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Molecular mechanisms associated with survival of *Salmonella enterica* in broiler feed are serovar and strain dependent

Ana Gissel Andino Dubón
University of Tennessee - Knoxville, aandinod@utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Ana Gissel Andino Dubón entitled "Molecular mechanisms associated with survival of *Salmonella enterica* in broiler feed are serovar and strain dependent." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Irene B. Hanning-Jarquin, Major Professor

We have read this thesis and recommend its acceptance:

Faith Critzer, Michael O. Smith

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Abstract

Food animals including poultry are known as a major reservoir for *Salmonella*. Poultry and poultry products are the leading sources of non-Typhi serotypes of *Salmonella enterica*. Feed has been recognized as a source of *Salmonella* in chickens. However, considering the fact that feed components have very low water activity of 0.4 approximately. The mechanisms of *Salmonella* survival in the feed and subsequent colonization of poultry are unknown. Given the conditions of the source of the main ingredients, processing, transportation and storage, poultry feed has a higher potential than other sources to become contaminated with *Salmonella*. Data indicate that prevalence of *Salmonella enterica* in human foodborne illness is not related to their prevalence of isolation from feed. Thus, it appears that survival in poultry feed may be an independent factor unrelated to virulence of specific serovars of *Salmonella*.

In this research, we examine the survival rates and gene expression of *Salmonella* in poultry feed. Fifteen different serovars isolated from human infections or poultry inoculated in poultry feed were assayed to determine survival rates at 0, 4, 8, 24 hours, 4 and 7 days. In addition, genes associated with colonization (*hilA, invA*) and survival via fatty acids synthesis (*cfa, fabA, fabB, fabD*) were evaluated using real-time PCR at four different time points, 0, 2, 4, and 24 hours after inoculation. This study demonstrated that the ability of *Salmonella enterica* to survive over storage time in poultry feed was serovar and strain dependent. Furthermore, the data indicate that the upregulation of short chain fatty acid synthesis and down regulation of virulence genes may be associated with survival in poultry feed.

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Introduction

Salmonella general characteristics

Salmonellae are facultative anaerobic Gram-negative rod-shaped bacteria generally 2-5 microns long by 0.5-1.5 microns wide and motile by peritrichous flagella. Genome sizes of *Salmonella* vary among serovars (Table 1) with ranges from 4460 to 4857 kb. Salmonellae belong to the family Enterobacteriaceae and are a medically important pathogen for both humans and animals. Salmonellae form a complex group of bacteria consisting of two species, six subspecies and include more than 2,579 serovars (Grimont and Weill, 2007; Malorny *et al.,* 2011). Two species are currently recognized in the genus *Salmonella*, *S. enterica* and *S. bongori* (Tindall *et al.,* 2005). *S. enterica* can be subdivided into the subspecies *enterica, salamae, arizonae, diarizonae, houtenae* and *indica* based on biochemical and genomic modifications (Brenner *et al.,* 2000). The majority of *Salmonella* are lactose fermenters, hydrogen sulfite producers, oxidase negative and catalase positive. Other biochemical properties that allow identification of *Salmonella* include the ability to grow on citrate as a sole carbon source, decarboxylate lysine, and ability to hydrolyze urea (Jensen and Hoorfar 2000; Abulreesh 2012).

The main niche of *Salmonella* serovars is the intestinal tract of humans and farm animals. It can also be present in the intestinal tract of wild birds, reptiles, and occasionally insects. Feedstuff, soil, bedding, litter and fecal matter are commonly identified as sources of *Salmonella* contamination in farms (Le Minor 1991; Sanchez, 2002; Rodriguez *et al.,* 2006; Hoelzer *et al.,* 2011). As *Salmonella* colonizes the gastrointestinal tract, the

organisms are excreted in feces from which they may be transmitted by insects and other animals to a large number of places and are generally found in polluted water. Salmonellae do not originate in water therefore their presence denotes fecal contamination (Albureesh 2012). Humans and animals that consume polluted water may shed the bacteria through fecal matter continuing of the cycle of contamination.

Foodborne Illness

Like many other infectious diseases, the course and outcome of the infection depends on variable factors including the dose of inoculation, the immune status of the host and the genetic background of both the host and the virulence of the pathogen (Sanderson and Nair, 2013). In the U.S., *Salmonella* is the leading foodborne pathogen, causing the largest number of deaths and has the highest cost burden (Batz *et al.*, 2011). The annual costs associated with salmonellosis for 2010 were estimated at $2.71 billion for 1.4 million cases (USDA, 2013). The highest numbers of *Salmonella* outbreaks from the past decade are related to land animals, with poultry as a major reservoir (Table 2). From 1998 to 2008, poultry and eggs were involved in the majority of *Salmonella* outbreaks. A considerable number of outbreaks are related to crops (Table 3). From 1998 to 2008 fruits and nuts were the largest source of *Salmonella* outbreaks in plant products, followed by vine stalk vegetables and sprouts. More than 70% of human salmonellosis in the US has been attributed to the consumption of contaminated chicken, turkey or eggs (CDC, 2013). In Batz *et al.* (2012) study, *Salmonella* appears eight times between the top 20 ranked pathogen-food combinations and is most notably associated with poultry, produce and eggs. It is not always easy to identify specific serovars in an outbreak, in many cases *Salmonella* cannot be linked to a specific food component due to complex food

preparations using a variety of ingredients. In data from foodborne outbreaks related to human illness collected from 2007 to 2011, 89% of serotypes were identified (CDC, 2013). Serovar Enteritidis was the most frequently isolated followed by Typhimurium, Newport, Heidelberg and Montevideo (Table 4). The food vehicles associated with these serovars include a wide variety of products such as eggs, chicken, pork, leafy greens, peanut butter, turkey, dairy products and vegetables (Table 4).

Specific to poultry

Close to 145 *Salmonella* outbreaks have been associated with poultry meat, while 117 outbreaks were sourced to eggs from 1998 to 2008, causing illness in 2580 and 2,938 people respectively (CDC, 2013). Salmonellae can enter and survive in the farm enviroment for long periods of time. Prevalence of *Salmonella* in farm enviroments ranges from 10 to 26% (Rodriguez *et al.,* 2006). Feed contamination with fecal matter has a great potential of incidence in conventional farms, being able to horizontally spread *Salmonella* contamination. Presence of *Salmonella* in feed and feed ingredients is well documented (Alali *et al.,* 2010; Bailey *et al.,* 2001; Maciorowski *et al.,* 2004; Rodriguez *et al.,* 2006). However, very low levels of *Salmonella* have been obtained from drinking water samples from broiler farms. Conversly, recovery of *Salmonella* was easily accomplished in samples from stagnant water where the bacteria can form biofilm layers in water pipes or hoses (Alali *et al.,* 2001; Bailey *et al.,* 2001; Lilebjelke *et al.,* 2005). Variety and prevalence of *Salmonella* serovars differs among studies in different regions and types of farms. Yet, there is some consistency in recovery rates of specific serovars: Heidelberg, Kentucky, Enteritidis, Typhimurium, Montevideo, Seftenberg and Thompson as these are the highest recovered serotypes (Bailey *et al.,* 2001; Roy *et al.*, 2002; Lilebjelke *et al.,* 2005). In a one

year experiment in a integrated operation, Bailey *et al.* (2001) found that hatchery transport pads, flies, drag swabs and boot swabs exhibited the highest prevalence of *Salmonella*. The most frequently identified serotypes from those farm samples were Seftenberg, Thompson and Montevideo. While in farms samples, serotypes Kentucky, Enteritidis, Heidelberg, Typhimurium and antigenic formula I 4, 5,12:i:- were commonly isolated from broilers (Table 5) and ground chicken (Table 6) according to reports from the monitoring system by the USDA through the Food and Safety Inspection Service (FSIS) from 2000 to 2009.

Shell eggs are a major vehicle for *S.* Enteritidis in humans. By 1994 *S.* Enteritidis became the most frequently serovar reported in US causing human salmonellosis. From 1985 to 2003 in 75% of *S.* Enteritidis outbreak cases, eggs were confirmed as the primary ingredient or food vehicle of contamination (CDC, 2013). A major outbreak occurred in 1994 where tanker trailers that previously carried *S.* Enteritidis contaminated liquid eggs caused the cross-contamination of ice-cream prepared at the same facility (Hennessy *et al.,* 1996). Serovar Enteriditis is known to be very well adapted to the hen house environment, the bird, and the egg. Most commonly, eggs are infected with *S.* Enteritidis by vertical transmission through transovarian infection from laying hens (Braden, 2006). *S.* Typhimurium and other serovars usually contaminate eggs externally by penetrating the egg shell (Martelli and Davies, 2012). Surveys conducted in US report *Salmonella* contamination in table eggs by other serovars including Heidelberg and Montevideo (Jones and Musgrove, 2007; Martelli and Davies, 2012). Enhanced biosecurity practices, post harvest intervention methods (sanitizing and decontamination) and egg pasteurization can reduce the risk factors for *Salmonella* infection in laying hen operations (Howard *et al.,* 2002).

Differences in *Salmonella* serovars

Diseases in chickens

Poultry are a specific host for *S.* Pullorum and *S.* Gallinarum and these rarely cause illness in humans. These *Salmonella* serovars are non-motile, host-specific that causes Pullorum disease (PD) and Fowl Typhoid (FT), respectively (Rettger 1909).

Pullorum disease was first described as "fatal septicemia" or "white diarrhea" (Rettger 1909). Clinical signs are predominantly observed in young chickens, showing lack of appetite, depression, respiratory distress, caseous core diarrhea and early death a few days after hatching. In laying hens symptoms include reduced egg production, fertility and hatchability (Bullis, 1977; Lister and Barrow, 2009; Hafez 2010). *S.* Pullorum may cause severe systemic lesions including peritonitis, liver and spleen enlargement, and organs may be streaked with hemorrhages. Furthermore, animals can also develop white focal necrosis in the case of young birds and abnormal color and shape in ovaries in older birds. Pullorum disease mortality rate is variable, but maybe as high as 100% in critical cases.

Fowl typhoid disease is caused by *S.* Gallinarum and affects chickens, turkeys, guinea fowl and birds of all ages and breeds (Shivaprasad *et al.,* 2013). The first described outbreak was characterized by high mortality and signs of the disease that began with yellow-to-green diarrhea with the birds dying a few days after infection (Rettger1909). Conversley to *S*. Pullorum, *S*. Gallinarum is more frequently seen in growers or older birds than young birds. One of the first signs of this disease is an increase in mortality rate, followed by a decline in feed consumption and therefore a drop in egg production and weight gain (Lister and Barrow, 2009). Histological examination reveals fatty degeneration

of the liver, occasionally accompanied by areas of necrosis, disintegration of muscle fibers and congestion and perivascular infiltration of mononuclear cells in the kidneys (Shivaprasad 2000).

Salmonella Pullorum and *S.* Gallinarum have been eradicated in developing regions including the U.S., Canada and Western Europe but are still problems in other parts of the world. Control programs that incorporated good hygiene management, biosecurity enforcement, serological tests and slaughter policies helped with the eradication of these pathogens. In 1935, the U.S. Federal Government executed the National Poultry Improvement Plan (NPIP) in order to reduce the mortality of chickens from Pullorum and Gallinarum disease. In the 1950's poultry breeders and hatchers in the U.S. implemented tests (blood analysis, tube agglutination and rapid serum test) for *S.* Pullorum and *S.* Gallinarum on a regular basis while uniform national management standards were adopted. Furthermore, in the 1950's vaccination was implemented to control pullorum disease and fowl typhoid. Two decades later both diseases were eradicated and by 1975 there was no evidence of infection in commercial poultry (Bullis 1977; Boyd 2001; Kabir 2010).

It has been suggested that clearing poultry flocks of *S.* Gallinarum and *S.* Pullorum opened a favorable niche for *S.* Enteritidis (Baulmer *et al.,* 2000; Cogan and Humphrey, 2003; Kumar *et al.,* 2009). The use of mathematical models with data from Europe and U.S. indicates that *S.* Gallinarum excluded *S.* Enteritidis from poultry (Rabsch *et al.*, 2000). Coincidently, *S.* Enteritidis detection was on the rise after eradication of *S.* Gallinarum and *S.* Pullorum, and by the 1990's it was the most frequently reported serovars in the U.S. Unlike avian *Salmonella* pathogens, serovar Enteritidis has rodents as reservoirs, making it more difficult to control on the farms. *S.* Enteritidis and *S.* Gallinarum

are antigenically similar, both belonging to serogroup D1 possessing a similar lipopolysaccharide structure and O9 antigens. When commercial flocks were cleared from *S.* Gallinarum, serovar Enteritidis was able to colonize chickens without noticeable signs of disease or without producing anti- O9 titers. It is believed that seropositive *S.* Pullorum chickens had an enhanced immunity dominant O9 antigen that protected against *S.* Enteritidis infection (Baulmer *et al.,* 2000).

Diseases in humans

Clinically, salmonellosis may be manifested as gastroenteritis, septicemia, or enteric fever. Enteric fevers are caused by the human-specific pathogens *S. enterica* serovars Typhi and Paratyphi. Infection severity may vary by the resistance of each individual and the immune system as well as the virulence of the *Salmonella* isolate (Gianella and Jay, 2008).

Typhoid and paratyphoid fevers

Salmonella Typhi is a motile, non-lactose fermenting bacillus that causes most endemic and epidemic cases of typhoid fever globally (Connor and Schwartz 2005; Crump *et al.,* 2008). Enteric fevers cause 200,000 deaths and 22 million illnesses per year, with the highest incidence happening in Southeast and Central Asia where it is endemic (Crump *et al.,* 2004). Doses from $10^3 – 10^9$ CFU of *Salmonella* Typhi are known to cause enteric fever. (Fangtham and Wilde, 2008).

Non-typhoidal salmonellosis

Like enteric fevers, non-typhoidal salmonellosis (NTS) are spread via the fecal-oral route, but estimated cases of NTS worldwide greatly surpass those for enteric fevers. Unlike Typhi and Paratyphi, non-typhoidal Salmonellae are not human-restricted. Many serovars closely related to foodborne outbreaks include *S.* Typhimurium, *S.* Enteritidis, *S.* Newport and *S.* Heidelberg and have reservoirs in farm animals (Rabsch *et al.,* 2001; Rodriguez *et al.,* 2006). Among other foodborne pathogens, NTS is the leading cause of death and hospitalizations (Scallan *et al*., 2011). In NTS, cases are characterized by gastroenteritis or bacteraemia, symptoms may involve nausea, vomiting, diarrhea, and are typically self-limiting lasting approximately 7 days. *Salmonella* can also induce chronic conditions including aseptic reactive arthritis and Reiter's syndrome.

Differences among serovars with respect to disease severity

Different *Salmonella* serovars may demonstrate unique reservoirs and pathogeneses. It is still poorly understood why a few *Salmonella* serovars are responsible for a majority of human diseases, but nearly all of them belong to subspecies *enterica*. In a 1995 global survey, serotypes Enteritidis and Typhimurium were the most prevalent serovars of all isolates (Herikstad *et al.,* 2002). The biggest difference among severity and treatment methods are between enteric fever salmonellae and non-typhoid salmonellae (Table 7). It is suggested that a combination of factors specific to each serovar including the presence of plasmid virulence genes (*spv*), surface cell structure, flagellin and pathogenity islands (SPIs) are involved in severity of salmonellosis. It has been demonstrated that *S.* Seftenberg and *S.* Litchfield have large deletions in invasion related genes, which might have been the result of a selective advantage in the intestinal

environment (Ginocchio *et al.,* 1997). Jones *et al.* (2008) analyzed data of more than 50 salmonellosis cases from 1996 to 2006 assesing differences among serovars in terms of severity (Table 8). From these data, the most common salmonellosis outcomes were related to serovars Typhimurium, Enteritidis and Newport, while fatality rates reported were in most cases related to serovars Dublin, Muenster and Choleraesuis.

Differences among serovars with respect to antibiotic resistance

Resistant *Salmonella* strains are commonly found in food animal sources (Swartz 2002; Su *et al.,* 2004). Mismanagement of antimicrobial agents for treatment in humans and animals and the use of growth promoters in livestock has promoted antimicrobial resistance in Salmonellae (Su *et al.,* 2004; Hur *et al.,* 2012). The occurrence of *Salmonella* serovars resistant to quinolones, fluoroquinones, and third generation cephalosporins which are medically significant treatments has increased (Rajashekara *et al.,* 2000; Martin *et al.,* 2004; Mather *et al.,* 2013). According to a NARMS report in 2010, the serovars with greater resistance to antimicrobials are Typhimurium specifically to ampicillin, chloramphenicol, streptomicin, sulfamethoxazole/sulfisoxazole, and tretracycline (ACSSuT), as well as Enteritidis with resistance to naldixic acid. Serovars Newport, Heidelberg, Dublin and I4, [5], 12:i:- were also shown to be resistant to various antimicrobial groups (Table 9). In terms of multidrug resistance (more than 5 antimicrobials) the most prevalent serovars of epidemiological importance are Typhimurium, Heidelberg, Dublin, Paratyphi B and I4, [5], 12:i:- (Table 10). Although *S.* Enteritidis is highly prevalent in human infections, it has lower antimicrobial resistance compared to other serovars. Antimicrobial resistance in *Salmonella* can be associated with horizontal transference of antibiotic resistant genes characteristically found on mobile

genetic elements among *Salmonella* strains and other Enterobacteria or by clonal spread of antimicrobial drug resistant serovars that are particularly effective in worldwide dissemination (Davies *et al.,* 2002; Butaye *et al.,* 2006; Michael *et al.,* 2006; Alcaine *et al.,* 2007). The mechanisms from which *Salmonella* develops resistance include production of enzymes that can degrade cell permeability to antibiotics, activation of antimicrobial efflux pumps, and production of β-lactamase to degrade the chemical structure of antimicrobial agents (Sefton 2002; Foley and Lynne 2008).

Farm animals have been a common source of isolation for antimicrobial resistant *Salmonella* serovars (Dunne *et al.,* 2000; Gupta *et al.,* 2003; Zhao *et al.,* 2003). A predominantly infectious *S.* Typhimurium DT104 emerged in the 1980's and has managed to spread worldwide. This serovar commonly carries chromosomally based resistance to five antimicrobials (ACSSuT) and it is believed that it was disseminated worldwide by human travel and then spread locally by the absence of effective antimicrobials (Glynn *et al.,* 1998; Acheson and Hohmann 2001; Davies *et al.,* 2002). *Salmonella* Newport has been identified to harbor plasmids encoding ACSSuT and produces β-lactamase, which inactivates cephalosporins, providing resistance to ampicillin and chloramphenicol (AmpC). In human isolates from *S.* Heidelberg showing high invasive infections, large plasmids (IncA/C and IncI1) were found to carry multiple resistance genes (Han *et al.,* 2011; Hur *et al.,* 2012). It is believed that horizontal transmission of virulence genes in multi-drug resistant *Salmonella* strains can increase virulence, invasiveness and cause higher mortality rates compared to susceptible *Salmonella* (Glynn *et al.,* 1998; Angulo and Molbak 2005; Varma *et al.,* 2005).

Prevalence

On the farm

Cattle

Salmonellosis in cattle is caused by numerous serovars, with *S.* Typhimurium and *S.* Dublin being the most common (La Ragione *et al.,* 2013). *Salmonella* Dublin serovar is commonly detected in calves and adult cattle. Most infections are introduced into *Salmonella* free herds by the purchase of infected animals that might have acquired infection on farm premises, in transit or on dealer's premises (Wray *et al.,* 1990). Another route of contamination can be water-borne infection. During the early stages of the acute enteric disease affected animals develop fever, dullness, loss of appetite, depressed milk yield and adult pregnant animals may abort (Kahrs *et al.,* 1972; La Ragione *et al.,* 2013). Infection with *S.* Dublin in humans is commonly developed after contact with carrier animals but can also be transmitted through contaminated food and may cause gastroenteritis (Fone and Barker, 1994; Uzzau *et al.,* 2000).

In samples taken by FSIS/USDA from 2000 to 2009 from cows and bulls, the increasing prevalence of serovars Montevideo, Newport, Agona, Kentucky and Mbandanka is notable over the last decade (Table 11). Furthermore, when steers and heifers were submitted to the same testing *S*. Dublin, *S.* Montevideo, *S.* Typhimirium, S. Anatum and *S.* Newport were more prevalent than other serovars (Table 12). Beef products are among the top five products related to *Salmonella* foodborne outbreaks (Table 2). When ground beef was tested, a constant increase in *S.* Montevideo and *S.* Dublin isolates was detected from 2004 to 2009, followed by serovars Newport, Typhimurium and

Anatum (Table 13). In the previous decade, a multistate sample collection from dairy cows revealed 7.3% of the samples were positive for *Salmonella* and the five most dominant serotypes were Meleagridis, Montevideo, Typhimurium, Kentucky and Agona (Blau *et al.,* 2005). However, 83% of the isolates were susceptible to all the antimicrobial drugs tested.

Pigs

Pigs are an important reservoir of human non-typhoidal salmonellosis and the isolation of the organism from pork and pork products is very common. Porcine salmonellae consist of two groups separated by host range and clinical presentation. The first group consists of the host-adapted serovar *S.* Choleraesuies, which tends to elicit systemic disease in the form of septicaemia with a high mortality rate in young pigs. The second group consists of all the other serovars, which have a broader host range and tend to produce momentary enteritis, for example *S.* Typhimurium. Like other animal farms, the prevalence of *Salmonella* from swine varies depending on the region and type of farm surveyed. Prevalence of *Salmonella* in samples taken from swine farm environments ranges from 3- 33% (Davies *et al.,* 1999; Rodriguez *et al.,* 2006; Foley *et al.,* 2007). When fecal samples were taken from grower and finisher pigs, the prevalence among serovars was higher for *S.* Derby and *S.* Typhimurium followed by Agona and Anatum, which are among the serovars with highest incidence in human foodborne outbreaks (APHIS/ USDA, 2009). Moreover, 79.6% isolates were resistant to at least one antibiotic (APHIS/ USDA, 2009). Antimicrobial resistance has been more likely associated with *S.* Typhimurium and *S.* Derby and pigs can become asymptomatic carriers (Boyen *et al.,* 2007).

In the US, from 2000 to 2009 the most prevalent serovars isolated from market hogs were Derby, Typhimurium, Johannesburg, Infantis and Anatum, two of which were

also in the top five serotypes isolated from humans in the same period (Haley *et al*., 2012). Other serovars commonly isolated from pigs in recent years include Heidelberg, Saintpaul and Agona (Table 14). Since the early 1990's there has been a shift in the predominant serovar isolated from swine, where Cholerasuis had a higher incidence this serovar has been replaced by *S*. Typhimurium.

Poultry

Chicks may acquire *Salmonella* via vertical transmission from the parent, but horizontal transmission from environmental facilities, transportation, feed, vectors including humans, rodents and insects can be a significant problem (Foley *et al.,* 2007; Wales and Davies, 2013). Among commercial layers, contaminated eggs will typically result from flock infections acquired via persistent environmental *Salmonella,* and are associated with the serovar Enteritidis (van de Giessen *et al.,* 1994; Kinde *et al.,* 1996; Wales *et al.,* 2006). In studies conducted in poultry farms, *Salmonella* prevalence ranges between 5 - 100% among various environmental and fecal samples (Jones *et al.,* 1991; Carramiñana *et al.,* 1997; Bailey *et al.,* 2002; Rodriguez *et al.,* 2006). It appears, *Salmonella* Enteritidis filled an ecological niche that was available after eradication of serovars Pullorum and Gallinarum. *S.* Enteritidis was the most prevalent serovar isolated from chickens during the 1990's but that has changed in the following decade. In recent years the serotypes commonly associated with chickens are Enteritidis, Kentucky, Heidelberg, Typhimurium and I 4, [5], 12:i:- (Table 5 and Table 6).

From food products

Salmonella outbreaks linked to consumption of non-meat foods has rapidly increased during the last decade. Recent data indicates that 13% of the *Salmonella* outbreaks in the US have been related to contaminated non-meat foods (Doyle and Erickson, 2008; Hanning *et al.,* 2009). *Salmonella* Saintpaul, *S.* Rubislaw and *S.* Javiana spread by paprika and paprika-powdered potato chips caused outbreaks with more than 1000 infected people (Lehmacher *et al.,* 1995). An increase of *S.* Oranienburg infections was registered in the early 2000's where multi-state control studies revealed the consumption of chocolate as the apparent cause of infection (Werber *et al.,* 2005). Epidemiological and environmental investigations indicate that cross-contamination in the manufacturing plants may be the cause of outbreaks associated with low moisture foods (Doyle and Buchanan 2013). *Salmonella* Typhimurium, *S.* Ofda, *S.* Tennessee and *S.* Poona were isolated from sesame paste and sesame seed which were sold for raw consumption in cereals (Brookmann *et al.*, 2004). It is known that bacteria on plant surfaces may form large biofilm with other bacteria (Cooke *et al.,* 2007). The persistence of these biofilms makes it difficult to clean and sanitize the crops. These factors are thought to contribute to outbreaks related to plant products including fruits, nuts and vine stalk vegetables (Table 3). Outbreaks of salmonellosis associated with seafood that occurred in the U.S. could be from cross-contamination during farming, processing, preparation and transportation. From 1999 to 2011, serovars Newport, Typhimurium, Dublin, Montevideo and Java were reported to have caused outbreaks associated with consumption of milk and cheese products in the US (Doyle and Buchanan 2013). The reason some *Salmonella* serovars are more prevalent in specific food products is not

completely understood. It is suggested that Salmonellae react in a serovar dependent manner to environmental stresses including differences in temperature, chemical and low-nutrient available conditions which can vary by food.

Survival (Different Stresses)

Temperature

Salmonella is considered to be mesophilic with some strains being able to survive at extreme low or high temperatures (2^{o}C to 54^{o}C). Sigma factors are proteins that compose fundamental subunits of prokaryotic RNA polymerase and provide a mechanism for cellular responses by redirecting transcription initiation (Kazmierczak *et al.,* 2005). Alternate sigma factors control the gene expression of bacteria by sensing the changes in the environment. The sigma factors can sense perturbation in the outer membrane and activate genes in response to heat stress in order to adapt to high temperatures. The mechanism used is by specific activation and transcription of *rpo*H genes under high temperature. RpoH is a virulence factor of *Salmonella* and other enteric bacteria and provides protection against heat stress in the cytoplasm (Spector and Kenyon, 2012). Transcription of *rpo*H genes in *S*. Enteritidis showed the highest level when cultured at 42^{o}C. Additionally all virulence genes were upregulated in response to high temperature (Brumell *et al*., 2001; Yang *et al.,* 2014).

Water activity (a_w) in foods is defined as the ratio of the vapor pressure of water in a food matrix compared to that of pure water at the same temperature. High time and temperature are required to kill 90% of *Salmonella* populations (*D*-value) in low a_w foods and may reflect the low efficiency of thermal inactivation in dry foods involved in

Salmonella related outbreaks including flour, nuts, butter, dry milk and chocolate (Scott *et al.,* 2009; Doyle and Buchanan 2013). The surrounding moisture and the conformation of the food matrix can influence the thermo tolerance of *Salmonella* by increasing the temperature required to inactivate the organism. Under low a_w conditions in high carbohydrate or high fat products, the heat resistance of *S.* Seftenberg strain 775W was greater than *S.* Typhimurium (Goepfert and Biggie 1968; Moats *et al.,* 1971; Gibson 1973; Mattick *et al.,* 2001). It is widely known that *S.* Seftenberg strain 775W has high resistance to heat, with a thermotolerance approximately 30 times more than *S.* Typhimurium. The thermotolerance of *Salmonella* in poultry products including liquid egg yolks and chicken meat highlights the distinctiveness of *S.* Seftenberg to survive high cooking temperatures. Other strains of *S.* Seftenberg and *S.* Bedford have shown similar inactivation temperatures to strain 775W. *Salmonella* Senftenberg and *S.* Typhimurium exhibited higher resistance to heat in chicken litter among other *Salmonella* serovars (Murphy *et al.,* 1999; Doyle and Mazzota, 2000; Chen *et al.,* 2013). Furthermore, heat stress encountered during feed processing increased the thermotolerance of *S.* Enteritidis strains and may induce expression of virulence gene *hil*A in *S.* Enteritidis, *S.* Typhimurium and *S.* Seftenberg (Churi *et al.,* 2010; Park *et al.,* 2011). It is believed that heat resistance development increases with pre-adaptation to temperature and it is influenced by the strain tested and culture conditions (Mañas *et al.*, 1991; Shah *et al.,* 1991).

Salmonella uses cold shock proteins (CSP) as a response for quick adaptation to a temperature downshift in the environment. The CSPs are created during the acclimation phase from 30^{o}C to 10^{o}C. During the downshift CSPs are synthesized for the cell to later resume growth (Jeffreys *et al.,* 1997; Craig *et al.,* 1998; Kim *et al.,* 2001). Many studies

have been conducted on the ability of salmonellae to increase its survival rate by expressing a CSP when treated at low temperature (5°C to 10°C) prior to freezing. *S.* Enteritidis was able to survive in chicken parts at 2°C, and in shell eggs at 4°C, while *S.* Typhimurium survived in minced chicken at 2°C. *Salmonella* Panama has also shown a elevated propensity to survive in agar at 4°C and *S.* Typhimurium and *S.* Tennessee had the ability to survive in estuarine environments below 10°C (Rhodes and Kator, 1988).

Chemicals

There are a wide variety of potential chemical stresses, including pH, oxidation, membrane disruption, and denaturation of critical macromolecules or metabolic poisons that can affect pathogenic bacteria (Lambert, 2008; Wales *et al.*, 2010). Chlorine, commonly used to disinfect water, can be antimicrobial to *Salmonella*. Salmonellae are capable of producing biofilms providing the organism with an exopolysaccharide matrix that inhibits chemical attack (McDonnell and Russell, 1999; Solano *et al.,* 2002; Lapidot *et al.,* 2006; White *et al,* 2006). Chlorine in recommended doses (2-5ppm of available chlorine) is able to control bacterial biofilm formation in poultry drinking systems and reduce incidence of *Salmonella* in the crop and ceca of broilers (Byrd *et al.,* 2003; Amaral, 2004). However, chlorination by itself is not enough to reduce *Salmonella* incidence and its degree of infection in birds. Other factors influencing the quality of drinking water for birds are the type of drinker system, pH (optimal pH 6-8) and overall contamination in the environment (Poppe *et al.,* 1986; Amaral, 2004). In chickens, *Salmonella* first reaches the crop (pH 4-5), as a result of bacterial lactic acid fermentation. If adaptation to that pH occurs, *Salmonella* can survive and adapt to a lower pH and therefore oppose antibacterial effects of the stomach (Rychlik and Barrow, 2005). Decontamination of broiler carcasses

occurs during immersion in the chilling tank and the bacterial load in each carcass is expected to be lower than the initial count. The use of chlorine at range of 20- 50 ppm in the chilling tank is enough to remove *Salmonella* biofilm on stainless steel. Chlorine is also used as a sanitizing method in poultry processing plants along with organic acids, inorganic phosphates and other organic preservatives. Treatments for decontamination of carcasses were performed on different strains of *Salmonella* in the presence of acidified sodium chlorite varied widely with serotype, the highest resistance levels were shown by serotypes Typhimurium, Newport, and Derby (Capita, 2007). Among organic acids the use of acetic and propionic acid have shown inhibitory effects against *Salmonella* (Chung and Goepfert 1970; Tamblyn and Conner 1996). Equipment sanitization is also important, and previous studies have shown the importance of combining sanitizing agents, including detergents and acids. Treatments with sanitizers and detergent successfully inactivated *S*. Enteritidis cells compared with a 50% inactivation by using sanitizers only (Zolotta and Sasahara, 1994). In general, chlorate preparations act as selective toxic agents to enteric pathogens by disrupting cell membrane causing the leakage of intracellular components in bacterium. In the case of organic acids their bactericidal activity is related to pH, affecting creation of un-dissociated acids that will acidify the cytoplasm and disrupt key biochemical processes.

Many virulence factors in bacteria, including *Salmonella*, are regulated via the PhoP/PhoQ system. PhoP genes act on the bacterial cell envelope by increasing the resistance to low pH and enhancing survival within the macrophage (Ernst *et al.,* 1999). *Salmonella* responds to acidic environmental challenges of pH 5.5 to 6.0 (pre-shock) followed by exposure of the adapted cells to pH 4.5 (acid shock), then activates a complex

acid tolerance response (ATR) that increases the potential of *Salmonella* survival under extremely acid environments (pH 3.0 to 4.0) (Alvarez-Ordoñez *et al.,* 2012). The ATR mechanism requires acid shock proteins including RpoS sigma factor and PhoPQ. It has been shown that RpoS and PhoPQ provide protection against inorganic acids, while regulators RpoS, iron regulatory protein Fur and adaptive response protein Ada had a major tolerance to stress in organic acids (Foster and Hall, 1992; Bearson *et al.,* 1998; Rychlik and Barrow, 2005). The PhoP locus is a crucial virulence determinant and *Salmonella pho*P strains are very sensitive to microbial peptides. Several genes, including *rpo*S, and some acid shock proteins and heat shock proteins are implicated in *Salmonella* virulence. Commonly isolated from chicken carcasses *S.* Kentucky shows more acid sensitivity (pH 5.5) than other *Salmonella* serovars (Enteritidis, Mbandaka and Typhimurium) (Joerger *et al*., 2009). When virulence gene presence was surveyed, acid adaptive stress genes including *rpo*S, *fur* and *pho*PQ were detected in *S.* Kentucky (Joerger *et al*., 2009). Virulent *S*. Typhimurium strains with mutations in the *rpoS* gene were unable to develop a full ATR and had significantly reduced virulence potential (Leyer and Johnson, 1993; Foster and Spector, 1995; Lee *et al.,* 1995).

It is known that virulence can be activated by acetic acid stress through the *hil*A gene. Virulence gene expression using *hil*A in response to pH showed up-regulation in strains Typhimurium 23595, Typhimurium 14028, Seftenberg, Heidelberg, Mbandanka, Montevideo and Infantis (Durant *et al.,* 2000; Gonzalez-Gil 2012).

Desiccation

Salmonella is heat tolerant, persistent in nature, survives long periods of time in dry products, but requires $a_w > 0.93$ for growth. Increasing numbers of multistate *Salmonella*

outbreaks associated with dry foods have occurred (Li *et al.,* 2012; Podolack *et al.,* 2010). Some of these outbreaks have been characterized by a low infectious dose. It is believed that enhanced virulence is induced by up-regulation of other stresses including acid and heat. Salmonellae can be exposed to desiccation stress in the poultry farm environment by numerous factors. Persistence of *Salmonella* cells in poultry house surroundings, dust, dry fecal matter, floor materials, and equipment remaining contaminated after cleaning and sanitization procedures can expose *Salmonella* to desiccation. The incapacity to detect dormant *Salmonella* cells may undermine routine hygiene checks (Sarlin *et al.,* 1998).

The genetic mechanism of *Salmonella* survival is related to the *proP* (Proline permease II) gene. When a *proP* deletion was assayed, mutants could not survive desiccation for long periods and became undetectable after four weeks. Sigma factor RpoS also plays a role in protecting cells from drying by stabilizing membranes and enzymes by threhalose synthesis, resulting in a more stable structure in the cell.

The formation of multicellular filamentous cells by *rdar* (red, dry and rough colony) morphology is a major change induced in *Salmonella* by low a_w exposure. *Rdar* morphology promotes formation of aggregative fimbriae and cellulose increases desiccation resistance in *Salmonella* cells, and these cells can remain viable for months (White and Surette 2006; Finn *et al.,* 2013). The a_w of food matrices, product formulation and storage temperature critically affect the survival of *Salmonella* in dry food matrices (Troller, 1986). When bacteria are exposed to desiccation stress, the a_w in the cell is lowered. Strains Enteritidis, Typhimurium, Mbandaka have been found to have greater persistence (over one year) than Seftenberg, but most authors agree than *S.* Seftenberg is the most tolerant to desiccation, surviving exposure to detergents and disinfectants up to 30

months (Derrick and Mackey 1982; Davies and Wray 1996; Kumar and Kumar 2003; Pedersen *et al.,* 2008).

More recently a cell shrinkage strategy for *Salmonella* has been studied as a mechanism of protection during desiccation. A scatter plot analysis showed that the conversion from rod shape to cocci occurred at a greater extent in *S.* Tennessee (strong desiccation resistance) than *S.* Typhimurium LT2 (weak desiccation resistance) responding to a 5 day desiccation treatment. Gene expression profile for the two strains significantly differed with *S.* Tennessee having no change in genes involved in cell elongation (rodA, rodZ, mrdB, mreB, mrdA, mrcA, and mrcB) after 24-hours of desiccation while S. LT2 cell morphology genes up-regulated from 38 to 91-fold (Megalis 2013).

Fatty acid associated genes

Adaptive mechanisms of *Salmonella* related to survival and virulence in low a_w foods include a modification of the fatty acid profile. *Salmonella* will induce and express genes encoding enzymes involved in the modification of the fatty acids, which will increase osmotolerance.

Increase in cyclopropane fatty acids is considered to be an indicator of starvation or desiccation stress (Kieft *et al.*, 1994). Fatty acid profiles affect the lipid membrane and increases osmotolerance. *Salmonella enterica* raises membrane fluidity via *fab*A and *fab*B pathway (Baysee and O'Gara 2007). The *cfa* gene encodes enzymes that increase membrane fluidity (Kim *et al.*, 2005). Up-regulation of short chain fatty acid related genes including, *fabA*, *fabB* and *cfa* was determined when *Salmonella* was inoculated in poultry feed (Andino *et al.*, 2014). Up-regulation of fatty acid catabolic genes has been identified

when *Salmonella* is exposed to dehydration stress under aerobic conditions (Li *et al.*, 2012; Finn *et al.,* 2013)

Cross-protection effects

It is believed that cross-protection between different factors including heat and acid stress can affect the virulence of *Salmonella*, although it is generally acknowledged that several genes, including *rpo*S, and some acid and heat shock proteins have related effects (Leyer and Johnson, 1992; Foster and Spector, 1995). For example, desiccation tolerance of *Salmonella enterica* can have a cross-tolerance effect for other stresses. *S*. Enteritidis, *S*. Newport, and *S*. Infantis and *S*. Typhimurium can show resistance to commonly used desinfectants, dry heat and UV irradiation when exposed to a previous dehydration stress. The interaction between temperature and pH is also important. Because cross protection effects can impact the survival and virulence of *Salmonella,* it is important evaluate these factors during formulation, processing and preservation of food products.

Conclusions

Salmonella is the leading foodborne pathogen, causing the largest number of deaths and the highest cost burden in the US. Poultry and poultry products have been related to a majority of *Salmonella* outbreaks in the past decade. *Salmonella* contamination in the farm environment and feed is a major concern.

Salmonella serovars are resilient microorganisms with a complex genomic system that makes the organism able to react to different harsh environmental conditions at the farm, during processing and in the gastrointestinal tract. Different stress factors include temperature, pH, osmotic shifts, and a_w beyond their normal growth range. These factors

pose a great risk to food safety during processing and storage of foods. Furthermore, more research is needed to understand why a few *Salmonella* serovars are responsible for a majority of human diseases and demonstrate such unique reservoirs and pathogenesis. With the description of stress mechanisms, mitigation methods can be implemented to contrast the probability of *Salmonella* contamination.

Chapter II. Survival of Salmonella enterica in poultry feed is serovar and strain dependent

A. Andino, S. Pendleton, N. Zhang, W. Chen, F. Critzer and I. Hanning*

Department of Food Science and Technology, University of Tennessee, 2605 River Dr., Knoxville TN, 37996.

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*Corresponding author: E-mail: ihanning@utk.edu; phone 865-946-1110; fax 865-974-7332.

Abstract

Feed components have low water activity making bacterial survival difficult. The mechanisms of *Salmonella* survival in feed and subsequent colonization of poultry are unknown. The purpose of this research was to compare the ability of *Salmonella* serovars and strains to survive in broiler feed and to evaluate molecular mechanisms associated with survival and colonization by measuring the expression of genes associated with colonization (*hilA, invA*) and survival via fatty acids synthesis (*cfa, fabA, fabB, fabD*). Feed was inoculated with one of 15 strains of *Salmonella enterica* consisting of 11 serovars (*S.* Typhimurium, *S.* Enteriditis, *S.* Kentucky, *S.* Seftenburg, *S.* Heidelberg, *S.* Mbandanka, *S.* Newport, *S.* Bairely, *S.* Javiana, *S.* Montevideo and *S.* Infantis). To inoculate feed, cultures were suspended in phosphate buffered saline (PBS) and survival was evaluated by plating samples onto XLT4 agar plates at specific time points (0h, 4h, 8h, 24h, 4d and 7d). To evaluate gene expression, RNA was extracted from the samples at the specific time points (0, 4, 8 and 24h) and gene expression measured with real time PCR (qRT-PCR). The largest reduction in *Salmonella* occurred at the first and third sampling time points (4 hours and 4 days) with the average reductions being 1.9 and 1.6 log cfu per g, respectively. For the remaining time points (8h, 24h, and 7d) the average reduction was less than 1 log cfu per g (0.6, 0.4, and 0.6, respectively). Most strains up-regulated *cfa* (cyclopropane fatty acid synthesis) within 8 hours which would modify the fluidity of the cell wall to aid in survival. There was a weak negative correlation between survival and virulence gene expression indicating down-regulation in order to focus energy on other gene expression efforts such as survival related genes. These data indicate the ability of strains to survive over time in poultry feed was strain dependent and that up-regulation of

cyclopropane fatty acid synthesis and down regulation of virulence genes were associated with a response to desiccation stress.

Introduction

Each year 31 identified pathogens caused an estimated 9.4 million episodes of foodborne illness in the U.S. (Scallan *et al*., 2011). Among these foodborne pathogens, nontyphoidal *Salmonella enterica* is the leading cause of death and hospitalizations (Scallan *et al*., 2011). Foodborne pathogens can be acquired by food producing animals, which may transmit zoonotic pathogens through the food chain and subsequently cause human foodborne illness (Crump *et al*., 2002). Poultry and poultry products are the leading source of non-Typhi serotypes of *S. enterica* in the U.S. (Braden, 2006). Poultry may be colonized with *S. enterica* but not cause any signs or symptoms of disease in the birds. Thus, if intestinal contents are released during processing, contamination of the carcasses may occur (Rigby, 1980).

The initial source of *S. enterica* to the birds can be transmitted from a number of vectors (Jarquin *et al*., 2009). Protein and by-product ingredients originating from animals, which are used in feed, have been suggested as a source of *S. enterica* (Williams, 1981; Davies *et al*., 2004). Given the conditions of the source of the main ingredients, processing, transportation and storage, poultry feed has a higher potential than other sources to become contaminated with *S. enterica* (Jones 2011).

Currently, *S. enterica* serovar Kentucky is the dominant serovar isolated from poultry and poultry products in the United States (Foley *et al*. 2008), but this serovar rarely causes foodborne illness. Conversely, even though isolation of serovar Enteritidis from poultry products has declined, infections with this serovar have increased (CDC 2010). Thus it appears that survival on the farm and in other poultry related environments including feed may not be related to the ability of *S. enterica* to cause disease (Foley *et al.*

2008). Therefore, the main objective of this study was to compare the survival capabilities of *S. enterica* serovars and strains in broiler feed over time in storage. A second objective was to investigate molecular mechanisms associated with survival and virulence by evaluating expression of specific genes associated with these characteristics.

Materials and Methods

Bacteria and culturing conditions

In these studies a total of 11 serovars consisting of 15 strains of *S. enterica* were utilized (Table 1). All *S. enterica* strains were initially cultured on tryptic soy agar (TSA, Becton, Dickinson and Company, Sparks, MD) and incubated at 37°C for 24h. After incubation, a 10µl loop of culture was inoculated into 30 mL of tryptic soy broth (TSB, Becton, Dickinson and Company; pH 7.2) and incubated in a shaking water bath at 37°C for 15h. From this culture, 1mL was inoculated into TSB and incubated in a shaking water bath at 37°C for 3h. The culture then was centrifuged at 8,000 x g for 5 min and the supernatant discarded. The culture was washed 3 times by resuspending the pellet in phosphate buffered saline (PBS, Becton, Dickinson and Company), centrifuging at 8000 x g for 5 min at 25^{o}C and finally resuspending in PBS. *Salmonella* suspensions were standardized to 0.15 at 630 nm by spectrophotometry so that all serovars were used at approximately the same concentrations (7 log CFU mL^{-1}). A dilution series was also conducted on the suspension to precisely determine the initial *S. enterica* concentration.

Spiking and analysis of feed sample

A Chick Starter/Grower-AMP BMD feed was purchased from a local Co-op (Knoxville, TN) and was sieved through a screen (No. 8; 2.38 mm openings) to remove

dust and small particles. The composition of the formulated starter feed is presented in Table 2. Water activity of the feed was measured using a water activity meter (Aqua Lab; Decagon Services, Inc. Pullman, WA). For the survival studies, 10µl aliquots of the *S. enterica* suspension prepared as described in the previous section were placed into 2 g of the feed in 5 mL tubes and mixed by agitation. The inoculated feed was stored at 25°C. At specific time points (0, 4, 8, 24 h, 4 and 7 d), *S. enterica* survival was evaluated using standard microbiological methods and a standard dilution series. We chose to use seven days because this is the average time of storage of poultry feed on poultry farms. Briefly, the sample was suspended in 2 mL of PBS, vortexed and a 100µl portion of the solution was used in a dilution series that was inoculated on XLT4 (xylose lysine tergitol-4, Becton, Dickinson and Company) agar which was incubated at 37°C for 24h. A unioculated sample of the poultry feed acted as the negative control. Triplicate samples were evaluated with two repetitions performed for each serovar.

RNA Preparation

Total RNA was isolated from the samples as described by Gonzalez-Gil *et al*. (2012) with some modification. At specific time points (0h, 4h, 8h and 24h) and equal volume of RNA protect bacterial reagent (Qiagen, Valenica, CA) was added to a 2ml microfuge tube containing the *Salmonella* feed suspensions and allowed to stand at room temperature for 5 min. Subsequently, RNA was extracted from the samples using the RNeasy mini kit (Qiagen) as directed by the manufacturer. After extraction, the RNA samples were subjected to a DNase treatment utilizing the Qiagen DNase kit (Qiagen) as directed by the manufacturer. All samples then were quantified using spectrophotometry (Nanodrop ND-1000; ThermoScientific; Pittsburgh, PA).

Quantitative Reverse Transcriptase Real Time PCR (qRT-PCR)

After purification, cDNA was synthesized from the RNA using the iScript™ cDNA Synthesis Kit (Bio Rad, Hercules, CA). All qRT-PCR reactions were performed as described by Gonzalez-Gil *et al*. (2012) using the ABI 7100 RT-PCR system (Applied Biosystems; Carlsbad, CA). Briefly, a 20µl total volume consisted of 10µl of Power SYBR® Green PCR Master Mix (Life Technologies Corporation, Carlsbad, CA), 300 nM of each primer, 100 ng of cDNA template and water to volume. With the exception of *hilA* and 16S rRNA, primers were designed using the NCBI Primer-BLAST tool and evaluated for specificity (Table 3). All primers were synthesized by Integrated DNA Technologies (Coralville, IA). The qRT-PCR reactions were optimized to the conditions of 95°C for 15 min for the initial activation of Taq polymerase. This was followed by 35 cycles of denaturation at 94°C for 15 sec., annealing at 55°C for 30 sec. and amplification at 60°C for 30 sec. with fluorescence being measured during the extension phase. Melting curves were conducted subsequently and consisted of 95°C for 15 sec., 60°C for 5 min. to a final temperature of 95°C for 15 sec. All reactions were performed independently and in triplicate.

Analysis of gene expression

Samples were normalized using the 16S rRNA gene as an internal standard (Table 3). The relative changes (n-fold) in gene expression between samples were calculated using the 2(-Delta Delta C(T)) method as described by Livak and Schimittgen (2001). Fold change in expression for specific target gene was determined and these data were utilized to generate heat maps within a Microsoft® Excel® 14.3.5 (Microsoft Corporation, Redmond, WA) spread sheet using the conditional formatting and color scale functions.

Statistical Analysis

For survival and water activity experiments, each strain was sampled in duplicate with triplicate repetitions, and culturable CFU counts were analyzed via mixed ANOVA analysis ($p < 0.05$) to determine statistical differences between strains. Results are expressed as least-square means with standard error of the means (SEM). For water activity measurements, each strain was sampled in triplicate for each time point and analyzed as above for the survival experiments. The software utilized was SAS® 9.3.

Results

The water activity of the sample of spiked feed was measured at specific times of 0, 4, 8, 24h, 4 and 7d (Table 4). This was done in order to correlate water activity in the feed with any impact on the survival of *S. enterica*. Not surprisingly, there was some correlation between the water activity in the spiked feed and the survival rates of the bacteria. Water activity consistently decreased over the course of the experiments, as did the counts of culturable *S. enterica*. However, the correlation coefficients indicated that there was no significant correlation between water activity and reduction in culturable *Salmonella*. This is most likely due to the large variation in reduction of *Salmonella* counts between each time point.

The culturable *S. enterica* populations (log CFU g^{-1}) were determined at 0, 4, 8, 24h, 4 and 7d, and differences in the survival of the bacteria were found to be dependent on serovar and strain (Table 5). After 7d, nearly 3 logs (CFU per g of feed) of *S. Enteriditis* (WT) and *S.* Typhimurium ATCC 23595 (LT2) were recovered from the feed samples. After 4d of incubation at room temperature, *S.* Typhimurium 14028 and *S.* Montevideo

could not be recovered. Both strains of *S.* Kentucky and *S.* Typhimurium 14028 had the most rapid decrease after 4h with approximately 3 logs (CFU per g of feed) less than the initial inoculum recovered from the feed. Both strains of *S.* Enteritidis, *S.* Seftenburg, *S.* Mbandanka and *S.* Infantis, had the lowest decrease (approximately 1 log CFU g^{-1}) in recoverable bacteria after 4 h. The remaining strains decreased by approximately 2 log CFU g^{-1} from the initial inoculum levels after 4 h of incubation at room temperature. Interestingly, data regarding strains of the same serovar was quite variable. The three Typhimurium strains had different patterns in reduction of *Salmonella*, while the strains of Kentucky and Enteritidis had similar patterns when comparing data of the same serovar.

Relative fold change in gene expression for each gene was calculated and heat maps generated for the 3 time points sampled over the course of the experiment (Figure 1). These maps then were sorted from ascending to descending for each gene. In this way, it was visually apparent that the *cfa* gene was up-regulated in most serovars after 4h. Furthermore, it appeared that there was a correlation between regulation of the *cfa* gene and the *fabB* gene at the 8 and 24 h time points (0.93 and 0.90, respectively). There were no other apparent gene regulation and gene correlations consistent among all strains.

Correlation analysis was performed to determine if survival of the *S. enterica* serovars was correlated to expression of specific genes. A low positive coefficient of correlation was obtained between bacterial survival and the genes *cfa, fabA* and *fabB* (0.23, 0.04, and 0.13, respectively). For the genes *invA, fabD and hilA,* a low negative correlation (-0.24, -0.04, and -0.28) was correlated with the survival capability of the *S. enterica* strains tested. Although the values of correlation were numerically different, they were not statistically significant ($P>0.05$).

Discussion

According to Ha *et al.* (1998), *S. enterica* survival in feed can vary and is dependent on formulation. In their study, Ha *et al*. (1998) also found that aerobic bacterial counts recovered from feeds containing meat and bone meal were greater than those containing soybean meals. However, Pektar *et al*. (2011) reported that there were no differences in the abilities of *S. enterica* to survive in conventional versus organic feed where the conventional feed contained bone and poultry meal which was replaced in the organic feed with alfala meal. *S. enterica* contamination on individual ingredients of the feed is also an important fact to consider, since *S. enterica* has been isolated from feed ingredients including, grains, oilseed meal, feather and fish meal and meat by-products (Maciorowski *et al*., 2004).

Survival of *S. enterica* in low water activity foods is well documented (Tamminga *et al*., 1976; Juven *et al*., 1984; Rowe *et al*., 1987; Lehmacher *et al*., 1995; Beuchat 2009). Interestingly, previous studies suggest that *S. enterica* survival is higher in foods with a_w between 0.43 and 0.55 than foods at a_w 0.75 (Juven *et al*., 1984 and Pektar *et al*. 2011). Since water activity did not drop below 0.61 in this study, water activity may have been suboptimal for the *S. enterica* strains we evaluated for survival in feed.

The *invA* gene allows *Salmonella* to enter epithelial cells, playing an important role in the invasion and disease process (Galán *et al*., 1992). The second virulence gene evaluated in this study, *hilA,* regulates the expression of invasion genes in response to environmental stimuli including osmolarity, oxygen levels, and pH (Durant *et al*., 2000; Fluit, 2005; Chuanchuen et al., 2010; Park *et al.* 2011; Gonzalez-Gil *et al*. 2012). In the present study, there was an overall negative correlation between survival and up-regulation

of these two genes indicating that perhaps efforts for virulence were shifted away from these genes and instead focused on up-regulation of stress responses (Gonzalez-Gil *et al*., 2012).

To survive the stress of desiccation, some bacteria increase membrane fluidity (Baysse and O'Gara 2007). For *S. enterica*, membrane fluidity can be modified with an increase in *de novo* synthesis of unsaturated fatty acids (UFA's), which occurs via the *fabA-fabB* pathway. Likewise, the *cfa* gene encodes CFA (cyclopropane fatty acid) synthase, an enzyme which cyclizes UFA to improve membrane fluidity (Kim *et al.* 2005). Conversely, *fabD* is activated to produce saturated fatty acids, which decrease membrane fluidity. Thus the up-regulation of *cfa* in this study at the 4 h time point was not surprising as an increase in CFAs is considered to be an indicator of starvation or desiccation stress (Kieft *et al*. 1994).

Low water activity food products can become cross contaminated after processing by factors including poor sanitization practices, poor equipment design and poor ingredient control, which presents a significant food safety risk (Podolack *et al*., 2010). Some research indicates the infectious dose of *S. enterica* is lower when infection occurs via a contaminated low a_w food (Greenwood and Hooper 1983; Rowe *et al*., 1987). The reason for this is not exactly known. However, data from this study indicates that this may not be due to up-regulation of virulence associated genes *hilA* and *invA* as our data showed a tendency for these genes to be down-regulated in lower water activity. Instead, the lower infectious dose may be an adaptive tolerance response where cells that survived the low water activity are more stress resistant making it easier for these cells to survive the subsequent stress of passage through the acidic gastrointestinal environment (Ma *et al.*

2009). It has also been suggested that pathogens in low water activity foods are typically metabolically inactive, and this metabolic state makes the cells less susceptible to stresses such as those encountered in the gastrointestinal environment (Barat *et al.* 2012).

Conclusions

The data indicate that differences in survival and gene expression vary by serovars of *S. enterica*, caution should be taken if applying the results of this study to other serovars of *S. enterica* that have not been evaluated. In addition, because only one type of feed and incubation temperature were used, additional experiments are necessary to understand how these variables may impact the results. In conclusion, this study demonstrated that the ability of *S. enterica* to survive over storage time in poultry feed was serovar and strain dependent. Furthermore, the data indicate that the upregulation of short chain fatty acid synthesis and down regulation of virulence genes may be associated with survival in the poultry feed component.

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Appendix

Table 1. Examples of some genomic characteristics of *Salmonella* serovars

Serovar	Genome size (kb)	G+C (%)	Plasmid size (kb)		Reference
Typhi CT18	4809	52.09	pHCM1: 218	pHCM2:106	Parkhill *et al*. 2001
Typhimurium LT2	4857	53	94		McClelland *et al.* 2001
[a] Typhi Ty2	4792	52.02			Deng *et al*. 2003
[a] Paratyphi A (ATCC 9150)	4585	53			McClelland *et al.* 2004
Choleraesuis SC-1367	4755	52.11	pSC: 138	pSCV: 50	Chiu *et al*. 2005
[a] Enteritidis PT4	4685	52.17			Thomson *et al*. 2008
[a] Gallinarum	4658	52.22			Thomson *et al.* 2008

[a] No plasmid

Table 2. Number of national *Salmonella* foodborne outbreaks linked to farm animals from 2006 to 2011 (CDC, 2013)

Food Animals	Number of outbreaks	Number of Illness
Poultry	145	2580
Eggs	117	2938
Pork	43	1043
Beef	37	1138
Dairy	21	682
Game	4	48

Table 3. Number of national *Salmonella* foodborne outbreaks linked to crops from 2006 to 2011 (CDC, 2013)

Food	Number of outbreaks	Number of Illness
Fruits/nuts	36	2359
Sprouts	21	711
Vine stalk vegetables	21	3216
Leafy vegetables	11	306
Roots	6	172
Grains/beans	5	259
Oil/sugar	1	14
Fungus	1	10

Table 4. Examples of *Salmonella* serovars isolated from foodborne outbreaks in humans and most common food items related to each serovar from 2007 to 2011. (CDC, 2013).

Serovar	# Outbreaks	%	Ill	Hospitalized	Deaths	Most common food vehicles
Enteritidis	167	27%	4972	394	2	Egg, chicken, pork, beef
Typhimurium	84	14%	2043	342	9	Chicken, leafy greens, peanut butter
Heidelberg	44	7%	1875	212	5	Chicken, turkey, dairy products
Newport	63	10%	1581	209	2	Sprouts, vegetables, tomatoes, pork, poultry
Montevideo	21	3%	1154	141	0	Beef, pepper, pork, cheese
Braenderup	19	3%	203	29	1	Pork, chicken, vegetables
Muenchen	17	3%	229	34	1	Sprouts, deli meat, fruit
Infantis	16	3%	363	34	0	Pork, turkey, beans
Javiana	14	2%	876	73	1	Chicken, pork, fruits, vegetables
Saintpaul	10	2%	1866	340	2	Peppers, tomatoes, poultry, beef

Table 5. Examples of *Salmonella* serovars (total % serotypes) profile of Pathogen Reduction/ Hazard Analysis and Critical Control Point (PR/HACCP) systems verification samples from broilers (USDA/FSIS, 2010)

***Salmonella* serovar**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**
Kentucky	25.49	33.59	36.28	35.96	42.74	45.18	48.97	47.14	36.83	39.61
Enteritidis	2.68	1.62	3.13	3.51	6.06	7.71	13.66	10.82	18.31	20.78
Heidelberg	23.05	24.81	24.88	19.85	15.15	14.52	11.34	13.43	12.96	14.07
[b] Typhimurium	6.4	6.39	4.37	6.05	5.22	9.45	8.08	8.96	11.52	6.49
[a]I 4,5,12:i:-					3.03	4.18	4.3	2.49	3.29	2.16
Montevideo	4.31	3.05	1.9	2.06	2.09	3.47	1.63	2.24	2.06	1.73
Schwarzengrund	2.91	3.05	1.71		2.82	2.83	1.29		1.44	1.3
Typhimurium (var. Copenhagen)	6.64	3.34	6.36	9.56	8.78					
Hadar	4.89	2.96	4.37	1.82		1.03				
Thompson	3.14	2.48	2.18	2.06		1.16				
Infantis			1.33		1.25		1.03	1.49	2.06	

[a] Prior to 2004, isolates fitting the designation were included in the unidentified isolates category.

[b] After 2005 Typhimurium includes Typhimurium 5- (formerly Copenhagen).

Table 6. Examples of *Salmonella* serovars profile of Pathogen Reduction/ Hazard Analysis and Critical Control Point (PR/HACCP) systems verification samples from ground chicken (USDA/FSIS, 2010)

	% Total Serotyped									
***Salmonella* serovar**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**
Kentucky	26.53	18	16	20	12.89	31.91	42	24.81	28.57	30.88
Enteritidis	4.08		4.8		1.8	31.91	16	25.56	20	29.41
Heidelberg	18.37	26	29.6	25.71	1.55	12.77	16	20.3	24.76	10.29
Typhimurium	12.24	10	9.6	0.95	1.8	6.38	4	6.02	5.71	7.35
[a] I 4,5,12:i:-					0.26	2.13	4	5.26	0.95	4.41
Braenderup					0.26					2.94
Infantis	4.08		3.2	3.81	0.52		3	2.26	1.9	1.47
Montevideo			4.8	1.9	1.29				1.9	
Schwarzengrund	2.04	20	3.2		1.29		3	1.5		
Hadar	6.12	4	3.2	27.62	0.26	2.13	1			
Thompson	4.08	4	3.2	5.71	1.03	2.13		2.26		

[a] Prior to 2004, isolates fitting the designation were included in the unidentified isolates category.

Table 7. Examples of characteristic features of enteric fever and non-typhoidal salmonellosis

	Enteric fever	**NTS**
Natural host	Humans	Food Animals, reptiles, insects
Common related serovars	Typhi and Paratyphi	Enteritidis, Typhimurium, Heidelberg
Incubation period	7 - 14 days	6 - 12 hours
Common symptoms	Fever, coated tongue, bradycardia, rose spots on chest, myalgia	Nausea, vomiting, fever, chills, abdominal pain, myalgia
Treatment	Fluoroquinone (5-7 days), chloramphenicol, amoxicillin [a]	Antibiotic treatment not recommended for systemic disease. Fluoroquinones [b]
Vaccination	Available in endemic areas [c]	Not available

[a] Depending on local patterns of antibiotic resistance, severity of the disease, availability and cost

[b] Fluroquinones are usually prefered if antibiotic treatment is appropiate

[c] Licensed available vaccines. Efficacy of the vaccine is 60 – 80% and protection for up to 7 year

Table 8. Examples of severity of disease and outcome from *Salmonella* serovars related to infection in humans from 1996 to 2006 (Adapted from Jones *et al.,* 2008)

Serovar	**Total**	**%**	**Hospitalization**	**Invasive disease**	**Death**
All	46,639	100	22.8	6.7	0.5
Typhimurium	10,894	23.4	24.2	5.7	0.6
Enteritidis	7572	16.2	20.6	6.7	0.5
Newport	4779	10.2	21.9	1.4	0.3
Heidelberg	2830	6.1	26.2	13.4	0.4
Sandiego	164	0.4	22.6	18.9	0
Tennessee	155	0.3	29.7	4.5	1.3
Dublin	100	0.2	67	64	3
Muenster	98	0.2	26.5	11.2	2
Cerro	55	0.1	16.4	7.3	1.8
Choleraesuis	55	0.1	60	56.4	1.8

Table 9. Examples of Non-Typhoidal *Salmonella* isolates from humans and resistance profile of specific antimicrobial agents (NARMS, 2010)

	Antimicrobial Agent group													
	Cephems		**Quinolones**		**Phenicols**		**Folate Pathway Inhibitors**		**Penicillins**		**Aminoglycosides**		**Tetracycline**	
Serovar	**Ceftriaxone**		**Naldixic Acid**		**Chloramphenicol**		**Sulfisoxale**		**Ampicillin**		**Streptomicin**		**Tetracycline**	
Newport	22	31%	1	2%	22	18%	23	10%	23	10%	25	12%	25	9%
Typhimurium	18	26%	5	10%	74	61%	105	47%	96	43%	94	44%	106	39%
Enteritidis			27	55%	3	2%	10	4%	12	5%	3	1%	11	4%
Heidelberg	15	21%			1	1%	7	3%	24	11%	17	8%	15	5%
Dublin	3	4%											22	8%
I 4,[5],12:i:-	2	3%	4.1	8%	1	1%	15	7%	17	8%	15	7%		
Montevideo											2	1%	3	1%
Cubana	1	1%	1	2%										
Kentucky	1	1%	1	2%										
Choleraesuis			1	2%										
Paratyphi B					8	7%	9	4%	9	4%	10	5%	10	4%
Other					11	9%	41		31	14%	42	20%	68	25%

Table 10. Examples of Non-Typhoidal *Salmonella* isolates from humans and their multidrug resistance profile (NARMS, 2010)

					Multidrug			
Serovar	**Resistant to >5 Antimicrobials**		**ACSSuT [1]**		**ACSSuTAuCx [2]**		**ACT/S [3]**	
Newport	22	17.2%	22	20.6%	22	66.7%	4	36.4%
Typhimurium	76	59.4%	68	63.6%	7	21.2%	4	36.4%
Heidelberg	6	4.7%	1	0.9%				
Dublin	3	2.3%	3	2.8%	3	9.1%	1	9.1%
I 4, [5],12:i:-	3	2.3%	1	0.9%				
Infantis	1	0.8%	1	0.9%	1	3.0%		
Cubana	2	1.6%	1	0.9%			1	9.1%
Concord	2	1.6%						
Denver	1	0.8%						
Kentucky	2	1.6%						
Choleraesuis	2	1.6%	1	0.9%			1	9.1%
Paratyphi B	7	5.5%	7	6.5%				
Unknown	1	0.8%	1	0.9%				

[1] ACSSuT: ampicillin, chloramphenicol, streptomicin, sulfamethoxazole/sulfisoxazole, and tretracycline

[2] ACSSuTAuCx: ACSSuT, amoxicillin-clavilinic acid, and ceftriaxone

[3] ACT/S: ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole

Table 11. Examples of *Salmonella* serovars profile of analyzed Pathogen Reduction/ Hazard Analysis and Critical Control Point (PR/HACCP) systems verification samples from cows and bulls (USDA/FSIS, 2010)

	% Total Serotyped									
Serovar	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**
Montevideo	10	13.46	5.48	2.63	4.17	11.5	15.79	9.52	16.67	25
Newport	15	5.77	24.66	13.16	8.33	3.85		16.67	8.33	16.67
Agona			6.85	5.26	4.17	7.69	10.53		16.67	8.33
Kentucky	7.5	9.62	6.85			7.69	21.05	2.38	8.33	8.33
Mbandaka	2.5	3.85	4.11				5.26	2.38		8.33
Cerro				7.89	8.33	7.69	5.26	11.9	16.67	
Anatum		9.62		2.63	4.17	7.69		16.67	8.33	
Muenster	12		10.96	18.42	8.33	7.69	10.53	9.52	8.33	
Typhimurium	10	7.69	6.85	7.89	8.33	11.54				
Dublin	2.5	5.77			8.33	3.85	5.26			
Meleagridis		3.85		5.26	4.17	3.85	5.26	2.38		
Infantis	2.5		5.48	2.63	4.17	7.69		4.76		
Derby	2.5		4.11	5.26	8.33	3.85				
Enteritidis							5.26	2.38		

Table 12. Examples of *Salmonella* serovars profile of analized Pathogen Reduction/ Hazard Analysis and Critical Control Point (PR/HACCP) systems verification samples from steers and heifers (USDA/FSIS, 2010)

	% Total Serotyped									
Serovars	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**
Dublin		18.18			8.33	16.67		22.22	22.22	
Montevideo	50	9.09	7.14	10.53			10	11.11	11.11	10
Typhimurium	25				8.33		10		11.11	10
Anatum				10.53	8.33		10	11.11	11.11	
Newport				5.26	8.33	8.32	20	11.11	11.11	
Mbandanka				5.26					11.11	
Muenster			7.14			8.32	10			10
Muenchen						16.67				10
Poona						16.67				10
Derby		36.36	7.14	15.79	33.33					
Heidelberg		9.09	7.14	5.26						
Kentucky		9.09	14.29	10.53				11.11		

Table 13. Examples of *Salmonella* serovars profile of analized Pathogen Reduction/ Hazard Analysis and Critical Control Point (PR/HACCP) systems verification samples from ground beef (USDA/FSIS, 2010)

	% Total Serotyped									
Serovars	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**
Montevideo	12.72	14.05	11.32	10	14.06	13.89	16.86	23.43	24.51	31.1
Dublin				5.31	4.95	4.17	5.14	9.81	12.25	12.8
Newport	8.25	10.91	10.69	11.02	7.52	6.48	6.86	5.99	7.35	9.15
[a] Typhimurium	6.31	5.53	4.07	5.51	4.16	9.26	6	5.18	6.62	8.54
Anatum	6.8	9.27	9.8	9.18	10.89	9.26	7.71	3.81	7.6	4.88
Cerro	5.05	3.89	3.82			3.7	6.29	4.9	5.15	4.88
Kentucky	4.27	6.88	4.83	4.69	4.16			2.72	4.41	4.88
Typhimurium var. Copenhagen)	7.77	3.74	6.49	5.51	3.56					
Muenster	4.47	7.77	8.27	4.9	9.31	7.87	9.71	7.63	3.92	
Mbandaka	4.37	5.38	4.58	4.49	3.37	5.56	4	6.27	4.17	
Agona			6.62	5.92	7.13	3.24		4.09		

[a] After 2005 Typhimurium includes Typhimurium 5- (formerly Copenhagen).

Table 14. Examples of *Salmonella* serovars profile of analized Pathogen Reduction/ Hazard Analysis and Critical Control Point (PR/HACCP) systems verification samples from market hogs (USDA/FSIS, 2010)

	% Total Serotyped									
Serovars	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**
Derby	22.6	33.01	30.38	17.22	28.34	29.8	18.49	13.3	21.1	19.44
[a] Typhimurium	3.08	2.94	2.95	3.97		13.47	8.22	20.69	10.09	16.67
Johannesburg	8.22	3.59	2.95	4.64	3.64	3.67	9.59	9.85	4.59	9.26
Infantis	6.85	8.5	5.91	7.28	7.69	8.98	5.48	8.37	12.84	7.41
Anatum	3.42	7.19	5.49	5.3	10.93	5.31	21.58	6.4	5.5	5.56
Adelaide					4.05	3.27		4.93		4.63
Agona							3.42	3.94	5.5	4.63
Heidelberg	5.82	4.25	2.95	6.62		2.45	4.45			3.7
Saintpaul	2.4	4.58	5.91	5.3		4.49	5.48	6.4	6.42	3.7
Typhimurium (var. Copenhagen)	16.1	6.86	13.08	10.6	17					
Reading	2.4	4.25	3.38	3.31	3.24	4.08				

[a] After 2005 Typhimurium includes Typhimurium 5- (formerly Copenhagen).

Table 15. *Salmonella enterica* serovars, source of the strains and references describing characteristics of the strains utilized in this work.

Salmonella enterica serovar	Source	Reference
S. Typhimurium DT104	Human infection	Threlfall 2000
S. Typhimurium ATCC 23595 (LT2)	Laboratory strain	Swords *et al*. 1997
S. Typhimurium ATCC 14028	Laboratory strain	None
S. Enteritidis (WT)	Human infection	None
S. Enteritidis ATCC 13076	Human infection	None
S. Kentucky	Poultry carcass	Clement *et al*. 2010
S. Kentucky	Poultry carcass	Clement *et al*. 2010
S. Seftenburg	Poultry farm	Rodriguez *et al.* 2006
S. Heidelberg	Poultry farm	Rodriguez *et al.* 2006
S. Mbandanka	Poultry carcass	Melendez *et al*. 2010
S. Newport	Poultry carcass	Melendez *et al*. 2010
S. Bairely	Poultry carcass	Melendez *et al*. 2010
S. Javana	Poultry Farm	Rodriguez *et al.* 2006
S. Montevideo	Swine farm	Rodriguez *et al.* 2006
S. Infantis	Poultry Farm	Rodriguez *et al.* 2006

Table 16. The formulation and ingredient list of the starter/grower feed (CO-OP Chick) feed used in this study:

Guaranteed Analysis Component	%
Crude Protein	19
Lysine	0.82
Methionine	0.27
Crude Fat	3.5
Crude Fiber	4.5
Calcium	0.80-1.30
Phosphorus	0.7
Salt	0.25-0.75

Active drug ingredients	g/t
Amprolium	125.11
Bactracin Methylene Disalicylate	220.46

Ingredients: Grain Products, Plant Protein Products, Processed Grain By-Products, Molasses Products, Propionic Acid, Calcium Carbonate, Calcium Phosphate, Salt, Choline Chloride, Yucca Schidegera Extract, Bacillus subtilis, Niacin Supplement, Vitamin E Supplement, Calcium Pantothenate, Riboflavin Supplement, Vitamin A Acetate, Menadione Dimethylpyrimidinol Bisulfite, Vitamin D-3 Supplement, Biotin, Vitamin B-12 Supplement, Pyridoxine Hydrochloride, Folic Acid, Thiamine, Ferrous Sulfate, Manganous Oxide, Zinc Oxide, Copper Oxide, Calcium Iodate, Sodium Selenite, Cobalt Carbonate.

Table 17. A list of the genes, primer sequences and references for the primers that were used to evaluate gene expression changes of *Salmonella enterica* strains used in this study.

Target gene	Sequence (5' to 3')	References
16S	Forward: GCGGCCCCCTGGACAAAGAC Reverse: TAGCTCCGGAAGCCACGCCT	Gonzalez-Gil *et al.* 2012
hilA	Forward: ATGCCATAGCATTTTTATCC Reverse: GATTTAATCTGTATCAGG	Park *et al*. 2011
invA	Forward: CTGTCTGGCGGTGACGCTGG Reverse: ACGCGCCATTGCTCCACGAA	Own design. NCBI Reference Sequence: NC_003198.1
cfa	Forward: GCTGGTGGGAATGCGAGCGT Reverse: CAGCACACGCATCCCCGGTT	Own design. NCBI Reference Sequence: NC_011294.1
fabA	Forward: ACTCCCTGCGCCGAACATGC Reverse: CACTTCGCCCACGCCCAGAG	Own design.NCBI Reference Sequence: NC_011294.1
fabB	Forward: CCGCGTGGTCTGAAAGCCGT Reverse: GGACAGTGCGCCCATCGCAT	Own design.NCBI Reference Sequence: NC_011294.1
fabD	Forward: ACCCAGCAAGGTCCAGCGG Reverse: TTCGCGCCAGCGGCTTTACA	Own design.NCBI Reference Sequence: NC_011294.1

Table 18. Measurement of water activity (a_w) in the poultry feed, before being spiked with *S. enterica* cultures, and after spiking at specific time points.

Sample	a_w
Un-spiked	0.35±0.001[a]
0h	0.74±0.001[b]
4h	0.70±0.003[c]
8h	0.69±0.003[d]
24h	0.67±0.001[e]
4d	0.65±0.002[f]
7d	0.61±0.001[g]

[1]Values of Standard Error of the Mean ± from triplicates from each *S. enterica* strain. Mean values within a column that do not have the same superscript letter are significantly different ($P < 0.05$).

Table 19. Changes in the counts of culturable *S. enterica* serovars (CFU/g feed) expressed in log recovered from artificially inoculated feed at specific time points.

Strain	Changes between time points[1]				
	0h to 4h	**4h to 8h**	**8h to 24h**	**24h to 4d**	**4d to 7d**
S. Typhimurium DT104	2.17±0.10^{a}	0.38±0.10^{bc}	0.51±0.12^{b}	2.71±0.49^{a}	-0.58±0.78^{abcd}
S. Typhimurium ATCC 23595 (LT2)	1.79±0.11^{ab}	0.03±0.14^{bc}	0.83±0.16^{bc}	0.73±0.27^{ab}	-0.22±0.28^{d}
S. Typhimurium ATCC 14028	3.47±0.80^{abc}	-0.15±0.80^{abc}	1.59±0.29^{a}	1.42±0.45^{b}	NC±0.00[2]^{d}
S. Enteritidis (WT)	1.40±0.10^{bc}	0.13±0.05^{c}	0.55±0.09^{b}	1.19±0.14^{a}	0.42±0.10^{cd}
S. Enteritidis ATCC 13076	1.03±0.05^{c}	0.74±0.28^{abc}	0.29±0.17^{bcd}	1.50±0.06^{a}	2.10±0.00^{a}
S. Kentucky A	3.01±0.81^{abc}	0.36±1.07^{abc}	0.69±0.74^{abc}	0.75±0.44^{ab}	0.7±0.44^{bcd}
S. Kentucky F	2.95±0.47^{ab}	0.20±0.64^{bc}	0.92±0.75^{abc}	0.00±0.94^{ab}	0.35±0.65^{abcd}
S. Seftenburg	0.97±0.21^{abc}	-0.22±0.27^{abc}	0.38±0.24^{bcd}	3.09±0.47^{a}	1.05±0.47^{abcd}
S. Heidelburg	1.57±0.35^{abc}	1.28±0.11^{a}	-0.38±0.09^{d}	1.75±0.54^{ab}	1.42±0.64^{b}
S. Mbandanka	1.35±0.14^{bc}	0.59±0.11^{b}	-0.02±0.08^{cd}	2.21±0.62^{ab}	0.33±0.72^{abcd}
S. Newport	2.30±0.27^{abc}	0.85±0.24^{abc}	0.87±0.11^{b}	1.15±0.43^{ab}	1.41±0.64^{abcd}
S. Bairely	1.97±0.20^{abc}	0.43±0.20^{abc}	0.18±0.21^{bcd}	0.94±0.22^{ab}	2.02±0.29^{ab}
S. Javiana	2.09±0.32^{abc}	0.77±0.35^{abc}	0.44±0.05^{b}	1.42±0.60^{ab}	1.94±0.63^{abcd}
S. Montevideo	1.94±0.27^{abc}	0.93±0.49^{abc}	1.21±0.71^{abcd}	2.16±0.68^{ab}	NC±0.00[1]^{d}
S. Infantis	0.82±0.16^{c}	0.71±0.20^{abc}	0.40±0.11^{bc}	2.14±0.43^{a}	1.75±0.5^{abcd}

[1]Values± standard error of the mean from triplicates with duplicate repetition samples. Mean values within a column that do not have the same superscript letter are significantly different ($P < 0.05$).

[2]NC: No change between timepoints.

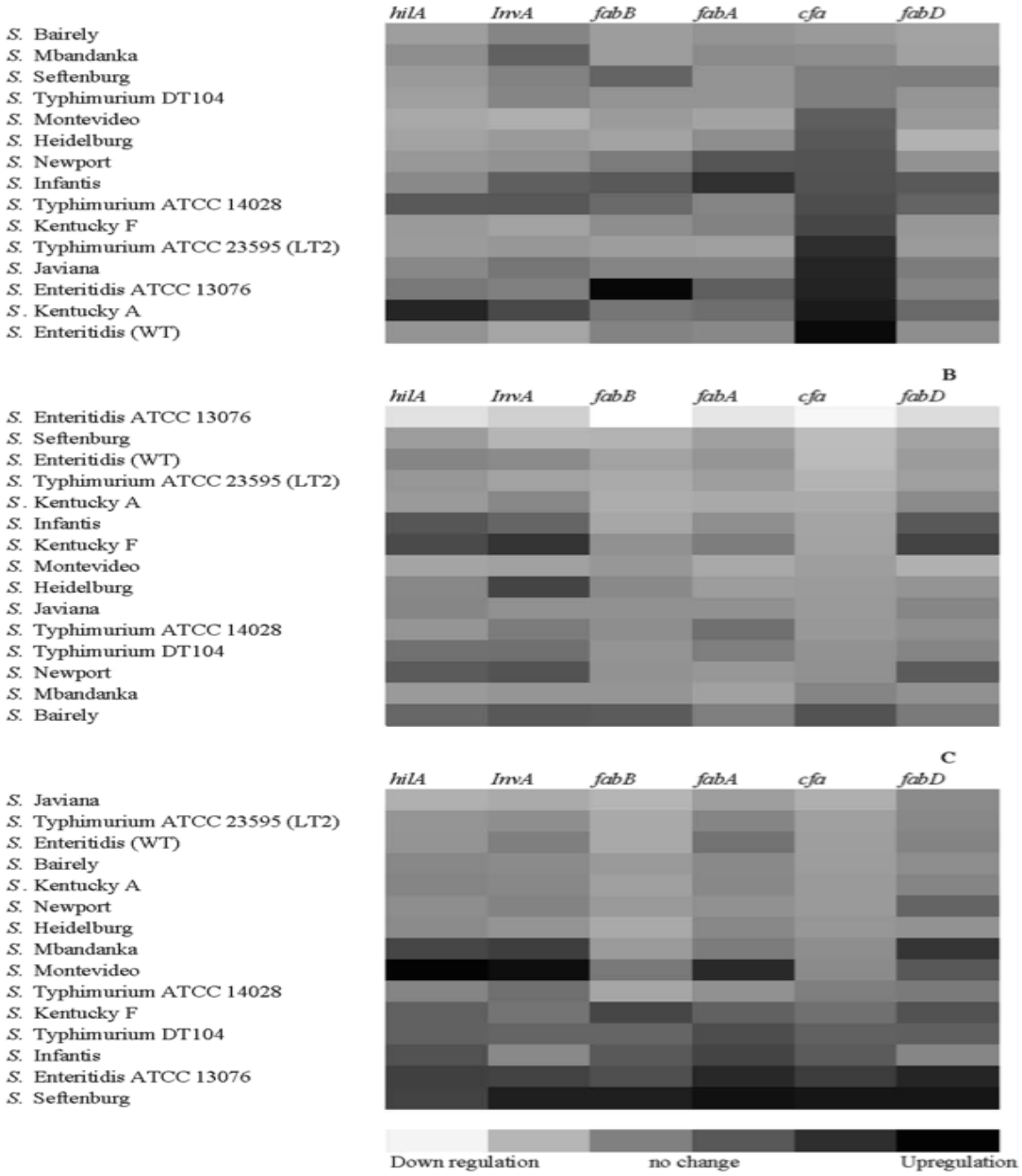

Figure 1. A heat map of relative fold change in gene expression of genes involved in virulence and colonization (*hilA, InvA*) and fatty acid synthesis (*cfa, fabB, fadD, fabA*) in 15 *S. enterica* serovars artifically inoculated into poultry feed and sampled after incubation at room temperature at 4h (panel A), 8h (panel B) and 24h (panel C). Maps are sorted based on the *cfa* gene in ascending order of regulation for each time point.

Vita

Ana Andino Dubón was born in Tegucigalpa, Honduras. She attended Zamorano University, where she received her B.S. degree in Agriculture Science and Production while working under Dr. Gernat. After graduation, she worked at Central Amenrican Poultry Company in Honduras for five years. In 2009 she received a Master's degree in Business Management from UNITEC, Honduras and IEDE Business School, Madrid. In 2011 she joined Dr. Hanning's research group to later enroll in the Master's Program. Ana received a Master's Degree in Food Science and Technology from the University of Tennessee in August, 2014. After graduation she moved to Miami, FL to start a job as Technical Sales Representative for Wincorp International, Inc.