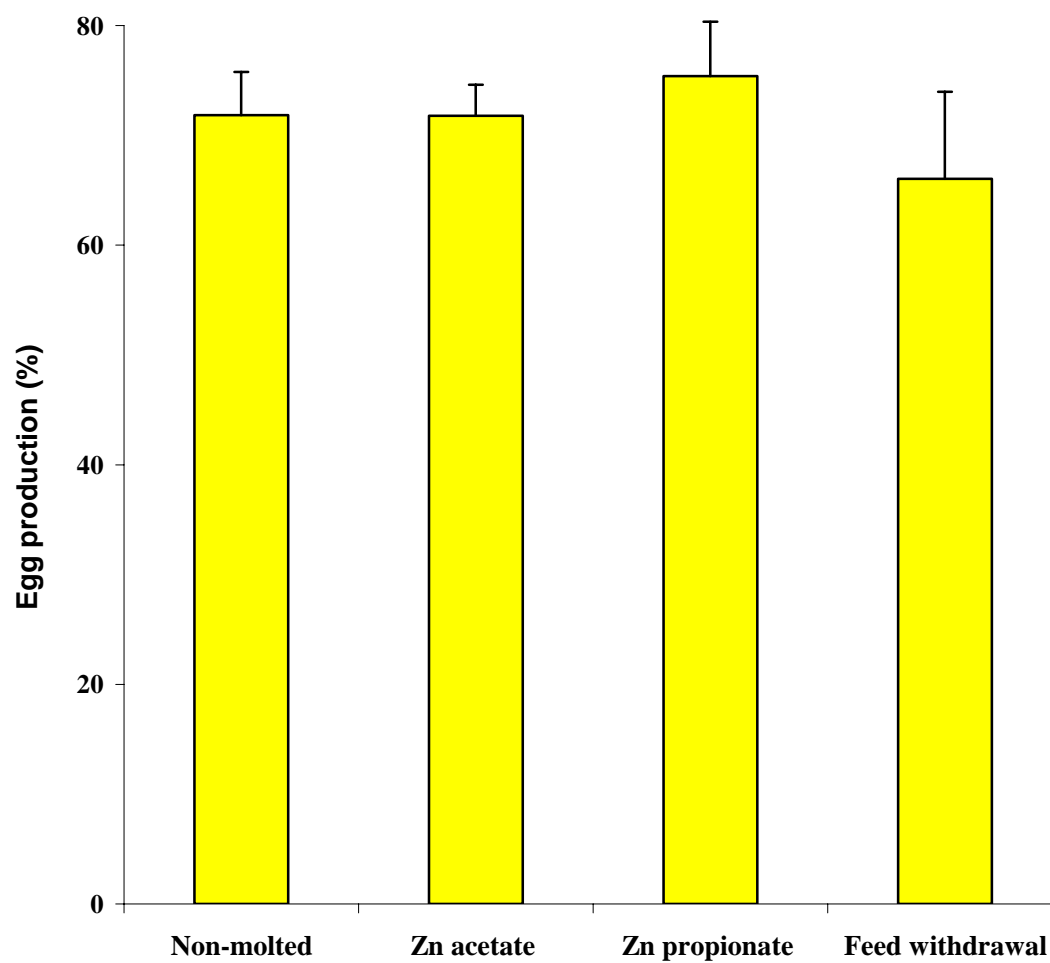


FIGURE 9-2. Percent average egg production after induced molting by four treatments (weeks 3 to 12). Hens were grouped into fasted, fed 1% zinc either as Zn acetate or Zn propionate, or fed non-molted control diet

molted hens. Shippee *et al.* (1979) reported that 1% zinc as Zn oxide or Zn acetate resulted in reduction of egg production from 60 to 0% within 6 days. Creger and Scott (1977) and Berry and Brake (1985) also reported that hens fed high zinc stopped ovulating a day earlier than fasted hens. The result may suggest that zinc has both a direct effect on reproduction organs distinct from that of fasting and a combination effect of the suppression of feed intake resulting in regressive reproduction organs and direct suppressive on the reproductive organs. Direct suppressive effect on the reproductive organs is independent of anorexia (Johnson and Brake, 1992). The overall egg production from by hens fed 1% zinc in the form of either Zn acetate or Zn propionate in the current study showed higher egg production than hens undergoing feed withdrawal.

Interior and Exterior Egg Quality

Interior and exterior egg quality in the current study were measured to investigate whether zinc feeding in the form of either Zn acetate or Zn propionate would alter interior or exterior quality in eggs. There were no significant ($P > 0.05$) differences in Hunter 'L' (lightness: 0 (black) to 100 (white)), 'a' (+: redness and -: greenness), or 'b' (+: yellowness, -: blue) or albumen height (mm) between molted hens and non-molted control hens during the postmolt periods (Table 9-6).

There were no significant ($P > 0.05$) differences in egg width, length, or breaking strength for any of the treatments (Table 9-7). Hens undergoing feed withdrawal (64.92 g) had significantly ($P < 0.05$) lower egg weights than Zn propionate fed hens (68.14 g) or non-molted control diet fed hens (68.37 g). Eggs from feed withdrawal hens (14.28

TABLE 9-6. Effect of molting and nonmolting diets on the internal egg quality of Single Comb White Leghorn (SCWL) hens during the postmolt

Diet Treatment	Hunter 'L' ¹	Hunter 'a' ²	Hunter 'b' ³	Albumen Height (mm)
Feed withdrawal ⁴	56.69±0.45	-2.00±0.14	42.55±0.48	7.36±0.26
Zn acetate ⁵	56.20±0.58	-1.97±0.11	41.35±0.74	7.56±0.28
Zn propionate ⁶	56.75±0.41	-2.07±0.11	41.90±0.51	7.10±0.37
Layer ration ⁷	55.67±0.42	-1.89±0.13	42.51±0.67	6.57±0.42

¹Hunter color 'L' values = lightness (0 = dark, 100 = bright)

²Hunter color 'a' values = redness/greenness (+ = red, - = green)

³Hunter color 'b' values = yellowness/blueness (+ = yellow, - = blue)

⁴Feed withdrawal = hens that were undergoing feed withdrawal for 9 days

⁵Zn acetate = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

⁶Zn propionate = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

⁷Layer ration =(nonmolted control) hens that received TAMU layer ration for 9 days

cm) were also significantly ($P < 0.05$) smaller in circumference than non-molted control hens (14.48 cm). Thick shell eggs exhibit higher specific gravity values (Keshavarz and Quimby, 2002). In the current study, specific gravity was not different in any of treatments (data not shown) and overall specific gravity of shell eggs was 1.07%. This was similar to values from the study by Nesbeth and coworkers (1976) who reported that specific gravity of eggs from both layer ration and no-salt layer ration (molting diet) after molting was 1.073%. Shippee and coworkers (1979) reported that no significant differences in shell thickness were observed for Zn acetate and Zn oxide molting treatments during the postmolt periods. However, Bar *et al.* (2003) recently reported that shell thickness was significantly increased by Zn oxide (2.5% zinc) molting treatment.

The values of breaking strength by Zn acetate hens (3.47 kg/g) were significantly ($P > 0.05$) higher from that of Zn propionate hens (3.14 kg/g), feed withdrawal hens (2.97 kg/g), or non-molted hens (2.88 kg/g)(Table 9-7). This may indicate that high zinc diets as Zn acetate are effective in improving shell quality based on shell strength.

Most interior and exterior quality parameters were not significantly different ($P > 0.05$) during 3 months in postmolt for any of the treatments. However, egg weights of hens fed 1% zinc as Zn propionate (68.14 g) were higher ($P < 0.05$) than those of feed withdrawal hens (64.92 g), but the egg weights of hens fed 1% zinc as Zn propionate were not significantly ($P > 0.05$) different from hens fed 1% zinc as Zn acetate (66.35 g) or the non-molted hens (68.37 g).

TABLE 9-7. Effect of molting and nonmolting diets on the external egg quality of Single Comb White Leghorn (SCWL) hens during the postmolt

Diet Treatment	Weight (g)	Width (cm)	Length (cm)
Feed withdrawal ¹	64.92±0.85 ^b	4.79±0.11	5.59±0.12
Zn acetate ²	66.35±0.78 ^{ab}	4.78±0.10	5.76±0.13
Zn propionate ³	68.14±0.67 ^a	4.96±0.13	5.64±0.13
Layer ration ⁴	68.37±1.20 ^a	4.88±0.14	5.70±0.12

Diet Treatment	Circumference (cm)	Breaking strength (kg/g)
Feed withdrawal ¹	14.28±0.06 ^b	2.97±0.15 ^b
Zn acetate ²	14.38±0.13 ^{ab}	3.47±0.23 ^a
Zn propionate ³	14.43±0.05 ^{ab}	3.14±0.15 ^b
Layer ration ⁴	14.48±0.08 ^a	2.88±0.24 ^b

^{a-b}Means within a column with no common superscripts differ significantly ($P < 0.05$)

¹Feed withdrawal = hens that were undergoing feed withdrawal for 9 days

²Zn acetate = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

³Zn propionate = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

⁴Layer ration = (nonmolted control) hens that received TAMU layer ration for 9 days

In general, egg size is larger during the second cycle than during the first cycle, and the shell quality and interior egg quality are better during the first cycle than during the second cycle (North and Bell, 1990). Zeelen (1975) also reported that egg size is increased significantly after induced molting with a higher percentage of eggs graded large. Zimmermann *et al.* (1987) reported that shell weight of eggs is only improved after molting. No significant differences were observed between feed and water restrictions and low-sodium diet in egg production, egg weight, interior egg quality, or shell quality during the postmolt periods (Naber *et al.*, 1980; Said *et al.*, 1984). However, Zn acetate and Zn oxide molting treatments resulted in improved egg Haugh units during the postmolt periods (Shippee *et al.*, 1979).

The results in the current study indicate that egg qualities were not significantly ($P > 0.05$) different in most parameters between all molted hens and full fed layer ration hens. However, hens fed 1% zinc as Zn acetate exhibited stronger shells, and hens fed 1% zinc as Zn propionate exhibited higher egg weights than hens undergoing feed withdrawal. This higher egg weight may be an advantage only if there is a consumer's preference for the larger and/or heavier eggs in market (North and Bell, 1990).

Summary

All laying hens treated with Zn propionate (1% zinc) in a layer diet had lower crop pH, completely stopped egg production by the fourth day in the molting process, and greatly reduced feed intake, body weight loss, and ovary regression. Zinc fed hens yielded either statistically higher, lower or similar values of relative organ weights that were dependent on organ type and yielded more tibia ash (%). Zinc concentrations in

the liver and kidney accumulated possibly due to zinc (1%) feeding. The overall egg qualities except for egg weight and egg production from Zn propionate were similar to other molting methods. Zinc accumulated in the liver and kidney. Therefore, dietary Zn propionate (1% zinc) as an alternative molting dietary zinc supplement was comparable in its effectiveness to induce molt. Future experiments are needed to examine the effect of Zn propionate on the *Salmonella* infection in molted laying hens because induced molting without feed may increase the incidence of *S. enteritidis* in these hens (Holt and Porter, 1992; Holt, 1993; Holt *et al.*, 1995).

CHAPTER X

**COMPARISON OF ZINC ACETATE AND PROPIONATE
ADDITION ON GASTROINTESTINAL TRACT FERMENTATION
AND SUSCEPTIBILITY OF LAYING HENS TO
SALMONELLA ENTERITIDIS DURING FORCED MOLT**

Overview

The method most commonly used to induce molting and stimulate multiple egg-laying cycles in laying hens for commercial egg production is feed deprivation. Unfortunately, an increased risk of *Salmonella enteritidis* may result from the use of this method. Methods to stimulate multiple egg-laying cycles without increasing the risk of SE are needed. In each of three experiments, hens over 50 wk of age were divided into groups of 12 hens and placed in individual laying cages. One wk prior to dietary changes, hens were put on an 8-h light and 16 h-dark photoperiod that continued for the 9-day experiments. Individual hens in all treatments were challenged orally with 10^4 CFU of SE on the fourth day. Treatments were full fed hens (non-molted, NM), non-fed hens (molted, M), a zinc acetate diet (ZAC), and a zinc propionate diet (ZPR). The zinc diets contained 10,000 mg zinc per kg of diet.

Body weight losses were significantly higher in the M, ZPR, and ZAC treatments than in the NM treatment. Ovary weights of ZAC or ZPR hens exhibited statistically ($P > 0.05$) the same level of reduction as those of NM hens (Trial 1 or Trial 2). Crop lactic

acid decreased more in M, ZPR, and ZAC treatments than in NM hens. Crop pH was significantly ($P < 0.05$) lower in NM hens than M, ZAC, or ZPR hens (Trial 2) and crop pH tended to be inversely related to feed consumption. Although cecal individual or total VFA, and lactic acid were not significantly ($P > 0.05$) different between NM hens and M, ZAC or ZPR hens in Trial 1, cecal total VFA or lactic acid were significantly ($P < 0.05$) higher in NM hens than in M, ZAC or ZPR hens (Trials 2 and 3). Colonization of SE in the crop and the ceca was higher in the M and ZPR hens (Trials 1 and 2). Liver, spleen, or ovary invasion by SE was higher in the M and ZPR hens (Trials 1 and 2) for NM hens. At the zinc concentration used in these studies, the ZAC regimen may be effective for inducing molt and stimulating multiple laying cycles without increasing the risk of SE.

Introduction

Salmonellosis is one of the most common foodborne diseases with an estimated 800,000 to 4 million human infections reported each year in the United States alone. During the past 10-15 years, the number of cases of gastroenteritis due to *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S. enteritidis*) infections has increased markedly in the United States and Europe and by 1995, *S. enteritidis* comprised 25% of all foodborne *Salmonella* isolates (Holt *et al.*, 1995). Between 1985 and 1991, 82% of *S. enteritidis* infections in the United States were associated with table eggs (St Louis *et al.*, 1988). *S. enteritidis* is invasive in poultry and therefore has the potential to contaminate eggs by transovarian transmission following colonization of the intestinal tract (Thiagarajan *et al.*, 1994).

It has been suggested that the high incidence of *S. enteritidis* infection may be linked to the specific stressful management practice of inducing a molt to stimulate multiple egg-laying cycles in hens. Over 94% of commercial laying facilities in the western United States use induced molting as a means of increasing productivity in flocks (USDA, 2000). Feed withdrawal is the primary method used in the layer industry to induce molting but has been shown experimentally to increase *S. enteritidis* recovery from crops, increase invasion of organs in chickens (Holt, 1993; Thiagarajan *et al.*, 1994; Holt *et al.*, 1995; Durant *et al.*, 1999), and increased horizontal transfer in flocks (Holt *et al.*, 1998).

The poultry industry needs assessment of alternative molting procedures that do not require feed withdrawal but allow layer house managers to retain the economic advantages of obtaining a second laying cycle with a high production rate of high quality eggs via molting without increasing the risk of *S. enteritidis* (Keshavarz and Quimby, 2002).

Several dietary alterations have been used to induce molt including feeding plant byproducts, alteration of the dietary mineral balance or feeding deficient salt or sodium diets (Rolon *et al.*, 1993). Including zinc at 1 to 2% (10,000 to 20,000 ppm) of the ration has received the most attention as a possible alternative to feed deprivation (Shippee *et al.*, 1979; Stevenson and Jackson, 1984; Berry and Brake, 1985; McCormick and Cunningham, 1987). Zinc oxide when fed at 20,000 ppm completely stops egg production within 5 days (Scott and Creger, 1976; Creger and Scott, 1977; Berry and Brake, 1985) and hens fed high zinc stop ovulating up to a full day sooner than fasted

hens (Creger and Scott, 1977; Berry and Brake, 1985). Similar results have been obtained using either zinc acetate or zinc oxide (10,000 ppm zinc) (Shippee *et al.*, 1979), zinc sulfate heptahydrate (≤ 2800 ppm zinc) in a low calcium diet (Breeding *et al.*, 1992) and either zinc acetate or zinc propionate (10,000 ppm zinc) (Park *et al.*, 2003).

Alternative molting diets than have been developed from wheat middlings (Seo *et al.*, 2001) or alfalfa (Kwon *et al.*, 2001; Medvedev *et al.*, 2001) and have been shown to limit SE colonization. This supports to the hypothesis that the key to inducing a molt that minimizes *S. enteritidis* infection of laying hens is to use a molt diet that not only retains a crop microflora near the population levels found in full-fed birds but maximizes crop bacterial fermentation activities antagonistic to *S. enteritidis* colonization (Ricke, 2003). Molt diets that retain protective microflora during induced molting would provide poultry producers with dietary approaches that would potentially avoid the more drastic measure of feed withdrawal that is accompanied by increases in *S. enteritidis* contamination (Holt, 2003; Ricke, 2003). The present study was conducted to determine if alternative molting diets, utilizing zinc as zinc acetate (ZAC) or zinc propionate salts (ZPR) at high concentrations of zinc (10,000 ppm), would reduce intestinal colonization by *S. enteritidis* in laying hens and to determine if key characteristic changes in the chicken intestinal microenvironment can be linked to diets, feed consumption and *S. enteritidis* colonization.

Materials and Methods

Bacterial Strain

A primary poultry isolate of *Salmonella enteritidis* (phage type 19A), obtained from the National Veterinary Services Laboratory, Ames, Iowa, selected for resistance to novobiocin and nalidixic acid (NO-NA) in the USDA-ARS, College Station, TX, was used. Media used to culture the resistant isolate in experimental studies contained 25 µg of NO and 20 µg of NA per ml. The challenge inocula were prepared from an overnight culture which had been previously transferred three times in Trypticase soy broth. The culture was serially diluted in sterile phosphate-buffered saline to a concentration of approximately 10⁴ colony forming units (CFU) per ml. The number of CFU in the challenge inoculum was confirmed by plating onto brilliant green agar (BGA) plates (Difco Laboratories, Detroit, MI).

Molt Procedure

Feed deprivation by a modification (Holt, 1993) of a previously described procedure (Brake *et al.*, 1982) was used to induce molt. Seven days before feed removal or feeding the high zinc diet, hens were exposed to an 8 h light-16 h dark photoperiod which was continued throughout the experiment. Beginning on day 0, feed was withdrawn for 9 days, or hens received their respective molting diets, after which the study was ended.

Experimental Protocol

Single Comb White Leghorn hens (Hyline International) over 50 weeks of age were obtained from a commercial laying flock. Cloacal swab samples were collected

from each hen and examined for salmonellae by successive culturing in tetrathionate (TT) broth (Difco Laboratories, Detroit, MI) and BGA as described by Andrews *et al.* (1992). *Salmonella* spp.-positive hens were eliminated from the study. Laying hens were placed in wire layer cages (2 hens per cage) and provided free access to water and a balanced, unmedicated, corn-soya-meal-based mash layer feed ration that met or exceeded the National Research Council Research nutritional requirements for nutrients (1994). This diet provided 2,818 kcal of metabolizable energy per kg, 16.5% crude protein, 3.5% calcium, and 0.48% available phosphorus. Before use, three randomly selected 25 g samples of the feed were cultured successively in buffered peptone water, TT broth, and BGA as described by Andrews *et al.* (1992) and examined for salmonellae. *Salmonella* spp.-positive feed was not found. The hens were allowed to acclimate for a minimum of 1 week, followed by random assignment to four treatment groups of 12 hens each, designated as follows: (1) Non-molted control (NM); (2) Non-fed (molted, M); (3) Zinc acetate diet (ZAC); or (4) Zinc propionate diet (ZPR) in 3 separate trials. The zinc diets contained 10,000 mg zinc per kg of diet (10,000 ppm). The hens were housed in approved facilities at the USDA-ARS, College Station, TX, under a protocol approved by the USDA-ARS Animal Use and Care Committee.

On day 4 of each study, all hens in each treatment group were challenged by crop gavage with 1 ml of inoculum containing approximately 10^4 CFU of NA-NO resistant *S. enteritidis*. The challenge dosage approximates the 5.6×10^4 CFU dosages reported previously to be the mean infectious dosage (ID₅₀) for *S. enteritidis* in non-molted hens (Holt, 1993). On the last day of each study, all hens challenged with *S. enteritidis* in

each group were euthanatized and the crop, ceca, liver, spleen, and ovary excised aseptically. The crop, ceca, liver, spleen and ovary of each hen were cultured for *S. enteritidis*.

Crop Lactic Acid Concentrations and Crop pH

Crop lactic acid concentrations and crop pH were determined as described previously (Durant *et al.*, 1999). Crop pH was determined by the insertion of a sterile glass pH electrode (Model 05669-20; Cole Palmer Model, Niles, IL) through an incision in the crop wall ensuring that the electrode remained in contact with the crop mucosal surface (Durant *et al.*, 1999). Each crop was excised, cut open aseptically, and the entire crop and contents together with 10 ml of sterile distilled water blended for 1 min in a Stomacher 80 Lab Blender (Stewart Medical, London, England). Samples of the blended crop were analyzed for lactic acid concentrations.

Cecal Volatile Fatty Acid and Lactic Acid Concentrations

The concentrations of VFAs (acetic, propionic, butyric, isobutyric, valeric, and isovaleric) in the ceca contents were determined by gas-liquid chromatography as described previously (Corrier *et al.*, 1990). Briefly, the analyses were conducted with a gas chromatograph equipped with a flame ionization detector and peak profiles integration-quantification integrator (Model 110 Gas Chromatograph, SR1 Instruments, Torrance, CA). Each sample peak profile was integrated and quantified relative to an internal standard of methylbutyric acid placed in the same sample. Analyses were conducted at an oven temperature of 200 °C and a flow rate of 85 ml/min. The

concentration of each acid was expressed as μmol per ml. Lactic acid concentrations were determined by an enzymatic method (Hohorst, 1965).

Crop Colonization by *S. enteritidis*

One ml of the blended crop sample was transferred into 10 ml of TT broth and incubated for 24 h at 37 °C. After incubation, the broth was streaked onto NO-NA-BGA plates and incubated for an additional 24 h at 37 °C and examined for the presence of suspect *S. enteritidis* colonies. Suspect colonies were confirmed by biochemical tests on triple sugar-iron agar and lysine-iron agar (Oxoid, Unipath Ltd., Hampshire, England) and further identified as *S. enteritidis* serologically using *Salmonella* O antiserum group D, factors 1, 9, 12 (Difco). Identification of the NO-NA-resistant *S. enteritidis* by the culture on NO-NA-BGA plates and by the biochemical and serological procedures described were considered confirmatory without further serotyping.

Cecal Colonization by *S. enteritidis*

One cecum from each hen was cut into several pieces, placed in 30 ml of TT broth, shaken vigorously, and incubated for 24 h at 37 °C. After incubation, the broth were streaked on NO-NA-BGA plates, incubated for an additional 24 h at 37°C, and examined for the presence of suspect *S. enteritidis* colonies. Suspect colonies were confirmed biochemically and serologically as described in the section on crop colonization.

Liver, Spleen, and Ovary Colonization by *S. enteritidis*

Liver, spleen and ovary specimens were minced with scissors and cultured. The organ samples were incubated for 24 h at 37 °C in TT broth. After incubation, the broth

was streaked onto NO-NA-BGA plates, incubated for an additional 24 h at 37 °C, and examined for the presence of *S. enteritidis* colonies. Suspect colonies were confirmed biochemically and serologically as described in the section on crop colonization.

***S. enteritidis* CFU per Gram of Crop and Cecal Contents**

The contents of crop and one cecum from each hen were serially diluted and spread plated on NO-NA-BGA plates at dilutions 10^{-1} through 10^{-4} . The plates were incubated for 24 h at 37°C, after which the number of CFU of *S. enteritidis* per gram of crop or cecal contents was determined and *S. enteritidis* colonies were confirmed biochemically and serologically as described in the section on crop colonization. Crop and cecal contents in which *S. enteritidis* was not detected at the 10^{-1} dilution on BGA plates and after TT broth enrichment and BGA plating were scored as 0 CFU. Crop and cecal contents negative at 10^{-1} dilution on BGA plates but positive after tetrathionate enrichment and BGA plating were arbitrarily assigned $\log 0.95$ CFU of *S. enteritidis* per g of crop or cecal contents.

Statistical Analysis

Chi-square analysis was used to determine significant differences between treatment groups for incidences of *S. enteritidis* colonization of the crop, ceca, liver, spleen and ovary (Luginbuke and Schlotzhauer, 1987). Differences in the crop pH, VFA concentrations and \log_{10} CFU of *S. enteritidis* counts among treatment groups were determined by analysis of variance using the general linear models procedures. Significant differences were further separated using Duncan's multiple range test and commercial statistical analysis software (SAS Institute, Cary, NC; Luginbuke and

Schlotzhauer, 1987). All data analyzed by statistical analyses were considered significant at ($P < 0.05$).

Results and Discussion

Feed Intake

Natural molting is caused by self-induced decreased feed intake and activity during the last summer and early fall when the day length naturally begins to shorten (Brake and Thaxton, 1979; Mrosovsky and Sherry, 1980). These physiological changes cause the cessation of reproduction (Brake and Thaxton, 1979; Mrosovsky and Sherry, 1980). Therefore, wild birds take a self-induced rest to rejuvenate body tissues and build up energy stores. Based on these characteristics using feed consumption to induce molt may be of importance for ensure that hens induce molt.

Full-fed (nonmolted; NM) hens had significantly ($P < 0.05$) greater feed intake (Trial 1: 67.35, Trial 2: 81.72, Trial 3: 88.40 g/hen/day) than did Zn acetate (ZAC) or Zn propionate (ZPR) fed hens in all three trials (Table 10-1). ZPR fed hens was observed to have less feed intake than ZAC fed hens in all three trials, but the ZPR diet hens (14.60 g/hen/day) had significantly ($P < 0.05$) less feed intake than did ZAC fed hens (57.20 g/hen/day) in trial 3. Compared to the feed intake of NM fed hens, there was reduced feed intake in ZPR fed hens (Trial 1: 79.44, Trial 2: 73.81, Trial 3: 83.48%) and ZAC fed hens (Trial 1: 37.34, Trial 2: 57.66, trial 3: 35.29%) in all three trials. A similar trend for feed intake in Trial 2 was observed previously in egg production studies by Park *et al.* (2003) who reported that no differences could be detected in the average

TABLE 10-1. Effects of nonmolting and molting diets on feed intake, body weight lost, and ovary weight of hens

Item	Treatment			
	NM ¹	M ²	ZAC ³	ZPR ⁴
Trial 1				
Feed intake (g/hen/day)	67.35±16.05 ^a	NA ⁵	42.20±0.42 ^{ab}	13.85±9.12 ^b
Body weight lost (%)	0.21±6.06 ^a	25.10±4.33 ^c	10.82±8.66 ^b	16.36±9.09 ^b
Ovarian weight (g)	29.94±24.68 ^a	7.35±5.40 ^b	10.30±5.05 ^b	9.18±5.08 ^b
Trial 2				
Feed intake (g/hen/day)	81.72±9.40 ^a	NA	34.40±1.70 ^b	21.40±1.85 ^b
Body weight lost (%)	1.53±7.01 ^a	21.63±4.70 ^c	10.99±7.18 ^b	14.62±6.16 ^b
Ovarian weight (g)	38.07±16.97 ^a	7.79±3.17 ^a	12.10±8.11 ^b	9.69±6.80 ^b
Trial 3				
Feed intake (g/hen/day)	88.40±8.20 ^a	NA	57.20±6.08 ^b	14.60±1.13 ^c
Body weight lost (%)	4.11±3.61 ^a	30.34±3.67 ^d	12.09±6.60 ^b	23.18±5.27 ^c
Ovarian weight (g)	40.26±5.86 ^a	7.04±3.08 ^c	15.57±12.69 ^b	8.46±5.15 ^c

^{a-c}Means within a row with no common superscripts differ significantly ($P < 0.05$)

¹NM, Nonmolted = hens that received TAMU layer ration for 9 days

²M, Molted = hens that were undergoing feed withdrawal for 9 days

³ZAC = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

⁴ZPR = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

⁵N/A = not applicable

daily feed intake when Zn acetate (1% Zn) fed hens (22.61) were compared with Zn propionate (1% Zn) fed hens (26.59 g/bird/day) during the molting period.

The reduced feed intake could be due to appetite depression (Brink *et al.*, 1950), or low palatability of high levels of propionic acid (Ryś and Koreleski, 1974; Cave, 1982, 1984) or zinc (Fox, 1989). It has also been reported that the reduced feed intake could be due to the ability of zinc cation (Zn^{2+}) to induce follicular atresia and halt egg laying (Scott and Creger, 1976; Shippee *et al.*, 1979; Berry and Brake, 1985; McCormick and Cunningham, 1987; Johnson and Brake 1992). Therefore, it is likely that the efficiency of high dietary zinc treatments as a method to induce molt in this study was directly related to the suppression of feed intake.

Body Weight Loss

Body weight loss has an influence on the successful results of an induced molting procedure (Brake and Thaxton, 1979; Brake *et al.*, 1981; Brake and McDaniel, 1981; Baker *et al.*, 1983). Baker *et al.* (1983) suggest that the extreme body weight loss during the induced molt is directly related to a hen's postmolt performance. Approximately 25% body weight loss in hens by the feed withdrawal has been directly attributed to decreased muscle weight, decreased liver weight, decreased use of adipose tissue, involution of the reproductive organs, and greater reproductive regression (Brake and Thaxton, 1979; Berry and Brake, 1985).

Non-fed (molted; M) hens (Trial 1: 25.10%, Trial 2: 21.63%) exhibited significantly ($P < 0.05$) more body loss than did ZAC or ZPR fed hens in both Trials 1 and 2 (Table 10-1). However, there was no significant ($P > 0.05$) difference in body

weight loss between ZAC fed hens (Trial 1: 10.82, Trial 2: 10.99%) and ZPR fed hens (Trial 1: 16.36, Trial 2: 14.62%) in both Trials 1 and 2. In Trial 3, there was significant ($P > 0.05$) difference of the body weight loss from all four treatments. NM fed hens had the least body weight loss compared to other treatments of molted hens in all three trials. Similar trends in hens' body weight loss in Trials 1 or 2 were observed by Park *et al.* (2003) for hens undergoing feed withdrawal (25.12), 1% zinc as Zn acetate (15.52) or Zn propionate hens (15.66), and non-molted control hens (1.15%). The extent of a hens' body weight loss by ZAC or ZPR feeding was also similar to values previous study by McCormick and Cunningham (1987) who reported that body weight loss during the 4 days for fasted and zinc fed hens was 16.4% and 15.2%, respectively.

Ovarian Weight

The regression of the ovary is the most important factor for induced molt because the reduced reproductive weight may be linked to the overall rejuvenation process (Brake and Thaxton, 1979). Therefore, ovarian weight was measured in the current study as an indication of molting. ZAC (Trial 1: 10.30, Trial 2: 12.10 g) or ZPR (Trial 1: 9.18, Trial 2: 9.69 g) fed hens did not have significantly ($P > 0.05$) different ovarian weights when compared to M hens (Trial 1: 7.35, Trial 2: 7.79 g) in both Trials 1 and 2. NM (Trial 1: 29.94, Trial 2: 38.07 g) fed hens had significantly ($P < 0.05$) higher ovarian weights when compared to all other molted hen dietary treatments in both Trials 1 and 2 (Table 10-1). Similar ovarian weight responses in Trial 1 was also seen by Park *et al.* (2003) where non-molted control hens had significantly higher ovarian weights

(31.04g) when compared to all other molted hen dietary treatments (Zn acetate, Zn propionate and feed withdrawal).

Berry and Brake (1985) reported that the ovarian weights of hens fed 2% zinc as Zn oxide for 8 days were 9.00 g. McCormick and Cunningham (1984) also reported that feeding of 1% zinc or 2% zinc as Zn oxide for 4 days resulted in reproductive involution (80% reduction in ovarian weights) of hens. Although we did not monitor or measure for reproductive involution in the current study, hens treated both by high dietary zinc as ZAC or ZPR may have experienced reproductive involution as indicated by substantial ovarian regression and reduced ovarian weight. The results may indicate that the major effects of high dietary zinc on the reproductive organs are potentially linked to general physiological state of high zinc fed hens (Berry and Brake, 1985).

Crop Lactic Acid and pH Level

Lactic acid is the main fermentation product by the *Lactobacillus* spp that are present in the crop (Fuller, 1977). The lactic acid concentrations were significantly ($P > 0.05$) lower in the crop of M hens (Trial 1: 11.05, Trial 2: 6.67 $\mu\text{mol/ml}$) than in that of NM hens (Trial 1: 29.53, Trial 2: 22.02 $\mu\text{mol/ml}$) in both Trials 1 and 2 (Table 10-2). The significant decrease in lactic acid in the M hens may have resulted from a significant decrease in the numbers of *Lactobacilli* in these birds although the numbers of lactobacilli were not determined in the current study. Compared to the NM or M hens, the ZAC or ZPR hens had lower or higher concentrations of lactic acid in all three Trials. The ZAC hens (Trial 1: 19.23, Trial 2: 15.54 $\mu\text{mol/ml}$) had higher concentrations of

TABLE 10-2. Effects of nonmolting and molting diets on the crop lactic acid concentration and pH of hens

Item	Treatment			
	NM ¹	M ²	ZAC ³	ZPR ⁴
Trial 1				
Lactic acid (μmol/ml)	29.53±37.33	11.05±3.00	19.23±11.0	16.98±4.81
Crop pH	5.19±0.45	5.42±0.72	5.82±1.15	5.90±0.54
Trial 2				
Lactic acid (μmol/ml)	22.02±12.96 ^a	6.67±1.49 ^c	15.54±5.14 ^b	9.55±5.18 ^{bc}
Crop pH	4.79±0.48 ^b	5.49±0.72 ^a	5.36±0.73 ^a	5.40±0.54 ^a
Trial 3				
Lactic acid (μmol/ml)	20.70±10.82 ^a	7.97±1.65 ^b	13.96±9.0 ^{ab}	17.81±13.31 ^a
Crop pH	4.29±0.52 ^{bc}	4.81±0.42 ^{ab}	4.06±0.98 ^c	5.17±0.61 ^a

^{a-c}Means within a row with no common superscripts differ significantly ($P < 0.05$)

¹NM, Nonmolted = hens that received TAMU layer ration for 9 days

²M, Molted = hens that were undergoing feed withdrawal for 9 days

³ZAC = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

⁴ZPR = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

lactic acid than did the ZPR hens (Trial 1: 16.98, Trial 2: 9.55 $\mu\text{mol/ml}$) in both Trials 1 and 2. However, the ZAC hens (13.96 $\mu\text{mol/ml}$) had lower concentrations of lactic acid than did the ZPR (17.81 $\mu\text{mol/ml}$) in Trial 3. There were no consistent trends in the concentrations of lactic acid in the crop throughout the Trials.

There were no consistent trends in the crop pH throughout the Trials (Table 10-2) and there were no significant ($P > 0.05$) differences in the crop pH in all four treatments. However, NM hens (Trial 1: 5.19, Trial 2: 4.79) had lower values of the crop pH than the other molted hens in trial 1 and had significantly ($P < 0.05$) lower values of crop pH than other molted hens in Trial 2. The ZAC hens (4.06) had significantly ($P < 0.05$) lower values of crop pH than the ZPR hens (5.17) although the ZAC hens (13.96 $\mu\text{mol/ml}$) had a lower concentration of lactic acid than in the ZPR (17.81 $\mu\text{mol/ml}$) in Trial 3. Reduced feed intake may be the main factor causing the decrease in the *Lactobacilli* population (Humphrey *et al.*, 1993; Corrier *et al.*, 1999; Durant *et al.*, 1999), thereby allowing the pH to rise in hens that are deprived of feed. Humphrey *et al.* (1993) reported that when chickens are undergoing malnutrition or starvation, the crop pH can increase due to decreased *Lactobacillus* fermentation within the crop. Feed withdrawal for 9 days resulted in a decrease in lactic acid in the crop, accompanied by an increase in crop pH (Durant *et al.*, 1999). ZAC or ZPR feeding may be inhibitory to *Lactobacilli* population due to the influence of zinc on microorganism growth and infectivity in animals. *Lactobacilli* that are the predominant microflora in crop, play an important role in maintaining a low pH that prevented coliform establishment in the crop (Fuller and Brooker, 1974) and in preventing the growth of *E. coli in vitro* (Fuller, 1977).

Consequently, the results in the current study may be linked to the observation that when ZAC hens consumed more feed ($P < 0.05$) than did the ZPR hens as subsequent increases in the lactic acid concentrations were accompanied by concomitant decreases in crop pH in the NM hens. However, decreases in the concentrations of lactic acid during feed withdrawal were not always accompanied by a significant increase in the crop pH in the M hens in the current study. Therefore, increases in crop pH by dietary molting treatments as either M, ZAC, or ZPR feeding may simply decrease lactic acid production by crop microflora without altering the population levels substantially. Hume *et al.* (2003) using denaturing gradient gel electrophoresis noted that in hens where addition of zinc (2800 mg/kg) was used to induce molting a typical microflora was still present despite the decrease in hen's feed intake caused by excess zinc levels in the feed. They also reported that even nonmolted and feed withdrawal molted hens exhibited 40% similarity between cecal communities, whereas diets with calcium (0.8% wt/wt) and zinc (2,800 mg/kg) exhibited 90% similarity between cecal communities.

Cecal Volatile Fatty Acids (VFA) and Lactic Acid

There were no significant ($P > 0.05$) differences in concentrations of acetic acid in the ceca of hens from all four treatments in three Trials (Table 10-3). Concentrations of propionic acid were significantly ($P < 0.05$) lower in the ceca of M hens (Trial 2: 21.12, Trial 3: 25.46 $\mu\text{mol/ml}$) than in that of NM hens (Trial 2:45.38, Trial 3:50.81 $\mu\text{mol/ml}$), ZAC (Trial 2: 45.08, trial 3: 52.13 $\mu\text{mol/ml}$), or ZPR fed hens (Trial 2:43.65, Trial 3:51.77 $\mu\text{mol/ml}$), but the concentrations of propionic acid were not significantly ($P > 0.05$) different in the ceca of hens from all four treatments Trial 2 (Table 10-3).

Concentrations of butyric acid were significantly ($P < 0.05$) lower in the ceca of M hens (13.11 $\mu\text{mol/ml}$) than in that of ZAC (18.86 $\mu\text{mol/ml}$) or NM (19.81 $\mu\text{mol/ml}$) hens in Trial 3, but concentrations of butyric acid were not significantly ($P > 0.05$) different in the ceca of hens from all four treatments in Trials 1 and 2 (Table 10-3). Concentrations of isobutyric acid were significantly ($P < 0.05$) higher in the ceca of ZPR fed hens (8.23 $\mu\text{mol/ml}$) than in that of M hens (5.71 $\mu\text{mol/ml}$) in Trial 3, but concentrations of isobutyric acid were not significantly ($P > 0.05$) different in the ceca of hens from all four treatments in both Trials 1 and 2 (Table 10-3). Concentrations of valeric acid were significantly ($P < 0.05$) lower in the ceca of M hens (5.37 $\mu\text{mol/ml}$) than in that of NM hens (7.62 $\mu\text{mol/ml}$), ZAC fed hens (7.68 $\mu\text{mol/ml}$), or ZPR fed hens (8.16 $\mu\text{mol/ml}$) in Trial 3, but concentrations of valeric acid were not significantly ($P > 0.05$) different in the ceca of hens from all four treatments in both Trials 1 and 2 (Table 10-3).

Concentrations of isovaleric acid were significantly ($P < 0.05$) lower in the ceca of M hens (5.08 $\mu\text{mol/ml}$) than in that of ZPR fed hens (7.04 $\mu\text{mol/ml}$) in Trial 3, but concentrations of isovaleric acid were not significantly ($P > 0.05$) different in the ceca of hens from all four treatments in both Trials 1 and 2 (Table 10-3). Concentrations of total VFA were significantly ($P < 0.05$) lower in the ceca of M hens (118.30 $\mu\text{mol/ml}$) than in that of NM hens (192.59 $\mu\text{mol/ml}$), ZAC hens (201.45 $\mu\text{mol/ml}$), or ZPR fed hens (207.03 $\mu\text{mol/ml}$) in Trial 3, but concentrations of total VFA were not significantly ($P > 0.05$) different in the ceca of hens from all four treatments in both Trials 1 and 2 (Table 10-3). Concentrations of lactic acid were significantly ($P > 0.05$) higher in the ceca of

TABLE 10-3. Effects of nonmolting and molting diets on cecal volatile fatty acid⁵ and lactic acid concentrations

Item	Treatment			
	NM ¹	M ²	ZAC ³	ZPR ⁴
Trial 1				
Acetic acid	69.05±25.02	50.50±37.52	77.73±30.38	76.23±39.74
Propionic acid	21.13±10.20	11.93±17.82	22.29±13.87	23.45±17.35
Butyric acid	6.33±4.88	5.01±8.05	6.81±5.25	8.27±7.17
Isobutyric acid	2.83±1.10	1.99±2.26	2.75±1.77	3.07±2.52
Valeric acid	2.68±1.24	1.83±2.52	2.94±1.91	3.10±2.06
Isovaleric acid	2.56±1.10	1.68±1.80	2.23±1.67	2.72±2.10
Total VFA	106.56±42.75	74.40±69.52	117.02±54.47	119.64±71.01
Lactic acid	6.08±1.62	4.07±1.47	4.32±1.28	5.23±3.28
Trial 2				
Acetic acid	103.70±27.30	91.39±48.61	112.63±30.82	112.79±55.45
Propionic acid	45.38±16.23 ^a	21.12±14.19 ^b	45.08±16.86 ^a	43.65±24.65 ^a
Butyric acid	21.26±6.93	18.08±9.68	20.52±8.41	15.59±9.57
Isobutyric acid	5.49±6.30	3.80±2.49	4.69±2.88	6.25±7.03
Valeric acid	4.88±1.96	3.48±2.24	5.54±2.62	5.28±2.72
Isovaleric acid	2.80±1.26	2.78±2.00	3.12±1.38	2.83±1.79
Total VFA	184.13±53.61	140.34±75.35	194.12±63.31	190.48±95.74
Lactic acid	12.03±4.17 ^a	7.19±3.25 ^b	6.67±1.83 ^b	8.26±6.35 ^b
Trial 3				
Acetic acid	98.56±27.03	61.80±24.89	106.71±36.05	210.72±355.9
Propionic acid	50.81±12.76 ^a	25.46±13.20 ^b	52.13±18.15 ^a	51.77±19.76 ^a
Butyric acid	19.81±3.46 ^a	13.11±5.64 ^b	18.86±6.68 ^a	17.19±4.96 ^b
Isobutyric acid	7.04±1.40 ^{ab}	5.71±1.66 ^b	6.74±2.71 ^{ab}	8.23±4.00 ^a
Valeric acid	7.62±1.48 ^a	5.37±2.26 ^b	7.68±2.00 ^a	8.16±2.86 ^a
Isovaleric acid	6.34±1.37 ^{ab}	5.08±1.57 ^b	6.19±1.44 ^{ab}	7.04±1.90 ^a
Total VFA	192.59±44.4 ^a	118.30±48.8 ^b	201.45±63.6 ^a	207.03±69.2 ^a
Lactic acid	16.09±11.72 ^a	4.59±4.39 ^b	9.90±6.07 ^{ab}	6.87±6.08 ^b

^{a-c}Means within a row with no common superscripts differ significantly ($P < 0.05$)

¹NM, Nonmolted = hens that received TAMU layer ration for 9 days

²M, Molted = hens that were undergoing feed withdrawal for 9 days

³ZAC = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

⁴ZPR = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

⁵Unit of cecum volatile fatty acid = $\mu\text{mol/ml}$

NM hens (Trial 1:12.03, Trial 2:16.09 $\mu\text{mol/ml}$) than M hens (Trial 2:7.19, Trial 3:4.59 $\mu\text{mol/ml}$), the ZAC fed hens (Trial 2:6.67, Trial 3: 9.90 $\mu\text{mol/ml}$; this value was not statistically different from those of NM hens), or the ZPR fed hens (trial 2:8.26, trial 3:6.87 $\mu\text{mol/ml}$) in both Trials 2 and 3, but concentrations of lactic acid were not significantly ($P > 0.05$) different in the ceca of hens from all four treatments in trial 1 (Table 10-3). Corrier *et al.* (1997) also reported that the concentrations of total VFA along with acetic, propionic, and butyric acid were significantly decreased in the ceca of the molted hens compared to unmolted hens, but there was no significant difference cecal pH or cecal oxidation-reduction potential between molted hens and unmolted hens.

***S. enteritidis* Colonization in the Crop**

Dietary zinc may influence growth and infectivity of bacterial pathogens in animal. Park *et al.* (2002) recently reported that zinc may inhibit *S. typhimurium* under *in vitro* aerobic or anaerobic atmospheric conditions. Earlier studies have shown that high zinc (10,000 ppm) (Kubena *et al.*, 2001) or low zinc (110 ppm) (Ricke *et al.*, 2001) containing molting diets decreased *S. enteritidis* colonization in laying hens when compared to hens undergoing feed withdrawal. Although compared with NM hens (0%), the number of *S. enteritidis*- crop- culture positive hens increased significantly ($P < 0.05$) in the M hens (91.7%), ZAC hens (33.3%), or ZPR hens (100%), which had a significant higher *S. enteritidis* number (2.31 CFU/g) in the crop contents than did the NM hens (0 CFU/g), M hens (0.52 CFU/g), or ZAC hens (0 CFU/g) in Trial 1 (Table 10-4). When compared with NM hens (16.7%), the number of *S. enteritidis*- crop- culture

positive hens increased significantly ($P < 0.05$) in the M hens (83.3%), or ZPR hens (100%), which had a significant higher *S. enteritidis* number (1.51 CFU/g) in the crop contents than did the NM hens (0 CFU/g) or ZAC hens (0 CFU/g) in trial 2 (Table 10-4). There were no significant ($P > 0.05$) differences in *S. enteritidis* colonization and number (CFU/g) in the crop contents from four treatments in Trial 3 (Table 10-4). The crop can be one of the main reservoirs for *Salmonella* (Hargis *et al.*, 1995), and feed withdrawal can increase in the number of chicken with crops colonized by *Salmonella* (Ramirez *et al.*, 1997). Humphrey *et al.* (1993) reported that an increase in the recovery of *S. enteritidis* from the crop of broilers resulted from feed deprivation for 24 h. Durant *et al.* (1999) reported that the introduction of *S. enteritidis* into the crop environment with high pH and lowered concentrations of lactate and total VFA were accompanied by the increased crop colonization.

***S. enteritidis* Colonization in the Ceca**

When compared with NM hens (8.3%), the number of *S. enteritidis*- cecal-culture positive hens increased significantly ($P < 0.05$) in the M hens (100%), ZAC hens (25%), or ZPR hens (100%) but there were no significant ($P > 0.05$) differences in *S. enteritidis* number (CFU/g) in the crop contents from four treatments in Trial 1 (Table 10-5). Likewise when compared with NM hens (41.7%), the number of *S. enteritidis*-crop-culture positive hens increased significantly ($P < 0.05$) in the M hens (91.7%) or ZPR hens (58.3%) but there were no significant ($P > 0.05$) differences in *S. enteritidis* number (CFU/g) in the crop contents from four treatments in Trial 2 (Table 10-5). In Trial 3, compared with NM hens (18.2%), the number of *S. enteritidis*- crop-culture

TABLE 10-4. Effects of nonmolting and molting diets on *S. enteritidis* crop colonization of hens

Item	Treatment			
	NM ¹	M ²	ZAC ³	ZPR ⁴
Trial 1				
Positive hens/total (%)	0/12(0)	11/12*(91.7)	4/12*(33.3)	12/12*(100)
Log ₁₀ CFU/g	0 ^b	0.52±1.31 ^b	0 ^b	2.31±2.95 ^a
Trial 2				
Positive hens/total (%)	2/12(16.7)	10/12*(83.3)	1/12(8.3)	12/12*(100)
Log ₁₀ CFU/g	0 ^b	0.63±1.03 ^{ab}	0 ^b	1.51±2.27 ^a
Trial 3				
Positive hens/total (%)	0/11(0)	1/12(8.3)	0/11(0)	0/12(0)
Log ₁₀ CFU/g	0	0.28±0.98	0	0

¹NM, Nonmolted =hens that received TAMU layer ration for 9 days

²M, Molted = hens that were undergoing feed withdrawal for 9 days

³ZAC = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

⁴ZPR = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

Hens were challenged by crop gavage with 10⁴ CFU of *S. enteritidis* on day 4 of molt and cultured for *Salmonella* on day 9 of molt.

*, $P < 0.05$, and **, $P < 0.01$, between treatment and control (NM) (n=12, or n=11)

positive hens increased significantly ($P < 0.05$) in the M hens (83.3%), which had a significant higher *S. enteritidis* number (CFU/g) in the crop contents than did other treatments (Table 10-5).

Besides the crop, the ceca is an alimentary tract site in poultry that is most likely to be colonized by *Salmonella* (Fanelli *et al.*, 1971) and the *S enteritidis* replicates and disseminates to various organs, including the ovaries (Gast and Beard, 1990; Shivaprasad *et al.*, 1990). Feed withdrawal may cause an increase on the level of cecal colonization *Salmonella* (Moran and Bilgili, 1990; Ramirez *et al.*, 1997). The native intestinal bacteria have a protective function against *Salmonella* colonization of the

cecum (Nurmi and Rantala, 1973; Barnes *et al.*, 1980; Nisbet *et al.*, 1994; Corrier *et al.*, 1995) in chicken. The importance of VFA and pH in preventing *Salmonella* colonization of the cecum has been positively correlated with increased VFA concentrations and decreased pH (Barnes *et al.*, 1979; Nisbet *et al.*, 1994; Corrier *et al.*, 1995). A lowered pH can promote the bacteriostatic action of VFA by increasing the concentrations of the undissociated state, which can permeate the cell membrane (Cherrington *et al.*, 1991). The decreased number of *Salmonella* in the cecal contents of chicks has been associated with elevated concentrations of propionic acid and total VFA (Hollister *et al.*, 1992; Ziprin *et al.*, 1993; Nisbet *et al.*, 1994; Corrier *et al.*, 1995). VFA in the cecal contents in chicken are fermentation products of normal indigenous anaerobic bacteria (Barnes *et al.*, 1979, 1980) and the concentrations of VFA may represent the degree of fermentation activity of bacteria in the ceca (Barnes *et al.*, 1980). Corrier *et al.* (1997) also reported that induced molting by feed withdrawal had no

TABLE 10-5. Effects of molting and nonmolting diets on *S. enteritidis* cecal colonization of hens

Item	Treatment			
	NM ¹	M ²	ZAC ³	ZPR ⁴
Trial 1				
Positive hens/total (%)	1/12(8.3)	12/12*(100)	3/12*(25)	12/12*(100)
Log ₁₀ CFU/g	0.53±1.27	1.14±2.07	0.56±1.35	0.67±1.61
Trial 2				
Positive hens/total (%)	5/12(41.7)	11/12*(91.7)	4/12(33.3)	7/12** (58.3)
Log ₁₀ CFU/g	0.37±1.27	1.89±2.96	0.89±2.14	2.26±2.51
Trial 3				
Positive hens/total (%)	2/11(18.2)	10/12*(83.3)	4/11 (36.4)	6/12(50)
Log ₁₀ CFU/g	0 ^b	2.77±3.44 ^a	0.55±1.27 ^{ab}	1.82±2.99 ^{ab}

¹NM, Nonmolted =hens that received TAMU layer ration for 9 days

²M, Molted = hens that were undergoing feed withdrawal for 9 days

³ZAC = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

⁴ZPR = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

Hens were challenged by crop gavage with 10⁴ CFU of *S. enteritidis* on day 4 of molt and cultured for *Salmonella* on day 9 of molt.

*, $P < 0.05$, and **, $P < 0.01$, between treatment and control (NM) (n=12, or n=11).

apparent effect on pH or on the oxidation-reduction potential of ceca. The lactic acid bacteria probably do not play a major role in the metabolic activity in the ceca because the concentration of fermentable carbohydrates that favors their growth is much lower in the ceca than in the crop. The native intestinal microflora functions as a protector against *Salmonella* colonization of the ceca (Nurmi and Rantala, 1973; Barnes *et al.*, 1980; Nisbet *et al.*, 1994; Corrier *et al.*, 1995) and crop (Barnes *et al.*, 1980) in chicken. Cecal contents of broilers normally contain very low concentrations of lactic acid (Hinton *et al.*, 1991), which is the major metabolic byproduct of these acid-producing bacteria.

***S. enteritidis* Colonization in Liver, Spleen, and Ovary**

Compared with NM hens (liver: 0%, spleen: 0%, ovary: 16.7%), the number of *S. enteritidis*- liver/spleen/ovary- culture positive hens increased significantly ($P < 0.05$) in the M hens (liver: 66.7%, spleen: 58.3%, ovary: 83.3%), or ZPR hens (liver: 91.71%, spleen: 91.7%, ovary: 100%) in Trial 1 (Table 10-6). Compared with NM hens (liver: 25%, spleen: 8.3%, ovary: 100%), the number of *S. enteritidis*- liver/spleen/ovary culture positive hens increased or decreased significantly ($P < 0.05/ P < 0.01$) in the M hens (liver: 75%, spleen: 50%, ovary: 58.3%), ZAC hens (liver: 8.3%, spleen: 41.7%, ovary: 66.7%), or ZPR hens (liver: 75%, spleen: 66.7%, ovary: 75%) in Trial 2 (Table 10-6). Compared with NM hens (liver: 9.1%, spleen: 0%, ovary: 0%), the number of *S. enteritidis*- liver/spleen/ovary- culture positive hens increased significantly ($P < 0.05/ P < 0.01$) in the M hens (liver: 50%, spleen: 16.7%, ovary: 50%) or ZPR hens (liver: 25%, spleen: 25%, ovary: 8.3%) in Trial 3 (Table 10-6).

TABLE 10-6. Effects of nonmolting and molting diets on *S. enteritidis* colonization of the liver, spleen, and ovary of hens

Item	Treatment			
	NM ¹	M ²	ZAC ³	ZPR ⁴
Trial 1				
Liver	0/12(0)	8/12*(66.7)	1/12(8.3)	11/12*(91.7)
Spleen	0/12(0)	7/12*(58.3)	1/12(8.3)	11/12*(91.7)
Ovary	2/12(16.7)	10/12*(83.3)	2/12(16.7)	12/12*(100)
Trial 2				
Liver	3/12(25)	9/12*(75)	1/12(8.3)	9/12*(75)
Spleen	1/12(8.3)	6/12*(50)	5/12** (41.7)	8/12*(66.7)
Ovary	12/12(100)	7/12*(58.3)	8/12*(66.7)	9/12** (75)
Trial 3				
Liver	1/11(9.1)	6/12*(50)	3/11(27.8)	3/12(25)
Spleen	0/11(0)	2/12(16.7)	2/12(16.7)	3/12** (25)
Ovary	0/11(0)	6/12*(50)	2/12(16.7)	1/12(8.3)

¹NM, Nonmolted =hens that received TAMU layer ration for 9 days

²M, Molted = hens that were undergoing feed withdrawal for 9 days

³ZAC = hens that received a diet containing 10,000 mg/kg zinc as zinc acetate for 9 days

⁴ZPR = hens that received a diet containing 10,000 mg/kg zinc as zinc propionate for 9 days

Hens were challenged by crop gavage with 10⁴ CFU of *S. enteritidis* on day 4 of molt and cultured for *Salmonella* on day 9 of molt.

*, $P < 0.05$, and **, $P < 0.01$, between treatment and control (NM) (n=12, or n=11)

The presence of feed in the gastrointestinal tract stimulates peristalsis and mucin production (Sturkie, 1965). However, feed deprivation may result in decreased peristalsis, decreased mucin production (Duke and Evanson, 1976) and increased opportunity for bacterial colonization (Holt and Porter, 1992; Holt, 1993; Holt *et al.*, 1994; Holt *et al.*, 1995). Holt *et al.* (1994) reported that hens infected with *S. enteritidis* and molted by feeding a ration low in calcium and energy had less severe cecal and colonic inflammation than hens molted by complete feed deprivation. Feed deprivation may also increase the severity of *S. enteritidis* infection by altering normal intestinal bacteria populations and pH. Carrier *et al.* (1990) demonstrated that lowered cecal pH, induced by administration of volatile fatty acid-producing cecal flora, protected against *Salmonella typhimurium* infection in chicks. Macri *et al.* (1997) reported that induced molting by feed deprivation shortened the time of onset and increased the severity of acute *S. enteritidis* inflammation in the cecum, colon, possibly, the ileum. Holt and Porter (1992) reported that molted infected chickens and unmolted infected chickens exhibited no significant difference in the spleen *S. enteritidis* counts. Cecum, liver, spleen, and ovary counts were significantly higher in the fasted hens and no differences in any of the *S. enteritidis* counts were observed in nonmolted versus wheat middling fed hens (Seo *et al.*, 2001). Kwon *et al.* (2001) also reported that the total number of *S. enteritidis* positive organs was decreased in alfalfa fed hens as compared to fasted hens, while there was no colonization in nonmolted hens. Carrier *et al.* (1997) suggested that increased susceptibility of molted hens to *S. enteritidis* colonization may be related to decreased fermentation and production of VFA-producing bacteria present in the ceca,

and addition of lactose in the drinking water during the water may enhance resistance to *S. enteritidis* colonization.

No consistent trends over the entire three trials were exhibited in the *S. enteritidis* colonization in each organ of M, NM, ZAC, or ZPR hens. However, *S. enteritidis* crop and cecal colonization in crop, in addition to liver, spleen, and ovary invasion, were more frequent in ZPR hens in all three trials when compared to either M hens or ZAC hens. Feed intake was depressed in ZPR hens in all three trials when compared to ZAC hens during the 9 days. Zinc feeding as either ZAC or ZPR may not alter the intestinal microenvironment of hens in the current study because no consistent trends were exhibited in the concentrations of crop or cecal lactic acid or individual and total VFA between NM, M, ZAC and ZPR hens. Therefore, the results indicated that *S. enteritidis* colonization in ZPR hens may be directly influenced by lower feed intake of hens not by fermentation of lactic acid or VFA.

Conclusions

The most commonly practiced method of molt induction is by the withdrawal of feed for a period of several days to enter into a second egg laying cycle (North and Bell, 1990). This is an efficient method to induce a molt because it is management friendly, economically advantageous and results in satisfactory postmolt performance for the commercial layer industry (Brake, 1993). However, increased public awareness of the animal stress associated with feed withdrawal has led researchers to investigate alternative molting processes. Additionally, the stress results in reduced resistance of

hens to *Salmonella enteritidis* colonization (Holt and Porter, 1992; Holt, 1993; Holt *et al.*, 1995; Carrier *et al.*, 1997); this may lead to increased risk of food borne illness to consumers of these products.

Under the conditions of these studies, the ZAC regimen has the potential to be used as an alternative method to feed deprivation to induce molting and a second laying cycle without significantly increasing the risk of *S. enteritidis* contamination. Although the ZPR feed hens exhibited lower ovary weights, they were also more susceptible to *S. enteritidis* contamination compared to ZAC hens. For ZPR to be an effective alternative method to induced molting and avoid a risk of *Salmonella* contamination lower ZPR concentrations will need to be examined. This research may provide the poultry industry with a scientifically based rationale for a possible management molting alternative that reduces molting as a major risk for *S. enteritidis* contamination but enhances the commercial benefits of a second laying cycle and is publicly more acceptable.

CHAPTER XI

**DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) AS
A RAPID METHOD FOR ASSESSING GASTROINTESTINAL
TRACT MICROFLORA RESPONSES IN LAYING HENS
FED SIMILAR ZINC MOLT INDUCTION DIETS**

Overview

Induced molting through feed withdrawal can change the microenvironment of crop and ceca sufficiently to allow them to be the main sites of *Salmonella* colonization in the chicken intestine and increases horizontal transfer in flocks. This study compares the denaturing gradient gel electrophoresis (DGGE) profiles of microbial crop and cecal communities among molted hens fed either zinc acetate or zinc propionate amended molt diets to hens either undergoing feed withdrawal or hens full fed and not molted. Dendrograms of DGGE amplicon patterns indicated over 85% similarity of cecal communities between zinc acetate fed hens and zinc propionate fed hens and over 60% similarity of crop communities between zinc acetate fed hens and zinc propionate fed hens. Rapid comparison of complex gastrointestinal microflora profiles in laying hens fed similar diets is possible using DGGE.

Introduction

Digestive microbial populations in the gastrointestinal tract of adult hens are considered complex but relatively stable (Mead, 1989). This complex microbial populations is considered resistant to colonization by foodborne pathogens (McNab, 1973; Freter, 1983a, 1983b). However, hens molted by conventionally feed withdrawal may be more susceptible to *Salmonella enteritidis* infection leading to increases in horizontal transfer in flocks (Holt, 1992; Holt and Porter, 1992; Holt, 1993; Holt *et al.*, 1994; Holt, 1995; Holt *et al.*, 1998). When chickens are undergoing malnutrition or starvation, the pH of crop can increase due to decreased *Lactobacillus* fermentation within the crop (Humphrey *et al.*, 1993). Feed withdrawal for 9 days decreases crop lactic acid in conjunction with an increase in crop pH (Durant *et al.*, 1999). Feed withdrawal can also lead to decreases in production of acetic, propionic, and total volatile fatty acids (VFA) in the ceca (Corrier *et al.*, 1997). These decreased fermentation and production of VFA-producing bacteria present in the ceca colonization may be related to increased susceptibility of molted hens to *S. enteritidis* colonization (Corrier *et al.*, 1997). Induced molting through feed withdrawal appears to alter the microenvironment of crop and ceca sufficiently to allow them to be the main sites of *Salmonella* colonization in the chicken intestine (Brownell *et al.*, 1970; Soerjadi *et al.*, 1981; Impey and Mead, 1989).

Given the importance of the microenvironment on poultry pathogen establishment in the gastrointestinal tract of poultry, it becomes imperative that rapid methods are available to profile the microbial population. Although diet has been

identified in molted hens as a key contributor to alterations in the gastrointestinal tract that make it more susceptible to *Salmonella* colonization the complexity of the bacterial population and the requirement for anaerobic cultivation techniques makes it difficult to correlate nutritional factors with shifts in specific microbial populations (Ricke and Pillai, 1999; Ricke, 2003). In addition, there is the potential for overlap in nutritional specificity among groups of gastrointestinal organisms making conventional substrate specific selective media less precise than is desirable for characterizing subtle shifts in microbial populations (Russell and Baldwin 1978, 1979; Russell, 1984; Ricke and Pillai, 1999).

Conventional DNA-based approaches have been used to provide an imprecise picture of the genetic relatedness of organisms but are less amendable to more precise characterization (Raskin *et al.*, 1997; Ricke and Pillai, 1999). More recently Zhu *et al* (2002) successively used temperature gradient gel electrophoresis to identify 16S rRNA-based gene sequences representing phylogenetic groups in broiler chickens. Hume *et al.* (2003) reported that molecular-based denaturing gradient gel electrophoresis (DGGE) could detect changes in the digestive microbial communities in young chicks and molted laying hens. Our overall objective is to examine detection approaches for potential rapid assessment of microbial profiles from the gastrointestinal tract that could serve as consistent indicators of the presence of a protective microflora against pathogen colonization. Therefore, the specific objective of the current study was to compare digestive microbial crop and cecal communities among molted hens fed by either zinc acetate or zinc propionate amended molting diets with hens undergoing feed withdrawal

or full fed nonmolted hens using DGGE as described by Hume *et al.* (2003) to assess the reproducibility between diets and independent bird trials.

Materials and Methods

Sample Collection

Approximately 0.3 g of cecal contents were collected aseptically in three replicate experiments from five hens each in four treatment groups from a previous study (Moore *et al.* 2003): Group 1 – Control non-molted hens (C); Group 2 – molted hens with feed removed (Mo); Group 3 – molted hens given a diet containing 10,000 mg of zinc (zinc acetate) per ton of feed (Za); and Group 4 – given a diet containing 10,000 mg of zinc (zinc propionate) per ton of feed (Zp). Volumes were brought to 1 ml with sterile distilled water and samples were stored at -70°C until used. Crops from the same hens were collected aseptically and stomached for 30 sec in 10 ml of Butterfield's buffer (0.62 mM potassium phosphate, pH 7.2) and 3-ml portions were stored at -70°C until used.

Denaturing Gradient Gel Electrophoresis

Methodology for DGGE analysis was conducted as described previously by Hume *et al.* (2003). Briefly, genomic DNA was extracted and isolated (QIAamp DNA Mini Kit, Protocol D; QIAGEN, Valencia, CA) from 1-ml sample volumes of cecal and crop contents. Isolated DNA (50 ng/cecum or crop sample) from each hen was combined to give a total of 250 ng of DNA per group. Primers (50 pmol of each per reaction mixture; primer 2 and primer 3 with a 40-base G-C clamp (Integrated DNA

Technologies, Inc., Coralville, IA) (Sheffield *et al.*, 1989; Muyzer *et al.*, 1993) for PCR are shown in Table 11-1 and mixed with Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO), according to methods described in the kit, and 5% (w/v) acetamide to eliminate preferential annealing (Reysenbach *et al.*, 1992). Run parameters for amplification (PTC-200 Peltier Thermal Cycler (MJ Research; MJ Research, Inc., Waltham, MA) were: 1) denaturation at 94.9 °C for 2 min; 2) denaturation at 94.0 °C for 1 min; 3) annealing at 67.0 °C for 45 sec; -0.5 °C per cycle (touchdown to minimize spurious by-products (Don *et al.*, 1991; Wawer and Muyzer, 1995); 4) extension at 72.0 °C for 2 min; 5) repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94 °C for 1 min; 7) annealing at 58.0 °C for 45 sec; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72.0 °C for 7 min; 10) 4.0 °C final.

Amplicons were separated on polyacrylamide gels 8% (vol/vol) acrylamide-bisacrylamide ratio 37.5:1 (Bio-Rad Laboratories; Richmond, CA) cast with a 35 to 60% urea-deionized formamide (Sigma) gradient; 100% denaturing acrylamide was 7 M urea and 40% deionized formamide. Samples were mixed with an equal volume of 2X loading buffer (0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol) and 4 ml of each was loaded in sample wells. Gel electrophoresis was run in a DCode Universal Mutation Detection System (Bio-Rad) with 0.5X TAE (20 mM Tris (pH 7.4), 10 mM sodium acetate, 0.5 M EDTA) run buffer at 59 °C for 17 h at 60 V. Bands were stained with SYBR Green I (Sigma) (1:10,000 dilution) and fragment pattern relatedness was determined with Molecular Analysis

TABLE 11-1. Oligonucleotide primer sequences

Primer Designation ¹	Primer Sequences (5'→3'')
Primer 2	ATTACCGCGGCTGCTGG
Primer 3	GCCCGCCGCGCGCGGGCGGGGCGGGGG- CACGGGGGCCTACGGGAGGCAGCAG

¹Based on published sequences (Sheffield *et al.* 1989; Muyzer *et al.* 1993) and primers were obtained from Integrated DNA Technologies, Inc. (Coralville, IA).

Fingerprinting Software, version 1.6 (Bio-Rad Laboratories, Hercules, CA) based on the Dice similarity coefficient and the unweighted pair group method using arithmetic averages for clustering.

Results and Discussion

Given the problems associated with conventional feed withdrawal induced molting and *S. enteritidis* colonization, there is a need to apply molecular characterization as a rapid detection tool for screening the effectiveness of alternative molting diets to select indigenous gastrointestinal microflora that consistently limit *S. enteritidis* colonization and invasion. Zinc propionate (10,000 ppm zinc) as an alternative molting diet additive has been recently demonstrated to induce molt (Moore *et al.*, 2003; Park *et al.*, 2003) but Zp fed hens were more susceptible to *S. enteritidis* colonization compared to Za fed hens (Moore *et al.*, 2003). Since these two compounds are both zinc-based organic acids the question arises as to whether detectable differences are present in the gastrointestinal microbial populations supported by these respective diets that could account for the differences in *S. enteritidis* colonization. Therefore, DGGE profiles were generated for the 2 key colonization sites for *S. enteritidis*, namely the crop and cecum. An important criteria for determining the gastrointestinal microbial population that may be an indicator of microflora selected by consumption of a particular diet is the consistency of the molecular patterns in independent trials. In the present study, laying hen trial crop and cecal samples were collected from 3 independent

laying hen molting trials conducted in a previous study (Moore *et al.*, 2003) to compare the microbial populations in the crops and ceca in birds undergoing molt induction via Za or Zp amended diets versus the more extreme dietary manipulation of either complete feed withdrawal or hens continued to be fed laying ration *ad libitum*.

The ceca is an alimentary tract site in poultry that is most likely to be colonized by *Salmonella* (Fanelli *et al.*, 1971) and *S. enteritidis* replicates and disseminates to various organs, including the ovaries (Gast and Beard, 1990; Shivaprasad *et al.*, 1990). An increase on the level of cecal colonization *Salmonella* can result from feed withdrawal (Moran and Bilgili, 1990; Ramirez *et al.*, 1997). The native intestinal microflora are believed to play an important role in preventing *Salmonella* colonization of the cecum (Nurmi and Rantala, 1973; Barnes *et al.*, 1980; Nisbet *et al.*, 1994; Corrier *et al.*, 1995) in the chicken. The importance of VFA and pH in preventing *Salmonella* colonization of the cecum has been associated with increased VFA concentrations and decreased pH (Barnes *et al.*, 1979; Nisbet *et al.*, 1994; Corrier *et al.*, 1995). However, Corrier *et al.* (1997) reported that induced molting by feed withdrawal had no apparent effect on pH or on the oxidation-reduction potential of ceca. This would indicate that changes in the cecal microflora are somewhat subtle in response to dietary changes and potentially difficult to detect metabolically.

Based on DGGE analysis, cecal populations changed as a result of feed removal (Mo) and by the inclusion of feed. Cecal populations from hens given Za and Zp shared the greatest similarities as indicated by coefficients of 86.9 and 85.3%, respectively, for trials 1 and 2 but in trial 3, cecal populations from hens undergoing Mo or fed Zp shared

the greatest similarity (90.1%). Microbial patterns from Za, Zp, and Mo hens formed a linked group for trials 1 and 3, but C, Za, and Zp hens formed a more related group for trial 2. When these ceca samples were examined for fermentation products from the same trials and reported in a previous study (Moore *et al.*, 2003) there were negligible differences in most cecal individual VFA, total VFA, and lactic acid concentrations among Za or Zp molted hens in all 3 trials. However, in trial 3, hens undergoing feed withdrawal exhibited similar lower levels of lactic and butyric acid concentrations as the Zp fed hens versus Za fed hens (Moore *et al.*, 2003). The DGGE relatedness of cecal populations among nonmolted hens, molted by feed withdrawal, and molted by Za or Zp determined in this study for the most part appeared to match the similarities in fermentation profiles observed previously for these same trials (Moore *et al.*, 2003).

The crop can be one of the main reservoirs for *Salmonella* (Hargis *et al.*, 1995), and feed withdrawal can increase the number of chickens with crops colonized by *Salmonella* (Ramirez *et al.*, 1997). Humphrey *et al.* (1993) reported that an increase in the recovery of *S. enteritidis* from the crop of broilers resulted from feed deprivation for 24 h. Durant *et al.* (1999) reported that the introduction of *S. enteritidis* into the crop environment with high pH and lowered concentrations of lactate and total VFA were accompanied by increased crop colonization. In the current study, DGGE analysis revealed the highest similarity in crop microbial populations (82.8, 79.1, and 73.2%) in C versus Za hens (trial 1), Za versus Zp hens (trial 2), and C versus Zp hens (trial 3), respectively. In all 3 trials, greater similarity in crop DGGE profiles was shared between control hens and hens fed dietary zinc than with profiles of crops from feed withdrawal

molted hens. When the crop samples were examined for pH and fermentation products from the same trials and reported (Moore *et al.*, 2003) there were negligible differences in crop pH and lactic acid production between hens fed Za and hens fed Zp in trials 1 and 2 and while only crop pH levels were similar in trial 3. Crop lactic acid was generally less for Mo hens than C hens or Za and Zp molted hens (Moore *et al.*, 2003). Consequently, the general similarity of crop microbial populations for hens receiving feed may in part be due to comparable fermentative crop microflora yielding similar lactic acid production and leading to similar pH levels. The DGGE detected difference in feed withdrawal hens may result from back of feed possibly leading to a decrease in the *Lactobacilli* population in hens that are deprived of feed (Durant *et al.*, 1999; Humphrey *et al.*, 1993).

Based on the results of the current study, molecular-based denaturing gradient gel electrophoresis (DGGE) method can be applied as a rapid screening tool to detecting similarities in the digestive microbial communities between molted hens by either Za or Zp amended feeds. However, greater molecular sensitivity may be needed to more precisely quantitate key indicator groups of gastrointestinal bacteria that reveal the potentially more subtle differences created by feeding similar molting diets.

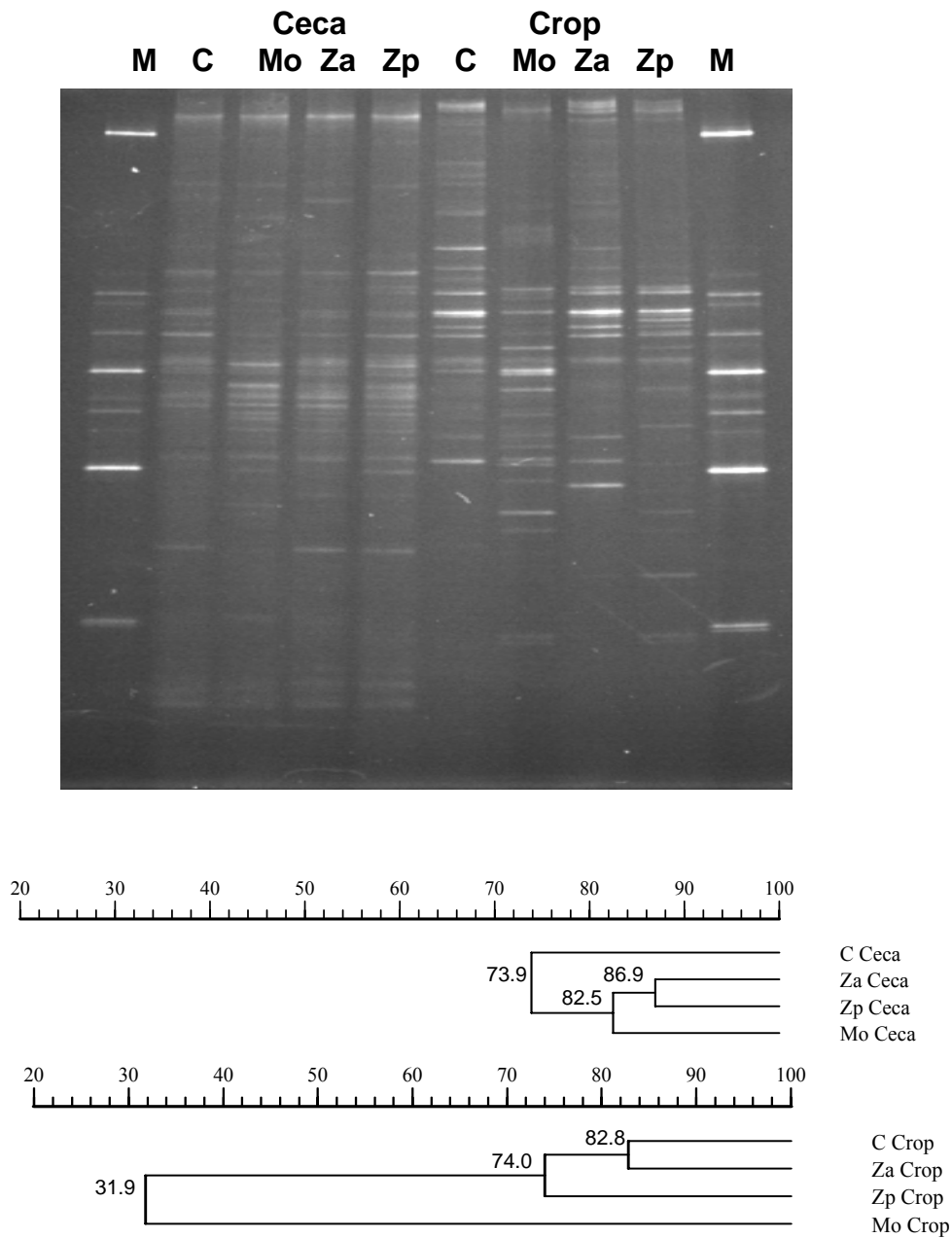


FIGURE 11-1. Denaturing gradient gel electrophoresis of ceca or crop bacterial 16S amplicon patterns from Leghorn hens on nonmolted control (C), molted feed withdrawal (Mo), zinc acetate (Zc), and zinc propionate (Zp) in trial 1. M refers reference amplicons. Relative similarity of band patterns is indicated by their grouping on the dendrogram and the percentage coefficient

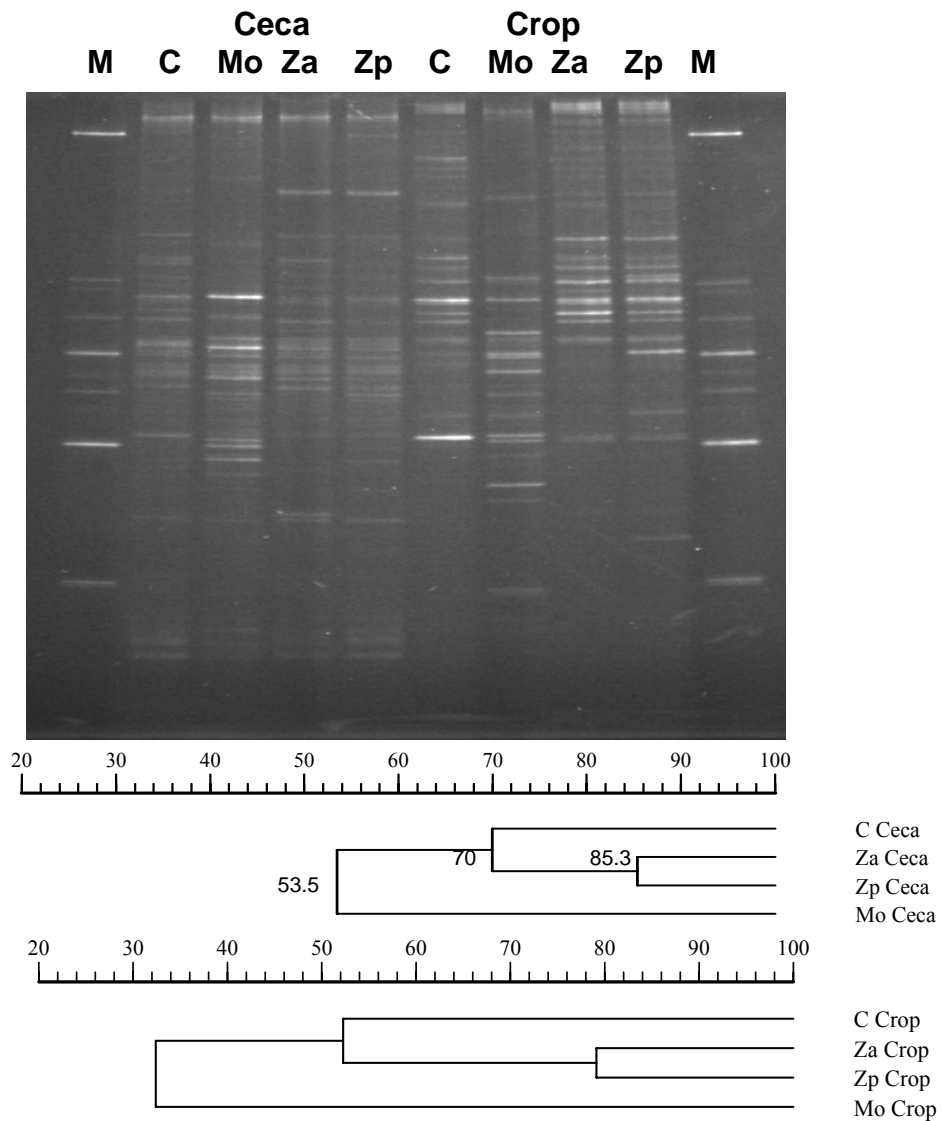


FIGURE 11-2. Denaturing gradient gel electrophoresis of ceca or crop bacterial 16S amplicon patterns from leghorn hens on nonmolted control (C), molted feed withdrawal (Mo), zinc acetate (Zc), and zinc propionate (Zp) in trial 2. M refers reference amplicons. Relative similarity of band patterns is indicated by their grouping on the dendrogram and the percentage coefficient

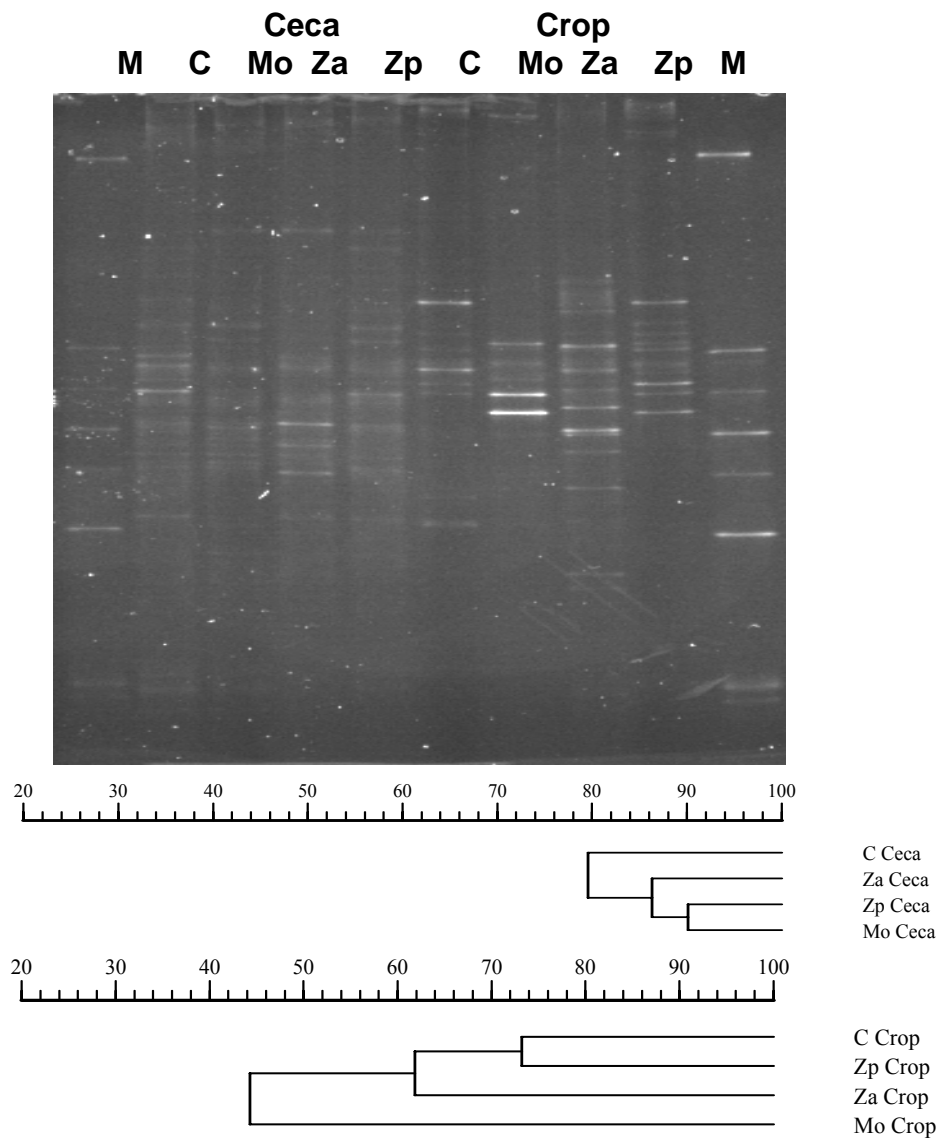


FIGURE 11-3. Denaturing gradient gel electrophoresis of ceca or crop bacterial 16S amplicon patterns from Leghorn hens on nonmolted control (C), molted feed withdrawal (Mo), zinc acetate (Zc), and zinc propionate (Zp) in trial 3. M refers reference amplicons. Relative similarity of band patterns is indicated by their grouping on the dendrogram and the percentage coefficient

CHAPTER XII

**EFFECTS OF STORAGE CONDITIONS ON BONE BREAKING
STRENGTH AND BONE ASH IN LAYING HENS AT
DIFFERENT STAGES IN PRODUCTION CYCLES***

Overview

This study was conducted to determine the effects of refrigeration and frozen storage on tibia breaking strength (kg/g) and tibia ash (%) in bones obtained from hens of different ages. A total of 75 Single Comb White Leghorn hens were divided into three equal groups according to age (72, 80, and 92 wk of age). Both tibia of each hen were harvested and immediately defleshed. The tibia from each hen were randomly assigned to either 1 day refrigeration or 7 days frozen storage. Tibia breaking strengths were determined using an Instron Universal Testing Machine with 50-kg-load cell at 50-kg-load range with a crosshead speed of 50 mm/min. Tibia ash weights were determined by ashing for 24 h at 615 °C. Bone breaking strength in 72 wk of age hens was significantly ($P < 0.05$) higher in refrigerated tibia (2.48 kg/g) than those in frozen storage (1.75 kg/g). However, there was no significant difference ($P > 0.05$) of bone ash (percentage of tibia ash) in all three groups between refrigerated (72 wks: 38.84%, 80 wks: 46.40%, 92 wks: 46.99%) and frozen storage (72 wks: 39.12%, 80 wks: 44.88%, 92 wks: 45.35%). This study indicated that frozen storage only influenced assessment of

* Reprinted with permission from: Effects of storage conditions on bone breaking strength and bone ash in laying hens at different stages in production cycles, by Park *et al.*, 2002, Poultry Sci. Accepted.

bone strength in 72 wk aged laying hens but could be used for sample storage of bones from older hens.

Introduction

During the laying cycles, changes in medullary bone have been investigated in avian species (Bloom *et al.*, 1958; Candlish, 1971). Medullary bone is the storage site for the calcium necessary for egg shell calcification (Mueller *et al.*, 1964). Dietary calcium depletion of laying hens cause a decrease in the amount in bone and an increase in the osteoid (Bloom *et al.*, 1958; Zambonin Zallone and Teti, 1981). Bone measurements such as bone-breaking force (Crenshaw *et al.*, 1981; Merkley, 1981; Ruff and Hughes, 1985), bone ash (Garlich *et al.*, 1982), bone ash concentration (Garlich *et al.*, 1982; Cheng and Coon, 1990), bone mineral content (Akpe *et al.*, 1987), and bone density (Watkins and Southern, 1992) have been used as indicators of bone status in the mineral nutrition of poultry and swine.

When large numbers of animals are involved, the logistics for immediately processing bone samples for measuring bone breakage and other parameters becomes difficult. Therefore, an effective procedure is needed to be able to preserve and stabilize the bone mineralization in medullary bone of mature hens when a large quantity of medullary bone can not be processed at the same time. Researchers may want to use refrigerated storage as a method of preservation. However, it is not known if the preservation method could influence estimation of bone mineralization or bone strength.

Therefore, the objective of the current study was to compare the refrigerated and

frozen storage among bone breaking strength and bone ash for tibias from pre or post-molt laying hens.

Materials and Methods

General Procedure

A total of seventy five of Single Comb White Leghorn (SCWL) hens were used in this study. These hens were divided into three groups of twenty five hens, 72 wk (in the first laying cycle), 80 wk (immediately following the molting process), or 92 wk (in the second laying cycle) of age. Hens were maintained under an artificial lighting program of hours of light (L) and dark (D) of 16L:8D and provided *ad libitum* access to a complete layer ration and water via nipple drinkers that prevented cross-contamination of water and feed. Hens were euthanized by cervical dislocation. The right or left tibia of each of hen was randomly chosen from each bird, excised from the fresh carcass, and defleshed without boiling. The tibias were individually sealed in a 4 oz Whirl-Pak bag (Nasco, Fort Atkinso, WI) to minimize moisture loss. The sample bags were contained into a plastic container and stored in a 4 °C walk-in cooler for one day or -20 °C work-in freezer for seven days. The tibias were dried at 105 °C for 24 h, placed in a desiccator, and bone weight was recorded. Tibia breaking strengths (breaking force divided by bone weight expressed as kilograms per gram) (Shafer *et al.*, 2001) were conducted using a Model 1011 Instron Universal Testing Machine (Instron Corp., Canton, MA) with 50-kg-load cell at 50-kg-load range with a crosshead speed of 50 mm/min with tibia supported on a 3.35-cm span (Shafer *et al.*, 2001). Moisture free tibia ash were

determined by ashing in tared ceramic crucibles for 24 h at 615 °C. Percentage tibia ash was calculated by dividing tibia ash weights by tibia dry weight and multiplying by 100 (AL-Batshan *et al.*, 1994).

Molting Procedure

Laying hens were housed two per cage for the molting procedure. The hens were assigned to each of four treatment groups: Zn acetate (1% of Zn added to layer ration) diet, Zn propionate (1% of Zn added to layer ration) diet, poultry layer ration (nonmolted control), and feed withdrawal (control). All birds were allowed *ad libitum* access to water and their respective diets. Hens were placed on an artificial lighting program of 8L: 16D prior to molting procedure. Treatments were placed throughout the house to ensure there was no variability in egg production or reproductive tract regression due to light stimulation.

During the molt procedure, hen weights were monitored at Day 1, 3, 5, 7, and 9. In accordance with Texas A&M University Lab Animal Care Committee (ULACC) animal use protocols, any hen reaching 25% weight loss prior to the end of the trial (Day 9) were removed from her respective diet and placed back on a complete layer ration. Data was not collected from these hens.

Statistical Analysis

Data were analyzed using a t test in SAS statistical analysis software program, version 6.11 (SAS Institute Inc., Cary, NC). All statistical analyses were considered significantly different at the $P < 0.05$ level.

Results and Discussion

Hens at ages of 72, 80, and 92 wk were chosen in this experiment because 72 wk of age of hens were at the end of the laying cycle, 80 wk age of hens immediately following the molting process, and 92 wk hens were in the second laying cycle. Therefore, this allowed for determination of whether or not two different preservation methods (refrigerated versus frozen storage) could influence bone breakage or ash values from hens processed in different production cycles.

When breaking strengths of the tibias held in refrigerated storage were compared to those of the tibia held in frozen storage, values from 72 wk age of hens were significantly ($P < 0.05$) stronger than those obtained from tibia held in refrigerated storage (Figure 12-1). The average of breaking strengths in refrigerated and frozen storage was 2.484 and 1.745 kg/g, respectively. However, post molt hens either 80 wks or 92 wks did not ($P > 0.05$) exhibit significantly different breaking strength of tibias between refrigerated and frozen storage. When percentage bone ash of the tibias in refrigerated storage was compared to those of the frozen storage hens, all three groups of hens were not ($P > 0.05$) significantly different between refrigerated and frozen storage (Figure 12-2). The average of percentage bone ash between refrigerated and frozen storage in 72, 80 and 92 wks was 38.84 (refrigerated) and 39.12% (frozen), 46.40 (refrigerated) and 44.88% (frozen), and 46.99 (refrigerated) and 45.35% (frozen), respectively (Figure 12-2). According to AL-Batshan *et al.* (1994), femur ash decreased from 50.8 (37 wk) to 47.6% (58 wk) and then increased up to 49% after molt (72 wk). Garlich *et al.* (1984) also reported that femur weight of laying hens decreased from

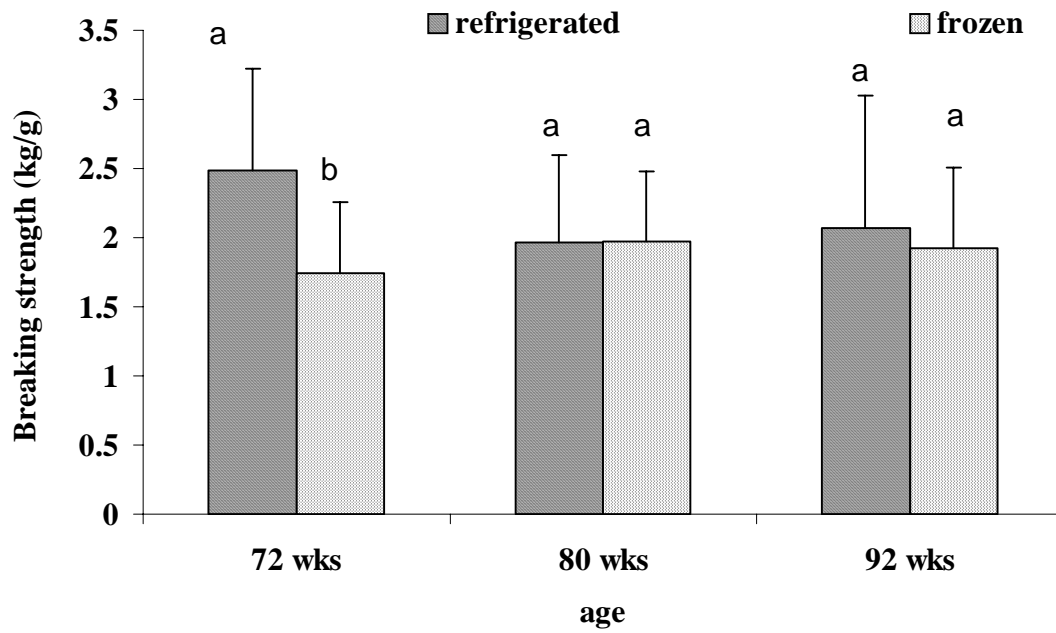


FIGURE 12-1. Effect of storage conditions on breaking strength (breaking force divided by bone weight expressed as kilograms per gram) of tibia obtained from hens of different ages. a, b values on top of bars are significantly different ($P < 0.05$)

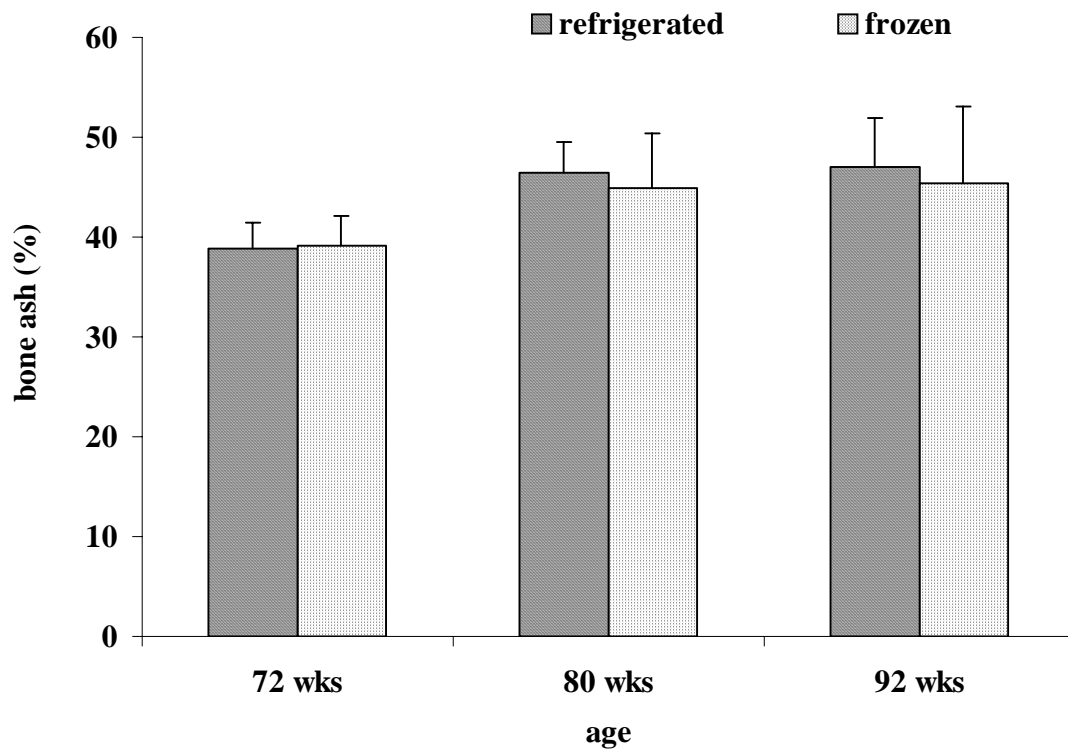


FIGURE 12-2. Effect of storage conditions on percentage bone ash (tibia ash weights divided by tibia dry weight and multiplying by 100) of tibia obtained from hens of different ages

4.58g (67 wk in the first laying cycle) to 4.18 (77 wk at molt) and then increased 4.37 g (83 wk in the second laying cycle). Although we did not statistically compare the breaking strength and bone ash of tibia obtained from different laying cycles of hens in refrigerated storage, breaking strength decreased from 2.48 (72 wk in the first laying cycle) to 1.97 (80 wk at molt) and then slightly increased 2.07 kg/g after molting (92 wk in the second laying cycle). Bone ash was increased from 38.84 (72 wk in the first laying cycle) to 46.40 (80 wk at molt) and then slightly increased 46.99% after molting (92 wk in the second laying cycle). The breaking strength in the current study showed same trend as a study in Garlich *et al.* (1984). Moreover, Lott *et al.* (1980) reported that breaking strength of fresh tibia was not different from that of frozen tibia obtained from 4 week old chicks. The breaking strength of fresh tibia and frozen tibia were 21.62 and 21.71 kg, respectively. Bone mineral content and density were not influenced by processing method and the fresh tibia was sheared only 4% more force than dry fat-free tibia by Orban *et al.* (1991).

In the current study, the processing methods were somewhat different from many studies. Before the tibia were stored at 4 °C or -20 °C, the fresh was cut from the bone and muscle residual was removed by scraping a knife. This method efficiently removed the muscle from tibia without boiling. This may reduce the cell damage caused by boiling. Therefore, this method may save processing time and may minimize the impact of sample preparation on the tibia breaking strength. The typical method which removes the flesh by boiling, may influence the breaking strength because the tibia may absorb some water and may have cell damaged due to boiling. And the two storage types of

moisture free tibia did not have significantly different amounts of bone ash. Therefore, the bone ash content was not affected by frozen storage.

Because this study only focused on preservation methods (refrigerated versus frozen), all three groups of hens were maintained under similar conditions including feeding with poultry layer ration and caging. All tibias were dry moisture-free. In this study, it was determined that tibia breaking strength was influenced by frozen storage, but tibia ash was not. An approximately 30% reduction in bone strength in 72 wk aged laying hens was affected by frozen storage. This reduction of bone strength in those hens could be the result of cell damage by frozen storage. Therefore, the frozen method could be used for sample storage of bones from older post-molted hens.

CHAPTER XIII

CONCLUSION

The results in the first phase of studies suggest that the further development of aerosol sampling systems may allow for continuous monitoring of microbial populations in a poultry layer house and aerosol sampling could be a potential diagnostic tool for the poultry layer house as a whole. The successful application of transposon footprinting method could lead to more rapid screening of mutants with potential genes associated with desiccation.

The *in vitro* results in the second phase of studies suggest that zinc compounds, or the combination of zinc compounds and an acidic condition effectively limit *Salmonella* growth. Future studies might include its application to limit the surface contamination of *Salmonella* in the poultry houses. Additionally, high dietary zinc amended poultry feeds may be a potential barrier to interrupt the transmission of *Salmonella* in a poultry layer house.

The *in vivo* results in the third phase of studies suggest that dietary zinc propionate is an effective new alternative method to induce molting, and dietary zinc acetate has the potential to be used as an alternative method to feed deprivation to induce molting and subsequent laying cycle without significantly increasing the risk of *Salmonella enteritidis* contamination. Both dietary Zn acetate and Zn propionate fed hens showed higher egg production than hens undergoing feed withdrawal. Egg weights from Zn propionate fed hens were significantly heavier than that from feed withdrawal treatment hens. Additionally, by comparing the dendrogram of amplicon patterns, there

were quite similar cecal and crop communities observed in between zinc acetate fed hens and zinc propionate fed hens.

In conclusion, the three phases of studies may provide the poultry industry with a scientifically based rationale for a possible production and processing management that can not only reduce the major risk for *Salmonella* contamination, but also enhance the commercial benefits from the safety of environments of poultry layer house process and poultry shell eggs.

REFERENCES

- Agranoff, D. D., and S. Krishna, 1998. Metal ion homeostasis and intracellular parasitism. *Molecular Microbiology*. 28:403-412.
- Akpe, M. E., P. E. Waibel, K. Larntz, A. L. Metz, S. L. Noll, and, M. M. Walser, 1987. Phosphorous availability bioassay using bone ash and bone densitometry as response criteria. *Poultry Sci.* 66:713-720.
- Al-Batshan, H. A., S. E. Scheideler, B. L. Black, J. D. Garlich, and K. E. Anderson, 1994. Duodenal calcium uptake, femur ash, and increase following molt. *Poultry Sci.* 73:1590-1596.
- Alvarez, A. J., M. P. Buttner, and L. D. Stetzenbaach, 1995. PCR for bioaerosol monitoring: Sensitivity and environmental interference. *Appl. Environ. Microbiol.* 61:3639-3644.
- Anderson, J. D., F. A. Dark, and S. Peto, 1968. The effect of aerosolization upon survival and potassium retention by various bacteria. *J. Gen. Microbiol.* 52:99-105.
- Andrews, D. K., W. D. Berry, and J. Brake, 1987a. Effect of lighting program and nutrition on reproductive performance of molted Single Comb White Leghorn hens. *Poultry Sci.* 66:1298-1305.
- Andrews, D. K., W. D. Berry, and J. Brake, 1987b. Effect of lighting program and nutrition on feather replacement of molted Single Comb White Leghorn hens. *Poultry Sci.* 66:1635-1639.

- Andrews, W. H., G. A. June, P. S. Sherrod, T. S. Hammack, and R. M. Amaguana, 1992. *Salmonella*. Pages 5.01-5.20 in: *Bacteriological Analytical Manual*. 8th ed. Association of Official Analytical Chemists, Arlington, VA.
- Angulo, F. J., and D. L. Swerdlow, 1998. *Salmonella enteritidis* infections in the United States. *J. Am. Vet. Med. Assoc.* 213:1729-1731.
- Anonymous, 1994. From the Centers for Disease Control and Prevention. Outbreaks for *Salmonella enteritidis* associated with homemade ice cream-Florida, 1993. *J. Am. Med. Assoc.* 272:1490-1492.
- Anonymous, 1999. AVAR's concerns about forced molting. *J. Am. Vet. Med. Assoc.* 215:1236.
- Anton, A., C. Grosse, J. Reissmann, T. Pribyl, and D. H. Nies, 1999. Czcd is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. Strain CH34. *J. Bacteriol.* 181:6876-6881.
- Arnouts, S., J. Buyse, M. Cokelaere, and E. Decuypere, 1993. Jojoba meal in the diet of broiler breeders: Physiological and endocrinological effects. *Poultry Sci.* 72:1741-1721.
- Arp, L. H., C. H. L. Graham, and N. F. Cheville, 1978. Comparison of clearance rates of virulent and avirulent *Escherichia coli* in turkeys after aerosol exposure. *Avian Dis.* 23:386-391.
- Asem, E. M., M. Molnar, and F. Hertelendy, 1987. Leutenizing hormone-induced intracellular calcium mobilization in granulose cells: Comparison with forskolin and 8-bromo-adenosine 3', 5'-monophosphate. *Endocrinology.* 120:853-859.

- Auerswald, E. A., G. Ludwig, and H. Schaller, 1981. Structural analysis of Tn5. *Cold Spring Harbor Symp. Quant. Biol.* 45:107-113.
- Bains, B. S., and M. A. MacKenzie, 1974. Transmission of *Salmonella* through an integrated poultry organization. *Poultry Sci.* 53:1114-1118.
- Baker, M., J. Brake, and G. R. McDaniel, 1983. The relationship between body weight loss during an induced molt and postmolt egg production, egg weight, and shell quality in cage layers. *Poultry Sci.* 62:409-413.
- Balch, W. E., and R. S., Wolfe, 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)- dependent growth of *Methanobacterim ruminantium* in a pressurized atmosphere. *Appl. Environ. Microbiol.* 32:781-791.
- Bannister, J., W. Bannister, and E. Wood, 1971. Bovine erthrocyte cupro-zinc protein. *European Journal of Biochemistry.* 18:178-186.
- Bar, A., V. Razaphkovsky, D. Shinder, and E. Vax, 2003. Alternative procedures for molt induction: Practical aspects. *Poultry Sci.* 82:543-550.
- Barnes, E. M., C. S. Impey, and B. J. H. Stevens, 1979. Factors affecting the incidence and anti-salmonella activity of the anaerobic caecal flora of the young chick. *J. Hyg.* 82:263-283.
- Barnes, E. M., C. S. Impey, and D. M. Cooper, 1980. Manipulation of the crop and intestinal flora of the newly hatched chick. *Am. J. Clin. Nutr.* 33:2426-2433.

- Barnhart, H. M., D. W. Dreesen, and J. L. Burke, 1993. Isolation of *Salmonella* from ovaries and oviducts from whole carcasses of spent hens. *Avian Dis.* 37:977-980.
- Barrow, P. A., 1993. *Salmonella* control-Past, present and future. *Avian Pathol.* 22:651-669.
- Barrow, P. A., and M. A. Lovell, 1991. Experimental infection of egg-laying hens with *Salmonella enteritidis* phage type 4. *Avian Pathol.* 20:335-348.
- Baskerville, A., T. J. Humphrey, R. B. Fitzgeorge, R. W. Cook, H. Chart, B. Rowe, and A. Whitehead, 1992. Airborne infection of laying hens with *Salmonella enteritidis* phage type 4. *Vet. Rec.* 130:395-398.
- Batal, A. B., T. M. Parr, and D. H. Baker, 2001. Zinc bioavailability in tetrabasic zinc chloride and the dietary zinc requirement of young chicks fed a soy concentrate diet. *Poultry Sci.* 80:87-90.
- Batič, M., D. Lenarčič, J. Štupar, and P. Raspor, 1996. Rapid method for screening the tolerance of yeasts to zinc (II) and chromium (III). *J. Rapid Meth. Auto. Microbiol.* 4:265-278.
- Baumler, A. J., B. M. Hargis, and R. M. Tsoils, 2000. Tracing the origins of *Salmonella* outbreaks. *Science.* 287:50-52.
- Beach, R. S., M. E. Gershwin, and L. S. Hurley, 1982. Reversibility of developmental retardation following murine fetal zinc deprivation. *J. Nutr.* 112:1169-1181.

- Beckers, H. J., F. M. Vanleusden, M. J. M. Meijssen, and E. H. Kampelmacher, 1985. Reference material for the evaluation of a standard method for the detection of *Salmonella* in foods and feeding stuffs. *J. Appl. Bacteriol.* 59:507-512.
- Begin, J., and T. Johnson, 1976. Effect of dietary salt on the performance of laying hens. *Poultry Sci.* 55:2395-2404.
- Bell, D., 1987. Is molting still a variable replacement alternative? *Poultry Trib.* 93:32-35.
- Bell, D., 2001. Economic implications of controversial layer management programs. Pages 83-91 *in*: Proceedings of the 50th western poultry disease conference, Davis, CA.
- Benbough, J. E., 1967. Death mechanisms in airborne *Escherichia coli*. *J. Gen. Microbiol.* 47:325-333.
- Berrang, M. E., N. A. Cox, and J. S. Bailey, 1995. Measuring air-borne microbial contamination of broiler hatching cabinets. *J. Appl. Poultry Res.* 4:83-87.
- Berry, W. D., and J. Brake, 1985. Comparison of parameters associated with molt induced by fasting, zinc, and low dietary sodium in caged layers. *Poultry Sci.* 64:2027-2036.
- Bhatia, T. R. S., G. D. McNabb, H. Wyman, and G. P. S. Nayar, 1980. *Salmonella* isolation from litter as an indicator of flock infection and carcass contamination. *Avian Dis.* 23:838-847.

- Bird, H. R., and M. L. Sunde, 1982. Evaluation of two forced moulting procedures and their effect on productive performance of two strains of white leghorns. *Poultry Sci.* 61:1418. (Abstr.).
- Blair, R., and A. B. Gilbert, 1973. The influence of supplemental phosphorous in a low-calcium diet designed to induced a resting phase in laying hens. *Br. Poultry Sci.* 14:131-135.
- Blalock, T. L., and C. H. Hill, 1988. Studies on the role of iron in zinc toxicity in chicks. *Biol. Trace Elem. Res.* 17:17-29.
- Bloom, M. A., L. V. Domm, A. V. Nalbandov, and W. Bloom, 1958. Medullary bone of laying chickens. *Am. J. Anat.* 102:411-451.
- Blumenthal, D., 1990, April. *Salmonella enteritidis* from the chicken or the egg, FDA Consumer, 7-10.
- Bohnhoff, M., and C. P. Miller, 1962. Enhanced susceptibility to *Salmonella* infection in streptomycin-treated mice. *J. Infect. Dis.* 111:117-127.
- Boonchuvit, B., and P. B. Hamilton, 1975. Interaction of aflatoxin and paratyphoid infections in broiler chickens. *Poultry Sci.* 54:1567-1573.
- Brake, J., and P. Thaxton, 1979. Physiological changes in caged layers during a forced molt. 2. Gross changes in organs. *Poultry Sci.* 58:707-716.
- Brake, J., P. Thaxton, and E. H. Benton, 1979. Physiological changes in caged layers during a forced molt. 3. Plasma tyroxine, plasma triiodothyronine, adrenal cholesterol and total adrenal steroids. *Poultry Sci.* 58:1345-1350.

- Brake, J., M. Baker, and J. G. Mannix, 1981. Weight loss characteristics of the body, liver, ovary, oviduct, and uterine lipid during a forced molt and their relationship to postmolt performance. *Poultry Sci.* 60:1628. (Abstr.).
- Brake, J., and G. R. McDaniel, 1981. Factors affecting broiler breeder performance. 3. Relationship of body weight during fasting to post-molt performance. *Poultry Sci.* 60:726-729.
- Brake, J., G. W. Morgan, M. Baler, and P. Thaxton, 1982. Physiological changes in caged layers during a forced molt. 4. Leucocyte and packed cell volume. *Poultry Sci.* 61:790-795.
- Brake, J., 1992. Mechanisms and metabolic requirements for complete and rapid reproductive rejuvenation during an induced molt-A brief review. *Ornis Scand.* 23:335-339.
- Brake, J., 1993. Recent advances in induced molting. *Poultry Sci.* 72:929-931.
- Breeding, S. W., J. Brake, J. D. Garlich, and A. L. Johnson, 1992. Molt induced by dietary zinc in a low-calcium diet. *Poultry Sci.* 71:168-180.
- Brenner, F. W., R. G. Villar, F. J. Angulo, R. V. Tauxe, and B. Swaminathan, 2000. *Salmonella* nomenclature. *J. Clin. Microbiol.* 38:2465-2467.
- Brink, M. F., D. E. Becker, S. W. Terrill, and A. H. Jensen, 1950. Zinc toxicity in the weaning pig. *J. Animal Sci.* 18:836-842.
- Brody, A. L., (Ed.) 1989. Microbiological safety of modified/controlled atmosphere/vacuum packaged feeds. Pages 159-174 *in*: Controlled/Modified

Atmosphere Vacuum Packaging of Food. A. L. Brody, ed. Food & Nutrition Press, Inc. Trumbull, CT.

- Brownell, J. R., W. W. Sadler, and M. J. Fanelli, 1970. Role of ceca in intestinal infections of chickens with *Salmonella typhimurium*. Avian Dis. 14:106-116.
- Butler, E. J., M. J. Curtis, and M. Watford, 1973. The effect of *Escherichia coli* endotoxin on the plasma iron concentration in the domestic fowl. Res. Vet. Sci. 15:267-269.
- Butler, E. J., and M. J. Curtis, 1973. The effects of *Escherichia coli* endotoxin and ACTH on the plasma zinc concentration in the domestic fowl. Res. Vet. Sci. 15:363-367.
- Buttner, M. P., and L. D. Stetzenbach, 1991. Evaluation of four aerobiological sampling methods for the retrieval of aerosolized *Pseudomonas syringae*. Appl. Environ. Microbiol. 57:1268-1270.
- Byrd, J. J., H. S. Xu, and R. R. Colwell, 1991. Viable but nonculturable bacteria in drinking water. Appl. Environ. Microbiol. 57:875-878.
- Byrd, J. A., D. E. Carrier, J. R. DeLoach, and D. J. Nisbet, 1997. Comparison of drag-swab environmental protocols for the isolation of *Salmonella* in poultry houses. Avian Dis. 41:709-713.
- Byrd, J. A., J. R. DeLoach, D. E. Carrier, D. J. Nisbet, and L. H. Stanker, 1999. Evaluation of *Salmonella* serotype distributions from commercial broiler hatcheries and grower houses. Avian Dis. 43:39-47.

- Caldwell, D. J., B. M. Hargis, D. E. Corrier, J. D. Williams, L. Vidal, and J. R. DeLoach, 1994. Predictive value of multiple drag-swab sampling for the detection of *Salmonella* from occupied or vacant poultry house. *Avian Dis.* 38:461-466.
- Caldwell, D. J., B. M. Hargis, D. E. Corrier, L. Vidal, and J. R. DeLoach, 1995. Evaluation of *Salmonella* serotype isolation from poultry farms using drag-swab sampling. *Avian Dis.* 39:617-621.
- Caldwell, D. J., B. M. Hargis, D. E. Corrier, and J. R. DeLoach, 1998. Frequency of isolation of *Salmonella* from protective foot covers worn in broiler houses as compared to drag-swab sampling. *Avian Dis.* 42:381-384.
- Campos, E. J. and N. C. Baiao, 1979. The effects of methods of forced molting on performance of commercial layers. *Poultry Sci.* 58:1040. (Abstr.).
- Candlish, J. K., 1971. The formation cortical and medullary bone during shell calcification. *Br. Poult. Sci.* 12:119-127.
- Carlson, H. C., and G. R. Whenham, 1968. Coliform bacteria in chicken broiler house dust and their possible relationship to coli-septicemia. *Avian Dis.* 12:297-302.
- Carlsson, J., G. P. D. Grandberg, G. K. Nyberg, and M. J. K. Edlund, 1979. Bactericidal effects of cysteine exposed to atmospheric oxygen. *Appl. Environ. Microbiol.* 37:383-390.
- Cason, J. A., N. A. Cox, and J. S. Bailey, 1994. Transmission of *Salmonella typhimurium* during hatching of broiler chicks. *Avian Dis.* 38:583-588.
- Cave, N. A. G., 1982. Effect of dietary short- and medium-chain fatty acids on feed intake by chicks. *Poultry Sci.* 61:1147-1153.

- Cave, N. A. G., 1984. Effect of dietary propionic and lactic acids on feed intake by chicks. *Poultry Sci.* 63:131-134.
- Centers for Disease Control and Prevention, 1999. Infection of foodborne illness: Preliminary data from the foodborne diseases active surveillance network (FoodNet)-United States, 1998. *MMWR.* 48:189-194.
- Charles, D. R., H. A. Elson, and M. S. P. Haywood, 1994. Poultry housing. Pages 249-272 *in*: *Livestock Housing*. C. M. Wathes and D. R. Charles, ed. CAB International, Wallingford, UK.
- Cheng, S., C. Fockler, W. M. Barnes, and R. Higuchi, 1994. Effective amplification of long targets from cloned inserts and human genomic DNA. *Proc. Natl. Acad. Sci. USA.* 91:5695-5699.
- Cheng, T. K., and C. N. Coon, 1990a. Sensitivity of various bone parameters of laying hens to different daily calcium intakes. *Poultry Sci.* 69:2209-2213.
- Cheng, T. K., and C. N. Coon, 1990b. Effect of calcium source, particle size, limestone solubility *in vitro*, and calcium intake level on layer bone status and performance. *Poultry Sci.* 69:2214-2219.
- Cherrington, C. A., M. Hinton, G. C. Mead, and I. Chopra, 1991. Organic acids: chemistry, antibacterial activity and practical applications. *Adv. Microbial Physiol.* 32:87-108.
- Chesters, J. K., 1992. Trace element-gene interactions. *Nutrition Reviews.* 50:217-223.

- Chlebowski, J. F., and J. E. Coleman, 1976. Zinc and its role in enzymes. Pages 1-140
in: Metal Ions in Biological Systems, Vol 6. H. Sigel, ed., Marcel Dekker, Inc.,
New York, NY.
- Choi, M. Y., and C. K. Chan, 2002. The effects of organic species on the hygroscopic
properties of inorganic aerosols. *Environ. Sci. Technol.* 36:2422-2428.
- Choudhury, R., and S. Srivastava, 2001. Mechanism of zinc resistance in *Pseudomonas*
putida strain S4. *World J. Micro. Biotechnol.* 17:149-153.
- Chung, K. C., and J. M. Goepfert, 1970. Growth of *Salmonella* at low pH. *J. Food Sci.*
35:326-328.
- Clark, S., R. Rylander, and L. Larsson, 1983. Airborne bacteria, endotoxin and
fungi in poultry and swine confinement buildings. *Am. Ind. Hyg. Assoc. J.*
44:537-541.
- Clemmer, D. I., J. L. S. Hickey, J. F. Bridges, D. J. Schliessman, and M. F. Shaffer,
1960. Bacteriological studies of experimental air-borne salmonellosis in chicks.
J. Infect. Dis. 106:197-210.
- Cohen, J. L., J. A. Bartlett, and G. R. Corey, 1987. Extra-intestinal manifestations of
Salmonella infections. *Medicine.* 66:349-388.
- Cohen, N. D., D. E. Wallis, H. L. Neibergs, A. P. McElroy, E. D. McGruder, J. R.
DeLoach, D. E. Corrier, and B. M. Hargis, 1994. Comparison of the polymerase
chain reaction using genus-specific oligonucleotide primers and microbiologic
culture for the detection of *Salmonella* in drag-swabs from poultry houses.
Poultry Sci. 73:1276-1281.

- Collins, F. M., 1974. Vaccines and cell-mediated immunity. *Bacteriol. Rev.* 38:371-402.
- Corrier, D. E., A. Hinton Jr., R. L. Ziprin, R. C. Beier, and J. R. DeLoach, 1990. Effect of dietary lactose on cecal pH, bacteriostatic volatile fatty acids and *Salmonella typhimurium* colonization of broiler chicks. *Avian Dis.* 34:617-625.
- Corrier, D. E., B. Hargis, A. Hinton Jr., D. Lindsey, D. Caldwell, J. Manning, and J. DeLoach, 1991. Effect of anaerobic cecal microflora and dietary lactose on colonization resistance of layer chicks to invasive *Salmonella enteritidis*. *Avian Dis.* 35:337-343.
- Corrier, D. E., D. J. Nisbet, C. M. Scanlan, A. G. Hollister, and J. R. DeLoach, 1995. Control of *Salmonella typhimurium* colonization in broiler chicks with a continuous-flow characterized mixed culture of cecal bacteria. *Poultry Sci.* 74:916-924.
- Corrier, D. E., D. J. Nisbet, B. M. Hargis, P. S. Holt, and J. R. DeLoach, 1997. Provision of lactose to molting hens enhances resistance to *Salmonella enteritidis* colonization. *J. Food Prot.* 60:10-15.
- Corrier, D. E., J. A. Byrd, B. M. Hargis, M. E. Hume, R. H. Bailey, and L. H. Stanker, 1999. Survival of *Salmonella* in the crop contents of market-age broilers during the feed withdrawal. *Avian Dis.* 43:453-460.
- Cousins, R. J., K. T. Smith, M. L. Failla, and L. A. Markowitz, 1978. Origin of low-molecular-weight zinc-binding complexes from rat intestine. *Life Sci.* 23:1819-1826.

- Cox, C. S., 1966. The survival of *Escherichia coli* sprayed into air and into nitrogen from distilled water and from solutions of protecting agents as a function of relative humidity. *J. Gen. Microbiol.* 44:15-31.
- Cox, C. S., and F. Baldwin, 1967. The toxic effect of oxygen upon the aerosol survival of *Escherichia coli* B. *J. Gen. Microbiol.* 48:15-21.
- Cox, C. S., 1989. Airborne bacteria and viruses. *Science Progress.* 73:469-499.
- Cox, C. S., and C. M. Wathes, 1995. Bioaerosols in the environment. Pages 11-14 *in*: Bioaerosols Handbook. C. S. Cox and C. M. Wathes, ed. CRC Press, Inc., Boca Raton, FL.
- Cox, D. H., and D. L. Harris, 1960. Effect of excess dietary zinc on iron and copper in the rat. *J. Nutr.* 70:514-520.
- Cox, N. A., J. S. Bailey, J. E. Thomson, and B. J. Juven, 1983. *Salmonella* and other Enterobacteriaceae found in economical poultry feed. *Poultry Sci.* 62:2169-2175.
- Cox, N. A., M. E. Berrang, and J. A. Cason, 2000. *Salmonella* penetration of egg shells and proliferation in broiler hatching eggs-A review. *Poultry Sci.* 79:1571-1574.
- Coyle, E. F., C. D. Ribiero, A. J. Howard, S. R. Palmer, H. I. Jones, L., Ward, and B. Rowe, 1988. *Salmonella enteritidis* phage type 4 infection: Association with hens' eggs. *Lancet.* 2:1295-1297.
- Creger, C. R., and J. R. Scott, 1977. Dietary zinc as an effective resting agent for the laying hen. *Poultry Sci.* 56:1706. (Abstr.).

- Crenshaw, T. D., E. R. Peo Jr., A. J. Lewis, and B. D. Moser, 1981. Bone strength as a trait for assessing mineralization in swine: A critique of techniques involved. *J. Anim. Sci.* 53:827-835.
- Crook, B., J. F., Robertson, S. A. Tavers Glass, E. M. Botheroyd, J. Lacey, and M. D. Topping, 1991. Airborne dust, ammonia, microorganisms, and antigens in pig confinement houses and the respiratory health of exposed farm workers. *Am. Ind. Hyg. Assoc. J.* 52:271-279.
- Cunningham-Rundles, S., and W. E. Cunningham-Rundles, 1988. Zinc modulation of immune system. Pages 197-214 *in: Nutrition and Immunology.* R. K, Chandra, ed. Alan R. Liss Inc, New York.
- Cutter, C. N., 2002. Microbial control by packaging: A review. *Crit. Rev. Food Sci. Nutr.* 42:151-161.
- Dardenne, M., and J. M. Bach, 1993. Rationale for the mechanism of zinc interaction in the immune system. Pages 501-509 *in: Nutrient Modulation of the Immune Response.* S. Cunningham-Rundles, ed. Marcel Dekker Inc, New York.
- Davies, R. H., and C. Wray, 1997. Distribution of *Salmonella* contamination in ten animal feedmills. *Vet. Microbiol.* 57:159-169.
- Davies, R. H., R. A. J. Nicholas, I. M. McLaren, J. D. Corkish, D. G. Lanning, and C. Wray, 1997. Bacteriological and serological investigation of persistent *Salmonella enteritidis* infection in an integrated poultry organization. *Vet. Microbiol.* 57:277-293.

- Davis, N. T., 1980. Studies on the absorption of zinc by rat intestine. *Br. J. Nutr.* 43:189-203.
- Davis, M. S., 1998. Effects of supplemental copper on cellular and humoral immune response and performance of newly weaned calves. M.S. thesis. Texas A&M University.
- De Jong, N., S. G. M. Adam, L. C. P. G. M. de Groot, and C. de Graaf, 2000. Variability of micronutrient content in enriched dairy and fruit products. *Int. J. Food Sci. Nutr.* 51:247-257.
- Derviese, L. A., A. H. Devos, and L. R. VanDamme, 1975. Quantitative aspects of the *Staphylococcus aureus* flora of poultry. *Poultry Sci.* 54:95-101.
- Dewar, W. A., P. A. L. Wight, R. A. Pearson, and M. J. Gentle, 1983. Toxic effects of high concentrations of zinc oxide in the diet of the chick and laying hens. *Br. Poult. Sci.* 24:397-404.
- Dibner, J. J., and P. Buttin, 2002. Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. *J. Appl. Poultry Res.* 11:453-463.
- Dilworth, B. C., and E. J. Day, 1976. Sodium depletion and repletion of White Leghorn hens in cages. *Poultry Sci.* 55:1593. (Abstr.).
- Dixon, R. C., and P. B. Hamilton, 1981. Effect of feed ingredients on the antifungal activity of propionic acid. *Poultry Sci.* 60:2407-2411.
- Don, R. H., P. T. Cox, B. J. Wainwright, K. Baker, and J. S. Mattick, 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acid Res.* 19:4008.

- Donham, K. J., W. Popenorf, U. Palmgren, and L. Larsson, 1986a. Airborne bacteria, endotoxin and fungi in poultry and swine confinement buildings. *Am. J. Ind. Med.* 10:294-297.
- Donham, K. J., L. J. Scallon, W. Popenorf, M. W. Treuhaft, and R. C. Roberts, 1986b. Characterization of dusts collected from swine confinement buildings. *Am. Ind. Hyg. Assoc. J.* 47:404-410.
- Donham, K. J., 1991. Association of environmental air contaminants with disease and productivity in swine. *Am. J. Vet. Res.* 52:1723-1730.
- Dorea, J. G., 2000. Zinc in human milk. *Nutr. Res.* 20:1645-1687.
- Dougherty, S. B., 2002. Effects of prenatal energy supplementation and prenatal prebreeding trace mineral/vitamine E supplemental on calf health and reproductive performance in beef cows. M.S. thesis. Texas A&M University.
- Dougherty, T. J., 1976. A study of *Salmonella* contamination in broiler flocks. *Poultry Sci.* 55:1811-1815.
- Drum, D. E., J. H. Harrison, and T.K. Li, 1967. Structure and functional zinc in horse liver and alcohol dehydrogenase. *Proc. Nat. Acad. Sci. USA.* 57:1434.
- Duke, G. E., and O. A. Evanson, 1976. Diurnal cycles of gastric motility in normal and fasted turkeys. *Poultry Sci.* 55:1802-1807.
- Duncan, G. D., L. F. Gray, and L. J. Daniel, 1953. Effect of zinc on cytochrome oxidase activity. *Proc. Soc. Exp. Biol. Med.* 83:625-627.

- Duncan, M. S., and A. W. Adams, 1972. Effects of a chemical additive and of formaldehyde-gas fumigation on *Salmonella* in poultry feeds. *Poultry Sci.* 51:797-802.
- Duncan, J. R., and L. S. Hurley, 1978. Thymidine kinase and DNA polymerase activity in normal and Zn-deficient developing rat embryos. *Proc. Soc. Exp. Biol. Med.* 159:39-43.
- Durant, J. A., D. E. Corrier, J. A. Byrd, L. H. Stanker, and S. C. Ricke, 1999. Feed deprivation affects crop environment and modulates *Salmonella enteritidis* colonization and invasion of Leghorn hens. *Appl. Environ. Microbiol.* 65:1919-1923.
- Edrington, T. S., L. F. Kubena, R. B. Harvey, and G. E. Rottinghaus, 1997. Influence of a superactivated charcoal on the toxic effects of aflatoxin or T-2 toxin in growing broilers. *Poultry Sci.* 76:1205-1211.
- Edwards III, H. M., and D. H. Baker, 2000. Zinc bioavailability in soybean meal. *J. Anim. Sci.* 78:1017-1021.
- El-Gazzar, F. E., and E. H. Marth, 1975. *Salmonellae*, salmonellosis, and dairy foods: A review. *J. Dairy Sci.* 75:2327-2343.
- Elia, M., C. Crozier, and G. Neale, 1984. Mineral metabolism during short-term starvation in man. *Clin. Chim. Acta.* 139:37-45.
- Elliot, I., 1990. *Salmonella* found in half of Canada's poultry barns. *Feedstuffs.* 62(7):1, 4.

- Etches, R. J., and F. J. Cunningham, 1976. The interrelationship between progesterone and leuteinizing hormone during the ovulation cycle of the hen (*Gallus domesticus*). J. Endocrinol. 71:51-58.
- Etches, R. J., and K. W. Cheng, 1981. Changes in the plasma concentrations of leuteinizing hormone, progesterone, oestradiol and testosterone and in the binding of follicle-stimulating hormone to the theca of follicles during the ovulation cycle of the hens (*Gallus domesticus*). J. Endocrinol. 91:11-22.
- Etches, R. J., 1987. Calcium logistics in the laying hen. J. Nutr. 117:619-628.
- Etches, R. J., 1990. The ovulatory cycles of the hen. Poultry Biology. 2:293-318.
- Evans, G. W., and P. E. Johnson, 1980. Characterization and quantitation of a zinc binding ligand in human milk. Pediatr. Res. 14:876-880.
- Fanelli, M. J., W. W. Sadler, C. E. Franti, and J. R. Brownell, 1971. Localization of salmonellae within the intestinal tract of chickens. Avian Dis. 15:366-375.
- Farkas, J., 2001. Physical methods of food preservation. Pages 567-591 *in*: Food Microbiology: Fundamentals and Frontiers, 2nd Ed. M. P. Doyle, L. R. Beuchat and T. J. Montville, ed. ASM Press, Washington, DC.
- Fell, G. S., A. Fleck, D. P. Cuthbertson, K. Queen, C. Morrison, R. G. Bessent, and S. L. Husain, 1973. Urinary zinc levels as an indication of muscle catabolism. Lancet 1:280-282.
- Ferket, P. R., and M. A. Qureshi, 1992. Effect of level of inorganic and organic zinc and manganese on the immune function of turkey toms. Poultry Sci. 71(Suppl. 1):60. (Abstr.).

- Finlay, B. B., 1994. Molecular and cellular mechanisms of *Salmonella* pathogenesis. *Curr. Top. Microbiol. Immunol.* 192:163-185.
- Fletcher, M. P., M. E. Gershwin, C. L. Keen, and L. Hurley, 1988. Trace element deficiencies and immune responsiveness in human and animal models. Pages 215-239 *in: Nutrition and Immunology.* R. K Chandra, ed. Alan R. Liss Inc, New York.
- Flinchum, J. D., C. F. Nockels, and R. E. Moreng, 1989. Aged hens fed zinc-methionine had chicks with improved performance. *Poultry Sci.* 68(Suppl. 1):55. (Abstr.).
- Folk, J. E., 1971. Carboxypeptidase B. Pages 57-79 *in: The Enzymes, Vol 3.* P. D. Boyer, ed. New York, Academic Press.
- Forbes, R. M., 1961. Excretory patterns and bone deposition of zinc, calcium, and magnesium in the rat as influenced by zinc deficiency, EDTA, and lactose. *J. Nutr.* 74:194-200.
- Fosmire, G. J., 1990. Zinc toxicity. *Am. J. Clin. Nutr.* 51:225-227.
- Fox, M. R. S., 1989. Zinc excess. Pages 365-370 *in: Zinc in Human Biology.* C. F. Mills, ed. Spring-Verlag, New York.
- Freter, R., E. Stauffer, D. Cleven, L. V. Holdeman, and W. E. C. Moore, 1983a. Continuous-flow cultures as in vitro models of the ecology of large intestinal flora. *Infect. Immun.* 39:666-675.
- Freter, R., H. Brickner, M. Botney, D. Cleven, and A. Aranki, 1983b. Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. *Infect. Immun.* 39:676-685.

- Freter, R., 1984. Interdependence of mechanisms that control bacterial colonization of the large intestine. *Microbial Therap.* 14:89-96.
- Fujioka, M, and I. Lieberman, 1964. A Zn^{2+} requirement of synthesis of deoxyribonucleic acid by rat liver. *J. Biol. Chem.* 239:1164-1167.
- Fuller, R., and B. E. Brooker, 1974. Lactobacilli which attach to the crop epithelium of the fowl. *Am. J. Clin. Nutr.* 27:1305-1312.
- Fuller, R., 1977. The importance of lactobacilli in maintaining normal microbial balance in the crop. *Br. Poult. Sci.* 18:85-94.
- Garber, L., M. Smeltzer, P. Fedorka-Cray, S. Ladely, and K. Ferris, 2003. *Salmonella enterica* serotype enteritidis in table egg layer house environments and in mice in U. S. layer houses and associated risk factors. *Avian Dis.* 47:134-142.
- Garlich, J. D., and C. R. Parkhurst, 1982. Increased egg production by calcium supplementation during the initial fasting period of a forced molt. *Poultry Sci.* 61:955-961.
- Garlich, J., C. Morris, and J. Brake, 1982. External bone volume, ash fat-free dry matter and femur of laying hens fed diets deficient or adequate in phosphorous. *Poultry Sci.* 61:1003-1006.
- Garlich, J., J. Brake, C. R. Parkhurst, J. P. Thaxton, C. Morris, and G. W. Morgan, 1984. Physiological profile of caged layers during one production year, molt, and postmolt: Egg production, egg shell quality, liver, femur, and blood parameters. *Poultry Sci.* 63:339-343.

- Gast, R. K., and C. W. Beard, 1990a. Production of *Salmonella enteritidis*-contaminated eggs by experimentally infected hens. *Avian Dis.* 34:348-446.
- Gast, R. K., and C. W. Beard, 1990b. Isolation of *Salmonella enteritidis* from internal organs of experimentally infected hens. *Avian Dis.* 34:991-993.
- Gast, R. K., and C. W. Beard, 1993. Research to understand and control *Salmonella enteritidis* in chickens and eggs. *Poultry Sci.* 72:1157-1163.
- Gast, R. K., 1994. Understanding *Salmonella enteritidis* in laying chickens: The contributions of experimental infections. *Int. J. Food Microbiol.* 21:107-116.
- Gatti, D., B. Mitra, and B. P. Rosen, 2000. *Escherichia coli* soft metal ion translocation ATPases. *J. Biol. Chem.* 275:34009-34012.
- Gilbert, A. B., and R. Blair, 1975. A comparison of the effects of two low-calcium diets on egg production in the domestic fowl. *Br. Poultry Sci.* 16:547-552.
- Gilbert, A. B., J. Peddie, G. G. Mitchell, and P. W. Teague, 1981. The egg-laying response of the domestic hen to variation in dietary calcium. *Br. Poultry Sci.* 22:537-548.
- Glynn, M. K., C. Bopp, W. Dewitt, P. Dabney, M. Mokhtar, and F. J. Angulo, 1998. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. *N. Engl. J. Med.* 338:1333-1338.
- Goodlow, R. J., and F. A. Leonard, 1961. Viability and infectivity of microorganisms in experimental airborne infection. *Bacteriol. Rev.* 25:182-187.

- Gotoh, N., K. Toshiyuki, T. Hideto, W. Takaomi, and N. Takeshi, 1999. Topological analysis of an RND family transporter, MexD of *Pseudomonas aeruginosa*. FEBS Letters. 458:32-36.
- Grimont, P. A. D., F. Grimont, and P. Bouvet, 2000. Taxonomy of the genus *Salmonella*. Pages 1-17 in: *Salmonella in Domestic Animals*. C. Wray and A. Wray, ed. CAB International, Wallingford, UK.
- Guard-Petter, J., D. J. Henzler, M. M. Rahman, and R. W. Carlson, 1997. On-farm monitoring of mouse-invasive *Salmonella enterica* serovar enteritidis and a model for its association with the production of contaminated eggs. Appl. Environ. Microbiol. 63:1588-1593.
- Guffanti, A. A., Y. Wei, S. V. Rood, and T. A. Krulwich, 2002. An antiport mechanism for a member of the cation diffusion facilitator family: Divalent cations efflux in exchange for K^+ and H^+ . Molecular Microbiology. 45:145-153.
- Guthrie, R. K., 1992. *Salmonella enteritidis* in eggs. Pages 117-129 in: *Salmonella*. CRC Press, Inc., Boca Raton, FL.
- Ha, S. D., K. G. Maciorowski, Y. M., Kwon, F. T. Jones, and S. C. Ricke. 1998a. Survivability of indigenous microflora and a *Salmonella typhimurium* marker strain in poultry mash treated with buffered propionic acid. Animal Feed Sci. Tech. 75:145-155.
- Ha, S. D., K. G. Maciorowski, Y. M., Kwon, F. T. Jones, and S. C. Ricke. 1998b. Indigenous feed microflora and *Salmonella typhimurium* marker strain survival

- in poultry mash diets containing varying levels of protein. *Animal Feed Sci. Tech.* 76:23-33.
- Hambleton, P., 1971. Repaired cell wall damage in *Escherichia coli* recovered from an aerosol. *J. Gen. Microbiol.* 69:81-88.
- Hantke, K., 2001. Bacterial zinc transporters and regulators. *BioMetals.* 14:239-249.
- Hargis, B. M., D. J. Caldwell, R. L. Brewer, D. E. Corrier, and J. R. DeLoach, 1995. Evaluation of the chicken crop as a source of *Salmonella* contamination of broiler carcasses. *Poultry Sci.* 74:1548-1552.
- Harry, E. G., 1964. The survival of *Escherichia coli* in the dust of poultry houses. *Vet. Rec.* 76:466-470.
- Hartung, J., 1994a. The effect of airborne particulates on livestock health and production. Pages 55-69 *in: Pollution in Livestock Production Systems.* I. A. P. Dewi, R. F. E. Axford, I. Fayed, M. Maria, and H. Omed, ed. CAB International, Wallingford, UK.
- Hartung, J. 1994b. Environment and animal health. Pages 25-48 *in: Livestock Housing.* C. M. Wathes, and D. R. Charles, ed. CAB International, Wallington, UK.
- Hassan, J. O., and R. Curtiss, 1997. Efficiency of a live avirulent *Salmonella typhimurium* vaccine in preventing colonization and invasion of laying hens by *Salmonella typhimurium* and *Salmonella enteritidis*. *Avian Dis.* 41:783-791.
- Hayes, J. R., L. E. Carr, E. T. Mallinson, L. W. Douglass, and S. W. Joseph, 2000. Characterization of the contribution of water activity and moisture content to the

- population distribution of *Salmonella* spp. in commercial poultry houses. *Poultry Sci.* 79:1557-1561.
- Hedberg, C. W., M. J. David, K. E. White, K. L. MacDonald, and M. T. Osterholm, 1993. Role of egg consumption in sporadic *S. enteritidis* and *S. typhimurium* infections in Minnesota. *J. Infect. Dis.* 167:107-111.
- Hentges, D. J., and R. Freter, 1961. *In vivo* and *in vitro* antagonism of intestinal bacteria against *Shigella flexneri*. 1. Correlation between various tests. *J. Infect. Dis.* 110:30-37.
- Henzler, D. J., and H. M. Opitz, 1992. The role of mice in the epizootiology of *Salmonella enteritidis* infection on chicken layer farms. *Avian Dis.* 36:625-631.
- Henzler, D. J., D. C. Kradel, and W. M. Sischo, 1998. Management and environmental risk factors for *Salmonella enteritidis* contamination of eggs. *Am. J. Vet. Res.* 59:824-829.
- Herber-McNeil, S. M., and M. E. Van Elswyk, 1998. Dietary marine algae maintain egg consumer acceptability while enhancing yolk color. *Poultry Sci.* 77:493-496.
- Herbert, J. A., and G. J. Cerniglia, 1978. Comparison of low sodium chloride, high zinc oxide and high potassium iodine for force pausing layers. *Poultry Sci.* 58:1015. (Abstr.).
- Heres, L., B. Engel, F. Van Knapen, M. C. M. De Jong, J. A. Wagenaar, and H. A. P. Urlings, 2003. Fermented liquid feed reduces susceptibility of broilers for *Salmonella enteritidis*. *Poultry Sci.* 82:603-611.

- Hermayer, K. L., P. E. Stake, and R. L. Shippe, 1977. Evaluation of dietary zinc, cadmium, tin, lead, bismuth and arsenic toxicity in hens. *Poultry Sci.* 56:1721-1722.
- Herremans, M., M. G. Verheyen, and E. Decuypere, 1988. Effect of temperature during induced molting on plumage renewal and subsequent production. *Br. Poult. Sci.* 29:853-861.
- Herrero, M., V. DeLorenzo, and K. N. Timmis, 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* 172:6557-6567.
- Hill, C. H., 1989. Effect of *Salmonella gallinarum* infection on zinc metabolism in chicks. *Poultry Sci.* 68:297-305.
- Hinton, A. Jr., D. E. Corrier, R. L. Ziprin, G. E. Spates, and J. R. DeLoach, 1991. Comparison of the efficacy of cultures of cecal anaerobes as inocula to reduce *Salmonella typhimurium* colonization in chicks with or without dietary lactose. *Poultry Sci.* 70:67-73.
- Hoffmans, C. M., and D. Y. C. Fung, 1993. Effective method for dry inoculation of bacterial cultures. *J. Rapid Meth. Auto. Microbiol.* 1:287-294.
- Hogue, A., P. White, J. Guard-Petter, W. Schlosser, R. Gast, E. Ebel, J. Farrar, T. Gomez, J. Madden, M. Madison, A. M. McNamara, R. Morales, D. Parham, P. Sparling, W. Sutherlin, and D. Swerdlow, 1997. Epidemiology and control of

- egg-associated *Salmonella enteritidis* in the United States of America. Rev. Sci. Tech. 16:542-553.
- Hohorst, J. J., 1974. Lactate. Pages 266-270 in: Methods of Enzymatic Analysis. H. U. Bergmeyer, ed. Academic Press, Inc., New York.
- Hollister, A. G., D. E. Corrier, D. J. Nisbet, and J. R. DeLoach. 1992. Effect of cecal microflora and dietary lactose on cecal volatile fatty acids concentrations and *Salmonella* colonization in broiler and layer chicks and turkey poults. Poultry Sci. 71 (Suppl. 1):46. (Abstr.).
- Holt, P. S., and R. E. Porter, 1991. Effects of induced molting on immunocompetence and susceptibility to *Salmonella enteritidis* (SE) infection in laying hens. Poultry Sci. 70 (Suppl. 1):53.
- Holt, P. S., 1992. Effects of induced moulting on immune responses of hens. Br. Poultry Sci. 33:165-175.
- Holt, P. S., and R. E. Porter Jr., 1992a. Microbiological and histopathological effects of an induced-molt fasting procedure on a *Salmonella enteritidis* infection in chickens. Avian Dis. 36:610-618.
- Holt, P. S., and R. E. Porter Jr., 1992b. Effect of induced molting on the course of infection and transmission of *Salmonella enteritidis* in white legorn hens of different ages. Poultry Sci. 71:1842-1848.
- Holt, P. S., 1993. Effect of induced molting on the susceptibility of White Leghorn hens to a *Salmonella enteritidis* infection. Avian Dis. 37:412-417.

- Holt, P. S., and R. E. Porter Jr., 1993. Effect of induced molting on the recurrence of a previous *Salmonella enteritidis* infection. *Poultry Sci.* 72:2069-2078.
- Holt, P. S., R. J. Buhr, D. L. Cunningham, and R. E. Porter Jr., 1994. Effect of two different molting procedures on a *Salmonella enteritidis* infection. *Poultry Sci.* 73:1267-1275.
- Holt, P. S., 1995. Horizontal transmission of *Salmonella enteritidis* in molted and unmolted laying chickens. *Avian Dis.* 39:239-249.
- Holt, P. S., N. P. Macri, and R. E. Porter Jr., 1995. Microbiological analysis of the early *Salmonella enteritidis* infection in molted and unmolted hens. *Avian Dis.* 39:55-63.
- Holt, P. S., B. W. Mitchell, and R. K. Gast, 1998. Airborne horizontal transmission of *Salmonella enteritidis* in molted laying chickens. *Avian Dis.* 42:45-52.
- Holt, P. S., 1999. Impact of induced molting on immunity and *Salmonella enterica* serovar Enteritidis infection in laying hens. Pages 367-375 in: *Salmonella enterica* serovar Enteritidis in Humans and Animals-Epidemiology, Pathogenesis, and Control. A. M. Saeed, R. K. Gast, M. E. Potter, and P. G. Wall, eds. Iowa State University Press, Ames, IA.
- Holt, P. S., 2003. Molting and *Salmonella enterica* serovar Enteritidis infection: The problem and some solutions. *Poultry Sci.* 82:1008-1010.
- Hoover, N. J., P. B. Kenney, J. D. Amick, and W. A. Hypes, 1997. Preharvest sources of *Salmonella* colonization in turkey production. *Poultry Sci.* 76:1232-1238.

- Hopper, S. A., and S. Mawer, 1988. *Salmonella enteritidis* in a commercial layer flock. Vet. Rec. 123:351.
- Hoshino, S., M. Suzuki, T. Kakegawa, K. Imai, M. Wakita, Y. Kobayashi, and Y. Yamada, 1988. Changes in plasma thyroid hormones, leutenizing hormone (LH), estradiol, progesterone and coricosterone of laying hens during a forced molt. Comparative Biochemistry & Physiology A-Comparative Physiology. 90:355-359.
- Hughes, B. O., and C. C. Whitehead, 1974. Sodium deprivation, feather picking and activity in laying hens. Br. Poultry Sci. 15:435-439.
- Hughes, B. O., and C. C. Whitehead, 1979. Behavior changes associated with the feeding of low-sodium diets to laying hens. Applied Animal Ethology. 5:255-266.
- Hume, M. E., D. E. Corrier, S. Ambrus, A. Hinton Jr., and J. R. DeLoach., 1993. Effectiveness of dietary propionic acid in controlling *Salmonella typhimurium* colonization in broiler chicks. Avian Dis. 37:1051-1056.
- Hume, M. E., L. F. Kubena, T. S. Edrington, C. J. Donskey, R. W. Moore, S. C. Ricke, and D. J. Nisbet, 2003. Poultry digestive microflora biodiversity as indicated by denaturing gradient gel electrophoresis. Poultry Sci. 82:1100-1107.
- Humphrey, T. J., and D. G. Lanning, 1988. The vertical transmission of salmonellas and formic acid treatment of chicken feed. Epidem. Infect. 100:43-49.
- Humphrey, T. J., A. Baskerville, S. Mawer, B. Rowe, and S. Hopper, 1989. *Salmonella enteritidis* phage type 4 from the contents of intact eggs: A study

- involving naturally infected hens. *Epidemiol. Infect.* 103:415-423.
- Humphrey, T. J., H. Chart, A. Baskerville, and B. Rowe, 1991. The influence of age on the response of SPF hens to infection with *Salmonella enteritidis* PT4. *Epidemiol. Infect.* 106:33-43.
- Humphrey, T. J., A. Baskerville, A. Whitehead, B. Rowe, and A. Henley, 1993. Influence of feeding patterns on the artificial infection of laying hens with *Salmonella enteritidis* phage type 4. *Vet. Rec.* 132:407-409.
- Hungate, R. E. 1966. *The Rumen and Its Microbes*. Academic Press, New York.
- Hunter, R. S., and R. W. Harold, 1987. *The Measurement of Appearance*. 2nd Ed. Wiley Interscience Pub., New York.
- Hurley, L. S., B. Lonnerdal, and A. G. Stanislawski, 1979. Zinc citrate, human acrodermatitis enteropathica. *Lancet* 1:677-678.
- Hurwitz, S., S. Bornstein, and Y. Lev, 1975. Some responses of laying hens to induced arrest of egg production. *Poultry Sci.* 54:415-422.
- Imai, K., 1973. Effects of avian and mammalian pituitary preparations on induction of ovulation in the domestic fowl, *Gallus domesticus*. *J. Reprod. Fertil.* 33:91-98.
- Impey, C. S., and G. C. Mead, 1989. Fate of salmonellas in the alimentary tract of chicks pretreated with a mature caecal microflora to increase colonization resistance. *J. Appl. Bacteriol.* 66:469-475.
- Ingram, D. R., H. R. Wilson, and F. B. Mather, 1982. The response of the oviduct to White Leghorn hens to two methods of induced resting. *Poultry Sci.* 61:1482 (Abstr.).

- Isobe, T., and H. S. Lillehoj, 1992. Effects of corticosteroids on lymphocyte subpopulations and lymphokine secretion in chickens. *Avian Dis.* 36:590-596.
- Jensen, P. A., W. F. Todd, G. N. Davis, and P. V. Scarpino, 1992. Evaluation of eight bioaerosol samples challenged with aerosols of free bacteria. *Am. Ind. Hyg. Assoc. J.* 53:660-667.
- Jensen, P. A., B. Lighthart, A. J. Mohr, and B. T. Shaffer, 1994. Instrumentation used with microbial bioaerosols. Pages 226-284 *in: Atmospheric Microbial Aerosols.* B. Lighthart and A. J. Mohr, ed. Chapman & Hall, Inc. New York.
- Johnson, A. L., and A. van Tienhoven, 1980. Plasma concentrations of six steroids and LH during the ovulatory cycle of the hen (*Gallus domesticus*). *Biol. Reprod.* 23:386-393.
- Johnson, A. L., and A. van Tienhoven, 1984. Effects of aminoglutethimide on luteinizing hormone and steroid secretion, and ovulation in the hen, *Gallus domesticus*. *Endocrinology.* 114:2276-2283.
- Johnson, A. L., 1990. Steroidogenesis and actions of steroids in the hen ovary. *Crit. Rev. Poult. Biol.* 2:319-346.
- Johnson, A. L., and J. Brake, 1992. Zinc-induced molt: Evidence for a direct inhibitory effect of granulosa cell steroidogenesis. *Poultry Sci.* 71:161-167.
- Jones, F. T., R. C. Axell, D. V. Rives, S. E. Scheideler, F. R. Tarver Jr., R. L. Walker, and M. J. Wineland, 1991. A survey of *Salmonella* contamination in modern broiler production. *J. Food Prot.* 54:502-507.

- Jones, F. T., D. V. Rives, and J. B. Carey, 1995. *Salmonella* contamination in commercial eggs and an egg production facility. *Poultry Sci.* 74:753-757.
- Jones, P. W., P. Collins, G. T. H. Brown, and M. Aitken, 1982. Transmission of *Salmonella mbandaka* to cattle from contaminated feed. *J. Hyg.* 88:255-263.
- Jones, W., K. Moring, S. A. Olenchock, T. Willams, and J. Hickey, 1984. Environmental study of poultry confinement buildings. *Am. Ind. Hyg. Assoc. J.* 45:760-766.
- Juven, B. J., N. A. Cox, J. S. Bailey, J. E. Thomson, O. W. Charles, and J. V. Shutze, 1984. Survival of *Salmonella* in dry food and feed. *J. Food Prot.* 47:445-448.
- Keilin, D., and T. Mann, 1940. Carbonic anhydrase. Purification and nature of the enzyme. *Biochem. J.* 34:1163.
- Keshavarz, K., and F. W. Quimby, 2002. An investigation of different molting techniques with an emphasis on animal welfare. *J. Appl. Poult. Res.* 11:54-67.
- Khan, M., and M. Katamay, 1969. Antagonistic effect of fatty acids against *Salmonella* in meat and bone meal. *Appl. Microbiol.* 17:402-404.
- Kidd, M. T., N. B. Anthony, and S. R. Lee, 1992. Progeny performance when dams and chicks are fed supplemental zinc. *Poultry Sci.* 71:1201-1206.
- Kidd, M. T., N. B. Anthony, L. A. Newberry, and S. R. Lee, 1993. Effect of supplemental zinc in either a corn-soybean or a milo and corn-soybean meal diet on the performance of young broiler breeders and their progeny. *Poultry Sci.* 72:1492-1499.

- Kidd, M. T., M. A. Qureshi, P. R. Ferket, and L. N. Thomas, 1994. Dietary zinc-methionine enhances mononuclear-phagocytic function in young turkeys. *Biol. Trac. Elem. Res.* 42:217-229.
- Kidd, M. T., P. R. Ferket, and M. A. Qureshi, 1996. Zinc metabolism with special reference to its role in immunity. *World's Poult. Sci. J.* 52:309-324.
- Killar, L., and T. K. Eisenstein, 1983. Strain dependent variation of delayed-type hypersensitivity in *Salmonella typhimurium* infected mice. *Adv. Exp. Med. Biol.* 162:297-302.
- Kim, B-K., E. C. Demacario, J. Nölling, and L. Daniels, 1996. Isolation and characterization of copper-resistant methanogens from a copper-mining soil sample. *Appl. Environ. Microbiol.* 62:2629-2635.
- Kingston, D. J., 1981. A comparison of culturing drag-swabs and litter for identification of infections with *Salmonella* spp. in commercial chicken flocks. *Avian Dis.* 25:513-516.
- Klasing, K. C., 1984. Effect of inflammatory agents and interleukin 1 on iron and zinc metabolism. *Am. J. Physiol.* 247:R901-904.
- Knudsen, G. R., and H. W. Spurr, 1987. Field persistence and efficacy of five bacterial preparations for control of peanut leaf spot. *Plant Disease* 71:442-445.
- Koutsolioutsou, A., E. A. Martins, D. G. White, S. B. Levy, and B. Demple, 2001. A *soxRS*-constitutive mutation contributing to antibiotic resistance in clinical isolate of *Salmonella enterica* (Serovar Typhimurium). *Antimicrob. Agents Chemother.* 45:38-43.

- Kowarski, S., C. Blair-Stanek, and D. Achachter, 1974. Active transport of zinc and identification of zinc-binding protein in rat jejunal mucosa. *Am. J. Physiol.* 226:401-407.
- Kubena, L. F., Y. M. Kwon, J. A. Byrd, C. L. Woodward, R. W. Moore, R. L. Ziprin, R. C. Anderson, D. J. Nisbet, and S. C. Ricke, 2001. Drinking water treatment and dietary treatment effects on *Salmonella enteritidis* in leghorn hens during forced molt. *Poultry Sci.* 80 (Suppl. 1):88. (Abstr.).
- Kwon, Y. M., S. D. Ha, and S. C. Ricke, 1998. Growth response of *Salmonella typhimurium* poultry isolate to propionic acid under aerobic and anaerobic conditions. *J. Food Safety.* 18:139-149.
- Kwon, Y. M., C. L. Woodward, J. Peña, D. E. Corrier, S. D. Pillai, and S. C. Ricke, 1999. Comparison of methods for processing litter and air filter matrices from poultry houses to optimize polymerase chain reaction detection of *Salmonella typhimurium*. *J Rapid Meth. Autom. Microbiol.* 7:103-111.
- Kwon, Y. M., and S. C. Ricke, 1999. *Salmonella typhimurium* poultry isolate response to propionic acid and sodium propionate under aerobic and anaerobic conditions. *International Biodeterioration & Biodegradation.* 43:161-165.
- Kwon, Y. M., and S. C. Ricke, 2000. Efficient amplification of multiple transposon-flanking sequences. *J. Microbiol. Meth.* 41:195-199.
- Kwon, Y. M., C. L. Woodward, S. D. Pillai, J. Peña, D. E. Corrier, J. A. Byrd, and S. C. Ricke, 2000. Litter and aerosol sampling of chicken houses for rapid detection of

- Salmonella typhimurium* contamination using gene amplification. J. Microbiology & Biotechnology. 24:379-382.
- Kwon, Y. M., L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2001. Functional analysis of bacterial genome by transposon footprinting. 101st American Society for Microbiology, Annual Meeting, Orlando, FL. (Abstr.).
- Kwon, Y. M., L. F. Kubena, C. L. Woodward, J. A. Byrd, R. W. Moore, D. J. Nisbet, and S. C. Ricke, 2001. Use of an alfalfa diet for molting in Leghorn hens to reduce *Salmonella enteritidis* colonization and invasion. Poultry Sci. 80 (Suppl. 1):90 (Abstr.).
- Kwon, Y. M., L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2002. Functional screening of bacterial genome for virulence genes by transposon footprinting. Methods Enzymol. 358:14-52.
- Lax, A. J., P. A. Barrow, P. W. Jones, and T. S. Wallis, 1995. Current perspectives in salmonellosis. Br. Vet. J. 151:351-377.
- Leach, S. A., A. Williams, A. C. Davies, J. Wilson, P. D. Marsh, and T. J. Humphrey, 1999. Aerosol route enhances the contamination of intact eggs and muscle of experimentally infected laying hens by *Salmonella typhimurium* DT104. FEMS Microbiol. Lett. 171:203-207.
- Lecos, C. W., 1986, February. Of microbes and milk: Probing America's worst *Salmonella* outbreak, FDA Consumer, 18-21.

- Lee, C. A., B. D. Jones, and S. Falkow, 1992. Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc. Natl. Acad. Sci. USA. 89:1847-1851.
- Lee, K., 1982. Effects of force molt period on postmolt performance of leghorn hens. Poultry Sci. 61:1594-1598.
- Lee, P., A., Golliver, and T. Morris, 1971. A quantitative analysis of the literature concerning the restricted feeding of growing pullets. Br. Poult. Sci. 12:413-437.
- Leeson, S., and J. D. Summers, 1997. Nutrition and world poultry production. Pages 1-9 in: Commercial Poultry Nutrition. S. Leeson and J. D. Summers, eds. University Books, Guelph, Ontario, Canada.
- Lev, M., and C. A. E. Briggs, 1956. The gut flora of the chick. II. The establishment of the flora. J. Appl. Bacteriol. 19:224-230.
- LeVan, T. D., J. A. Robinson, J. Ralph, R. C. Greening, W. J. Smolenski, J. A. Z. Leedle, and D. M. Schaefer, 1998. Assessment of reductive acetogenesis with indigenous ruminal bacterium populations and *Acetitomaculum ruminis*. Appl. Environ. Microbiol. 64:3429-3436.
- Lever, M. S., and A. Williams, 1996. Cross-infection of chicks by airborne transmission of *Salmonella enteritidis* PT4. Lett. Appl. Microbiol. 23:347-349.
- Lieberman, I., R. Abrams, N. Hunt, and P. Ove, 1963. Levels of enzyme activity and deoxyribonucleic acid synthesis in mammalian cells cultured from the animal. J. Biol. Chem. 238:3955-3962.

- Lien, R. J., and T. D. Siopes, 1989. Turkey plasma thyroid hormone and prolactin concentrations throughout an egg laying cycle and in relation to photorefractoriness. *Poultry Sci.* 68:1409-1417.
- Lighthart, B., and A. J. Mohr, 1987. Estimating down wind concentrations of viable airborne microorganisms in dynamic atmospheric conditions. *Appl. Environ. Microbiol.* 53:1580-1583.
- Lister, S. A., 1988. *Salmonella enteritidis* infection in broilers and broiler breeders. *Vet. Rec.* 123:350.
- Lott, B. D., F. N. Reece, and J. H. Drott, 1980. Effect of preconditioning on bone breaking strength. *Poultry Sci.* 59:724-725.
- Luck, M. R., and C. G. Scanes, 1980. Ionic and endocrine factors influencing the secretion of lutenizing hormone by chicken anterior pituitary cells *in vitro*. *General Comp. Endocrinol.* 41:260-265.
- Luginbuke, R., and S. D. Schlotzhauer, 1987. *SAS/SAT Guide for Personal Computers*, 6th ed. SAS Institute, Inc., Cary, NC.
- Lundholm, I. Monica, 1982. Comparison of methods for quantitative determinations of airborne bacteria and evaluation of total viable counts. *Appl. Environ. Microbiol.* 44:179-183.
- Lutsenko, S., and J. H., Kaplan, 1995. Organization of P-type ATPases: Significance of structural diversity. *Biochemistry.* 34:15607-15613.
- Maciorowski, K. G., J. Peña, S. D. Pillai, and S. C Ricke, 1998. Application of gene amplification in conjunction with a hybridization sensor for rapid detection of

- Salmonella* spp. and fecal contamination indicators in animal feed. J. Rapid Methods Auto. Microbiol. 5:225-238.
- MacKenzie, M. A., and B. S. Bains, 1976. Dissemination of *Salmonella* serotypes from raw feed ingredients to chicken carcasses. Poultry Sci. 55:957-960.
- Macri, N. P., R. E. Porter Jr., and P. S. Holt, 1997. The effects of induced molt on the severity of acute inflammation caused by *Salmonella enteritidis*. Avian Dis. 41:117-124.
- Madelin, T., and C. M. Wathes, 1989. Air hygiene in a broiler house: Comparison of deep litter with raised netting floor. Br. Poultry Sci. 30:23-27.
- Mallinson, E. T., C. R. Tate, R. G. Miller, B. Bennet, and E. Russek-Cohen, 1989. Monitoring poultry farms for salmonella by drag-swab sampling and antigen-capture immunoassay. Avian Dis. 33:684-690.
- Marthi, B., 1994. Resuscitation of microbial bioaerosols. Pages 192- 225 in: Atmospheric Microbial Aerosols. B. Lighthart and A. J. Mohr, eds. Chapman and Hall, New York.
- Mather, F. B., D. R. Ingram, and H. R. Wilson, 1982. Performance and oviductal histology of hens as influenced by low calcium and/or low sodium diets during a force moult. Poultry Sci. 61:1385. (Abstr.).
- Matlho, G., S. Himathongkham, H. Riemann, and P. Kass, 1997. Destruction of *Salmonella enteritidis* in poultry feed by combination of heat and propionic acid. Avian Dis. 41:58-61.

- McClelland, M., K. E. Sanderson, J. Spdeth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Ssott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan., R. Waterston, and R. K. Wilson, 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature*. 413:852-856.
- McClintock, B., 1950. The origin and behavior of mutable loci in maize. *Nat. Acad. Sciences*. 36:344-355.
- McCord, J. M., B. B. Keele Jr., and I. Fridovich, 1971. An enzyme-based theory of obligate anaerobiosis: The physiological function of superoxide dismutase. *Proc. Natl. Acad. Sci. USA*. 68:1024-1027.
- McCormick, C. C., 1984. The tissue-specific accumulation of hepatic zinc metallothionin following parenteral iron loading. *Proc. Soc. Exp. Biol. Med*. 176:392-402.
- McCormick, C. C., and D. L. Cunningham, 1984a. High dietary zinc and fasting as methods of forced resting: a performance comparison. *Poultry Sci*. 63:1201-1206.
- McCormick, C. C., and D. L. Cunningham, 1984b. Forced resting by high dietary zinc: Tissue zinc accumulation and reproductive organ weight changes. *Poultry Sci*. 63:1207-1212.

- McCormick, C. C., and D. L. Cunningham, 1987. Performance and physiological profiles of high dietary zinc and fasting as methods of inducing a forced rest: A direct comparison. *Poultry Sci.* 66:1007-1013.
- McNab, J. M., 1973. The avian caeca: A review. *World's Poultry Science Journal.* 29: 251-263.
- Mead, G. C., 1989. Microbes of the avian cecum: Types present and substrates utilized. *J. Exp. Zool. Suppl.* 3:48-54.
- Mead, G. C., and P. A. Barrow, 1990. Salmonella control in poultry by competitive exclusion or immunization. *Lett. Appl. Microbiol.* 10:221-227.
- Mead, G. C., 1993. Problems of producing safe poultry: discussion paper. *J. Royal Soc. Medicine.* 86:39-42.
- Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe, 1999. Food-related illness and death in the United States. *Emerg. Inf. Dis.* 5:607-625.
- Medvedev, K., C. Woodward, X. Li, L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2001. Egg production and quality response of commercial laying hens molted with alfalfa diets. *Poultry Sci.* 80 (Suppl. 1):54.
- Menard, M. P., and R. J. Cousins, 1983. Zinc transport by brush border membrane vesicles from rat intestine. *J. Nutr.* 113:1434-1442.
- Merkley, J. W., 1981. A comparison of bone strengths from broilers reared under various conditions in cages and floor pens. *Poultry Sci.* 60:98-106.

- Miller, J. H., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Mishu, B., J. Koehler, L. A. Lee, D. Rodrigue, F. H. Berenner, P. Blake, and R. V. Tauxe, 1994. Outbreaks of *Salmonella enteritidis* infections in the United States, 1985-1991. *J. Infect. Dis.* 169:547-552.
- Mitchell, E., M. O'Mahony, D. Lynch, L. R. Ward, B. Rowe, A. Uttley, T. Rogers, D. G. Cunningham, and R. Watson, 1989. Large outbreak of food poisoning caused by *Salmonella typhimurium* definitive type 49 in mayonnaise. *Br. Med. J.* 298:99-101.
- Monnet, D. L., 1999. European recommendations to respond to the threat of antimicrobial-resistant microorganisms. *Am. Soc. Microbiol. News.* 65:390-391.
- Monsi, A., and H. L. Enos, 1977. The effects of low dietary salt on egg production. *Poultry Sci.* 56:1373. (Abstr.).
- Moore, R. W., S. Y. Park, L. F. Kubena, J. A. Byrd, J. L. McReynolds, M. R. Burham, M. E. Hume, S. G. Birkhold, D. J. Nisbet, and S. C. Ricke, 2003. Comparison of zinc acetate and propionate addition on gastrointestinal tract fermentation and susceptibility of laying hens to *Salmonella enteritidis* during forced molt. *Poultry Sci.* (Submitted).
- Moran, E. T. Jr., and S. F. Bilgili, 1990. Influence of feeding and fasting broilers prior to marketing on cecal access or orally administered *Salmonella*. *J. Food Prot.* 53:205-207.

- Morris, G. K., B. L. McMurray, M. M. Galton, and J. G. Wells, 1969. A study of the dissemination of salmonellosis in a commercial broiler chicken operation. *Am. J. Vet. Res.* 30:1413-1421.
- Mrosovsky, N., and D. F. Sherry, 1980. Animal anorexias. *Science.* 207:837-842.
- Mueller, W. J., R. Schraer, and H. Schraer, 1964. Calcium metabolism and skeletal dynamics of laying pullets. *J. Nutr.* 84:20-26.
- Murase, T., K. Senjyu, T. Maeda, M. Tanaka, H. Sakae, Y. Matsumoto, Y. Kaneda, T. Ito, and K. Otsuk, 2001. Monitoring of chicken houses and an attached egg-processing facility in a laying farm for *Salmonella* contamination between 1994 and 1998. *J. Food Prot.* 64:1912-1916.
- Muyzer, G., E. C. De Waal, and A. G. Uitterlinden, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase-chain reaction –amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59:695-700.
- Naber, E. C., J. D. Latshae, and G. A. Marsh, 1980. Use of low sodium diets for recycling of laying hens. *Poultry Sci.* 59:1643 (Abstr.).
- Nakamura, M., N. Nagamine, T. Takahashi, S. Suzuki, M. Kijima, Y. Tamura, and S. Sato, 1994. Horizontal transmission of *Salmonella enteritidis* and effect of stress on shedding in laying hens. *Avian Dis.* 38:282-288.
- National Research Council, 1994. *Nutrient Requirements of Poultry*, 8th rev. ed. National Academy Press, Washington, DC.

- Nayak, R., P. B. Kenney, and G. K. Bissonnette, 2001. Inhibiting and reversal of *Salmonella typhimurium* attachment to poultry skin using zinc chloride. *J. Food Prot.* 64:456-461.
- Nesbeth, W., C. Douglas, and R. Harms, 1974. Response of laying hens to a low salt diet. *Poultry Sci.* 53:1961 (Abstr.).
- Nesbeth, W., C. Douglas, and R. Harms, 1976a. Response of laying hens to a low salt diet. *Poultry Sci.* 55:2128-2133.
- Nesbeth, W., C. Douglas, and R. Harms, 1976b. The potential use of dietary salt deficiency for the force resting of laying hens. *Poultry Sci.* 55:2375-2379.
- Neurath, H., 1960. Carboxypeptidase A and B. Pages 11-36 *in*: *The Enzymes*, Vol 4. P. D. Boyer, , H. Lardy, and K. Myrback, ed. Academic Press, New York.
- Nevalainen, T. J., 1969. The effect of calcium-deficient diet on the reproductive organs of the hen (*Gallus domesticus*). *Poultry Sci.* 48:653-659.
- Ngou Ngoupayou, F., P. Maiorino, and B. Reid, 1982. Jojoba meal in poultry diets. *Poultry Sci.* 61:1692-1696.
- Nies, D. H., 1995. The cobalt, zinc, and cadmium efflux system CzcABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *Escherichia coli*. *J. Bacteriol.* 177:2707-2712.
- Nies, D. H., 1999. Microbial heavy-metal resistance. *Appl. Microbiol. Biotechnol.* 51:730-750.
- Nikaido, H., 1996. Multidrug efflux pumps of gram-negative bacteria. *J. Bacteriol.* 178:5853-5859.

- Nisbet, D. J., D. E. Corrier, and J. R. DeLoach, 1993. Effect of mixed cecal microflora maintained in continuous culture and of dietary lactose on *Salmonella typhimurium* colonization in broiler chicks. *Avian Dis.* 37:528-535.
- Nisbet, D. J., S. C. Ricke, C. M. Scanlan, D. E. Corrier, A. G. Hollister, and J. R. DeLoach, 1994. Inoculation of broiler chicks with a continuous-flow derived bacterial culture facilitates early cecal bacterial colonization and increases resistance to *Salmonella typhimurium*. *J. Food Prot.* 57:12-15.
- North, M. O., and D. D. Bell, 1990. *Commercial Chicken Production Manual-4th Edition*. Chapman & Hall, New York, NY.
- North, M. O., and D. D. Bell, 2002. *Commercial Chicken Production Manual-5th Edition*. Kluwer Academic Publishers, Norwell, MA.
- Nurmi, E., and M. Rantanla, 1973. New aspects of *Salmonella* infection in broiler production. *Nature* 241:210-211.
- Ocaktan, A., H. Yoneyama, and T. Nakae, 1997. Use of fluorescence probes to monitor function of the subunit proteins of the MexA-MexB-OprM drug extension machinery in *Pseudomonas aeruginosa*. *J. Biol. Chem.* 272:21964-21969.
- O'Dell, B. L., 1992. Zinc plays both structural and catalytic roles in metalloproteins. *Nutrition Reviews.* 50:48-50.
- Opara, O. O., L. E. Carr, E. Russek-Cohen, C. R. Tate, E. T. Mallinson, R. G. Miller, L. E. Stewart, R. W. Jonhson, and S. W. Joseph, 1992. Correlation of water activity and other environmental conditions with repeated detection of salmonella contamination on poultry farms. *Avian Dis.* 36:664-671.

- Orban, J. I., D. A. Roland Sr., and M. M. Bryant, 1991. Influence of the method of processing and physical state of bone (wet vs dry) on various bone quality criteria in chickens. *Poultry Sci.* 70(Suppl. 1):90 (Abstr.).
- Ortega-Benito, J. M., and P. Langridge, 1992. Outbreak of food poisoning due to *Salmonella typhimurium* DT4 in mayonnaise. *Public Health.* 106:203-208.
- Outten, C. E., and O'Halloran, T. V., 2001. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Sci.* 292:2488-2491.
- Pageaux, J. F., C. Laugier, D. Pal, and H. Pacheco, 1983. Analysis of progesterone receptor in the quail oviduct. Correlation between plasmatic estradiol and cytoplasmic progesterone receptor concentrations. *J. Steroid Biochemistry.* 18:209-214.
- Park, S. Y., Y. M. Kwon, S. G. Birkhold, L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2002a. Application of a transposon footprinting technique for rapid identification of *Salmonella typhimurium* Tn5 mutants required for survival under desiccation stress condition. *J. Rapid Meth. Auto. Microbiol.* 10:197-206.
- Park, S. Y., C. L. Woodward, S. G. Birkhold., L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2002b. *In vitro* comparison of anaerobic and aerobic growth response of *Salmonella typhimurium* to zinc addition. *J. Food Safety.* 22:219-229.
- Park, S. Y., S. G. Birkhold, L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2003. Effects of high zinc diets using zinc propionate on molt induction, organs, and postmolt egg production and quality in laying hens. *Poultry Sci.* (Accepted).

- Paster, N., 1979. A commercial scale study of the efficiency of propionic acid and calcium propionate as fungistats in poultry feed. *Poultry Sci.* 58:572-576.
- Patzer, S. I., and K. Hantke, 1998. The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. *Mol. Microbiol.* 28:1199-1210.
- Paulsen, I. T., J. H. Park, P. S. Choi, and M. H. Saier Jr., 1997. A family of gram-negative bacterial outer membrane factors that function in the export of proteins, carbohydrates, drugs, and heavy metals from gram-negative bacteria. *FEMS Microbiol. Lett.* 156:1-8.
- Péczely, P., 1992. Hormonal regulation of feather development and molting on the level of feather follicles. *Ornis. Scand.* 23:837-842.
- Pekarek, R. S., M. C. Powanda, and R. W. Wannemacher Jr., 1972. The effect of leukocytic endogenous mediator (LEM) on serum copper and ceruloplasmin concentrations in the rat (36926). *Proc. Soc. Exp. Biol. Med.* 141:1029-1031.
- Peña, J., S. C. Ricke, C. L. Shermer, T. Gibbs, and S. D. Pillai, 1999. A gene amplification-hybridization sensor based methodology to rapidly screen aerosol samples for specific bacterial gene sequences. *J. Env. Sci. Health. A* 34:529-556.
- Peng, C., M. N. Chan, and C. K. Chan, 2001. The hygroscopic properties of dicarboxylic and multifunctional acids: Measurements and UNIFAC predictions. *Environ. Sci. Technol.* 35:4495-4501.
- Pethes, G. Y., Z. Szelnyi, and P. Peczelu, 1982. Changes in the plasma concentrations of thyroid hormones and sexual steroids during forced molt of male and female

- domestic chickens. *Acta Veterinaria Academic Scientiarum Hungaricae*.
30:193-201.
- Petersen, C. F., E. A. Sauter, J. F. Parkinson, J. E. Doxon, and R. C. Stroh, 1978.
Microflora of air samples from poultry houses. *Poultry Sci.* 57:1180 (Abstr.).
- Petter, J. G., 2001. The chicken, the egg and *Salmonella enteritidis*. *Environ. Microbiol.*
3:421-430.
- Pillai, S. D., S. C. Ricke, D. J. Nisbet, D. E. Corrier, and J. R. DeLoach, 1994. A rapid
method for screening for *Salmonella typhimurium* in a chicken cecal microbial
consortium using gene amplification. *Avian Dis.* 38:598-604.
- Pillai, S. D., and S. C. Ricke, 1995. Strategies to accelerate the applicability of gene
amplification protocols for pathogen detection in meat and meat products. *Crit.*
Rev. Microbiol. 21:239-261.
- Pillai, S. D., K. W. Widmer, S. E. Dowd, and S. C. Ricke, 1996. Occurrence of airborne
bacteria and pathogen indicators during land application of sewage sludge. *Appl.*
Environ. Microbiol. 62:296-299.
- Pitcher, D. G., N. A. Saunders, and R. J. Owen, 1989. Rapid extraction of bacterial
genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* 8:151-156.
- Pivnick, H., and E. Nurmi, 1982. The Nurmi concept and its role in the control of
salmonellae in poultry. Pages 41-70 *in: Developments in Food Microbiology-1.*
R. Davies, ed. Applied Science Publishers. Ltd., Essex, England.

- Porter Jr., R. E., and P. S. Holt, 1993. Effects of induced molting on the severity of intestinal lesions caused by *Salmonella enteritidis* infection in chickens. *Avian Dis.* 37:1009-1016.
- Potter, M., 1987. Poultry egg related *Salmonella enteritidis* infections. *Zootec. Int.* 12:44-46.
- Prasad, A. S., 1982. Zinc deficiency in human subjects. Pages 3-62 *in*: Clinical, Biochemical, and Nutritional Aspects of Trace Elements, A. S. Prasad, ed. Alan R. Liss Inc, New York,
- Prasad, A. S., 1993. Acquired zinc deficiency and immune dysfunction in sickle cell anemia. Pages 393-410 *in*: Nutrient Modulation of the Immune Response, S. Cunningham-Rundles, ed. Marcel Dekker Inc, New York.
- Proudman, J. A., and Opel, H., 1981. Turkey prolactin: Validation of a radioimmunoassay measurement of changes associated with broodiness. *Biology of Reproduction.* 25:573-580.
- Rabsch, W., T. Helmut, and A. J. Bäumler, 2001. Non-typhoidal salmonellosis: Emerging problems. *Microbes and Infection* 3:237-247.
- Rama, R., and J. Blanas, 1981. Effects of dietary zinc on iron metabolism in chickens. *Biol. Trace. Elem. Res.* 3:287-299.
- Ramirez, G. A., L. L. Sarlin, D. J. Caldwell, C. R. Yezak Jr., M. E. Hume, D. E. Corrier, J. R. DeLoach, and B. M. Hargis, 1997. Effect of feed withdrawal on the incidence of *Salmonella* in the crops and ceca of market age broiler chickens. *Poultry Sci.* 76:654-656.

- Raskin, L., W. C. Campan, R. Sharp, L. K. Poulsen, and D. A. Stahl, 1997. Molecular ecology of gastrointestinal ecosystems. Pages 243-298 *in*: Gastrointestinal Microbiology vol.2 –Gastrointestinal Microbes and Host Interactions. R. I. Macki, B. A. White, and R. E. Isaacson, eds. Chapman & Hall, New York.
- Rensing, C., T. Pirbyl, and D. H. Nies, 1997. New functions for the three subunits of the CzcCBA cation-proton antiporter. *J. Bacteriol.* 179:6871-6879.
- Reysenach, A.-L., L. J. Giver, G. S. Wickham, and N. R. Pace, 1992. Differential amplification of rRNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* 58:3417-3418.
- Richards, M. P., and Cousins, R. J., 1975. Mammalian zinc homeostasis: Requirement for RNA and metallothionine synthesis. *Biochem. Biophys. Res. Commun.* 64:1215-1223.
- Ricke, S. C., and D. M. Schaefer, 1991. Growth inhibition of the rumen bacterium *Selenomonas ruminantium* by ammonium salts. *Appl. Microbiol. Biotechnol.* 36:394-399.
- Ricke, S. C., Y. M. Kwon., C. L. Woodward, J. A. Byrd, J. A., Nisbet, and L. F. Kubena, 2001. Limitation of *Salmonella enteritidis* colonization by diets containing and low zinc. *Poultry Sci.* 80 (Suppl. 1):262. (Abstr.).
- Ricke, S. C., 2003a. Perspective on the use of organic acids and short chain fatty acids as antimicrobials. *Poultry Sci.* 82:632-639.
- Ricke, S. C., 2003b. The gastrointestinal tract ecology of *Salmonella enteritidis* colonization in molting hens. *Poultry Sci.* 82:1003-1007.

- Riemann, H., S. Himathongkham, D. Willoughby, R. Tarbell, and R. Breitmeyer, 1998. A survey for *Salmonella* by drag swabbing manure piles in California egg ranches. *Avian Dis.* 42:67-71.
- Riordan, J. F., and B. L. Vallee, 1976. Structure and function of zinc metalloenzymes. Pages 227-251 *in: Trace Elements in Human Health and Disease, Vol. 1.* A. S. Prasad, ed. Academic Press, New York,.
- Roberson, R. H., and P. J. Schaible, 1960. The tolerance of growing chicks for high levels of different forms of zinc. *Poultry Sci.* 39:893-896.
- Roberson, R. H., and D. W. Francis, 1979. The effect two molting methods on performance of Hy-line and Shaver hens. *Poultry Sci.* 58:1098. (Abstr.).
- Rodriguez, E. M., A. Rodriguez, M. Sanz Alaejos, and C. Diaz Romero, 2000. Concentrations of iron, copper and zinc in human milk and powdered infant formula. *Int. J. Food Sci. Nutr.* 51:373-380.
- Roland Sr., D. A., D. J. Holcombe, and R. H. Harms, 1977. Further studies with hens producing a high incidence of non-calcified and partially calcified egg shells. *Poultry Sci.* 61:2473-2481.
- Roland Sr., D. A., and J. Brake, 1982. Influence of premolt production on postmolt performance with explanation for improvement in egg production due to force molting. *Poultry Sci.* 61:2473-2481.
- Rolon, A. R., J. Buhr, and D. L. Cunningham, 1993. Twenty-four-hour feed withdrawal and limited feeding as alternative methods for induction of molt in laying hens. *Poultry Sci.* 72:776-785.

- Ross, E., and R. B. Herrick, 1981. Forced rest induced by moult or low-salt diet and subsequent hen performance. *Poultry Sci.* 60:63-67.
- Rozenboim, I., J. L. Silsby, C. Tabibzadeh, G. R. Pitts, O. M., Youngren, and M. E. El Halawani, 1993. Hypothalamic and posterior pituitary content of vasoactive intestinal peptide, gonadotropin releasing hormone I and II in the turkey hen. *Biol. Reprod.* 49:622-627.
- Ruff, C. R., and B. L. Hughes, 1985. Bone strength of height restricted broilers as affected by levels of calcium, phosphorous and manganese. *Poultry Sci.* 64:1628-1636.
- Russell, J. B., and R. L. Baldwin, 1978. Substrate preferences in rumen bacteria: evidence of catabolite regulatory mechanisms. *Appl. Environ. Microbiol.* 36:319-329.
- Russell, J. B., and R. L. Baldwin, 1979. Comparison of substrate affinities among several rumen bacteria: A possible determinant of rumen bacterial competition. *Appl. Environ. Microbiol.* 37:531-536.
- Russell, J. B., 1984. Factors influencing competition and composition of the rumen bacterial flora. Pages 31-345 *in: Herbivore Nutrition in the Subtropics and Tropics*, Gilchrist, F. M.C. and Mackie, R.I., eds. The Science Press (PTY), Pretoria, South Africa.
- Russell, J. B., and F. Diez Gonzale, 1998. The effects of fermentation acids on bacterial growth. *Adv. Microb. Physiol.* 39:205-234.

- Ruszler, P. L., 1998. Health and husbandry considerations of induced molting. *Poultry Sci.* 77:1789-1793.
- Ryś, R., and J. Koreleski, 1974. The effect of dietary propionic acid on the requirement of chicks for vitamin B₁₂. *Br. J. Nutr.* 31:143-146.
- Sahagian, B. M., I. Harding-Barlow, and H. M. Perry Jr., 1967. Transmural movements of zinc, manganese, cadmium and mercury by rat small intestine. *J. Nutr.* 93:291-300.
- Said, N. W., and T. W. Sullivan, 1984. A comparison of the effect of two force molting methods on performance of two commercial strains of laying hens. *Poultry Sci.* 63:2399-2403.
- Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989. *Molecular Cloning. A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
- Sandoval, M., P. R. Henry, C. B. Ammerman, R. D. Miles, and R. C. Littell, 1997. Relative bioavailability of supplemental inorganic zinc sources for chicks. *J. Anim. Sci.* 75:3195-3205.
- SAS Institute, 1979. *SAS User's Guide*. SAS Inst., Inc., Raleigh, NC.
- SAS Institute, 1985. *SAS® User's Guide: Statistics*. SAS Institute Inc., Cary, NC.
- SAS Institute, 1988. *SAS® User's Guide*. SAS Institute Inc., Cary, NC.
- Sattar, S. A., and M. K. Ijaz, 1987. Spread of viral infections by aerosols. *CRC Critical Reviews in Environmental Control.* 17:89-131.
- Sauter, E. A., C. F. Petersen, E. E. Steele, and J. F. Parkinson, 1981. The airborne microflora of poultry houses. *Poultry Sci.* 60:569-574.

- Savage, D. C., 1987. Factors influencing biocontrol of bacterial pathogens in the intestine. *Food Technol.* 41:82-87.
- Scanes, C. G., P. M. Godden, and P. J. Sharp, 1977. An homologous radioimmunoassay for chicken follicle-stimulating hormone: Observations on the ovulatory cycle. *J. Endocrinol.* 73:473-481.
- Scott, J. T., and C. R. Creger, 1976. The use of dietary zinc as an effective molting agent in laying hens. *Poultry Sci.* 55:2089. (Abstr.).
- Seinfeld, J. H., 1989. Urban air pollution: State of the science. *Science.* 243:745-752.
- Seo, K-H., P. S. Holt, and R. K. Gast, 2001. Comparison of *Salmonella enteritidis* infection in hens molted via long-term feed withdrawal versus full-fed wheat middling. *J. Food Prot.* 64:1917-1921.
- Shafer, D. J., R. P. Burgers, K. A. Conrad, J. F. Prochaska, and J. B. Carey, 2001. Characterization of alkaline hydroxide-preserved whole poultry as a dry byproduct meal. *Poultry Sci.* 80:1543-1548.
- Shahabi, N. A., J. M. Bahr, and A. V. Nalbandov, 1975. Effect of LH injection on plasma and follicular steroids in the chicken. *Endocrinology.* 96:969-972.
- Shaw, B. W., P. P. Buharivala, C. B. Parnell Jr., and M. A. Demny, 1997. Emission factors for grain receiving and feed loading operations at feed mills. *Am. Soc. Agri. Engin.* 41:757-765.
- Sheffield, V. C., D. R. Cox, L. S. Lerman, and R. M. Myers, 1989. Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the

- polymerase chain reaction results in improved detection of single-base changes. Proc. Natl. Acad. Sci. USA. 86:232-236.
- Shippee, R. L., P. R. Stake, U. Koehn, J. L. Lambert, and R. W. Simmons, 1979. High dietary zinc or magnesium as force-resting agents for laying hens. Poultry Sci. 58:949-954.
- Shivaprasad, H. L., J. F. Timoney, S. Morales, B. Lucio, and R. C. Baker, 1990. Pathogenesis of *Salmonella enteritidis* infection in laying chickens. I. Studies on egg transmission, clinical signs, fecal shedding, and serologic responses. Avian Dis. 34:548-557.
- Simkiss, K., 1961. Calcium metabolism and avian reproduction. Biol. Rev. 36:321-367.
- Smith, K. T., and R. J. Cousins, 1980. Quantitative aspects of zinc absorption by isolated, vascularly perfused rat intestine. J. Nutr. 110:316-323.
- Snoeyenbos, G. H., V. L. Carlson, B. A. McKie, and C. F. Smyser, 1967. An epidemiological study of salmonellosis of chickens. Avian Dis. 11:653-667.
- Snoeyenbos, G. H., C. F. Smyser, and H. VanRockel, 1969. *Salmonella* infections of the ovary and peritoneum of chickens. Avian Dis. 13:668-670.
- Snyder, L., and W. Champness, 1997. Transposition and nonhomologous recombination. Pages 195-214 *in*: Molecular Genetics of Bacteria. ASM Press, Washington, DC.

- Sobocinski, P. Z., W. J. Canterbury Jr., and M. C. Powanda, 1977. Differential effect of parenteral zinc on the course of various bacterial infections. *Proc. Soc. Exp. Biol. Med.* 156:334-339.
- Soerjadi, A. S., S. M. Stehman, G. H. Snoyenbos, O. M. Weinack, and C. F. Smyser, 1981. Some measurements of protection against paratyphoid *Salmonella* and *Escherichia coli* by competitive exclusion in chickens. *Avian Dis.* 25:706-712.
- Song, M. K., and N. F. Adham, 1979. Evidence for an important role of prostaglandins E2 and F2 in the regulation of zinc transport in the rat. *J. Nutr.* 109:2152-2159.
- Song, M. K., 1987. Low-molecular weight zinc-binding ligand: A regulatory modulator for intestinal zinc transport. *Comp. Biochem. Physiol.* 87:223-230.
- Squibb, R. L., W. R. Beisel, and K. A. Bostain, 1971. Effect of Newcastle disease on serum copper, zinc, cholesterol, and carotenoid values in the chick. *Appl. Microbiol.* 22:1096-1099.
- Srinivas, U., J. H. Braconier, B. Heppson, and L. Hansson, 1989. Influence of zinc deficiency and malnutrition on organ uptake of *Escherichia coli* during gram-negative sepsis in rats. *Nutr. Res.* 9:455-463.
- Stahl, J. L., J. L. Greger, and M. E. Cook, 1989. Zinc, copper, and iron utilization by chicks fed various concentrations of zinc. *Br. Poultry Sci.* 30:123-134.
- Stark, K. D., J. Nicolet, and J. Frey, 1998. Detection of *Mycoplasma hyopneumoniae* by air sampling with a nested PCR assay. *Appl. Environ. Microbiol.* 64:543-548.

- Stavric, S., and J. -Y. D'Aoust, 1993. Undefined and defined bacterial preparations for the competitive exclusion of *Salmonella* in poultry- A review. *J. Food Prot.* 56:173-180.
- Stersky, A. K., and T. I. Hedrick, 1972. Inhibition of growth of airborne coliforms and other bacteria on selective media. *J. Milk Food Technol.* 35:156-162.
- Stevenson, M. H., and N. Jackson, 1984. Comparison of dietary hydrated copper sulphate, dietary zinc oxide and a direct method for inducing a moult in laying hens. *Br. Poultry Sci.* 25:505-517.
- Stewart, R. G., R. D. Wyatt, and J. Kiker, 1977. Effect of commercial antifungal compounds on the performance of broiler chickens. *Poultry Sci.* 56:1664-1666.
- St. Louis, M. E., D. L. Morse, M. E. Potter, T. M. DeMelfi, J. J. Guzewich, R. V. Tauxe, and P. A. Blake, 1988. The emergence of graded A eggs as a major source of *Salmonella enteritidis* infections. New implications for the control of salmonellosis. *J. Am. Med. Assoc.* 259:2103-2107.
- Stuart, J. C., 1984. The introduction of *Salmonella* into poultry flocks. *J. Sci. Food Agric.* 35:632-633.
- Sturkie, P. D., 1965. *Avian Physiology*. 2nd ed. Ballière Tindall & Cassell. London, UK
- Sturkie, P. D., 1976. Endocrine influences on lipid metabolism. *in: Avian Physiology*. 3rd ed. P. D. Sturkie, ed. Springer-Verlag, New York.

- Suso, F. A., and H. M. Edwards, 1968. Influence of various chelating agents on the absorption of ^{60}Co , ^{59}Fe , ^{54}Mn and ^{65}Zn by chickens. *Poultry Sci.* 47:1417-1425.
- Swanson, K. M. J., F. Busta, E. H. Peterson, and M. G. Johnson, 1992. Colony count methods. Pages 75-95 *in*: Compendium of Methods for the Microbiological Examination of Foods 3rd ed. C. Vanderzant and D. F. Splittstoesser, eds. American Public Health Association, Washington, DC.
- Tauxe, R. V., 1991. *Salmonella*: A postmodern pathogen. *J. Food Prot.* 54:563-568.
- Taylor, T. G., 1965. Calcium-endocrine relationship in the laying hens. *Proc. Nutr. Soc.* 24:49-54.
- Terzieva, S., J. Donnelly, V. Ulevicius, S. A. Grinshpun, K. Willeke, G. N. Stelma, and K. P. Brenner, 1996. Comparison of methods for detection and enumeration of airborne microorganisms collected by liquid impingement. *Appl. Environ. Microbiol.* 62:2264-2272.
- Thiagarajan, D., A. M. Saeed, and E. K. Asem. 1994. Mechanism of transovarian transmission of *Salmonella enteritidis* in laying hens. *Poultry Sci.* 73:89-98.
- Thorne, P. S., M. S. Kiekhaefer, P. Whitten, and K. J. Donham, 1992. Comparison of bioaerosol sampling methods in barns housing swine. *Appl. Environ. Microbiol.* 58:2543-2551.
- Tietjen, M., and D. Y. C. Fung, 1995. Salmonellae and food safety. *Critical Reviews in Microbiology.* 21:53-83.

- Timoney, J. F., H. I. Shivaprasad, R. C. Baker, and B. Rowe, 1989. Egg transmission after infection of hens with *Salmonella enteritidis* phage type 4. *Vet. Rec.* 125:600-601.
- Todd, E. C. D., 1989. Preliminary estimates of costs of foodborne disease in the United States. *J. Food Prot.* 52:595-601.
- Tortuero, F., 1973. Influence of implantation of *Lactobacillus acidophilus* in chicks on the growth, feed conversion, malabsorption of fats syndrome and intestinal flora. *Poultry Sci.* 52:1848-1851.
- Tsoils, R., and F. Heffron, 1994. Mutagenesis and variant selection in *Salmonella*. *Methods in Cell Biol.* 45:79-106.
- Tufft, L. S., C. F. Nockels, and M. J. Fettman, 1988. Effects of *Escherichia coli* on iron, copper, and zinc metabolism in chicks. *Avian Dis.* 32:779-786.
- Turnbull, P. C. B., and G. H. Snoeyenbos, 1974. Experimental salmonellosis in the chicken. 1. Fate and host response in alimentary canal, liver, and spleen. *Avian Dis.* 18:153-177.
- Underwood, E. J., 1977. Zinc: Intermediary metabolism *in: Trace Elements in Human and Animal Nutrition.* 4th ed. Academic Press, New York.
- U. S. Department of Agriculture, Animal and Plant Health Inspection Service, 1994. *Salmonella enteritidis* (SE) control program. SE pilot project summary October 1994. U. S. Government Printing Office, Washington, DC.

- U. S. Department of Agriculture, Food Safety and Inspection Service, 1998. *Salmonella enteritidis* risk assessment. Shell eggs and egg products. U. S. Government Printing Office, Washington, DC.
- U. S. Department of Agriculture, National Animal Health Monitoring System, 2000. Layers '99: Part II: Reference of 1999 table egg layer management in the U.S. p 29 in APHIS Veterinary Services Misc. Report #N323.0100. USDA, Washington, DC.
- Vallee, B. L., and F. H. Hoch, 1955. Yeast alcohol dehydrogenase, a zincmetalloenzyme. *J. Am. Chem. Soc.* 77:821-822.
- Vallee, B. L., and D. S. Auld, 1990. Active site zinc ligands and activated H₂O of zinc enzymes. *Proc. Nat. Acad. Sci. USA.* 87:220-224.
- Vanderwal, P., 1979. *Salmonella* control of feedstuffs by pelleting or acid treatment. *World's Poultry Sci.* 35:70-79.
- Veldman, A., H. A. Vahl, G. J. Borggreve, and D. C. Fuller, 1995. A survey of the incidence of *Salmonella* species and Enterobacteriaceae in poultry feeds and feed components. *Vet. Rec.* 135:169-172.
- Verheyen, G., E. Decuypere, R. B. Chiasson, J. Verrloesem, E. R. Kuhn, and H. Michels, 1987. Gonadal function in underfed rats: II. Effect of estrogen on plasma gonadotropins after pinealectomy or constant light exposure. *Biol. Reprod.* 17:630-634.

- Vermaut, S., K. DeConinck, G. Flo, M. Cokelaere, M. Onagbesan, and E. Decuypere, 1997. The effect of deoiled jojoba meal on feed intake in chickens: Satiating or taste effect? *J. Agri. Food Chem.* 45:3158-3163.
- Vohra, P., E. Krantz, and F. H. Kratzer, 1965. Formation constants of certain zinc-complexes by ion-exchange methods. *Proc. Soc. Exp. Biol. Med.* 121:422-425.
- Vohra, P., and F. H. Kratzer, 1966. Influence of various phosphates and other complexing agents on the availability of zinc for turkey poults. *J. Nutr.* 89:106-112.
- VonWartburg, J. P., J. L. Bethune, and B. L. Vallee, 1964. Human liver alcohol dehydrogenase. Kinetics and physiochemical properties. *Biochem.* 3:1775-1782.
- Vruwink, K. G., C. L. Keen, M. E. Gershwin, J. P. Mareschi, and L. S. Hurley, 1993. The effect of experimental zinc deficiency on development of the immune system. Pages 263-279 *in: Nutrient Modulation of the Immune Response*. S. Cunningham-Rundles, ed. Marcel Dekker Inc, New York.
- Walter, M. V., B. Marthi, V. P. Fieland, and L. M. Ganio, 1990. Effect of aerosolization on subsequent bacterial survival. *Appl. Environ. Microbiol.* 56:3468-3472.
- Waltman, W. D., and A. M. Horne, 1993. Isolation of *Salmonella* typhoid agglutination test. *Avian Dis.* 37:805-810.

- Wapnir, R. A., D. E. Khani, M. A. Bayne, and A. Lifshitz, 1983. Absorption of zinc by the rat ileum: Effects of histidine and other low molecular weight ligands. *J. Nutr.* 113:1346-1354.
- Wathes, C. M., W. A. Zaidan, G. R. Pearson, M. Hinton, and N. Todd, 1988. Aerosol infection of calves and mice with *Salmonella typhimurium*. *Vet. Rec.* 123:590-594.
- Wathes, C. M., 1994. Air and surface hygiene. Pages 123-148 *in*: Livestock Housing. C. M. Wathes and D. R. Charles, ed. CAB International, Wallingford, UK.
- Wathes, C. M., 1995. Bioaerosol in animal houses. Pages 547-577 *in*: Bioaerosols Handbook. C. S. Cox and C. M. Wathes, ed. CAB International, Wallingford, UK.
- Wathes, C. M., 1998. Aerial emissions from poultry production. *World's Poultry Sci. J.* 54: 241-251.
- Watkins, B. A., B. F. Miller, and D. H. Beil, 1982. *In vivo* inhibitory effects of *Lactobacillus acidophilus* against pathogenic *Escherichia coli* in gnotobiotic chicks. *Poultry Sci.* 61:1298-1308.
- Watkins, B. A., and B. F. Miller, 1983. Competitive gut exclusion of avian pathogens by *Lactobacillus acidophilus* in gnotobiotic chicks. *Poultry Sci.* 62:1772-1779.
- Watkins, K. L., and L. L. Southern, 1992. Effect of dietary sodium zeolite A and graded levels of calcium and phosphorous on growth, plasma, and tibia characteristics of chicks. *Poultry Sci.* 71:1048-1058.

- Wawer, C., and G. Muyzer, 1995. Genetic diversity of *Desulfovibrio* spp. in environmental samples analyzed by denaturing gradient gel electrophoresis of [NiFe] hydrogenase gene fragments. *Appl. Environ. Microbiol.* 61:2203-2210.
- Webb, S. J., 1959. Factors affecting the viability of airborne bacteria. I. Bacteria aerosolized from distilled water. *Can. J. Microbiol.* 5:649-669.
- Wedekind, K. J., and D. H. Baker, 1990. Zinc bioavailability in feed-grade sources of zinc. *J. Anim. Sci.* 68:684-689.
- Whitehead, C. and D. W. F. Shannon, 1974. The control of egg production using a low-sodium diet. *Br. Poultry Sci.* 15:429-434.
- Whitehead, C. and P. J. Sharp, 1976. An assessment of the optimal range of dietary sodium for inducing a pause in laying. *Br. Poultry Sci.* 17:601-611.
- White, P. L., W. Schlosser, C. E. Benson, C. Maddox, and A. Hogue, 1997. Environmental survey by manure drag sampling for *Salmonella enteritidis* in chicken layer houses. *J. Food Prot.* 60:1189-1193.
- Williams, J. E., and S. T. Benson, 1978. Survival of *Salmonella typhimurium* in poultry feed and litter at three temperatures. *Avian Dis.* 22:742-747.
- Willams, J. E., 1981. *Salmonellas* in poultry feeds-A worldwide review. *World's Poultry Sci.* 37:6-25, 97-105.
- Wilson S. C., and F. J. Cunningham, 1980. Concentrations of corticosterone and leutenizing hormone in plasma during the ovulatory cycle of the domestic hen and after the administration of gonadal steroids. *J. Endocrinology.* 85:209-218.

- Xiong, A., and R. K. Jayaswal, 1998. Molecular characterization of a chromosomal determinant conferring resistance to zinc and cobalt ions in *Staphylococcus aureus*. *J. Bacteriol.* 180:4024-4029.
- Yongyi, T., and B. Lighthart, 1998. Life state of airborne bacteria. *Recent Res. Devel. Microbiol.* 2:499-517.
- Zambonin Zallone, A., and A. Teti, 1981. The osteoclasts of hen medullary bone under hypocalcaemic conditions. *Anat. Embryol.* 162:379-392.
- Zeelen Hr., H. H. M., 1975. Technical and economic results from forced molting of laying hens. *World's Poultry Sci. J.* 31:57-67.
- Zhu, X. Y., T., Zhong, Y. Pandya, and R. D. Joerger, 2002. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Appl. Environ. Microbiol.* 68:124-137.
- Zimmermann, N. G., D. K. Andrews, and J. McGinnis, 1987. Comparisons of several induced molting methods on subsequent performance of Single Comb White Leghorn hens. *Poultry Sci.* 66:408-417.
- Ziprin, R. L., D. E. Corrier, A. Hinton Jr., R. C. Beier, G. E. Spates, J. R. DeLoach, and M. H. Elissadle, 1990. Intracloacal *Salmonella typhimurium* infection of broiler chickens: Reduction of colonization with anaerobic organisms and dietary lactose. *Avian Dis.* 34:749-753.
- Ziprin, R. L., D. E. Corrier, and J. R. DeLoach, 1993. Control of established *Salmonella typhimurium* intestinal colonization with *in vivo*-passaged anaerobes. *Avian Dis.* 37:183-188.

Zinpro, Corp, 1993. Differentiation of organic trace minerals *in*: Poultry Nutrition

Seminar, Zinpro Corporation, Edina, MN.

APPENDIX

1. Axial Fan Puralator Filter Sampling: Commercial Puralator 2000 filters used in household air conditioning air return units (Puralator Inc, Henderson, NV) (7.6 cm x 7.6 cm sq.) as modified from a system described in Kwon *et al.* (1999) having a larger pore size than 0.2 μm were randomly fastened to seven mesh plastic canvas (Uniek, Wavnaakee, WI) which was attached to a commercial 20 inch household box fan (Lakewood Eng & Mfg. Chicago, IL). Average airflow across the axial fan was measured by anemometer (Extech Instrument Corp., Walthman, MA). This sampling was run for 85 min (time frame analysis of airborne samplers) or 30 min (comparison of airborne samplers) an average calculated flow rate of 673.7 L (0.67 m^3)/min.
2. For total bacterial colony forming units, the samples were brought to the laboratory within an hour after sampling. To each bioaerosol sample 20 ml of 2% peptone (time frame analysis of airborne samplers) or 0.1% peptone (comparison of airborne samplers, except for the drag swab samples which were 40 ml of 0.1%) was added and stomached for 30 seconds. A 0.5 ml aliquot of the original dilution was serially diluted in peptone for plating. Tryptic Soy Agar (TSA; Difco, Detroit, MI) was used to enumerate total aerobic bacteria. Plates were incubated for 48 hours at 37 $^{\circ}\text{C}$.
3. An autoclaved all-glass impinger (AGI-30) (Ace Glass Inc. Vineland, NJ) was filled with 20 ml of 2% peptone (time frame analysis of airborne samplers) or 0.1% peptone (for comparison of airborne samplers). Sampling was conducted for 20 min at a flow rate of 12L (0.012 m^3)/min.

4. Axial Fan Glass Fiber Filter Sampling: Three glass fiber filters (7.6 cm x 7.6 cm sq.) (Anderson, Instr., Smyrna, Georgia) having a 0.2 μm pore size were randomly fastened to plastic canvas which was attached to a commercial 20 inch household box fan (Lakewood Eng & Mfg. Chicago, IL). This sampling was run for 30 min at an average flow rate of 673.7 L (0.67 m^3)/min.

5. Circulating Fan Collection: An impaction system for the evaluation of bioaerosol sampling was developed and described by Shaw *et al.* (1997). A sterilized glass fiber filter (8 inch x 10 inch) having a 0.2 μm pore size was fastened to a frame mounted onto a funnel device attached to a circulating fan with adjustable flow rate. Sampling was conducted for 15 min at a flow rate of 730.5 L (0.731 m^3)/min.

6. Drag swab assemblies were made according to previously published methods with minor alterations in materials (Kingston, 1981; Caldwell *et al.*, 1994; Byrd *et al.*, 1997). Drag-swab assemblies were made from two 4 x 4 inch (10.2 x 10.2 cm) sterile cotton cheese cloth (American Fiber & Finishing Inc. Burlington, MA) and 6-ft (182.9 cm) length of size 18 cotton twine (Ace hardware, Oakbrook, IL). The gauze pads were securely tied at 5 ft (152.4 cm) and 6 ft (182.9 cm) on the cotton twine. Each assembly was placed in a glass bottle and autoclaved. Each assembly was wetted in 20 ml of 0.1 % peptone and dragged the entire length of the poultry layer house four times in separate vertical lines.

7. Enumerations of total airborne bacteria was calculated by multiplying Colony Forming Units (CFU) in 20ml, the original sample, and divided by the total volume air

(m³) passing through the sampling system device. The CFU/m³ of air values were transformed into log₁₀ CFU/ m³ values.

Plate enumerations were expressed as logarithmic functions and analyzed by the GLM procedure of SAS, version 6.11 (SAS Institute Inc., Cary, NC 27511-8000). Interactions and variables with $P > 0.25$ were considered nonsignificant and removed from the models. Means were separated by the Pdiff option of SAS.

Tables are presented with data of impinger, dragswab and circulating fan samples are single samples taken at each day and time; all axial fan filters (puralator and glass) are averages of three filters removed at the same time and day. Graphics are of paired samples at each time and day, done by SPSS (SPSS for Windows, standard version 10.0, SPSS, Inc., Chicago, IL). Correlations and standard deviations are also done by SPSS.

VITA

SHINYOUNG PARK (shinyoungpark321@hotmail.com)
306-2, #301, Yatop-Dong, Bundang-Ku,
Sungnam, KyungKi City, Korea

EDUCATION

B.S. Human Nutrition, Korean Samyook University (1989-1993)
M.S. Food Science and Technology, Mississippi State University (1996-1998)
Ph.D. Food Science and Technology, Texas A&M University (1998-2003)

PROFESSIONAL AND ACADEMIC APPOINTMENTS

1998-2003 Graduate Research/Teaching Assistant/Laboratory Teaching Instructor,
Poultry Science Department, Texas A&M University

SELECTED PUBLICATIONS

- Y. M. Kwon, **S. Y. Park**, S. G. Birkhold, and S. C. Ricke, 2000. Induction of resistance of *Salmonella typhimurium* to environmental stresses by exposure to short-chain fatty acids. *J. Food Sci.* 65:1037-1040.
- S. Y. Park**, C. L. Woodward, S. G. Birkhold, L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2002. *In vitro* comparison of anaerobic and aerobic growth response of *Salmonella typhimurium* to zinc addition. *J. Food Safety.* 22:219-229.
- S. Y. Park**, Y. M. Kwon, S. G. Birkhold, L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2002. Application of a transposon footprinting technique for rapid identification of *Salmonella typhimurium* Tn5 mutants required for survival under desiccation stress condition. *J. Rapid Meth. Auto.Microbiol.* 10:197-206.
- S. Y. Park**, S. G. Birkhold, L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2003. Effect of storage condition of bone breaking strength and bone ash in laying hens at different stages in production cycles. *Poultry Sci.* (Accepted)
- S. Y. Park**, S. G. Birkhold, L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2003. Effects of high zinc diets using zinc propionate on molt induction, organs, and postmolt egg production and quality in laying hens. *Poultry Sci.* (Accepted)
- S. Y. Park**, C. L. Woodward, S. G. Birkhold, L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2004. The combination of zinc compounds and acidic pH limits aerobic growth of a *Salmonella typhimurium* poultry isolate marker strain in rich and minimal media. *J. Environ. Sci. Health, Part B.* B39 (1) (Accepted).
- S. Y. Park**, S. G. Birkhold, L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2003. Survival of a *Salmonella typhimurium* poultry marker strain added as a dry inoculum to zinc and sodium organic acid amended feeds. *J. Food Safety.* (Submitted)
- S. Y. Park**, C. L. Woodward, S. G. Birkhold, L. F. Kubena, D. J. Nisbet, and S. C. Ricke, 2003. Influence of oxidation-reduction media reductants on *Salmonella typhimurium* growth kinetics response in an anaerobic atmosphere after initial pH Adjustment and zinc compound addition. *J. Food Processing and Preservation.* (Submitted).