8-2013

Development of Rapid Detection Methods and Novel Control Measures for Salmonella in Poultry

Si Hong Park
University of Arkansas, Fayetteville

DEVELOPMENT OF RAPID DETECTION METHODS AND NOVEL CONTROL MEASURES FOR *SALMONELLA* IN POULTRY

DEVELOPMENT OF RAPID DETECTION METHODS AND NOVEL CONTROL MEASURES FOR *SALMONELLA* IN POULTRY

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

By

Si Hong Park
Kyung Hee University
Bachelor of Science in Food Science and Technology, 2004
Kyung Hee University
Master of Science in Food Biotechnology, 2006

August 2013
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

_____
Dr. Steven C. Ricke
Dissertation Director

_____
Dr. David McNabb
Committee Member

_____
Dr. Young Min Kwon
Committee Member

_____
Dr. Michael Slavik
Committee Member

_____
Dr. Rajesh Nayak - ex officio
Committee Member

ABSTRACT

This dissertation consists of three research parts: **1)** development of rapid detection methods for foodborne pathogens; **2)** immune response of chicken cells against *Salmonella* and bacteriophage P22; **3)** evaluation of novel control measures for poultry productions. In order to develop rapid and accurate detection methods for foodborne pathogens, two types of PCR assays were utilized. Three foodborne pathogens included *Campylobacter*, *Escherichia coli* and *Salmonella* in watershed were qualitatively and quantitatively detected by multiplex PCR and qPCR (**chapter 2**). Since *Salmonella* species are commonly present in poultry and poultry products as well as most popular foodborne pathogen in the United States, we have developed multiplex PCR for simultaneous detection of *Salmonella* genus, *Salmonella* subspecies I, *S.* Enteritidis, *S.* Heidelberg, and *S.* Typhimurium. In addition, low numbers of *Salmonella* were quantified via qPCR (**chapter 3**). To evaluate the immune responses in chicken macrophage cells against *Salmonella* and bacteriophage P22 invasion, cell culture models were utilized. The productions of cytokines such as IL-4, IL-8, IL-10, and IFN-γ were measured by ELISA and qRT-PCR (**chapter 4**). Prebiotics is a non-digestible food component that provides beneficial effects on the host by stimulating the growth and activity of selected bacteria in the lower intestinal tract. In this study, we evaluated a production performance in pasture flock raised broilers after treatment with three different prebiotics. Furthermore, microarray was conducted to evaluate different gene expressions according to prebiotics treatments using small intestinal cells and ingenuity pathway analysis (IPA) software was used to analyze functional networks among up- or down-regulated genes based on microarray data (**chapter 5**). Lastly, DGGE was performed to evaluate gastrointestinal microflora shifts in pasture flock raised chickens supplemented with prebiotics (**chapter 6**).

ACKNOWLEDGMENTS

I would like to deeply express gratitude to my advisor, Dr. Steven C. Ricke, for his infinite supports and guidance during my doctoral program in the University of Arkansas. I could not accomplish my degree program without his great encouragement and efforts. I also would like appreciate my committee member, Dr. Michael Slavik, Dr. David McNabb, Dr. Young Min Kwon and Dr. Rajesh Nayak for their direction and suggestion on my dissertation.

I am very thankful to Dr. Irene Hanning for her help on my research and living in Fayetteville. Without her help, I could not adapt myself to new circumstance in the USA. Also, I am very thankful to Dr. Sun-Ok Lee as a mentor. I would like to thank Dr. Debabrata biswas (Debu) and Dr. Kristen Gibson for their collaboration. Special thanks to former and current Center for Food Safety lab members, Dr. Philip Crandall, Dr. Corliss O'Bryan, Dr. Jody Lingbeck, Dr. Ok Kyung Koo, Ashley, Ann, Erin, Juliany, Giselle, Michelle, Nathan, Peter, Sean for their friendship and help. Also, I am very thankful to Dr. Jeong Yoon Lee for his experimental advice and help.

I would like to thank Cell and Molecular Biology program for funding and Department of Food Science and Poultry science all staffs for kindness and handling my paperwork.

Most of all, I very appreciate my lovely wife, Seong-Deok Joo for her never ending support, advice, and encouragement as well as my daughter, Erin. Lastly, I would like to express deeply gratitude to my parent and sister for their support and encouragement. I love all of my family and friends.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

LIST OF PUBLISHED PAPERS

CHAPTER 1 (Poultry Science, 92: 546-561, 2013)

Title: Modifying the Gastrointestinal Ecology in Alternatively Raised Poultry and the Potential for Molecular Assessment

CHAPTER 2 (FEMS Microbiology Letters, 316: 7-15, 2011)

Title: Multiplex Polymerase Chain Reaction Assay for Detection and Quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* Serotypes in Water Samples

CHAPTER 4 (FEMS Microbiology Letters, 339: 137-144, 2013)

Title: Enhancement of Macrophage Chicken Cytokine Response to *Salmonella* Typhimurium When Combined with Bacteriophage P22

CHAPTER 5 (Poultry Science 91: 3295-3299, 2012)

Title: Assessment of Production Performance in 2 breeds of broilers fed prebiotics as feed additives

INTRODUCTION

The increase of foodborne diseases leads to develop not only rapid and accurate molecular detection methods in foods but also alternative food additives to reduce foodborne pathogens in poultry. The outbreaks caused by foodborne pathogens were estimated to be approximately 9.4 million illnesses and more than 55,961 persons are hospitalized because in many cases food consumption occurred without the respective individual being aware that the contaminated food can potentially cause disease. Both *Campylobacter* and *Salmonella* can be present in the GI tract of chickens without exhibiting external symptoms while these bacteria can cause disease in humans by ingestion of contaminated poultry. To detect these foodborne pathogens in poultry products, we developed multiplex PCR and quantitative PCR based on specific region of genomic DNA.

The consumer demands for organic and natural poultry products continue to increase because of organic or natural products are better than their conventional counterparts in terms of safety, taste, and increased health benefits. In order to reduce foodborne pathogens in poultry, prebiotics and bacteriophage are utilized widely as biological alternatives in the pre-harvest control of enteric foodborne pathogens. In this study, we have evaluated the effects of prebiotics and bacteriophage for not only the reduction of *Salmonella* in poultry but also alterations in gut microflora using microarray and DGGE.

CHAPTER 1

LITERATURE REVIEW

Modifying the Gastrointestinal Ecology in Alternatively Raised Poultry and the Potential for Molecular Assessment

Si Hong Park[1,2], Irene Hanning[3,4] and Steven C. Ricke[1,2*]

[1]Cell and Molecular Biology Program, Department of Food Science, University of Arkansas, Fayetteville, AR

[2]Center for Food Safety and Department of Food Science, University of Arkansas, Fayetteville, AR

[3]Department of Food Science and Technology, University of Tennessee, Knoxville, TN

[4]Department of Genomic Sciences and Technology, University of Tennessee, Knoxville, TN

Poultry Science 92: 546-561, 2013

1. Abstract

The demands for nonconventional poultry products by consumers continue to increase in the United States. In pasture flock and organic poultry production, probiotics and prebiotic feed additives have potential advantages because they promote intestinal health and may offer a replacement for current intervention strategies that are not considered acceptable for these production systems. Prebiotics have been demonstrated to produce effects on the gastrointestinal (GI) tract including modulation of microflora by promoting selective increases in beneficial bacteria concomitant with decreases in undesirable bacteria. In-depth assessment of microbial community changes during host growth and development as well as the establishment of beneficial microbial species by adding biologicals such as probiotics and prebiotics is important to achieve predictable and consistent improvements in chicken health and productivity. To analyze microflora shifts and metabolites produced by bacteria in the gut as well as host responses to biological additives, sophisticated molecular techniques are now available and are becoming more widely used. Polymerase chain reaction (PCR) assays, denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) offer approaches for detecting microbial shifts in the gut. Likewise, the employment of microarrays and analysis by the Ingenuity Pathway Analysis (IPA) programs on gut tissues can reveal insight into gut physiological and responses to dietary and other changes. Combining all these technologies will provide a plenary understanding of poultry gut health in alternative production systems.

2. Introduction

The demands for organic and natural poultry products by consumers continue to increase in the United States (US) because of an ongoing perception that organic or natural products are better than their conventional counterparts in terms of safety, taste, and increased health benefits (Harper and Makatouni, 2002; Van Loo et al., 2012b). The general term "organic" foods is utilized to define foods that are produced without using chemical fertilizers, additives, and synthetic pesticides as well as not processed with irradiation (DeSoucey, 2007). Among organic foods, the overall organic meat market size is small compared to the conventional meat industries in the US. However, according to the Organic Trade Association (OTA), the organic meat industry has grown $29 billion in 2010 compared to $ 3.6 billion in 1997 (OTA, 2011). Specifically, organic poultry productions have increased from 2 million in 2000 to over 5 million in 2005 and the numbers of laying hens have increased from approximately 1.1 million in 2000 to 5.6 million in 2008 (ERS, 2010). With increases in organic poultry products in the US, new management approaches are needed to compensate for potential food safety concerns and bird health (O'Bryan et al. 2008).

In this review, an overview will be presented on alternative poultry production systems and some of the key characteristics. This will be followed by a general discussion on the primary foodborne pathogens associated with poultry and much of this will focus on conventional poultry studies since less has been published on these topics for alternative poultry production systems. The primary biologic intervention that will be discussed in any detail will be prebiotics and for other interventions the reader is referred to several review sources (O'Bryan et al., 2008; Sirsat et al., 2009; Ricke et al., 2012). Finally, some description of microbiome developments will be provided as suggestions for potential applications to alternative poultry production systems.

3. Poultry Rearing Systems

3.1. Conventional Production Systems

Conventional rearing systems for poultry products have been commonly utilized in animal industry and represent over 95% of overall poultry production in the US (MacDonald, 2008). The word “conventional” in the poultry industry essentially refers to commercial broiler chickens such as rapidly growing Cornish and White Rock species that have high feed conversion rates and are raised in housing units up to 6 to 8 weeks to achieve an average market weight (6.5 to 8.5 lbs) (Fanatico et al., 2008; National Chicken Council, 2010). The poultry growth and environmental conditions are critical factors for commercial poultry industry to diminish economic loss due to mortalities. In general, conventional broiler chickens are grown in poultry grower houses ranging from 6.5 to 8.5 lbs/ft^2 (National Chicken Council, 2010). Furthermore, conventional chickens are raised in standard indoor housing and fed commercial antimicrobials and dietary supplements, while undergoing standard management practices such as beak trimming to prevent diseases and increase productivity (Henderson et al., 2009; Van Loo et al., 2012a).

3.2. Organic Production Systems

An increased awareness for high quality foods has resulted in continued demands for non-conventionally produced foods (Van Loo et al., 2012b). Although many consumers perceive that organically grown poultry products such as broiler chickens and eggs are safer and more nutritious than when grown under traditional conditions, the USDA defines organic foods as being different from conventionally produced products only in growing, handling and processing methods, not in safety and nutrition aspects (Bailey and Cosby, 2005; USDA National Organic

Program, 2008; Ricke et al., 2012; Van Loo et al., 2012b). The USDA has developed guidelines to standardize and regulate organic foods in accordance with the Organic Foods Production Act of 1990 (AMS/USDA, 2008; Pittman et al., 2012). Essentially by these guidelines animal production systems in the organic program should be reared without synthetic pesticides, antibiotics, hormones, and mammalian byproducts in the feed (AMS/USDA, 2008). However, prebiotics, probiotics, and vaccines are allowed to replace antibiotic growth promoters (AMS/USDA, 2008; O'Bryan et al., 2008; Fanatico et al., 2009; Van Loo et al., 2012c). In addition, living conditions, feeds, and breeder sources are important factors that are taken into consideration when acquiring organic certification (USDA National Organic Program, 2008; Fanatico et al., 2009). The birds should have unrestricted access to outside environments for exercise, fresh air, and sunlight except during inclement weather. Only organic and non-genetically modified feed ingredients are permitted for organic production (Fanatico et al., 2009; Chalova and Ricke, 2012). To prevent potential growth deficiencies from insufficient dietary sources of trace elements, minerals approved by the Food and Drug Administration (FDA) can be used in organic diets for chickens (Chalova and Ricke, 2012). Since there are no certified organic hatcheries in the US, non-organic chicks must be managed and grown under organic system conditions after the second day of hatch (Fanatico et al., 2008, 2009).

3.3. Pasture Flock Production Systems

The term "pasture raised" or "free range" is generally used to infer that birds have been grown outside so that they can utilize fresh grass, air, and sunlight (Plamondon, 2003; Siemen et al., 2007). The containment areas can be divided into categories of fixed and movable pen types. Due to the fixed pen being fairly large and constructed of solid materials, birds can be protected

from predators such as coyotes, minks, and foxes as well as inclement weather (Plamondon, 2003; Fanatico et al., 2009). In contrast, lightweight movable pens are considered efficient at providing ongoing fresh pasture access because they are convenient to move either daily or weekly depending on the management of the individual grower and the pasture space available (Plamondon, 2003; Fanatico et al., 2009; Van Loo et al., 2012a).

4. Foodborne Pathogens

In the US, a wide range of foodborne pathogens are reported to cause various human diseases each year. The outbreaks caused by these pathogens were estimated to be 37.2 million illnesses, 228,744 hospitalizations and 2,612 deaths (Scallan et al., 2011). Each year approximately 9.4 million illnesses occur, more than 55,961 persons are hospitalized and 1,351 mortalities occur from foodborne illness because in many cases food consumption occurred without the respective individual being aware that the contaminated food can potentially cause disease (Scallan et al., 2011). In developing countries, 15 to 34% of deaths due to diarrhea occur in children (Girad et al., 2006; Haddad et al., 2010). There are various foodborne infections caused by different foodborne microorganisms including viruses (59%), bacteria (39%), and parasites (2%), and more than 250 different foodborne illnesses have been classified (Frenzen 2005; Scallan et al., 2011). Pathogenic bacteria or toxins invade small intestines through the gastrointestinal (GI) tract and may cause diseases with symptoms such as vomiting, nausea, diarrhea, and abdominal cramps.

In the poultry industry, both *Campylobacter* and *Salmonella* can be present in the GI tract of chickens without the infected bird exhibiting external symptoms (Lafont et al., 1983; Newell and Fearnley, 2003; Horrocks et al., 2009). However, these bacteria can cause disease in humans

by ingestion of contaminated poultry products which may have become contaminated during slaughter or processing. In addition, these foodborne pathogens can be transmitted via incoming contaminated animals (Saito et al., 2009). Historically, several antibiotics have been used to control foodborne pathogens but emergence of multidrug resistant (MDR) pathogens has led to concerns over antibiotic resistance potentially impacting human health (Jones and Ricke, 2003; O'Bryan et al., 2008; Boerlin, 2010). In the past, conventional rearing systems have utilized various antibiotics regularly to stimulate growth performance, whereas certified organic poultry production have always been prohibited from using antibiotics (Jones and Ricke, 2003; Siemon et al., 2007; O'Bryan et al., 2008). Both *Campylobacter* and *Salmonella* isolated from conventional farms in some studies have exhibited a greater frequency of antibiotic resistance than organic flocks (Cui et al., 2005; Lestari et al., 2009; Alali et al., 2010).

4.1. *Campylobacter*

Campylobacter are Gram-negative, spiral, motile, oxidase positive, and can be cultured under microaerophilic (5% O_2, 10% CO_2, and 85% N_2) conditions (Snelling et al., 2005). *Campylobacter* are zoonotic, water-, and food-borne pathogens and are ubiquitous in animals, birds, and the environments. *Campylobacter* species consist of several subspecies including *C. jejuni*, *C. coli*, *C. lari*, and *C. fetus*. Among these species, *C. jejuni* and *C. coli* are the most common in animals and humans (Friedman et al., 2000). *Campylobacter* can be divided into serotypes based on the Penner and Lior system which targets thermo-stable and thermo-labile antigens (Lior et al., 1982; Patton et al., 1985). Both heat-stable and heat-labile antigens have been used for determining *Campylobacter* serotypes (Patton et al., 1985). Penner and Hennessy developed *Campylobacter* serotyping methods based on soluble heat-stable antigens and have

identified over 60 serotypes (Penner and Hennessy, 1980; Walker et al., 1988; de Zoete et al., 2007). The Lior system, composed of 108 serotypes, was specifically developed to detect heat-labile antigens by using live, whole cells on a glass slide (Lior et al., 1982; Walker et al., 1988; de Zoete et al., 2007).

Since *C. jejuni* are naturally present in the GI tract of poultry without causing disease to the host, poultry products can be contaminated during processing if intestinal contents are ruptured (Ringoir et al., 2007; Horrocks et al., 2009). The *Campylobacter* infectious dose in humans is approximately 500 cells and lower numbers can cause the disease in children, senior, and immune-compromised persons (Jacob-Reitsma, 2000). The symptoms of human *Campylobacter* infections, referred to as campylobacteriosis include fever, diarrhea with blood, abdominal pain which may continue for 24 h to a week with varying severity based on the individual's health status (Black et al., 1988). *Campylobacter* species are one of the most prevalent foodborne pathogens causing enteric disease in the US and worldwide (Scallan et al., 2011). There are 18 million cases of human campylobacteriosis and 13 laboratory-confirmed cases per 100,000 persons reported annually and infections in children under 4 years old are the most common in the US (Kirkpatrick and Tribble, 2010). To prevent *C. jejuni* infections in humans, several therapies have been utilized such as antibiotic administration, phage therapy, and the use of vaccines in poultry have been proposed (de Zoete et al., 2007; Buckley et al., 2010).

In addition, *C. jejuni* infections have been related to a rare autoimmune nervous disorder referred to as Guillain-Barré syndrome (GBS) (Hahn, 1998). The symptoms of GBS can occur following *C. jejuni* infections and target the peripheral nervous systems (Nachamkin, 2001). According to serological and culture tests, approximately 30 to 40% of GBS patients exhibited

evidence of a prior *C. jejuni* infections (Nachamkin et al., 2000). The occurrence of GBS is thought to be due to the molecular mimicry of the lipooligosaccharide (LOS) of *C. jejuni* which reacts with human gangliosides (Monteiro et al., 2009; Israeli et al., 2010). Although the incidence of GBS is considered fairly low, 0.6 to 4/100,000 persons per year, it remains a major concern because of its life-threatening nature to humans when it does occur (Israeli et al., 2010).

4.2. *Salmonella*

Salmonella are Gram-negative, facultative, motile bacteria of the enterobacteria group and are divided taxonomically into *Salmonella enterica* and *Salmonella bongori* (V) (Brenner et al., 2000; Grimont et al., 2000; Kim et al., 2006; Park et al., 2009). *Salmonella enterica* species are subsequently divided into six subspecies: *S. enterica* subsp. *enterica* (I), *salamae* (II). *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (Brenner et al., 2000; Grimont et al., 2000; Kim et al., 2006). *Salmonella enterica* subspecies can cause a variety of diseases, commonly referred to as salmonellosis in humans and other animals (Grimont et al., 2000; D'Aoust et al., 2007). However, *S. bongori* species is non-pathogenic and rarely detected in humans (D'Aoust et al., 2007; Park et al., 2009). *Salmonella enterica* species are composed of more than 2500 serotypes according to the Kauffmann-White scheme method based on somatic (O), flagellar (H), and capsular (K) antigens (Grimont and Weill, 2007).

Salmonella infection symptoms are divided into two general categories: typhoid fever in humans which is caused by *S.* Typhi and Paratyphi, and gastroenteritis in humans and animals caused by other *S. enterica* serovar (Kim et al., 2006; Nester et al., 2009). Typhoid fever is a systemic disease where the infected individual exhibits high fever, abdominal pains, and general weakness (Nath and Maurya, 2010). Numerous typhoid fever outbreaks continue to be reported

annually in the world with high mortality (15%) rates (Park et al., 2009). Salmonellosis caused by pathogenic *Salmonella* strains produce gastroenteritis symptoms characterized by nausea, headache, diarrhea, and fever (Bäumler et al., 2000; D'Aoust and Maurer, 2007; Park et al., 2009). In the US, approximately 40,000 cases are reported annually and result in approximately a 1% mortality level. Most people recover within a few days without medical treatments; however for immunodeficient individuals, elderly, or young children, the resulting infections may be more serious and even fatal.

Most *Salmonella* infections are transmitted by contaminated foods, water, and fecal routes, but rarely person-to-person transmission (Murray, 2000; Park et al., 2008). At least 10^6 to 10^9 bacteria are required to cause salmonellosis in healthy adults (Nester et al., 2009). *Salmonella* are sensitive to acidic conditions, and they rarely persist in the small intestine or stomach but can adapt and tolerate lower pH levels as well as high concentrations of fermentation organic acids (Foster, 1991; Foster and Spector, 1995; Kwon and Ricke, 1998; Ricke, 2003a; Dunkley et al., 2009; Nester et al., 2009). *Salmonella* can survive severe acid conditions lower than pH 3 and express acid-shock proteins via acid tolerance response systems at log or stationary growth phases (Foster, 1991; Foster and Spector, 1995). When surviving *Salmonella* reach the lumen of small intestine, adhesion of the bacterial cell occurs when it attaches to a specific receptor on the surface of the epithelial cells (Lamont, 2004). The contact with epithelial cells activates a type III secretion system (Lamont, 2004). From this stage, *Salmonella* may penetrate into various deep tissue locations of the body including the liver, bile, the bloodstream, and the spleen within a few days (Raskin et al., 1997; Nester et al., 2009).

In poultry, more than 200 different *Salmonella* serovars are capable of colonizing the GI tract (Gast, 2007; Foley et al., 2011). *Salmonella* infections often lead to different outcomes for

newly hatched poultry versus more mature stock except for instances where the GI tracts of adult birds are experiencing substantial stress such as removal of feed and a subsequent alteration of the gut microflora and fermentation (Durant et al., 1999; Ricke, 2003b). In susceptible young chicks and poults, *Salmonella* infections can sometimes lead to illness and death at high frequencies (Smith and Tucker, 1980; Barrow et al., 1987). In older birds, infections are often subclinical, causing production losses that are often undetectable to producers (Smith and Tucker, 1980). Nevertheless, the bacterium can be transmitted to humans through contaminated food, with poultry carcasses, and eggs serving as important sources for amplification of the bacteria to infectious levels (Ricke et al., 2001; Ricke, 2003b; Finstad et al., 2012; Howard et al., 2012).

4.3. *Campylobacter* and *Salmonella* Incidences in Alternative Poultry Production

Since *Campylobacter* species are fairly common commensal microorganisms in chickens, most studies have reported *Campylobacter* presence in poultry regardless of whether they originated from conventional, organic or pasture flock poultry (Newell and Fearnley, 2003; Cui et al., 2005; Esteban et al., 2008; Han et al., 2009; Hanning et al., 2010). Furthermore, most of these studies have detected similarities in *Campylobacter* prevalence between conventional and organic flocks, indicating that environmental conditions have minimal influence on overall *Campylobacter* contamination levels (Cui et al., 2005; Han et al., 2009). Hanning et al. (2010) screened 242 samples from 2 pasture flocks, facilities and retail carcasses for 8 months, and isolated 105 *Campylobacter* species (43%). Han et al. (2009) also detected a 43.3% *Campylobacter* contamination level in birds raised in conventional and organic chickens in Louisiana. However, Griggs et al. (2006) and Luangtongkum et al. (2006) reported an even greater percentage of *Campylobacter* prevalence in organically raised broiler chickens with 96%

and 89%, respectively. Stern and Line (1992) reported that 67 to 98% of retail chicken meats were contaminated by *C. jejuni* during the slaughtering process.

Only limited studies have been performed to compare *Salmonella* prevalence in chicken meat between conventional and organic birds at retail stores in the US (Van Loo et al., 2012a, b). Cui et al. (2005) collected conventional and organic chicken meat from retail stores in Maryland and reported that *Salmonella* prevalence in organic birds (61%) was greater than conventional (44%) chickens. Lestari et al. (2009) also compared *Salmonella* incidence in organic (20.8%) and conventional (22%) chicken carcasses in Louisiana. However, several groups evaluated *Salmonella* prevalence using only organic or pastured raised broiler chickens. Melendez et al. (2010) recovered *Salmonella* isolates from two pasture chicken farms, a local processing plant, and a retail natural food market. They isolated 18 *Salmonella* strains from carcasses (n=36) and 41 strains from pasture farm facilities (n=164) such as feed, water, and sponges. Bailey and Cosby (2005) collected a total of 53 all-natural chickens from 8 lots and 135 free-range chickens from four different commercial producers in 14 different lots to evaluate the presence of *Salmonella*. They reported that three of 8 lots and 25% (n=53) of the chickens were positive for *Salmonella* in all-natural chickens, also nine of 14 lots and 31% (n=135) of the chickens were contaminated by *Salmonella*.

5. Gut Microflora

The microbial composition of the chicken GI tract and its roles in health, development, and responses to feeding trials has been the subject of numerous studies (Zhu et al.,2002; Lu et al., 2003; Xu et al., 2003; Biggs et al., 2007; Donalson et al., 2008a). Based on these studies, a better understanding of how the microbial communities are temporally altered during host

growth and development and resist important intestinal pathogens can potentially be attributed to the presence of specific beneficial microbial species as well as by an earlier establishment of a more stable and diverse adult cecal community (Ricke and Pillai, 1999; Gong et al., 2002; Zhu et al., 2002; Lu et al., 2003; Patterson and Burkholder, 2003; Ricke et al., 2004; Chaucheyras-Durand and Durand, 2010; Torok et al., 2011; Siragusa and Ricke, 2012). The ceca are considered the primary site of focus because they not only contain one of the most diverse and abundant bacterial communities in the chicken including strict anaerobes such as methanogens, but also may harbor pathogens such as *S. enterica* and *C. jejuni* where these organisms can be the most numerous (Zhu et al., 2002; Saengkerdsub et al., 2007a, b; Dunkley et al., 2009; Horrocks et al., 2009; Foley et al., 2011).

5.1. Functions

The GI tract is a highly complex ecosystem with the mucosal surface of the small intestine providing a site for colonization by numerous microorganisms (Lu et al., 2008; Davis et al., 2010). To understand the interaction between host and microorganisms in complex ecosystems, various studies have been conducted but are still somewhat limited in scope (Holzapfel and Schillinger, 2002; Gibson et al., 2004). The immense microbial populations in the human GI tract have diverse autochthonous bacterial genera and have the potential to elicit regulatory effects on body functions (Holzapfel and Schillinger, 2002). In poultry, the ceca contain the largest number of microorganisms in the GI tract (Callaway et al., 2009; Kim and Mundt, 2011). More than 200 different bacteria have been isolated and most of these are strict anaerobes (Kim and Mundt, 2011). In poultry, extensive strict anaerobic activities including formation of short-chain fatty acids (SCFA) and methanogenesis occurs in the ceca of birds fed a

variety of diets (Ricke et al., 2004; Saengkerdsub et al., 2006, 2007a, b). Some GI tract microorganisms in various animal species including poultry GI microflora have the potential to hydrolyze and ferment dietary fiber into oligosaccharides and other low molecular weight carbohydrates (Kass et al., 1980; Ricke et al., 1982; Sunvold et al., 1995; Dunkley et al., 2007a, c).

5.2. Benefits

In the poultry industry, feed withdrawal is a procedure used to reduce fecal material in the intestinal content and fecal contamination of carcasses when intestines are ruptured during processing (Finstad et al., 2012). In laying hens, it has been shown that the removal of feed for long periods of time can lead to histological changes in the GI tract of poultry and alter the indigenous microbial population levels and fermentation activities that results in colonization opportunities for pathogens including *Salmonella* (Durant et al., 1999; Ricke, 2003b; Dunkley et al., 2007b, 2009). Dietary fiber can be utilized preferentially by *Bifidobacteria* and *Lactobacillus* species leading to the production of lactic acid and SCFA, both of which are inhibitory to *Salmonella* (Kaplan and Hutkins, 2000). Furthermore, the presence of fiber can lead to the maintenance of a normal microbial population in the bird GI tract (Fuller and Turvey, 1971; Bird, 2000; Woodward et al., 2005; Dunkley et al., 2007c).

5.3. Prebiotics and Probiotics

Prebiotics and probiotics represent biological alternatives in the pre-harvest control of enteric pathogens such as *Campylobacter*, *Salmonella* and *Escherichia coli* (Holzapfel and Schillinger, 2002; Patterson and Burkholder, 2003, Siragusa and Ricke, 2012). Prebiotics can be

defined as non-digestible food components that provide beneficial effects on the host by stimulating the growth and activity of selected bacteria in the lower intestinal tract (Schrezenmeir and de Vrese, 2001). In previous reports, prebiotics were demonstrated to produce several detectable effects on the chicken GI tract including increases in stool volume and modulation of colonic microflora by selective stimulation of beneficial bacteria as well as inhibition of undesirable bacteria (Holzapfel and Schillinger, 2002; Patterson and Burkholder, 2003; Jacob and Pescatore., 2012). Prebiotics are not hydrolyzed by digestive enzymes in the upper GI tract of the respective host but are selectively utilized by beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* which are generally regarded as safe (GRAS) (Roberfroid, 1998; Swennen et al., 2006). The definition of a probiotic is a product that contains sufficient numbers of viable bacteria which can alter the microflora in the host and exert detectable beneficial health effects in this host (Schrezenmeir and de Vrese, 2001; Siragusa and Ricke, 2012). In general, lactic acid bacteria such as *Bifidobacteria* and *Lactobacillus* have been traditionally used as probiotics added to fermented milk products or lyophilized forms (Ziemer and Gibson, 1998). More complex microbial consortia have been successfully applied to poultry to limit colonization of *Salmonella* in the GI tract (Ricke and Pillai, 1999; Nisbet, 2002; Siragusa and Ricke, 2012).

5.4. Prebiotic Applications in Poultry

Poultry may be exposed to *Salmonella* at or soon after hatching. Exposure and infection of poultry with *Salmonella* at the early stages of development can result in a diminished ability to clear *Salmonella* and eventually spread contamination throughout the slaughtering facility during processing (Gast and Holt, 1997; Park et al., 2008; Finstad et al., 2012). According to several

reports, antibiotic therapy is ineffective in the control of enteric *Salmonella* colonization in poultry, and antibiotics can disturb the beneficial protective microflora, and consequently increase susceptibility of poultry to *Salmonella* colonization (Seuna et al., 1980; Manning et al., 1994; Angulo et al., 2000; Threlfall et al., 2001). For these reasons, more recent research has focused on alternative methods for the control of *Salmonella* infections in poultry (Siragusa and Ricke, 2012). In recent years, the rising concern associated with increased MDR bacterial pathogens and the increased interest in organic poultry production systems has led research towards the application of non-antibiotic interventions capable of either killing or retarding growth of pathogenic microorganisms (Jones and Ricke, 2003; Ricke, 2003a; Berghman et al., 2005; Ricke et al., 2005; O'Bryan et al., 2008; Sirsat et al., 2009).

Prebiotics have become popular due to the ease of application. Although many different types of prebiotics such as peptides, proteins, and lipids can be utilized, oligosaccharides are the primary prebiotics because they can be hydrolyzed and fermented by gut bacteria (Gibson and Roberfroid, 1995; Ziemer and Gibson, 1998; Sako et al., 1999). In general, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and mannan-oligosaccharides (MOS) have been used widely in humans and animals (Gibson and Roberfroid, 1995; Malinen et al., 2002; Biggs et al., 2007). Fructo-oligosaccharides are naturally occurring oligosaccharides, usually of plant origin, and are the only product recognized and used as a food ingredient and prebiotics (Gibson and Roberfroid, 1995; Bomba et al., 2002). Since FOS include β-linkages as part of their chemical structures, they can resist adsorption and enzymatic degradation in the upper GI tract to reach the ceca, where the majority of fermentation occurs in chickens (Gibson and Roberfroid, 1995; Xu et al., 2003; Júskiewicz et al., 2004). Numerous reports have demonstrated that beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* were increased in

the large intestines of broilers when supplemented with FOS consistently (Roberfroid et al., 1998; Fukuta et al., 1999; Xu et al., 2002). Donalson et al. (2007, 2008a, b) used FOS alfalfa and layer ration combinations to assess laying hen cecal microflora fermentation and potential to inhibit *Salmonella* in *in vitro* incubations and feeding studies. Based on these studies, it appeared that the presence of FOS led to increase levels of acetate, propionate, butyrate, volatile fatty acid and lactic acid concentrations, limiting *S.* Enteritidis colonization in the ovaries and liver (Donalson et al., 2007, 2008a, b).

The prebiotic GOS have also been used to control intestinal microflora added to feeds. Although GOS have been less investigated in the poultry industry compared to FOS, they do produce bifidogenic effects in humans (Malinen et al., 2002; Gopal et al., 2003). Jung et al. (2008) demonstrated that GOS preferentially stimulated *Bifidobacteria* and significantly modified intestinal microflora in broiler chickens. Ito et al. (1990) and other groups (Rowland and Tanaka, 1993) reported similar results using human feces. Mannan-oligosaccharides are commonly present in yeast cell walls and have been reported to promote microbial changes in poultry (Biggs et al., 2007). In general, the mechanism of MOS in the small intestines of poultry is distinguishable from other prebiotics' functions because they interfere with binding site attachment by pathogens rather than serving as substrates for GI tract bacterial metabolism (Ofek et al., 1977). Since most pathogens possess mannose-specific type-1 fimbriae antigen on their cell walls, they can potentially bind MOS instead of small intestine surfaces and consequently, move through the intestine without colonization (Newman, 1994).

In poultry, oligosaccharides reach the hindgut and alter lower intestinal tract physiology and function, which could be beneficial in preventing bacterial contamination on broiler carcasses (Orban et al., 1997). The SCFA also have a bacteriostatic effect on some enteric

bacteria including *S.* Typhimurium and reduce intestinal pH to change environments favorable for beneficial GI tract bacteria such as *Bifidobacteria* and *Lactobacillus* (Van der Wielen et al., 2000; Ricke, 2003a; Forchielli and Walker, 2005). McHan and Shotts (1993) reported that *in vitro* toxic effect of SCFA to some *Enterobacteriaceae* generated a 50 to 80% reduction in *S.* Typhimurium populations in the presence of SCFA. It has been suggested that propionic acid was more effective in inhibiting pathogenic bacteria (Nisbet et al., 1996b; Marounek et al., 1999), whereas others observed that acetate was more effective (Van der Wielen et al., 2000).

Although both prebiotics and probiotics can be useful for reducing *Salmonella* colonization, prebiotics offer several advantages over probiotics including application and governmental approval for use (Holzapfel and Schillinger, 2002; Gibson et al., 2004). A problem arises with probiotics in that only defined cultures are allowed to be used per FDA regulations. Nisbet (2002) summarized a series of studies involving competitive exclusion cultures (CE) including a 29 bacterial consortia isolated from ceca of broiler chickens that originated from continuous-flow cultures and was approved by FDA. However, many of the probiotics that have been determined to be effective have not been defined, which makes them unsuitable due to unpredictable candidate probiotic strains, loss of activity by storage quality during clinical trials and high costs of clinical trials (O'Sullivan et al. 1992; Klaenhammer, 2000). A second issue with probiotics arises in application because they are typically live cultures and questions arise as to whether or not they survive passage through intestinal tract and if they can colonize once they reach the intestinal tract (Casey et al., 2004; Higgins et al., 2004). Depending on the type of probiotic culture not only is survival of the bacteria *in vivo* important but ensuring that their specific metabolic properties associated with their beneficial characteristics remain intact is equally important. This has been shown to be particularly true for defined probiotic cultures

which consist of a large number of bacterial strains where it has been demonstrated that maintaining the metabolic relationship among the microbial consortia was essential to retaining their efficacy against *Salmonella* (Nisbet et al., 1996a, b). Consequently, combining a prebiotic with a probiotic where the prebiotic serves as selective substrate for the probiotic *in vivo* has recently received more consideration as a means to ensure successful establishment of the resulting "synbiotic' (Patterson and Burkholder, 2003). In addition, the dosage of probiotics a bird may receive can be variable if delivered in the drinking water (Watkins and Kratzer, 1984; Timmerman et al., 2006). Conversely, prebiotics are usually feed additives that can be mixed during the feed milling process so all the birds receive the same feed to prebiotic dose ratio (Davis et al., 2010). Overall, prebiotics are easier to use than probiotics because producers do not have to adjust any rearing conditions (Patterson and Burkholder, 2003; Davis et al., 2010).

In summary, biological dietary amendments such as prebiotics show considerable promise for benefiting not just alternative poultry production systems but conventional systems as well. However, both application and predictable outcomes continue to be somewhat inconsistent and hamper more universal recommendations for routine use. This is no doubt due at least partially to differing management systems, bird breeds and types as well as environmental exposure. However, a key issue is the complexity of the bird GI tract and the influence of dietary manipulation on the microbial consortia that make up the collective microbiome. Historically, comprehensive assessment of the gut microbiome was fairly superficial due to lack of experimental tools that offered detailed analysis of the microflora and the corresponding metabolic activities (Ricke and Pillai, 1999). However, the advent of high throughput sequencing and metabolomics has changed this perception and offers the first opportunities to truly conduct detailed and extensive comparative analysis of poultry gut ecology

(van der Werf et al., 2005; Crhanova et al., 2011; Danzeisen et al., 2011; Kwon and Ricke, 2011). Consequently, for the first time there are tremendous opportunities to potentially develop a much more complete understanding of gut ecosystem dynamics in their entirety (microbiome and host interface). This is critical because even though commercial poultry production systems have diverged between conventional and alternative systems, both are now seeking research advances on alternative treatments to promote health and well being of their respective grower systems. Identifying common and universal responses in all poultry gut ecosystems versus those unique to a particular management system is needed to be effective commercial implementation. The remainder of this review focuses on some of the analytical tools that have become available and how they might be used to address these issues.

6. Bacteriophage

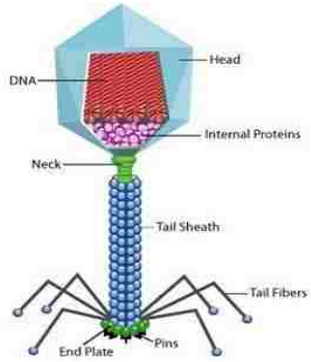


Figure 1. The structure of general bacteriophage

Bacteriophages were discovered in 1917 by Felix D'Herelle, who suggested using bacteriophages as a method for bacterial infection treatment. He introduced the name bacteriophage which originated from 'bacteria' and the Greek phagein which means "to eat" (Sabour and Griffiths, 2010). Also, he used the word 'plaque' to describe the clear zone caused by infection of single bacteriophage to bacteria on the agar plates (Sabour and Griffiths, 2010).

Bacteriophages are viruses that are host-specific killers of bacterial cells and can be defined as obligate intracellular parasites lacking an independent metabolism. They are typically composed of head, neck, tail sheath, and tail fibers (Figure 1). Bacteriophages are able to reproduce in the bacteria and lyse the bacteria using bacteriophage particles such as holin and endolysin. The

schematic overview of bacteriophage infection cycles are described in Figure 2. The bacteriophages are bound to specific surface proteins such as lipooligosaccharides of Gram negative bacteria and complex murein of Gram positive bacteria or capsules on host cells (Sabour and Griffiths, 2010). After irreversible adsorption, the bacteriophage genomic materials are injected to host cells through bacterial barriers and internalization. The host cell RNA polymerase can recognize promoter on bacteriophage genome and subsequently lead to expression for synthesis of bacteriophage virions. When optimal conditions for metabolism are established, the replication of bacteriophage genome is initiated to multiply inside the host cells and newly formed particles are assembled to form mature bacteriophage. Finally, newly formed bacteriophages are released from the host cells in search of other host cells as prey.

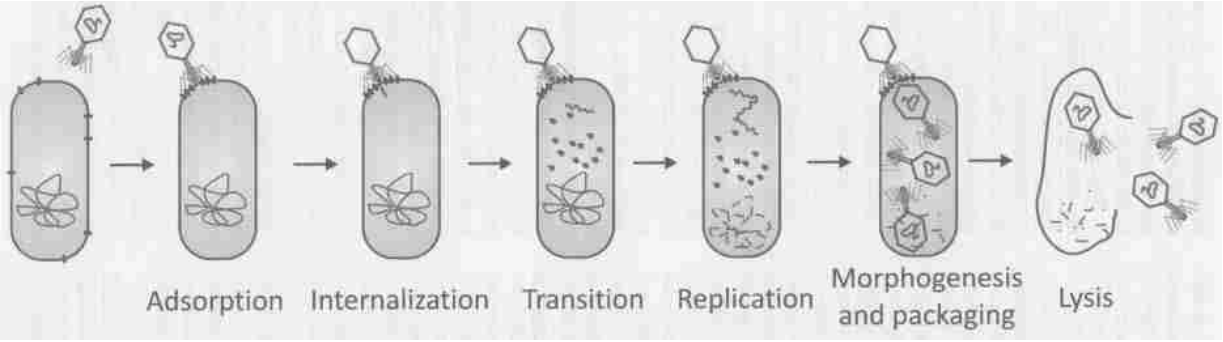


Figure 2. Schematic overview of bacteriophage infection cycle (Sabour and Griffiths, 2010)

6.1. Classification of Bacteriophage

A single or double strand of DNA or RNA molecule is a component of bacteriophage particles or virions, which is coated with proteins and lipoproteins. According to the International Committee on the Taxonomy of Viruses (ICTV), the bacteriophages can be classified into 13 families based on virion nucleic acid compositions and morphologies. Among these families, double strand DNA bacteriophages of the order *Caudovirales* represent over 95% and are mainly associated with foodborne pathogens. In addition, the order of *Caudovirales* can

be distinguished by distinct tail morphologies; long flexible tails (60%), double layered with contractile tails (25%) and short stubby tails (15%) (Ackermann, 2007; Sabour and Griffiths, 2010).

The bacteriophages can be divided into two major types based on life cycle differences, namely lytic (virulent) and lysogenic (temperate) bacteriophage. Lytic bacteriophages immediately use the host metabolism for the production of new bacteriophage virions and release their particles by lysis of host cells. In contrast, lysogenic bacteriophages are able to replicate in the host cells by two ways; lysing host cells such as lytic bacteriophages or stable combining with host DNA (Sabour and Griffiths, 2010). Lysogenic bacteriophage DNA can be integrated into the host chromosomal DNA and replicated along with the bacterial genetic material as well as be induced by an environmental trigger to excise from the host's chromosome and enter into a lytic cycle (Sulakvelidze, 2011).

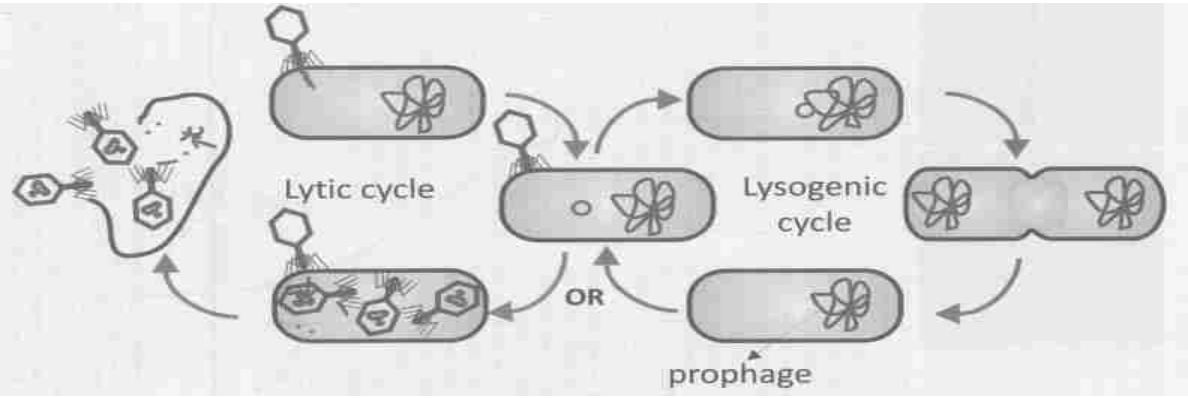


Figure 3. Two types of bacteriophage life cycle (Sabour and Griffiths, 2010)

6.2. Virulence Factors of Bacteriophage

Bacteriophages and their protein production such as endolysin have been used widely to control foodborne pathogens. They have own cell wall hydrolases (lytic enzymes) called endolysin which are highly effective molecules to digest bacterial peptidoglycans, teichoic acids

and lipopolysaccharides (LPS) immediately (Goode et al., 2003; Sabour and Griffiths, 2010). The endolysin degrades the peptidoglycan layers until the cells are unable to maintain the internal pressure and the mature bacteriophage particles are released. In addition, holin which is one of the virulence factors of bacteriophage is important for host cell lysis because it creates channels in the host cell walls to export bacteriophage particles (Sabour and Griffiths, 2010).

In developed countries, bacteriophage therapy was abandoned in favor of the development and widespread use of antibiotics. Antibiotic treatments have predominated over the usage of bacteriophage in last few decades due to several advantages such as easy production scale up, stability of the resulting preparation and broad spectrum capabilities (Levin and Bull, 2004). However, the interest in phage therapy is now gaining momentum in animal productions due to food safety concerns and the emergence of multidrug-resistant veterinary pathogens (Nakai and Park, 2002; Levin and Bull, 2004; Ricke et al., 2012). Bacteriophages can be administered directly to poultry or their respective endolysins. The endolysin gene was cloned into a vector and transformed to host cells for expression, and the endolysins were subsequently administered to poultry orally or by adding to feeds. The advantages and disadvantages of two different therapies using bacteriophages are summarized in Table 1.

Table 1. Comparative advantages and disadvantages between bacteriophage and lysin therapy (Sabour and Griffiths, 2010)

Therapy	Advantages	Disadvantages
Bacteriophage	- Self-replication - Both gram negative and positive - Many uses (humans, animals, and food) - Specific target - No harmful to normal microflora	- Resistance easily evolved - Limited host range - Potential transfer of toxin genes - Consumer acceptance
Lysin	- Protein therapeutic - Resistance not yet reported - Many uses (humans, animals, and food) - Specific target - No harmful to normal microflora	- No self-replication - Protein stability - Limited host range

6.3. Therapeutical Use of Bacteriophage

Several studies have shown that bacteriophages may be useful in reducing the number of bacterial foodborne pathogens including *Escherichia coli* O157 (O'Flynn et al., 2004), *Campylobacter jejuni* (Goode et al., 2003), *Listeria* species (Leverentz et al., 2003) and *Salmonella* serovars (Andreatti Filho et al., 2007) contaminating the surface of food. Studies have also recently sought to utilize bacteriophages to treat airsacculitis in chickens (Huff et al., 2003) and infections of fish (Nakai and Park, 2002). Utilization of bacteriophages as surface prophylactic agents will present different challenges than the use of bacteriophages as therapeutic agents. In addition to understanding the pharmacodynamics of the therapeutics, use of bacteriophages to modulate pathogen loads in complex ecosystems such as the intestine will present additional logistical challenges (Ricke et al., 2012). Several studies have investigated the use of bacteriophages to reduce *Campylobacter* and *Salmonella* loads in the poultry intestine; however, its application has resulted only in modest success (Higgins et al., 2007; Toro et al., 2005).

Campylobacter and *Salmonella* have been found in poultry products and eggs and are considered one of the primary sources of foodborne diseases (Ricke, 2003a, b; Park et al., 2008; Dunkley et al., 2009; Horrocks et al., 2009; Foley et al., 2011; Finstad et al., 2012; Howard et al., 2012). *Campylobacter* is a zoonotic, water-, and food-borne pathogen that is ubiquitous in animals, birds and the environment as well as naturally present in the intestinal tract of poultry (Horrocks et al., 2009). For this reason poultry food products can be contaminated during processing if intestinal contents are ruptured. Also, *Salmonella* is a major cause of foodborne infection after consumption of contaminated poultry products. Bacteriophage therapy has been conducted in live poultry to prevent the foodborne pathogen contaminations (McCrea et al.,

2006). Single bacteriophage or cocktails of 4 different bacteriophages for *Salmonella* Enteritidis were inoculated to newly hatched chicks through oral administration and exhibited significant reduction in *S*. Enteritidis after 24 h (Andreatti Filho et al., 2007). Although several studies based on bacteriophages have been performed to reduce *Salmonella*, it has not eliminated them due to multiple routes for *Salmonella* transmission in the flocks and environment (Sabour and Griffiths, 2010). In addition to *Salmonella* bacteriophage treatment, *Campylobacter* therapy in poultry has been also investigated by many researchers (Horrocks et al., 2009). Wagenaar et al. (2005) have conducted two experiments that are therapeutic and preventative aspects of bacteriophages for control of *Campylobacter* in broiler chickens. In the therapeutic experiments, although *Campylobacter* numbers were drastically reduced for several days, the numbers eventually stabilized 1 log lower than control. Also, it showed that the bacteriophages have the ability to delay the growth of *Campylobacter* in the preventative experiment. Therefore, bacteriophage therapy for control of *Campylobacter* potentially is useful immediately before slaughter (Wagenaar et al., 2005).

Bacteriophages can be used to control bacterial populations at any stage of the food chains. In the pre-harvest application, bacteriophages can be added to food and water directly to inhibit the spread of foodborne diseases at the farm (Joerger, 2003). Also, additional advantages for bacteriophage interventions are the lower development costs compared to new antibiotics (Brüssow, 2002). Bacteriophage therapies for meats and poultry productions are particularly attractive because bacteriophages represent an acceptable intervention that can be made fairly specific for targeting foodborne pathogens on the meat surfaces. Bacteriophages have been directly applied to meat surfaces for reducing specific foodborne pathogens. Bacteriophage

applications to meats have merits for commercialization but will require regulatory approval (Ricke et al., 2012).

6.4. Optimizing Bacteriophage Sources for Therapeutic Application

As discussed previously, since bacteriophages are specific to target bacteria and have no harmful effects on normal microbial populations in the gut, the usage of bacteriophages has a great advantage instead of broad spectrum antibiotics. However, bacteriophage therapies have disadvantages as well. When closely related bacterial strains are mixed in a sample, bacteriophages may only infect some of bacterial cells with specificity in a sample. Minor mutations in the LPS structures or bacterial surface proteins can make the difference between bacteriophage attachments and infections versus resistances (Tanji et al., 2004). According to a previous report, bacteriophage isolated from bovine fecal samples which is specific to *E. coli* O157:H7 was bound to the O157 serotype antigen and not to other common antigens such as flagella, pili, fimbrae, or lipopolysaccharide core. Strains of *E. coli* with absent or altered O157 antigen cannot be infected by these phages (Tanji et al., 2004).

Multiple bacteriophages that are targeting the same bacterial species on several surface receptors are able to reduce bacteria released from bacateriophage infection. When a mixture of three bacteriophages isolated from chickens were given to young broiler chicks, it was reported that 3.5 fold reductions occurred in *S*. Enteritidis levels until 25 days after treatment (Fiorentin et al., 2005)

6.5. Animal Host Response to Phage Entry

Since bacteriophage structure is composed of outer protein coats, bacteriophages are considered as antigenic and thus are recognized by antibodies (Dabrowska et al., 2005). This might have a significant influence on bacteriophage therapy. For example, Huff et al. (2010) detected increased bacteriophage specific IgG serum levels in birds pretreated with an intramuscular injection of a phage specific for the *E. coli* causing colisepticemia. When bacteriophage reacted with the antibody, the bacteriophage activity was inhibited and led to increasing mortalities of birds indicating that the bacteriophage therapy encountered immune interference. When bacterial infections are dealt with bacteriophage administered to the animal host through one of the routes previously discussed, the target bacteria are theoretically removed either by direct bacteriophage lysis or via an immuno-stimulation of antibodies in response to the target bacterial cell lysates generated by the bacteriophage (Borysowski and Górski, 2008).

7. Analysis and Molecular Tools

More recently, researchers have focused on changes in the gut ecosystem and quantification of microbial population shifts attributable to added prebiotics as well as chicken host responses to evaluate the effects of prebiotics (Xu et al., 2003; Ibuki et al., 2010; Torok et al., 2011). Because prebiotics introduced into the gut may lead to decrease in the pathogenic bacteria contamination levels in birds during production there is also interest in the metabolites produced by the microflora selected by the presence of prebiotics that may directly inhibit pathogen establishment in the gut.

Historically, microbiologists developed a variety of techniques to detect and identify GI tract microflora from the small intestine, large intestine, ceca, and feces based on a series of biochemical, specific microbiological, immunological, and molecular biological techniques

(Ricke and Pillai, 1999; Dwivedi and Jaykus, 2011). Traditionally, culture-based methods based on the growth media were commonly used to detect viable cells. Since most GI tract microflora are more likely to be strictly anaerobic and the GI tract microbial consortia correspondingly complex, it is difficult to identify and characterize individual species using traditional culture methods such as selective media (Ricke and Pillai, 1999; Dwivedi and Jaykus, 2011). To analyze the microflora changes, metabolites and chicken response to prebiotics, molecular techniques such as PCR, denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), microarrays, next generation sequencing (NGS), and ultra pressure liquid chromatography-mass spectroscopy (UPLC-MS) have been widely used. As more data is generated the resulting increase in genome sequences including both bacteria and animals should expedite identification of gut microflora and the physiological relationship(s) between them and the chicken host in a much more detailed manner.

7.1. PCR

Since the PCR technique was developed in 1983 by Kary Mullins, there have been tremendous advancements in molecular biology technology and applications. The PCR technique has been used for biological, medical, and various molecular applications included cloning, sequencing, identification of functional genes, detection of infectious pathogens, and gut indigenous organisms such as methanogens (Saengkerdsub et al., 2007a; O'Regan et al., 2008; Park et al., 2009, 2011; Kollanoor-Johny et al., 2012). This technique is able to amplify few copies of DNA fragment to millions of copies of DNA within few hours. All PCR reactions consist of template DNA fragment, primer pair (forward and reverse) for initiation of specific region amplification, *Taq* polymerase for extending of the DNA fragment, dNTP (dATP, dTTP,

dCTP, dGTP) for adding nucleotides during extension, and $MgCl_2$ for helping the reaction. In addition, a thermocycler which can adjust and repeat the heating and cooling of the reaction is needed. After PCR reactions, the amplicons are electrophoresed onto agarose gel including fluorescence dye such as SYBR green or Ethidium Bromide (EtBr), and can be visualized on the transilluminator. The PCR is used widely for the detection of foodborne pathogens due to sensitivity, and accuracy. The entire experimental process, including sample preparation, can be completed within 5 h. Furthermore this technique can identify various pathogens and discriminate individual species simultaneously in a single reaction (Park et al., 2009, 2011).

7.2. Multiplex PCR

Multiplex PCR is a further developed technique of normal PCR to identify pathogens simultaneously in a mixed sample as well as detect gene mutations and deletions among same genomic DNA template. The reagents cost and sample preparation time are less in a multiplex PCR than single PCR. In general, one primer pair can amplify multiple regions in a template DNA or over two primer pairs are used to amplify each specific target sequences. Several factors should be considered to develop optimized multiplex PCR from primer design to PCR conditions. The primer pairs used in multiplex PCR should be designed based on detailed sequences to avoid non-specific reactions, contain similar G/C contents for specific annealing temperature and produce visually a distinguishable amplicon size in agarose gel electrophoresis (Edwards and Gibbs, 1994). Thermocycler parameters included annealing temperature and extension time is usually determined by the characterization of primer pairs and expected product size.

Multiplex PCR was widely utilized to identify and discriminate *Salmonella* serotypes in human clinical samples, foods, poultry and poultry products (Malkawi and Gharaibeh, 2003;

Cortez et al., 2006; Kim et al., 2006; Park et al., 2009, 2011). Cortez et al. (2006) have developed multiplex PCR to differentiate *Salmonella* spp., *S*. Typhimurium and *S*. Enteritidis in chicken abattoirs. From these data, 29 (10%) out of 288 samples were positive for *Salmonella* spp. and 16 (5.6%) and 7 (2.4%) samples were identified as *S*. Typhimurium and *S*. Enteritidis, respectively. Also, Park et al. (2009) have optimized multiplex PCR to detect whole *Salmonella* genus and discriminate genetically related *Salmonella* strains including *S*. Typhi, *S*. Typhimurium, and *S*. Enteritidis. They have added internal amplification control (IAC) in a multiplex PCR mixture to prevent false-positive and false-negative results. Kim et al. (2006) have applied two five-plex PCR assays to *S. enterica* subspecies serotyping method using 30 different serotypes that commonly isolated from clinical samples. The results showed that 97% were correct among 111 clinical samples.

7.3. Quantitative Real-Time PCR (qPCR)

Quantitative real-time PCR (qPCR) has been developed to quantify genomic DNA copy number changes and differential gene expression levels between wild type and mutant strain. In general, there are two distinct qPCR methods based on detection mechanisms using fluorophore. TaqMan probe system application was developed by Applied Biosystems (Foster City, CA) and this probe consists of short length oligonucleotides including fluorophore reporter and quencher dye at the 5’ and 3’-end, respectively. The 5’ to 3’ exonuclease activity of *Taq* polymerse cleaves a probe which hybridizes with target sequences and fluorescence signals are released for quantitative measurements during the exponential stages of qPCR reactions. In contrast, SYBR green can bind double-stranded DNA and release signals to quantify the amounts. Although the TaqMan probe system showed more sensitivity and specificity than SYBR green system, there

are several limitations such as difficulty in probe construction and high cost per assay. SYBR green-based qPCR has more commonly utilized due to the convenience and low cost. However, melting curve analysis should be incorporated with SYBR green-based qPCR assay in order to discriminate between target amplicons and non-specific products ranging from 60 ˚C to 95 ˚C as well as compensate for low specificity.

A qPCR has been used widely to detect *Salmonella* in various foods such as eggs, meats, milk, poultry products and raw sausage (Malorny et al., 2004; Perelle et al., 2004; Seo et al., 2004; Wang et al., 2004; McCarthy et al., 2009). O'Regan et al (2008) developed real-time multiplex PCR to detect multiple *Salmonella* serovars in chicken samples and this assay reduced total assay time from 114 h to 31 h compared with traditional method (ISO 6579:2002). They also evaluated the relative accuracy, relative sensitivity, and relative specificity of optimized assay with naturally contaminated chicken samples and determined to be 89, 94 and 87%, respectively. McCarthy et al (2009) established multiplex PCR and qPCR based on TaqMan probe system to discriminate *S.* Typhimurium and *S.* Heidelberg in food and clinical samples. The qPCR detection limit of both *Salmonella* strains was 60 CFU/ml and total assay time was in less than 48 h. Interestingly, selective broth (Rappaport-Vassiliadis, RV) was more sensitive than non-selective broth (buffered peptone water, BPW) for *Salmonella* detection with a limitation of 61 CFU/ml in RV and 6,100 CFU/ml in BPW.

7.4. DGGE and TGGE

Both DGGE and TGGE techniques have been used to compare and analyze bacterial populations in complex ecosystems such as GI tracts and fecal samples (Muyzer, 1999; Hume et al., 2003; Dunkley et al., 2007; Hill et al., 2008; Hanning and Ricke, 2011). TGGE separates

DNA molecules which have different G+C contents based on temperature changes while DGGE is dependent on different concentrations of denaturing agents (Muyzer, 1999). In general, both TGGE and DGGE techniques amplify a common region of the 16S ribosomal DNA (rDNA) using a genomic DNA mixture and amplicons are subsequently separated on a polyacrylamide gel by different temperatures and containing a gradient of denaturant, respectively. In this way, amplicons are separated based on G+C content and the resulting banding pattern can be analyzed to identify microbial populations according to the treatments and corresponding time of collection as well as determining the complexity of the microflora (Owens et al., 2008). Because bands are separated based on G+C contents of a partial 16S rDNA sequence, the exact bacterial diversity is difficult to determine because some bacterial species may have very similar G+C contents and subsequently appear as one band (Palys et al., 1997; Muyzer, 1999; Hanning and Ricke, 2011). Sequencing can be conducted to alleviate this problem. Single bands that are sequenced will have only one DNA sequence and can be assumed to be a single band. In addition, the recovered DNA fragments from the gel can be sequenced to identify species by searching comprehensive databases such as the basic local alignment search tool (BLAST) (Altschul et al., 1990). Although DGGE has been reported to be a suitable technique for qualitative analysis, it is limited as a quantitative analysis of each bacterial species (Hill et al., 2008; Hanning et al., 2011). Because there may be a PCR bias towards amplification of some specific 16S rDNA sequences, caution must be taken when implying quantitative results based on band intensity (McCracken et al., 2001).

7.5. Microarrays

Microarrays based on DNA, RNA and proteins represent innovative techniques for the detection and characterization of bacteria in food matrices as well as for the assessment of differential gene expression levels of bacterial cells after exposure to a wide variety of conditions of experimental interest (Ibuki et al., 2010; Sirsat et al., 2010; Higgins et al., 2011). Microarrays are usually composed of artificially synthesized short length (25 to 80 bp) oligonucleotides, referred to as probes, that are specific for a selected target bacterium and these numerous probes are arrayed on the slide glass or silicon (Eom et al., 2007). Bacterial genomic DNA or cDNA synthesized from total RNA is hybridized with these probes with high specificity. The completion of probe-target hybridization is detected and quantified by signals emitted from fluorescence dyes such as Cy3 and Cy5 chemicals (Kim et al., 2006). Microarrays have been developed traditionally in close conjunction with available genome sequences and various methods for the detection of foodborne pathogens (Goldschmidt, 2005; Kim et al., 2006; Sirsat et al., 2010). However, there are some limitations for application of microarrays such as sensitivity, reproducibility, and probe homology with target sequences. To overcome these limitations and perform a microarray successfully, factors should be considered including genomic DNA or total RNA purity, concentration, prior amplification through PCR, removal of hybridization inhibitors to enhance the sensitivity, and over 90% of probe sequence identity (Kim et al., 2002; Arota et al., 2006; Eom et al., 2007; Sirsat et al., 2011a).

Microarrays can be applied to identify differential gene expression levels in the chicken host (*in vivo*) and using chicken cell lines (*in vitro*). For the *in vivo* assays, microarray results can represent gene expression changes by selected experimental treatments at different time points during the chicken lifespan up to 8 weeks. Ibuki et al. (2010) fed β-1, 4-mannobiose to one-day-old chicks for 4 weeks to confirm the effects of β-1, 4-mannobiose on chicken gene expression

levels in the small intestines and the mucosal immune systems using ileal samples. de Greeff et al. (2010) designed a microarray to evaluate the effects of conventionally and organically produced diets to assess jejunal gene expressions of different chickens. Furthermore, Higgins et al. (2011) performed microarrays to characterize chicken gene expression levels in the presence of *Salmonella* at early time points and assess the influence of probiotic treatments. To do this, *Salmonella* and the probiotic culture were inoculated to day-of-hatch chicks and the ceca were isolated and analyzed 24 h post-treatment (Higgins et al., 2011). Both regulated genes at each time point were associated with apoptosis and the nuclear factor kappa B complex (Higgins et al., 2011).

Microarrays have also shown utility for rapidly assessing overall transcriptomic responses with *in vitro* cell line models. Specific chicken cells such as lung, liver and macrophage can be cultivated through cell culture and treated by pathogens to evaluate immune response. Lee et al. (2010, 2012) reported transcriptional profiling of chicken embryo lung cells infected with laryngotracheitis virus (ILTV) based on microarray results using a cell culture model. Furthermore, these identified genes were associated with cancer, cellular growth, death and genetic disorders (Lee et al., 2010). Sirsat et al. (2011b) evaluated pathogenic gene expression levels of *S.* Typhimurium under sublethal heat stress towards Caco-2 cells using a *Salmonella* microarray chip. The heat stress enhanced the ability of adhesion of bacterial cells to Caco-2 cell and promoted microbial virulence (Sirsat et al., 2011b). In addition, Milillo et al. (2011) reported differential gene expression levels in *Salmonella* by combining of two organic acids (sodium acetate and sodium propionate) to achieve true multiple-hurdle effectiveness.

8. Conclusions

The market demands for organic and alternative poultry production have continued to expand in the past few decades due to the respective consumers' perception of these products being a source of safer and healthier foods. Conventional chickens may be reared with traditional commercial antimicrobials and dietary additives to increase productivity, while chemicals and antimicrobial usage in organic and pasture flock chickens are much more strictly controlled. However, depending on environmental exposure alternative poultry production systems have an added challenge with respect to control of foodborne pathogen contamination due to reduced biosecurity which increases bird contact with potential vectors of foodborne pathogens. *Campylobacter* and *Salmonella* are the most common pathogenic bacteria present in the chicken ceca which also contains diverse and abundant bacterial communities. Since MDR pathogens have emerged, alternatives such as prebiotics to decrease pathogens in chickens are greatly needed. Prebiotics exhibit several effects on the GI tract and colonic microflora by selective stimulation of beneficial bacteria as well as inhibition of undesirable bacteria. However, effectively and economically administering such compounds to achieve a consistent and predictable outcome will require a more in-depth analysis of the host and its corresponding microbiome. For the first time, such analytical tools are in-hand and have potential application.

To identify and detect GI tract microflora from the gut and feces, numerous techniques have been developed based on biochemical, microbiological, immunological and molecular biological features. The DGGE/TGGE approached have been utilized to compare and analyze bacterial communities in complex GI tract ecosystems by amplification of common 16s rDNA sequences. Microarrays represent a comprehensive approach for the detection and characterization of foodborne pathogens in food matrices as well as the identification of differential gene expression levels in the chicken host when exposed to different experimental or

environmental conditions. In conclusion, as the organic and alternative poultry production systems continue to become more popular, there will be an increased need for efficient methods to rapidly and accurately detect host, microbiome and metabolome responses to derive predictable responses that allow for routine formulation in commercial settings. Such standardization is needed if there is to be less risk due to exposure from foodborne pathogens and potentially improved bird performance originating from these alternative systems.

9. Acknowledgment

This review was partially supported by a United States Department of Agriculture - National Integrated Food Safety Initiative (USDA-NIFSI) grant # 2008-51110-04339 to author Steven C. Ricke.

10. References

Ackermann, H. W. 2007. 5500 Phages examined in the electron microscope. Arch. Virol. 152:227-243.

Alali, W. Q., S. Thakur, R. D. Berghaus, M. P. Martin, and W. A. Gebreyes. 2010. Prevalence and distribution of *Salmonella* in organic and conventional broiler poultry farms. Foodborne Pathog. Dis. 7:1363-1371.

Altschul, S., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Amit-Romach, E., D. Sklan, and Z. Uni. 2004. Microflora ecology of the chicken intestine using 16S ribosomal DNA primers. Poult. Sci. 83:1093-1098.

AMS/USDA. 2008. The national organic program. Washington DC: Agricultural marketing service/United States Department of Agriculture. http://www.ams.usda.gov/AMSv1.0 Accessed July 2012.

Andreatti Filho, R. L., J. P. Higgins, S. E. Higgins, G. Gaona, A. D. Wolfenden, G. Tellez, and B. M. Hargis. 2007. Ability of bacteriophages isolated from different sources to reduce *Salmonella* enterica serovar Enteritidis *in vitro* and *in vivo*. Poult. Sci. 86:1904-1909.

Angulo, F. J., K. R. Johnson, R. V. Tauxe, and M. L. Cohen. 2000. Origins and consequences of antimicrobial-resistant nontyphoidal *Salmonella*: Implications for the use of fluoroquinolones in food animals. Microb. Drug Resist. 6:77-83.

Arota, K., S. Chand, and B. D. Malhotra. 2006. Recent developments in bio-molecular electronics techniques for food pathogens. Anal. Chim. Acta. 568:259-274.

Bailey, J. S., and D. E. Cosby. 2005. *Salmonella* prevalence in free-range and certified organic chickens. J. Food Prot. 68:2451-2453.

Barrow, P. A., M. B. Huggins, M. A. Lovell, and J. M. Simpson. 1987. Observations on the pathogenesis of experimental *Salmonella typhimurium* infection in chickens. Res. Vet. Sci. 42:194-199.

Bäumler, A. J., R. M. Tsolis, and F. Heffron. 2000. Virulence mechanisms of *Salmonella* and their genetic basis. Pages 57-72 in *Salmonella* in Domestic Animals. C. Wray and A. Wray, ed. CABI Publishing, Wallingford, UK.

Berghman, L. R., D. Abi-Ghanem, S. D. Waghela, and S. C. Ricke. 2005. Antibodies: An alternative for antibiotics? Poult. Sci. 84:660-666.

Biggs, P., C. M. Parsons, and G. C. Fahey. 2007. The effects of several oligosaccharides on growth performance, nutrient digestibilities, and cecal microbial populations in young chicks. Poult. Sci. 86:2327-2336.

Bird, A. R., I. L. Brown, and D. L. Topping. 2000. Starches, resistant starches, the gut microflora and human health. Curr. Issues Intest. Microbiol. 1:25-37.

Black, R. E., M. M. Levine, M. L. Clements, T. P. Hughes, and M. J. Blaser. 1988. Experimental *Campylobacter jejuni* infection in humans. J. Infect. Dis. 157:472-479.

Boerlin, P. 2010. Implication of antimicrobial agents as therapeutics and growth promoters in food animal production. Pages 1-9 in Bacteriophages in the Control of Food- and Waterborne Pathogens. P. M. Sabour and M. W. Griffiths, ed. ASM Press, Washington DC, USA.

Bomba, A., R. Nemcová, S. Gancarcíková, R. Herich, P. Guba, and D. Mudronová. 2002. Improvement of the probiotic effect of micro-organisms by their combination with maltodextrins, fruco-oligosaccharides and polyunsaturated fatty acids. Br. J. Nutr. 88:S95-S99.

Borysowski, J., and A. Górski. 2008. Is phage therapy acceptable in immunocompromised host? Int. J. Infect. Dis. 12:466-471.

Brenner, F. W., R. G. Villar, F. J. Angulo, R. Tauxe, and B. Swaminathan. 2000. *Salmonella* nomenclature. J. Clin. Microbiol. 38:2465-2467.

Brüssow, H., and R.W. Hendrix. 2002. Phage genomics: Small is beautiful. Cell 108:13-16.

Buckley, A. M., J. Wang, D. L. Hudson, A. J. Grant, M. A. Jones, D. J. Maskell, and M. P. Stevens. 2010. Evaluation of live-attenuated *Salmonella* vaccines expressing *Campylobacter* antigens for control of *C. jejuni* in poultry. Vaccine. 28:1094-1105.

Callaway, T. R., S. E. Dowd, R. D. Wilcott, Y. Sun, J. L. McReynold, T. S. Edrington, J. A. Byrd, R. C. Anderson, N. Krueger, and D. J. Nisbet. 2009. Evaluation of the bacterial diversity in cecal contents of laying hens fed various molting diets by using bacterial tag-encoded FLX amplicon pyrosequencing. Poult. Sci. 88:298-302.

Casey, P. G., G. D. Casey, G. E. Gardiner, M. Tangney, C. Stanton, R. P. Ross, C. Hill, and G. F. Fitzgerald. 2004. Isolation and characterization of anti-*Salmonella* lactic acid bacteria from the porcine gastrointestinal tract. Lett. Appl. Microbiol. 39:431-438.

Chalova, V. I., and S. C. Ricke. 2012. Organic animal nutrition and feed supplementations. Pages 157-1758 in Organic Meat Production and Processing. S. C. Ricke, E. J. Van Loo, M. G. Johnson and C. A. O'Bryan, ed. John Wiley & Sons Inc, New Jersey, USA.

Chaucheyras-Durand, F., and H. Durand. 2010. Probiotics in animal nutrition and health. Beneficial Microbes. 1:3-9.

Cortez, A. L. L., A. C. F. B. Carvalho, A. A. Ikuno, K.. P. Bürger, and A. M. C. Vidal-Martins. 2006. Identification of *Salmonella* spp. isolates from chicken abattoirs. Res. Vet. Sci. 81:340-344.

Crhanova, M., H. Hradecka, M. Faldynova, M. Matulova, H. Havlickova, F. Sisak, and I. Rychlik. 2011. Immune response of chicken gut to natural colonization by gut microflora and to *Salmonella* enterica serovar Enteritidis infection. Infect. Immun. 79:2755-2763.

Cui, S., B. Ge, and J. Zhang. 2005. Prevalence and antimicrobial resistance of *Campylobacter* spp. and *Salmonella* serovars in organic chickens from Maryland retail stores. Appl. Envrion. Microbiol. 71:4108-4111.

Dabrowska, K., K. Switala-Jelen, A. Opolski, B. Weber-Dabrowska, and A. Górski. 2005. Bacteriophage penetration in vertebrates. J. Appl. Microbiol. 98:7-13.

Danzeisen, J. L., H. B. Kim, R. E. Isaacson, Z. J. Tu, and T. J. Johnson. 2011. Modulation of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. PLoS one. 6:e27949.

D'Aoust, J. Y., and J. Maurer. 2007. *Salmonella* species. Pages 187-236 in Food Microbiology - Fundamentals and Frontiers, 3rd Edition. M. P. Doyle and L. R. Beuchat, ed. ASM Press, Washington DC, USA.

Davis, L. M. G., I. Martínez. J. Walter, and R. Hutkins. 2010. A dose dependent impact of prebiotic galactooligosaccharides on the intestinal microbiota of healthy adults. Int. J. Food Microbiol. 144:285-292.

DeSoucey, M. 2007. Organic food. In The Business of Food: Encyclopedia of the Food and Drink Industries. G. J. Allen and K. Albala, ed. ABC-CLIO Greenwood, Santa Barbara, USA.

de Greeff, A., M. Huber, L. van de Vijver, W. Swinkels, H. Parmentier, and J. Rebel. 2010. Effect of organically and conventionally produced diets on jejunal gene expression in chickens. Br. J. Nutr. 103:696-702.

de Zoete, M. R., J. P. M. van Putten, and J. A. Wagenaar. 2007. Vaccination of chickens against *Campylobacter*. Vaccine 25:5548-5557.

Donalson, L. M., W.K. Kim, V.I. Chalova, P. Herrera, C. L. Woodward, J. L. McReynolds, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2007. *In vitro* anaerobic incubation of *Salmonella enterica* serotype *Typhimurum* and laying hen cecal bacteria in poultry feed substrates and a fructooligosacharide prebiotic. Anaerobe 13:208-214.

Donalson, L. M., J. L. McReynolds, W. K. Kim, V. I. Chalova, C. L. Woodward, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2008a. The influence of a fructooligosaccharide prebiotic combined with alfalfa molt diets on the gastrointestinal tract fermentation, *Salmonella* enteritidis infection, and intestinal shedding in laying hens. Poult. Sci. 87:1253-1262.

Donalson, L. M., W. K. Kim, V. I. Chalova, P. Herrera, J. L. McReynolds, V. G. Gotcheva, D. Vidanović, C. L. Woodward, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2008b. In vitro fermentation response of laying hen cecal bacteria to combinations of fructooligosaccharide prebiotics with alfalfa or a layer ration. Poult. Sci. 87:1263-1275.

Dunkley, K. D., C. S. Dunkley, N. L. Njongmeta, T. R.Callaway, M. E. Hume, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2007a. Comparison of in vitro fermentation and molecular microbial profiles of high-fiber feed substrates (HFFS) incubated with chicken cecal inocula. Poult. Sci. 86:801-810.

Dunkley, K. D., J. L. McReynolds, M. E. Hume, C. S. Dunkley, T. R. Callaway, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2007b. Molting in *Salmonella* Enteritidis challenged laying hens fed alfalfa crumbles I. *Salmonella* Enteritidis colonization and virulence gene *hilA* response. Poult. Sci. 86:1633-1639.

Dunkley, K. D., J. L. McReynolds, M. E. Hume, C. S. Dunkley, T. R. Callaway, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2007c. Molting in *Salmonella* Enteritidis-challenged laying hens fed alfalfa crumbles. II. Fermentation and microbial ecology response. Poult. Sci. 86:2101-2109.

Dunkley, K.D., T. R. Callaway, V. I. Chalova, J. L. McReynolds, M. E. Hume, C. S. Dunkley, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2009. Foodborne *Salmonella* ecology in the avian gastrointestinal tract. Anaerobe 15:26-35.

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.

Dwivedi, H. P., and L. -A. Jaykus. 2011. Detection of pathogens in foods: the current state-of-the-art and future directions. Crit. Rev. Microbiol. 37:40-63.

Economic Research Services (ERS). 2010. Organic production. http://ers.usda.gov/data-products/organic-production.aspx Accessed Aug. 2012.

Edwards, M. C., and R. A. Gibbs. 1994. Mulplex PCR: advantages, development, and applications. Genome Res. 3:S65-S75.

Eom, H. S., B. H. Hwang, D. -H. Kim, I. -B. Lee, Y. H. Kim, and H. J. Cha. 2007. Multiple detection of food-borne pathogenic bacteria using a novel 16S rDNA-based oligonucleotide signature chip. Biosens. Bioelectron. 22:845-853.

Esteban, J. I., B. Oporto, G. Aduriz, R. A. Juste, and A. Hurtado. 2008. A survey of food-borne pathogens in free-range poultry farms. Int. J. Food Microbiol. 123:177-182.

Fanatico, A. C., C. M. Owens, and J. L. Emmert. 2009. Organic poultry production in the United States: Broilers. J. Appl. Poult. Res. 18:355-366.

Fanatico, A. C., P. B. Pillai, P. Y. Hester, C. Falcone, J. A. Mench, C. M. Owens, and J. L. Emmert. 2008. Performance, livability, and carcass yield of slow- and fast-growing chicken genotypes raised indoors or with outdoor access. Poult. Sci. 87:1012-1021.

Finstad, S., C. A. O'Bryan, J. A. Marcy, P. G. Crandall, and S. C. Ricke. 2012. *Salmonella* and broiler processing in the United States: relationship to foodborne salmonellosis. Food Res. Int. 45:789-794.

Fiorentin, L., N. D. Vieira, and W. Barioni Jr. 2005. Oral treatment with bacteriophages reduces the concentration of *Salmonella* Enteritidis PT4 in caecal contents of broilers. Avian Pathol. 34:258-263.

Foley, S. L., R. Nayak, I. B. Hanning, T. J. Johnson, H. Jing, and S. C. Ricke. 2011. Population dynamics of *Salmonella* enterica serotypes in commercial egg and poultry production. Appl. Envrion. Microbiol. 77:4273-4279.

Forchielli, M. L., and W. A. Walker. 2005. The role of gut-associated lymphoid tissues and mucosal defence. Br. J. Nutr. 93:S41-48.

Foster, J. W. 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. 173:6896-6902.

Foster, J. W., and M. P. Spector. 1995. How *Salmonella* survive against the odds. Annu. Rev. 49:145-174.

Frenzen, P. D. 2005. Death due to unknown foodborne agents. Emerg. Infect. Dis. 10: 1536-1542.

Friedman, C. R., J. Neimann, H. C. Wegener, and R. V. Tauxe. 2000. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. Pages 121-138 in *Campylobacter*. I. Nachamkin and M. J. Blaser, ed. ASM Press, Washington DC, USA.

Fukuta, T., K. Sasai, T. Miyamoto, and E. Baba. 1999. Inhibitory effects of competitive exclusion and fructooligosaccharide, singly and in combination, on *Salmonella* colonization of chicks. J. Food Prot. 62:229-233.

Fuller, R., and A. Turvey. 1971. Bacteria associated with the intestinal wall of the fowl (*Gallus domesticus*). J. Appl. Microbiol. 34:617-622.

Gast, R. K. 2007. Serotype-specific and serotype-independent strategies for preharvest control of food-borne *Salmonella* in poultry. Avian Dis. 51:817-828.

Gast, R. K., and P. S. Holt. 1997. Persistence of *Salmonella enteritidis* from one day of age until maturity in experimentally infected layer chickens. Poult. Sci. 77:1759-1762.

Gibson, G.R., and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota-introducing the concept of prebiotics. J. Nutr. 125:1401-1412.

Gibson, G. R., H. M. Probert, J. Van Loo, R. A. Rastall, and M. B. Roberfroid. 2004. Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. Nutr. Res. Rev. 17:259-275.

Girad, M. P., D. Steele, C. -L. Chaignat, and M. P. Kieny. 2006. A review of vaccine research and development: human enteric infections. Vaccine 24:2732-2750.

Goldschmidt, M. C. 2006. The use of biosensor and microarray techniques in the rapid detection and identification of salmonellae. J. AOAC Int. 89:530-537.

Gong, J., R. J. Foster, H. Yu, J. R. Chambers, P. M. Sabour, R. Wheatcroft, and S. Chen. 2002. Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen. FEMS Microbiol. Lett. 208:1-7.

Goode, D., V. M. Allen, and P. A. Barrow. 2003. Reduction of experimental *Salmonell*a and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. Appl. Environ. Microbiol. 69:5032-5036.

Gopal, P. K., J. Prasad, and H. S. Gill. 2003. Effects of the consumption of *Bifidobacterium lactis* HN019 (DR10TM) and galacto-oligosaccharides on the microflora of the gastrointestinal tract in human subjects. Nutr. Res. 23:1313-1328.

Griggs, J. P., J. B. Bender, and J. P. Jacob. 2006. Microbial safety of chickens raised without antibiotics. J. Appl. Poult. Res. 15:475-482.

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. CABI Publishing, Wallingford, UK.

Grimont, P. A. D., and F. -X. Weill. 2007. Antigenic formulae of the *Salmonella* serovars, 9th edition. Institut Pasteur. Paris, France.

Haddad, N., C. Marce, C. Magras, and J. -M. Cappelier. 2010. An overview of methods used to clarify pathogenesis mechanisms of *Campylobacter jejuni*. J. Food Prot. 73:786-802.

Hahn, A. F. 1998. Guillain-Barré sysdrome. Lancet. 352:635-641.

Han, F. S., I. Lestari, S. Pu, and B. Ge. 2009. Prevalence and antimicrobial resistance among *Campylobacter* spp. in Louisiana retail chickens after the enrofloxacin ban. Foodborne Pathog. Dis. 6:163-171.

Hanning, I., and S. C. Ricke. 2011. Prescreening methods of microbial populations for the assessment of sequencing potential. Pages 159-170 in High-Throughput Next Generation Sequencing: Methods and Applications. Y. M. Kwon and S. C. Ricke, ed. Springer Protocols, Humana Press, New York, USA.

Hanning, I., D. Biswas, P. Herrera, M. Roesler, and S. C. Ricke. 2010. Prevalence and characterization of *Campylobacter jejuni* isolated from pasture flock poultry. J. Food Sci. 75:M496-M502.

Harper, G. C., and A. Makatouni. 2002. Consumer perception of organic food production and farm animal welfare. Br. Food J. 104:287-299.

Henderson, S. N., J. T. Barton, A. D. Wolfenden, S. E. Higgins, J. P. Higgins, W. J. Kruenzel, C. A. Lester, G. Tellez, and B. M. Hargis. 2009. Comparison of beak-trimming methods on early broiler breeder performance. Poult. Sci. 88:57-60.

Higgins, J. P., S. E. Higgins, A. D. Wolfenden, S. N. Henderson, A. Torres-Rodriguez , J. L. Vicente, B. M. Hargis, and G. Tellez. 2004. Effect of lactic acid bacteria probiotic culture treatment timing on *Salmonella* Enteritidis in neonatal broilers. Poult. Sci. 89:243-247.

Higgins, S. E., A. D. Wolfenden, G. Tellez, B. M. Hargis, and T. E. Porter. 2011. Transcriptional profiling of cecal gene expression in probiotic- and *Salmonella*-challenged neonatal chicks. Poult. Sci. 90:901-913.

Higgins, S. E., J. P. Higgins, L. R. Bielke, and B. M. Hargis. 2007. Selection and application of bacteriophages for treating *Salmonella* Enteritidis infections in poultry. Int. J. Poult. Sci. 6:163-168.

Hill III, J. G., I. Hanning, S. J. Beaupre, S. C. Ricke, and M. M. Slavik. 2008. Denaturing gradient gel electrophoresis for the determination of bacterial species diversity in the gastrointestinal tracts of two crotaline snakes. Herpetol. Rev. 39:433-438.

Holzapfel, W. H., and U. Schillinger. 2002. Introduction to pre- and probiotics. Food Res. Int. 35:109-116.

Horrocks, S. M., R. C. Anderson, D. J. Nisbet, and S. C. Ricke. 2009. Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. Anaerobe 15:18-25.

Howard, Z. R., C. A. O'Bryan, P. G. Crandall, and S. C. Ricke. 2012. *Salmonella* Enteritidis in shell eggs: Current issues and prospects for control. Food Res. Int. 45:755-764.

Huff, W. E., G. R. Huff, N. C. Rath and A. M. Donoghue. 2010. Immune interference of bacteriophage efficacy when treating colibacillosis in poultry. Poult. Sci.89: 895-900.

Huff, W. E., G. R. Huff, N. C. Rath, J. M. Balog, and A. M. Donoghue. 2003. Bacteriophage treatment of a severe *Escherichia coli* respiratory infection in broiler chickens. Avian Dis. 47:1399-1405.

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. Poult. Sci. 82:1100-1107.

Ibuki, M., J. Kovacs-Nolan, K. Fukui, H. Kanatani, and Y. Mine. 2010. Analysis of gut immune-modulating activity of β-1,4-mannobiose using microarray and real-time reverse transcription polymerase chain reaction. Poult. Sci. 89:1894-1904.

Israeli, E., N. Agomon-Levin, M. Blank, J. Chapman, and Y. Shoenfeld. 2012. Guillain-Barré syndrome - a classical autoimmune disease triggered by infection or vaccination. Clin. Rev. Allerg. Immun. 42:121-130.

Ito, M., Y. Deguchi, A. Miyamori, H. Kikuchi, K. Matsumoto, Y. Kobayashi, T. Yajiama, and T. Kan. 1990. Effects of administration of galactooligosaccharides on human faecal microflora, stool weight and abdominal sensation. Microb. Ecol. Health Dis. 3:285-292.

Jacob, J., and A. Pescatore. 2012. Prebiotics. Pages 379-405 in Organic Meat Production and Processing. S. C. Ricke, E. J. Van Loo, M. G. Johnson and C. A. O'Bryan, ed. John Wiley & Sons Inc, New Jersey, USA.

Jacob-Reitsma, W. 2000. *Campylobacter* in the food supply. Pages 467-481 in *Campylobacter*. I. Nachamkin and M. J. Blaser, ed. ASM Press, Washington DC, USA.

Jones, F. T., and S. C. Ricke. 2003. Observations on the history of the development of antimicrobials and their use in poultry feeds. Poult. Sci. 82:613-617.

Joerger, R. D. 2003. Alternatives to antibiotics: Bacteriocins, antimicrobial peptides and bacteriophages. Poult. Sci. 82:640-647.

Jung, S. J., R. Houde, B. Baurhoo, X. Zhao, and B. H. Lee. 2008. Effects of galacto-oligosaccharides and a *Bifidobacteria lactis*-based probiotic strain on the growth performance and fecal microflora of broiler chickens. Poult. Sci. 87:1694-1699.

Júskiewicz, J., Z. Zduńczyk, and J. Jankowski. 2004. Selected parameters of gastrointestinal tract metabolism of turkeys fed diets with flavomycin and different inulin content. World's Poult. Sci. J. 60:177-185.

Kaplan, H., and R. W. Hutkins. 2000. Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. Appl. Environ. Microbiol. 66:2682-2684.

Kass, M. L., P. J. Van. Soest, and W. G. Pond. 1980. Utilization of dietary fiber from alfalfa by growing swine. II. Volatile fatty acid concentrations in a disappearance from the gastrointestinal tract. J. Anim. Sci. 50:192-197.

Kim, C. C., E. A. Joyce, K. Chan, and S. Falkow. 2002. Improved analytical methods for microarray-based genome composition analysis. Genome Biol. 3:research0065.1-0065.17.

Kim, H. J., S. H. Park, T. H. Lee, B .H. Nahm, Y. H. Chung, K. H. Seo, and H. Y. Kim. 2006. Identification of *Salmonella enterica* serovar Typhimurium using specific PCR primers obtained by comparative genomics in *Salmonella* serovars. J. Food Prot. 69:1653-1661.

Kim, S., J. G. Frye, J. Hu, P. J. Fedorka-Cray, R. Gautom, and D. S. Boyle. 2006. Multiplex PCR-based method for identification of common clinical serotypes of *Salmonella enterica* subsp. *enterica*. J. Clin. Microbiol. 44:3608-3615

Kim, T., and E. Mundt. 2011. Metagenomic analysis of intestinal microbiomes in chickens. Pages 185-194 in High-Throughput Next Generation Sequencing: Methods and Applications. Y. M. Kwon and S. C. Ricke, ed. Springer Protocols, Humana Press, New York, USA.

Kirkpatrick, B. D., and D. R. Tribble. 2010. Update on human *Campylobacter jejuni* infections, Curr. Opin. Gastroenterol. 27:1-7.

Klaenhammer, T. R. 2000. Probiotic bacteria: today and tomorrow. J. Nutr. 130:415s-416s.

Kollanoor-Johny, A., T. Mattson, S. A. Baskaran, M. A. Amalaradjou, S. Babapoor, B. March, S. Valipe, M. Darre, T. Hoagland, D. Schreiber, M. I. Khan, A. Donoghue, D. Donoghue, and K. Venkitanarayanan. 2012. Reduction of *Salmonella enterica* serovar Enteritidis colonization in 20-day-old broiler chickens by the plant-derived compounds trans-cinnamaldehyde and eugenol. Appl. Environ. Microbiol. 78:2981-2987.

Kwon, Y. M., and S. C. Ricke. 1998. Induction of acid resistance of *Salmonella typhimurium* by exposure to short-chain fatty acids. Appl. Environ. Microbiol. 64:3458-3463.

Kwon, Y. M., and S. C. Ricke. 2011. High-Throughput Next Generation Sequencing: Methods and Applications. Springer Protocols, Humana Press, New York, USA.

Lafont, J. P., A. Bree, M. Nacir, P. Yvore, J. F. Guillot, and E. Chaslus-Dancla. 1983. Experimental study of some factors limiting “competitive exclusion” of salmonella in chicken, Res. Vet. Sci. 34:16-20.

Lamont, R. J. 2004. Bacterial Invasion of Host Cells. Pages 1-24. Cambridge University Press, Cambridge, UK.

Lee. J. Y., J. J. Song, A. Wooming, X. Li, H. Zhou, W. G. Bottje, and B. -W. Kong. 2010. Transcriptional profiling of host gene expression in chicken embryo lung cells infected with laryngotracheitis virus. BMC Genomics 11:445.

Lee. J. Y., W.G. Bottje, and B. -W. Kong. 2012. Genome-wide host responses against infectious laryngotracheitis virus vaccine infection in chicken embryo lung cells. BMC Genomics. 13:143.

Lestari, S. I., F. Han, and F. Wang. 2009. Prevalence and antimicrobial resistance of Salmonella serovars in conventional and organic chickens from Louisiana retail stores. J. Food Prot. 72:1165-1172.

Leverentz, B., W. S. Conway, M. J. Camp, W. J. Janisiewicz, T. Abuladze, M. Yang, R. Saftner, and A. Sulakvelidze. 2003. Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. Appl. Environ. Microbiol. 69:4519-4526.

Levin, B. R., and J. J. Bull. 2004. Population and evolutionary dynamics of phage therapy. Nat. Rev. Microbiol. 2:166-173.

Lior, H., D. L. Woodward, J. A. Edgar, L. J. Laroche, and P. Gill. 1982. Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors, J. Clin. Microbiol. 15:761-768.

Lu, J., U. Idris, B. Harmon, C. Hofacre, J. J. Maurer, and M. D. Lee. 2003. Diversity and succession of the intestinal bacterial community of the maturing broiler chicken. Appl. Environ. Microbiol. 69:6816-6824.

Luangtongkum, T., T. Y. Morishita, A. J. Ison, S. Huang, P. F. McDermont, and Q. Zhang. 2006. Effect of conventional and organic production practices on the prevalence and antimicrobial resistance of *Campylobacter* spp. in poultry. Appl. Environ. Microbiol. 72:3600-3607.

MacDonald, J. M. 2008. The economic organization of U.S. broiler production. P.26 in Economic Information Bulletin, No 38. Economic Research Service, USDA, Washington DC, USA.

Malkawi, H. I., and R. Gharaibeh. 2003. Multiplex PCR for the direct detection of *Salmonella enterica* from chicken, lamb and beef food products. J. Basic Microbiol. 4:328-336.

Malinen, E., J. Mättö, M. Salmitie, M. Alander, M. Saarela, and A. Palva. 2002. PCR-ELISA II: Analysis of *Bifidobacterium* populations in human faecal samples from a consumption trial with *Bifidobacterium lactis* Bb-12 and a galacto-oligosaccharide preparation. Syst. Appl. Microbiol. 25:249-258.

Manning, J. G., B. M. Hargis, A. Hilton Jr., D. E. Corrier, J. R. DeLoach, and C. R. Greger. 1994. Effect of selected antibiotics and anticoccidials on Salmonella enteritidis cecal colonization and organ invasion in Leghorn chicks. Avian Dis. 38:256-261.

Marounek, M., O. Suchorska, and O. Savka. 1999. Effect of substrate and feed antibiotics on in vitro production of volatile fatty acids and methane in caecal contents of chickens. Anim. Feed Sci. Technol. 80:223-230.

McCarthy, N., F. J. Reen, J. F. Buckley, J. G. Frye, E. F. Boyd, and D. Gilroy. 2009. Sensitive and rapid molecular detection assays for *Salmonella enterica* serovars Typhimurium and Heidelberg. J. Food Prot. 72:2350-2357.

McCracken, V. J., J. M. Simpson, R. I. Mackie, and H. R. Gaskins. 2001. Molecular ecological analysis of dietary and antibiotic-induced alterations of the mouse intestinal microbiota. J. Nutr. 131:1862-1870.

McCrea, B. A., K. H. Tonooka, C. VanWorth, C. L. Boggs, E. R. Atwill, and J. S. Schrader. 2006. Prevalence of *Campylobacter* and *Salmonella* species on farm, after transport, and at processing in specialty market poultry. Poult. Sci. 85:136-143.

McHan, F., and E. B. Shotts. 1993. Effect of short-chain fatty acids on the growth of Salmonella typhimurium in an in vitro system. Avian Dis. 37:396-398.

McSweeny, S., and R. I. Mackie. 1997. Gastrointestinal detoxification and digestive disorders in ruminant animals. Pages 588-621 in Gastrointestinal Microbiology. R. I. Mackie and B. A. White, ed. Champman & Hall, International Thomson Publishing, New York, USA.

Melendez, S. N., I. Hanning, J. Han, R. Nayak, A. R. Clement, A. Wooming, P. Hererra, F. T. Jones, S. L. Foley, and S. C. Ricke. 2010. *Salmonella enterica* isolates from pasture-raised poultry exhibit antimicrobial resistance and class I integron. J. Appl. Microbiol. 109:1957-1966.

Monteiro, M. A., S. Baqar, E. R. Hall, Y. -H. Chen, C. K. Porter, D. E. Bentzel, L. Applebee, and P. Guerry. 2009. Capsule polysaccharide conjugate vaccine against diarrheal disease caused by *Campylobacter jejuni*, Infect. Immun. 77:1128-1136.

Murray, C. J. 2000. Environmetal aspects of *Salmonella*. Pages 265-283 in *Salmonella* in Domestic Animals. C. Wray and A. Wray, ed. CABI Publishing, Wallingford, UK.

Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. Curr. Opin. Microbiol. 2:317-322.

Nachamkin, I. 2001. *Campylobacter* enteritis and the Guillian-Barré Syndrome. Curr. Infect. Dis. Rep. 3:116-122.

Nachamkin, I., B. M. Allos, and T. W. Ho. 2000. *Campylobacter jejuni* infection and the association with Guillain-Barré syndrome. Pages 155-175 in *Campylobacter*. I. Nachamkin and M. J. Blaser, ed. ASM Press, Washington DC, USA.

Nakai, T., and S.C. Park. 2002. Bacteriophage therapy of infectious diseases in aquaculture. Res. Microbiol. 153:13-18.

Nath, G., and P. Maurya. 2010. Drug resistance patterns in *Salmonella enterica* subspeices *enterica* serotype Typhi strains isolated over a period of two decades, with special reference to ciprofloxacin and ceftriaxone. Int. J. Antimicrob. Ag. 35:482-485.

National Chicken Council. 2010. National chicken council animal welfare guidelines and audit checklist for broilers. Washington DC, http://www.nationalchickencouncil.org/wp-content/uploads/2012/01/NCC-Animal-Welfare-Guidelines-2010-Revision-BROILERS.pdf Accessed Aug. 2012.

Nester, E. W., D. G. Anderson, C. E. Roberts Jr, and M. T. Nester. 2009. Pages 601-603 in Microbiology (A human perspective) 6th edition. McGrawHill, New York, USA.

Newell, D. G., and C. Fearnley. 2003. Sources of *Campylobacter* colonization in broiler chickens. Appl. Environ. Microbiol. 69:4343-4351.

Newman, K. 1994. Manna-oligosaccharides: Natural polymers with significant impact on the gastrointestinal microflora and the immune system. Pages 167-174 in Biotechnology in the Feed Industry. Proc. Alltech's 10th Annu. Symp. T. P. Lyons and K. A. Jacques, ed. Nottingham University Press, Nottingham, UK.

Nisbet, D. 2002. Defined competitive exclusion cultures in the prevention of enteropathogen colonisation in poultry and swine. Antonie van Leeuwenhoek. 81:481-486.

Nisbet, D. J., D. E. Corrier, S. C. Ricke, M. E. Hume, J. A. Byrd II, and J. R. DeLoach. 1996a. Maintenance of the biological efficacy in chicks of a cecal competitive-exclusion culture against *Salmonella* by continuous-flow fermentation. J. Food Prot. 59:1279-1283.

Nisbet, D. J., D. E. Corrier, S. C. Ricke, M. E. Hume, J. A. Byrd II, and J. R. DeLoach. 1996b. Cecal propionic acid as a biological indicator of the early establishment of a microbial ecosystem inhibitory to *Salmonella* in chicks. Anaerobe 2:345-350.

O'Bryan, C. A., P. G. Crandall, and S. C. Ricke. 2008. Organic poultry pathogen control from farm to fork. Foodborne Pathog. Dis. 5:709-720.

Ofek, I., D. Mirelman, and N. Sharon. 1977. Adherance of *Escherichia coli* to human mucosal cells mediated by mannose receptors. Nature. 265:623-625.

O'Flynn, G., R. P. Ross, G. F. Fitzgerald, and A. Coffey. 2004. Evaluation of a cocktail of three bacteriophages for biocontrol of *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 70:3417-3424.

Orban, J. I., J. A. Patterson, A. L. Sutton, G. N. Richards. 1997. Effect of sucrose thermal oligosaccharide caramel, dietary vitamin-mineral level, and brooding temperature on growth and intestinal bacterial populations of broiler chickens. Poult. Sci. 76:482-490.

O'Regan, E., E. McCabe, C. Burgess, S. McGuinness, T. Barry, G. Duffy, P. Whyte, and S. Fanning. 2008. Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. BMC Microbiology 8:156.

Organic Trade Association (OTA). 2011. U.S. organic industry valued at nearly $29 billion in 2010. http://www.organicnewsroom.com/2011/04 Accessed Aug. 2012.

O'Sullivan, M. G, G. Thorton, G. C. O'Sullivan, and J. K. Collins. 1992. Probiotic bacteria: myth or reality? Trends Food Sci. Technol. 3:309-314.

Owens, B., L. Tucker, M. A. Collins, and K. J. McCracken. 2008. Effects of different feed additives alone or in combination on broiler performance, gut microflora and ileal histology. Br. Poult. Sci. 49:202-212.

Palys, T., L. K. Nakamura, and F. M. Cohan. 1997. Discovery and classification of ecological diversity in the bacterial world: The role of DNA sequence data. Int. J. Syst. Bacteriol. 47:1145-1156.

Park, S. H., H. J. Kim, W. H. Cho, J. H. Kim, M. H. Oh, S. H. Kim, B. K. Lee, S. C. Ricke, and H. Y. Kim. 2009. Identification of *Salmonella enterica* subspecies I, *Salmonella enterica* serovars Typhimurium, Enteritidis and Typhi using multiplex PCR. FEMS Microbiol. Lett. 301:137-146.

Park, S. H., I. Hanning, R. Jarquin, P. Moore, D.J. Donoghue, A.M. Donoghue, and S.C. Ricke. 2011. Multiplex PCR assay for the detection and quantification of *Camplylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes in water samples. FEMS Microbiol. Lett. 316:7-15.

Park, S. Y., C. L. Woodward, L. F. Kubena, D. J. Nisbet, S. G. Birkhold, and S. C. Ricke. 2008. Environmental dissemination of foodborne *Salmonella* in preharvest poultry production: reservoirs, critical factors, and research strategies. Crit. Rev. Env. Sci. Tech. 38:73-111.

Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82:627-631.

Patton, C. M., T. J. Barrett, and G. K. Morris. 1985. Comparison of the Penner and Lior methods for serotyping *Campylobacter* spp.. J. Clin. Microbiol. 22:558-565.

Penner, J. L., and J. N. Hennessy. 1980. Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *Jejuni* on the basis of soluble heat-stable antigens. J. Clin. Microbiol. 12:732-727.

Pittman, H. M., K. C. Boling, and S. J. Mirus. 2012. Regulatory issues in domestically raised and imported organic meats in the United States. Pages 23-51 in Organic Meat Production and Processing. S. C. Ricke, E. J. Van Loo, M. G. Johnson and C. A. O'Bryan, ed. John Wiley & Sons Inc, New Jersey, USA.

Plamondon, R. 2003. Range poultry housing; Livestock production guide. https://attra.ncat.org/attra-pub/summaries/summary.php?pub=233 Accessed July 2012.

Raskin, L., W. Capman, R. Sharp, L. Poulsen, and D. Stahl. 1997. Molecular ecology of gastrointestinal ecosystems. Pages 243-298 in Gastrointestinal Microbiology. R. I. Mackie and B. A. White, ed. Champman & Hall, International Thomson Publishing, New York, USA.

Ricke, S.C. 2003a. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poult. Sci. 82:632-639.

Ricke, S. C. 2003b. The gastrointestinal tract ecology of *Salmonella* Enteritidis colonization in molting hens. Poult. Sci. 82:1003-1007.

Ricke, S. C., and S. D. Pillai. 1999. Conventional and molecular methods for understanding probiotic bacteria functionality in gastrointestinal tracts. Crit. Rev. Microbiol. 25:19-38.

Ricke, S. C., S. G. Birkhold, and R. K. Gast. 2001. Eggs and egg products. Pages 473-481 in Compendium of Methods for the Microbiological Examinations of Foods, 4th Edition. F. P. Downes and K. Ito, ed. American Public Health Association, Washington DC, USA.

Ricke, S. C., E. J. Van Loo, M. G. Johnson, and C. A. O'Brian. 2012. Organic Meat Production and Processing. John Wiley & Sons Inc, New Jersey, USA.

Ricke, S. C., L. Casendra, L. Woodward, Y. M. Kwon, L. F. Kubena, and D. J. Nisbet. 2004. Limiting avian gastrointestinal tract *Salmonella* colonization by cecal anaerobic bacteria, and a potential role for methanogens. Pages 141-150 in Preharvest and Postharvest Food Safety, Contemporary Issues and Future Direction. R. C. Deier, S. D. Pillai and T. D. Phillips, ed. Blackwell Publishing Professional, Ames, USA.

Ricke, S. C., M. M. Kundinger, D. R. Miller, and J. T. Keeton. 2005. Alternatives to antibiotics: Chemical and physical antimicrobial interventions and foodborne pathogen response. Poult. Sci. 84:667-675.

Ricke, S. C., P. Hererra, and D. Biswas. 2012. Chapter 23. Bacteriophages for potential food safety applications in organic meat production. In: S.C. Ricke, E.J. Van Loo, M.G. Johnson

and C.A. O'Bryan (Eds.), Organic Meat Production and Processing. Wiley Scientific/IFT, New York, NY. pp. 407-424.

Ricke, S. C., P. J. Van der Aar, G. C. Fahey Jr, and L. L. Berger. 1982. Influence of dietary fibers on performance and fermentation characteristics of gut contents from growing chicks. Poult. Sci. 61:1335-1443.

Ricke, S. C., R. Jarquin, and I. Hanning. 2012. Antimicrobials in feed: benefits and limitations. Pages 411-431 in Animal Feed Contamination: Effects on Livestock and Food Safety. J. Fink-Gremmels, ed. Woodhead Publishing Ltd, Cambridge, UK.

Ringoir, D. D., D. Szylo, and V. Korolik. 2007. Comparison of 2-day-old and 14-day-old chicken colonization models for *Campylobacter jejuni*. FEMS Immuno. Med. Microbiol. 49:155-158.

Roberfroid, M. B. 1998. Prebiotics and synbiotics: concepts and nutrional properties. Br. J. Nutr. 80:S197-S202.

Rowland, I. R., and R. Tanaka. 1993. The effects of transgalactosylated oligosaccharides on gut flora metabolism in rats associated with a human faecal microflora. J. Appl. Bacteriol. 74:667-674.

Sabour, P. M., and M. W. Griffiths. 2010. Bacteriophages in the control of food- and waterborne pathogens. ASM Press. Washington, DC.

Saengkerdsub, S., P. Herrera, C. L. Woodward, R. C. Anderson, D. J. Nisbet, and S. C. Ricke. 2007a. Detection of methane and quantification of methanogenic archaea in faeces from young broiler chickens using real-time PCR. Lett. Appl. Microbiol. 45:629-634.

Saengkerdsub, S., R. C. Anderson, H. H. Wilkinson, W. -K. Kim, D. J. Nisbet, and S. C. Ricke. 2007b. Identification and quantification of methanogenic archaea in adult chicken ceca. Appl. Environ. Microbiol. 73:353-356.

Saengkerdsub, S., W. -K. Kim, R. C. Anderson, D. J. Nisbet, and S. C. Ricke. 2006. Effects of nitrocompounds and feedstuffs on in vitro methane production in chicken cecal contents and rumen fluid. Anaerobe 12:85-92.

Saito, N., D. Hirohara, M. Miyaji, A. Ito, Y. Uzawa, and K. Nomura. 2009. Validity of gram staining of stool samples for diagnosing *Campylobacter* Enteritis in patient with acute diarrhea. Gen. Med. 10:17-21.

Sako, T., K. Matsumoto, and R. Tanaka. 1999. Recent progress on research and applications of non-digestible galacto-oligosaccharides. Int. Dairy J. 9:69-80.

Scallan E, R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. -A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States-major pathogens. Emerg. Infect. Dis. 17:7-15.

Schrezenmeir, J., and M. de Vrese. 2001. Probiotics, prebiotics, and synbiotics-approaching ad definition1-3. Am. J. Clin. Nutr. 73:361S-364S.

Sergeant, M. J., C. Constantinidou, T. Cogan, C. W. Penn, and M. J. Pallen. 2012. High-throughput sequencing of 16S rRNA gene amplicons: Effects of extraction procedure, primer length and annealing temperature. PLoS one 7:e38094.

Seuna, E., C. Schneitz, and E. Nurmi. 1980. Combined therapy of Salmonella infection in chickens by antimicrobial agents followed by cultured cecal bacteria. Poult. Sci. 59:1187-1192.

Siemon, C. E., P .B. Bahnson, and W. A. Gebreyes. 2007. Comparative investigation of prevalence and antimicrobial resistance of *Salmonella* between pasture and conventionally reared poultry. Avian Dis. 51:112-117.

Siragusa, G. R. and S. C. Ricke. 2012. Probiotics as pathogen control agents for organic meat production. Pages 331-349 in Organic Meat Production and Processing. S. C. Ricke, E. J. Van Loo, M. G. Johnson and C. A. O'Bryan, ed. Wiley Scientific/IFT, New York, USA.

Sirsat, S. A., A. Muthaiyan, and S. C. Ricke. 2009. Antimicrobials for pathogen reduction in organic and natural poultry production. J. Appl. Poult. Res. 18:379-388.

Sirsat, S. A., A. Muthaiyan, and S.C. Ricke. 2011a. Optimization of RNA extraction method for transcriptome studies of *Salmonella* inoculated on commercial raw chicken breast samples. BMC Res. Notes. 4:60.

Sirsat, S. A., K. M. Burkholder, A. Muthaiyan, S. E. Dowd, A. K. Bhunia, and S.C. Ricke. 2011b. Effect of sublethal heat stress on *Salmonella* Typhimurium virulence. J. Appl. Microbiol. 110:813-822.

Sirsat, S. A., A. Muthaiyan, S. E. Dowd, Y. M. Kwon, and S. C. Ricke. 2010. The potential for application of foodborne *Salmonella* gene expression profiling assays in postharvest poultry processing. Pages 195-222 in Perspectives on Food-Safety Issues of Animal-Derived Foods. S. C. Ricke and F. T. Jones, ed. University of Arkansas Press. Fayetteville, USA.

Smith, H. W., and J. F. Tucker. 1980. The virulence of salmonella strains for chickens: Their excretion by infected chickens. J. Hyg. 84:479-488.

Snelling, W. J., M. Matsuda, J. E. Moore, and J. S. G. Dooley. 2005. *Campylobacter jejuni*, Lett. Appl. Micorbiol. 41:297-302.

Stern, N. J., and J. E. Line. 1992. Comparison of three methods for recovery of *Campylobacter* spp. from broiler carcasses. J. Food Prot. 55:663-666.

Sulakvelidze, A. 2011. Safety by nature: Potential bacteriophage applications. Microbe 6:122-126.

Sunvold, G. D., H. S. Hussein, G. C. Fahey, Jr., N. R. Merchen, and G. A. Reinhart. 1995. In vitro fermentation of cellulose, beet pulp, citrus pulp, and citrus pectin using fecal inoculum from cats, dogs, horses, humans, and pigs and ruminal fluid from cattle. J. Anim. Sci. 73:3639-3648.

Swennen, K., C. M. Courtin, and J. A. Delcour. 2006. Non-digestible oligosaccharides with prebiotic properties. Crit. Rev. Food Sci. 46:459-471.

Tanji, Y., T. Shimada, M. Yoichi, K. Miyanaga, K. Hori, and H. Unno. 2004. Toward rational control of *Escherichia coli* O157:H7 by a phagecocktail. Appl. Microbiol. Biotechnol. 64:270-274.

Threlfall, E. J., J. A. Skinner, and L. R. Ward. 2001. Detection of decreased susceptibility to ciprofloxacin in *Salmonella enterica* serotypes Typhi and Paratyphi A. J. Antimicrob. Chemother. 48:740-741.

Timmerman, H. M., A. Veldman, E. van den Elsen, F. M. Rombouts, and A. C. Beynen. 2006. Mortality and growth performance of broilers given drinking water supplemented with chicken-specific probiotics. Poult. Sci. 85:1383-1388.

Toro, H., S. B. Price, A. S. McKee, F. J. Hoerr, J. Krehling, M. Perdue, and L. Bauermeister. 2005. Use of bacteriophages in combination with competitive exclusion to reduce *Salmonella* from infected chickens. Avian Dis. 49:118-124.

Torok, V. A., R. J. Hughes, L. L. Mikkelsen, R. Perez-Maldonado, K. Balding, R. MacAlpine, N. J. Percy, and K. Ophel-Keller. 2011. Identification and characterization of potential performance-related gut microbiotas in broiler chickens across various feeding trials. Appl. Environ. Microbiol. 77:5868-5878.

USDA National Organic Program. 2008. Agricultural Marketing Service 7. Code of Federal Regulations (CFR), Part 205: the National Organic Program.

van der Werf, M. J., R. H. Jellema, and T. Hankemeier. 2005. Microbial metabolomics: replacing trial-and-error by the unbiased selection and ranking of targets. J. Ind. Microbiol. Biotechnol. 32:234-252.

van der Wielen, P. W. J. J., S. Biesterveld, S. Notermans, H. Hofstra, B. A. P. Urlings, and F. van Knapen. 2000. Role of volatile fatty acids in development of the cecal microflora in broiler chickens during growth. Appl. Environ. Microbiol. 66:2536-2540.

Van Loo, E. J., S. N. Melendez, I. B. Hanning, and S. C. Ricke. 2012a. Food-borne pathogen occurrence in organically and naturally raised poultry. Pages 315-328 in Organic Meat

Production and Processing. S. C. Ricke, E. J. Van Loo, M. G. Johnson and C. A. O'Bryan, ed. John Wiley & Sons Inc, New Jersey, USA.

Van Loo, E. J., V. Caputo, R. M. Nayga Jr., M. Canavari, and S. C. Ricke. 2012b. Orgainc meat marketing. Pages 67-85 in Organic Meat Production and Processing. S. C. Ricke, E. J. Van Loo, M. G. Johnson and C. A. O'Bryan, ed. John Wiley & Sons Inc, New Jersey, USA.

Van Loo, E. J., W. Alali, and S. C. Ricke. 2012c. Food safety and organic meats. Annu. Rev. Food Sci. Technol. 3:203-225.

Wagenaar, J. A., M. A. P. Van Bergen, M. A. Mueller, T. M. Wassenaar, and R. M. Carlton. 2005. Phage therapy reduces *Campylobacter jejuni* colonization in broilers. Vet. Microbiol. 109:275-283.

Walker, R. I., E. A. Schmauder-Chock, J. L. Parker, and D. Burr. 1988. Selective association and transport of *Campylobacter jejuni* through M cells of rabbit Peyer's patches. Can. J. Microbiol. 34:1142-1147.

Watkins, B. A., and F. H. Kratzer. 1984. Drinking water treatment with a commercial preparation of a concentrated *Lactobacillus* culture for broiler chickens. Poult. Sci. 63:1671-1673.
Woodward, C. L., Y. M. Kwon, L. F. Kubena, J. A. Byrd, R. W. Moore, D. J. Nisbet, and S. C. Ricke. 2005. Reduction of *Salmonella enterica* serovar Enteritidis colonization and invasion by an alfalfa diet during molt in Leghorn hens. Poult. Sci. 84:185-193.

Xu, Z. R., C. H. Hu, M. S. Xia, X. A. Zhan, and M. Q. Wang. 2003. Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. Poult. Sci. 82:1030-1036.

Zhu, X.Y., T. Zhong, Y. Pandya, and R. D. Joerger. 2002. 16S rRNA-based analysis of microbiota from the ceum of broiler chickens. Appl. Environ. Micrbiol. 68:124-137.

Ziemer, C.J., and G. R. Gibson. 1998. An overview of probiotics, prebiotics, synbiotics in the functional food concept: perspectives and future strategies. Int. Dairy J. 8:473-479.

11. Appendix

11.1. Chapter 1 Publication by Journal

Next Generation Sequencing: Applications for Food Safety and Poultry Production

Modifying the gastrointestinal ecology in alternatively raised poultry and the potential for molecular and metabolomic assessment[1]

S. H. Park,*† I. Hanning,‡§ A. Perrota,# B. J. Bench,‖ E. Alm,¶ and S. C. Ricke*†[2]

*Cell and Molecular Biology Graduate Program, and †Center for Food Safety and Department of Food Science, University of Arkansas, Fayetteville 72704; ‡Department of Food Science and Technology, and §Department of Genomic Sciences and Technology, University of Tennessee, Knoxville 37996; #Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, Cambridge 02139; ‖Tyson Foods Inc., Food Safety and Research Laboratory, Springdale, AR 72762; and ¶Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge 02139

ABSTRACT Consumer demand for nonconventional poultry products continues to increase in the United States. In pasture flock and organic poultry production, probiotics and prebiotic feed additives have potential advantages because they are thought to promote intestinal health and may offer a replacement for current intervention strategies that are not considered acceptable for these production systems. Prebiotics have been demonstrated to produce effects on the gastrointestinal tract including modulation of microflora by promoting selective increases in beneficial bacteria concomitant with decreases in undesirable bacteria. In-depth assessment of microbial community changes during host growth and development as well as the establishment of beneficial microbial species by adding biologicals such as probiotics and prebiotics is important to achieve predictable and consistent improvements in chicken health and productivity. To analyze microflora shifts and metabolites produced by bacteria in the gut as well as host responses to biological additives, sophisticated molecular techniques are now available and are becoming more widely used. Polymerase chain reaction assays, denaturing gradient gel electrophoresis, and temperature gradient gel electrophoresis offer approaches for detecting microbial shifts in the gut. Likewise, the employment of microarrays and molecular analysis of gut tissues can reveal insight into gut physiological and responses to dietary and other changes. Recent application of 16S rDNA sequencing and analysis utilizing basic local alignment search tool (BLAST) and FASTA databases on poultry gut samples have the potential to provide a much more in-depth assessment of the gut microbiome. Utilizing ultra pressure liquid chromatography-mass spectroscopy profiling, metabolomic assessment of gut contents will also allow for parallel comparisons of changes in the gut contents with microbiome and physiological responses. Combining all these technologies will provide a plenary understanding of poultry gut health in alternative production systems.

Key words: alternatively raised poultry, prebiotics, foodborne pathogen, gastrointestinal ecosystem, genomics

2013 Poultry Science 92:546–561
http://dx.doi.org/10.3382/ps.2012-02734

INTRODUCTION

Consumer demand for organic and natural poultry products continues to increase in the United States because of an ongoing perception that organic or natural products are better than their conventional counterparts in terms of safety, taste, and increased health benefits (Harper and Makatouni, 2002; Van Loo et al., 2012b). The general term organic foods is used to define foods that are produced without using chemical fertilizers, additives, and synthetic pesticides as well as not being processed with irradiation (DeSoucey, 2007). Among organic foods, the overall organic meat market size is small compared with the conventional meat industries in the United States. However, according to the Organic Trade Association, the organic meat industry has grown $29 billion in 2010 compared with $3.6 billion in 1997 (OTA, 2011). Specifically, organic poultry broiler production has increased from 1.9 million in 2000 to more than 10.4 million broilers in 2005 and the numbers of laying hens have increased from 1.1 million in 2000 to 2.4 million in 2005 (ERS, 2010).

Received August 30, 2012.
Accepted October 27, 2012.
[1]Presented as part of the Next Generation Sequencing: Applications for Food Safety and Poultry Production Symposium at the Poultry Science Association's annual meeting in Athens, Georgia, July 10, 2012.
[2]Corresponding author: sricke@uark.edu

11.2. Copyright for Figures in Chapter 1

ASM PRESS LICENSE
TERMS AND CONDITIONS

Jan 15, 2013

This is a License Agreement between SI HONG PARK ("You") and ASM Press ("ASM Press") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by ASM Press, and the payment terms and conditions.

License Number	3070410053146
License date	Jan 15, 2013
Licensed content publisher	ASM Press
Licensed content publication	ASM Book
Licensed content title	Bacteriophages in the Control of Food- and Waterborne Pathogens
Licensed content author	Editors: Parviz M. Sabour and Mansel W. Griffiths
Licensed content date	August 2010
Type of Use	Journal
Requestor type	academic/educational
Format	electronic
Portion	figures/tables/images
Number of figures/tables/images	2
Figure number(s) you are reusing from this book	Figure 2 (P. 16) and Figure 3 (P. 19) in chapter 2
Will you be translating?	No
Order reference number	
Title of new article	Bacteriophage: Alternatives for Control of Foodborne Pathogens
Publication the new article is in	Research Collection of Institute of Life Sciences & Resources
Publisher of new article	Institute of Life Sciences & Resources, Kyung Hee University
Author of new article	Si Hong Park, Steven C. Ricke, and Hae-Yeong Kim
Expected publication date of new article	Jan 2013
Estimated size of new article (pages)	5
Billing Type	Credit Card
Credit card info	Visa ending in 6277
Credit card expiration	10/2014
Total	172.50 USD
Terms and Conditions	

ASM Press Terms and Conditions for Rightslink Licenses

11.3. Do Not Need Copyright for Published Paper in Chapter 1

Rights and Permissions

Permissions requests are processed through the Copyright Clearance Center. [click here to request permission]

General Information about Permissions

Written permission to republish whole or parts of works owned by the Poultry Science Association must be obtained prior to publication. Please do not contact the authors initially for permission, instead request permission from the Copyright Clearance Center. Permissions fees or other charges are calculated based on intended use and number of reproductions.

Authors requesting permission to use their own material

Without express permission, authors may do the following after their work has been published by *Poultry Science*.

- Present the work orally (partially or in its entirety) for noncommercial, educational purposes.
- Use the article (or parts of) in book you edit or chapter you author.
- Use the article in a thesis or dissertation.
- Posting articles on institutional repositories is prohibited.

11.4. Authorship Statement for Chapter 1

Si Hong Park is the first author of the paper and has written at least 51% of the paper among coauthors which the title is "**Modifying the Gastrointestinal Ecology in Alternatively Raised Poultry and the Potential for Molecular Assessment**" in chapter 1.

Major Advisor: Dr. Steven C. Ricke

Date: June 4th, 2013

CHAPTER 2

Multiplex Polymerase Chain Reaction Assay for Detection and Quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* Serotypes in Water Samples

Si Hong Park[1,2], Irene Hanning[2], Robin Jarquin[3], Philip Moore Jr[4], Dan J. Donoghue[1,3], Ann M. Donoghue[1,4] and Steven C. Ricke[1,2,3*]

[1]Cell and Molecular Biology Program, Department of Food Science, University of Arkansas, Fayetteville, AR

[2]Center for Food Safety-IFSE and Department of Food Science, University of Arkansas, Fayetteville, AR

[3]Department of Poultry Science, University of Arkansas, Fayetteville, AR

[4]Poultry Production and Poultry Safety Research Unit, Agricultural Research Services, USDA, Fayetteville, AR

FEMS Microbiology Letters 316: 7-15, 2011

1. Abstract

Three pathogens, *Campylobacter*, *Salmonella*, and shiga-toxin producing *Escherichia coli* (STEC) are leading causes of bacterial gastroenteritis in the United States and worldwide. Although these three bacteria are typically considered foodborne pathogens, outbreaks have been reported due to contaminated drinking water and irrigation water. The aim of this research was to develop two types of PCR assays that could detect and quantify three pathogens, *Campylobacter* spp., *E. coli* O157:H7, and *Salmonella* spp. in watershed samples. In conventional PCR, three target strains were detected by multiplex PCR using each specific primer pairs simultaneously. Under optimized multiplex PCR conditions, the assay produced a 90-bp product for *Campylobacter jejuni*, a 150-bp product for *E. coli* O157:H7, and a 262-bp product for *Salmonella* Typhimurium and the limitation of detection was approximately 700 copies for all three bacteria. In addition, real-time PCR was performed to quantify the three pathogens using SYBR green fluorescence. The assay was designed so that each target had a different melting temperature (*C. jejuni* (80.1˚C), *E. coli* O157:H7 (83.3˚C), and *S.* Typhimurium (85.9˚C)). Therefore, this system could quantify and distinguish three pathogens simultaneously in a single reaction.

2. Introduction

Three pathogens, *Campylobacter* spp., shiga toxin-producing *Escherichia coli* (STEC), and *Salmonella* spp. are leading causes of bacterial gastroenteritis in the United States (US) and worldwide (Shelton *et al*., 2006; Botteldoorn *et al*., 2008; D'Souza *et al*., 2009). *Campylobacter* spp. have been estimated to affect 2.4 million people annually, causing approximately 124 deaths and costing $1.2 to $6 billion (Mead *et al*., 1999; CDC, 2008). *Campylobacter* spp. are responsible for 17% of all hospitalizations related to illness, and although *Campylobacter* spp. have a much lower case fatality rate than *Salmonella* spp. and *E. coli* O157:H7, they account for 5% of food-related deaths (Zhao *et al*., 2001). The Centers for Disease Control estimates 73,000 cases of *E. coli* O157 STEC infections occur annually and are transmitted by food or other vehicles (Rangel *et al*., 2005). The annual cost of this disease is estimated at $405 million in terms of premature death, medical care and lost productivity. In the US, disease caused by an estimated 1.4 million non-typhoidal *Salmonella* spp. infections (Rabsch *et al*., 2001), resulted in 168,000 visits to physicians, 15,000 hospitalizations and 580 deaths annually in the US. The total cost associated with illnesses due to *Salmonella* spp. infection is estimated at $3 billion annually in the US. (Faúndez *et al*., 2004).

These pathogens can inhabit the gastrointestinal tract of agricultural animals, including cattle, swine and poultry, as commensals without causing any signs or symptoms of disease in the animals. While inhabiting the gastrointestinal tract, pathogens can be shed into the environment and may subsequently contaminate water sources (Topp *et al*., 2009). Other animals including wild birds, rodents, reptiles, amphibians, and deer can carry and shed these pathogens into water sources as well (Pasmans *et al*., 2008; Pickering *et al*., 2008). Feces from birds and animals, including cattle, contaminated with *Campylobacter* spp. have been detected in surface

water supplies used as drinking water sources (Bopp *et al*., 2003). In addition, sewage leaks into ground water have led to contamination of drinking water and outbreaks of *Salmonella* spp. and *Campylobacter* spp. gastroenteritis (O'Reilly *et al*., 2007).

Although these three pathogens are typically considered food-borne, outbreaks have been reported due to contaminated drinking water. An estimated 20% of cases of illness caused by *C. jejuni* and 15% of salmonellosis cases are due to vehicles of infection other than food, including water (Mead *et al*., 1999). In many rural areas, water derived from groundwater may be the only practical source of drinking water (Pedley and Howard, 1997) and rural waterborne disease outbreaks have been associated with contaminated groundwater (Clark *et al*., 2003; Kussi *et al*., 2004**)**. All three pathogens have been associated with large waterborne outbreaks in North America territory (Bopp *et al*., 2003; Clark *et al*., 2003; O'Reilly *et al*., 2007). Considering the large impact that these three pathogens have on the health of humans, it is important to prevent potential illnesses. Given that water can be a source of these pathogens either directly (drinking water) or indirectly (irrigation water), prevention of illnesses could be accomplished by consistent monitoring of water supplies. Detection of bacteria in water samples can be complicated by factors such as fecal inhibitors of nucleic acid based detection assays (Loge *et al*., 2002), viable but non-culturable bacteria (Leskinen and Lim, 2008), inhibitors from soil suspension in water samples (Juen and Traugott, 2006), and low quantities of cells requiring a large volume of sample. The aim of this research was to develop multiplex PCR and real-time PCR assays that could simultaneously detect and quantify three pathogens, *Campylobacter* spp., Enterohemorrhagic *E. coli* (EHEC), and *Salmonella* spp. in a single reaction. Methods to overcome factors that inhibit analysis of samples were also addressed.

3. Materials and Methods

3.1. Bacteria and Culturing Conditions

For development and optimization of the two PCR assays, *Campylobacter jejuni* NCTC 11168, *Escherichia coli* O157:H7 American Type Culture Collection (ATCC) 43888, and *Salmonella enterica* Typhimurium ATCC 14028 were used. *Campylobacter jejuni* was cultured on *Campylobacter* enrichment agar (Acumedia Manufacturers Inc, Lansing, MI, USA) and incubated at 42˚C for 48 h under microaerophilic (5% O_2, 10% CO_2, and 85% N_2). Both *E. coli* O157:H7 and *S.* Typhimurium were cultured on Tryptic Soy Agar (EMD Chemicals Inc, Gibbstown, NJ, USA) and plates were incubated at 37˚C for 24 h. In addition 14 strains of bacteria were used to qualify the specificity of the primer pairs (Table 1), and were cultured on the appropriate media and under the appropriate growth conditions.

3.2. DNA Extraction for multiplex PCR and Real-Time PCR Assays

Freshly cultured cells were collected from an agar plate with a sterile loop and suspended in 2 mL of Phosphate Buffered Saline (PBS), pH 7.4. Of the 2 mL suspension, 100 μL was utilized for a dilution series to enumerate the cells in suspension. One mL of each cell suspension was subsequently frozen at -20˚C. After samples were firmly frozen (at least 1 h), genomic DNA was extracted from the samples first by thawing frozen samples at room temperature. The samples were centrifuged at 16,000 x g for 3 min and 900 μL of the supernatant was discarded. After vortexing the samples were boiled in a water bath for 10 min and subsequently refrigerated at 4˚C for 10 min. Finally, samples were centrifuged at 16,000 x g for 2 min and 100 μL of the supernatant was used as template DNA. All samples were immediately used for multiplex and real-time PCR assays after preparation.

3.3. Conventional Multiplex PCR Assay

The PCR assay was optimized using an MJ PTC 100 thermocycler (Bio-Rad, Hercules, CA, USA). Primer sets for the PCR assay are listed in Table 2. All primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). The reactions resulted in a 90-bp fragment for *C. jejuni*, a 150-bp fragment for *E. coli* O157:H7 (Sharma *et al*., 1999), and a 262-bp fragment for *S.* Typhimurium (Cheng *et al*., 2008). The *Campylobacter* spp. primers were designed by targeting a conserved region of the *hsp60* gene. Reactions specific for each pathogen were first done independently and each reaction consisted of a 25 μL total volume mixture with 12.5 μL of SYBR Green Premix Ex Taq^{TM} (Takara, Fisher Scientific, Pittsburg, PA, USA), 800 nM of each primer, 1.6 μL of bovine serum albumin (BSA, 20 mg mL^{-1}), 1 μL of DNA template and water to volume. After each PCR reaction was optimized independently, a multiplex PCR reaction was optimized to detect all three pathogens simultaneously and three independent experiments were performed to verify the reproducibility. The multiplex PCR reaction consisted of 25 μL total volume mixture with 12.5 μL of SYBR Green Premix Ex Taq^{TM} (Takara, Fisher Scientific, Pittsburg, PA, USA), 400 nM of *Campylobacter* spp.-specific primers, 400 nM of *E. coli* O157:H7-specific primers, 960 nM of *Salmonella* spp.-specific primers, 1.6 μL of BSA (20 mg mL^{-1}), 3 μL of three DNA template and water to volume. The PCR reaction was optimized to conditions of 94˚C for 2 min. then 35 cycles of 94˚C for 30 s, 55˚C for 30 s and 72˚C for 30 s with a final extension cycle at 72˚C for 5 min. The PCR products were separated in a 2% agarose gel at 100 V for 20 min. Gels were stained with ethidium bromide (10 mg mL^{-1}) and viewed with a UV transilluminator.

3.4. SYBR Green Real-Time PCR Assay

The SYBR green real-time PCR assay was optimized using an Eppendorf Masterplex thermocycler ep (Eppendorf, Westbury, NY, USA). Gradient Technology in the Eppendorf unit was used to optimize annealing and extension temperatures and times. Real-time PCR assays were conducted as three independent experiments and triplicate samples per each experiment. The same primer sets utilized for conventional PCR, listed in Table 1, were also used for the SYBR green real-time PCR reaction. A 25 μL total volume reaction mixture consisted of 12.5 μL of SYBR Green Premix Ex Taq^{TM} (Takara, Fisher Scientific, Pittsburg, PA, USA) 800 nM of each primer, 1.6 μL of BSA (20 mg mL^{-1}), 1 μL of DNA template and water to volume. The PCR reaction was optimized to the conditions of 95˚C for 2 min. followed by 40 cycles of 95˚C for 15 s, 55˚C for 15 s and 68˚C for 20 s with fluorescence being measured during the extension phase. Melting curves were subsequently done and consisted of 95˚C for 15 s, 60˚C for 20 minutes increasing in 0.5˚C increments to 95˚C. The real time PCR results were confirmed further through agarose gel electrophoresis.

3.5. Construction of SYBR Green Real-Time PCR Standard Curve

To create the standard curve for the SYBR green real-time PCR assay, serial dilutions of DNA were prepared from DNA of *C. jejuni*, *E. coli* O157:H7 and *S.* Typhimurium as described in the previous section. The 10-fold serial dilutions of three independent experiments were used to determine the initial starting concentration of cells and template DNA copy numbers. The fluorescence along with the DNA template number results were used to construct a linear curve that correlated the first cycle number at which fluorescence was detected to the number of cells per mL. For each reaction, the threshold cycle number (Ct) was determined to be the cycle number at which fluorescence was greater than 400 of fluorescence units. The efficiency of the

reactions were calculated with the formula $E=10^{(-1/slope)}$ -1. Melting curves were created and analyzed with the Eppendorf realplex software (version 2.0).

3.6. Spiking and Analysis of Watershed Samples

Watershed samples were collected on 10 occasions and prepared as previously described (Metcalf *et al*., 2009). All samples were analyzed for the presence of *Campylobacter* spp., *E. coli* O157:H7, and *Salmonella* spp. using conventional plating techniques. To spike watershed samples for analysis, 2 mL of a cell suspension in PBS was prepared. Of the 2 mL suspension, 100 µL was utilized for a dilution series to enumerate cells in each suspension. One milliliter of the cells then was pelleted by centrifugation at 16,000 x g for 2 min. The supernatant was discarded and the cells were resuspended in 10 mL of watershed sample, and 1 mL aliquots were made. The cell suspensions were frozen at -20˚C and DNA was extracted in the same manner as described for cells suspended in PBS as well as stored at 4˚C for 7 days to confirm viability difference according to storage period and conventional plating methods were employed as three independent experiments. All PCR assays also were performed using the spiked watershed samples. The reaction components were the same with the exception of the addition of 1.6 µL of BSA (20 mg mL^{-1}).

4. Results

4.1. Primer Specificity and Sensitivity

To evaluate the specificity of three primer pairs used in this study, 22 strains were selected including target microorganisms (Table 1). *Campylobacter* spp.-specific primer pairs were synthesized using *hsp60* gene to fit multiplex PCR conditions and the other two primer

pairs were adopted from previous reports (Sharma *et al*., 1999; Cheng *et al*., 2008). Although each primer pair showed high specificity for target bacteria in a uniplex PCR, primer dimers caused by *Salmonella* spp.-specific primers emerged with a low concentration of template DNA in the multiplex PCR and real-time PCR.

4.2. Optimization of Multiplex PCR

In this study, the concentrations of the three primer pairs were adjusted to yield similar band intensities; 400 nM of *Campylobacter* spp.-specific primers, 400 nM of *E. coli* O157:H7-specific primers, 960 nM of *Salmonella* spp.-specific primers. Under this optimized multiplex PCR condition, three types of PCR were performed; uniplex (Fig. 1, lanes 1-3), duplex (Fig. 1, lanes 4-6), and triplex (Fig.1, lane 7). Each PCR results exhibited high specificity and sensitivity of target products and the amplicon size was the same as the expected value.

4.3. Detection Limits of Multiplex PCR in Pure Culture

Each target genomic DNA was prepared from 1 ml of pure culture bacteria containing 7.33×10^7 copies, and was diluted 10-fold until 7.33×10^0 copies. In a uniplex PCR, the *Campylobacter* spp.-specific primer pair was more sensitive than the other two primer pairs in detecting target microorganisms. The detection limit of *C. jejuni* was 7.33×10^1 copies, while *E. coli* O157:H7, and *S.* Typhimurium were 7.33×10^2 copies in pure culture samples (Table 3). In contrast to uniplex PCR, multiplex PCR showed detection limits of 7.33×10^3 copies in mixed culture sample detection of the three bacteria due to primer competition as well as dimer formation (Fig. 2-A) and all results were based on triplicate experiments.

4.4. Application of Multiplex PCR to Spiking Watershed Samples

Watershed samples were collected from a local farm and analyzed using traditional selective media to confirm whether samples were contaminated naturally. Samples were aliquoted and analyzed immediately by conventional plate method and PCR and also analyzed after 7 days storage at 4˚C. By conventional plating, the number of *C. jejuni*, *E. coli* O157:H7, and *S.* Typhimurium in samples stored for 7 days decreased by 1 to 2 logs compared to initial inoculation levels (Table 4). *C. jejuni* was reduced from 5.3×10^9 CFU mL^{-1} to 2.2×10^7 CFU mL^{-1}, *E. coli* O157:H7 was reduced from 9.3×10^8 CFU mL^{-1} to 6.7×10^7 CFU mL^{-1}, and *S.* Typhimurium was reduced from 3.2×10^9 CFU mL^{-1} to 4.3×10^8 CFU mL^{-1} (Table 4).

To evaluate multiplex PCR assay, different concentrations of each bacteria were inoculated into the watershed samples; 0-day samples of *C. jejuni* contained 5.3×10^9 to 5.3×10^2 CFU mL^{-1}, *E. coli* O157:H7 contained 9.3×10^8 to 9.3×10^1 CFU mL^{-1}, *S.* Typhimurium contained 3.2×10^9 to 3.2×10^2 CFU mL^{-1} and 7 day samples (*C. jejuni* (2.2×10^7 to 2.2×10^0 CFU mL^{-1}), *E. coli* O157:H7 (6.7×10^7 to 6.7×10^0 CFU mL^{-1}), *S.* Typhimurium (4.3×10^8 to 4.3×10^1 CFU mL^{-1})). Uniplex and multiplex PCR results showed that there was no obvious difference between 0 and 7-days samples (Fig. 2-B, C) in detection limitation. Only the detection limitation of *C. jejuni* was decreased by 4 fold in a uniplex PCR (data not shown).

4.5. Real-Time PCR for Standard Curve Using Pure Culture DNA

Purified genomic DNA of *C. jejuni*, *E. coli* O157:H7, and *S.* Typhimurium were used to design standard curves and the calculated DNA copy numbers ranged from 7.33×10^7 copy μL^{-1} to 7.33×10^1 copy μL^{-1}. Only the *C. jejuni* standard curve could be constructed to start at 7.33×10^0 copy μL^{-1} due to high sensitivity of primer pair. As a result, the lowest copy number was

determined as the detection limit in pure culture DNA for each bacterium. The melting temperature of *C. jejuni* was approximately 80.1˚C, *E. coli* O157:H7 was 83.3˚C, and *S.* Typhimurium was 85.9˚C, respectively. The *Salmonella* spp.-specific primer pair dimer exhibited a melting temperature peak at 76.5˚C at low template concentrations, but this did not influence identification of target products.

4.6. Application of Real-Time PCR to Watershed Samples

Both 0- and 7-day samples were analyzed three times through independent experiments. Each bacterium cell number was calculated based on standard plate count method that was averaged among the three plates. In 0 day samples, the detection limits of the SYBR green real-time PCR assay were determined by using the threshold (Ct) values from three independent reactions. For *C. jejuni*, the assay detected 53 CFU ml^{-1}. For *E. coli* O157:H7, the assay could detect 93 CFU ml^{-1}. For *S.* Typhimurium, the assay detected 3,200 CFU ml^{-1} (Table 5). In 7-day samples, the detection limit of *C. jejuni* was 2.2 CFU ml^{-1}, *E. coli* O157:H7 was 67 CFU ml^{-1}, and *S.* Typhimurium was 430 CFU ml^{-1} (Table 5). The Ct values of each bacterium are shown in table 5 and these values were averaged from three independent experiments. The melting temperatures of the amplicons for *C. jejuni*, *E. coli* O157:H7, and *S.* Typhimurium were the same for spiked watershed samples and pure cultures in PBS; *C. jejuni* was 80.1˚C, *E. coli* O157:H7 was 83.3˚C, and *S.* Typhimurium was 85.9˚C, respectively (Fig. 3). The differences of melting temperatures allowed more specific identification of the three bacteria.

5. Discussion

Numerous types of media have been developed to enumerate microorganisms including pathogens important to the food industry. Selective media for pathogens has been useful to detect viable cells associated with human illnesses in food matrices (Gracias and Mckillip, 2004). Although culture based methods have been used traditionally and are employed widely, there are many limitations such as length of time (minimum of 24 h), false-negative results and the necessity for conformational assays (Gracias and Mckillip, 2004; Cheng *et al*., 2008). In addition, pre-enrichment steps are necessary to recover stressed and injured cells. Accurate quantification of *Salmonella* spp. by plating from watershed samples was not possible in these experiments because direct plating would underestimate the true cell concentration due to the inability to recover injured, stressed cells (Gracias and Mckillip, 2004). Furthermore, because enrichment is necessary to detect these populations, quantification from enriched samples would result in gross overestimation of the actual concentration of cells (O'Leary *et al*., 2009).

To overcome culturing limitations, molecular approaches have been prepared as a means to identify and quantify the pathogens rapidly and accurately. Molecular methods that have been developed and modified accordingly to detect and quantify pathogens simultaneously using DNA include multiplex PCR (m-PCR) and quantitative real-time PCR (qPCR). The m-PCR approach has utility for identifying subspecies in genus among unknown mixed samples in a single reaction while qPCR has been developed to quantify DNA with high accuracy and sensitivity from environmental water samples (Fey *et al*., 2004). In addition, qPCR using SYBR green fluorescence is more convenient and economical than a primer. In this study, m-PCR and qPCR assays were optimized to analyze watershed samples, because m-PCR has the advantage of identifying three pathogens simultaneously in a single reaction and utilize qPCR for quantifying the pathogens.

Both culturing and qPCR detected a reduction of viable cells after 7 days in spiked watershed samples. This implies that 4˚C was biocidal to the pathogens (Mizunoe *et al*., 1999; Matches and Liston 2006), especially *C. jejuni*, which is more sensitive to low temperatures than the other two pathogens (Chan *et al*., 2001). The difference in viable cells at 0 and 7 day in spiked watershed samples did not change the detection limit of m-PCR, because the visible PCR amplicons on agarose gel are limited to detecting 5 ng or more of DNA. However, after the watershed samples were spiked, the sensitivity of the qPCR assay increased after samples were stored at 4˚C for 7 days (Table 5) because the DNA of nonviable cells was detected. The discrepancy between plating and qPCR may be result of genomic DNA from nonviable cells being detected.

An inability to distinguish between viable and non-viable cells has been a criticism of DNA- based detection methods. To alleviate this problem, mRNA was isolated from total RNA and used in the PCR method. However, several limitations have been emerged in application of mRNA to these assays. The short life span due to rapid degradation, the instability of mRNA, the difficulty of recovery, and increased assay time all result in a reduction in the accuracy of quantification (Guy *et al*., 2006).

In this study, genomic DNAs were prepared from samples using a boiling method without a clean-up step in order to conserve DNA. Although purifying DNAs through a column would reduce PCR inhibitors, a loss of template DNA would reduce the PCR assay sensitivity. Deletion of PCR inhibitors is crucial to increase PCR sensitivity and specificity. Chemicals including tannic, humic, fulvic acids and acidic plant polysaccharides derived from plant are plentiful in natural water and can inhibit the *Taq* polymerase binding affinity (Kreader, 1996; Demeke and Jenkins, 2010). Bovine serum albumin (BSA) has been used extensively to break

down many substances binding lipids by hydrophobic reaction and anions due to its high lysine content, thus preventing the interference of inhibitors with PCR as well as preserving *Taq* polymerase activation (Kreader, 1996). In this study, we found the addition of BSA to our spiked watershed samples reduced inhibitors and allowed the assay to be as sensitive as the pure bacterial cultures samples prepared in PBS.

The molecular assays developed in this research provide several advantages over currently published methods. The time to detect and identify the three pathogens was reduced from 48 hours with culturing to just 4 hours with the m-PCR. To the best of our knowledge, this is the first m-PCR method published to detect *Campylobacter*, *E. coli* O157:H7 and *Salmonella* simultaneously from watershed samples. The m-PCR assay allowed less time and reagents to be used. Because quantification with plating was not possible with these watershed samples, the qRT-PCR method reported here allows pathogens to be quantified rapidly and accurately. Inhibitors present in water and soils are both present in watershed run-off and our method was optimized so that the assay was just as sensitive as using pure cultures in PBS.

6. Acknowledgment

This research was funded by a USDA National Research Initiative (NRI) grant #6226-63000-001-16.- awarded to Phillip Moore, Ann Donoghue, Dan Donoghue, Steven C. Ricke, and Andrew Sharpley.

7. References

Bopp DJ, Sauders BD, Waring AL *et al*. (2003) Detection, isolation, and molecular subtyping of *Escherichia coli* O157:H7 and *Campylobacter jejuni* associated with a large waterborne outbreak. *J Clin Microbio*l **41**: 174-180.

Botteldoorn N, Coillie EV, Piessens V, Rasschaert G, Debruyne L, Heyndrickx M, Herman L & Messens W (2008) Quantification of *Campylobacter* spp. in chicken carcass rinse by real-time PCR. *J Appl Microbiol* **105**: 1909-1918.

Centers for Disease Control and Prevention (CDC) (2008) *Campylobacter* general information (www.cdc.gov/nczved/divisions/dfbmd/diseases/campylobacter/technical.html.).

Chan KF, Tran HL, Kanenaka RY & Kathariou S (2001) Survival of clinical and poultry-derived isolates of *Campylobacter jejuni* at a low temperature (4˚C). *Appl Environ Microbiol* **67**: 4186-4191.

Cheng CM, Lin W, Van KT, Phan L, Tran NN & Farmer D (2008) Rapid detection of *Salmonella* in foods using real-time PCR. *J Food Protect* **71**: 2436-2441.

Clark CG, Price L, Ahmed R, Woodward DL, Melito PL, Rodgers FG, Jamieson F, Ciebin B, Li A & Ellis A (2003) Characterization of waterborne outbreak-associated *Campylobacter jejuni*, Walerton, Ontario. *Emerg Infect Dis* **9**: 1232-1241.

Demeke T & Jenkins GR (2010) Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. *Anal Bioanal Chem* **396**: 1977-1990.

D'Souza DH, Critzer FJ & Golden DA (2009) Real-time reverse-transcriptase polymerase chain reaction for the rapid detection of *Salmonella* using *invA* primers. *Foodborne Pathog Dis* **6**: 1097-1106.

Faúndez G, Troncoso M, Navarrete P & Figueroa G (2004) Antimicrobial activity of copper surfaces against suspensions of *Salmonella* enterica and *Campylobacter jejuni*. *BMC Microbiol* **4**: 19.

Fey A, Eichler S, Flavier S, Christen R, Höfle MG & Guzmán CA (2004) Establishment of a real-time PCR-based approach for accurate quantification of bacterial RNA targets in water, using *Salmonella* as a model organism. *Appl Environ Microbiol* **70**: 3618-3623.

Gracias KS & Mckillip JL (2004) A review of conventional detection and enumeration methods for pathogenic bacteria in food. *Can J Microbiol* **50**: 883-890.

Guy RA, Kapoor A, Holicka J, Shepherd D & Horgen PA (2006) A rapid molecular-based assay for direct quantification of viable bacteria in slaughterhouses. *J Food Protect* **69**: 1265-1272.

Juen A & Traugott M (2006) Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. *Soil Biol Biochem* **38**: 1872-1879.

Kreader CA (1996) Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl Environ Microbiol* **62**: 1102-1106.

Kussi M, Klemets P, Miettinen I, Laaksonen I, Sarkkinen H, Hanninen ML, Rautelin H, Kela E & Nutri JP (2004) An outbreak of gastroenteritis from a non-chlorinated community water supply. *J Epidemiol Community Health* **58**: 273-277.

Leskinen SD & Lim DV (2008) Rapid ultrafiltration concentration and biosensor detection of Enterococci from large volumes of Florida recreational water. *Appl Environ Microbiol* **74**: 4792-4798.

Loge FJ, Thompson DE & Call DR (2002) PCR detection of specific pathogens in water: A risk-based analysis. *Environ Sci Technol* **36**: 2754-2759.

Matches JR & Liston J (1968) Low temperature growth of *Salmonella*. *J Food Sci* **33**: 641-645.

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM & Tauxe RV (1999) Food-related illness and death in the United States. *Emerg Infect Dis* **5**: 607-625.

Metcalf JH, Reyes-Herrera I, Blore P, Moore Jr PA, Donoghue AM, Hanning I, Ricke SC & Donoghue DJ (2009) Development and validation of methods for the recovery of *Salmonella* and *Campylobacter* from watershed runoff after poultry litter application. 2009 Poultry Science Association (PSA) Annual Meeting Abstract, 86-87.

Mizunoe Y, Wai SN, Takade A & Yoshida S (1999) Restoration of culturability of starvation-stressed and low-temperature-stressed *Escherichia coli* O157 cells by using H_2O_2-degrading compounds. *Arch Microbiol* **172**: 63-67.

O'Leary J, Corcoran D & Lucey B (2009) Comparison of the EntericBio multiplex PCR system with routine culture for detection of bacterial enteric pathogens. *J Clin Microbiol* **47**: 3449-3453.

O'Reilly CE, Bowen AB, Perez NE *et al*. (2007) A waterborne outbreak of gastroenteritis with multiple etiologies among resort island visitors and residents: Ohio, 2004. *Clin Infect Dis* **44**: 506-512.

Pasmans F, Blahak S, Martel A & Pantchev N (2008) Introducing reptiles into a captive collection: The role of the veterinarian. *Vet J* **175**: 53-68.

Pedley S & Howard G (1997) The public health implications of microbiological contamination of groundwater. *Q J Eng Geo* **30**: 179-188.

Pickering LK, Marano N, Bocchini JA & Angulo FJ (2008) Exposure to nontraditional pets at home and to animals in public settings: Risks to children. *Pediatrics* **122**: 876-886.

Rabsch W, Tschäpe H & Bäumler AJ (2001) Non-typhoidal salmonellosis: emerging problems. *Microbes Infect* **3**: 237-247.

Rangel JM, Sparling PH, Crowe C, Griffin PM & Swerdlow DL (2005) Epidemiology of *Escherchia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerg Infect Dis* **11**: 603-609.

Sharma VK, Dean-Nvstrom EA & Casey TA (1999) Semi-automated fluorogenic PCR assays (Taqman) for rapid detection of *Escherichia coli* O157:H7 and other shiga toxigenic *E. coli*. *Mol Cell Probes* **13**: 291-302.

Shelton DR, Karns JS, Higgins JA, Kessel JASV, Perdue ML, Belt KT, Russell-Anelli J & DebRoy C (2006) Impact of microbial diversity on rapid detection of enterohemorrhagic *Escherichia coli* in surface waters. *FEMS Microbiol Lett* **261**: 95-101.

Topp E, Scott A, Laper DR, Lyautey E & Duriez P (2009) Livestock waste treatment systems for reducing environmental exposure to hazardous enteric pathogens: Some considerations. *Bioresour Technol* **100**: 5395-5398.

Zhao C, Ge B, Villena JD, Sudler R, Yeh E, Zhao S, White DG, Wagner D & Meng J (2001) Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the greater Washington, D.C., area. *Appl Environ Microbiol* **67**: 5431-5436.

Table 1. Confirmation of each specific primer pair

Species	PCR results		
	C[#]	E*	S[†]
Campylobacter jejuni NCTC 11168	+[§]	-[ʃ]	-
Campylobacter coli	+	-	-
Escherichia coli ATCC 25922	-	+	-
Escherichia coli O157:H7 ATCC 43888	-	+	-
Salmonella Typhimurium ATCC 14028	-	-	+
Salmonella Heidelberg	-	-	+
Salmonella Infantis	-	-	+
Salmonella Montevideo	-	-	+
Bacillus cereus ATCC 10987	-	-	-
Bacillus cereus ATCC 11778	-	-	-
Bacillus licheniformis ATCC 12579	-	-	-
Citrobacter freundii ATCC 11168	-	-	-
Enterobacter aerogenes	-	-	-
Listeria monocytogenes ATCC 15313	-	-	-
Listeria monocytogenes ATCC35152	-	-	-
Listeria innocua ATCC 33090	-	-	-
Listeria grayi ATCC 19120	-	-	-
Listeria ivanovii ATCC 19119	-	-	-
Listeria welshimeri ATCC 35897	-	-	-
Staphylococcus aureus ATCC 25923	-	-	-
Staphylococcus aureus ATCC 6538	-	-	-
Staphylococcus epidermidis ATCC12228	-	-	-

[#]C: *Campylobacter* spp. specific primer, *E: *Escherichia coli* O157:H7 specific primer
[†]S: *Salmonella* spp. specific primer, [§]+: Positive result in PCR, [ʃ]-: Negative result in PCR

Table 2. Sequence of primer pairs used in this study

Species	Primer	Sequence (5'-3')	Amplicon size (bp)	Target gene	Reference
C. jejuni	campsh	CAA GTT GCT ACA ATC TCA GCC A GAT AAC ACC ATC TTT GCC CAC T	90	*hsp60*	This study
E. coli O157:H7	eae150	GGC GGA TTA GAC TTC GGC TA CGT TTT GGC ACT ATT TGC CC	150	*Eae*	Sharma *et al*., 1999
S. Typhimurium	invA3	AAC GTG TTT CCG TGC GTA AT TCC ATC AAA TTA GCG GAG GC	262	*invA*	Cheng *et al*., 2008

Table 3. Detection limits of uniplex and multiplex PCR in watershed samples

Type	Strain	Pure culture (CFU mL^{-1})	Watershed sample (CFU mL^{-1})[#]	
			0-day	7-day*
Uniplex PCR	*C. jejuni*	7.33×10^1	5.3×10^1	2.2×10^2
	E. coli O157:H7	7.33×10^2	9.3×10^2	6.7×10^2
	S. Typhimurium	7.33×10^2	3.2×10^3	4.3×10^3
Multiplex PCR	*C. jejuni*	7.33×10^3	5.3×10^2	2.2×10^2
	E. coli O157:H7	7.33×10^3	9.3×10^4	6.7×10^3
	S. Typhimurium	7.33×10^3	3.2×10^3	4.3×10^3

[#]Watershed sample (CFU mL^{-1}): N=3 for plates, calculated the average on three independent plates, *7-day: Cells from frozen stock

Table 4. The difference in bacterial cell concentrations according to the storage period

Strain	Watershed sample (CFU mL^{-1})[#]	
	0-day (Initial inoculation)	**7-day***
C. jejuni	5.3×10^9	2.2×10^7
E. coli O157:H7	9.3×10^8	6.7×10^7
S. Typhimurium	3.2×10^9	4.3×10^8

[#]Watershed sample (CFU mL^{-1}): N=3 for plates, calculated the average on three independent plates, *7-day: Cells from frozen stock

Table 5. Sensitivity of SYBR green real-time PCR for detection of *C. jejuni*, *E. coli* O157:H7, and *S.* Typhimurium in watershed samples at 0- and 7-days

Strain	0-day		7-day[#]	
	CFU*	Ct value (mean±SD[†])	CFU	Ct value (mean±SD)
C. jejuni	5.3×10^1	31.95 ± 0.32	2.2×10^0	27.42 ± 0.45
	5.3×10^2	28.50 ± 0.14	2.2×10^1	23.96 ± 0.06
	5.3×10^3	24.57 ± 0.27	2.2×10^2	20.89 ± 0.23
	5.3×10^4	21.02 ± 0.88	2.2×10^3	17.35 ± 0.01
	5.3×10^5	17.54 ± 0.22	2.2×10^4	14.32 ± 0.09
	5.3×10^6	14.53 ± 0.79	2.2×10^5	11.29 ± 0.29
E. coli O157:H7	9.3×10^1	33.26 ± 1.77	6.7×10^0	29.73 ± 0.30
	9.3×10^2	30.91 ± 0.37	6.7×10^1	26.23 ± 0.04
	9.3×10^3	27.73 ± 0.33	6.7×10^2	22.78 ± 0.07
	9.3×10^4	24.56 ± 0.26	6.7×10^3	19.57 ± 0.10
	9.3×10^5	21.46 ± 0.18	6.7×10^4	16.43 ± 0.10
	9.3×10^6	18.71 ± 0.49	6.7×10^5	13.83 ± 0.49
S. Typhimurium	3.2×10^3	29.53 ± 0.57	4.3×10^2	31.61 ± 1.84
	3.2×10^4	25.87 ± 0.03	4.3×10^3	27.58 ± 0.42
	3.2×10^5	22.18 ± 0.29	4.3×10^4	24.18 ± 0.22
	3.2×10^6	18.93 ± 0.05	4.3×10^5	20.88 ± 0.01
	3.2×10^7	16.73 ± 0.12	4.3×10^6	17.66 ± 0.18

[#]7-day: Cells from frozen stock, *CFU; Calculated cell number based on plate count

[†]SD; N=3 for plates and Ct value, standard deviation was calculated using Microsoft Office Excel program

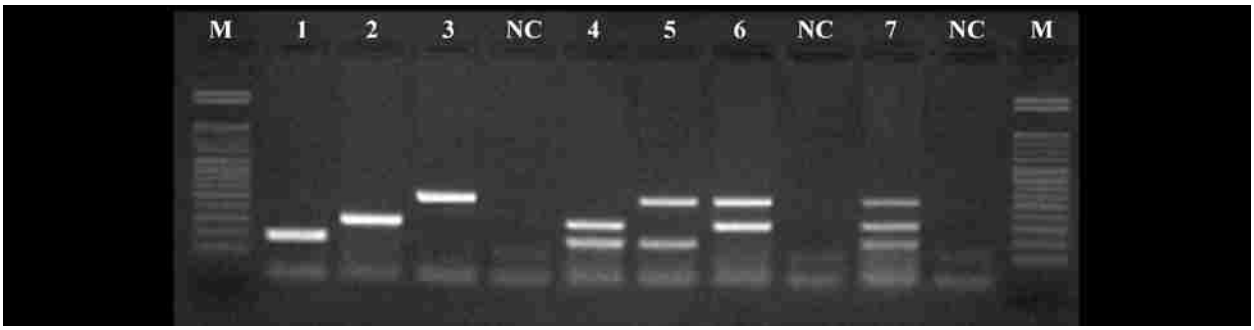


Figure 1. Multiplex PCR products from genomic DNA of *C. jejuni*, *E. coli* O157:H7, and *S.* Typhimurium were electrophoresed in 2% agarose gel at 100 V for 20 min. Lane M showed 50 bp DNA ladder and lane NC was negative control. One of each bacterium was presented in a reaction tube; one PCR amplicon was emerged (lane 1; *C. jejuni*, lane 2; *E. coli* O157:H7, lane 3; *S.* Typhimurium). Two bacteria were presented in a reaction tube; two PCR amplicons were emerged (lane 4; *C. jejuni*, *E. coli* O157:H7, lane5; *C. jejuni*, *S.* Typhimurium, lane 6; *E. coli* O157:H7, *S.* Typhimurium), Three bacteria were presented in a reaction tube; three PCR amplicons were emerged (lane 7; *C. jejuni*, *E. coli* O157:H7, *S.* Typhimurium)

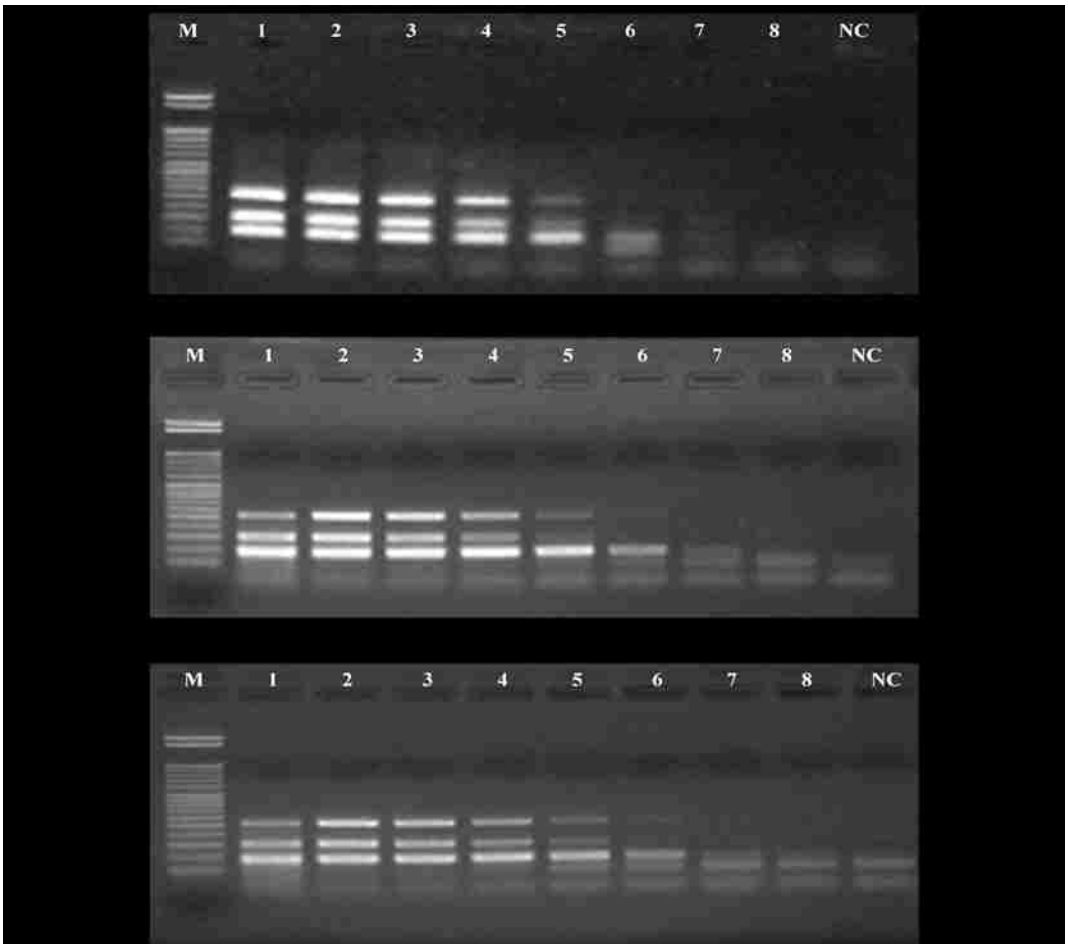


Figure 2. Multiplex PCR products from PBS, 0- and 7-days watershed samples were electrophoresed in 2% agarose gel at 100 V for 20 min to verify the limitation of detection. Lane M showed 50 bp DNA ladder and lane NC was negative control. In a PBS sample (A), lane 1 to 8 represented the genomic DNA range from 7.33×10^7 to 10^0 copies. The inoculation levels of each bacterium in watershed 0-day sample (B) was 5.3×10^9 to 10^2 CFU mL^{-1} for *C. jejuni* (lanes 1 to 8), 9.3×10^8 to 10^1 CFU mL^{-1} for *E. coli* O157:H7 (lanes 1 to 8), and 3.2×10^9 to 10^2 CFU mL^{-1} for *S.* Typhimurium (lanes 1 to 8). The inoculation levels of each bacterium in watershed 7-day sample (C) was 2.2×10^7 to 10^0 CFU mL^{-1} for *C. jejuni* (lanes 1 to 8), 8.2×10^7 to 10^1 CFU mL^{-1} for *E. coli* O157:H7 (lanes 1 to 8), and 4.3×10^8 to 10^1 CFU mL^{-1} for *S.* Typhimurium (lanes 1 to 8).

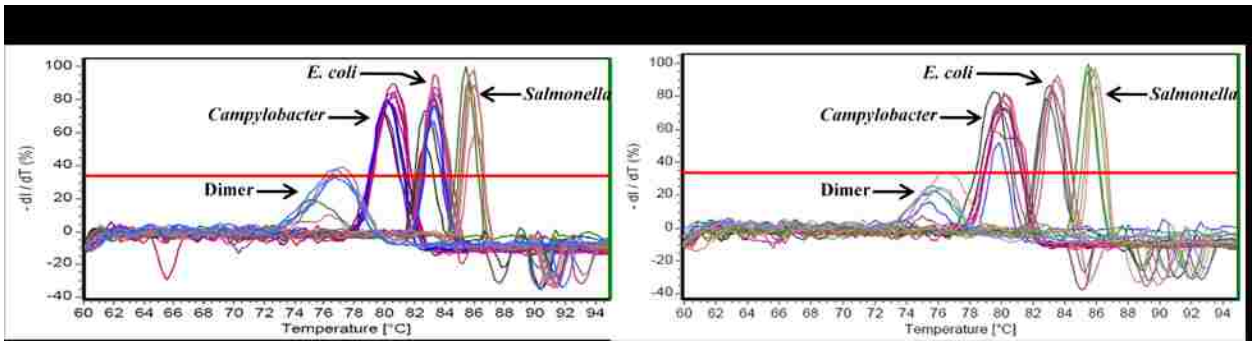


Figure 3. Comparison of melting temperature in real-time PCR between 0- and 7-days (frozen) watershed samples. Each 0- and 7-days sample showed distinct melting temperature, *C. jejuni* was 80.1˚C, *E. coli* O157:H7 was 83.3˚C, and *S.* Typhimurium was 85.9˚C. Also dimer caused by *Salmonella* spp.-specific primers represented peak at 76.5˚C.

8. Appendix

8.1. Chapter 2 Publication by Journal

FEMS MICROBIOLOGY LETTERS

RESEARCH LETTER

Multiplex PCR assay for the detection and quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes in water samples

Si Hong Park[1,2], Irene Hanning[2], Robin Jarquin[3], Philip Moore[4], Dan J. Donoghue[1,3], Ann M. Donoghue[1,4] & Steven C. Ricke[1,2,3]

[1]Cell and Molecular Biology Program, University of Arkansas, Fayetteville, AR, USA; [2]Center for Food Safety–IFSE and Department of Food Science, University of Arkansas, Fayetteville, AR, USA; [3]Department of Poultry Science, University of Arkansas, Fayetteville, AR, USA; and [4]Poultry Production and Poultry Safety Research Unit, Agricultural Research Services, USDA, Fayetteville, AR, USA

Correspondence: Steven C. Ricke, Department of Food Science, University of Arkansas, 2650 North Young Avenue, Fayetteville, AR 72704, USA. Tel.: +1 479 575 4678; fax: +1 479 575 6936; e-mail: sricke@uark.edu

Received 2 August 2010; revised 2 November 2010; accepted 26 November 2010.
Final version published online 14 January 2011.

DOI:10.1111/j.1574-6968.2010.02188.x

Editor: Reggie Lo

Keywords
Campylobacter spp.; *E. coli* O157:H7; *Salmonella* spp.; multiplex PCR; real-time PCR; watershed.

Abstract

Three pathogens, *Campylobacter*, *Salmonella*, and Shiga-toxin-producing *Escherichia coli*, are leading causes of bacterial gastroenteritis in the United States and worldwide. Although these three bacteria are typically considered food-borne pathogens, outbreaks have been reported due to contaminated drinking water and irrigation water. The aim of this research was to develop two types of PCR assays that could detect and quantify three pathogens, *Campylobacter* spp., *E. coli* O157:H7, and *Salmonella* spp., in watershed samples. In conventional PCR, three target strains were detected by multiplex PCR (m-PCR) using each specific primer pair simultaneously. Under optimized m-PCR conditions, the assay produced a 90-bp product for *Campylobacter jejuni*, a 150-bp product for *E. coli* O157:H7, and a 262-bp product for *Salmonella* Typhimurium, and the limitation of detection was approximately 700 copies for all three bacteria. In addition, real-time PCR was performed to quantify the three pathogens using SYBR green fluorescence. The assay was designed so that each target had a different melting temperature [*C. jejuni* (80.1 °C), *E. coli* O157:H7 (83.3 °C), and *S.* Typhimurium (85.9 °C)]. Therefore, this system could quantify and distinguish three pathogens simultaneously in a single reaction.

Introduction

Three pathogens, *Campylobacter* spp., Shiga toxin-producing *Escherichia coli* (STEC), and *Salmonella* spp., are leading causes of bacterial gastroenteritis in the United States and worldwide (Shelton *et al.*, 2006; Botteldoorn *et al.*, 2008; D'Souza *et al.*, 2009). *Campylobacter* spp. have been estimated to affect 2.4 million people annually, causing approximately 124 deaths and costing $1.2–6 billion (Mead *et al.*, 1999; CDC, 2008). *Campylobacter* spp. are responsible for 17% of all hospitalizations related to illness, and although *Campylobacter* spp. have a much lower case fatality rate than *Salmonella* spp. and *E. coli* O157:H7, they account for 5% of food-related deaths (Zhao *et al.*, 2001). The Centers for Disease Control estimates that 73 000 cases of *E. coli* O157 STEC infections occur annually and are transmitted by food or other vehicles (Rangel *et al.*, 2005). The annual cost of this disease is estimated at $405 million in terms of premature death, medical care, and lost productivity. In the United States, disease caused by an estimated 1.4 million nontyphoidal *Salmonella* spp. infections (Rabsch *et al.*, 2001) resulted in 1 68 000 visits to physicians, 15 000 hospitalizations, and 580 deaths annually. The total cost associated with illnesses due to *Salmonella* spp. infection is estimated at $3 billion annually in the United States (Faúndez *et al.*, 2004).

These pathogens can inhabit the gastrointestinal tract of agricultural animals, including cattle, swine, and poultry, as commensals without causing any signs or symptoms of disease in the animals. While inhabiting the gastrointestinal tract, pathogens can be shed into the environment and may subsequently contaminate water sources (Topp *et al.*, 2009). Other animals including wild birds, rodents, reptiles, amphibians, and deer can carry and shed these pathogens into

8.2. Copyright for Published Paper in Chapter 2

JOHN WILEY AND SONS LICENSE
TERMS AND CONDITIONS

Feb 26, 2013

This is a License Agreement between SI HONG PARK ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3096610722866
License date	Feb 26, 2013
Licensed content publisher	John Wiley and Sons
Licensed content publication	FEMS Microbiology Letters
Licensed content title	Multiplex PCR assay for the detection and quantification of Campylobacter spp., Escherichia colia??O157:H7, and Salmonella serotypes in water samples
Licensed copyright line	A?? 2011 Federation of European Microbiological Societies. Published by Blackwell Publishing Ltd. All rights reserved
Licensed content author	Si Hong Park,Irene Hanning,Robin Jarquin,Philip Moore,Dan J. Donoghue,Ann M. Donoghue,Steven C. Ricke
Licensed content date	Jan 14, 2011
Start page	7
End page	15
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Order reference number	
Total	0.00 USD
Terms and Conditions	

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or a society for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at http://myaccount.copyright.com)

8.3. Authorship Statement for Chapter 2

Si Hong Park is the first author of the paper and completed at least 51% of the studies among coauthors which the title is "**Multiplex Polymerase Chain Reaction Assay for Detection and Quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* Serotypes in Water Samples**" in chapter 2.

Major Advisor: Dr. Steven C. Ricke

Date: June 4th, 2013

CHAPTER 3

Development of Novel Multiplex and Quantitative PCR Assays for Simultaneous Detection of *Salmonella* Genus, *Salmonella* Subspecies I, *S*. Enteritidis, *S*. Heidelberg, and *S*. Typhimurium

Si Hong Park[1,2] and Steven C. Ricke[1,2*]

[1]Cell and Molecular Biology Program, Department of Food Science, University of Arkansas, Fayetteville, AR

[2]Center for Food Safety-IFSE and Department of Food Science, University of Arkansas, Fayetteville, AR

1. Abstract

The *Salmonella* genus is divided taxonomically into six *Salmonella enterica* subspecies and *Salmonella bongori*. In general, *Salmonella* subspecies I can cause foodborne diseases, commonly referred to as salmonellosis in humans and animals. The majority of the *Salmonella* serovars involved subspecies I can colonize in the intestinal tracts of humans as well as poultry, and the consumption of contaminated poultry and poultry products is one of the primary sources of human salmonellosis. Thus, strategies for the rapid detection of *Salmonella* serovars in poultry are needed to further reduce the incidence of salmonellosis in humans. The aim of this research was to develop multiplex PCR and quantitative real-time PCR (qPCR) assays that could simultaneously detect *Salmonella* genus, *Salmonella* subspecies I, *S*. Enteritidis, *S*. Heidelberg, and *S*. Typhimurium since these three *Salmonella* serovars can cause disease in humans and are most common isolates associated with poultry and poultry products. Five primer pairs were utilized to establish multiplex PCR and the assay consisted of a 423 bp product for *Salmonella* genus, a 137 bp product for *Salmonella* subspecies I, a 171 bp product for *S*. Enteritidis, a 216 bp product for *S*. Heidelberg, and a 310 bp product for *S*. Typhimurium, respectively. The optimized multiplex PCR technique was applied to 66 *Salmonella* isolates from conventional, organic and pasture flock raised chickens and environmental samples from farm. We also spiked three *Salmonella* strains to chicken breast meats to evaluate the specificity and sensitivity of multiplex PCR as well as qPCR was optimized to quantify *Salmonella* strains in samples. These results confirmed that multiplex PCR and qPCR approaches would provide rapid and consistent results and would be useful for the detection and quantification of *Salmonella* in contaminated poultry, foods and environmental samples.

2. Introduction

The *Salmonella* genus is divided taxonomically into six *Salmonella enterica* subspecies (subsp.) and *Salmonella bongori*. The majority of *Salmonella* serovars involved in *S. enterica* subsp. I can colonize in the intestinal tracts of humans as well as poultry, and cause foodborne diseases in humans, commonly referred to as salmonellosis. Annually, salmonellosis costs an estimated 2.3 billion dollars for medical care costs and loss of productivity (Scallan et al., 2011). Foodborne salmonellosis originating from poultry and poultry products is a major problem and feed continues to be regarded as an important source of contamination in poultry as well as a potential risk to humans (Crump et al., 2002; Maciorowski et al., 2006; Soria et al., 2011). Numerous selective media based methodologies have been examined over the years for counting *Salmonella* in foods but unfortunately they have shown limitations in specificity and sensitivity for accurate detection. Rapid and advanced techniques based on nucleic acids such as PCR have been developed over the last few decades to overcome these disadvantages (Bansal et al., 2006; O'Regan et al., 2008; McCarthy et al., 2009).

A multiplex PCR assay for simultaneous detection and discrimination of various *Salmonella* species in foodstuffs, poultry products and watershed samples was developed and widely utilized among several types of conventional PCR methods (Hong et al., 2009; McCarthy et al., 2009; Park et al., 2009, 2011; Saeki et al., 2013). Since multiplex PCR can amplify specific DNA sequences and discriminate each target strain in a sample simultaneously, we can save considerable time and cost (reduce the total assay time to within 3 h, multiple assays at one time and 60% less cost versus a single PCR-based assay) for each assay. Considering these advantages in development of rapid and accurate detection techniques, a novel multiplex PCR assay is needed to cover the entire spectrum of *Salmonella* possibilities by simultaneously

detecting the general presence of *Salmonella* (universal conserved sequence for entire genus covering 2,579 serovars), a conserved sequence that can detect as a group all *Salmonella* subsp. I (1,531 serovars) which cause diseases in warm blood animals including humans (Grimont and Weill, 2007). Finally, specific sequences for distinguishing each of the three key *Salmonella* serovars (Enteritidis, Heidelberg and Typhimurium) which represent the most common three serovars in the United States and originating from poultry that result in foodborne disease in humans (Vugia et al., 2004). In addition, quantitative real-time PCR (qPCR) has been widely used to detect low copy numbers (10 cells) of *Salmonella* due to much higher sensitivity over conventional PCR methods.

In this study, we developed multiplex PCR and qPCR assays to detect and quantify *Salmonella* in artificially contaminated chicken breast meat. Multiplex PCR was optimized for detection of overall *Salmonella* genus, *Salmonella* subsp. I as a group and each of the three *Salmonella* serovars (Enteritidis, Heidelberg and Typhimurium) individually, and qPCR for quantification of *Salmonella* as low as possible.

3. Materials and Methods

3.1. Bacterial Strains

A total 23 *Salmonella* serovars and 12 non-*Salmonella* pathogens were used in this study to develop multiplex PCR as well as qPCR, and listed in Table 1. *Salmonella* serovars were grown on Tryptic Soy Agar (EMD Chemicals Inc, Gibbstown, NJ, USA) at 37˚C for 24 h and other bacteria were cultivated on appropriate media under optimal growth conditions. One colony of each strain was inoculated to 5 ml of broth and incubated 18 h under appropriate conditions and then, grown cultures were subsequently harvested for DNA extraction.

3.2. DNA Extraction and Primer Specificity

A 3 ml of bacterial cells were collected from overnight grown cultures and extracted genomic DNA using the Qiagen DNeasy Blood Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. The isolated genomic DNA concentration and purity were measured using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) and DNA samples were subsequently stored at -20˚C until used.

A total of five primer pairs were used to establish multiplex PCR in this study and listed in Table 2. Four primer pairs which are specific for *Salmonella* genus, *Salmonella* subsp. I, *S.* Enteritidis *S.* Typhimurium were adopted from previous reports and one primer pair specific for *S.* Heidelberg was constructed in this study to optimze multiplex PCR condition (Bronowski et al., 2009). All primer pairs were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA) and the specificity of each primer pair was evaluated using 23 *Salmonella* serovars and 12 non-*Salmonella* pathogens listed in Table 1.

3.3. Multiplex PCR and Specificity

The novel multiplex PCR assay was optimized using a MJ PTC 100 thermocycler (Bio-Rad, Hercules, CA, USA) and five primer pairs which confirmed the specificity via single PCR. The internal amplification control (IAC) was constructed following previous report (Park et al., 2009) and added to the PCR mixture to confirm the multiplex PCR results. A total of 30 μl of multiplex PCR mixture consisted of 15 μl of Premix Ex Taq^{TM} (Takara, Fisher Scientific, Pittsburg, PA, USA), five primer pairs with different concentrations, 75 ng of template DNA mixture (25 ng per each strain), 5 ng of IAC, 1 μl of bovine serum albumin (BSA, 20 mg/ml) and RNase-DNase free water to final volume. The multiplex PCR reaction was optimized to

conditions of 94˚C for 2 min. then 30 cycles of 94˚C for 30 s, 63˚C for 30 s and 72˚C for 30 s with a final extension cycle at 72˚C for 5 min. The PCR amplicons were separated in a 3.5% of agarose gel at 100 V for 25 min., subsequently stained with ethidium bromide (10 mg/ml) and viewed with a UV transilluminator.

The established multiplex PCR detection limit was determined using *S.* Enteritidis, *S.* Heidelberg and *S.* Typhimurium genomic DNA mixture. Each *Salmonella* serovar genomic DNA was prepared from containing 4.68×10^6 copies, and subsequently diluted as 10-fold until 4.68×10^2 copies was obtained. A total of 66 *Salmonella* strains isolated from conventional, organic and pasture flock raised chickens were used to evaluate multiplex PCR specificity. These isolates were acquired from previous studies and identified the serovars using Kauffman-White scheme serotyping method (Clement et al., 2010; Melendez et al., 2010).

3.4. Quantitative Real-Time PCR (qPCR)

The *Salmonella* genus specific primer pair (SG) which was the same used in the multiplex PCR was utilized to optimize qPCR. A total 20 µl of reaction mixture consisted of 10 µl of SYBR® Green Premix Ex Taq™ II, 800 nM of primer, 1 µl of BSA (20 mg/ml), 5 µl of template DNA and RNase-DNase free water to bring to the final volume. Positive and negative reactions were run simultaneously. The qPCR assay based on SYBR green was established using an Eppendorf Masterplex thermocycler ep. Gradient Technology (Eppendorf, Westbury, NY, USA) and the conditions of 95˚C for 2 min. followed by 40 cycles of 95˚C for 15 s, 60˚C for 15 s and 68˚C for 20 s with fluorescence being measured during the extension phase. Melting curves were subsequently generated and consisted of 95˚C for 15 s, 60˚C for 20 min. increasing in 0.5˚C increments to 95˚C. The qPCR results were confirmed further through 1.5% of agarose gel

electrophoresis at 100 V for 20 min., gels were stained with ethidium bromide (10 mg/ml) and viewed on a UV transilluminator. The qPCR assay was conducted as triplicate samples per each experiment and three independent experiments.

3.5. Spiked Three *Salmonella* to Chicken Breast Meat

A *Salmonella* presence in chicken breast meat was examined using xylose lysine tergitol (XLT-4) selective media in prior to experiment. A 25 g samples of chicken breast meat was inoculated with 2.2×10^8 to 2.2×10^0 CFU/g of *S.* Enteritidis, *S.* Heidelberg and *S.* Typhimurium mixture and subsequently incubated at room temperature for 1 h. Subsequently, 225 ml of buffered peptone water (BPW) was added for enrichment, homogenized using a stomacher and incubated at 37˚C. To evaluate the multiplex PCR detection limit at different enrichment time points, 1 ml aliquots were taken from enrichment samples at each 0, 4, 8, 18 h during incubation. One ml of enrichment samples was pelleted by centrifugation at 16,000 x g for DNA extraction. The supernatant was discarded and added 100 μl of DNase-RNase free water for cell suspension and then, genomic DNA was isolated by a boiling method (Park et al., 2011). A five μl of isolated genomic DNA was utilized for multiplex PCR and qPCR. Alternatively, enrichment samples were plated on XLT-4 selective media to calculate *Salmonella* cell numbers.

4. Results

4.1. Primer Specificity

The specificity of total five primer pairs were evaluated using 23 *Salmonella* strains and 12 non-*Salmonella* pathogens. *Salmonella* genus and *Salmonella* subsp. I primer pairs produced a 423 bp and a 137 bp of expected product size in 23 *Salmonella* strains respectively, and no

amplification with non-*Salmonella* pathogens. Three strains of each *Salmonella* serovar specific primer pair also only amplified a 171 bp for *S.* Enteritidis, a 216 bp for *S.* Heidelberg, and a 310 bp for *S.* Typhimurium, respectively (Table 1). Since the primer pairs used in this study showed high specificity toward target species, multiplex PCR and qPCR assays were further optimized using these primer pairs.

4.2. Multiplex PCR

The multiplex PCR using 5 primer pairs was optimized to detect *Salmonella* genus, *Salmonella* subsp. I, *S.* Enteritidis, *S.* Heidelberg, and *S.* Typhimurium in a single reaction. The multiplex PCR mixture including only *S.* Typhimurium genomic DNA generated four bands after PCR reaction; 100 bp for IAC, 137 bp for *Salmonella* subsp. I, 310 bp for *S.* Typhimurium and 423 bp for *Salmonella* genus specific products (Figure 1, lane 1). The samples included each *S.* Enteritidis and *S.* Heidelberg also amplified four bands but the species-specific bands were different (Figure 1, lanes 2 and 3). Three target *Salmonella* serovars were present in a sample; six bands were produced corresponding to *Salmonella* genus, *Salmonella* subsp. I, *S.* Enteritidis, *S.* Heidelberg, *S.* Typhimurium and IAC (Figure 1, lane 4).

Each *Salmonella* serovar genomic DNA ranging from 4.68×10^6 copies to 4.68×10^2 copies was prepared to evaluate the detection limit and multiplex PCR showed detection limit of 4.68×10^4 copies (Figure 2, lane 3) with six bands. A total of 66 *Salmonella* isolates from conventional, organic and pasture flock raised chicken carcass were used to confirm optimized multiplex PCR specificity. These isolates serovars were identified by conventional serotyping methods (Clement et al., 2010; Melendez et al., 2010). The multiplex PCR was performed using 66 isolates, identified as *Salmonella* subsp. I and 16 out of 66 isolates were classified as *S.*

Enteritidis. No *S*. Heidelberg or *S*. Typhimurium was detected and this result corresponded with previous serotyping results (Table 3) (Clement et al., 2010; Melendez et al., 2010).

4.3. Quantitative Real-Time PCR (qPCR)

The qPCR based on SYBR green was established to detect the entire *Salmonella* genus using SG primer pair. A standard curve was constructed using three *Salmonella* DNA mixture ranging from 4.68×10^6 copies to 4.68×10^1 copies and each sample were prepared in triplicate per experiment. The correlation coefficient (R^2) value and efficiency were calculated and showed 0.998 and 92%, respectively (Figure 3A). The melting temperature was generated to identify correct PCR products and the value was approximately 91.5˚C (Figure 3B).

4.4. Analysis of Spiked Chicken Breast Meats

In order to validate the multiplex PCR and qPCR established in this study, we tested artificially contaminated chicken breast meats. A 25 g of chicken breast meat was spiked with three *Salmonella* (Enteritidis, Heidelberg and Typhimurium) serovars ranging from 2.2×10^8 to 2.2×10^0 CFU/g. Figure 4 showed multiplex PCR results using different enrichment time point samples. Although the multiplex PCR was unable to amplify all target bands without enrichment (0 h), *Salmonella* subsp. I, *S*. Enteritidis and *S*. Typhimurium specific bands were produced at the level of 2.2×10^7 CFU/g (Figure 4A, lane 2). At 4 h and 8 h enrichment samples, all six bands were amplified at the level of 2.2×10^6 CFU/g and 2.2×10^1 CFU/g, respectively (Figure 4B, lane 3, and Figure 4C, lane 8). In addition, optimized multiplex PCR could detect approximately 2 CFU of *Salmonella* per gram after 18 h enrichment (Figure 4D, lane 9). There was no amplification in negative control sample except one band for IAC.

In qPCR results using spiked samples, the detection limit varied depending on different enrichement time points. A 2.2×10^5 CFU/g of *Salmonella* was detected after 4 h enrichment. However, 22 CFU and 2.2 CFU per gram of *Salmonella* was determined to be the detection limit after 8 h and 18 h enrichment, respectively (Table 4). In addition, there was no PCR amplicons except primer dimers. With the qPCR optimized in this study, 22 CFU of *Salmonella* can be detected within total 11 h assay time including 8 h enrichment, 1 h for DNA preparation and 2 h for qPCR assay.

5. Discussion

Since *Salmonella* species are one of the prominent foodborne pathogens causing disease in humans, the principal aims of this study were the development of rapid and accuarate detection methods. Several methods based on nucleic acids and antigens have been developed to identify *Salmonella* in foodstuffs such as PCR, ELISA, microarray and next generation sequencing (NGS). In this study, we established multiplex PCR to detect *Salmonella* genus, *Salmonella* subsp. I and three most prominent *Salmonella* serovars in a single reaction considering assay time, cost and accuracy.

In order to establish the multiplex PCR successfully, specific primer construction for each target species and no cross-reaction with other primer pairs are important factors. Therefore, the specificity of primer pairs should be evaluated prior to multiplex PCR using various organisms which posess high genetic homology via single PCR as well as the BLAST algorithm. Four primer pairs adopted from previous reports were constructed based on comparative genomics and evaluated for their specificity using over 100 *Salmonella* species (Kim et al., 2006). Since *S.* Heidelberg serogroup (1,4,[5]12:r:1,2) is similar to *S.* Typhimurium (1,4,[5],12:i:1,2),

traditional serotyping method might be difficult to discriminate each other because only the difference is the Phase I antigen (Grimont and Weill, 2007). In this study, we have designed *S.* Heidelberg specific primer pair and the specificity was confirmed by 25 *S.* Heidelberg isolates from turkey farm (data not shown). Furthermore, the multiplex PCR results that applied to 66 *Salmonella* isolates which were serotyped in a previous report showed that this assay can be useful to detect *Salmonella* subsp. I and three most *Salmonella* strains (Enteritidis, Heidelberg and Typhimurium) in poultry and poultry products (Clement et al., 2010; Melendez et al., 2010). In a previous reports, McCarthy et al (2009) developed multiplex PCR and qPCR to discriminate *S.* Typhimurium and *S.* Heidelberg in different food matrices using specific primer pairs constructed by comparative genomics. However, they used *S.* Typhimurium specific primer pair for differentiation of *S.* Heidelberg and failed to design *S.* Heidelberg specific primer pairs.

Rapid and accurate *Salmonella* detection methods in foods are needed for food safety issue. In general, a non-selective enrichment step (8-24 h) is combined with PCR-based methods to detect as low as viable *Salmonella* present in foods and increase assay sensitivity (Soumet et al., 1994; Maciorowski et al. 2000; Ferretti et al., 2001; Myint et al., 2006). Maciorowski et al. (2000) have evaluated the *Salmonella* detection limit in animal feeds by different enrichment time using PCR. Although they could not detect any *Salmonella* within 7 h enrichment, 2 out of 8 samples (25%) included at least 30 CFU/g of *Salmonella* were positive after 13 h enrichment as well as 4 out of 8 samples (50%) were *Salmonella* positive after 24 h enrichment. Therefore, enrichment step is necessary in order to detect viable *Salmonella* in foods using PCR (Keer and Birch, 2003; Cocolin et al., 2011).

Soumet et al. (1994) have developed PCR assays for *Salmonella* detection in chicken products using six different DNA isolation methods. After 10 h enrichment, *Salmonella* can be

detected in spite of PCR inhibitors and DNases. Ferretti et al. (2001) also optimized PCR assay with a 6 h nonselective enrichment for detection of various *Salmonella* serotypes in Italian salami at the level of as low as 1 CFU in 100 ml of food homogenate. Myint et al. (2006) evaluated naturally *Salmonella* contaminated poultry tissue samples using PCR without enrichment and reported all negative results. However, *Salmonella* was detected in all samples after enrichment. In contrast, Wolffs et al. (2006) developed real-time PCR to detect and quantify *Salmonella* in biological samples without enrichement. They utilized two step filtration systems; 40 um filter to remove large food particle and durapore 0.22 um filter (Millipore Corporation, Bedford, MA, USA) to capture *Salmonella* then extracted DNA on the membrane. Through this system, 220 CFU of *Salmonella* in 100 ml of sample could be detected via real-time PCR.

A total of five primer pairs used in this study showed no cross reaction with normal microflora present in chicken breast meat and 22 CFU of *Salmonella* was detected via multiplex PCR after 8 h enrichment. To the best of our knowledge, this is the first multiplex PCR assay to detect *Salmonella* genus, *Salmonella* subsp. I, *S*. Enteritidis, *S*. Heidelberg and *S*. Typhimurium simultaneously from chicken breast meats.

6. References

Bansal, N. S., V. Gray, and F. McDonell. 2006. Validated PCR assay for the routine detection of *Salmonella* in food. J. Food Prot. 69:282-287.

Bronowski, C., and C. Winstanley. 2009. Identification and distribution of accessory genome DNA sequences from an invasive African isolate of *Salmonella* Heidelberg. FEMS Microbiol. Lett. 298:29-36.

Carrique-Mas, J. J., S. Bedford, and R. H. Davies. 2007. Organic acid and formaldehyde treatment of animal feeds to control *Salmonella*: efficacy and masking during culture. J. Appl. Microbiol. 103:88-96.

Clement, A., I. Hanning, S. H. Park, S. M. Melendez, S. Pendleton, A. Woo-Ming, E. E. Scott, and S. C. Ricke. 2010. Processing treatments and rearing conditions effects on *Salmonella*, *Campylobacter*, and aerobic bacteria present on whole carcass chickens. American Society for Microbiology 110th General Meeting. San Diego, CA. 2010.

Cocolin, L., A. Rajkovic, K. Rantsiou, and M. Uyttendaele. 2011. The challenge of merging food safety diagnostic needs with quantitative PCR platforms. Trends in Food Sci. 22:30-38.

Crump, J. A., P. M. Griffin, and F. J. Angulo. 2002. Bacterial contamination of animal feed and its relationship to human foodborne illness. Clin. Infect. Dis. 35:859-865.

Ferretti, R., I. Mannazzu, L. Cocolin, G. Comi, and F. Clementi. 2001. Twelve-hour PCR-based method for detection of *Salmonella* spp. in food. Appl. Environ. Microbiol. 67:977-978.

Grimont, P. A. D., and F. -X. Weill. 2007. *Antigenic formulae of the Salmonella serovars*. (9th ed.). Paris; Institut Pasteur.

Hong, Y., T. Liu, M. D. Lee, C. L. Hofacre, M. Maier, D. G. White, S. Ayers, L. Wang, R. Berghaus, and J. Maurer. 2009. A rapid screen of broth enrichments for *Salmonella* enterica serovars Enteritidis, Hadar, Heidelberg, and Typhimurium by using an allelotyping multiplex PCR that targets O- and H-antigen alleles. J. Food Prot. 72:2198-2201.

Keer, J. T., and L. Birch. 2003. Molecular methods for the assessment of bacterial viability. J. Microbiol. Methods. 53:175-183.

Kim, H. -J., S. -H. Park, and H. -Y. Kim. 2006. Comparison of *Salmonella enterica* serovar Typhimurium LT2 and non-LT2 *Salmonella* genomic sequence, and genotyping of salmonellae by using PCR. Appl. Environ. Microbiol. 72:6142-6151.

Maciorowski, K. G., S. D. Pillai, and S. C. Ricke. 2000. Efficacy of a commercial polymerase chain reaction-based assay for detection of *Salmonella* spp. in animal feeds. J. Appl. Microbiol. 89:710-718.

Maciorowski, K. G., P. Herrera, F. T. Jones, S. D. Pillai, and S. C. Ricke. 2006. Cultural and immunological detection methods for *Salmonella* spp. in animal feeds- A review. Vet. Res. Comm. 30:127-137.

McCarthy, N., F. J. Reen, J. F. Buckley, J. G. Frye, E. F. Boyd, and D. Gilroy. 2009. Sensitive and rapid molecular detection assays for *Salmonella* enterica serovars Typhimurium and Heidelberg. J. Food Prot. 72:2350-2357.

Melendez, S. N., I. Hanning, J. Han, R. Nayak, A. R. Clement, A. Wooming, P. Hererra, F. T. Jones, S. L. Foley, and S. C. Ricke. 2010. *Salmonella enterica* isolates from pasture-raised poultry exhibit antimicrobial resistance and class I integrons. J. Appl. Microbiol. 109:1957-1966.

Myint, M. S., Y. J. Johnson, N. L. Tablante, and R. A. Heckert. 2006. The effect of preenrichment protocol on the sensitivity and specificity of PCR for detection of naturally contaminated *Salmonella* in raw poultry compared to conventional culture. Food Microbiol. 23: 599-604.

O'Regan, E., E. McCabe, C. Burgess, S. McGuinness, T. Barry, G. Duffy, P. Whyte, and S. Fanning. 2008. Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples, BMC Microbiol. 8:156.

Park, S. H., H. J. Kim, W. H. Cho, J. H. Kim, M. H. Oh, S. H. Kim, B. K. Lee, S. C. Ricke, and H. Y. Kim. 2009. Identification of *Salmonella enterica* subspecies I, *Salmonella enterica* serovars Typhimurium, Enteritidis and Typhi using multiplex PCR, FEMS Microbiol. Lett. 301:137-146.

Park, S. H., I. Hanning, R. Jarquin, P. Moore, D. J. Donoghue, A. M. Donoghue, and S. C. Ricke. 2011. Multiplex PCR for detection and quantification of *Campylobacter* spp., *Escherichia coli* O157:H7, and *Salmonella* serotypes in water samples. FEMS Microbiol. Lett. 316:7-15.

Petkar, A., W. Q. Alali, M. A. Harrison, and L. R. Beuchat. 2011. Survival of *Salmonella* in organic and conventional broiler feed as affected by temperature and water activity. Agric. Food. Anal. Bacteriol. 1:175-185.

Saeki, E. K., J. Alves, R. C. Bonfante, E. Y. Hirooka, and T. C. R. M. de Oliveira. 2013. Multiplex PCR (mPCR) for the detection of *Salmonella* spp. and the differentiation of the Typhimurium and Enteritidis serovars in chicken meat. J. Food. Saf. 33:25-29.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. -A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States-major pathogens. Emerg. Infect. Dis. 17:7-15.

Soria, M. C., M. A. Soria, D. J. Bueno, and J. L. Colazo. 2011. A comparative study of culture methods and polymerase chain reaction assay for *Salmonella* detection in poultry feed. Poult. Sci. 90:2606-2618.

Soumet, C., G. Ermel, P. Fach, and P. Colin. 1994. Evaluation of different DNA extraction procedures for the detection of *Salmonella* from chicken products by polymerase chain reaction. Lett. Appl. Microbiol. 19:294-298.

Vugia, D. J., M. Samuel, M. M. Farley, R. Marcus, B. Shiferaw, S. Shallow, K. Smith, and F. J. Angulo. 2004. Invasive *Salmonella* infections in the United States, FoodNet, 1996-1999: incidence, serotype distribution, and outcome. Clin. Infect. Dis. 21:1065.

Wolffs, P. F. G., K. Glencross, R. Thibaudeau, and M. W. Griffiths. 2006. Direct quantitation and detection of Salmonellae in biological samples without enrichment, using two-step filtration and real-time PCR. Appl. Environ. Microbiol. 72:3896-3900.

Table 1. Bacterial strains used in this study

	Strains	Source	Primer				
			SG[a]	SS-I[b]	SE[c]	SH[d]	ST[e]
Salmonella	*S.* Enteritidis	ATCC 13076	+	+	+	-	-
	S. Heidelberg	ATCC 8326	+	+	-	+	-
	S. Typhimurium	ATCC 14028	+	+	-	-	+
	S. Kentucky	Isolates from chicken, turkey and farm	+	+	-	-	-
	S. Mbandaka		+	+	-	-	-
	S. Newport		+	+	-	-	-
	S. Muenster		+	+	-	-	-
	S. Agona		+	+	-	-	-
	S. Senftenberg		+	+	-	-	-
	S. Montevideo		+	+	-	-	-
	S. Worthington		+	+	-	-	-
	S. Anatum		+	+	-	-	-
	S. Rough		+	+	-	-	-
	S. Infantis		+	+	-	-	-
	S. Alachua		+	+	-	-	-
	S. Barranquilla		+	+	-	-	-
	S. Georgia		+	+	-	-	-
	S. Give		+	+	-	-	-
	S. Manhattan		+	+	-	-	-
	S. Oranienburg		+	+	-	-	-
	S. Rubislaw		+	+	-	-	-
	S. Taksony		+	+	-	-	-
	S. Tennessee		+	+	-	-	-
Non-Salmonella	*B. cereus*	ATCC 11778	-	-	-	-	-
	B. licheniformis	ATCC 12579	-	-	-	-	-
	C. freundii		-	-	-	-	-
	E. aerogenes		-	-	-	-	-
	E. coli	ATCC 25922	-	-	-	-	-
	L. grayi	ATCC 19120	-	-	-	-	-
	L. innocua	ATCC 33090	-	-	-	-	-
	L. ivanovii	ATCC 19119	-	-	-	-	-
	L. monocytogenes	ATCC 35152	-	-	-	-	-
	L. welshimeri	ATCC 25897	-	-	-	-	-
	Staph. aureus	ATCC 6538	-	-	-	-	-
	Staph. epidermidis	ATCC 12228	-	-	-	-	-

SG[a]: *Salmonella* genus, SS-I[b]: *Salmonella* subsp. I, SE[c]: *S.* Enteritidis, SH[d]: *S.* Heidelberg, ST[e]: *S.* Typhimurium

Table 2. Primer sequences used in this study

Target	Primer	Sequence (5' to 3')	Size (bp)	Target gene	Conc. (uM)	Reference
Salmonella genus	SG	TTTGG CGGCG CAGGC GATTC GCCTC CGCCT CATCA ATCCG	423	STM3098	0.27	Kim *et al*., 2006
Salmonella subsp. I	SS-I	GGTGG CCTCG ATGAT TCCCG CCCAC TTGTA GCGAG CGCCG	137	STM4057	0.27	Kim *et al*., 2006
S. Typhimurium	ST	AACAA CGGCT CCGGT AATGA GATTG ATGAC AAACT CTTGA TTCTG AAGAT CG	310	STM4497	0.2	Park *et al*., 2009
S. Enteritidis	SE	GCCGA GCTTG ATGAC AAACC TG GCGCT TCGCT TTTCC AACTG CC	171	SEN0997	0.2	Park *et al*., 2008
S. Heidelberg	SH	TGTTT GGAGC ATCAT CAGAA GCTCA ACATA AGGGA AGCAA	216	Restriction enzyme (ACF69659)	5	This study

Table 3. Application of multiplex PCR to *Salmonella* strains isolated from conventional, organic and pasture flock raised chickens

Source	**Strain No.**	**Identification**	**Reference**
Conventional chickens	1	*Salmonella* subsp. I	Clement et al. (2010)
Organic chickens	11 (2)	*Salmonella* subsp. I (*S.* Enteritidis)	Clement et al. (2010)
Pasture flock raised chickens and farm	54 (14)	*Salmonella* subsp. I (*S.* Enteritidis)	Melendez et al. (2010)

Table 4. Comparison of *Salmonella* genus detection limit between multiplex PCR and qPCR

Strain	Enrichment time point											
	0 h			4 h			8 h			18 h		
	C[a]	M[b]	Q[c]	C	M	Q	C	M	Q	C	M	Q
Salmonella mixture (Enteritidis, Heidelberg, and Typhimurium)	2.2×10^8	-[d]	+[e]	2.4×10^8	+	+	1.4×10^9	+	+	1.3×10^9	+	+
	2.2×10^7	-	+	5.0×10^7	+	+	1.1×10^9	+	+	1.2×10^9	+	+
	2.2×10^6	-	-	9.0×10^6	+	+	6.7×10^8	+	+	1.1×10^9	+	+
	2.2×10^5	-	-	3.0×10^5	-	+	3.1×10^8	+	+	9.9×10^8	+	+
	2.2×10^4	-	-	3.3×10^5	-	-	1.1×10^8	+	+	9.8×10^8	+	+
	2.2×10^3	-	-	1.2×10^5	-	-	7.0×10^7	+	+	9.9×10^8	+	+
	2.2×10^2	-	-	2.0×10^5	-	-	4.2×10^7	+	+	9.9×10^8	+	+
	2.2×10^1	-	-	1.4×10^4	-	-	3.2×10^7	+	+	1.0×10^9	+	+
	2.2×10^0	-	-	6.0×10^3	-	-	2.5×10^6	-	+	8.9×10^8	+	+

C[a]: Average *Salmonella* numbers of triplicate plates using XLT-4 media (CFU/g)
M[b]: Multiplex PCR results showing *Salmonella* genus specific product (423 bp)
Q[c]: qPCR results using *Salmonella* genus specific primer pair (SG)
-[d]: PCR negative
+[e]: PCR positive

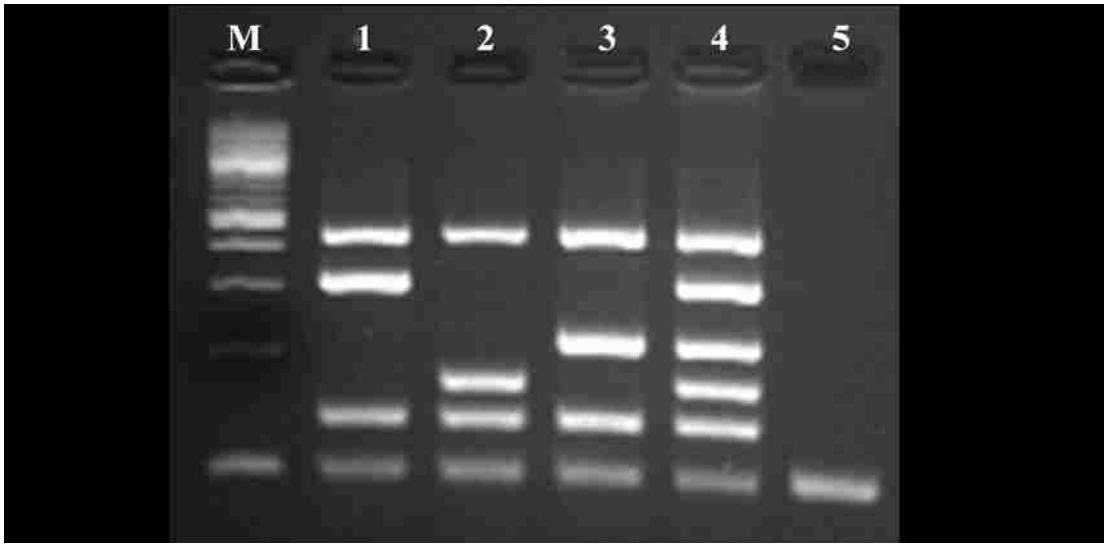


Figure 1. Multiplex PCR results using pure genomic DNA of *Salmonella* type strains Lane M: 100 bp ladder, lane 1: *S*. Typhimurium (ATCC 14028), lane 2: *S*. Enteritidis (ATCC 13076), lane 3: *S*. Heidelberg (ATCC 8326), lane 4: *S*. Typhimurium (ATCC 14028), *S*. Enteritidis (ATCC 13076), and *S*. Heidelberg (ATCC 8326), lane 5: negative control

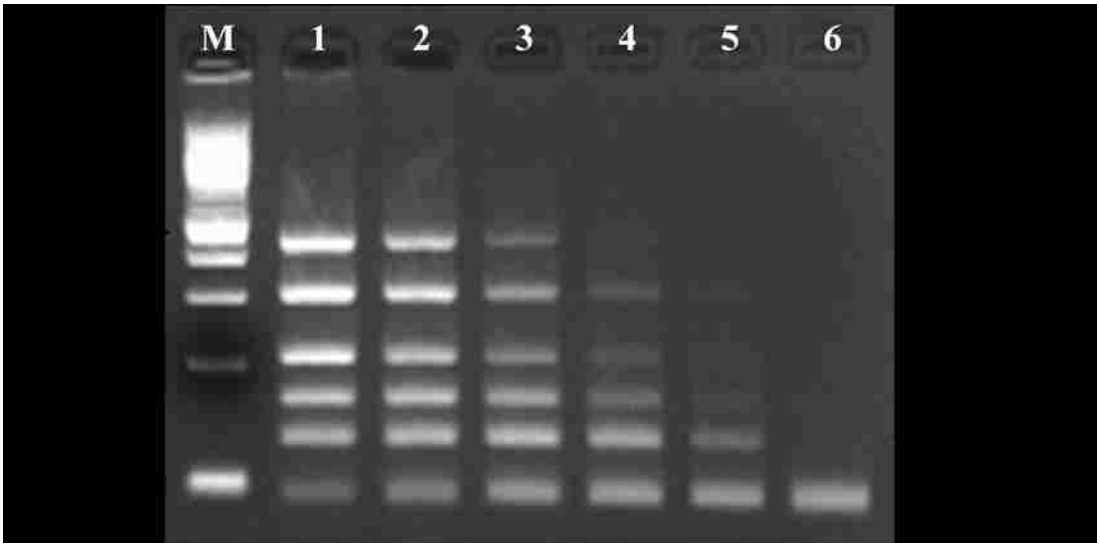


Figure 2. Evaluation of multiplex PCR detection limit using three *Salmonella* pure genomic DNA mixture

M: 100-bp DNA ladder, lanes 1-5: 4.68×10^6 to 4.68×10^2 copies of *S*. Typhimurium (ATCC 14028), *S*. Enteritidis (ATCC 13076), and *S*. Heidelberg (ATCC 8326), lane 6: negative control

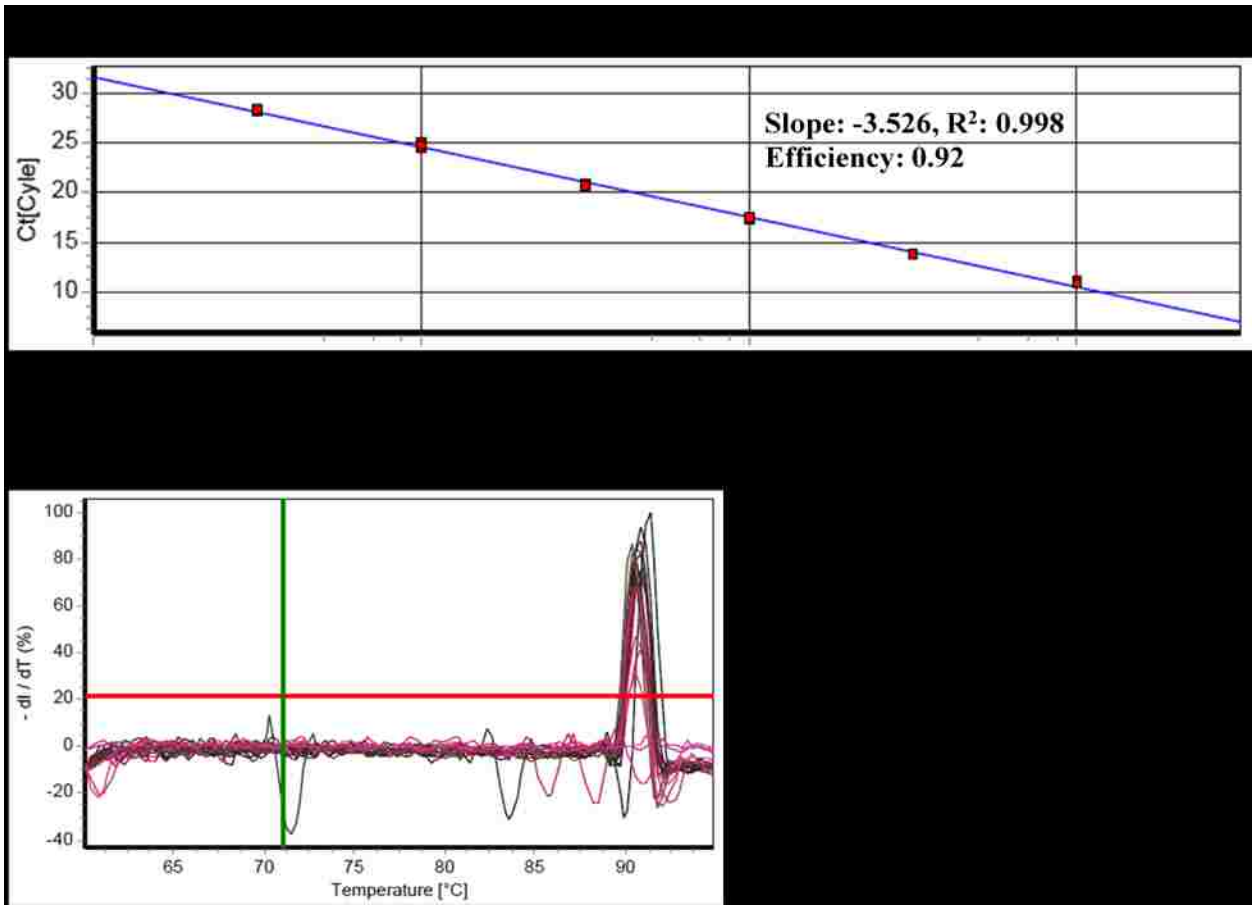


Figure 3. Standard curve construction and melting temperature of qPCR.

(A) Standard curve was generated using three *Salmonella* genomic DNA ranging frm 4.68×10^1 to 4.68×10^6 copies.

(B) Melting temperature showed approximately 91.5˚C.

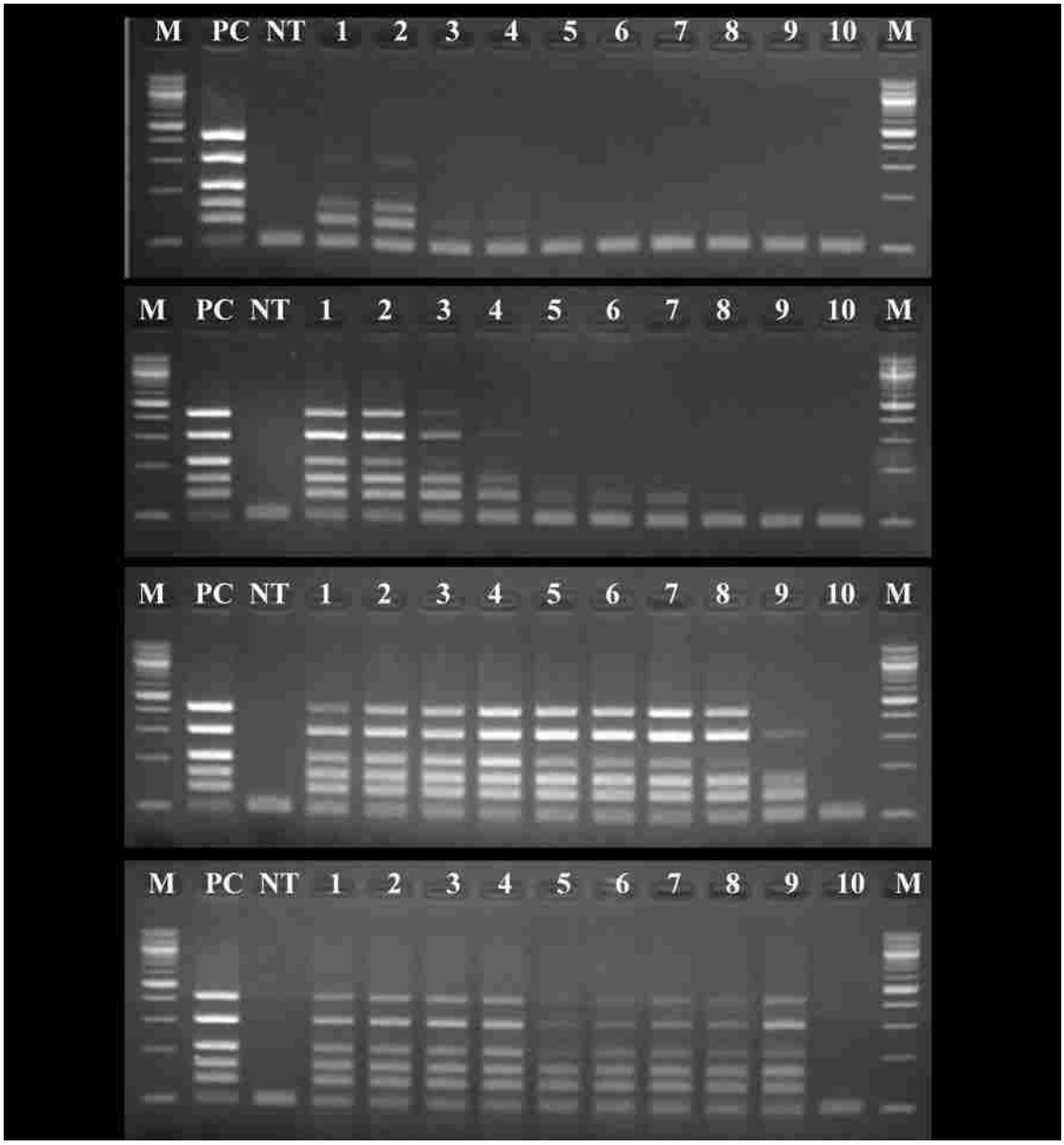


Figure 4. Application of multiplex PCR to artificially inoculated chicken breast meats

(A): 0 h enrichment, (B): 4 h enrichment, (C): 8 h enrichment, (D): 18 h enrichment

M: 100 bp DNA ladder, PC: positive control (pure DNA mixture of three *Salmonella* strains), NT: no template for PCR, lanes 1-9: 2.2×10^8 to 2.2×10^0 CFU/g of *S*. Typhimurium (ATCC 14028), *S*. Enteritidis (ATCC 13076), and *S*. Heidelberg (ATCC 8326), lane 10: negative control

7. Appendix

7.1. Authorship Statement for Chapter 3

Si Hong Park is the first author of the paper and completed at least 51% of the studies among coauthors which the title is "**Development of Novel Multiplex and Quantitative PCR Assays for Simultaneous Detection of *Salmonella* Genus, *Salmonella* Subspecies I, *S*. Enteritidis, *S*. Heidelberg, and *S*. Typhimurium**" in chapter 3.

Major Advisor: Dr. Steven C. Ricke

Date: June 4th, 2013

CHAPTER 4

Enhancement of Macrophage Chicken Cytokine Response to *Salmonella* Typhimurium When Combined with Bacteriophage P22

Si Hong Park[1,2], Debabrata Biswas[3], Jody Lingbeck[2], Ok Kyung Koo[2] and Steven C. Ricke[1,2*]

[1]Cell and Molecular Biology Program, Department of Food Science, University of Arkansas, Fayetteville, AR

[2]Center for Food Safety and Department of Food Science, University of Arkansas, Fayetteville, AR

[3]Current address: Department of Animal and Avian Sciences and Center for Food Safety and Security Systems, University of Maryland, College Park, MD

FEMS Microbiology Letters 339: 137-144, 2013

1. Abstract

Salmonella infections are reported as the second most common pathogen causing foodborne disease in the United States and several *Salmonella* serovars can colonize the intestinal tracts of poultry. Reducing *Salmonella* in poultry is crucial to decrease the incidence of salmonellosis in humans. In this study, we evaluated the immune response of chicken macrophage cells (HD-11) and effects of bacteriophage P22 against the extra- and intracellular *S.* Typhimurium LT2. Four treatments; 1) HD-11 cells as control, 2) HD-11 cells with LT2, 3) HD-11 cells with LT2 and P22 and 4) HD-11 cells with P22 were administered and IL-8 responses of HD-11 cells were measured using an ELISA. Also, four cytokine (IL-4, 8, 10 and IFN-γ) gene expression levels in the presence of LT2 and/or P22 were quantified by qRT-PCR. We found that P22 lysed the extra- and intracellular LT2 which adhered and were taken up by the HD-11 cells. The ELISA assay indicated that HD-11 cells produced significantly higher IL-8 cytokine levels in the supernatant during the intracellular lyses of LT2 by P22 ($P < 0.05$). The IL-8 expression levels measured by qRT-PCR also exhibited similar results with the IL-8 production based on ELISA measurements.

2. Introduction

Salmonella has the potential to cause fatal bacterial infections in infants and individuals with a suppressed immune system (Scallan *et al*., 2011; Finstad *et al*., 2012). The majority of the foodborne *Salmonella* serovars can colonize in the intestinal tracts of humans and one of the major routes of human salmonellosis is believed to be consumption of contaminated poultry and poultry products (Finstad *et al*., 2012). Thus, strategies for the control and prevention of poultry colonization are needed to further reduce the incidence of salmonellosis in humans. Currently, there are several preventative measures for limiting *Salmonella* establishments in poultry flocks including dietary alterations, prebiotics, probiotics, antimicrobials such as organic acids and the administration of vaccine strains (Ricke, 2003a, b; Vandeplas *et al*., 2010). However, there are very limited options for reducing already established *Salmonella* in the avian gastrointestinal tract (Toro *et al*., 2005; Atterbury *et al*., 2007).

In many developed countries, bacteriophage therapy was abandoned in favor of the development and widespread production of antibiotics (Stone, 2002). However, interest in phage therapy has gained momentum in animal productions over the past few years as antibiotics have fallen out of favor (Nakai & Park, 2002; Joerger, 2003; Levin & Bull, 2004; Atterbury *et al*., 2007; Ricke *et al*., 2012). The bacteriophage P22 used in this study is able to bind specific somatic antigen structures of lipopolysaccharide (LPS) present in *Salmonella* serogroup A, B and D1 included *S*. Typhiumurium via tailspike proteins (TSP) (Marietto-Gonçalves *et al*., 2011). P22 utilize TSP enzymes to penetrate the outer membrane of *S*. Typhimurium and allow P22 to inject genetic materials into host cells. The virions produced by replication of P22 DNA inside the host cells assemble to form mature P22 and subsequently lyse the respective host cells.

Since cytokines are considered as crucial regulators or mediators against antigens in the host immune system, the change of cytokine expression levels in the presence of *Salmonella* and bacteriophage is important for understanding roles in inflammation and apoptosis during pathogen infections (Liu *et al*., 2010). Research on avian cytokines has expanded due to the increased interest in avian immune responses against pathogens and advanced techniques that are now available for studying these responses in detail (Giansanti *et al*., 2006).

In this study, we hypothesized that P22 could enhance reduction of intracellular *S.* Typhimurium if the phage was allowed to come in contact with the host cell. To test this hypothesis we used an *in vitro* host chicken cell model (HD-11 macrophage) to differentiate the cellular immune response against *S.* Typhimurium with or without P22. As part of this study, HD-11 cytokine expression levels were assessed using an enzyme-linked immunosorbent assay (ELISA) and quantitative reverse transcriptase polymerase chain reaction assays (qRT-PCR).

3. Methods and Materials

3.1. Bacterial Strains and Growth Conditions

Salmonella Typhimurium LT2 (ATCC 19585) and ST55, a reduced motility mutant, were used in this study (Aswad & Koshland Jr., 1975). One loop of each *S.* Typhimurium strain was taken from frozen stock and streaked onto Luria-Bertani (LB) (EMD Chemicals Inc, Gibbstown, NJ, USA) plate. After incubation for 24 h at 37˚C, one colony was selected and grown in 5 mL of LB broth for 24 h at 37˚C under aerobic growth conditions.

3.2. Propagation and Enumeration of Bacteriophage P22

The *Salmonella* Typhimurium LT2 strain was grown on LB plates overnight at 37˚C under aerobic incubation conditions and cells were collected in phosphate buffer saline (PBS) solution. The OD_{600} adjusted LT2 strain was subsequently added to 50 mL of LB broth in a conical flask and triplicate cultures were grown to late log phase including approximately 10^8 colony forming unit (CFU) at which point P22 including 10^6 plaque forming unit (PFU) was added to an approximate multiplicity of infection (MOI) of 0.01. Incubation with shaking was continued overnight and grown cultures were filtered to remove LT2 cells and bound phages. Unbound free phages were enumerated on host lawns of LT2 strain and stored at -20˚C. P22 stocks were evaluated the contamination of phage resistant bacteria prior to use.

3.3. Cell Culture

Chicken macrophage (HD-11) cells were maintained in minimal essential medium (MEM) with 10% fetal bovine serum (FBS) and grown routinely in a 75-cm^2 flask at 37˚C in a 5% CO_2-humidified incubator. Confluent stock cultures were treated with trypsin to release the attached cells and new stock cultures were seeded with 10^5 cells per mL. For the adherence and uptake assays, 24-well tissue culture plates (BD Biosciences, Franklin Lakes, NJ, USA) were seeded with 10^5 cells per mL of HD-11 cells and incubated at 37˚C in a 5% CO_2 humidified incubator for 18 to 20 h and a semi-confluent monolayer was obtained. Prior to the experiment, the monolayer was washed and incubated in MEM containing 10% FBS without antibiotic.

3.4. Adherence and Uptake Assays

Adherence and uptake assays were performed using a modified procedure derived from Biswas *et al* (2006). One loop of LT2 and ST55 grown overnight was collected from LB plates

and suspended in MEM with 10% FBS. The OD of each strain suspension was subsequently adjusted to an absorbance value of 0.2 at 600 nm. A 100 µL of the suspension containing approximately 10^7 CFU (MOI of 100) was inoculated into duplicate wells of a 24-well tissue culture plate containing semi-confluent monolayers of HD-11 cells. The concentration of each strain was determined simultaneously on LB plates as described previously. Infected monolayers were incubated for 2 h at 37˚C under a 5% CO_2 humidified atmosphere to allow LT2 and ST55 adherence and uptake by the cells. One plate was washed five times with PBS and P22 including 10^7 PFU (MOI of 1) was added followed by incubation for 4, 8 and 16 h to lyse bacterial cells. After incubation, the HD-11 cells were lysed with 0.1% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS for 15 min to enumerate the number of extra- (adherence) and intracellular (uptake) LT2 and ST55. Other plates were re-incubated for another 2 h in fresh media containing 250 µg mL^{-1} of gentamicin to kill the extracellular bacteria. After incubation, the number of intracellular (uptake) LT2 and ST55 were evaluated using the same method described previously. Three wells with only HD-11 cells and the other three wells infected with only P22 were prepared as controls. Results were expressed as the average number of adhered and invaded cells by LT2 and ST55 in three to five independent assays.

3.5. Detection of IL-8 Cytokine Produced by HD-11 Cells Using ELISA

A total of four treatments were utilized in this study, 1) HD-11 cells as control, 2) HD-11 cells with LT2, 3) HD-11 cells with LT2 and P22 and 4) HD-11 cells with P22, to evaluate IL-8 production levels along with a negative control of added with PBS instead of HD-11 cells. The ELISA was performed following the protocol provided by BD Biosciences (Catalog No. 555244,

BD Biosciences). Visible light absorbance readings of four different treatments were taken at a wavelength of 450 nm to quantify the level of IL-8 cytokine.

3.6. Sample Treatments for Cytokine Expression

Five treatments were prepared to evaluate the different cytokine expression levels of HD-11 cells in the presence of LT2 and P22. Treatment A consisted of only HD-11 cells as the control, treatment B was HD-11 cells with P22, treatment C was HD-11 cells with LT2, treatment D was HD-11 cells with P22 and LT2, and treatment E was the same as treatment D except for adding gentamicin. Since gentamicin is unable to kill LT2 cells that have already entered into HD-11 cells, treatment E was used to compare cytokine production levels against intracellular LT2 (Durant *et al*., 2000). All five treatments were used for qRT-PCR analysis to verify the expressions of the four cytokines (IL-4, IL-8, IL-10 and IFN-γ).

3.7. Total RNA Isolation

Total RNAs from five treatment samples were isolated using Trizol reagent (Sigma) to perform qRT-PCR. A 1 mL aliquot of Trizol reagent was added to each sample and collected immediately using a scraper. Total RNA was extracted and subsequently DNase I (New England Biolabs, Ipswich, MA, USA) treatment was performed for 1 h at 37˚C to remove possible contaminating genomic DNA. Total RNA was purified by Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction and the concentration was measured by a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA).

3.8. Quantitative Reverse Transcriptase PCR (qRT-PCR) Assay

The qRT-PCR assay was optimized using an Eppendorf Masterplex thermocycler ep Gradient Technology (Eppendorf, Westbury, NY, USA). Three primer pairs for IL-4, IL-10 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were adopted from previous work (Abdul-Careem *et al*., 2007; Lee *et al*., 2010) and the other two primer pairs for IL-8 and IFN-γ were synthesized in this study (Table 1). The specificity of designed primer pairs was confirmed by a basic local alignment search tool (BLAST) program. The 20 μL of reaction mixture consisted of 10 μL of EXPRESS SYBR GreenER^{TM}qPCRSuperMix with Premixed ROX (Invitrogen, Carlsbad, CA, USA), 0.5 μL of EXPRESS SuperScript Mix for One-Step SYBR GreenER (Invitrogen), 500 nM of each primer, 500 ng of total RNA template and DEPC treated water to volume. The qRT-PCR was optimized for the reaction conditions of 50˚C for 5 min for the synthesis of cDNA. This was followed by 40 cycles of 95˚C for 15 s, 57˚C for 15 s and 68˚C for 20 s. Melting curves were subsequently created which consisted of 95˚C for 15 s, 60˚C for 20 min. increasing by 0.5˚C per minute to a final temperature of 95˚C. Each experiment was performed in triplicate.

3.9. Statistical Analysis

The GAPDH gene was used as an internal standard to normalize the qRT-PCR and the Ct values were calculated with the Eppendorf realplex software (version 2.0). The relative gene expression changes in transcription levels of the four cytokines between the control and treatments were determined using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The data were generated by three independent experiments and each trial was carried out in triplicate. Statistical analysis was performed using JMP® Genomics 5.0 software (SAS Institute Inc., Cary, NC, USA).

The experimental data were analyzed using a T test and a calculated *P* value of < 0.05 was used to delineate significant differences.

4. Results

4.1. Adherence and Uptake of *S*. Typhimurium

In this series of experiments, we measured the adherence and uptake of LT2 and ST55 and demonstrated that our cell culture model could be used to screen for extra- and intracellular survival of both strains. Based on Fig. 1, it appears that both LT2 and ST55 adhered to HD-11 cells at 3.6 ± 0.2% and 2.5 ± 0.3% of their initial inoculation levels, respectively. Similarly, LT2 (0.39 ± 0.04%) were taken up by HD-11 cells more than ST55 (0.25 ± 0.02%). Since LT2 exhibited greater difference in adherence and uptake than ST55 strain ($P < 0.05$), we continued the study with LT2.

4.2. Evaluation of Extra- and Intracellular Killing of LT2 by P22

In a follow-up study utilizing the HD-11 cell culture model, we observed that P22 was capable of killing both extra- and intracellular LT2. Figure 2 showed relative recovery of LT2 strains at each time point. By the first 8 h after inoculation, no significant differences in both extra- and intracellular recoveries of LT2 occurred between 4 h and 8 h while almost all LT2 cells were eliminated at 16 h.

We also observed that HD-11 cells produced significantly higher amounts of cytokine (IL-8) in the supernatant in the presence of LT2 with P22 ($P < 0.05$). This finding indicates that intracellular lysis of LT2 strains enhanced the cell mediated immune response of chicken macrophages. In this study, we detected the IL-8 produced by LT2-infected HD-11 cells during

killing of extra- and intracellular bacterial cells by P22. The expression patterns of IL-8 in the LT2-infected HD-11 cells are illustrated in Fig. 3. The IL-8 expression level in the HD-11 cell increased over two fold in the presence of LT2 compared to HD-11 cells alone and P22 also stimulated the expression of IL-8. In addition, IL-8 expression level increased significantly in the presence of both LT2 and P22.

4.3. Cytokine Expression Levels by qRT-PCR

The primer pairs amplified PCR products with high specificity for each target gene with the respective melting curve. Electrophoresis of PCR products was conducted on an agarose gel to confirm the exact PCR result as well as each amplicon size as shown in Table 1. Infection of LT2 and P22 increased cytokine expression levels in HD-11 cells. The fold changes in cytokine expression levels due to the four different treatments compared to the naïve cells are presented in Table 2. The GAPDH gene served as the reference gene to normalize cytokine expression levels as fold changes. The IL-4 gene exhibited increases when LT2 and P22 were administered to the chicken cells but adding gentamicin caused a decrease in IL-4 gene expression. For the IL-8 gene, all treatments significantly increased gene expression levels ($P < 0.05$) and treatment E in particular exhibited a more than two fold increase compared to other treatments. The expression of IL-10 gene was markedly increased for treatment B ($P < 0.05$) with no significant differences in any of the other treatments. Finally, IFN-γ gene appeared to be highly up-regulated for treatment B.

5. Discussion

The significance of this study was the detection of changes in immune responses to LT2 on chicken macrophage cells (HD-11) when combined with P22 since macrophages play important roles in the innate immune system. Cytokines produced by innate immune cells influence the adaptive immune response and cell signaling molecules in intracellular communication (Witheanage *et al*., 2004). The cytokines evaluated in this study were selected for the important roles they play in innate and adaptive immunity. Furthermore, they interact with a wide variety of cell products during the immune response (Schroder *et al*., 2004). In the present study, the effects of P22 on the host (LT2) and the production of four different cytokines in chicken macrophage cells were evaluated by adherence and uptake assays as well as ELISA and qRT-PCR. The specific killing ability of P22 to LT2 has been reported in several studies (Pope *et al*., 2004; Toro *et al*., 2005). The phage utilized in this study was able to initiate killing of extra- and intracellular *S*. Typhimurium within a few hours after infection and completed bacterial lysis within 16 h.

Cytokines are generally divided into several categories by the activity and/or effects they produce (Giansanti *et al*., 2006). Pro-inflammatory cytokines IL-8 is produced by stimulation of macrophages and IFN-γ can also be induced by natural killer (NK) cells as well as T cells, and both cytokines are associated with innate immune response (Giansanti *et al*., 2006; Apte *et al*., 2008). In contrast, IL-10 produced by mast cells inhibits both NK cell activity and pro-inflammatory cytokine synthesis (Pestka *et al*., 2004). The cytokine IL-4 which is a key regulator in humoral and adaptive immune response decreases the production of macrophages and IFN-γ (Apte *et al*., 2008). There were no significant differences in IL-4 expression levels among any of the treatments. IL-4 is a key cytokine for humoral immunity and stimulates B- and T-cell proliferation and is characterized as a signal for decreasing production and deactivation of

macrophages (Bogdan & Nathan, 1993; He *et al*., 2011). The expression levels of IL-4 on each treatment exhibited no significant changes statistically when compared to control. IL-10 is an anti-inflammatory cytokine and inhibits the ability of antigen presenting cells (APCs). Therefore, the presence of LT2 in HD-11 cells (treatments C, D and E) led to no significant differences in expression levels of IL-10. The IFN-γ is an important cytokine in host defense mechanism against viral and intracellular pathogens. It is stimulated by macrophages and induces antimicrobial as well as antiviral activities (Liu *et al*., 2010).

In addition, the expression levels of IL-8 have been shown to be greatly increased by *S.* Typhimurium infection at multiple organs in chicken such as liver, cecal tonsil and jejuna (Witheanage *et al*., 2004). Since IL-8 is an important chemokine in immune system against bacterial and viral infections, the expression level of IL-8 was investigated by both indirect ELISA and qRT-PCR. Although individual infection of LT2 and P22 stimulated IL-8 expression levels, the presence of both increased IL-8 production significantly more than either individual treatment. These two results were supported by both the ELISA and qRT-PCR. In addition, the drastic over 40 fold increase in IL-8 production compared to control in all treatments implied that the infection of LT2 and P22 could stimulate IL-8 production in HD-11 cells. IL-8 mRNA expression levels in treatment B were higher than treatment C (Table 2) however, both treatments showed reverse results in protein expression levels (Fig. 3). The difference in correlation between mRNA and protein expression levels may be due to several factors such as various and complicated post-transcriptional mechanisms in translation from mRNA to protein, different half lives of protein as well as both mRNA and protein experimental limitations (Greenbaum et al., 2003). IL-8 is one of the CXC chemokines produced by macrophages and is an important mediator for initiation of innate immune response in the infected cells.

The utilization of bacteriophages to modulate pathogen load in complex ecosystems such as the intestine represents additional logistical challenges (Barrow, 2001; Ricke *et al*., 2012). Nonetheless, in the late 1980s, Smith and coworkers successfully used bacteriophages to control *E. coli* diarrhea in calves (Smith *et al*., 1987). Their study demonstrated the potential effectiveness of bacteriophage use to treat intestinal bacterial infections even in the complex milieu of the gastrointestinal system. In previous reports, several studies have shown that bacteriophages may be useful in reducing the number of bacterial foodborne pathogens including *Escherichia coli* O157:H7 (Sheng *et al*., 2006), *Campylobacter jejuni* (Goode *et al*., 2003), *Listeria monocytogenes* (Leverentz *et al*., 2003) and *Salmonella* serovars (Higgins *et al*., 2005; Wall *et al*., 2010) contaminating the surface of food, poultry and swine. In addition, bacteriophages have been investigated for their ability to reduce *Salmonella* already established in the poultry intestine; however, this application has resulted in only modest success (Toro *et al*., 2005; Atterbury *et al*., 2007; Higgins *et al*., 2007). Huff *et al* (2010) examined the immune response of chicken against bacteriophage SPR02 by IgG level titers in serum. Prior exposure to the same bacteriophage increased IgG levels in the chicken such that the therapeutic effectiveness of bacteriophage was believed to be decreased by the avian immune response.

Bacterial infections in animal hosts theoretically can be controlled by bacteriophage treatment through two mechanisms; direct bacteriophage lysis or immune response via bacterial lysate produced by bacteriophages (Merril *et al*., 1996; Borysowski & Górski, 2008). Thus, bacterial infection in a host can be directly eliminated by adding bacteriophages. However, bacteriophage itself can increase specific IgG serum levels in the animal host by intramuscular injection because outer protein structures of bacteriophage are recognized as antigens in the host cells thus they can be neutralized by antibodies (Huff *et al*., 2010; Ricke *et al*., 2012). As a result,

bacteriophage specific antibodies decreased the antibacterial phage activities and increased the mortalities of animal host (Huff *et al*., 2010).

In this study, we evaluated several chicken macrophage cell (HD-11) cytokine responses to the presence of either bacteriophage or *S*. Typhimurium, and were able to detect differential immune responses by the host cells. However, since this was an *in vitro* model system this does not ensure that such direct cell to phage interactions would occur *in vivo*. To assess such interactions when using bacteriophage for systemic treatments in food animals such as chickens, it will be essential in future studies to investigate the overall animal host immune response against the bacteriophage activities as well as responses at the cellular levels of the host.

6. Acknowledgment

We thank Dr. Gary Acuff, Department of Animal Sciences, Texas A&M University, College Station, Texas, USA, for providing bacterial strains used in this study. This study was supported by National Integrated Food Safety Initiative (NIFSI) grant # 2008-51110-04339 and a USDA Food Safety Consortium Grant at the University of Arkansas, Division of Agriculture.

7. References

Abdul-Careem MF, Hunter BD, Parvizi P, Haghighi HR, Thanthrige-Don N & Sharif S (2007) Cytokine gene expression patterns associated with immunization against Marek's disease in chickens. *Vaccine* **25**: 424-432.

Apte SH, Baz A, Groves P, Kelso A & Kienzle N (2008) Interferon-γ and interleukin-4 reciprocally regulate CD8 expression in $CD8^+$ T cells. *Proc Natl Acad Sci USA* **105**: 17475-17480.

Aswad D & Koshland Jr DE (1975) Isolation, characterization and complementation of *Salmonella typhimurium* chemotaxis mutants. *J Mol Biol* **97**: 225-235.

Atterbury RJ, Van Bergen MAP, Ortiz F, Lovell MA, Harris JA, De Boer A, Wagenaar JA, Allen VM & Barrow PA (2007) Bacteriophage therapy to reduce *Salmonella* colonization of broiler chickens. *Appl Environ Microbiol* **73**: 4543-4549.

Barrow PA (2001) The use of bacteriophages for treatment and prevention of bacterial disease in animals and animal models of human infection. *J Chem Tech Biotech* **76**: 677-682.

Biswas D, Reiman C, Wilson P, Potter A & Allan B (2006) Effect of cytolethal distending toxin of *Campylobacter jejuni* on adhesion and internalization in cultured cells and in colonization of the chicken gut. *Avian Dis* **50**: 586-593.

Bogdan C & Nathan C (1993) Modulation of macrophage function by transforming growth factor β, interleukin-4, and interleukin-10. *Ann N Y Acad Sci* **685**: 713-739.

Borysowski J & Górski A (2008) Is phage therapy acceptable in immunocompromised host? *Int J Infect Dis* **52**: 64-67.

Durant JA, Lowry VK, Nisbet DJ, Stanker LH, Corrier DE & Ricke SC (2000) Short chain fatty acids alter Hep-2 cell association and invasion by stationary growth phase *Salmonella typhimurium*. *J Food Sci* **65**: 1206-1209.

Finstad S, O'Bryan CA, Marcy JA, Crandall PG & Ricke SC (2012) *Salmonella* and broiler processing in the United States: relationship to foodborne salmonellosis. *Food Res Int* **45**: 789-794.

Giansanti F, Giardi MF & Botti D (2006) Avian cytokines - An overview. *Curr Pharm Des* **12**: 3083-3099.

Goode D, Allen VM & Barrow PA (2003) Reduction of experimental *Salmonella* and *Campylobacter* contamination of chicken skin by application of lytic bacteriophages. *Appl Environ Microbiol* **69**: 5032-5036.

Greenbaum D, Colangelo C, Williams K & Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol* **4:** 117.

He H, Genovese KJ & Kogut MH (2011) Modulation of chicken macrophage effector function by TH1/TH2 cytokines. *Cytokine* **53**: 363-369.

Higgins JP, Higgins SE, Guenther KL, Huff W, Donoghue AM, Donoghue DJ & Hargis BM (2005) Use of a specific bacteriophage treatment to reduce *Salmonella* in poultry products. *Poult Sci* **84**: 1141-1145.

Higgins SE, Higgins JP, Bielke LR & Hargis BM (2007) Selection and application of bacteriophages for treating *Salmonella* Enteritidis infections in poultry. *Int J Poult Sci* **6**: 163-168.

Huff WE, Huff GR, Rath NC & Donoghue AM (2010) Immune interference of bacteriophage efficacy when treating colibacillosis in poultry. *Poult Sci* **89**: 895-900.

Joerger RD (2003) Alternatives to antibiotics: bacteriocins, antimicrobial peptides and bacteriophages. *Poult Sci* **82**: 640-6407.

Lee JY, Song JJ, Wooming A, Li X, Zhou H, Bottje WG & Kong B-W (2010) Transcriptional profiling of host gene expression in chicken embryo lung cells infected with laryngotracheitis virus. *BMC Genomics* **11**: 445.

Leverentz B, Conway WS, Camp MJ, Janisiewicz WJ, Abuladze T, Yang M, Saftner R & Sulakvelidze A (2003) Biocontrol of *Listeria monocytogenes* on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Appl Environ Microbiol* **69**: 4519-4526.

Levin BR & Bull JJ (2004) Population and evolutionary dynamics of phage therapy. *Nat Rev Microbiol* **2**: 166-173.

Liu H, Zhang M, Han H, Yuan J & Li Z (2010) Comparison of the expression of cytokine genes in the bursal tissues of the chickens following challenge with infectious bursal disease viruses of varying virulence. *Virol J* **7**: 364.

Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**: 402-408.

Marietto-Gonçalves GA, Lima ET, Donato TC, Rocha TS, Álvarez LMC, Sequeira JL & Filho RLA (2011) Eradication of *Salmonella* Typhimurium in broiler chicks by combined use of P22 bacteriophage and probiotic. *Microbiology Research* **3**: e2

Merril CR, Biswas B, Carlton R, Jensen NC, Creed GJ, Zullo S, Adhya S (1996) Long-circulating bacteriophage as antibacterial agents. *Proc Natl Acad Sci USA* **93**: 3188-3192.

Nakai T & Park SC (2002) Bacteriophage therapy of infectious diseases in aquaculture. *Res Microbiol* **153**: 13-18.

Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y & Fisher PB (2004) Interleukin-10 and related cytokines and receptors. *Annu Rev Immunol* **22**: 929-979.

Pope WH, Haase-Pettingell C & King J (2004) Protein folding failure sets high-temperature limit on growth of phage P22 in *Salmonella enterica* serovar Typhimurium. *Appl Envrion Microbiol* **70**: 4840-4847.

Ricke SC (2003a) Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. *Poult Sci* **82**: 632-639.

Ricke SC (2003b) The gastrointestinal tract ecology of *Salmonella* Enteritidis colonization in molting hens. *Poult Sci* **82**: 1003-1007.

Ricke SC, Hererra P & Biswas D (2012) Ch 23. Bacteriophages for potential food safety applications in organic meat production. (*Ricke SC, Van Loo EJ, Johnson MG* & *O'Bryan CA* eds), pp. 407-424. Wiley Scientific/IFT, New York, NY.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones JL & Griffin PM (2011) Foodborne illness acquired in the United States-major pathogens. *Emerg Infect Dis* **17**: 7-15.

Schroder K, Hertzog PJ, Ravasi T & Hume DA (2004) Interferon-γ: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**: 163-189.

Sheng H, Knecht HJ, Kudva IT & Hovde CJ (2006) Application of bacteriophages to control intestinal *Escherichia coli* O157: H7 levels in ruminants. *Appl Envrion Microbiol* **72**: 5359-5366.

Smith HW, Huggins MB & Shaw KM (1987) The control of experimental *Escherichia coli* diarrhoea in calves by means of bacteriophages. *J Gen Microbiol* **133**: 1111-1126.

Stone R (2002) Stalin's forgotten cure. *Science* **298**: 728-731.

Toro H, Price SB, McKee AS, Hoerr FJ, Krehling J, Perdue M & Bauermeister L (2005) Use of bacteriophages in combination with competitive exclusion to reduce *Salmonella* from infected chickens. *Avian Dis* **49**: 118-124.

Vandeplas S, Dauphin RD, Beckers Y, Thonart P & Théwis A (2010) *Salmonella* in chicken: current and developing strategies to reduce contamination at farm level. *J Food Prot* **73**: 774-785.

Wall SK, Zhang J, Rostagno MH & Ebner PD (2010) Phage therapy to reduce preprocessing *Salmonella* infections in market-weight swine. *Appl Envrion Microbiol* **76**: 48-53.

Witheanage GSK, Kaiser P, Wigley P, Powers C, Mastroeni P, Brooks H, Barrow P, Smith A, Maskell D & McConnell I (2004) Rapid expression of chemokines and proinflammatory cytokines in newly hatched chickens infected with *Salmonella enterica* serovar Typhimurium. *Infect Immun* **72**: 2152-2159.

Table 1. Primer pair sequences used in this study

Target	Primer	Sequence (5’ - 3’)	Amplicon size (bp)	Reference
IL-4	CIL-4 (AC2007)	TGT GCT TAC AGC TCT CAG TG TGG AGT AGT GTT GCC TGC TG	212	Abdul Carem *et al*., 2007
IL-8	CIL-8 (SHP-1)	GCT CTG TCG CAA GGT AGG AC GGC CAT AAG TGC CTT TAC GA	231	This study
IL-10	CIL-10 (AC2007)	AGC AGA TCA AGG AGA CGT TC ATC AGC AGG TAC TCC TCG AT	103	Abdul Carem *et al*., 2007
INF-γ	CINF-γ (SHP-1)	AGC CGC ACA TCA AAC ACA TA TCC TTT TGA AAC TCG GAG GA	192	This study
GAPDH	chGAPDH	GGC ACT GTC AAG GCT GAG AA TGC ATC TGC CCA TTT GAT GT	99	Lee *et al*., 2010

Table 2. Cytokine gene expression in response to 5 treatments

Treatment		Fold changes[§]			
		IL-4	**IL-8**	**IL-10**	**IFN-γ**
A	Näive cell	1^{E}	1^{E}	1^{E}	1^{E}
B	HD-11 + bacteriophage (P22)	1.1^{E}	52.3^{BC}	17.4^{DE}	17.5^{DE}
C	HD-11 + S. Typhimurium LT2	1.6^{E}	40.3^{CD}	2.3^{E}	9.3^{E}
D	HD-11 + S. Typhimurium LT2 + bacteriophage (P22)	1.5^{E}	68.2^{B}	2.7^{E}	5^{E}
E	HD-11 + S. Typhimurium LT2 + bacteriophage (P22) + gentamicin	-1.3^{E}	201.1^{A}	1.2^{E}	2.5^{E}

[§]Fold changes: Values with different superscript capital letters (A to E) in columns and rows are significantly different ($P < 0.05$).

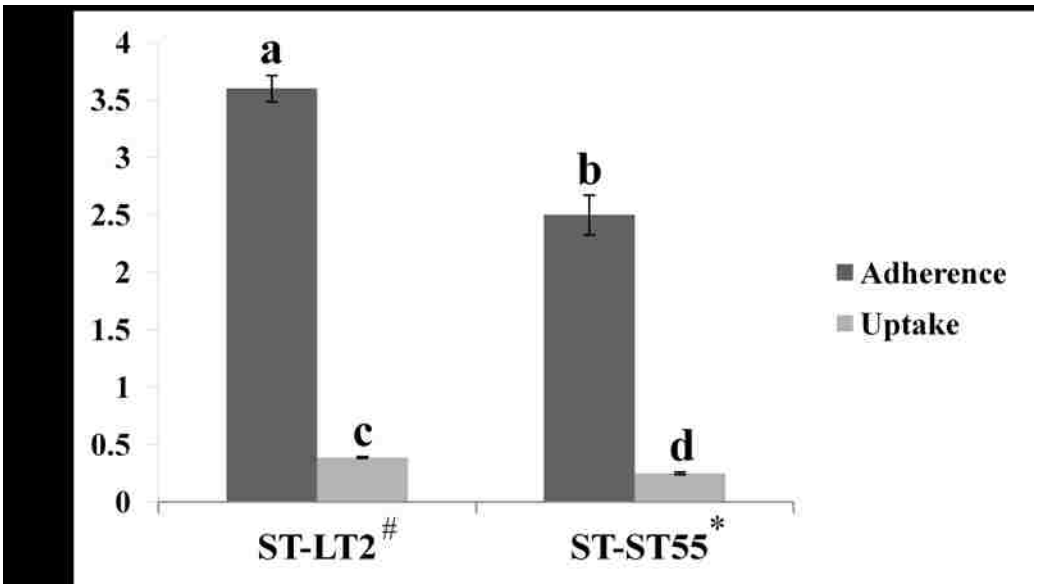


Figure 1. Adherence and uptake rate of HD-11 by LT2 and ST55 strains. Results were expressed as the percentage of adhered and invaded bacteria cells for three to five individual experiments. Different lower case letters (a to d) indicate significant differences within experiments ($P < 0.05$). ST-LT2[#]: *Salmonella* Typhimurium LT2 strain, ST-ST55*: *Salmonella* Typhimurium ST55 strain

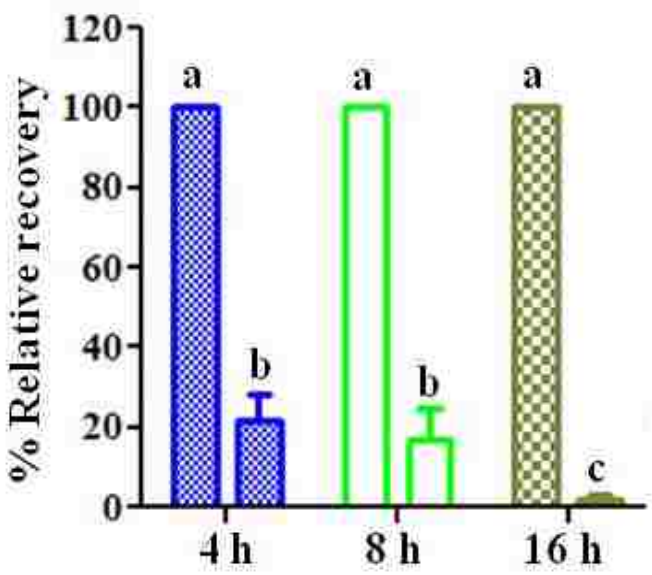


Figure 2. Effects of P22 on both extra- and intracellular LT2 recovery. Data showed the time period dependence of recovery. The relative percentage of recovery was determined as recovery presence of P22 divided by the recovery in the absence of P22 (i.e., 100% relative recovery). Different lower case letters (a to c) indicate significant differences within experiments ($P < 0.05$). Left bar stands for LT2 recovery without P22 and right bar stands for LT2 recovery with P22 at each time point

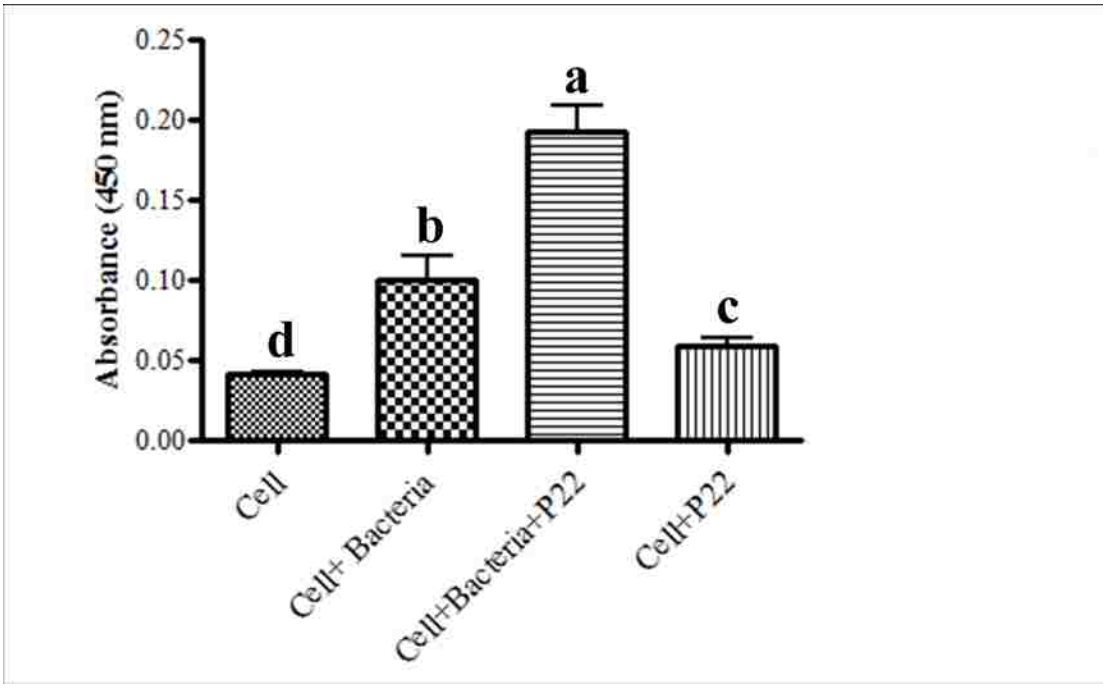


Figure 3. Supernatant IL-8 levels of HD-11 (cell) infected with LT2 and/or P22. Supernatant was collected from only HD-11, HD-11 infected with LT2, HD-11 cells infected with initially LT2 and later killed by P22 and HD-11 infected with P22. Different lower case letters (a to d) indicate significant differences within experiments ($P < 0.05$).

8. Appendix

8.1. Chapter 4 Publication by Journal

FEMS MICROBIOLOGY LETTERS

RESEARCH LETTER

Enhancement of chicken macrophage cytokine response to *Salmonella* Typhimurium when combined with bacteriophage P22

Si Hong Park[1,2], Debabrata Biswas[2], Jody Lingbeck[2], Ok Kyung Koo[2] & Steven C. Ricke[1,2]

[1]Cell and Molecular Biology Program, University of Arkansas, Fayetteville, AR, USA; and [2]Center for Food Safety, Department of Food Science, University of Arkansas, Fayetteville, AR, USA

Correspondence: Steven C. Ricke, Center for Food Safety, Department of Food Science, University of Arkansas, Fayetteville, AR 72704, USA. Tel.: +1 479 575 4678; fax: +1 479 575 6936; e-mail: sricke@uark.edu

Present address: Debabrata Biswas, Department of Animal and Avian Sciences, Center for Food Safety and Security Systems, University of Maryland, College Park, MD 20742, USA

Received 23 August 2012; revised 2 November 2012; accepted 13 December 2012. Final version published online 7 January 2013.

DOI: 10.1111/1574-6968.12064

Editor: Jeff Cole

Keywords
Salmonella Typhimurium; bacteriophage; immune response; ELISA; qRT-PCR.

Abstract

Salmonella infections are reported as the second most common pathogen caused foodborne disease in the United States, and several *Salmonella* serovars can colonize in the intestinal tracts of poultry. Reducing *Salmonella* in poultry is crucial to decrease the incidence of salmonellosis in humans. In this study, we evaluated the immune response of chicken macrophage cells (HD-11) and effects of bacteriophage P22 against the extra- and intracellular *S.* Typhimurium LT2. Four treatments, (1) HD-11 cells as control, (2) HD-11 cells with LT2, (3) HD-11 cells with LT2 and P22, and (4) HD-11 cells with P22, were administered, and IL-8 responses of HD-11 cells were measured using an ELISA. Also, four cytokine (IL-4, IL-8, IL-10, and IFN-γ) gene expression levels in the presence of LT2 and/or P22 were quantified by qRT-PCR. We found that P22 lysed the extra- and intracellular LT2, which adhered and were taken up by the HD-11 cells. The ELISA indicated that HD-11 cells produced significantly higher IL-8 cytokine levels in the supernatant during the intracellular lyses of LT2 by P22 ($P < 0.05$). The IL-8 expression levels measured by qRT-PCR also exhibited similar results with the IL-8 production based on ELISA measurements.

Introduction

Salmonella has the potential to cause fatal bacterial infections in infants and individuals with a suppressed immune system (Scallan *et al.*, 2011; Finstad *et al.*, 2012). The majority of the foodborne *Salmonella* serovars can colonize in the intestinal tracts of humans, and one of the major routes of human salmonellosis is believed to be consumption of contaminated poultry and poultry products (Finstad *et al.*, 2012). Thus, strategies for the control and prevention of poultry colonization are needed to further reduce the incidence of salmonellosis in humans. Currently, there are several preventative measures for limiting *Salmonella* establishments in poultry flocks including dietary alterations, prebiotics, probiotics, antimicrobials such as organic acids, and the administration of vaccine strains (Ricke, 2003a, b; Vandeplas *et al.*, 2010). However, there are very limited options for reducing already established *Salmonella* in the avian gastrointestinal tract (Toro *et al.*, 2005; Atterbury *et al.*, 2007).

In many developed countries, bacteriophage therapy was abandoned in favor of the development and widespread production of antibiotics (Stone, 2002). However, interest in phage therapy has gained momentum in animal production systems over the past few years as antibiotics have fallen out of favor (Nakai & Park, 2002; Joerger, 2003; Levin & Bull, 2004; Atterbury *et al.*, 2007; Ricke *et al.*, 2012). The bacteriophage P22 used in this study is able to bind specific somatic antigen structures of lipopolysaccharide (LPS) present in *Salmonella* serogroup A, B, and D1 including *S.* Typhimurium via tailspike proteins (TSP) (Marietto-Gonçalves *et al.*, 2011). P22

8.2. Copyright for Published Paper in Chapter 4

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Feb 26, 2013

This is a License Agreement between SI HONG PARK ("You") and John Wiley and Sons ("John Wiley and Sons") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by John Wiley and Sons, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3096610446917
License date	Feb 26, 2013
Licensed content publisher	John Wiley and Sons
Licensed content publication	FEMS Microbiology Letters
Licensed content title	Enhancement of chicken macrophage cytokine response to Salmonella Typhimurium when combined with bacteriophage P22
Licensed copyright line	A�� 2012 Federation of European Microbiological Societies. Published by Blackwell Publishing Ltd. All rights reserved
Licensed content author	Si Hong Park,Debabrata Biswas,Jody Lingbeck,Ok Kyung Koo,Steven C. Ricke
Licensed content date	Jan 7, 2013
Start page	137
End page	144
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Order reference number	
Total	0.00 USD

Terms and Conditions

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or a society for whom a Wiley Company has exclusive publishing rights in relation to a particular journal (collectively WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your Rightslink account (these are available at any time at http://myaccount.copyright.com)

Terms and Conditions

8.3. Authorship Statement for Chapter 4

Si Hong Park is the first author of the paper and completed at least 51% of the studies among coauthors which the title is "**Enhancement of Macrophage Chicken Cytokine Response to *Salmonella* Typhimurium When Combined with Bacteriophage P22**" in chapter 4.

Major Advisor: Dr. Steven C. Ricke

Date: June 4th, 2013

CHAPTER 5

Assessment of Production Performance and Transcriptomic Analysis in Small Intestines of Pasture Flock Raised Broiler Chickens Fed with Prebiotics

Si Hong Park[1,2], Irene Hanning[3], Jeong Yoon Lee[1], Byung-Whi Kong[1,4], Walter G. Bottje[4], Steven C. Ricke[1,2,4*]

[1]Cell and Molecular Biology Program, Department of Food Science, University of Arkansas, Fayetteville, AR

[2]Center for Food Safety and Department of Food Science, University of Arkansas, Fayetteville, AR

[3]Department of Food Science and Technology, University of Tennessee, Knoxville, TN

[4]Department of Poultry Science, University of Arkansas, Fayetteville, AR

Poultry Science 91: 3295-3299, 2012 (partial)

1. Abstract

Pasture flock raised poultry are becoming an increasingly popular product, but only limited options are currently available for maintaining gut health. For these producers, prebiotics are an attractive option because they are generally recognized as safe (GRAS) and can be mixed into the feed and thus do not require adjustments to production protocols. However, if prebiotic treatments reduce production performance, they would not be useful to producers. Thus, the objectives of this study were to measure performance of pasture flock raised broilers as well as transcriptomic analysis of small intestines fed one of three prebiotic treatments. The experimental design was replicated and birds were split into 4 groups, each group fed one feed additive 1) galactoligosaccharides (GOS; 2% W/W); 2) fructooligosacchrides (FOS; 1% W/W); 3) plum fibers (1% W/W); or 4) no additives. During the 6 week rearing period, 10 birds from each group were selected and euthanized to collect the small intestine. Throughout the study, mortality was monitored and body weight (BW) measurements were taken at 2 week intervals. There were no significant differences in BW at 2 wk of age bird. At 6 wk of age, those birds fed the GOS had a lower BW than the other 3 groups, and the group receiving feed supplemented with FOS had the highest final BW. Parts yields were also measured and results showed that there were no significant differences in parts yields among the treatments. In microarray data, a total of 1182, 2192 and 1845 differentially expressed genes in plum fibers, FOS and GOS were identified, respectively. Also, each 376, 713 and 628 genes were identified as functionally known genes and clustered into 63, 77, and 71 of biological functional group by IPA program.

2. Introduction

Organic and pasture flock poultry producers utilize a non-conventional system approach to poultry production. Some producers utilize mobile pens and move the pens on a routine basis in order to provide fresh pasture for the birds. Other producers may have a non-mobile housing unit with access to an enclosed outdoor area. There can be many variations of the design, but access to outdoors is given to the birds in all of these production systems (Tuytenns et al., 2011). Organic and pasture flock poultry products are becoming more popular and likewise the demand for these products is also increasing. Many consumers are attracted to these types of products because they believe organic and pasture raised poultry products will improve consumer health as compared to conventionally raised poultry (Van Loo et al., 2011). One of the factors driving this attitude is the fact that organic and pasture flock producers reduce or eliminate the use of antibiotics and vaccines. As such, producers are limited in the types of intervention measures that can be utilized to keep birds healthy. Many producers have considered prebiotics and probiotics as potential feed additives to improve gut health and overall health of the flock (Donalson et al., 2007; 2008a, b).

Historically, several approaches such as antibiotics, probiotics and prebiotics have been widely used to improve broiler chicken performance and reduce enteric diseases caused by poultry products (Ricke and Pillai, 1999; Jones and Ricke, 2003; Patterson and Burkholder, 2003; Biggs et al., 2007; Huyghebaert et al., 2011; Callaway and Ricke, 2012; Siragusa and Ricke, 2012). Generally, antibiotics have been more widely used than other growth promoters due to the prominent features in improving growth rates and acting as prophylactic agents (Jones and Ricke, 2003; Huyghebaert et al., 2011). However, the emergence of multidrug resistant (MDR) pathogens and consumer demands for antibiotic free chickens has led to a push to the

development of alternatives in the poultry industry. Prebiotics have been considered as one of the potential replacements for antibiotics to resolve these concerns. A prebiotic can be defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson and Roberfroid, 1995). Prebiotics are not hydrolyzed by digestive enzymes in upper gastrointestinal tracts of the respective host but are selectively utilized by beneficial bacteria such as *Bifidobacteria* and *Lactobacillus* which are generally regarded as safe (GRAS) (Roberfroid, 1998; Swennen et al., 2006). Although many different types of prebiotics such as peptides, proteins and lipids can be utilized, oligosaccharides are the primary prebiotics because oligosaccharides can be hydrolyzed and fermented by gut bacteria (Gibson and Roberfroid, 1995; Ziemer and Gibson, 1998; Sako et al., 1999).

A variety of oligosaccharides prebiotics have been investigated to promote gut health in chickens (Patterson and Burkholder, 2003; Park and Oh, 2010). The prebiotic oligosaccharide, fructooligosaccharides (FOS) has been shown to increase beneficial bacteria and inhibit *Clostridium perfringens*, *Escherichia coli* and *Salmonella* colonization in the large intestines of poultry (Bailey et al., 1991; Hofacre et al., 2005; Biggs et al., 2007; Donalson et al. 2007, 2008a, b). Galactooligosaccharides (GOS) ranges from 2 to 6 sugar moieties created by enzymatic reaction using lactose as substrate and acquired from degradation of galactan side chains in pectin (Jones et al., 1997; Park and Oh, 2010). Since several prebiotics possess common physiological features with dietary fibers, many studies have been performed to establish specific mechanisms attributable to prebiotics and gut microflora in humans and animals. Shifts to higher levels of short chain fatty acids (SCFA) such as acetate, propionate and butyrate produced by increasing beneficial bacteria and/or enhancing fermentation activities of specific gut microflora

metabolizing dietary prebiotics can lead to decreases pathogenic bacteria (Delzenne and Williams, 1998; Ricke, 2003). Furthermore, prebiotics have also been associated with lipid metabolism in a variety of animals that exhibit decreased hepatic and triglycerol levels in blood serum (Cheng and Lai, 2000; Delazenne and Kok, 2001).

To our knowledge, although prebiotic effects on gut microflora shifts in humans and animals have been extensively investigated, understanding the relationship between prebiotics and host metabolism at the molecular level has remained elusive. Notably, lipid metabolism is highly associated with prebiotics and needs to be studied at the molecular level. The goal of this study was to evaluate performance productions of broiler chickens after treatment with three different prebiotic supplements. Additionally, lipid metabolism in broiler chickens given prebiotics supplements was investigated. To do this, microarrays were conducted to evaluate different gene expressions according to prebiotic treatments using samples of small intestinal cells and ingenuity pathway analysis (IPA) software was used to analyze functional networks among up- or down-regulated genes based on microarray data.

3. Materials & Methods

3.1. Experimental Birds and Housing

A total of 340 day-of-hatch Cornish Cross White Plymouth Rock commercial broiler chicks were obtained from a local hatchery (Cobb 500; Cobb-Vantress, Fayetteville, AR, USA). The birds were split into 4 groups for a total of 85 birds in each pen. For the first 2 wk of life, the birds were placed in conventional housing pens measuring approximately 50 ft^2 (4.65 m^2). The floors of the pens were lined with wood shavings, nipple drinking units to provide water, and floor pans to provide feed. At 2 wk of age, birds were moved into outdoor pens measuring

approximately 80 ft^2 (7.23 m^2) which consisted of a wood base with wire mesh sides and roofing. Plastic tarps were used on the roofs to shield the birds from excess sun and rain. The feed and water access was similar to the indoor access. Birds had access to feed and water *ad libitum* for the duration of the experiment with the exception of a 12 h feed withdrawal at the end of the experiment. On a weekly basis, the pens and birds were moved to fresh pasture that was located 10 feet from the previous locations and had not been previously used for poultry rearing purposes.

3.2. Prebiotic and Feed Formulation

Three prebiotics were added to the starter and finisher feeds in each group and fed with water *ad libitum*. Each group consisted of 1) control (no prebiotic), 2) plum fibers (California Dried Plum Board, Sacramento, CA, USA) added at 1 Kg per ton of feed, 3) fructooligosacharrides (FOS; GTC Nutrition, Golden, CO, USA) added at 1 Kg per ton of feed and galactooligosacharrides (GOS; GTC Nutrition) added at 2 Kg per ton of feed respectively. Feed was supplemented with the prebiotics consistently during the experimental period. Every 2 wk, a total 10 birds from each group were selected randomly for necropsy, transported to the laboratory and euthanized humanely using CO_2. The extracted small intestines were immediately transferred to Trizol reagent (Invitrogen, Carlsbad, CA, USA) for RNA isolation as described in the following section.

3.3. Performance Measurement and Processing

Throughout the duration of the study, mortality was noted in each group. Every 2 wk, a total of 10 birds from each treatment group were removed from the pens, euthanized humanely,

and weighed. On 42 day of the study, the birds were processed for this portion of the study using commercial methods (Mehaffey et al., 2006). From each of the 4 treatment groups, all remaining birds were processed (n = 36 to 46). Tagged carcasses were weighed after evisceration and the carcasses were sectioned into 5 pieces consisting of legs, wings, breasts, tenders, and the remaining rack including skin. Separate weight data points were collected for each of these pieces. Carcasses were chilled for 4 h at 4˚C before deboning. Yield was expressed as a percentage of ready-to-cook carcasses (without giblets).

3.4. Statistical Analysis

Data were analyzed by ANOVA using the GLM procedure of SAS (SAS Institute Inc., 2002). The mean of treatments were separated by LSMEANS analysis. A probability of $P < 0.05$ was prerequisite for statistical significance. Each pen was considered as one experimental unit.

3.5. Total RNA Isolation

Total RNA of small intestine from each treatment was isolated using Trizol reagent to perform microarray and quantitative reverse transcriptase PCR (qRT-PCR). One mililiter of Trizol reagent was added to 200 mg of small intestines and homogenized immediately. Total RNA was isolated according to Linton's method (Linton et al., 2010) and DNase I (New England Biolabs, Ipswich, MA, USA) was treated for 1 h at 37˚C to eliminate possible contaminating genomic DNA. Total RNA was subsequently purified by Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturer's instruction, and the concentration and purity were measured by a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA). The quality of total RNA was evaluated by 1% agarose gel electrophoresis.

3.6. Microarray

The microarray system was designed to compare the control and each of the three different prebiotics treatments. A two color labeling system was applied to generate complementary RNA (cRNA) probes labeled with fluorescence using a Two Color Microarray Quick Labeling kit (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's instruction. In order to evaluate dye effects on labeled cRNA, the spike-in controls including two sets of ten synthesized RNA mixtures were used following the manufacturer's instruction (Zahurak et al., 2007). Prepared spike-in mixtures were added to either control or prebiotics treatments and co-hybridized with arrays.

In brief, 2 μg of total RNA mixed with spike-in were transcribed into cDNA by reverse transcriptase and oligo dT primers and subsequently, T7 RNA polymerase synthesized cRNA and labeled with Cy3 for control or Cy5 for prebiotic treatment. The labeled cRNA probes were purified using the Qiagen RNeasy Mini Kit (Qiagen). The concentrations and labeled cRNA probes quality were measured by NanoDrop ND-1000 (Thermo Scientific). Each 815 ng of Cy3 and Cy5 labeled cRNA were used for hybridization onto a 4 X 44 Agilent custom chicken oligo microarray chip. After 16 h hybridization, the slides were washed using commercial wash buffers (Agilent Technologies) and scanned by a GenePix 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA, USA). This experiment was repeated as four replicates.

3.7. Microarray Data Analysis

To normalize background-corrected red and green color intensities of each spot, locally weighted scatterplot smoothing linear regression (LOWESS) normalization method was utilized

to remove unexpected systematic variations during microarray experiments. Each spot showing both foreground intensity of >100 and signal to noise ratio (SNR) of >3 were screened as reliable signals as well as Spike-ins were considered as reference ratios reported previously (Zahurak et al., 2007). Normalized genes were analyzed by one-way ANOVA test to identify significant differential expressed genes over time. All statistically analyses were performed using Microsoft Excel 2010 and JMP Genomics 5.0 (SAS Institute, Cary, NC, USA) licensed with Cell and Molecular Biology Graduate Program, University of Arkansas, Fayetteville, AR, USA.

3.8. Quantitative Reverse Transcriptase PCR (qRT-PCR)

The qRT-PCR assay was optimized using an Eppendorf Masterplex thermocycler ep Gradient Technology (Eppendorf, Westbury, NY, USA). High up- or down-regulated genes at 6 week as well as presented in three treatments were selected to evaluate expression levels with microarray data. Primer pairs for selected genes were designed using Primer 3 program (http://frodo.wi.mit.edu/primer3/input.htm), and primer pair based on GAPDH gene was adopted from previous report as endogenous control for relative quantification (Lee et al., 2010). All primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA, USA, USA) and information is listed in Table 1. The total 20 μl of reaction mixture consisted of 500 ng of total RNA, 500 nM of each primers, 10 μl of EXPRESS SYBR GreenER^{TM}qPCR SuperMix with Premixed ROX (Invitrogen), 0.5 μl of EXPRESS SuperScript Mix for One-Step SYBR GreenER (Invitrogen) and DEPC water to final volume. The qRT-PCR was performed with the reaction conditions of 50˚C for 5 min for the synthesis of cDNA from total RNA, then followed by 40 cycles of 95˚C for 15 s, 57˚C for 15 s and 68˚C for 20 s. Melting curves were subsequently created which consisted of 95˚C for 15 s, 60˚C for 20 min. increasing by 0.5˚C per minute to a

final temperature of 95˚C. All PCR products were electrophoresed onto 1% agarose gel for confirmation as well as each experiment was repeated in triplicate. The relative differential gene expressions were calculated by the $2^{-\Delta\Delta CT}$ method.

3.9. Bioinformatics Using Ingenuity Pathway Analysis (IPA)

The Ingenuity Pathways Analysis (IPA; Ingenuity Systems®) 6.5 software was used in this study to interpret the functional connections among differentially expressed genes and molecular networks. The IPA program can be used as a bioinformatics tool to identify biological mechanisms at molecular levels in biological researches. The differentially expressed genes based on microarray data in each treatment over time were categorized by biological functions with an appropriate fold-change values and *p*-values based on the IPA database and the crucial associated genes were identified. Since the IPA program can potentially generate numerous networks with selected molecules, numbers of generated networks were limited to 10 and 35 molecules in each network, respectively.

4. Results and Discussion

4.1. Body Weight (BW), Mortality and Yield

Body weight (BW) of the birds was dependent upon the feed additive and time of BW measurement (Table 2). There were no significant differences in BW at 2 wk of age bird. At 6 wk of age, those birds fed the GOS had a lower BW than the other 3 groups and the group receiving feed supplemented with FOS had the highest final BW and average mortality was 4.4% (data not shown). With respect to feed treatments, average mortality was the lowest ($P < 0.05$) for birds given feed supplemented with GOS (2%), and there were no significant differences in

mortality among the other 3 treatment groups. Parts yields were also measured and results show that there were no significant differences in parts yields among the treatments (Table 3).

Gut health has been equated to and may be used as a measurement of overall health of birds (Manning et al. 2007). To improve gut health, products such as probiotics and prebiotics are used to enhance the concentrations of beneficial bacteria in the host gut. Gut health and overall health can be measured using performance standards such as weight gain, mortality, and intestinal histological measurements (Thompson and Applegate, 2006; Manning et al., 2007). However, other measurements including carcass yield and feed conversion are critical for producer profits. Treatments that decrease any performance measurements are not useful for producers. For example, Jarquin et al. (2007) used organic acids and probiotics as treatments in broilers to eliminate *Salmonella* from the gastrointestinal tract and crop, but found that weight was significantly reduced in the treatment groups. The authors concluded that the birds were refusing the water due to the off flavor and odor, which subsequently reduced weight gain. Additional studies have been performed with various organic acids and some studies reported similar results but varied depending on the concentration and acid used (Dibner and Buttin, 2002). For feed additives specifically, some feed supplements can reduce the passage rate of feeds (Cave, 1984; Dibner and Buttin, 2002). This can reduce the intake of feed and result in a numerical increase in feed efficiency. However, the end result is a smaller bird, which may not necessarily improve profitability for a producer. The data from this study indicated that GOS, FOS, and plum fibers could be used and did not reduce production performance. Feed additives have been evaluated for improving overall yield. Typically, proteases are added to improve the amino acid utilization, which in turn may increase the size of the muscle (Fisher, 1993; Cafe et al., 2002).

The feed additives used in this study were nondigestible carbohydrates and should not have any improvement on amino acid utilization, but could possibly reduce the performance in ways such as reducing passage rate or reducing amino acid utilization. However, the data indicated that none of the treatments reduced the yield of the bird performance parameters measured in this study. Additional studies should be done to confirm these results over several seasons and with more flocks. In conclusion, the prebiotic feed additives used in this study did not reduce the final weight gain, had no effect on mortality and did not decrease yield of the carcass. Thus, these feed additives can be used by producers without reducing production parameters. Future studies include investigating other effects the prebiotics may have on other factors including assessing any changes in the gut microflora.

4.2. Microarray

To identify different transcriptional gene expression levels between control (no prebiotics) and each prebiotics treatment, microarrays including whole chicken genomes were conducted using total RNA isolated from small intestine. A total of 44K probes on slides were evaluated in each microarray assay using 2, 4 and 6 weeks samples of each prebiotics treatment. The dye-swaps in two of four total replicates were performed and no possible dye effects were detected (data not shown). At least 2 fold up- or down-regulated genes at one of the time points were selected and confirmed as significant differences via one-way ANOVA test with the JMP Genomics 5.0. A total of 1182, 2192 and 1845 differentially expressed genes in plum fibers, FOS and GOS were identified, respectively. These selected genes were analyzed by IPA software to generate gene networks and functional annotations.

4.3. qRT-PCR

To confirm the microarray results, 15 genes commonly present in three treatments were randomly chosen and qPCR with GAPDH gene as internal control was performed with the same total RNA originally used in the microarrays. The microarray values were calculated by $\log_2$ fold changes while those of qPCR were determined by $2^{-\Delta\Delta Ct}$ and then converted to $\log_2$ value to compare with microarray values. The fold changes comparison between microarray and qRT-PCR were well-matched (Table 4).

4.4. Functional gene ontology

Recently, several bioinformatics tools have been developed for analysis of biological relationship between diffentially expressed fuctional genes acquired from microarray data. The IPA, one of bioinformatics programs, was utilized to analyze relevance of functional gene ontologies and genetic networks. Each ot the 376, 713 and 628 genes were identified as functionally known genes and clustered into 63, 77 and 71 biological functional group by IPA program in plum fibers, FOS and GOS treatments, respectively. Each of the top 10 groups that included the greatest number of genes is represented in Figure 1. Six out of 10 groups were commonly presented in three treatments and associated with small molecule biochemistry, lipid metabolism, molecular transport, genetic disorder, dermatological diseases and conditions and gastrointestinal disease. Among these functional groups, the lipid metabolism group was further investigated to verify the correlation with administration of the prebiotics.

In the development of numerous bioinformatics database utilization, several studies for transcriptomic profiling in chickens have been accomplished (Désert et al., 2008; Zheng et al., 2009; Ciraci et al., 2010; Higgins et al., 2011; Sibut et al., 2011). Désert et al. (2008)

investigated metabolic responses including energy-metabolic response in the liver of 4 wk male chickens using microarray and bioinformatics tools. They found 1162 of differentially expressed genes in chicken liver at feeding-to-fasting transition and analyzed genetic networks using IPA. After 16 h of fasting, genes associated with glucogenesis, peroximal fatty acid beta oxidation and ketogenesis were up-regulated while genes involved in fatty acid and cholesterol systhesis were down-regulated. Zheng et al. (2009) identified 543 genes involved in regulation of muscle growth at 1, 2, 4, 6, and 8 wks of broilers and layer chickens as well as gene ontology analysis was performed using GOEAST software. Interestingly, Sibut et al. (2011) utilized lean and fat chickens for transcriptomic analysis to investigate differentially expressed genes associated with muscle glycogen contents and meat quality. This is the first study for understanding of molecular mechanisms in chicken meat quality.

4.5. Gene Networks Analysis

Gene networks mapped by IPA program represented the interaction among focus molecules with fold change values. Although various assay setting conditions for generating networks were presented, the simplest assay condition consisted of 10 networks and 35 focus molecules was adopted to analyze the large number of genes. Of the networks generated 6, 4 and 4 out of each of the 10 networks in plum fibers, FOS and GOS treatments respectively were associated with lipid metabolism (Table 5), because many prebiotics were involved in lipid metabolism with a putative influence in animals and humans (Delzenne and Wiliams 2002). Thus, networks related to lipid metabolism are discussed in the following section.

4.5.1. Plum Fibers Treatment

Network #1 generated based on plum fibers supplemented chicken is closely related to lipid metabolism, small molecule biochemistry and molecular transport pathway with 29 focus molecules over time (Figure 2). Interestingly, acetyl-CoA C-acyltransferase appeared to associate with four core molecules (ACAA1, ACAA2, HADHA and HADHB). The expression level of ACAA1 (acetyl-CoA acyltransferase 1) and ACAA2 (acetyl-CoA acyltransferase 2) molecules which are associated with catabolism of long chain fatty acids through beta-oxidation were increased over 4 fold changes at 6 wk compared to 2 and 4 wks samples (He et al., 2011; Schlüter et al., 2011). Both HADHA (hydratase alpha subunit) and HADHB (hydratase beta subunit) molecules were also increased over time. Since these two molecules play important roles in long chain fatty acids metabolism, the fatty acids without sufficient level of both enzymes cannot be converted to energy and cause disease such as lethargy and hypoglycemia (Choi et al., 2007). FABP6 (fatty acid binding protein 6) which is involved in fatty acid uptake and transport is a highly conserved cytoplasmic protein that binds long chain fatty acids and bile acids (Hughes and Piontkivska, 2011). This molecule was increased over time along with SCARB1 (scavenger receptor class B, member 1) that is an integral membrane transporter protein identified in liver. The principal role of SCARB1 in the liver is to take up cholesterol from lipoprotein (Daniels et al., 2011).

4.5.2. FOS Treatment

Network #6 in FOS treatment is associated with lipid metabolism, small molecule biochemistry and molecular transport pathway with 29 focus molecules (Figure 3) as well as all molecules were highly up-regulated at 6 wk compared to 2 and 4 wk chickens. Both PPARA (peroxisome proliferator-activated receptor alpha) and PPARD (peroxisome proliferator-

activated receptor delta) acted directly on ACOX1 (acyl-CoA oxidase 1) which is the first enzyme for fatty acid beta oxidation-pathway. ACOX1 is responsible for pseudoneonatal adrenoleukodystrophy caused by accumulation of long chain fatty acids (El Hajj et al., 2012). PPARA is a ligand-dependent nuclear receptor and major regulator for lipid metabolism in liver. In general, PPARA is activated through ligand-binding and associated with fatty acid transport and peroximal fatty acid beta-oxidation (Harris and Finck, 2011). Both ACADS (acyl-CoA dehydrogenase short chain) participated in long chain fatty acid to generate acyl-CoA and ELOVL6 (elongation of long chain fatty acids) were affected by PPARA (He et al., 2010; Schlüter et al., 2011).

4.5.3. GOS Treatment

Network #1 in GOS treatment is related to lipid metabolism, small molecule biochemistry and molecular transport pathway with 29 focus molecules (Figure 4). Although most of the molecules associated with lipid metabolism are the same compared to plum and FOS treatments, differential expression levels of each molecule are higher than both treatments. FABP5 (fatty acid binding protein 5) found in epidermal cells and FABP6 (fatty acid binding protein 6) in ileal cells are up-regulated over a 9 fold change at 6 wk sample. Both molecules are highly conserved cytoplasmic transporters that bind long-chain fatty acids and hydrophobic ligands (Veerkamp et al., 1999; Hughes and Piontkivska, 2011) and ACAA1 molecule also increased in the 6 wk sample. PHYH (phytanoyl-CoA hydroxylase), one of cytoplasmic enzyme interact with ABCD3 (ATP-binding cassette, subfamily D) to transport enzyme into cells and this enzyme is associated with alpha-oxidation of branched chain fatty acids in peroxisomes (Mihalik et al., 1997).

5. Conclusion

In this study, we have evaluated the effect of three prebiotics on the bird performance and trancriptomic analysis of small intestines using molecular techniques and bioinformatics tools. Since there were no significant differences in BW, mortality and parts yield among treatment, prebiotics used in this study have no side effects on productivity. Furthermore, prebiotics can increase several protein expressions associated with lipid metabolism in small intestines. The breakdown of long chain fatty acids in the gut might increase beneficial bacteria as well as promote gut health. In order to confirm the effects of fatty acids on gut microflora, next generation sequencing needs to be performed.

6. References

Baily, J.S., L. C. Blankenship, and N. A. Cox. 1991. Effect of fructooligosaccharide on *Salmonella* colonization of the chicken intestine. Poult. Sci. 79:2433-2438.

Biggs, P., C. M. Parson, and G. C. Fahey. 2007. The effects of several oligosaccharides on growth performance, nutrient digestibilities, and cecal microbial population in young chicks. Poult. Sci. 86:2327-2336.

Cafe, M. B., C. A. Borges, C. A. Fritts, and P. W. Waldroup. 2002. Avizyme improves performance of broilers fed corn-soybean meal-based diets. J. Appl. Poult. Res. 11:29-33.

Callaway, T. R. and S. C. Ricke (Eds.), 2012. Direct Fed Microbials/Prebiotics for Animals: Science and Mechanisms of Action, Springer Science, New York, NY, 206 pp.

Cave, N.A. 1984. Effect of propionic and lactic acids on feed intake by chicks. Poult. Sci. 63:131-4.

Cheng, H. H., and M. H. Lai. 2000. Fermentation of resistant rice starch produces propionate reducing serum and hepatic cholesterol in rats. J. Nutr. 130:1991-1995.

Choi, J. -H., H. -R. Yoon, G. -H. Kim, S. -J. Park, Y. -L. Shin, and H. -W. Yoo. 2007. Identification of novel mutations of the *HADHA* and *HADHB* genes in patients with mitochondrial trifunctional protein deficiency. Int. J. Mol. Med. 19:81-87.

Ciraci, C., C. K. Tuggle, M. J. Wannemuehler, D. Nettleton, and S. J. Lamont. 2010. Unique genome-wide transcriptome profiles of chicken marcrophages exposed to *Salmonella*-derived endotoxin. BMC Genomics. 11:545.

Daniels, T. F., X. -L. Wu, Z. Pan, J. J. Michal, R. W. Wright Jr., K. M. Killinger, M. D. MacNeil, and Z. Jiang. 2011. The reverse cholesterol transport pathway improves understanding of genetic networks for fat deposition and muscle growth in beef cattle., PLoS ONE 5:e15203.

Delzenne, N. M., and N. Kok. 2001. Effects of fructan-type prebiotics on lipid metabolism, Am. J. Clin. Nutr. 73(Suppl): 456S-458S.

Delzenne, N. M., and C. M. Williams. 1998. Prebiotics and lipid metabolism. Cur. Opin. Lipidol. 13:61-67.

Désert, C., M. J. Duclos, P. Blavy, F. Lecerf, F. Moreews, C. Klopp, M. Aubry, F. Herault, P. L. Roy, C. Berri, M. Douaire, C. Diot, and S. Lagarrigue. 2008. Transcriptome profiling of the feeding-to-fasting transition in chicken liver. BMC Genomics 9:611.

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. Poult. Res. 11:453-463.

Donalson, L. M., W. -K. Kim, V. I. Chalova, P. Herrera, C. L. Woodward, J. L. McReynolds, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2007. In vitro anaerobic incubation of *Salmonella enterica* serotype Typhinmurium and laying hen cecal bacteria in poultry feed substrates and a fructooligosaccharide prebiotic. Anaerobe. 13: 208-214.

Donalson, L. M., W. K. Kim, V. I. Chalova, P. Herrera, J. L. McReynolds, V. G. Gotcheva, D. Vidanović, C. L. Woodward, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2008a. In vitro fermentation response of laying hen cecal bacteria to combinations of fructooligosaccharide prebiotics with alfalfa or a layer ration. Poult. Sci. 87: 1263-1275.

Donalson, L. M., J. L. McReynolds, W. -K. Kim, V. I. Chalova, C. L. Woodward, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2008b. The influence of a fructooligosaccharide prebiotic combined with alfalfa molt diets on the gastrointestinal tract fermentation, *Salmonella* Enteritidis infection, and intestinal shedding in laying hens. Poult. Sci. 87:1253-1262.

El Hajj, H. I., A. Vluggens, P. Andreoletti., K. Ragot, S. Mandard, S. Kersten, H. R. Waterham, G. Lizard, R. J. A. Wanders, J. K. Reddy, and M. Cherkaoui-Malki. 2012. The inflammatory response in acyl-CoA oxidase 1 deficiency (pesudoneonatal adrenoleukodystrophy). Endocrinology. 153:2568.

Fisher, C. 1993. Impact of amino acids on carcass quality in broilers. Pages 33-46 in Proc. Ark. Nutr. Conf. (Tech. Symp.). Fayetteville, AR. Univ. Arkansas, Fayetteville.

Gibson, G. R., and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota-Introducing the concept of prebiotics. J. Nutr. 125:1401-1412.

Harris, T. E., and B. N. Finck. 2011. Dual function lipin proteins and glycerolipid metabolism. Trends Endocrinol. Metab. 22:226-233.

He, M., Z. Pei, A. -W. Mohsen, P. Watkins, G. Murdoch, P. P. Van Veldhoven, R. Ensenauer, and J. Vockley. 2011. Identification and characterization of new long chain Acyl-CoA dehydrogenases. Mol. Genet. Metab. 102:418-429.

Higgins, S. E., A. D. Wolfenden, G. Tellez, B. M. Hargis, and T. E. Porter. 2011. Transcriptional profiling of cecal gene expression in probiotic- and *Salmonella*-challenged neonatal chicks. Poult. Sci. 90:901-913.

Hofacre, C. L., G. F. Mathis, and M. A. Quiroz. 2005. Natural alternatives to prevent necrotic enteritis. Int. Poult. Prod. 13:7-9.

Hughes, A. L., and H. Piontkivska. 2011. Evolutionary diversification of the avian fatty acid-binding proteins. Gene. 490:1-5.

Huyghebaert, G., R. Ducatelle, and F. van Immerseel. 2011. An update on alternatives to antimicrobial growth promoters for broilers. Vet. J. 187:182-188.

Jarquin R., G. M. Nava, A. D. Wolfenden, A. M. Donoghue, I. Hanning, S. Higgins, and B. M. Hargis. 2007. The evaluation of organic acids and probiotic cultures to reduce *Salmonella* enteriditis horizontal transmission and crop infection in broiler chickens. Int. J. Poult. Sci. 6:182-186.

Jones, F. T., and S. C. Ricke. 2003. Observation on the history of the development of antimicrobials and their use in poultry feeds. Poult. Sci. 82:613-617.

Jones, L., G. B. Seymour, and J. P. Knox. 1997. Localization of pectin galactan in tomato cell walls using a monoclonal antibody specific to (1[->]4)-[beta]-D-galactan. Plant Physiol. 113:1405-1412.

Linton, K. M., Y. Hey, S. Dibben, C. J. Miller, A. J. Freemont, J. A. Radford, and S. D. Pepper. 2010. Extraction of total RNA from fresh/frozen tissue (FT). Biotechnique Protocol Guide. p.53.

Lee. J. Y., J. J. Song, A. Wooming, X. Li, H. Zhou, W. G. Bottje, and B. -W. Kong. 2010. Transcriptional profiling of host gene expression in chicken embryo lung cells infected with laryngotracheitis virus. BMC Genomics. 11:445.

Manning, L., S. A. Chadd, and R. N. Baines. 2007. Key health and welfare indicators for broiler productions. World's Poultry Sci. J. 63:46-62.

Mehaffey, J. M., S. P. Pradhan, J. F. Meullenet, J. L. Emmert, S. R. McKee, and C. M. Owens. 2006. Meat quality evaluation of minimally aged broiler breast fillets from five commercial genetic strains. Poult. Sci. 85:902-908.

Mihalik, S. J., J. C. Morrell, D. Kim, K. A. Sacksteder, P. A. Watkins, and S. J. Gould. 1997. Identification of PAHX, a Refsum disease gene. Nat. Genet. 17:185-189.

Park, A. R., and D. K. Oh. 2010. Galacto-oligosaccharide production using microbial beta-galactosidase: current state and perspectives. Appl. Microbiol. Biotechnol. 85:1279-1286.

Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82:627-631.

Ricke, S. C. 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poult. Sci. 82:632-639.

Ricke, S. C., and S. D. Pillai. 1999. Conventional and molecular methods for understanding probiotic bacteria functionality in gastrointestinal tracts. Crit. Rev. Microbiol. 25:19-38.

Roberfroid, M. B. 1998. Prebiotics and synbiotics: concepts and nutrional properties. Brit. J. Nutr. 80:S197-S202.

Sako, T., K. Matsumoto, and R. Tanaka. 1999. Recent progress on research and applications of non-digestible galacto-oligosaccharides. Int. Dairy J. 9:69-80.

Schlüter, A., I. Ruiz-Trillo, and A. Pujol. 2011. Phylogenomic evidence for a myxococcal contribution to the mitochondrial fatty acid beta-oxidation. PLoS ONE. 6:e21989.

Siragusa, G. R., and S. C. Ricke. 2012. Probiotics as pathogen control agents for organic meat production. In: Ricke SC, Van Loo EJ, Johnson MG, O'Bryan CA, editors. Organic meat production and processing. 1st ed. New York: Wiley Scientific/IFT. p 331-49.

Sibut, V., C. Hennequet-Antier, E. L. Bihan-Duval, S. Marthey, M. J. Duclos, and C. Berri. 2011. Identification of differentially expressed genes in chickens differing in muscle glycogen content and meat quality. BMC genomics. 12:112.

Swennen, K., C. M. Courtin, and J. A. Delcour. 2006. Non-digestible oligosaccharides with prebiotic properties. Crit. Rev. Food Sci. Nutr. 46:459-471.

Thompson, K. L., and T. J. Applegate. 2006. Feed withdrawal alters small-intestinal morphology and mucus of broilers. Poul. Sci. 85:1535-1540.

Tuytenns, F. A. M., B. Sonck, M. Staes, S. Van Gansbeke, T. Van den Bogaert, and B. Ampe. 2011. Survey of egg producers on the introduction of alternative housing systems for laying hens in Flanders, Belgium. Poult. Sci. 90:941-950.

Van Loo, E., V. V. Caputo, R.M. Nayga, Jr., J.-F. Meullenet, and S.C. Ricke. 2011. Consumers' willingness to pay for organic chicken breast: Evidence from choice experiment. Food Qual. Preference. 22:603-613.

Veerkamp, J. H., H. T. B. van Moerkerk, C. F. M. Prinsen, and T. H. van Kuppevelt. 1999. Structural and functional studies on different human FABP types. Mol. Cell. Biochem. 192:137-142.

Zahurak, M., G. Parmigiani, W. Yu, R. B. Scharpf, D. Berman, E. Schaeffer, S. Shabbeer, and L. Cope. 2007. Pre-processing Agilent microarray data. BMC Bioinformatics. 8:142.

Zheng, Q., Y. Zhang, Y. Chen, N. Yang, X. -J. Wang, and D. Zhu. 2009. Systematic identification of genes involved in divergent skeletal muscle growth rates of broiler and layer chickens. BMC Genomics. 10:87.

Ziemer, C. J., and G. R. Gibson. 1998. An overview of probiotics, prebiotics, synbiotics in the functional food concept: perspectives and future strategies. Int. Dairy J. 8:473-479.

Table 1. Primer pair sequences used in this study

Primers name	Accession number	Sequence	Gene symbol
PT-1	NM_205147	GGACTCATTGATTGGGCACT TCCAGCTCTGCCTGAATCTT	CYP1A4
PT-2	BU124208	TGGGAGCATGAACACGAATA CACCAGCAGTTGTAGGCAAA	LOC424523
PT-12	NM_001001751	AGCTCTGTCCCTGTGAAGGA TGGCCATAAGAGGAGTGAGG	CYP3A7
PT-14	NM_001001203	CATTCAGAACTCGTCCGACA GAATGCTGCAGGAAGTCACA	EREG
PT-18	Z68489	TGGTATGCCAACAGAAGCTG TTCACGTGATGTAGCCCAAA	LCT
PT-19	XM_414163	ACTGGGTATCCATCCATCCA CCAAAGCCCCATTGTTCTTA	BCMO1
PT-23	XM_414111	ACAAAAGCCTGCAAGGAAGA AGCCACATATCCGTTTCCAG	GPT2
PT-25	NM_205513	CAGGGTGTCAAAATGTGTGC AAGCTTCCCTCCATCAGACA	CALB1
PT-27	XM_416675	ACCAACAGGGTCTCAACCAG AGCTCCAGCTGACAGCATCT	LIPI
PT-53	NM_204322	GCAAACATTGAAGCTGTGGA CCATCCTGCCTTCCTCTATG	GLDC
PB-10	BX936187	AAGGTTGCAAACCTCAATGG CGCTGTTTGAATCTCTCACG	RDH5
PB-11	CR389474	CCACTGGAAAACCAGAAGGA ACAGGAGACTGTGGGGACAC	LRCH2
PB-14	NM_001008463	CCAGAAGGAGCACACAGGTT AGCAATTCACCAGCTCCTGT	PGS1
PB-16	NM_205240	GTGAAACCAGTTGCCAAGGT ATGCTGGGAAGTGCTATCGT	ST6GALNAC1
PB-27	NM_205268	TGGCTGCATACAGACAGGAG GGCTCTTCGTTTTCACAAGG	NOV

Table 2. Body weight of birds fed[1,2] one of 3 feed supplements 1) galactoligosaccharides[3]; 2) fructooligosacchrides[4]; 3) plum fibers[5] or 4) no feed additives

		Feed Additive			
	Bird age	**Control**	**Plum**	**GOS**	**FOS**
Cornish White rock cross broilers	2wks	250.7 ± 19.9[a]	208 ± 10.6[b]	227.4 ± 10.9[a]	231.8 ± 13.9[a]
	4wks	976.7 ± 43.2[a]	940.9 ± 30.9[a]	974.5 ± 49.9[a]	873.6 ± 52.3[b]
	6wks	1866 ± 74.8[a]	1867 ± 70.2[a]	1697 ± 64.6[b]	1924 ± 55.1[c]

[a,b] Within a row and at each age, means without a common superscript are significantly different ($P < 0.05$)

[1]Values are means ± SEM; n = 10

[2]For the first two weeks of life, chick starter formula was utilized and for the remaining 4 weeks of the experiments, a grower formula was utilized. Feed additives were given for the duration of the experiment (6 weeks).

[3]GOS; 2% W/W; GTC nutrition, Golden, CO, USA

[4]FOS; 1% W/W; GTC Nutrition, Golden, CO, USA

[5]Plum fibers; 1% W/W; California Dried Plum Board, Sacramento, CA, USA

Table 3. Parts yield[1] of carcasses expressed as a percentage of carcass yield without giblets (WOG) of birds fed[2] one of 3 feed supplements 1) galactoligosaccharides[3]; 2) fructooligosacchrides[4]; 3) plum fibers[5] or 4) no feed additives.

	Control N=43	**Plum N=37**	**FOS N=46**	**GOS N=36**
Wing %	11.1 ± 1.66[a]	10.6 ± 1.7[a]	11.0 ± 1.6[a]	10.66 ± 1.7[a]
Breast %	22.1 ± 3.3[a]	22.5 ± 3.6[a]	22.2 ± 3.2[a]	22.5 ± 3.6[a]
Tender %	5.7 ± 0.9[a]	5.6 ± 0.9[a]	5.4 ± 0.8[a]	5.6 ± 0.9[a]
Legs %	5.6 ± 0.9[a]	5.6 ± 0.9[a]	5.4 ± 0.8[a]	5.6 ± 0.9[a]
Rack %	28.8 ± 4.3[a]	28.8 ± 4.7[a]	28.8 ± 4.2[a]	28.8 ± 4.6[a]

[a]Means without a common superscript are significantly different ($P < 0.05$).
[1]Yield expressed as percentage of ready-to-cook carcass
[2]For the first two weeks of life, chick starter formula was utilized and for the remaining 4 weeks of the experiments, a grower formula was utilized. Feed additives were given for the duration of the experiment (6 weeks).
[3]GOS; 2% W/W; GTC nutrition, Golden, CO, USA
[4]FOS; 1% W/W; GTC Nutrition, Golden, CO, USA
[5]Plum fibers; 1% W/W; California Dried Plum Board, Sacramento, CA, USA

Table 4. Comparison of fold changes between microarray and qRT-PCR

GenBank Accession Number	Gene Symbol	Plum						FOS						GOS					
		2 week		4 week		6 week		2 week		4 week		6 week		2 week		4 week		6 week	
		M[a]	P[b]	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P
NM_205147	CYP1A4	-0.4	-2.06	-0.79	-1.36	4.32	3.4	0.5	0.23	-0.63	-1.15	5.32	4.38	0.89	0.1	-1.27	-0.45	4.73	2.93
BU124208	LOC424523	0.37	0.14	0.74	2.46	5.11	8.24	0.08	0.14	-0.31	1.07	4.76	7.24	0.46	0.01	-1.67	-0.74	4.57	6.87
NM_001001751	CYP3A7	0.96	-0.17	0.49	1.22	4.21	3.64	1.1	0.29	-0.02	0.96	4.07	2.54	1.05	1.8	-0.68	0.6	3.76	2.7
NM_001001203	EREG	0.41	-0.84	0.27	1.25	2.96	1.77	0.23	-0.42	-0.09	0.83	4.2	3.01	0.02	1.24	-0.04	1.21	2.31	1
Z68489	LCT	-0.35	-1.32	0.11	0.85	3.46	2.56	0.58	0.26	-0.01	0.68	3.54	2.32	0.42	1.58	-0.63	0.58	4.11	2.72
XM_414163	BCMO1	0.73	0.49	0.39	1.49	3.83	3.02	0.6	0.01	0.07	1.49	3.48	2.89	0.22	1.49	-0.71	0.77	4.11	3.32
XM_414111	GPT2	1.06	0.61	0.48	0.59	3.53	2.18	1.03	0.86	0.1	0.45	4.03	2.32	1.3	0.85	-0.3	0.42	3.82	1.88
NM_205513	CALB1	0.55	1.21	-0.16	0.19	3.21	1.81	0.66	1.3	0.73	0.55	3.67	1.92	0.37	0.79	-0.62	0.24	4	2.3
XM_416675	LIPI	0.69	0.07	0.06	0.68	3.44	1.58	0.61	0.44	-0.21	0.12	3.98	1.7	0.41	-0.2	-0.62	0.39	3.28	1.11
NM_204322	GLDC	0.43	-0.23	-0.88	-0.27	3.55	2.18	0.72	0.54	0.12	0.65	3.46	1.43	1.42	0.69	-1.09	-0.4	3.53	1.45
BX_936187	RDH5	-3.18	-4.64	0.6	0.79	0.16	0.3	-2.7	-1.18	-0.09	0.14	1.5	1.12	-3.04	-3.18	-0.5	0.48	0.83	-0.38
CR389474	LRCH2	-0.27	-1.32	-1.59	0.64	0.34	0.12	-0.21	0.9	-1.85	0.24	0.41	-0.64	-0.01	-0.56	-1.91	0.44	0.34	-1.43
NM_001008463	PGS1	-1.08	-0.12	-1.76	1.15	0.55	1.02	-0.08	-0.88	-1.31	0.5	1.87	-1.26	-0.29	-0.54	-1.75	0.03	2.62	0.22
NM_205240		-1.29	-2.23	1.35	1.07	1.76	-0.23	0.55	-0.1	1.94	1.92	0.69	-2.77	-0.52	-1.24	1.55	2.22	2.56	-1.47
NM_205268	NOV	1.23	1.04	-2.22	-2	2.53	1.75	0.41	0.54	-2.65	-1.9	3.28	0.92	1.18	1.04	-2.29	-1.08	1.35	-1.58

M[a]: Microarray, P[b]: qRT-PCR

Table 5. List of functional networks associated with lipid metabolism

Treatment	ID	Networks functions	Focus molecules	Score
Plum	1	Lipid metabolism, Small molecule biochemistry, Molecular transport	29	46
	3	Lipid metabolism, Small molecule biochemistry, Energy production	25	37
	4	Lipid metabolism, Drug metabolism, Endocrine system development and function	25	37
	6	Lipid metabolism, Molecular transport, Small molecule biochemistry	22	30
	7	Lipid metabolism, Dermatological diseases and conditions, molecular transport	21	29
	10	Lipid metabolism, Small molecule biochemistry, Cardiovascular disease	19	24
FOS	2	Lipid metabolism, Genetic disorder, Metabolic disease	30	39
	3	Lipid metabolism, Cellular assembly and organization, Nervous system development and function	28	35
	4	Lipid metabolism, Nucleic acid metabolism, Small molecule biochemistry	28	35
	6	Lipid metabolism, Molecular transport, Small molecule biochemistry	27	33
GOS	1	Lipid metabolism, Small molecule biochemistry, Molecular transport	32	46
	4	Lipid metabolism, Nucleic acid metabolism, Small molecule biochemistry	28	36
	5	Lipid metabolism, Drug metabolism, Cellular assembly and organization	28	36
	9	Lipid metabolism, Small molecule biochemistry, Molecular transport	23	27

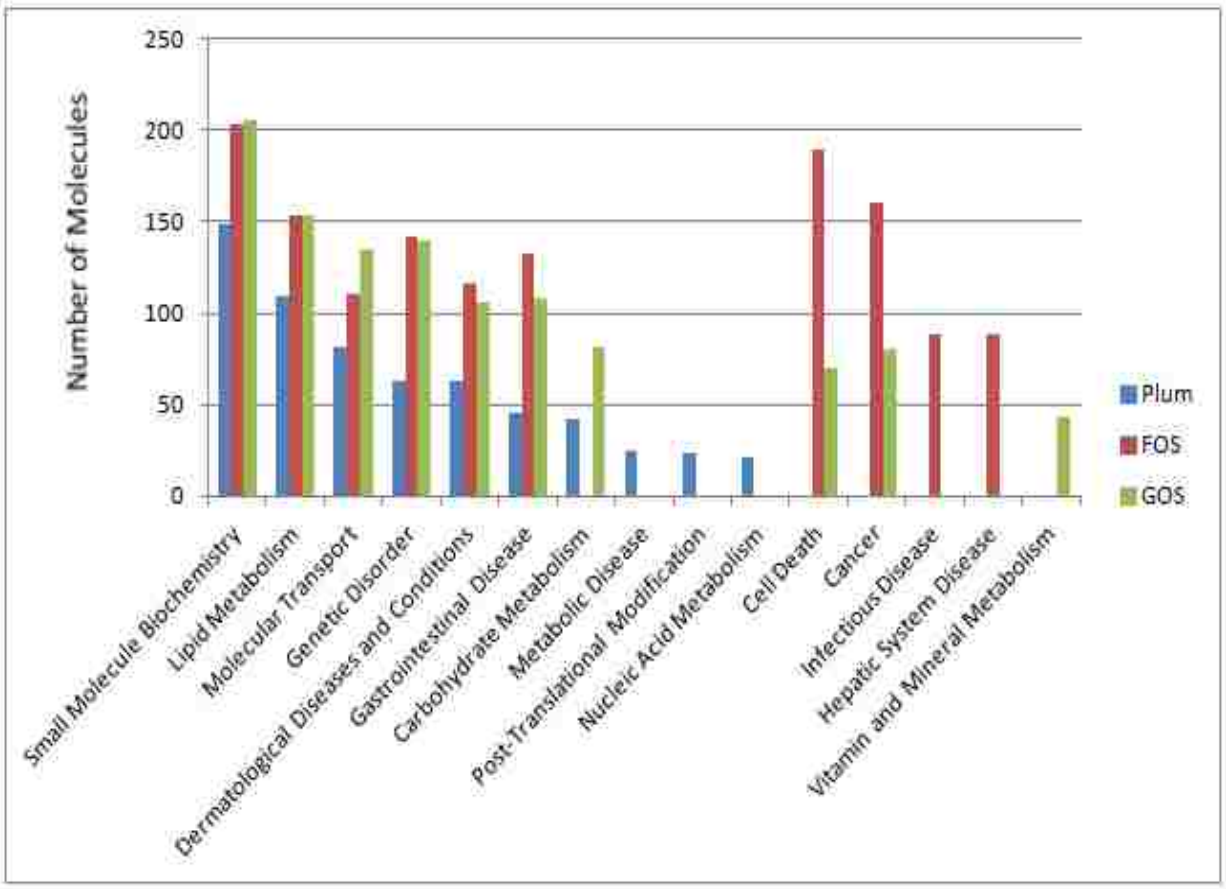


Figure 1. Top 10 functional gene ontologies of each treatment based on differentially expressed genes. The 376, 713 and 628 genes of plum, FOS and GOS treatment were categorized into functional groups by associated molecules using IPA program. The X-axis represented functional groups name and Y-axis showed number of molecules.

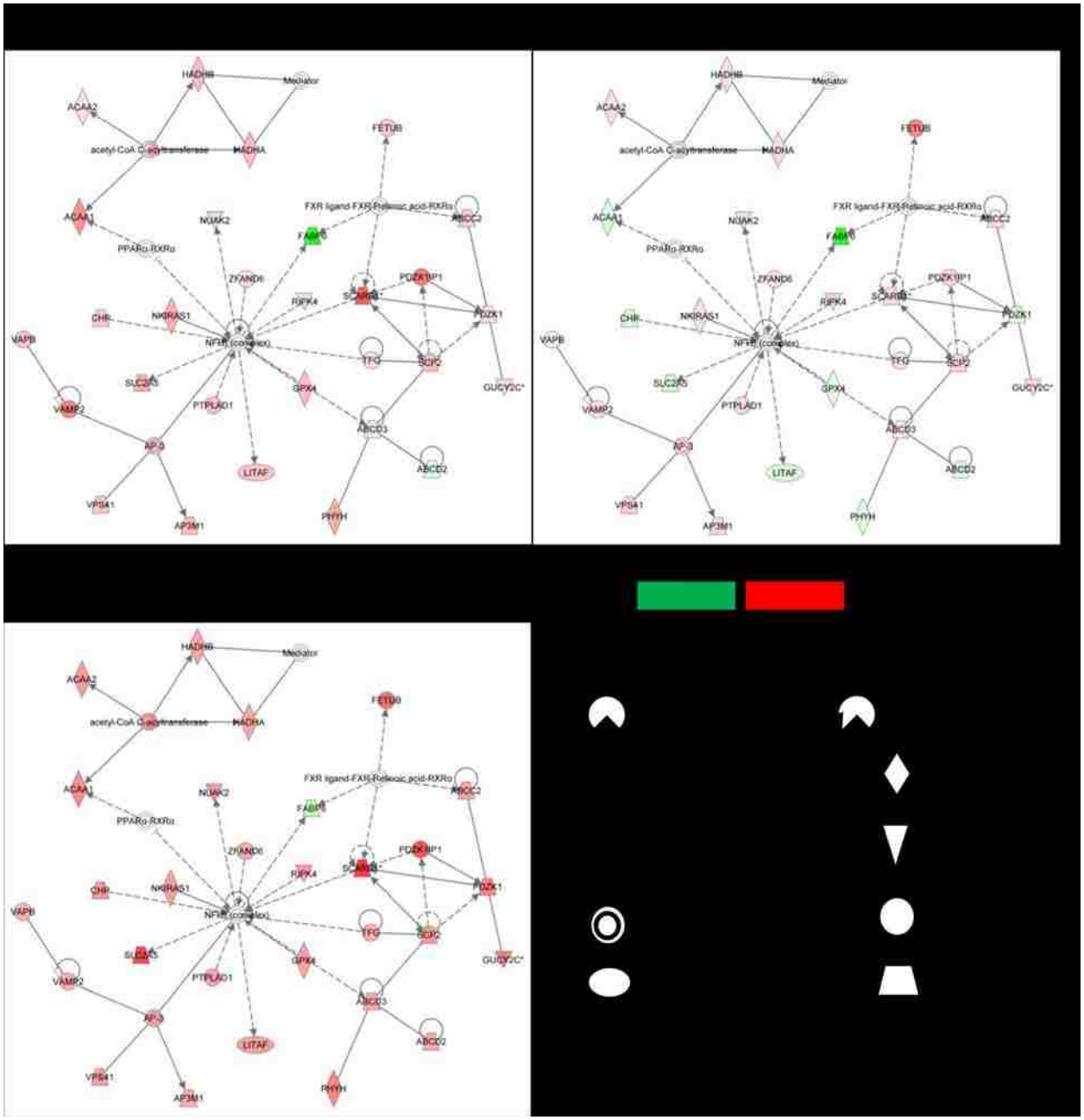


Figure 2. Network #1 of selected gene analysis associated with supplementing plum fiber. The interactions among molecules are represented with symbols and color. Red shows up-regulated genes while green color represents down-regulated genes. The intensities of color depicts the fold change values over time.

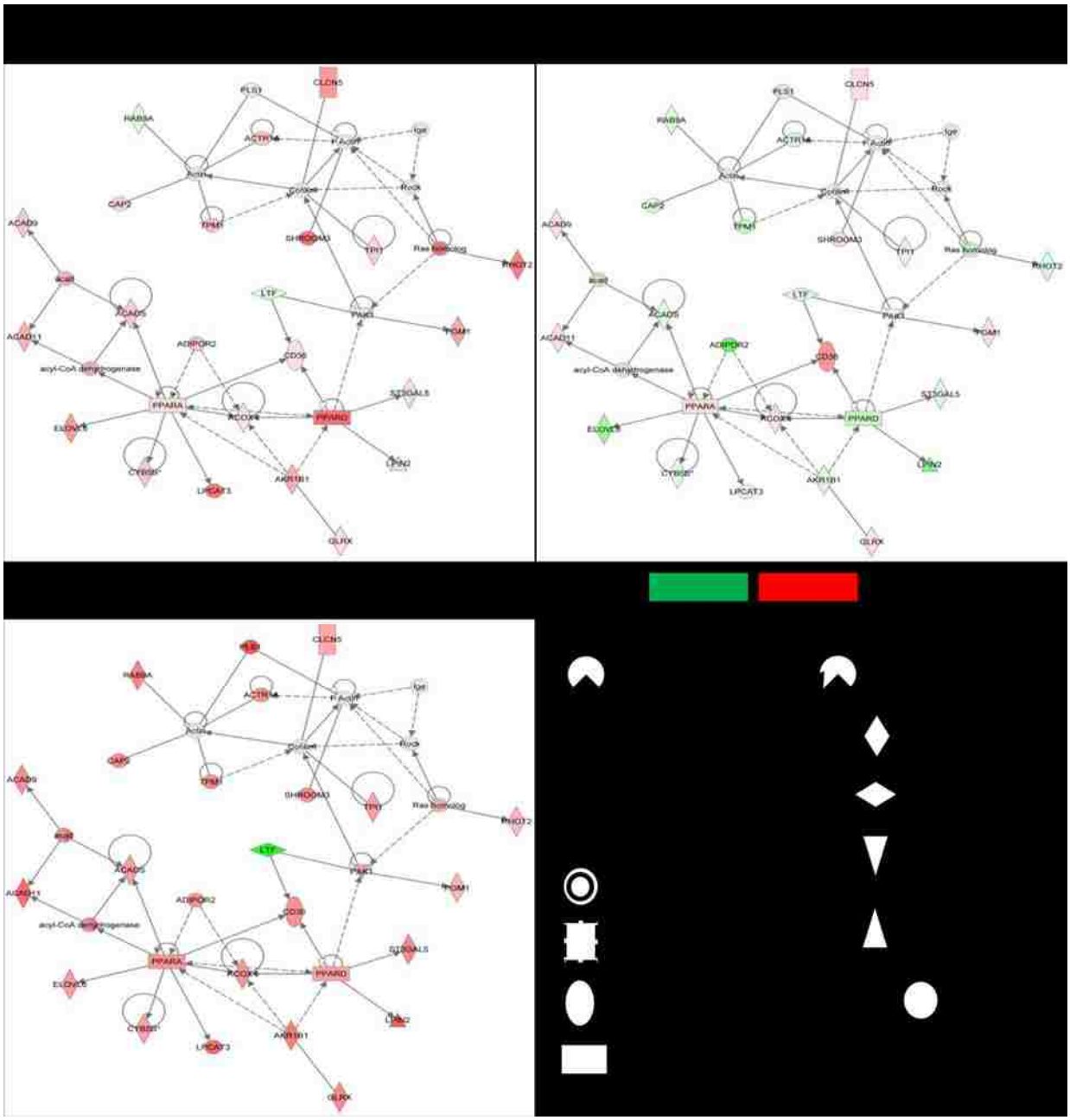


Figure 3. Network #6 of selected gene analysis associated with supplementing FOS. Interaction, symbols and color are same scheme described in Figure 2.

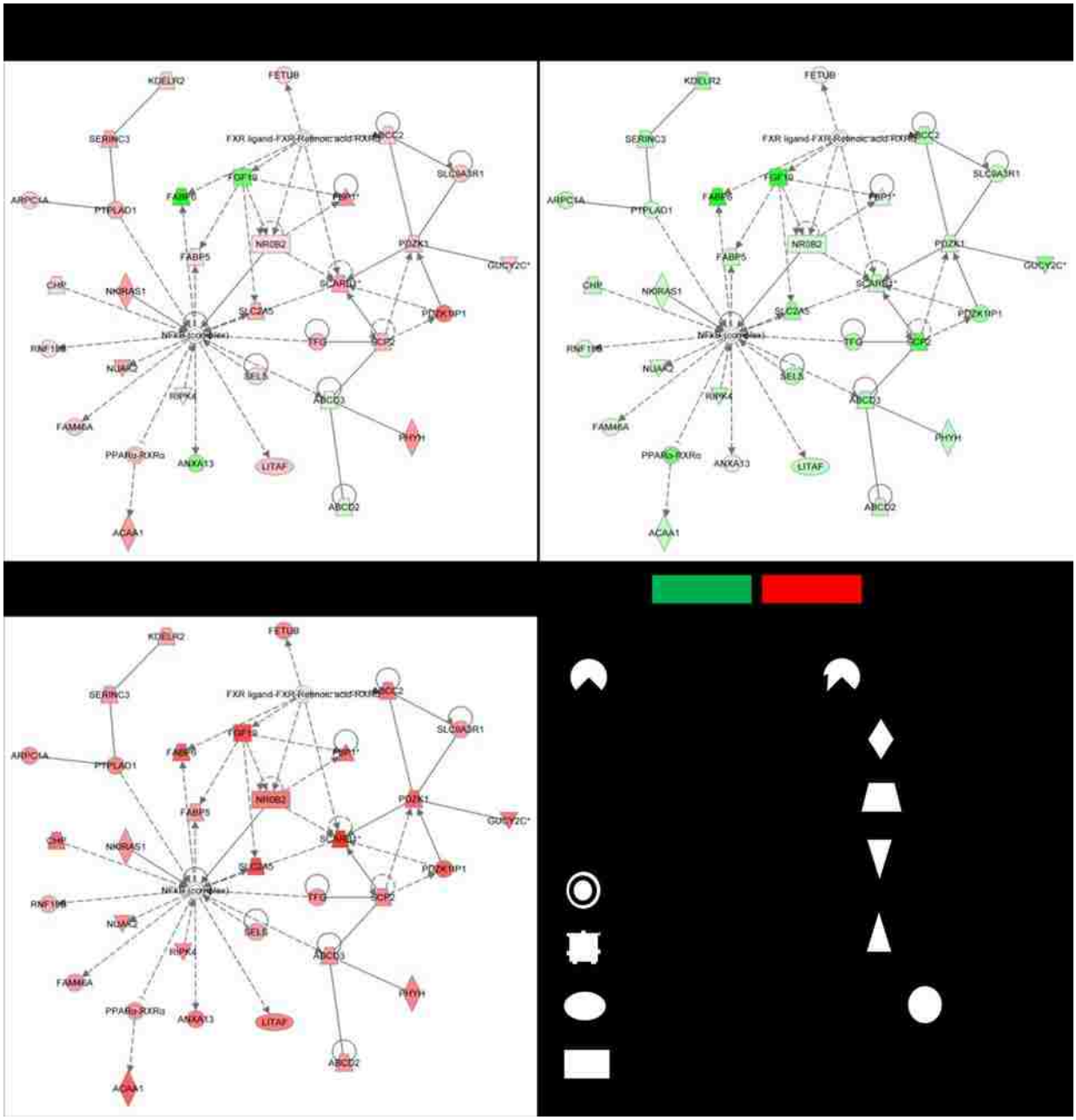


Figure 4. Network #1 of selected gene analysis associated with supplementing GOS. Interaction, symbols and color are same scheme described in Figure 2.

7. Appendix

7.1. Chapter 5 Publication by Journal (Partial)

Research Note

Assessment of production performance in 2 breeds of broilers fed prebiotics as feed additives

I. Hanning,*[1] A. Clement,†‡ C. Owens,‡ S. H. Park,† S. Pendleton,*† E. E. Scott,† G. Almeida,† F. Gonzalez Gil,* and S. C. Ricke†‡

*Department of Food Science and Technology, University of Tennessee, Knoxville 37996; and †Center for Food Safety and Department of Food Science, and ‡Department of Poultry Science, University of Arkansas, Fayetteville 72704

ABSTRACT Pasture-flock-raised poultry are becoming an increasingly popular product, but only limited options are currently available for maintaining gut health. For these producers, prebiotics are an attractive option because they are generally recognized as safe (GRAS) and can be mixed into the feed and thus do not require adjustments to production protocols. However, if prebiotic treatments reduce production performance, they would not be useful to producers. Thus, the objective of this study was to measure performance of pasture-raised broilers fed 1 of 3 prebiotic treatments. For these trials, 2 breeds of birds were used: Naked Neck slow-growing breeds and Cornish White Rock cross fast-growing breeds. The experimental design was replicated for each breed. A total of 340 birds were split into 4 groups, each group fed one feed additive: 1) galactoligosaccharides (2% wt/wt), 2) fructooligosaccharides (1% wt/wt), 3) plum fibers (1% wt/wt), or 4) no additives. During the 8-wk rearing period, 10 birds from each group were collected and euthanized to take small intestine samples. Histological preparations were made from the small intestine tissue, and 4 measurements of villi height and crypt depth from each cross section were taken. Throughout the study, mortality was monitored and BW measurements were taken at 2-wk intervals. For the Cornish White Rock cross, the group receiving the feed supplemented with fructooligosaccharides had higher ($P < 0.05$) 8-wk BW than those fed Plum; control and birds fed galactoligosaccharides were intermediate. For the Naked Neck breed, the group receiving the plum fibers had the highest final BW. It appears that all 3 feed supplements offered some protective effect for alterations in villi length and crypt depth due to feed withdrawal, but only for the Naked Neck breed. The data indicate the 3 prebiotics utilized in this study could be used without risk of decreasing production performance, but only for Naked Neck breeds.

Key words: pasture, poultry, feed additives, prebiotics

2012 Poultry Science 91:3295–3299
http://dx.doi.org/10.3382/ps.2012-02557

INTRODUCTION

Organic and pasture flock poultry producers utilize a nonconventional systems approach to poultry production. Some producers use mobile pens and move the pens on a routine basis to provide fresh pasture for the birds. Other producers may have a nonmobile housing unit with access to an enclosed outdoor area. The design can have many variations, but access to outdoors is given to the birds in all of these production systems (Tuyttens et al., 2011).

Organic and pasture flock poultry products are becoming more popular, and the demand for these products is also increasing. Many consumers are attracted to these types of products because they believe organic and pasture-raised poultry products will improve consumer health compared with conventionally raised poultry (Van Loo et al., 2011). One of the factors driving this attitude is the fact that organic and pasture flock producers reduce or eliminate the use of antibiotics and vaccines. As such, producers are limited in the types of intervention measures that can be used to keep birds healthy. Many producers have considered prebiotics and probiotics as potential feed additives to improve gut health and overall health of the flock (Donalson et al., 2007, 2008a,b).

Prebiotics are indigestible substances that can be used by specific groups of healthy bacteria and thus are aimed at increasing the populations of healthy bacteria in the gut. Probiotics are beneficial bacterial cultures that are fed directly to the bird to increase specific populations of these beneficial bacteria. Beneficial bacteria can improve gut and overall health through many

Received June 22, 2012.
Accepted September 4, 2012.
[1]Corresponding author: ihanning@utk.edu

7.2. Do Not Need Copyright for Published Paper in Chapter 5 (Partial)

Rights and Permissions

Permissions requests are processed through the Copyright Clearance Center. [click here to request permission]

General Information about Permissions

Written permission to republish whole or parts of works owned by the Poultry Science Association must be obtained prior to publication. Please do not contact the authors initially for permission, instead request permission from the Copyright Clearance Center. Permissions fees or other charges are calculated based on intended use and number of reproductions.

Authors requesting permission to use their own material

Without express permission, authors may do the following after their work has been published by *Poultry Science*.

- Present the work orally (partially or in its entirety) for noncommercial, educational purposes.
- Use the article (or parts of) in book you edit or chapter you author.
- Use the article in a thesis or dissertation.
- Posting articles on institutional repositories is prohibited.

7.3. Authorship Statement for Chapter 5

Si Hong Park is the first author of the paper and completed at least 51% of the studies among coauthors which the title is "**Assessment of Production Performance and Transcriptomic Analysis in Small Intestines of Pasture Flock Raised Broiler Chickens Fed with Prebiotics**" in chapter 5.

Major Advisor: Dr. Steven C. Ricke

Date: June 4th, 2013

CHAPTER 6

Assessment of Gastrointestinal Microflora in Pasture Flock Raised Chickens Fed with Two Commercial Prebiotics

Si Hong Park[1,2], Kristen Gibson[1,2], Steven C. Ricke[1,2*]

[1]Cell and Molecular Biology Program, Department of Food Science, University of Arkansas, Fayetteville, AR

[2]Center for Food Safety and Department of Food Science, University of Arkansas, Fayetteville, AR

1. Abstract

Prebiotics include nondigestible carbohydrate dietary additives and other biological components that stimulate the growth of one or more beneficial bacteria in the gastrointestinal (GI) tract that are beneficial to the host. The beneficial bacteria can inhibit colonization of pathogenic bacteria by producing antimicrobial substances and competing for niches within the gut. In this study, we have evaluated the effects of both Biolex® MB40 and Lieber® ExCel which are commercial prebiotics derived from brewer's yeast cell walls. The two prebiotics were added to GMO-free normal chicken feeds in the starter and finisher feeds in each group. Each group consisted of 1) control (no prebiotic), 2) Biolex® MB40 with 0.2%, 3) Leiber® ExCel with 0.2%. Feeds were consistently supplemented with the prebiotics during the experimental period. At 8 week, a total 15 of birds from each group were randomly selected for necropsy. The polymerase chain reaction based denaturing gradient gel electrophoresis (PCR-based DGGE) technique was utilized to compare microbial populations in control and both treatment groups. Feeds supplemented with either Biolex® MB40 or Leiber® ExCel prebiotics showed more consistent compared to control group. For Biolex® MB40 supplemented group, all samples were clustered with over 74% of relatedness. In Leiber® ExCel supplemented group showed 77% of relatedness among 4 samples except for one as an outlier. According to sequencing results, *Bacteriodes salanitronis* was constantly present in all groups, and *Barnesiella ciscericola* and *Firmicutes* were detected in both treatment groups.

2. Introduction

Prebiotics have been used for new alternatives in general gut health promotion as well as utilized for reducing pathogen colonization (Patterson and Burkholder, 2003, Siragusa and Ricke, 2012). Prebiotics include nondigestible carbohydrate dietary additives and other biological components that stimulate the growth of one or more beneficial bacteria in the gastrointestinal (GI) tract that are beneficial to the host (Schrezenmeir and de Vrese, 2001). In general, prebiotics are mixed with feeds as additives during the milling process so all birds can access the same feeds, including prebiotics over the entire feeding cycle (Callaway and Ricke, 2012). Prebiotics can be utilized preferentially by beneficial bacteria such as *Lactobacillus* and *Bifidobacteria* species (Callaway and Ricke, 2012), which leads to the production of lactic acid and short chain fatty acids (SCFA) both of which are inhibitory to pathogens (Kaplan and Hutkins, 2000; Ricke et al., 2013). In addition, the presence of prebiotics can lead to the maintenance of a normal microbial population (Kaplan and Hutkins, 2000; Callaway and Ricke, 2012). The beneficial bacteria can inhibit colonization of pathogenic bacteria by producing antimicrobial substances and competing for niches within the gut (Ricke and Pillai, 1999).

The polymerase chain reaction based denaturing gradient gel electrophoresis (PCR-based DGGE) technique has been widely utilized to compare microbial populations in various environments including feces and gut samples (Hume et al., 2003; Dunkley et al., 2007; Hill et al., 2008; Hanning and Ricke, 2011). This technique amplifies a common region of the 16S ribosomal RNA gene and amplicons are subsequently separated on a gradient polyacrylamide gel (Park et al., 2013). Double strands of PCR amplicons are partially unwinded due to denaturant concentrations and separated based on G+C contents. The resulting banding pattern can be compared to identify microfloral shifts between control and treatments (Owens et al., 2008).

Furthermore, the recovered DNA fragments from the gel can be sequenced to identify species by searching comprehensive databases, such as the basic local alignment search tool (BLAST) (Altschul et al., 1990).

Considering the prebiotic influences on the gastrointestinal microflora and the impact of microflora have on host health, the purpose of this study was to determine whether supplemented prebiotics caused shifts in gastrointestinal bacteria. In this study, we have evaluated the effects of both Biolex® MB40 and Lieber® ExCel, which are commercial prebiotics derived from brewer's yeast cell walls. Biolex® MB40 contains high concentration of beta-D-glucan and mannanoligosaccharides (MOS) which have been shown to bind detrimental substances for pathogenic bacteria (Oyofo et al., 1989). The components in Lieber® ExCel are similar to Biolex® MB40 as well as include natural RNA components (i.e. nucleotides) (http://www.leibergmbh.de/int/animal-nutrition/products). The first step to achieve this objective was utilizing the culture independent technique, PCR-based DGGE, to analyze and compare the microflora profiles. These microbial profiles were subsequently compared to determine microflora shifts based on DGGE gel banding patterns. Finally, selected bands were excised from the gel for further sequencing analysis to identify specific bacteria of interest that indicated which type of prebiotics supported the growth of specific bacteria.

3. Materials and Methods

3.1. Pasture Flock Chicken Study

A total of 147 day-of-hatch naked neck chicks were acquired from a local hatchery (Peterson Farms, Decatur, AR, USA). The birds were randomly distributed to 3 pens for a total of 49 birds per each pen. Birds had access to feed and water *ad libitum* for the duration of the

experiment. Also, the pens and birds were moved twice a week to fresh pasture that had not been previously used for poultry rearing purposes.

Two prebiotics were added to GMO-free normal chicken feeds (Hiland Naturals, Killbuck, OH, USA) in the starter and finisher feeds in each group. Each group consisted of 1) control (no prebiotic), 2) Biolex® MB40 with 0.2% (Leiber GmbH, Hafenstraße, Germany), 3) Leiber® ExCel with 0.2% (Leiber GmbH). Feeds were consistently supplemented with the prebiotics during the experimental period. At week 8, a total 15 birds from each group were randomly selected for necropsy, transported to the Poultry Health Corelaboratory (Fayetteville, AR, USA) and euthanized humanely using CO_2 gas. The cecal samples were extracted immediately and stored in -20˚C for microbial analysis.

3.2 DNA Extraction

DNA was isolated from 15 birds of each group using the Qiagen stool mini kit (Qiagen, Valencia, CA, USA) with some modifications to enhance DNA yields. In brief, 0.7 mm garnet beads (Mo Bio Laboratories Inc., Carlsbad, CA, USA) were added to cecal samples to lyse cells with vortexing vigorously for 1 min. Samples were centrifuged to remove unhomogenized materials and the supernatant was transferred into a fresh 2 ml of microcentrifuge tube containing 0.1 mm glass beads (Mo Bio laboratories Inc.). Beads beating was performed for 10 min by horizontal vortexing and the samples were incubated at 95˚C heating block for 6 min. The remainder of the DNA extraction protocol was performed according to the manufacturer's instructions. The extracted DNA concentration and purity were measured using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA) and DNA was subsequently stored at -20˚C until used.

3.3. PCR Reaction for DGGE

The conventional PCR assay was optimized using an MJ PTC 100 thermocycler (Bio-Rad, Hercules, CA, USA). A 50 μl of total reaction volume comprised of 50 ng of template DNA, 800 nM of each primer (Muyzer et al. 1993) (IDT, Coralville, IA, USA), 25 μl of Jump Start Ready Mix (Sigma, St. Louis, MO, USA), and was brought to a final volume with DNase-RNase free water. The PCR conditions consisted of pre-denaturation at 95˚C for 2 min, then 17 cycles of denaturation at 94˚C for 1 min., annealing at 67˚C for 45 s decreasing by -0.5˚C per cycle to a touchdown temperature of 59˚C, and annealing at 72˚C for 2 min. The reaction was followed with 12 cycles of denaturation at 94˚C for 1 min, annealing at 58˚C for 45 s with a final extension step at 72˚C for 7 min. The PCR products were confirmed on 1.5% of agarose gel and visualized on transilluminator (Bio-Rad, Hercules, CA, USA).

3.4. DGGE

PCR-based DGGE was performed using a 10 μl of the PCR products mixed with 5 μl of loading buffer. The samples were loaded into the wells of a polyacrylamide gradient gel composed of acrylamide:bisacrylamide (37:1) (Bio-Rad), with a 35% to 60% gradient of urea (Amersham Biosciences, Piscataway, NJ, USA) and formamide (Sigma). Electrophoresis was carried out using the DCode Universal Mutation Detection System (Bio-Rad) in 1X TAE buffer at 59˚C and 55 V for 17 h. The polyacrylamide gel was stained with SYBR Green (Cambrex Bioscience, Walkersville, MD, USA) in 1X TAE for 40 min. with gently shaking, destained in distilled water for 10 min. and viewed on a transilluminator. DGGE banding patterns among individual samples in each treatment as well as between treatments were analyzed using UPGMA algorithm (Bio-Rad) to determine the correlation.

3.5. DNA Recovery from Excised Gel for Sequence Analysis

Comparing banding patterns among groups, common or specific bands were excised from the polyacrylamide gel for sequence analysis. Briefly, excised fragments were disrupted via pinhole tube, transferred in 300 μl of TE buffer and incubated for 15 min. at 65˚C heating block for dissolving DNA. The suspension was transferred to a Spin-X® centrifuge tube (Corning, Tewksbury, MA, USA) and centrifuged at 16,000 x g for 5 min. to isolate DNA from the polyacrylamide gel. In order to precipitate DNA, the filtrate was mixed with 900 μl of ethanol, 133 μl of 7.5 M ammonium acetate, 3 μl of glycogen (20 mg/ml) and vortexing vigorously, and then incubated at -80˚C for 1 h. The mixture was pelleted via centrifugation at 16,000 xg for 15 min. and the pellet was washed with 70% of cold ethanol. The isolated DNA was subsequently sequenced using ABI 3100 capillary analyzing system (Applied Biosystems, Foster City, CA, USA) and the sequences were compared with database in GenBank using the BLAST algorithm.

4. Results

4.1. Analysis of Microbial Population Shifts in Chicken Cecum Using PCR-Based DGGE

All chicken cecal samples produced 233-bp of amplicons via conventional PCR in prior to DGGE and these products were subsequently used for DGGE analysis (Figure 1). DGGE were performed to verify microbial population shifts by supplemented one of prebiotics (Biolex® MB40 and Leiber® ExCel) using UPGMA algorithm (Figure 2). Fifteen chicken cecal samples in control group and 14 samples in both treatment groups (one sample per treatment was discarded due to contamination) were utilized for DGGE analysis. Based on DGGE banding patterns, each group phylogenetic tree was generated by the UPGMA algorithim, which illustrated the correlation among individual chickens (Figure 2). Fifteen individual chicken cecal

samples in the control were clustered with over 58% homology (Figure 2A). Also, each of the 14 cecal samples supplemented with Biolex® MB40 or Leiber® ExCel showed over 66% and 51% homology, respectively (Figure 2B and 2C). The Biolex® MB40 group showed more consistency with a greater homology than other two groups.

In order to compare the correlation between control and both treatment groups, cecal samples in each group were pooled into 5 samples considering individual DGGE banding pattern similarities and subsequently DGGE was performed using pooled samples (Figure 3). A phylogenetic tree was generated based on DGGE results and shown in Figure 4. Interestingly, the phylogenetic tree of control and two treatment groups exhibited 3 distinct clusters in each group except one outlier of Leiber® ExCel treatment (Figure 4). In the control group, four sample banding patterns were exhibited over 68% relatedness and one sample clustered in the Leiber® ExCel group with 70% relatedness. Feeds supplemented with either Biolex® MB40 or Leiber® ExCel prebiotics showed more consistent compared to control group. For the Biolex® MB40 supplemented group, all samples were clustered with over 74% of relatedness. In Leiber® ExCel supplemented group showed 77% of relatedness among 4 samples except for one as an outlier.

4.2. Sequencing

DGGE banding patterns showed high similarities within control and both treatment groups (Figure 3). However, some bands were specific in one group and the intensity of several common or specific bands were different among groups. For instance, band number 4 and 16 are specific for Biolex® MB40 treatment and control group, respectively (Figure 3). In addition, band number 2, 9, and 19 are common and appeared constant intensity over all groups (Figure 3).

These specific and common bands were excised from a polyacrylamide gel for sequencing analysis and identification results were shown in Table 2.

From the sequencing results, although several bands (12, 21, 22, 23, and 24) were identified as an uncultured bacterium and failed for sequencing (3, 5, 7, 8, and 18), remainder of bands were identified as a specific species with high homology. *Bacteriodes salanitronis* (2, 9, 13, and 19) was constantly present in all groups, and *Barnesiella ciscericola* and *Firmicutes* (6) were detected in both treatment groups. Interestingly, *Helicobacter ganmani* (1) and uncultured *porphyromonadaceae* (4) were only present in Biolex® MB40 treatment group. In only control group, *Paraprevotella clara* (17) and *Alistipes* species (20) were present with high intensity as well as *Bacteriodes coprocola* (16) was identified.

5. Discussion

The significance of this study was to evaluate the microbial population shifts in broiler chickens fed with one of two commercial prebiotics including beta-D-glucan and MOS. The beta-D-glucan polysaccharides are composed of D-glucose monomers joined by glycosidic bonds. They are used for medical treatment because of their antimicrobial properties (Balzarini, 2007). The MOS have been widely used as a nutritional additive to preserve gut health since they were initially introduced in the late 1980s (Oyofo et al., 1989). The mannose sugar site in MOS can bind to *Salmonella* fimbriae and inhibit *Salmonella* colonization in the broiler intestinal cells (Oyofo et al., 1989). Since the advantages of MOS in pathogenic bacteria inhibition and performance improvement in broilers, MOS has been used in poultry rearing systems (Biggs et al., 2007).

The microflora in gastrointestinal tract plays crucial roles by not only preventing pathogen colonizations but contributing to the complexity of the gut ecosystems that can generate antimicrobial metabolites such as SCFA to inhibit other species (Ricke and Pillai, 1999; Ricke, 2003b). In addition, this microbial population in birds can be changed with several alterations in feed additives and other factors (Ricke, 2003a; Dunkley et al., 2007; Siragusa and Ricke, 2012).

In this study, the gastrointestinal bacteria *Firmicutes* and the *Bacteriodes* were identified in all groups (Figure 3 and Table 2). *Firmicutes* are phylum of bacteria presenting Gram-positive cell wall structure are commonly present in gastrointestinal tracts, and are composed of over 250 genera including *Bacilli* and *Clostridia* (Bajzer and Seeley, 2006). *Bacteriodes* genus is a Gram-negative bacterium and utilizes plant glycans as their main energy sources (Martens et al., 2008). Furthermore, *Bacteroides* species show an additional benefit in the host by preventing colonization of pathogens (Hentges, 1989). *Bacteriodes* genus is one of the predominant anaerobic bacteria found in chicken cecum (Lan et al., 2006). In this study, *Bacteriodes salanitronis* was identified in all groups with great band intensity and *Bacteriodes coprocola* was found only in the control group. Identification of these bacteria was conrrespondence compared with previous reports (Bajzer and Seeley, 2006). *Helicobacter ganmani* found in Biolex® MB40 treatment was first isolated from intestines of laboratory mice and showed similar features as other *Helicobacter* species (Robertson et al., 2001). *Campylobacter* species is a commensal bacterium colonizing the gastrointestinal tract in poultry (Horrocks et al, 2009) and both treatment groups represented *C. jejuni*, *C. coli*, and *C. lari*.

Although PCR-based DGGE technique has several limitations for analysis of whole bacterial populations in gastronintestinal tracts, this assay proved to be useful for comparing

microbial population shifts influenced by prebiotic treatments. Furthermore, additional sequencing data provided concerning specific species of bacteria that may have been specifically impacted due to prebiotic supplements. In conclusion, microflora in both prebiotic supplemented groups are consistent than the control group based on phylogenetic tree analysis. Prebiotics might control microflora in cecum with increasing beneficial bacteria and decresing pathogens. Also, further studies to analyze all microbial populations using whole genome sequencing for confirmation are warranted.

6. Acknowledgment

This study was supported by Cell and Molecular Biology program in University of Arkansas to Si Hong Park and funding awarded to Drs. Ricke and Gibson from Leiber company.

7. Reference

Altschul, S., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Bajzer, M., and R. J. Seeley. 2006. Obesity and gut flora. Nature, 444:1009-1010.

Balzarini, J. 2007. Targeting the glycans of glycoproteins: a novel paradigm for antiviral therapy. Nature Rev. Microbiol. 5:583-597.

Biggs, P., C. M. Parsons, and G. C. Fahey. 2007. The effects of several oligosaccharides on growth performance, nutrient digestibilities, and cecal microbial populations in young chicks. Poult. Sci. 86:2327-2336.

Callaway, T. R. and S. C. Ricke (Eds.), 2012. Direct Fed Microbials/Prebiotics for Animals: Science and Mechanisms of Action, Springer Science, New York, NY, 206 pp.

Dunkley, K.D., J. L. McReynolds, M. E. Hume, C. S. Dunkley, T. R. Callaway, L. F. Kubena, D. J. Nisbet, and S. C. Ricke. 2007. Molting in *Salmonella* Enteritidis-challenged laying hens fed alfalfa crumbles. II. Fermentation and microbial ecology response. Poult. Sci.86:2101-2109.

http://www.leibergmbh.de/int/animal-nutrition/products/biolex-mb40/

Hanning, I., and S. C. Ricke. 2011. Prescreening methods of microbial populations for the assessment of sequencing potential. In Y. M. Kwon, S. C. Ricke (Eds.), Methods in Molecular Microbiology 733 - High-Throughput Next Generation Sequencing: Methods and Applications. New York: Humana Press. p 159-170.

Hentges, D. J. 1989. Anaerobes as normal flora. In Anaerobic Infections in Humans, pp. 37-53. Edited by S. M. Finegold & W. L. George. San Diego: Academic Press.

Hill III, J.G., I. Hanning, S. J. Beaupre, S. C. Ricke, and M. M. Slavik. 2008. Denaturing gradient gel electrophoresis for the determination of bacterial species diversity in the gastrointestinal tracts of two crotaline snakes. Herpetol. Rev. 39:433-438.

Horrocks, S. M., R. C. Anderson, D. J. Nisbet, and S. C. Ricke. 2009. Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. Anaerobe. 15:18-25.

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. Poult. Sci. 82:1100-1107.

Kaplan, H., and R. W. Hutkins. 2000. Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. Appl. Environ. Microbiol. 66:2682-2684.

Lan, P. T. N., M. Sakamoto, S. Sakata, and Y. Benno. 2006. *Bacteriodes barnesiae* sp. nov., *Bacteriodes salanitronis* sp. nov. and *Bacteriodes gallinarum* sp. nov., isolated from chicken caecum. Int. J. Sys. Evol. Microbiol. 56:2853-2859.

Martens, E. C., H. C. Chiang, and J. I. Gordon. 2008. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. Cell Host Microbe. 13: 447-457.

Muyzer, G., E. C. de Wall, 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.

Oyofo, B. A., J. R. DeLoach, D. E., Corrier, J. O. Norman, R. L. Zoprin, and H. H. Mollenhauer. 1989. Prevention of *Salmonella typhimurium* colonization of broilers with D-mannose. Poult. Sci. 68:1357-1360.

Owens, B., L. Tucker, M. A. Collins, and K. J. McCracken. 2008. Effects of different feed additives alone or in combination on broiler performance, gut microflora and ileal histology. Br. Poult. Sci. 49: 202-212.

Park, S. H., I. Hanning, W. Gilbert, M. Munro, L. Devareddy, and S. C. Ricke. 2013. Feeding mice aged and fresh blackberries powder supplements result in shifts in the gastrointestinal microflora. Food Bioscience. 1:66-72.

Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. 82:627-631.

Ricke, S.C. 2003a. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poult. Sci. 82:632-639.

Ricke, S. C. 2003b. The gastrointestinal tract ecology of *Salmonella* Enteritidis colonization in molting hens. Poult. Sci. 82:1003-1007.

Ricke, S.C., and S. D. Pillai. 1999. Conventional and molecular methods for understanding probiotic bacteria functionality in gastrointestinal tracts. Crit. Rev. Microbiol. 25: 19-38.

Ricke, S.C., C. S. Dunkley, and J. A. Durant. 2013. A review on development of novel strategies for controlling *Salmonella* Enteritidis colonization in laying hens: Fiber-based molt diets. Poult. Sci. 92: 502-525.

Robertson, B. R., J. L. O'Rourke, P. Vandamme, S. L. W. On, and A. Lee. 2001. Helicobacter ganmani sp. nov., a urease-negative anaerobe isolated from the intestines of laboratory mice. Int. J. Sys. Evol. Microbiol. 51:1881-1889.

Schrezenmeir, J., and M. de Vrese. 2001. Probiotics, prebiotics, and synbiotics-approaching ad definition1-3. Am. J. Clin. Nutr. 73:361S-364S.

Siragusa, G. R. and S. C. Ricke. 2012. Probiotics as pathogen control agents for organic meat production. Pages 331-349 in Organic Meat Production and Processing. S. C. Ricke, E. J. Van Loo, M. G. Johnson and C. A. O'Bryan, ed. Wiley Scientific/IFT, New York, USA.

Table 1. Primer pair sequences used in this study

Primer	Sequence (5’ to 3’)	Gene	Size	Reference
Hume-F	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCCTAC GGG AGG CAG CAG	16s rRNA	233 bp	Muyzer et al., 1993
Hume-R	ATT ACC GCG GCT GCT GG			

Table 2. Identification of DGGE bands via sequencing between control and two treatments

Band No.	**Identification**
1	*Helicobacter ganmani*
2, 9, 13, 19	*Bacteriodes salanitronis*
4	Uncultured *porphyromonadaceae*
6	*Barnesiella viscericola, Firmicutes*
10	*Barnesiella viscericola*
11	*Campylobacter jejuni, C. coli, C. lari*
14	Uncultured rumen bacterium
15	Uncultured *Rikenellaceae*
16	*Bacteriodes coprocola*
17	*Paraprevotella clara*
20	*Alistipes* sp.
12, 21, 22, 23, 24	Uncultured Bacterium

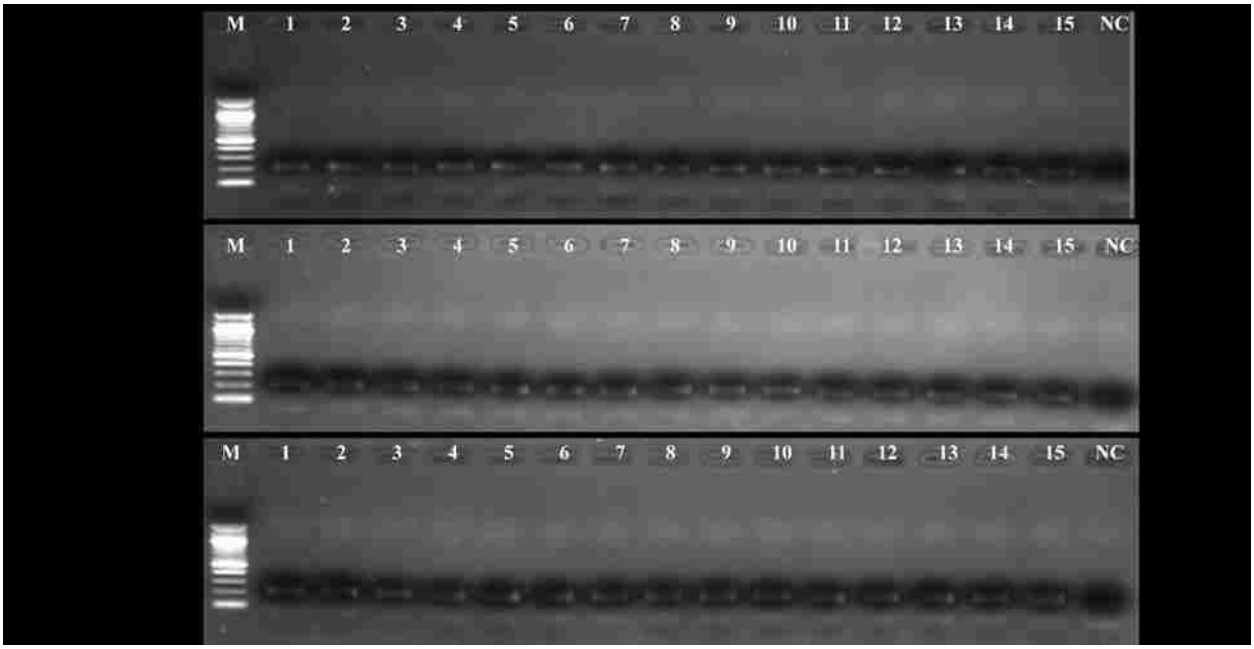


Figure 1. PCR results using DNA isolated from chicken cecal samples prior to DGGE

Lane M: 100 bp DNA ladder, lanes 1 to15: Individual chicken samples, NC: negative control

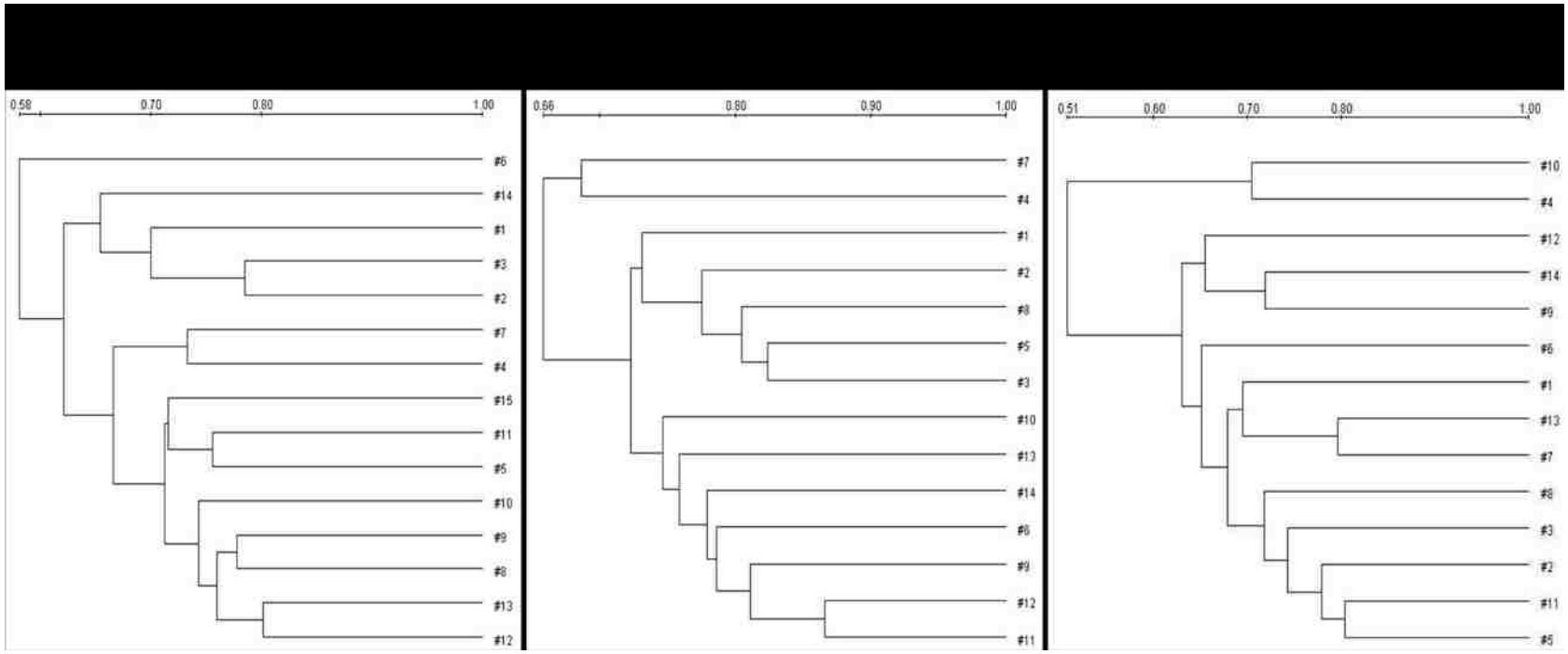


Figure 2. Analysis of phylogenetic tree based on individual chicken samples

(A) Control showed 15 of individual chickens; (B) Biolex® MB40 and (C) Leiber® ExCel showed 14 of individual chickens

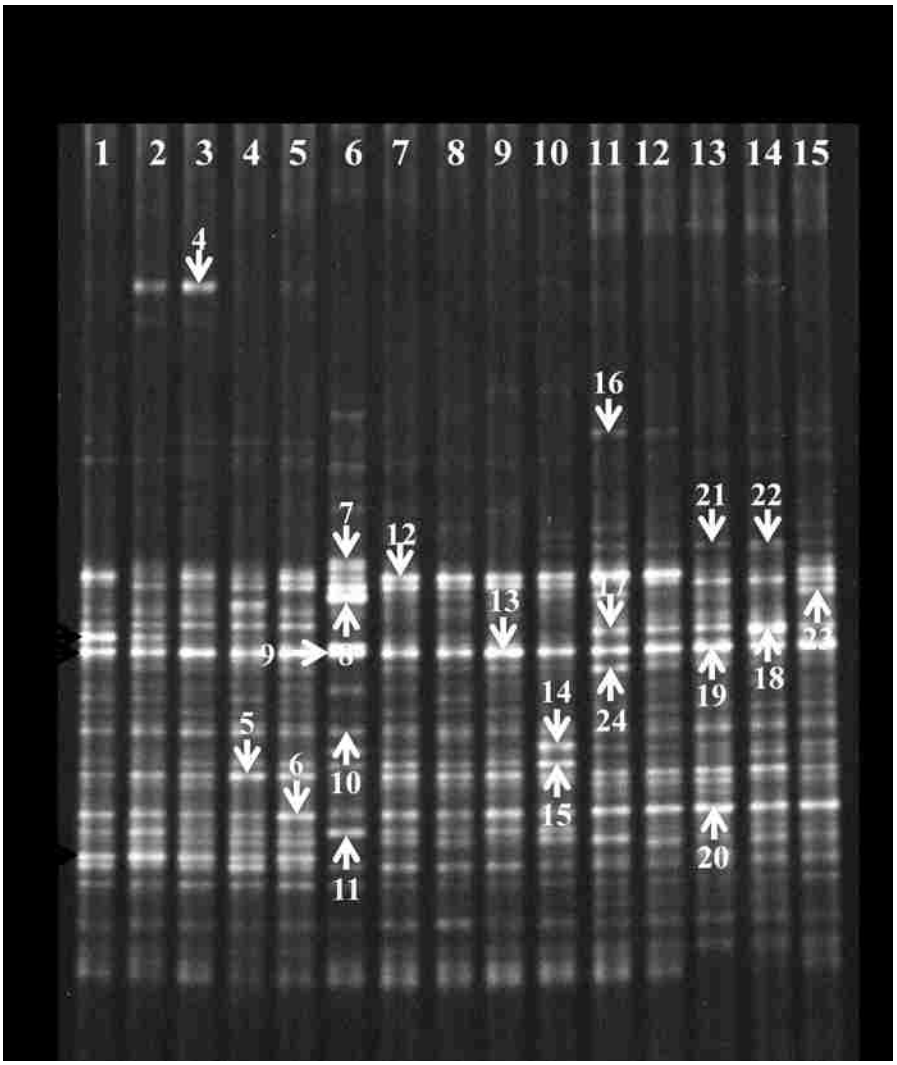


Figure 3. DGGE result using pooled samples

Lanes 1 to 5: Biolex® MB40, lanes 6 to 10: Leiber® ExCel, lanes 11 to 15: control

Each band number on the gel is corresponding to number in Table 2.

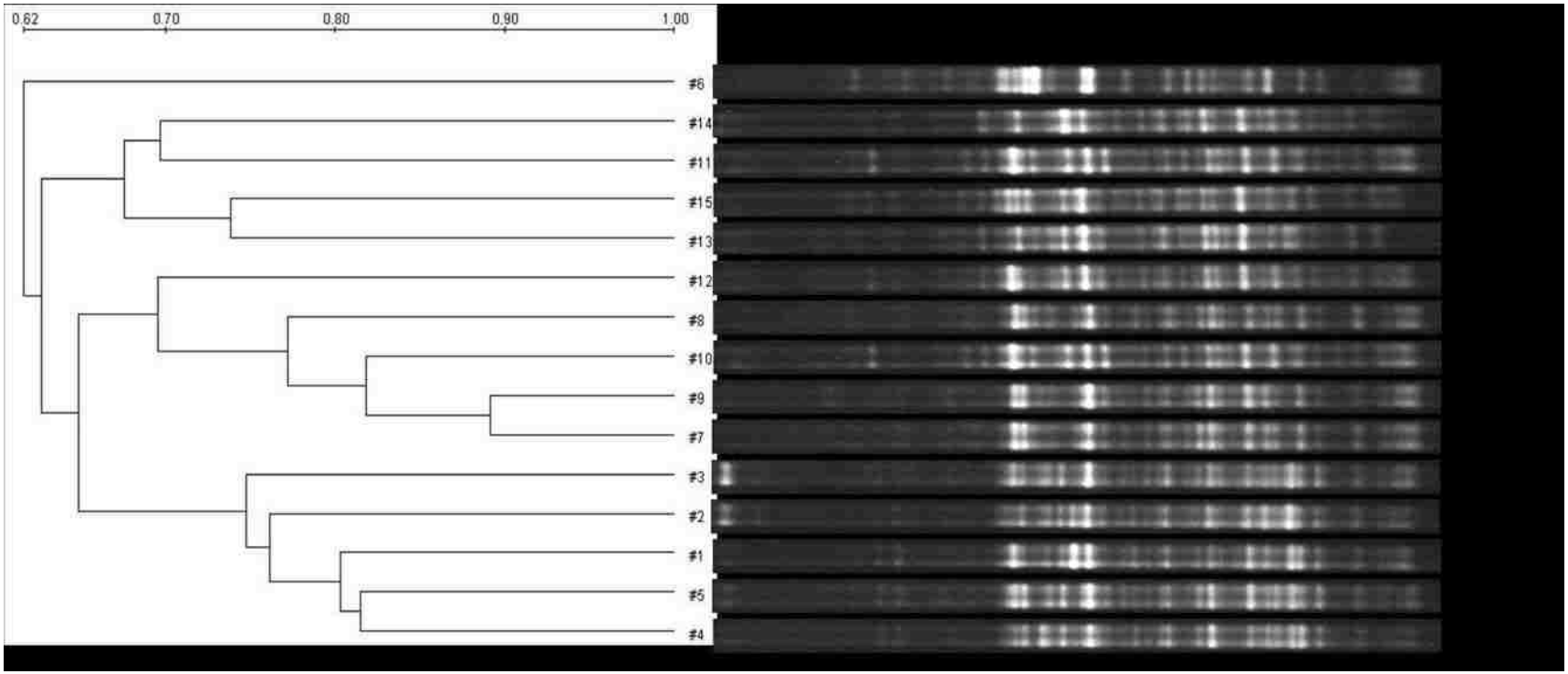


Figure 4. Analysis of phylogenetic tree based on DGGE results using pooled samples

T1: Leiber® MB40 (#1 to #5), T2: Leiber® ExCel (#6 to #10), C: Control (#11 to #15)

8. Appendix

8.1. Authorship Statement for Chapter 6

Si Hong Park is the first author of the paper and completed at least 51% of the studies among coauthors which the title is "**Assessment of Gastrointestinal Microflora in Pasture Flock Raised Chickens Fed with Two Commercial Prebiotics**" in chapter 6.

Major Advisor: Dr. Steven C. Ricke

Date: June 4th, 2013

CONCLUSIONS

The demands for organic and alternative poultry production by consumers have continued to expand in the past few decades since these products being a source of safer and healthier foods. Both *Campylobacter* and *Salmonella* are the most common foodborne pathogens pressent in the chicken ceca which also contains diverse and abundant bacterial communities. Prebiotics represent several effects on the GI tract by selective stimulation of beneficial bacteria as well as inhibition of undesirable bacteria.

The molecular methods developed in this research provide several advantages over currently published methods. The time to detect and identify the three pathogens was reduced from 48 hours with culturing to just 4 hours with the multiplex PCR. The multiplex PCR assay allowed less time and reagents to be used. The DGGE approached have been utilized to compare and analyze bacterial communities in complex GI tract ecosystems by amplification of common 16s rDNA sequences. Microarrays represent a comprehensive approach for the detection and characterization of foodborne pathogens in food matrices as well as the identification of differential gene expression levels in the chicken host when exposed to different experimental or environmental conditions.

In conclusion, as the organic and alternative poultry production systems continue to become more popular, there will be an increased need for efficient methods to rapidly and accurately detect host, microbiome and metabolome responses to derive predictable responses that allow for routine formulation in commercial settings. Such standardization is needed if there is to be less risk due to exposure from foodborne pathogens and potentially improved bird performance originating from these alternative systems.

APPENDIX

1. Other publications - Si Hong Park

- Journal of Applied Microbiology, 111: 426-432, 2011

ORIGINAL ARTICLE

Detection of *Salmonella* spp. survival and virulence in poultry feed by targeting the *hilA* gene

S.H. Park[1], R. Jarquin[2], I. Hanning[3], G. Almeida[1] and S.C. Ricke[1]

1 Center for Food Safety – Department of Food Science, University of Arkansas, Fayetteville, AR, USA
2 Department of Poultry Science, University of Arkansas, Fayetteville, AR, USA
3 Department of Food Science and Technology, University of Tennessee, Knoxville, TN, USA

- Journal of Food Protection, 75: 174-178, 2012

Characterization of *Staphylococcus aureus* Isolates from Retail Chicken Carcasses and Pet Workers in Northwest Arkansas

IRENE HANNING,[1]* DAVID GILMORE,[2] SEAN PENDLETON,[1] SCOTT FLECK,[1] ASHLEY CLEMENT,[1] SI HONG PARK,[1] ERIN SCOTT,[1] AND STEVEN C. RICKE[1]

[1]*Center for Food Safety, 2650 Young Avenue, University of Arkansas, Fayetteville, Arkansas 72704; and* [2]*Department of Biological Sciences, Arkansas State University, Jonesboro, Arkansas 72467, USA*

- Food Bioscience, 1: 66-72, 2013

Feeding mice aged and fresh blackberries powder supplements result in shifts in the gastrointestinal microflora

Si Hong Park[a,b,d], Irene Hanning[c], William Gilbert[d], Michelle Munro[b,1], Latha Devareddy[d], Steven C. Ricke[a,b,d,]*

[a]Cell and Molecular Biology Graduate Program, University of Arkansas, Fayetteville, AR, USA
[b]Center for Food Safety, Department of Food Science, University of Arkansas, Fayetteville, AR, USA
[c]Department of Food Science and Technology, University of Tennessee, Knoxville, TN, USA
[d]Department of Food Science, University of Arkansas, Fayetteville, AR, USA

- Journal of Nutrition and Food Sciences, 3: 178, 2013

Analysis of Microbial Populations and Metabolism of Anthocyanins by Mice Gut Microflora Fed with Blackberry Powder

Joseph Salyer[1], Si Hong Park[1,2], Steven C. Ricke[1,2] and Sun-Ok Lee[1*]

[1]*Department of Food Science, University of Arkansas, Fayetteville, AR, USA*
[2]*Cell and Molecular Biology Graduate Program, University of Arkansas, Fayetteville, AR, USA*

2. Institutional Animal Care and Use Committee (IACUC)

- Regarding the IACUC approval for chicken study in chapter 5 and chapter 6, we do not need the IACUC approval for these studies.

FW: IACUC results
4 messages

Corliss Ann Obryan < cobryan@uark.edu> Thu, Nov 8, 2012 at 9:51 AM
To: Si Hong Park <parksh@uark.edu>

Si Hong,

Here is the email on the 2 projects not needing IACUC approval.

Corliss

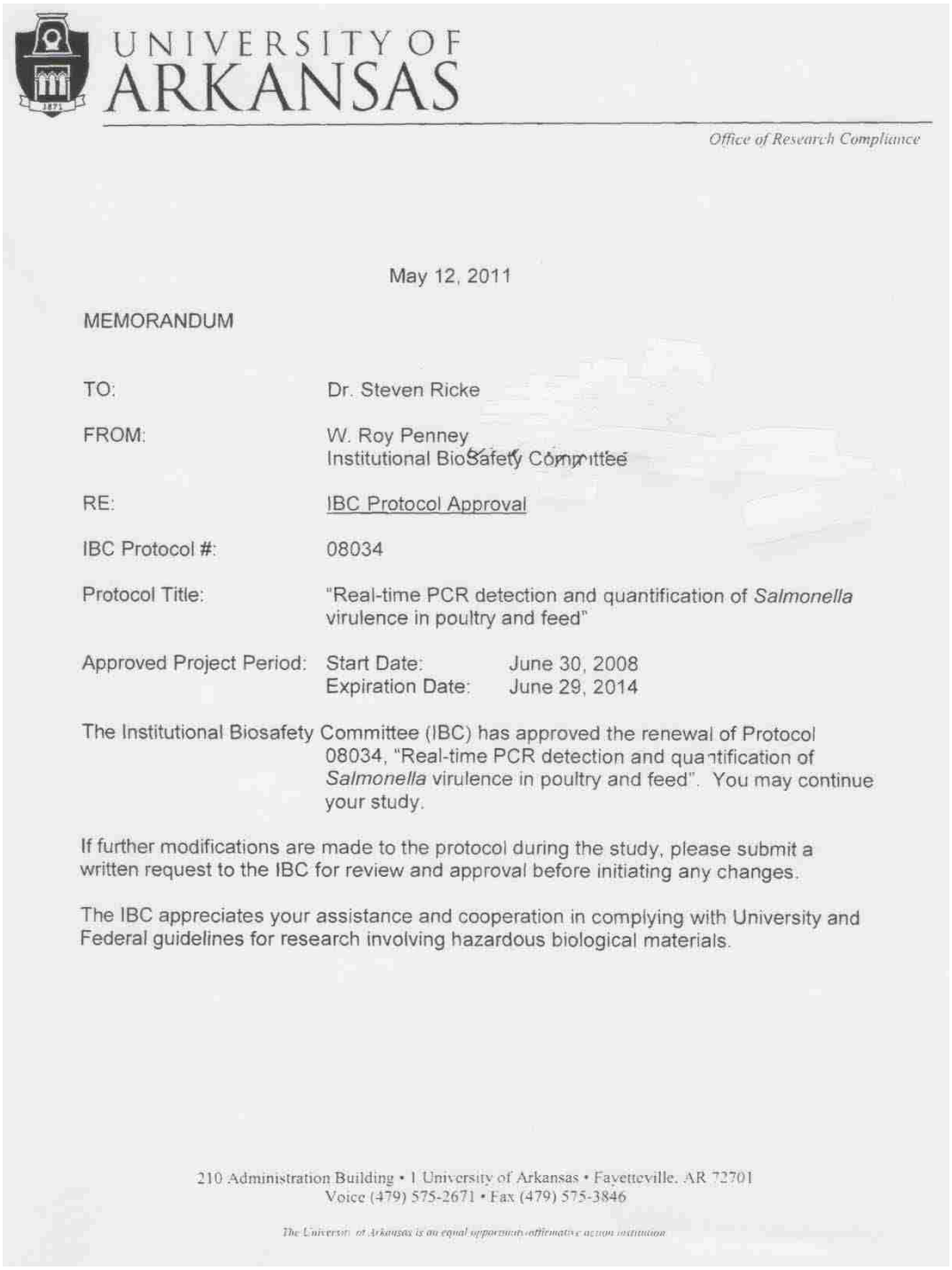
From: Carol Ann Rodlun
Sent: Friday, November 02, 2012 3:14 PM
To: Steven C. Ricke; Kristen Elizabeth Gibson; Corliss Ann Obryan
Cc: Billy M. Hargis; Craig N. Coon
Subject: IACUC results

To All: After much discussion at the meeting and input from both Billy Hargis, who was familiar with the nature of your projects and Jason Apple, who was familiar with the Jeff Chewning's operation (he had high praise for it) ; it was decided to return the two protocols with a decision of " Of No Action Required of the IACUC" with the request, citing the following portion of the UAF Policy on Use of Animals in Research and Teaching "*There is one exception to this policy, which is that specific Animal Use Protocols shall not be required for agricultural teaching applications involving the non-stressful observation of farm animals, demonstration of judging techniques, demonstration of accepted farm management practices, or normal use of farm animals in production.* ***Instead, standard operating procedures detailing such practices and procedures shall be kept on file in the office of Research Support and Sponsored Programs [Office of Research Compliance] and of the Associate Vice President for Agriculture-Research, and shall be incorporated into the Policies and Procedures of the Dale Bumpers College of Agricultural, Food and Life Sciences and the Agricultural Experiment Station***", that you send a memo to the IACUC (which I will distribute to the Committee) that includes a Standard Operating Procedure for the transport and euthanasia of birds that will be taken as samples from the production birds set for these kinds of studies. The IACUC would like for you to consult with Dr. Hargis as to the preparation of this memo and the SOP and how best to proceed with these kind of projects in the future.

This has certainly been a learning experience for all of us!

3. Institutional Biosafety Committee (IBC) Number

- This is an IBC number approval letter for chapter 2, 3 and 4.



UNIVERSITY OF ARKANSAS

Office of Research Compliance

May 12, 2011

MEMORANDUM

TO:	Dr. Steven Ricke
FROM:	W. Roy Penney Institutional BioSafety Committee
RE:	IBC Protocol Approval
IBC Protocol #:	08034
Protocol Title:	"Real-time PCR detection and quantification of *Salmonella* virulence in poultry and feed"
Approved Project Period:	Start Date: June 30, 2008 Expiration Date: June 29, 2014

The Institutional Biosafety Committee (IBC) has approved the renewal of Protocol 08034, "Real-time PCR detection and quantification of *Salmonella* virulence in poultry and feed". You may continue your study.

If further modifications are made to the protocol during the study, please submit a written request to the IBC for review and approval before initiating any changes.

The IBC appreciates your assistance and cooperation in complying with University and Federal guidelines for research involving hazardous biological materials.

210 Administration Building • 1 University of Arkansas • Fayetteville, AR 72701
Voice (479) 575-2671 • Fax (479) 575-3846

The University of Arkansas is an equal opportunity-affirmative action institution

08034 renewal IBC#: 08034

Please check the boxes for each of the forms that are applicable to the research project you are registering. The General Information Form - FORM 1 (this form) MUST be completed on all submitted project registrations, regardless of the type of research.

- [x] Recombinant DNA (EVEN IF IT IS EXEMPT from the NIH Guidelines.) (FORM 2)
- [ ] Pathogens (human/animal/plant) (FORM 3)
- [ ] Biotoxins (FORM 4)
- [ ] Human materials/nonhuman primate materials (FORM 5)
- [ ] Animals or animal tissues and any of the above categories; transgenic animals or tissues; wild vertebrates or tissues (FORM 6)
- [ ] Plants, plant tissues, or seed and any of the above categories; transgenic plants, plant tissues, or seeds (FORM 7)
- [ ] CDC regulated select agents (FORM 8)

To initiate the review process, you must attach and send all completed registration forms via email to ibc@uark.edu. All registration forms must be submitted electronically. To complete the registration, print page 1 of this form, PI sign, date, and mail to: Compliance Coordinator-IBC, 120 Ozark Hall, Fayetteville, AR 72701, or FAX it to 479-575-3846.

As Principal Investigator:

- [x] I attest that the information in the registration is accurate and complete and I will submit changes to the Institutional Biosafety Committee (IBC) in a timely manner.
- [x] I am familiar with and agree to abide by the current, applicable guidelines and regulations governing my research, including, but not limited to: the NIH Guidelines for Research Involving Recombinant DNA Molecules and the Biosafety in Microbiological and Biomedical Laboratories manual.
- [x] I agree to accept responsibility for training all laboratory and animal care personnel involved in this research on potential biohazards, relevant biosafety practices, techniques, and emergency procedures.
- [x] If applicable, I have carefully reviewed the NIH Guidelines and accept the responsibilities described therein for principal investigators (Section IV-B-7).
- [x] I will submit a written report to the IBC and to the Office of Recombinant DNA Activities at NIH (if applicable) concerning: any research related accident, exposure incident, or release of rDNA materials to the environment; problems implementing biological and physical containment procedures; or violations of NIH Guidelines.
- [x] I agree that no work will be initiated prior to project approval by the IBC.
- [x] I will submit my annual progress report to the IBC in a timely fashion.

Principal Investigator Typed/Printed Name: Dr. Steven C. Ricke

Signature (PI): _____ Date: _____

CONTACT INFORMATION:

Principal Investigator:

Name:	Steven C. Ricke
Department:	Food Science
Title:	Professor
Campus Address:	FDSC E-27
Telephone:	479-575-4678
*After Hours Phone:	479-387-4433
Fax:	479-575-6936
E-Mail:	sricke@uark.edu

Co-Principal Investigator:

Name:	Click here to enter text.
Department:	Click here to enter text.
Title:	Click here to enter text.
Campus Address:	Click here to enter text.
Telephone:	Click here to enter text.
*After Hours Phone:	Click here to enter text.
Fax:	Click here to enter text.
E-Mail:	Click here to enter text.

*Required if research is at Biosafety Level 2 or higher

PROJECT INFORMATION:

Have you registered ANY project previously with the IBC? Choose an item.

Is this a new project or a renewal?

☐ New Project ☐ Renewal

Project Title:	Real-time PCR detection and quantification of Salmonella virulence in poultry and feed
Project Start Date:	7/1/2008
Project End Date:	6/30/2014
Granting Agency:	Cobb-Vantress

Indicate the containment conditions you propose to use (check all that apply):

☑ Biosafety Level 1 Ref: 1 2 ☐ Biosafety Level 1A Ref: 1 2 ☐ Biosafety Level 1P Ref: 1 2

☑ Biosafety Level 2 Ref: 1 2 ☐ Biosafety Level 2A Ref: 1 2 ☐ Biosafety Level 2P Ref: 1 2

☐ Biosafety Level 3 Ref: 2 ☐ Biosafety Level 3A Ref: 2 ☐ Biosafety Level 3P Ref: 2

References:

1: Biosafety in Microbiological and Biomedical Laboratories (BMBL) 4th Edition

2: NIH Guidelines for Research Involving Recombinant DNA Molecules

3: University of Arkansas Biological Safety Manual

If you are working at Biosafety Level 2 or higher, has your laboratory received an onsite inspection by the Biosafety Officer or a member of the IBC?

◉ Yes ○ No

If yes, enter date if known: Click here to enter a date.
If no, schedule an inspection with the Biological Safety Officer.

Please provide the following information on the research project (DO NOT attach or insert entire grant proposals unless it is a Research Support & Sponsored Programs proposal).

Project Abstract:

The proposed research aims at reducing and preventing Salmonella colonization in poultry. Salmonella is a leading cause of foodborne bacterial diarrhea in the U.S. Poultry and poultry products are considered to be a major source of Salmonella infections in humans. Salmonella can colonize the gut of the chicken without causing any symptoms of disease.Infection of poultry breeder flocks with Salmonella is not tolerated and infected flocks are destroyed causing a large loss of profits. Poultry feed is considered to be a major source of Salmonella and therefore control of this initial contamination is crucial to preventing flock colonization. Processing of feed aims at eliminating Salmonella, but may not always be effective. Detection of Salmonella in feed may be hindered by inadequate sampling procedures, levels of Salmonella being too low to detect and / or inhibitors of PCR present in the feed.

Specific Aims:

1) To develop a nucleic-acid based PCR assay for the detection of Salmonella in feed to be used to determine if the feed process reduces or eliminates Salmonella. 2) Determine if processing of feed contaminated with Salmonella enhances virulence of Salmonella using a reverse transcriptase PCR assay and a bird model.

Relevant Materials and Methods (this information should be specific to the research project being registered and should highlight any procedures that involve biohazardous or recombinant materials):

Handling:a. Feed processing simulation experiments. A cryogenic vial containing a bacterial culture in glycerol will be removed from the freezer and placed in a biological safety cabinet. A loop full of the bacterial culture will be inoculated into the appropriate media and allowed to grow in the incubator (37C or 42C). After 24 to 48 hours, the cultures will be washed by centrifugation and resuspended in fresh broth or saline solution. Samples will be taken and enumerated on plates to determine the exact starting concentration. Feed components, such as corn and soy bean, will be soaked in Salmonella cultures to allow bacteria to attach and penetrate the foods. After incubation, feed components will be rinsed to remove any unattached cells with sterile PBS. Feed components then will be dried, by freezing or vacuuming. Feed components will be treated by heating to 70C for 2 minutes to simulate heat treatment in a feed mill processing. The feed components then will be suspended in an enrichment broth of Rappaport medium for 24 h at 37C. Serial diluted portions of the enriched samples will be inoculated onto Brilliant Green agar to determine the viability of Salmonella.b. Real-time and reverse transcriptase PCR assays. A cryogenic vial containing a bacterial culture in glycerol will be removed from the freezer and placed in a biological safety cabinet. A loop full of the bacterial culture will be inoculated into the appropriate media and allowed to grow in the incubator (37C or 42C). After 24 to 48 hours, the cultures will be washed by centrifugation and resuspended in fresh broth or saline solution. Salmonella will be inoculated into feed and dried as described above. The feed will be processed under simulated conditions as described above. Feed will be sampled and samples will be used for DNA preparations utilized in real-time and reverse transcriptase PCR assays. A sample will also be taken and inoculated into pre-enrichment broth as described above to validate the real-time PCR assays. c. Preparation and challenge of Salmonella. A cryogenic vial containing a bacterial culture in glycerol will be removed from the freezer and placed in a biological safety cabinet. A loop full of the bacterial culture will be inoculated into the appropriate media and allowed to grow in the incubator (37C or 42C). After 24 to 48 hours, the cultures are washed by centrifugation and resuspended in fresh broth or saline solution. Samples will be taken and enumerated on plates to determine the exact starting concentration. Poultry will be inoculated with Salmonella by utilizing feed prepared as described above at the Arkansas Veterinary Farm and personnel are required to wear surgical gloves and laboratory coats or overalls during the procedure.d. Isolation of Salmonella from the tissues. Tissue samples will be collected at the University of Arkansas Veterinary farm and personnel are required to wear surgical gloves and laboratory coats or overalls. Tissues will be transported to the lab at POSC (L-311) for determination of pathogen content. Samples will be inoculated into the appropriate media and allowed to grow in an incubator (37 or 42, 48 hours) for enumeration. No bird challenges will be conducted prior to IACUC approval. Because Salmonella is a BSL-2 pathogen, all the same precautions taken in the laboratory will also be taken at the poultry health farm. These procedures will include:For security purposes, the birds will be housed in an isolator access limited to authorized personnel. Only personnel that have been trained and working on this experiment will be permitted to enter the isolator where birds are being housed. On door, warning signs will be posted which reads – “Biohazard, No Eating, Smoking, or Drinking. This is a restricted area”. Emergency contact information with phone numbers will be posted on the doors. A list of biohazardous agents in use also will be posted on the door. In the isolator room, a list of emergency phone numbers will be posted which includes phone numbers for medical emergency, poison control center, chemical emergency, chemical/biological spill and the University Health Center.In the event of personnel exposure, depending on the nature of exposure, the lab personnel are trained to take simple measures such as washing using tap water, etc. to decontaminate first and then contact PI and office of Environmental Health and Safety and Pat Walker Health Center for further instruction and treatment. The PI, Pat Walker Health Center, Office of Environmental Health and Safety and Fire Department’s contact information will be posted on the front door of the isolator room and by the telephone.

The information requested above can be entered directly or cut & pasted into the space provided, or can be provided as an attached word document. If you provide an attachment, please indicate "See Attached" and list the file name(s) in the space below:

Click here to enter text.

PERSONNEL QUALIFICATIONS & FACILITY INFORMATION:

List all personnel (including PI and Co-PI) to be involved in this project:

Name (First and Last) - Position (Title, academic degrees, certifications, and field of expertise)	Qualifications/Training/Relevant Experience (Describe previous work or training with biohazardous and/or recombinant DNA; include Biosafety Levels)
Example: Bob Biohazard - Associate Professor, PhD- Microbiology	14 yrs working with E. coli at BL1, Salmonella enterica at BL2, 8 yrs working with transgenic mice.
Dr. Steven Ricke	20+ years of experience working as a PI, running research laboratory, and working with BSL-2 pathogens
Robin Jarquin, MSc, graduate student	Graduate Assistant trained under Dr. Steven Ricke, 8 years working with BSL-2 level pathogens and 8 years working with chicken models
Arunachalam Muthaiyan, Ph.D., Post Doctoral Associate, Food Science	11 years working in BL-1 and BL-2 labs and working with E. coli, Acinetobacter calcoaceticus, Staphylococcus aureus and Listeria monocytogenes
Corliss O'Bryan, PhD, Post Doctoral Assoc, Food Science	30 years working with BSL1 and BSL2 microorganisms, 15 years lab supervisory experience
Ashley Clement, Grad student, Program Assoc.	4 years working with BSL2 pathogens
Si Hong Park, graduate student	3 years working with BSL2 pathogens
Robert Story, MA, Program Associate	20 years working with BSL1 and 2 organisms
Click here to enter text.	Click here to enter text.

Additional Personnel Information (if needed):

Click here to enter text.

List all the laboratories/facilities where research is to be conducted:

Building:	**Room #:**	**Category:**	***Signage Correct?**
POSC	L-311	**Laboratory**	**Yes**
POSC	L-344	**Autoclave/BioStorage**	**Yes**
POSC	L-341	**Autoclave/BioStorage**	**Yes**
Poultry Vet Farm	Determined by Vet. Farm	**Animal Care**	**Yes**
Biomass	132	**Laboratory**	**Yes**
Biomass	101	**Autoclave/BioStorage**	**Yes**

		Choose an item.	Choose an item.
		Choose an item.	Choose an item.
		Choose an item.	Choose an item.
		Choose an item.	Choose an item.

* Biohazard signs are required for entrances to Biosafety Level 2 (including Animal Biosafety Level 2) areas. EH&S will supply these signs. If an updated biohazard sign is required, please indicate the location and what agents/organisms/hazards should be listed on the sign:

Click here to enter text.

Additional Facility Information (if needed):

Click here to enter text.

SAFETY PROCEDURES:

Please indicate which of the following personal protective equipment (PPE) will be used to minimize the exposure of laboratory personnel during all procedures that require handling or manipulation of registered biological materials.

Gloves:

- ☑ Latex
- ☑ Nitrile
- ☐ Other
- ☐ Vinyl
- ☐ Leather

Specify: Click here to enter text.

Face & Eye Protection:

- ☐ Face Shield
- ☑ Safety Glasses
- ☐ Other
- ☐ Safety Goggles

Specify: Click here to enter text.

Clothing Protection:

- ☑ Re-usable Lab Coat
- ☑ Disposable Clothing Protection
- ☐ Other
- ☑ Re-usable Coverall

Specify: Click here to enter text.

Dirty or contaminated protective clothing cleaning procedures: (Check all that apply)

- ☐ Autoclaved prior to laundering or disposal
- ☐ Laundered by qualified commercial service
- ☐ Other
- ☑ Laundered on site using bleach

Specify: Click here to enter text.

Outline procedures for routine decontamination of work surfaces, instruments, equipment, glassware and liquid containing infectious materials. Autoclaving or using fresh 10% bleach as a chemical disinfectant are preferred treatments; please specify and justify any exceptions:

Work surfaces will be decontaminated with a freshly prepared 10% bleach solution before and after working. Exception is biosafety cabinets which will be disinfected before and after use with Lysol® No Rinse Sanitizer in order to avoid the corrosiveness of the bleach on the metal of the biosafety cabinets. Instruments and equipment will be decontaminated by wiping down with 10% bleach. Paper towels used for these purposes will be discarded in biohazard bags. Glassware, waste, and disposable tubes will be autoclaved under standard conditions (15 psi, 121 C, 20 min). Disposable items (pipette tips, pipets, etc) will be discarded into 10% bleach. After 30 minutes it will be permissible to place these items in a biohazard bag for autoclaving before disposal.

Describe waste disposal methods to be employed for all biological and recombinant materials. Include methods for the following types of waste: (ref: UofA BiosafetyManual)

Sharps:

Placed into 10% bleach solution for decontamination followed by discarding into sharps waste container.

Cultures, Stocks and Disposable Labware:

Placed into biohazard bags and autoclaved before disposal. Liquids will be disposed of in drains after autoclaving. Disposable glass will be placed in glass disposal after autoclaving.

Pathological Waste:

Liquid biological waste will always be discarded into freshly made 10% bleach and then autoclaved for decontamination treatment before it is discarded. Other biological waste will be placed carefully into biohazard waste bags, autoclaved at 15 psi, 1210C for 20 min.

Other:

Click here to enter text.

Autoclave(s), to be used in this project, location(s) and validation procedures:

Autoclaves are located in L-344 and BIOR 101. All the materials and disposables contaminated with the pathogens will be either 1) disposed into biohazard bags (procedures conducted in L311) or 2) burned in the farm incinerator (performed at the farm). The glassware and containers as well as the biohazard bags will be autoclaved at 121C and 15psi for 15 to 45 min in the autoclave in POSC L-344. Autoclaved dishware is washed with detergent for future use. For biohazard bags, the autoclave tapes will be checked after autoclave to ensure sterilization. The bags then will be placed in an ordinary trash bag for disposal.Autoclaves conditions are also validated using a sterilization integrator (VWR catalog # 34010-019).

Will biological safety cabinet(s) be used?

Yes

If yes, please provide the following information:

Make/Model	Serial Number	Certification Expiration	**Location** (bldg/room)
Lab Conco/Delta Series	011117862E	10-11	L-313
Lab Conco/Delta Series	050334974	10-11	L-313
Biosafety Cabinet Level II ThermoForma Model 1186	100663	**11/30/2011**	Biomass Res. Center, Room 132
Biosafety Cabinet Level II FormaScientific Model 1000	13324-539	**11/30/2011**	Biomass Res. Center, Room 132
Biosafety Cabinet Level II FormaScientific Model 1126	12118-128	**11/30/2011**	Biomass Res. Center, Room 132
Biosafety Cabinet Level II Forma Scientific Model 1284	104294-5978	**11/30/2011**	Biomass Res. Center Room 136
Biosafety Cabinet Level II Baker Model VBM 400	SP7888V	**11/30/2011**	Biomass Res. Center, Room 132

Additional Biological Safety Cabinet Information (if needed):
Click here to enter text.

Indicate if any of the following aerosol-producing procedures will occur: (check all that apply)

- ☑ Centrifuging
- ☐ Blending
- ☐ Sonic Disruption
- ☐ Dissection
- ☐ Stomacher
- ☐ Other
- ☐ Grinding
- ☐ Vigorous Shaking or Mixing
- ☑ Pipetting
- ☐ Innoculating Animals Intranasally

Describe: Click here to enter text.

Describe the procedures/equipment that will be used to prevent personnel exposure during aerosol-producing procedures:

All personnel are required to wear surgical gloves and laboratory coats or overalls during procedures involving infectious the agent. Any procedure involving pipetting will be done under a biosafety cabinet to prevent personnel exposure to aerosols. All centrifuges are contained units to prevent exposure to aerosols.

EMERGENCY PROCEDURES:

In the event of personnel exposure (e.g. mucous membrane exposure or parenteral inoculation), describe what steps will be taken including treatment, notification of proper supervisory and administrative officials, and medical follow up evaluation or treatment:

In the event of exposure, the affected area will be rinsed or washed thoroughly (eyes, nose, mouth or skin abrasion). The PI will immediately be informed. The individual will be encouraged to consult with the physicians in the University of Arkansas Center for any symptoms related to the diseases that could be caused by the agent. In the event of personnel exposure, depending on the nature of exposure, the lab personnel are trained to take simple measures such as washing using tap water, etc. to decontaminate first and then contact PI and office of Environmental Health and Safety and Pat Walker Health Center for further instruction and treatment. The PI, Pat Walker Health Center, Office of Environmental Health and Safety and Fire Department's contact information is posted on the front door of L-311 and by the telephone.A list of Emergency phone numbers is posted in the laboratory (L-311, BIOR 132). This includes the phone numbers for medical emergency, poison control center, chemical emergency, chemical/biological spill and the University of Arkansas Health Center.

In the event of environmental contamination, describe what steps will be taken including a spill response plan incorporating necessary personal protective equipment (PPE) and decontamination procedures.

In case of spills, the responsible researcher will wear gloves and cover the spill area with paper towels (small spills) or chemical sorbent pads and soak in disinfectant for 5 minutes. The materials will be discarded into the biohazard bag. Material Safety Data Sheets are located in L-311 and BIOR 132 for reference. In addition, a first aid kit, biohazardous spill kit, and chemical spill kit are located in L-311 and BIOR 132. A spill kit for large chemical spills is located at the end of the hall inside the lab wing and in BIOR 132. A list of Emergency phone numbers is posted in the laboratory (L-311, BIOR 132). This includes the phone numbers for medical emergency, poison control center, chemical emergency, chemical/biological spill and the University of Arkansas Health Center. All researchers handling the infectious agent are encouraged to consult a physician in the University Health Center for any symptoms related to the disease that could be caused by the agent.

TRANSPORTATION/SHIPMENT OF BIOLOGICAL MATERIALS:

Transportation of Biological Materials: The Department of Transportation regulates some biological materials as hazardous materials; see 49 CFR Parts 171 - 173. Transporting any of these regulated materials requires special training for all personnel who will be involved in the shipping process (packaging, labeling, loading, transporting or preparing/signing shipping documents).

Will you be involved in transporting or shipping human or animal pathogens off campus?

No

If yes, complete the remaining:

- ☐ Cultures of Human or Animal Pathogens
- ☐ Environmenatl samples known or suspected to contain a human or anumal pathogen

☐ Human or animal material (including excreta, secreta, blood and its components, tissue, tissue fluids, or cell lines) containing or suspected of containing a human or animal pathogen.

Transportation/Shipment Training: Have any project personnel who will be involved in packaging, labeling, completing, or signing shipping documents received formal training to ship infectious substances or diagnostic specimens within the past 3 years?
Choose an item.

If yes, please provide the following information:

Name	Date Trained	Certified Shipping Trainer
Click here to enter text.	Click here to enter a date.	Click here to enter text.
Click here to enter text.	Click here to enter a date.	Click here to enter text.
Click here to enter text.	Click here to enter a date.	Click here to enter text.
Click here to enter text.	Click here to enter a date.	Click here to enter text.
Click here to enter text.	Click here to enter a date.	Click here to enter text.