8-2013

Comparison of Potential Salmonella Portals of Entry and Tissue Distribution Following Challenge of Poultry

Gopala Krishna Kallapura Sheshagiri
University of Arkansas, Fayetteville

Comparison of Potential *Salmonella* Portals of Entry and Tissue Distribution Following Challenge of Poultry

Comparison of Potential *Salmonella* Portals of Entry and Tissue Distribution Following Challenge of Poultry

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Poultry Science

by

Gopala Krishna Kallapura Sheshagiri
Bangalore University
Bachelor of Science in Biotechnology, 2005
Bangalore University
Master of Science in Biotechnology, 2007

August 2013
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

_____
Dr. Guillermo Tellez
Dissertation Director

_____
Dr. Billy M. Hargis
Committee Member

_____
Dr. Lisa R. Bielke
Committee Member

_____
Dr. Neil R. Pumford
Committee Member

_____
Dr. Luc R. Berghman
Ex-Officio Member

Abstract

The following studies evaluated our hypothesis that transmission by the fecal-respiratory route may be a viable portal of entry for *Salmonella* and could explain some clinical impressions of relatively low-dose infectivity under field conditions in relation to the requisite high oral challenge dose that is typically required for infection of poultry through the oral route in laboratory studies. Initial field reports indicating tracheal sampling to be a sensitive tool for monitoring *Salmonella* infection in commercial flocks, suggested that tracheal contamination could be a good indicator of *Salmonella* infection under commercial conditions. Further, a usual assumption regarding airborne *Salmonella* reaching the upper respiratory tract, would ultimately involve oral ingestion, due to the presence of the mucociliary clearance was evaluated. Suspension in 1% mucin failed to increase the infectivity at any dose of *Salmonella* when compared to OR administration without mucin and intratracheal (IT) challenge, which was also recovered from lung tissue. IT administration was more effective or at least as effective at colonizing the ceca of 7d chickens, suggesting that the respiratory tract may be an overlooked potential portal of entry for *Salmonella*e. Finally, the hypothesis was evaluated through IT administration of *Salmonella*, in comparison with oral administration. A significantly higher or equivalent cecal recovery of *Salmonella*, with a clear dose response curve, with the IT groups as compared to groups challenged OR, added further support to the hypothesis. Both the cecal CFU recovery data and organ invasion incidence data from these experiments provided evidence for the subsequent fate of *Salmonella* exposed to the respiratory system, potentially involving a systemic route. Overall, our data suggests that the respiratory route might be a viable portal of entry for *Salmonella* in poultry. Clarification of the potential importance of the respiratory tract for *Salmonella* transmission under field conditions may be of critical importance as efforts to develop intervention strategies to reduce transmission of these pathogens in poultry continue.

Table of Contents

Chapter 1

Introduction

Introduction

It is well accepted that many serotypes and isolates of the genus *Salmonella* are frequently able to colonize the gastrointestinal (GI) tract of vertebrate animals, with less host-specificity than many pathogens. Several serotypes are adapted to colonize humans, causing a range of clinical pathologies, frequently characterized by gastroenteritis associated with intestinal inflammation and diarrhea. Historically, the source of human *Salmonella* infection has frequently, been related to poultry, or poultry products. However, recent evidence, from salmonellosis outbreaks in many countries indicates that *Salmonella* sources frequently include unconventional food sources such as fresh horticultural products, peanut butter, vegetables, vegetarian snack food, dry puffed breakfast cereal, microwaveable pot pies, and hot peppers [108].

These unusual sources of *Salmonella* were perceived to be connected to various manure processes involving fertilizer use of large amounts waste material from animal feeding operations (AFO). Beef and dairy cattle, chicken, turkey, and swine operations are considered to be the major producers of manure [1], generating an estimated 500 million wet tons of manure annually, in the U*S.* Commercial poultry are known reservoirs of some zoonotic pathogens such as *Salmonella,* and associated manure has been implicated with contamination and infection [2 3]. Animal production facilities processing these manures were investigated to be managed in one of two ways, composting or biogas production through anaerobic fermentation, or a combination of the above, were considered to be proper management strategies involving these manures, but not infallible. Animal manures in solid, semi-solid, and liquid forms, traditionally used as soil conditioners and as a source of nutrients for crop production [4 5] were eventually observed contaminating the unconventional food sources mentioned above. On the other hand, it was often observed to be a general practice that the dry and wet manures were stocked as piles or within holding ponds for mechanical dewatering, which were observed polluting surface and ground waters with nutrients and pathogenic microorganisms [6], acting as a source of contamination. Thus, an area of study related to zoonotic pathogens generated as byproducts from these AFOs and land application of manures have gained interest recently [7 8 9 10 11 4].

From these observations it is possible to highlight the importance of unappreciated levels of understanding as to how *Salmonella,* and potentially other pathogens, adapt and survive. The ability of the pathogen to contaminate these new sources is explained by the presence of bacterial genetic determinants that can be modulated under different environmental changes, hence making it a highly adaptable species. There is good experimental and epidemiological evidence that primary infection by *Salmonella* is through the oral-fecal route, along with an established requisite infectious dose. Previously, published data has suggested that direct ingestion and contact are considered the most common routes of transmission for many zoonotic enteropathogens, and, also the inhalation of infectious particles should not be neglected [12] [13]. Airborne transmission of microbes is not restricted to agents causing respiratory disease, because enteric and other pathogens can also be transmitted by this route. While it is well documented that some communicable and noncommunicable human diseases are transmitted through airborne routes, the potential airborne transmission of zoonotic enteric pathogens, such as *Salmonella,* has not been well studied, even after repeated efforts with reports suggesting that airborne transmission of *Salmonella* is possible [14] [15] [16]. Much of the uncertainty regarding the airborne transmission and infection, by *Salmonella* and other enteric pathogens, has been associated with the relationship between bioaerosol particle size and number, and the dose of the pathogen it is carrying [17] [18] [19]. Also, it is an established impression that bioaerosol generation and airborne transmission of a pathogen might be possible, but the infection eventually has to be fecal-oral, because settlement of the microbe on the feed and other surfaces will lead to infection through ingestion [16].

Our laboratory has recently hypothesized that transmission of *Salmonella* by the fecal-respiratory route can a be a viable portal of entry for *Salmonella* and could explain some clinical impressions of relatively low dose infectivity under field conditions related to the high oral challenge dose that is typically required for infection of poultry through the oral route in laboratory studies. The present review discusses the possibilities of airborne *Salmonella* infection pertaining to poultry, followed by discussion involving accepted fecal oral route of *Salmonella* transmission. Established poultry practices potentially leading to the generation of *Salmonella* bioaerosols at every stage of commercial poultry production will be discussed, and possible mechanisms of survival and transmission of *Salmonella* in bioaerosols will be

reviewed. Furthermore, the role of avian respiratory anatomy, physiology and immunology in airborne infection of *Salmonella* will briefly be discussed. The present doctoral project was initiated to evaluate the possibility that a new mechanism of *Salmonella* infection should be considered. A more complete understanding of the role of airborne transmission of *Salmonella* in poultry may allow for more effective prophylaxes and interventions for this important economic issue for the poultry industry and, ultimately, this important source of human infections with this pathogen.

Chapter 2

Review of Literature

***Salmonella,* Disease and Poultry**

Salmonella is a genus of Gram-negative bacilli, non-spore forming, predominantly motile bacteria, belonging to the family enterobateriaceae. They are facultative anaerobes and chemoorganotrophs, obtaining their energy from oxidation and reduction of organic sources in their ecological niche. Genus *Salmonella* are divided into only two species: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* has six subspecies including *enterica, salamae, arizonae, diarizonae, houtenae, and indica*, each of which consists of various serovars, for a total of more than 2500 different serovars [13]. There are various ways to name each serovar, for example *S. enterica* subspecies *enterica* serovar Typhimurium, also referred as *Salmonella* Typhimurium or simply *S.* Typhimurium [20].

Various *Salmonella* serovars have been described as host restricted (e.g., *S.* Typhi and *S.* Paratyphi in humans and *S.* Gallinarum in poultry), host adapted (*S.* Dublin in cattle and *S.* Choleraesuis in swine), or ubiquitous (*S.* Typhimurium and *S.* Enteritidis), based on host range and adaptability [21]. *S.* Gallinarum, the causative agent of fowl typhoid, is a predominantly avian-restricted serovar. Interestingly, in common with the human-restricted serovar *S.* Typhi, the chicken-adapted *S.* Gallinarum causes an invasive typhoid-like disease. Thus, in this case, host adaptation appears to have coevolved with loss of intestinal colonization and the acquisition of the ability to cause systemic infection [22].

In humans, salmonellosis, the pathological condition caused by *Salmonella,* includes different clinical disease such as typhoid-like disease, where infectious agents *S.* Typhi and *S.* Paratyphi may cause human death, and non-typhoid disease which is usually limited to infection of the lining of the small intestine causing gastroenteritis often by *S.* Enteritidis and *S.* Typhimurium, although the latter causes systemic disease in mice [13], and other serotypes have been known to cause occasional systemic disease in humans [23]. The ability to transmit between and within particular host populations is centrally important in dictating the epidemiology of infections and the emergence of new diseases. Further, the acquisition or loss of certain genes plays an important role in the evolution of different serovars [22].

According to the Centers for Disease Control and Prevention (CDC) and the World Health Organization Food and Agriculture Organization of the United Nations (WHOFAO, 2004), *S. enterica* subspecies *enterica* is a widely spread group of zoonotic serotypes, mainly infecting humans as well as various animal species [22] [20]. Although members of the genus *Salmonella* are genetically close, there are wide variations in the host-specificity, virulence, and disease manifestations.

Zoonotic pathogens, particularly those associated with food production animals, are some of the most important causes of infectious diseases in humans [12] [24]. Pathogens associated with zoonotic infections exhibit a promiscuous phenotype in that they maintain the ability to colonize and potentially cause infections in more than one host species. Promiscuous, in the sense that they are comprised of sublineages that differ greatly in antigenic representation, virulence, and antimicrobial resistance phenotypes and hence can cause infections in mice, retain the ability to colonize the tissues of chickens, and cause gastroenteritis in humans. In contrast, some pathogenic agents are significantly host restricted, or adapted, and are normally only able to cause disease in one host. *Salmonella enterica* is a single bacterial species that includes examples of both promiscuous and host-adapted pathotypes [22] [25]. Isolates from serovars such as *S. enterica* serovars Typhimurium and Enteritidis predominantly retain the ability to infect more than one mammalian host, including humans, whereas serovars such as *S. enterica* serovars Typhi and Gallinarum are restricted to humans and chickens, respectively [21].

Worldwide public health systems consider foodborne *Salmonella enterica* infections to be a significant burden, even with extensive surveillance and intervention programs [21]. In the United States, *S. enterica* is responsible for an estimated 1.4 million cases of infection, 15,000 hospitalizations, and more than 400 deaths annually and is considered a category B pathogen and a potential food safety threat by the CDC [26] [27] [28]. Poultry has been considered the most important source of *Salmonella* infections in humans causing self-limiting gastroenteritis. In immunocompromised patient populations, however, the same serovars can cause life threatening systemic infections that require immediate and effective antimicrobial therapy.

At a global level, more than 27 million cases of typhoid fever have been estimated worldwide each year,

resulting in an estimated 217, 000 deaths [29]. Infections with non-typhoidal *Salmonella* (NTS) serovars, such as *S. enterica* serovar Typhimurium and Enteriditis, also cause a significant disease burden, with an estimated 93.8 million cases worldwide and 155, 000 deaths each year [30] [31]. In recent decades, *S.* Enteritidis and *S.* Typhimurium have topped the list of emerging pathogens and are re-emerging in Latin America [32], and are considered an endemic disease in Argentina [32].

The costs associated with non-typhoidal *Salmonella* infections are estimated to be about $2.4 billion dollars annually in the United States alone. Poultry producers suffer a large share of these losses due to *Salmonella* infection of the flock, including loss of birds, increased production time and reduced performance. In the United States loss due to productivity has been calculated to be close to $114 million per year, excluding the losses due to loss of eggs and other consumable poultry products [33]. *S. enterica* outbreaks, as well as individual cases of *S. enterica* infections are linked to various food sources. However, there is a general acceptance that poultry and poultry products are an important source of human food borne salmonellosis [32] and thus emphasis needs to be placed on efforts to reduce *Salmonella* levels during production.

Salmonella contaminated poultry meat has been associated frequently and consistently with the transmission of enteric pathogens (FAO/WHO 2002) [34]. Most frequent sources of infections are associated with contaminated animal products, especially raw or poorly cooked foods containing egg, emphasizing the importance of controlling food processing in general and, specifically for poultry products [35] [36] [37]. Callaway et al. stated that the "link between human salmonellosis and host animals is most clear in poultry and that raw eggs and undercooked poultry are considered by the entire community to be hazardous" [38]. Numerous outbreaks of salmonellosis in the early days were associated with eggs, a major vehicle of transmission of strains of *Salmonella* serovar Enteritidis, although the incidence of disease associated with this particular mode of transmission has decreased in recent times [37].

Prior to the pandemic of *S.* Enteritidis, this bacterium had been isolated in low numbers from a range of animals, including poultry, since 1900. *Bacterium Enteritidis* or Gaertner's Bacillus (now termed *S.*

Enteritidis) was first cultured in 1888, from the meat of a slaughtered cow with diarrhea thought to be responsible for fifty-eight cases of human food poisoning in Germany, via the illegal distribution of meat. This was one of the first reports of food poisoning linked to *Salmonella*. *Salmonella* Enteritidis was sporadically isolated from poultry and eggs in the 1930s, and contaminated eggs were found to be a result of infection of the avian oviduct, and in some cases, penetration of the shell. Interestingly, the potential of egg based dishes, such as mayonnaise, as sources of food poisoning was already recognized at this time [23]. Nevertheless, for eighty years or more, *S.* Enteritidis behaved like the many hundreds of *Salmonella* serotypes that were isolated regularly from animals at low baseline levels and had very little impact on human or animal health. Eradication of host specific *S.* Gallinarum and *S.* Pullorum from commercial poultry flocks, because of control programs like National Poultry Improvement Plan (NPIP) in the United States, consequently led to an increase in the incidence of broad host range serovars such as *S.* Enteritidis and *S.* Typhimurium. Baumler and co-workers (2000) saw a direct correlation between decreases in the incidence of *S.* Gallinarum with an increase in *S.* Enteritidis in commercial poultry. Since then, in order to monitor these cases along with *Salmonella* populations from clinical, veterinary, and food sources, the Food and Drug Administration has maintained an intensive active and passive *S. enterica* surveillance system that characterizes hundreds of isolates per year [21]. Australia, which has very strict regulations on the importation of animal products, including regulations on the importation of poultry and hatching eggs, has remained largely free of *S.* Enteritidis, indicating the importance of strict regulations in poultry. Despite these efforts, *S. enterica* continues to be an important public health threat worldwide, with several outbreaks occurring every year (CDC) [39].

Fecal - Oral Transmission

***Salmonella*, Zoonosis and Entry into Food Chain**

Domesticated livestock harbor a variety of bacterial, viral, and protozoal pathogens, some of which pose a risk to other animals and humans. Infectious diseases that are transmissible from animals to humans and vice versa are known as zoonoses. These diseases can be transmitted to humans through direct contact with skin wounds and mucous membranes, fecal-oral route via ingestion of contaminated food and water, or airborne routes such as aerosolized dust particles or contaminated vents [12]. Those pathogens that are transmissible through oral fecal route, eventually infecting the enteric system of the hosts, are called zoonotic enteropathogens. Well recognized zoonotic enteropathogens include *Escherichia coli* O157:H7, *Salmonella* spp., and *Campylobacter jejuni*. Many of these pathogens in addition to being endemic in commercial livestock are also highly adaptable to the environment of production facilities, hence are very difficult to eradicate from animals [23].

Salmonella, the most prevalent enteropathogen, is common in most livestock animals but also in wild birds, pets and rodents within production facilities, and other animals. However, nontyphoidal *Salmonella* occurs in both humans and animals and hence is considered a zoonotic pathogen [23]. Human infections by *Salmonella* generally occur through the ingestion of foodstuffs like poultry, which were infected directly or contaminated through the excretions from infected animals, resulting in human salmonellosis. Furthermore, most cases of foodborne salmonellosis in humans involve further amplification in mishandled food products, with temperature and/or time abuse allowing for further amplification of a generally low level contamination of fresh products [12].

The entry of *Salmonella* into poultry and subsequent colonization may happen at any stage of poultry production, with pedigree lines and great grandparents becoming infected, further trickling down the production pyramid [34]. Further, it is crucial to keep production flocks at the apex free from *Salmonella,* as this organism can be vertically transmitted from hen to egg. A colonized breeder flock can spread

Salmonella to a large number of commercial flocks descending from them. Further, unlike production flocks, with a relatively short life span of approximately forty-two days, these elite flocks, including breeders, may have a lifespan in excess of one year, wherein they can become colonized by *Salmonella* or other pathogens. Hence, it has long been recognized that breeding stock of poultry play a crucial role in the dissemination of *Salmonella* infection and contamination of the food chain [33].

Young chicks in the hatchery are more susceptible to infection with *Salmonella* due to an absence of protective gut microflora and an immature immune system. For this reason, day old chicks can be colonized with as few as five cells of *Salmonella*, but colonization of two week old birds which have protective microflora is inconsistent and requires higher doses [40]. Furthermore, the susceptibility of these young chicks results in rapid horizontal transmission. Surveys and estimates of *Salmonella*-positive chicks leaving the hatchery range from 4.8 to 9% [41 42]. Infected chicks from a hatchery that are placed on a grow-out farm can act as sources of infection and contamination to the farm environment. *Salmonella* contamination on broiler grow-out farms is complex and can come from multiple sources in the environment such as feed, feed ingredients, water, litter, and from breeding stock [27]. The major source of horizontal transmission of *Salmonella* is chickens, either directly or indirectly from the feces, which settles on the litter. Chickens, which have a natural tendency to peck the litter, are proven to become infected in a cyclical fecal-oral manner. Further, *Salmonella* has been demonstrated to persist in farm environments for over a year when poultry are present [43]. Total disinfection of grow-out farms may be impossible to achieve due to cleaning difficulties and environmental reservoirs such as mice and wild birds.

Salmonella contamination of poultry before slaughter may include exposure in the last few hours before slaughter during loading, transportation, and holding stages of final poultry processing. In commercial turkey production, turkeys rest in holding pens on trucks in the cooling sheds for two h or more prior to slaughter. The first point of increased exposure, prior to slaughter, may occur when birds are taken off feed for three to six h before loading. This can increase the flock's contact with *Salmonella* through increased oral consumption of the litter in the barn in search of food [44 45] which eventually leads to fecal oral route of infection [14]. Hence, catching and transportation of poultry from primary production units to

processing plants can be a significant stage in the dissemination of enteric pathogens, through contamination and subsequent cross-contamination because of the use of dirty crates, trucks and catching / pickup crews. Based on cecal sampling, Cox et al estimated that up to 31% of turkeys coming into the processing plant may harbor *Salmonella* [46]. Overall, *Salmonella* contaminated or infected live poultry entering the processing plant has been recognized as the principal source of processing plant contamination. Although plant interventions, including chlorinated water in the chill tank, can reduce the level of *Salmonella* on carcasses, incoming birds may affect the microbial quality of the end product [47] [48].

Through epidemiological studies it has been shown that poultry products are important sources of consumer exposure to pathogens such as *Salmonella* [34,49,50]. The relative contribution of different exposure pathways finally leading to entry of *Salmonella* into the food chain has been assessed. Eventual ingestion and infection of *Salmonella* in humans can thus be expected to be partially dependent on the distribution of *Salmonella* on poultry meat or poultry food commodities, especially when cross contamination and temperature or storage time abuse occurs. Generally, two pathways for human exposure to enteropathogens in poultry meat and eggs exist, undercooking and cross-contamination (FAO/WHO, 2002, 2003) [51]. During frying or other cooking procedures for meat, fairly high temperatures occur on the outside of the meat and there is a high probability that it will kill the bacteria located on the surface. If the poultry meat is undercooked, some of the internal bacterial pathogens could survive and viable bacteria may be ingested while consuming the undercooked meat.

Whereas undercooking is mainly a risk associated with bacterial pathogens located in eggs and inside of chicken or turkey meat, cross-contamination events transferring *Salmonella* from poultry meat or egg shells directly or indirectly to other foods in the majority of cases will involve bacteria located on the surface of poultry meat and eggs after amplification in improperly handled foodstuffs [52]. The relative risks of internal and external contamination of poultry meat and eggs were drawn from various risk assessment studies performed for *Salmonella* in combination with its related food product. Cross-contamination events from kitchen activities such as use of the same cutting board for chicken meat and salad without intermediate cleaning or spreading of pathogens via the kitchen environment, were found to be of far

greater significance than the risk associated with undercooking of poultry meat or eggs [49]. In conclusion, epidemiological data suggests that poultry consumption is the largest contributor to salmonellosis cases in the population, particularly when associated with contaminated or cross-contaminated and mishandled food products.

Salmonellosis includes different clinical cases such as: i) typhoid-like disease, whose infectious agents, *S.* Typhi and *S.* Paratyphi, may cause human death. For every ten cases of *S.* Typhi infection, there are one or two cases of paratyphoid fever, caused by the human-adapted *S. enterica* serovars Paratyphi A, Paratyphi B and Paratyphi C. Since paratyphoid fever is indistinguishable from typhoid fever in its clinical course, *S. enterica* serovars Typhi, Paratyphi A, Paratyphi B and Paratyphi C are collectively referred to as typhoidal *Salmonella* serovars. These typhoidal and paratyphoid infections are generally associated with human to human transmission, rather than implications of animal zoonotic sources. ii) Non-typhoid disease, generally limited to infection in the lining of the small intestine causing gastroenteritis, mainly by *S.* Enteritidis and *S.* Typhimurium, although the latter causes systemic disease in mice [13]

Process of *Salmonella* Infection and Transmission

Infection and transmission of *Salmonella* in poultry may occur vertically, via eggs, with the hatch of infected chicks or horizontally by means of ingestion of water, feed, fecal material, contaminated bedding material or dust, or by oral, nasal, conjunctival, cloacal and umbilicus routes [53]. Transmission of *Salmonella* to humans generally occurs by means of contaminated food and water, although person-to-person transmission may take place, mainly in hospitals. Transmission by contact with infected animals, mostly among veterinarians and farm workers is also possible [51]. In both cases it causes an enteric disease of varying severity, depending on the host species, health conditions, age, and on the pathogenicity of the microorganism, and usually involves diarrhea. Young chickens less than 2 weeks of age are often said to become infected easily at low doses and often develop systemic infection with varying degrees of disease, due to its immunological susceptibilities at that age. Whereas most adult hens, with a fully functional gut immune system, colonization with *S.* Enteritidis frequently results in

asymptomatic intestinal carriers [54]. Estimates of the number of organisms required to cause infection are quite variable, ranging from about 30 to more than 10^9 infectious organisms [55]. This variability in infectious dose is partly due to the feed source contamination to varying degrees with *Salmonella* and to intrinsic factors of the infecting hosts [56].

Bacterial Ingestion and Survival

Upon ingestion, in order to reach their sites of colonization, *Salmonella* must be able to survive the various antimicrobial properties of the stomach (proventriculus in birds), including low pH and the presence of enteric organic acids, as well as the innate immune system of the host [57] [58]. *Salmonella* have evolved mechanisms for survival at low pH for efficient transit through the upper GI, with the employment of several acid shock tolerance regulatory factors that include the RpoS σ-factor, PhoPQ, and Fur proteins [59]. The RpoS and PhoPQ proteins are shown to play an important role in the regulation of survival at low pH environment created by inorganic acids, whereas Fur and RpoS are said to be involved in the regulation of organic acid tolerance [60]. The exact mechanism that each protein plays in acid tolerance is not well understood, however, it is clear that multiple factors contribute to the overall process of acid resistance including molecular chaperones, cellular regulatory transcription and translation factors, envelope proteins, and fimbriae [60] [59] [26]. Overall, this acid tolerance is shown to provide only a partial resistance to the *Salmonella* transit, and these acidic factors decrease the overall number of ingested *Salmonella* by varying degrees.

The surviving population of *Salmonella* reaches the beginning of the intestinal lumen where they are exposed to bile, the role of which is which is well recognized with regard to *Salmonella* pathogenesis [61]. Information is available on bile tolerance of Gram-negative bacteria, and it is believed that they are inherently more resistant than Gram-positive bacteria and bile salts are often used in their selective enrichment, for example Mac Conkey agar, *Salmonella-Shigella* agar, violet red bile agar and bile esculin agar all include bile salts in their formulation. One of the initial reports of bile adaptation in a Gram-negative bacterium was for *S.* Typhimurium by Van Velkinburgh and Gunn [61]. *Salmonella* outer

membrane is a formidable barrier to agents such as bile, which are essentially membrane active compounds. Lipopolysaccharide (LPS) is observed to play a key role in resistance, resulting in loss of the O-antigen, resulting in a ‘‘rough’’ colony phenotype on selective media involving bile. Multidrug efflux pumps can remove bile that penetrates through the outer membrane, *S.* Typhimurium AcrAB pump is absolutely required for bile resistance [62 63]. Further, the transcription of *S.* Typhimurium invasion gene regulators on *Salmonella* pathogenicity island 1 (SPI-1) has been seen to be repressed in presence of bile, resulting in marked decrease gene expression involved in epithelial cell entry (discussed below). As a result, bacteria grown in the presence of bile are less able to invade epithelial cells than those grown without bile. *Salmonella* therefore uses bile as an environmental signal to repress its invasive capacity in the intestinal lumen where concentrations are high [64 61]. Interestingly, invasion is said to be initiated later, when bile concentrations have decreased, which is after transit through the mucus layer covering the epithelium in the lower small intestinal tract or large intestine (monogastric mammals) and the ceca of birds. Thus, only after transiting through these harsh innate conditions, the surviving population reaches the intestinal lumen where they can infect epithelial cells, paradoxically with enhanced virulence in many cases.

The host–pathogen interaction is considered to be initiated at this point and infection is established once the pathogen reaches the appropriate localization to survive in the host, including the cecal tonsils in poultry as a key site, where they can infect epithelial cells. *Salmonella* adhesion to host epithelial cells is the most crucial step of infection, which enables bacteria to colonize the host intestine, and these steps are the result of coordination, in time and space, of expression of many virulence genes. As an enteric pathogen, one of the most formidable barriers that *Salmonella* has to overcome is the intestinal epithelial lining. Several epithelial cell types can be invaded by *Salmonella*, and Peyer’s patches represent the main portal of entry in early *Salmonella* infection [65]. Peyer’s patches are an aggregation of lymphoid follicles found as a part of the gut associated lymphoid tissue (GALT). They are characterized by a dome-like architecture covered by the follicle associated epithelium (FAE). The FAE is distinguished from the adjoining villous epithelium by a lack of goblet cells and more importantly by the presence of membranous or microfold cells (M cells). Overall, *Salmonella* is known to invade all three kinds of

epithelial cells, regular villous epithelial cells or enterocytes, FAE and most importantly, M cells. However, the kind of epithelial cell invaded depends on stage of infection and type of *Salmonella*, typhoidal or non-typhoidal. Briefly, M cells and FAE are said to be involved for virulence during initial infection whereas *Salmonella* entry into regular villus epithelial cells is required for virulence during the intestinal phase of infection [24 66].

The intestinal infection process involves bacterial adhesion, invasion, SCV maturation and replication within the epithelial cells. At later stages pathogens must overcome defense mechanisms of the host in order to develop an intracellular lifestyle [67]. These adaptations occur by coordinated expression of many genes in response to specific signals present in the host. A large number of genes encoding virulence factors have been identified by the role they play in *Salmonella* pathogenesis. Interestingly, most of these genes are located closely to each other in the bacteria genome, in groups denominated as SPI [68]. Twenty-one SPIs have been identified in *Salmonella*, which were classified from SPI-1 to SPI-21, however, only a small set are said to be involved with a specific serotype of *Salmonella.* For example, the SPIs present in *S.* Typhimurium are SPI-1 to 6, 9, 11, 12, 13, 14 and 16 [69 70 71].

Bacterial Adhesion

Salmonella is known to prefer a shorter route across the intestinal lining. Early studies of *Salmonella* infection have revealed that, immediately after infection, *Salmonella* are preferentially associated with Peyer's patches in the terminal ileum. M cells are specialized antigen sampling cells within the FAE that were first identified due to their morphological characteristics based upon irregular microvillus or microfolds and near absence of glycocalyx as compared to the enterocytes present in the FAE. They also display an unusual invagination in their basolateral membrane through which they establish a close association with the underlying lymphocytes [24 66]. These peculiar physical characteristics of M cells allow them to efficiently internalize intestine luminal antigens which are then transcytosed to the underlying lymphocytes associated with the submucosa, thus making them a crucial player in the induction of a mucosal immune response. This very property of M cells that helps in the development of mucosal

immune response also leaves them highly susceptible to attack by various enteropathogens. Their lack of glycocalyx and brush border makes their surface freely accessible for adherence and internalization of enteropathogens such as *Yersinia*, *Shigella* and *Salmonella* [66 72 73].

M cells represent only a small proportion of the specialized FAE overlying GALT. However, their density and number is known to increase during *Salmonella* infection through unknown mechanisms. Further, even short term exposure to *Salmonella* leads to a significant increase in the antigen uptake capacity of M cells and a possible effect on the maturation of immature M cells within the FAE [72]. Using *in vitro* and *in vivo* infection models, it was demonstrated that the *S.* Typhimurium type III effector protein SopB induces an epithelial-mesenchymal transition (EMT) of FAE enterocytes into M cells. Thus, *S.* Typhimurium, astonishingly, transformed primed FAE into M cells to promote host colonization and invasion [72].

As an extension, there are suggestions that, despite the inherent phagocytic properties of M cells, the exclusive affinity of *Salmonella* to M cells points towards a specific cell-cell interaction leading to invasion. SPI1 is known to play an important role during epithelial invasion [13]. It has been observed that invasion gene mutants show reduced invasion of M cells suggesting that the *inv* locus is involved in specific binding to M cells. However, many of these mutant studies have demonstrated that invasion does not occur exclusively through SPI1 mediated mechanisms [66]. Also, the role of dynamin in *Salmonella* internalization was discovered, suggesting that it might occur through a process of macropinocytosis [24].

At this stage, host epithelial cell receptors interact with several adhesion factors of *Salmonella*, including fimbrial, flagella, LPS and capsule [74]. At least 13 different fimbriae and 3 nonfimbrial adhesins for *S.* Typhimurium colonization have been found, which are transiently expressed in a very controlled way. Finally, full adherence of *Salmonella* to M cells is brought about by a significant production of flagella, the propulsion and motility apparatus, probably mediated by chemotactic responses [73 75].

Bacterial Invasion

Upon adherence to M cells, a new step of infection is initiated, referred to as invasion. This step involves a successful mechanism named “trigger process”, where many new bacterial effectors, regulated by SPI1 genes, are encoded and poured into the cytoplasm of the host epithelial cells. The effectors induce cytoskeletal reorganization at the apical surface of M cells causing a ruffling process, resulting in bacterial internalization [76]. Inside the epithelial cell, *Salmonella* are enclosed within an intracellular phagosomal compartment termed *Salmonella*-containing vacuole (SCV) [77]. *Salmonella* can be found within M cells as early as 5 minutes pi. Following the engulfment process, the host cell cytoskeleton is repaired by a SptP-mediated process opposed to the activity of SopE which causes membrane ruffling [78].

SCV Maturation and Replication

Salmonella within internalized SCVs has to face a hostile environment within host epithelial cells. In eukaryotic cells, membrane trafficking events that involve signaling cascades occur extremely rapidly. For example, molecules internalized from the cell surface via clathrin-coated vesicles can be found within sorting endosomes within 1 minute, and undesirable molecules have been reported to be recycled back to the cell surface with a half-time of 2-8 minutes [79]. Also, similarities found between phagocytosis in specialized cells like macrophages and bacterially-induced uptake in epithelial cells. In each case, foreign particles that are destined for degradation in the lysosome are found in late endosomes within 10-15 minutes [80]. Few pathogens are known to be able to survive in the hostile environment of the cellular degradative pathway. To avoid degradation, some pathogens produce secreted proteins that lyse the vacuole and release the bacteria into the cytoplasm. However, *Salmonella* survive and replicate within the SCV, enduring by modifying the vacuole and preventing lysosomal fusion or delivery of lysosomal enzymes. These mechanisms have indicated that *Salmonella* can modify their intracellular environment rapidly and that, by escaping the archetypal endocytic pathway at an early stage, they effectively avoid the subsequent delivery of lysosomal enzymes [78 26].

Hosts have multiple mechanisms to combat this *Salmonella* attack. Other than epithelial cells acting primary as a physical barrier against *Salmonella* infection, paneth cells produce several kinds of anti-microbial peptides such as defensins and cryptidin which can easily kill invading *Salmonella* [24] [63]. Several cytokines and chemokines have been shown to increase upon infection. For example, IL-8, IL-6, TNFα increase significantly upon infection and various other chemokines, altogether contribute to the anti-microbial peptides [81] [82] [83] [84]. With high levels of complexity involved in the multiple interactions that occur between *S.* Typhimurium and epithelial cells the internalized SCVs begin to mature within the host cells. Once the SCV maturation is finished, the vacuole travels through the host cytoplasm to reach a perinuclear region near the Golgi apparatus. This localization allows SCVs to capture nutrients from endocytic and exocytic transport vesicles of Golgi apparatus. The bacterial replication starts after SCV is located in the perinuclear region and enough nutrients have been incorporated [62].

These initial phases involving *Salmonella* infection are almost the same for various typhoidal and non-typhoidal *Salmonella* serovars (NTS), essentially transmitted by oral fecal route, either in poultry or humans. However, it is only later that they diverge in their mode of infection and end up causing entirely different clinical manifestations. From a clinical perspective, localized gastroenteritis is caused by NTS which is characterized by a rapid onset after a short incubation period of 12-72h and a brief duration of symptoms typically less than ten days. Typhoid fever or fowl typhoid, with a systemic infection, has a considerably longer incubation period with a median of 5 -9 days and longer duration of symptoms that may persist for weeks [85] [28].

Non-typhoidal *Salmonella* - The Orchestration of Exudative Inflammation and Gastroenteritis

Gastroenteritis caused *Salmonella* is characterized by a classic exudative inflammatory response leading to intestinal pathology and diarrhea. The inflammation accompanied by the infiltration of phagocytic mononuclear cells such as neutrophils, or heterophils in avian systems, cause a typical diarrheal disease characterized by exudative inflammation in the terminal ileum and colon of humans, and the lower ileum and ceca in poultry [86].

Pattern Recognition and Polymorphonuclear Cell recruitment

The innate immune system senses microbial translocation within SCVs into tissue by detecting conserved microbe-specific molecular patterns using three major pattern recognition systems: Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain (Nod)-like receptors (NLRs), and complement [87] [88].

TLRs are located on the host cell surface or in a vesicular compartment and function as a ‘‘bar code reader’’ that distinguish bacterial patterns from those associated with host cells. For example, TLR5, which recognizes bacterial flagellin, is expressed at the basolateral surface of human intestinal epithelial cells, while TLR4, which recognizes the lipid a moiety of LPS from all Gram-negative bacteria, is found in vesicular structures in the cytoplasm of these cells. This functional compartmentalization of TLRs enables the epithelium to serve as a sentinel for microbial translocation from the intestinal lumen [89].The second family of pattern recognition systems, termed NLRs, is located in the host cell cytosol. Some NLRs function in detecting the presence of foreign nucleic acids in the cytoplasm. NLRs function as an important sensory system for bacterial ‘patterns of pathogenesis’ detecting, for instance, the presence of virulence factors such as toxins and type III secretion systems (T3SS) produced by *Salmonella* that do not enter the cytoplasm [90].

The third pattern recognition system, complement, is a humoral component of innate immune surveillance. After crossing the epithelial lining, bacteria become exposed to complement component 3 (C3), which can distinguish bacterial surfaces from those of host cells. This pattern recognition event represents the first step in the alternative pathway of complement activation by bacterial surface carbohydrates, which stimulates exudative inflammation through the generation of anaphylatoxins. C5a being the most potent factor, stimulates basophils and mast cells to release TNF-α and histamine, which contributes to inflammation by promoting vasodilation. Furthermore, C5a, C3a, and histamine are mediators of endothelial contraction, thereby contributing to the increased vascular permeability and the formation of tissue exudates characteristic of exudative inflammation. C5a is a potent neutrophil chemo-

attractant and can, therefore, contribute directly to recruiting these cells [13 91 92].

The development of exudative inflammation is, in part, orchestrated by cytokines. The detection of bacteria by TLRs and NLRs results in an initiating cytokine response that is limited in scope, but includes the release of mediators, which in turn help to amplify responses in tissue. One mediator initiating the amplification of cytokine responses in tissues is IL-23, which can be released by dendritic cells [93] or CD14+ intestinal macrophages [94] residing beneath the M cells and FAE. IL-23 amplifies cytokine responses by stimulating antigen-experienced T helper (Th)17 cells and natural killer (NK) T cells to produce IL-17 and IL-22 [95 96]. The resulting increase in cytokine production becomes apparent within 2–5 h after infection with invasive enteric pathogens, such as *S.* Typhimurium, where the products of the above amplification mechanisms, IL-17, IL-22, and IFN-ɣ, are among the most prominently induced cytokines in intestinal tissue during the early phases of inflammation [97 96].

The mechanism by which *Salmonella* triggers a pro-inflammatory response has been an area of intense research. Secretion of effectors by either SPI1 or SPI2, that were responsible for inducing actin cytoskeletal rearrangements and which facilitate the entry of bacteria into epithelial cells, and initiate the signal transduction cascades leading to migration of polymorphonuclear leukocyte (PMN) toward the basolateral aspect of the intestinal epithelium [98]. Studies show that SPI1 effectors have a partial role in neutrophil recruitment while no significant role for SPI2 effectors has been found. Neutrophil recruitment is triggered by bacterial adherence to cell surface rather than its internalization and occurs in a MyD88-dependent fashion. The resulting neutrophil influx and inflammatory diarrhea are a stereotypic host response to bacterial invasion of the intestinal mucosa [85 25 99].

Collateral Damage and Exudate Formation

One characteristic of exudative inflammation is the formation of exudates that can accumulate in intestinal tissue, resulting in swelling and edema, or when accompanied by significant injury to the intestinal epithelium, can transfer into the lumen where they contribute to diarrhea. Severe acute exudative

inflammation is caused by invasive *S.* Typhimurium in mice, is associated with extensive epithelial injury and necrosis of the upper mucosa, affecting large areas of the colon and/or the terminal ileum [100]. In addition, a recent study demonstrates the ability of *Salmonella* to compromise the tight junctions in the intestinal epithelium, which may lead not only to enhanced bacterial translocation, but also initiate neutrophil transepithelial migration. These findings suggest that neutrophils are largely responsible for the collateral tissue damage accompanying exudative inflammation, thereby contributing to intestinal fluid accumulation and diarrhea. The resulting dehydration caused by diarrhea can become life threatening, which illustrates that the protection against bacterial translocation and dissemination conferred by exudative inflammation can come at a high cost and needs to be tightly controlled to limit the damage inflicted on the host [93].

Exploitation of Host Responses by *Salmonella*

Interestingly, the fraction of *S.* Typhimurium population that remains in the intestinal lumen thrives in an inflamed gut and becomes a sizeable fraction of the intestinal microbiota [87 93 101]. It has therefore been proposed that induction of an inflammatory response by the invading fraction of the *S.* Typhimurium population, through self-destructive cooperation, empowers the luminal fraction of the *S.* Typhimurium population to bloom [102 103 104 105].

Interestingly, growth of *S.* Typhimurium is aided by the very inflammatory responses that are aimed at controlling luminal bacteria, including the epithelial release of antimicrobials and epithelial transmigration of neutrophils into the intestinal lumen. *Salmonella,* triggering intestinal inflammation, actually is known to take advantage of at least two environmental changes that accompany this host response [93]. The first notable alteration of the intestinal environment occurring during inflammation is the removal of luminal contents by the flushing action of diarrhea. The remaining microbes in this niche rely predominantly on nutrients found in the mucus layer to support their growth. The mechanisms by which pathogens can take advantage of this situation are still poorly understood, but initial studies suggest that the ability to colonize mucus layer plays an important role in their ability to outgrow non-pathogenic microbiota. For example, *S.*

Typhimurium uses motility and chemotaxis toward mucus carbohydrates to colonize this niche, and both properties are essential for benefiting from inflammation by outgrowing the intestinal microbiota [106] [98].

A second important change in the gut environment encountered during acute exudative inflammation is the epithelial release of antimicrobials into the lumen. For example, the antimicrobial protein lipocalin-2 is released into the intestinal lumen, where it binds enterobactin, a low molecular weight iron chelator produced by many enteric bacteria. By sequestering enterobactin, lipocalin-2 exerts a bacteriostatic activity on bacteria that depend on this siderophore for iron acquisition. In addition to enterobactin, *S.* Typhimurium produces a glycosylated derivative of enterobactin, termed salmochelin, which is no longer bound by lipocalin-2, thereby conferring resistance to this antimicrobial action. Salmochelin bestows a growth advantage upon *S.* Typhimurium in the lumen of the inflamed intestine, presumably because lipocalin-2 suppresses growth of competing microbes [87] [107] [108].

In healthy individuals, the gut lumen is thought to be fairly anaerobic, with traces of oxygen being readily consumed by the microbiota. The majority of microbiota is strictly anaerobic bacteria belonging to the classes Bacteroidetes and Clostridiales that rely on fermentation of amino acids and complex polysaccharides. One fermentation end product generated by the microbiota is hydrogen sulfide (H_2S), a cytotoxic compound that is converted to thiosulfate ($S_2O_3^{2-}$) by the colonic epithelium. During inflammation, neutrophils that transmigrate into the intestinal lumen release reactive oxygen species (ROS) in an attempt to kill bacteria. A by-product of releasing ROS is the oxidation of thiosulfate to tetrathionate ($S_4O_6^{2-}$). In contrast to the fermenting microbiota, *S.* Typhimurium can use tetrathionate as a terminal electron acceptor to support its growth by anaerobic respiration, which is more efficient for energy production than fermentation. Also anaerobic tetrathionate respiration facilitates growth on poorly fermentable carbon sources [93].

Collectively, these processes enable NTS to establish an intestinal lifestyle and outgrow competing gut flora, resulting in a marked increase in numbers of pathogens within the cecal contents. In turn, enhanced growth in the intestinal lumen promotes transmission of NTS by the fecal-oral route. In summary, the

benefit NTS harnesses from intestinal inflammation are largely based on an improved access to nutrient sources. It can thus be speculated that NTS virulence evolved predominantly as a means to access nutrient resources in its host and then disseminate further by the fecal-oral route [85] [28]. Generally, NTS are demonstrated to be unable to overcome mucosal immune defenses, limiting the bacterial dissemination from the intestinal mucosa to systemic sites of infection [109] [24]. However, NTS bacteremia and subsequent systemic infection have been observed in patients with impaired immunity, such as individuals with AID*S.* In contrast, *S.* Typhi, with an established systemic infection process, was shown to have any prerequisite of an immunodeficient host to cause a systemic infection, demonstrated by its ability to cause typhoid fever in both healthy individuals and AIDS patients with similar efficiency. Thus, unlike NTS, it is likely that *S.* Typhi possesses unique virulence traits that allow it to overcome mucosal barrier functions in immunocompetent hosts [85] [28]

Typhoidal *Salmonella* Infection and Disease

In contrast to nontyphoidal gastroenteritis, typhoid fever in is not a typical diarrheal disease and the intestinal pathology is characterized by interstitial inflammation with predominantly mononuclear infiltrates, while neutrophils are observed to be scarce, supporting the absence of any exudative inflammation. Onset of fever was seen approximately in one third of typhoid fever patients, and diarrhea was seen associated with fecal leukocyte populations, with mononuclear cells dominating the exudate [85]. It was interesting and necessary to understand the fact that typhoidal *Salmonella*, being very invasive in its infection process, does not trigger the stereotypic host response upon penetration of the intestinal mucosa (i.e. neutrophil influx) which is typically observed during infection with other enteroinvasive pathogens. In this regard, several tissue culture studies supported the concept that *S.* Typhi infection results in reduced inflammatory responses when compared to NTS, such as *S.* Typhimurium [85]. Gene expression analysis in intestinal epithelial cells showed that unlike *S.* Typhimurium, *S.* Typhi did not trigger a pro-inflammatory response through TLR5 stimulation. Also, while *S.* Typhimurium triggered neutrophil transmigration across a monolayer of colonic epithelial cells, *S.* Typhi did not. Furthermore, infection of macrophage-like cells with *S.* Typhi resulted in markedly reduced production of the neutrophil

chemo-attractant IL-8 compared to infection with *S.* Typhimurium. Collectively, these observations raised the possibility that *S.* Typhi expresses virulence mechanisms, allowing it to down-regulate a PRR-mediated host response in the intestinal mucosa that results in the absence of neutrophil infiltration and inflammatory diarrhea [64 109 85].

Intracellular Survival and Systemic Dissemination

Besides repressing the pro-inflammatory response of the host by reduced PRR signaling and delayed neutrophil recruitment, *S.* Typhi also follows an unusual intracellular lifestyle which explains the inability of neutrophils to contain systemic *Salmonella* infection [110 85]. With many survival strategies explained earlier, the transport of SCVs continues through the mucosa, leading to direct access of underlying macrophages and dendritic cells to *Salmonella* for establishment of a systemic infection [62]. M cells do not recover from these rearrangements resulting in cytotoxicity. This effect is visible within 30 minutes post-infection, and by 120 minutes M cells are fully destroyed, which results in the breakdown of FAE architecture, allowing *Salmonella* to further infect the adjoining enterocytes [111 66].

Survival within Dendritic cells

Dendritic cells (DC) are specialized antigen-presenting cells that function at the dividing line of innate and adaptive immunity that capture antigens in the peripheral tissues and present the processed antigens via MHC-I and II [112]. Invading *Salmonella* from the intestinal epithelial layer can reach the subepithelial compartment where they interact with DC and macrophages that reside under Peyer's patches. Intestinal DC sample bacteria and facilitate their penetration across the gut and subsequently serve as an alternative mechanism for *Salmonella* entry into the host via DCs in Peyer's patches or lamina propria that breach the epithelial barrier and sample luminal bacteria [113 24 114]. After *Salmonella* infection, DCs are recruited to the intestinal lymphoid tissues and ingest the bacteria, resulting in varied outcomes. In some cases, an effective adaptive immune response generated by these infected DCs, either in the T cell zone of Peyer's patches, or in the draining lymph node (not seen in avian systems) [113 64 24]. Alternatively,

Salmonella sometimes utilize DCs as a vehicle to systemically disseminate within an infected host. The intracellular life of *Salmonella* in DC is interesting. *Salmonella* does not get killed nor does it proliferate in DC populations. Intracellular survival of *S.* Typhimurium in murine DC was independent from the function of virulence factors known to be important for survival in macrophages, such as the SPI2-T3SS [113]. Surprisingly, the bacterial LPS O antigen is found to be crucial in maintaining the bacterial steady burden in DC [24 113].

Survival within Macrophages

Monocytes form a major subset of MLNs that migrate to the intestinal epithelium during *Salmonella* infection, in addition to locally residing macrophages beneath M cells. Inflammatory monocytes, upon entering a tissue, are a source of molecules important in controlling bacterial infection, particularly iNOS and TNF-α. Chemokines are the main mediators involved in the recruitment and migration of leukocytes to and within tissues [115 28]. An array of chemokines is induced by inflammation and recruit monocytes to sites of infection. Chemokine receptors CCR2 are required for monocytes to egress from the bone marrow into the blood during steady state and infection [116]. Moreover, both MyD88-dependent and MyD88/TLR4-independent pathways are involved in phagocyte recruitment to the intestinal lymphoid tissue during oral *Salmonella* infection [88 114 108]. *Salmonella* can enter these macrophages and other phagocytic cells by host cell mediated phagocytosis or by SPI1 dependent invasion and survival inside macrophages is a prerequisite for establishing systemic infection [24 64 117]. During this step, a host antibacterial cascade is activated as a defense barrier. Host defense includes acidification of the phagosome lumen, activation of cationic proteins and production of antimicrobial peptides such as defensins, ROS and nitrogen intermediates, starvation, lysosomal enzymes, or other activities. *Salmonella*'s ability to counter the multiple hurdles posed by infected cells is indeed fascinating. To begin with, virulent *Salmonella*, as described before, is known to inhibit the fusion of SCV with lysosomal vesicles containing NADPH oxidase and thereby leading to an increased bacterial survival in macrophages [118]. The pathogen responds to this defense by activating the global regulator, involved in acid tolerance and resistance to cationic peptides (described above), which was also demonstrated to be

activated during transit of *Salmonella* under acidic pH conditions in the gut [119]. Also by inducing the activation of the pagD and pagC SPI-11 genes, intramacrophage survival was enhanced [120]. In addition to SsrAB, PhoP/PhoQ system controls the expression of SPI2-T3SS effectors, which in turn reduces exposure of *Salmonella* to the host defense battery such as antimicrobial defense, antigen presentation and ROS and nitrogen species generation [119 121 122].

Systemic Dissemination into Organs

While macrophages are major effector cells eliciting innate immunity, they also transport *Salmonella* to host tissues [122], particularly to organs of the reticulo-endothelial system such as the liver, spleen, kidney, blood, and reproductive tract [123]. Macrophages provide a protected site for intracellular bacterial replication and further act as a vehicle of dissemination. Intracellular *Salmonella* are found in SCVs, which localize around the endoplasmic reticulum and survive the host defense by the concerted strategies described above. Further, within SCVs in macrophages, *Salmonella* also down-regulates synthesis of flagellar proteins in response to low pH. Many studies have postulated that the pathogen is able to cause cell death and systemic spread after replication by mechanisms that involve the SPI-1 effectors in a number of cell types [124]. This hypothesis was supported by the observation that the SipB SPI1 effector induced cell death in a cytokine-dependent pathway [125]. SipB is encoded by SPI1 and induces rapid macrophage cell death through activation of the host protein caspase-1, resulting in apoptosis, autophagy via degradation of cytosolic components, pyroptosis and plasma membrane blebbing [126 107].

Although apoptosis and necrosis are terms that are widely used to define eukaryotic cell death, and the two processes are said to be individually different and not connected, recently, a new concept has emerged, that apoptosis and necrosis are not mutually exclusive but rather necrosis is the end stage of any cell death, including apoptosis. This was based on report that *Salmonella* induced cell death of macrophages was also caspase-3-dependent, along with caspase-1, which, in concert, triggered swelling of macrophages, an event termed oncosis (from Greek, “onkos, ” meaning swelling). Further, oncotic

macrophages were found often packed with motile *Salmonella* and these flagellated *Salmonella* were observed escaping irregularly from these cells. These observations were against classical understandings of *Salmonella* infection and the whole process was observed to end with necrotic cell death of macropahges [127]. Considering the adaptive abilities of *Salmonella*, this novel strategy by which it survives in, accumulates in, and escapes from macrophages seems remarkable.

Irrespective of the pathway followed, the overall process results in release of *Salmonella* from macrophages into the location, or more specifically, into the organ the macrophages have migrated to, leading to systemic infection. Colonization of the gall bladder by *Salmonella* is of special importance, since the organ is recognized to be a chronic site of infection in the carrier state [61]. *Salmonella* outer membrane is an excellent barrier to membrane active agents such as bile. Multidrug efflux pumps can remove bile that penetrates the outer membrane, e.g. the *S.* Typhimurium AcrAB pump is absolutely required for bile resistance. *Salmonella*e have the ability to colonize the gallbladder where bile concentrations are extremely high and it has been observed that it stays and multiplies in gallbladder epithelial cells without getting translocated to the mucosa. *Salmonella* can further form biofilms on gallstones during persistent infection and is one of its potent virulence strategies [61 58]. In support of this hypothesis, enhanced fecal shedding and colonization of gall bladder tissue and bile was observed where *Salmonella* formed biofilms on gallstones. Also, biofilm formations that occur *in vivo* not only protect bacteria from high levels of bile in the gallbladder but it was also seen as limiting the effectiveness of certain antibiotics. This ability of *Salmonella* to reside in the gall bladder allows its host to become an asymptotic carrier and spread the disease. The chronic asymptomatic carrier state observed in 3–5% of infected humans was attributed to this colonization [61].Collectively, all these processes enable typhoidal *Salmonella* to persistently infect the host, resulting in a marked increase in relative abundance of the pathogen in intestinal contents. In turn, enhanced growth in the intestinal lumen along with a systemic spread promotes effective transmission of *Salmonella* by the fecal-oral route.

Poultry Practices and Bioaerosol Generation

Introduction

The aims of this section are to provide an overview of the poultry industry, with an emphasis on end to end poultry meat production and to consider the possible entry points of enteric pathogens throughout the production chain. These entry points are largely considered to be fecal-oral. However, there is a possibility and process for airborne transmission and fecal- respiratory route is a potential mechanism of transmission for *Salmonella* in poultry, of unknown importance. Here, we highlight the current knowledge of bioaerosol generation, fate and transport, with a specific focus on *Salmonella* bioaerosols generated at each stage of commercial poultry production. Additional emphasis is placed on survivability of *Salmonella* in these bioaerosols as a means to assess the transport of bioaerosols and subsequent risk of exposure and infection of poultry.

Primary production of commercial poultry begins with pedigree lines, which become great grandparents at the apex of the production pyramid. The eggs from these hens yield, in turn, the grandparents and parents, and ultimately the broilers or layers in multiplier houses. Control of *Salmonella* infection is vital at the apex of production, as this organism can be vertically transmitted from hen to egg [128]. Further, it is crucial to keep breeder production flocks free from *Salmonella* because a colonized flock will spread the bacteria to a large number of commercial flocks. Unlike production flocks, which are relatively short-lived with a total lifespan of approximately 42 days, elite flocks, including breeders, may have a lifespan in excess of 1 year, so careful management is needed to ensure ongoing freedom from colonization by *Salmonella* or other pathogens [34].

In this regard, Hazard Analysis and Critical Control Point (HACCP) -based intervention strategies have been developed and are used extensively. These strategies begin with elite breeder flocks and filter down the production pyramid. These strategies include those already established, such as biosecurity,

vaccination, competitive exclusion, pre- and probiotics, feed and water control, and those more experimental, such as bacteriophage or immunoglobulin therapy [33].

While many of these strategies have proven effective in laboratory or limited field trials, implementation in extensive trials or true commercial operations has proven inconsistent in some cases, especially towards the lower end of the production pyramid. There have been constant reports of *Salmonella* infections, with most hurdles beginning at the commercial hatcheries, followed by expansion of the incidence to processing plants [129 51 130]. With increasing reports, we are forced to question the present intervention strategies established based on fecal oral route as major route of infection. In this regard, it is possible that airborne *Salmonella* has a major unappreciated role.

The Hatchery

Commercial poultry hatcheries are ideal environments for the contamination and dissemination of enteropathogens [46 41]. Ideally, fertile eggs received from farms should be enteropathogen free; however, this is not the commercial reality in many countries. Dust and bioaerosols, generated from contaminated eggs within a hatchery incubator, a critical control point, can spread enteropathogens to other areas of the hatchery depending upon airflow [41]. Presence of *Salmonella* in air samples of hatcheries is irrefutable and has been proven by various sampling and identification methods [131 41]. Furthermore, it is a general notion that dust carried by airflow settles down elsewhere in the hatchery on uninfected eggs and hatched egg shells, and is then assumed to follow a typical oral fecal route of transmission, with hatchlings pecking these contaminated egg shells. However, the possibility of entry and transmission of *Salmonella* through the respiratory route has historically received little attention [41 132 15 133].

Primary breeding flocks are largely more valuable than other poultry stock and, therefore, hatchery design is usually state-of-the-art with a one-way movement of clean to dirty flow to reduce contamination, thus incidence of *Salmonella* is typically lower in broiler breeder hatcheries. These breeder flocks are much smaller and hatching eggs are gathered more frequently and disinfected shortly after being gathered.

While many of these strategies have proven effective in breeder hatcheries, implementation in true commercial operations have proven problematic and ineffective.

Further, in modern poultry production, separation of the hatchery from the production facility means that hatchlings will spend a period of time without provision of feed and water. The time period between hatch and placement is highly variable, which depends on the availability of transport equipment, distance to the placement facility and hatchery practices. Producers in some parts of the world, with good transportation facilities, strive to place neonates within a period of hours. This provides feed and water to birds in the brooding environment, reducing stress and eventually resulting in better performance. However, a major part of the world follows a practice that holds birds in the hatcheries for about 12 - 24 h to allow them to mature and to initiate a vaccine response, while the birds are under low immunological challenge from other antigens [50] [134]. However, in these situations, hatched birds experience prolonged high density confinement in the hatchery where they are exposed to circulating air which can carry *Salmonella* and other pathogens. Furthermore, the transportation of hatchlings from hatcheries to placement facilities adds additional confinement time. Under practical conditions, the whole process can sometimes require as much as 36 to 48 h after hatching, during which birds do not have access to feed and water [134].

It is during this time that body weight decreases and a direct correlation has been reported between the time involved to reach placement and body weight loss in broilers [135] [136]. In the absence of feed and water under incubating conditions, a linear reduction in body weight has been reported at 0.17 g/h [137]. Delay of placement exacerbates this condition with further decreases in weight [138] [139]. Furthermore, early feed deprival is associated with delayed gut maturation [140] [141] [142], which in turn causes inefficient absorption of nutrients at later feeding stages.

These effects compound to cause reduced immunity [143] [144], increased susceptibility to pathogens, and reduction in overall performance [135] [145] [146]. Under such stressed conditions, the anatomical, physiological and immunological state of hatchlings increases vulnerability to infections. Considering these factors, it is necessary that we regard respiratory route as a potential portal of entry for enteropathogens and

Salmonella, in particular. Overall, we hypothesize that the hatchery environment is one of the most critical parts of commercial poultry production, with respect to airborne transmission of *Salmonella* and hence various intervention strategies have to be developed at this stage to ensure prevention.

Litter

Infected chicks from a hatchery that are placed on a grow-out farm can act as sources of infection and contamination to the farm environment. It has long been recognized that breeding stock of poultry play a crucial role in the dissemination of *Salmonella* infection and contamination. *Salmonella* contamination on broiler grow-out farms is complex and can come from multiple sources in the environment such as feed, feed ingredients, water, and litter. *Salmonella* has been demonstrated to persist in farm environments for over a year with or without poultry being present. Furthermore, total disinfection of grow-out farms may be impossible to achieve due to cleaning difficulties and environmental reservoirs such as mice, insects and wild birds [43] [33].

With anticipation that hatchlings are not infected with any enteropathogens, they are transported from hatcheries to placement facilities, in to an appropriate brooding environment, with provision for feed, water, air and temperature along with a bed of litter. Absorbent material is used to line the floor of the poultry house and, depending upon local availability, may consist of non-sterile wood shavings, peanut or rice hulls or other similar material. A study has suggested that broilers grown on litter, compared to broiler cage housing, had lower caecal populations of *Salmonella*, as nonstarch polysaccharides in the litter modulate the intestinal microflora, increasing the competitive exclusion (CE) of microorganisms [147].

However, litter may introduce pathogens into the primary production environment, although treatment and testing prior to use can greatly reduce or eliminate pathogen carriage [148] [149]. Prolonged use of litter, with multiple sequential flocks of birds, is considered a far greater problem, as it can harbor pathogens [150]. Sources of *Salmonella* for hatchlings are primarily chickens, either directly or indirectly from the feces, which accumulates in the litter. Chickens have a natural tendency to peck litter, and this has proven to

sometimes result in infection, as described above. On the other hand, dust originating from litter due to air circulation and movement of birds leads to bioaerosol generation from the production system. In automated chicken egg layer management systems, the main sources of bioaerosols are live birds with both feces and birds linked to the contribution of both *Salmonella* and *E. coli* in associated bioaerosols. Today's large-scale poultry production, with densely stocked and enclosed production buildings, is often accompanied by very high concentrations of airborne microorganisms [12 151]. Modern broiler houses reflect considerable progress in design, with the majority of poultry houses in countries such as the United States and Australia being tunnel ventilated. In these systems, large volumes of air are moved through the house, by negative pressure, to provide the optimal temperature and superior ventilation for broiler growth. These large volumes of moving air may potentially contain a range of bacteria sourced from the internal environment of the house, including pathogens such as *Salmonella*. Hence, there is a possibility that air circulation within poultry housing environments provides opportunities for transfer of these pathogens throughout the production house and to the surrounding air environment [50 152].

Studies relevant to the presence and levels of airborne *E. coli*, *Salmonella*, and *Campylobacter* inside poultry shed environments are very limited. To date, there have been few studies specifically examining the levels of bacteria, including pathogens, in the air either inside or outside tunnel ventilated broiler sheds. In a Bulgarian study of mechanically ventilated sheds, levels of 1.68×10^7 CFU/m^3 of air were found inside the sheds [153]. *Salmonella* was recovered but was not quantified in the air inside a room containing experimentally infected laying hens in a trial carried out by Gast et al. [154]. Airborne movement of dust and fluff has been implicated in transfer of this organism in layer houses [155]. *Salmonella* was isolated from 63 of 206 samples in bioaerosols in processing plant environments at levels of approximately 10^3 CFU/m^3 of air [129]. Other than these few studies, there appear to have been no reports of studies attempting to quantify the levels of key pathogens such as *Salmonella* in the air in and around broiler houses through the production cycle [156 130]. Such studies would allow an assessment of the quantifiable risks to poultry health via the aerosol pathway.

A study by Chinivasagam and co-workers (2009), addressed this issue of aerosolized bacterial pathogens

in terms of assessing levels, by observing patterns of distribution, as well as the possible interrelationships, leading to pathogen presence in bioaerosols. More specifically, they quantified the levels of *Salmonella*, *Campylobacter*, and *E. coli,* which served as an indicator organism, within the chicken production environment, through whole production cycles, in both internal and external bioaerosols on four broiler farms. The study identified the dynamics of pathogen transfer within Australian mechanically ventilated production systems, which do not vary markedly from U.*S.* production systems. However, this may not be necessarily considered applicable as a worldwide pattern [27]. In this study, the aerosol transfer process was seen to occur via the litter-dust-air interface within the housing environment. Studies on the recovery of *S.* Typhimurium from infected chickens demonstrated that sampling bioaerosols was a representative way of understanding overall flock contamination, alleviating the need to examine large numbers of litter samples [157]. The study ranged over a 3-year period on four poultry farms and consisted of six trials across a boiler production cycle of around 55 days. Weekly testing of litter and bioaerosols was carried out through the cycle. A key point that emerged from these studies was that the levels of airborne bacteria were linked to levels of these bacteria in litter. This hypothesis was demonstrated by *E. coli*, the indicator organism, used in this trial. The typical levels of *E. coli* in litter were 10^8 CFU/g of litter and, as a consequence, ranged from 10^2 to 10^4 CFU /m^3 of air in bioaerosols, both inside and outside the housing environment. *Salmonella* serovars isolated in litter were generally also isolated from bioaerosols and dust, with the *Salmonella* serovars Chester and Sofia being the dominant serovars across these interfaces. In this study, *Salmonella* was isolated from settled dust and dust levels peaked during the middle of the cycle, thus showing a link to chicken activity [11].

Transportation and Processing

The goal of on-farm pathogen reduction strategies is to deliver poultry to the processing plant with undetectable levels of *Salmonella*. However, catching and transportation of poultry from primary production to processing can be significant in the dissemination of enteric pathogens, through contamination and subsequent cross-contamination because of the use of dirty crates, trucks and the catching / pickup crews. Based on cecal sampling, Cox et al. estimated that up to 31% of turkeys entering

the processing plant may harbor *Salmonella*. Hence, the potential for horizontal spread of *Salmonella* from farm to farm is very high. Spread is managed through washing of crates and truck tires, as well as quarantine of colonized flocks for end-of-day processing. Washing and drying of catching crates are critical processes, influenced by the nature and concentration of sanitizing agents [158] [159]. Flocks can be sampled and tested for *Salmonella* as close as possible to transportation to allow additional management processes such as late processing, freezing or commercial cooking to be instigated if the pathogen is present.

Further, *Salmonella* contaminated or infected live poultry entering the processing plant are the primary source of processing plant contamination. Although plant interventions, including chlorinated water in the chill tank, can reduce levels of *Salmonella* on carcasses, incoming birds may affect the microbial quality of the end product [48] [47] [41]. Controlling *Salmonella* contamination of poultry prior to slaughter may include limiting the poultry's exposure to bacteria in the last few hours before slaughter during on-farm loading, transportation, and holding at the processing plant. In commercial turkey production, turkeys rest in holding crates on trucks in cooling sheds for 2 h or more before slaughter. Very little work has been done to show if rapid airborne infection from contaminated fecal dust in poultry is possible and if it is responsible for increasing the number of *Salmonella*-contaminated birds entering commercial slaughter houses. Understanding the kinetics of *Salmonella* transmission to chickens and turkeys from the environment during the last few h before slaughter may identify a critical control point to reduce the number of contaminated turkeys entering the processing plants. If rapid airborne transmission by inhalation occurs immediately prior to slaughter, controlling dust and therefore reducing transmission at this time, will be important [160].

In this regard, Harbaugh et al. hypothesized that it may be possible that *Salmonella* contamination occurs in turkeys, and in general poultry, prior to slaughter, relating to the prevalence of airborne *Salmonella* in commercial poultry. They predicted multiple points in the last few hours before slaughter that may increase contamination of turkeys, which in turn may be extrapolated to other poultry [14]. The first point of increased exposure, prior to slaughter, may occur when birds are taken off feed for 3 to 6 h before

loading. This can increase the flock's contact with *Salmonella* through increased ingestion of the litter within the barn in search of food [45] [44] which may eventually lead to fecal-oral route of infection. Feed withdrawal is also connected to stress, somewhat similar to that of the hatchery conditions addressed above. Second, this disturbance of litter increases the amount of dust in the air, which may increase the risk of infection due to inhalation of *Salmonella*-contaminated particles or bioaerosols [14] [45]. Third, a large amount of dust is generated during the load-out process that facilitates potential inhalation of *Salmonella*-bioaerosols. In addition, turkeys are loaded on to crates that are often contaminated with *Salmonella* due to improper cleaning and disinfection [41]. Finally, once turkeys arrive at the slaughter plant, they are kept in cooling sheds until shackled. Cooling sheds are structures with a roof, no walls, dirt or concrete floors, and accommodate as many trucks as needed for that day's slaughter. Large fans blow air on turkeys to keep them cool during the summer months. These sheds are also used during winter to protect the birds from the cold, although the fans are not used in cold environments. Turkeys arriving at cooling sheds may harbor *Salmonella* and may transfer the organism to the environment. From there, it may spread through dust blown by fans to uninfected birds. These events occur in the last few hours, and may increase exposure to *Salmonella* laden dust [160] [14].

These steps have been identified as points of increased *Salmonella* infection in pigs due to rapid infection during the last few hours before slaughter. Recovery rates for *Salmonella* in pigs are reported to be 3 to 10 times higher after slaughter at the processing plant than those from cohorts slaughtered on the farm, suggesting that pigs become infected sometime during loading, transportation, and holding [161]. In commercial pork production, pigs rest in holding pens for 2 h or more before slaughter, further, *Salmonella* in dried feces remained viable for up to 13 h and was infectious to pigs via either the intranasal or oral route [162]. In addition, *Salmonella* transmission, based on cultures of the intestinal tract and lymph nodes, has been demonstrated to occur solely by the respiratory route in hogs in which the esophagus was transected [163].

Pathogen contamination of the respiratory tract of birds entering processing plants may contribute to carcass contamination, and *Campylobacter* was detected in the respiratory tract of broilers prior to

entering the scald tank. The amount of dust in grow-out houses during catching and transport led Berrang et al. to hypothesize that presence of *Campylobacter* in the respiratory tract contaminates eviscerated carcasses without leakage of the gut during processing [164]. An excellent support to this hypothesis of airborne transmission and contamination of poultry meat products by *Salmonella* was found in an additional study [49]. The study involved risk assessments addressing the various exposure pathways of enteropathogens in poultry meat and eggs infecting humans. An analysis of the relative contribution of different exposure pathways finally leading to ingestion of the pathogens and resulting in illness was performed, with a specific focus on *Salmonella* spp. and *Campylobacter jejuni* and their occurrence in poultry meat and chicken table eggs [49].

One investigation assessed eight studies, each with two datasets concentrated on *Campylobacter* spp., and two studies which examined *Salmonella* only [52]. Quantitative dataset analysis showed the prevalence of *Salmonella* on the surface of poultry meat products involving 2936 samples, and estimated the average prevalence of *Salmonella* on the surface of poultry meat at 22.6%. Only one study looked at the percentage of duck breasts that were internally contaminated with *Salmonella*, where 3.8% of 53 samples were internally contaminated. Especially high prevalence rates were found in a study from India [165] and from Trinidad and Tobago [166]. With respect to table eggs, only half of the eight studies showed *Salmonella* contamination of egg contents. With the exception of the study from Trinidad and Tobago, the contamination rate inside of eggs was found to be significantly lower than on the shell surface. In combination with the observed higher prevalence of surface contamination of poultry meat and eggs with both pathogens, this study concluded that risk management activities aiming to reduce the number of human campylobacteriosis and salmonellosis cases should focus on applying management measures that will reduce the number of *Salmonella* and *Campylobacter* on the surface of poultry meat products and on egg shells. Considering the hypothesis that it may be possible that increased airborne *Salmonella* contamination occurs in poultry, prior to slaughter in a span of little time, as described above, surface contamination of poultry meat and eggs appear to be of primary importance [49 50].

Ultimately, the direct risk of poultry acquiring infections from bioaerosols containing these pathogens is

what matters. This in turn depends on various factors like, survivability of *Salmonella* in bioaerosol, size of bioaerosols that can carry *Salmonella* and dose at which it enters the respiratory tract of birds for the infection process to unfold.

Survival of Bacteria in Bioaerosol

As a general pattern, *Salmonella* can be isolated from a variety of sources other than the bird, and at various stages of the production cycle [167]. It is also likely that prevalence of these pathogens within the production environment could vary. For *Salmonella*, estimates of incidence have been quite variable. As an example, there was 42% prevalence for *Salmonella* in 198 U.*S.* broiler houses, potentially leading to bioaerosol generation [130], however, Gast et al. (2004) detected airborne *S.* Enteritidis up to 75% using an electrostatic device, over a 4 week study.

The aerosolization process begins from three interfaces - litter, dust, and air, during any production cycle [11]. However, aerosolization and its transfer is a traumatic process for most microorganisms, and survival can be dependent on the mechanisms of aerosolization, the climate into which these organisms are launched, the distance they are travelling and, of course, the time involved in the whole process [168169170]. It is hard to have a set number and combination of these parameters to be expressed as best for one particular type of bacteria or pathogen of interest, since they essentially have a combined effect on the process and are continuously variable in any production facility. However, analogies can be drawn from the survival patterns of these organisms in other aerosol environments such as processing plants or hatcheries, or even laboratory-generated conditions.

Salmonella has been shown to be viable in laboratory generated bioaerosols for more than 2 h [168]. Additionally, it has been shown that the death rate of *Salmonella* was influenced by the protective nature of media during aerosolization, along with overall prevailing relative humidity and temperature of the air [12]. D Value, a measure of survival of bacteria, defined as decimal reduction time, is the time required, at a certain temperature, to kill 90% of the organisms being studied in a particular sample, has been

established for *Salmonella*. The D values for *S.* Newbrunswick aerosolized in skim milk at 10 °C ranged from 245 minutes to 404 minutes at 30 and 90% relative humidity respectively. At 21 °C the time ranged from 164 minutes to 470 minutes at similar humidity conditions. This work, though carried out under laboratory conditions, does emphasize the link between environmental parameters, whether it be the internal poultry housing or the external atmosphere, and the impact on survival of airborne organisms [11] [171].

It has been demonstrated that strains of *S.* Enteritidis which have been maintained in aerosols for 2 h remain at least as viable as respiratory pathogens such as *Legionella pneumopilia* [168] and therefore present a potential risk in conditions such as poultry houses where significant numbers of *S.* Typhimurium, and other chicken pathogens, may be present in the air [168]. Airborne transmission of *S.* Enteritidis and other chicken pathogens has long been recognized [16] and the persistence of cases of *S.* Enteritidis may be linked in part to airborne cross-infection within the poultry house. Also, it should be interesting to note that the time range of 2-4 h observed in this study is close to the time turkeys, and in general any poultry, rest in holding pens on trucks in cooling sheds before slaughter, under commercial processing conditions. The time range can also be correlated to prior slaughter time, where birds are taken off feed for 3 to 6 h before loading, which in turn increase flock contact with *Salmonella* through increased scratching of litter in the barn in search of feed and increased bioaerosol generation [44] [45]. In a cage production situation, excreted *Salmonella* have been suggested to show increased survival in nest boxes, presumably due to the body heat of birds and protection via organic matter in feed or dust, a possible reason for the prolonged survival of pathogens in litter [129]. Similarly, temperatures within litter may have a role in supporting the continued survival of the organism in dust aerosols.
Continued survival of *Salmonella* in source material such as dust or litter and subsequent survivability in aerosol, can be dependent on serovar of *Salmonella* as well. It is possible that some serovars can be more resilient than others in a poultry environment. A Danish study found that a *S.* Senftenberg clone persisted for more than 2 years, despite cleaning, disinfection, desiccation, and depopulation, and was subsequently able to infect newly placed *Salmonella*-free layers [172]. In this study, the fact that *S.* Senftenberg was detected in litter and aerosols suggested that the serovars detected in the air were

resilient enough to be captured in the aerosol environment, as well as litter, and were potent enough to infect a flock of birds. In a study discussed above by [11], *S.* Chester was by far the dominant serovar captured in all three interfaces through two consecutive production cycles. Thus, this study was by far the most resilient serovar in terms of entering and surviving in an aerosol environment.

Humidity is known to play a major role in survivability of *Salmonella* in bioaerosols and dependence on humidity has known to be the characteristic of many Gram-negative organisms [131], but is one of the many factors that determine its survivability in bioaerosols. In one of the earliest studies by Wathes et al. (1988), survivability of *S.* Typhimurium was examined at three different relative humidities. Once aerosol was generated from a suspension, samples were collected at regular intervals and numbers of bacteria were determined, with humidity and time as variables. The experiment was carried at an average temperature of 17.6 °C with 32, 53 and 72 % relative humidities. The change in viability of *Salmonella* as the age of the microbial aerosol increased was determined based on microbial inactivation kinetics model by Cox et al. (2010). The viability of *Salmonella* was affected by both relative humidity and time. Viabilities predicted based on the model at 32, 53 and 72% relative humidity were 4, 12.3 and 24.8 % of the initial value respectively, at an aerosol age of 5 minutes. The viability value decreased to 1% of its initial value for the relative humidity of 72% at an aerosol age of 90 minutes [168] [12].

Overall, after microbes are launched from litter to the dust and air interface, there will be an initial sharp fall in the number of viable microbes, which is also true for *Salmonella*. However the rate of fall was observed to decrease as the bioaerosol aged. On average, the number of surviving *Salmonella* in bioaerosol 120 minutes after aerosolization was evaluated to be 1% of the initial value in the litter [16]. So theoretically, if there are 10^6 *Salmonella* in litter, which is reasonable considering the concentration of *Salmonella* required for fecal-oral transmission, after aerosolization a dose of about 10^3 to 10^4 can be expected. This duration of survivability of *Salmonella* is suggested sufficiently long to cause significant hazard by airborne spread [16] [14].

Avian Upper Respiratory Tract and Bronchial Airway

Introduction:

Among air-breathing vertebrates, the avian respiratory organ is considered the most structurally complex and most functionally efficient gas exchanger and its uniqueness has captured the interest of investigators for well over four centuries [173 174 175 176 177 178 179 180 181 182 183]. The avian respiratory system exhibits a distinctive architecture adapted for efficient extraction of oxygen from the environment in order to maintain aerobic metabolism. To perform its primary functions, delivery of oxygen to and the removal of carbon dioxide from the body, the respiratory system continually ventilate the lungs. As a consequence, particles and particle-associated microorganisms are inhaled as unavoidable constituents of tidal air flow. Intensive management systems for poultry are frequently associated with high levels of dust and pathogens in the environment and therefore pose a particular stress to the respiratory system [184]. The generation and possible impact of bioaerosols in poultry production was discussed extensively in the previous section.

Architecture of the avian respiratory tract is an important component to susceptibility and resistance to infectious agents. Here, explanation of the respiratory system will be divided in to four anatomical sections including i) upper respiratory tract, including nares through terminal trachea; ii) primary and secondary bronchi, which together constitute the bronchial system; iii) parabronchi, the gas exchange region; and iv) air sacs. Anatomical structure, physiological functions and immunological defense are described collectively for each anatomical section to provide a view of the conduit a pathogen has to travel. Overall, the objective of this section of the review is to understand the respiratory system and emphasize the incongruity inherent in each component that would support our investigation regarding respiratory route as a possible portal of entry for *Salmonella* in poultry. The mucosa of the entire respiratory system is covered by epithelial cells, which form a barrier separating the rapidly changing environment outside of a bird's body from the highly stable internal environment within the body. Coexistence of these two environments is made possible by the consorted functioning of anatomical,

physiological and mucosal immune systems [185] [186].

The Upper Respiratory Tract

The upper respiratory system begins at the nares or mouth and consists of passages that lead inhaled air and bioaerosol to the larynx. Barrier integrity is maintained by tight junctions in the epithelial linings within these structures [187] [188]. Physiologically, these oro-nasal structures function to heat and humidify inspired air and, hence, can be expected to do the same to bioaerosols containing *Salmonella*, which may provide favorable conditions for recovery and growth for *Salmonella* after the traumatic transfer.

Immune structures involved in the respiratory system of chickens and turkeys include Harderian glands (HG), conjunctival associated lymphoid tissue (CALT), paranasal gland (PG) and nasal associated lymphoid tissue (NALT), which all together are often referred to as head associated lymphoid tissue. Though these structures do not have a direct anatomical connection with the respiratory system, they are functionally important components of local immunity, especially in the upper airways. Local immunity of the respiratory mucosal system is ensured by non-specific defensive reactions with a formidable arsenal of mechanical and cellular defenses, supplemented when necessary by inflammatory and immune responses [91] [185] [186].

Harderian Gland (HG)

Lymphoid tissue of the HG is organized into two histologically separate structures consisting of lymphoid structures of the head and body of the gland. The body is filled with plasmatic cells in different stages of maturation. After contact with an antigen, they migrate into the epithelium of the HG head central canal and produce immunoglobulins. Central canals within the epithelium of the HG head can therefore be classified as lympho-epithelial tissue, where small and medium-sized lymphocytes are accompanied by dendritic-like cells and scarce macrophages. A secretory duct leads out of the HG, also referred to as the deep gland of the third eyelid, to the conjunctival sac, from which its excretion returns to the beak cavity

through the nasolacrimal duct [91 186 189].

Paranasal Gland (PG)

The PG is a structure associated with the nasal cavity and its excretion is exuded to the nasal cavity through secretory ducts. The PG lymphoid structures present the secretory ducts of chickens are localized similarly as in the nasal atrium epithelium. Numerous T cells, and some B cells, have been shown to be present in the nasolacrimal duct, which suggests a certain relationship between NALT and HG [190].

Conjunctival Associated Lymphoid Tissue (CALT)

Various immune structures have been shown to exist in the lymphoid tissue of the chickens' conjunctiva and are generally referred to as CALT of the lower eyelid. Progression from randomly clustered lymphocytes in one week old chicks, to fully functional lymphoid-like structures with germinal centers (GC), to plasmatic cells observed in birds over four weeks of age have been noted [186 191]. This suggested that CALT, due to the presence of immunocompetent cells, may function as a structure that neutralizes pathogen factors at their entrance gate, and that the importance of CALT to immune function of the respiratory mucosal system in birds is age related [186 192].

Nasal Associated Lymphoid Tissue (NALT)

Mucosal tissue of the nose is first to come into contact with bioaerosols or microorganisms during inhalation. Large particles and microorganisms can be removed mechanically by mucus, but invasive pathogens require suppression by the immune system. A major characteristic of organized chicken NALT is the formation of circumscribed B-cell areas occasionally displaying GC, covered by a CD4+ T-cell cap [193 91]. In both organized NALT and epithelium, the dominant isotype is IGY, whereas IgM^+ cells are less frequent and IgA^+ cells are relatively rare [91]. IgY-containing cells are more frequently observed than the

other isotypes, so in contrast to the importance of IgA in the mammalian mucosal immune system, IgY may be more important at avian mucosal surfaces, at least in the nasal cavity. These lymphocytes are present in the nasal mucosa both intraepithelially and under the respiratory epithelium, as well as in the paranasal gland and in their secretory ducts. $CD8^+$ T cells were observed in the epithelium and lamina propria of the nasal cavity mucosa [190].

Trachea

The oro-nasal cavity and associated lymphoid structures are separated from trachea by the larynx, which opens into trachea through a slit-like glottis. Laryngeal muscles contract with breathing, to open the glottis during inspiration and decrease resistance to the inspiratory air flow. This rhythmic opening of the glottis is useful when attempting to intubate birds for intratracheal challenge. The trachea extends from the larynx, made of cartilaginous rings and smooth muscles, hence is easy to compress, at least in chickens [173].

There is no constitutive lymphoid tissue present in trachea. However, studies investigating respiratory system infections have shown reactivity of the tracheal mucosa to infections, despite a lack of essential lymphoid tissue [194]. The tracheal mucosa is highly responsive to infection and reacts with extensive lymphocyte infiltration followed by proliferation. Tracheal lesions characteristic for *Mycoplasma* infections predominantly consist of proliferating B cells similar to IBV infection, the cells were found to be mainly located in the lamina propria and submucosa.

Immune responses within trachea are age-dependent, as B cells were not detected in trachea of *Mycoplasma gallisepticum* infected SPF layer-type chickens until three weeks post–infection [91]. Further, it has been shown that the tracheal mucosa was mainly infiltrated by B cells in birds, at varying times post–infection regardless of vaccination status. Vaccinated birds reacted to *Mycoplasma* infections by producing secondary lymphoid nodules as B-cell clusters covered by a coat of CD4+cells. Nodules first appeared on day four, were fully formed by 12 dpi and were accompanied by a mild lymphocytic

infiltration without heterophils. Unvaccinated birds reacted to infection through thickening of the tracheal mucosa, caused by infiltration of a large number of lymphocytes, histiocytes, plasmatic cells and a considerable number of heterophils in the lamina propria. They were accompanied by deciliation as well as degeneration of epithelial cells of the mucosa [91].

The relationship between development of these immune structures and age is an important detail that requires further consideration for understanding immune function related to the respiratory system. As mentioned above, NALT and CALT, which are at the forefront of defense, are not fully functional GCs and plasmatic cells at four weeks of age [186 193]. Further, B-cell responses were not detected in trachea until three weeks post-infection in layer type chickens, which is typically more than half the lifespan of the average broiler. However, these structures may play an important role in immunity of elite flocks, including breeders, which have a lifespan in excess of one year. Further, having assessed the high risk of contamination of chicks through the aerosol route in hatcheries, these structures appear to have a very minute role in preventing infections acquired through inhalation [91].

Airway (Bronchial) System - Primary Bronchus

The trachea branches into two extrapulmonary primary bronchi, each of which connects to a lung and its associated air sacs. The primary bronchus enters the lungs at the border of the anterior and medial third of the ventral lung surface and extends to the caudal margin, where it opens into the abdominal air sac. The intrapulmonary primary bronchus runs next to the pulmonary artery and pulmonary vein and through the lung with a rather curved trajectory, changing greatly in cross-sectional area as it passes through the lung proximo-distally, and secondary bronchi branch from various parts of the circumferential surface of the intrapulmonary primary bronchus. Lined with pseudostratified columnar epithelium with mucous secreting goblet cells and supported by cartilaginous plates, the intrapulmonary primary bronchus terminates at the caudal margin of the lung where it enters the abdominal air sac [180 173]

Secondary Bronchi

The secondary bronchi can be designated into two functional groups based on their origin from the primary bronchus, or according to the part of the lung they supply with air. Four sets of secondary bronchi occur in avian lungs. The cranial group consists of 4 or 5 medioventral secondary bronchi that arise from the dorsomedial wall of the cranial third of the intrapulmonary primary bronchus. These cranial secondary bronchi branch further to form a fan covering the medioventral surface of the lung. The rest of the secondary bronchi on the caudal side consist of 6 to 10 mediodorsal secondary bronchi, lateroventral secondary bronchi, and laterodorsal secondary bronchi arise from the caudal two-thirds of the intrapulmonary primary bronchus [180].

Both primary and secondary bronchi are called the conducting airways because they do not participate in gas exchange. Cartilaginous semi-rings and smooth muscle support primary bronchi, however walls of the secondary bronchi are flaccid and need to be supported by the surrounding lung. Multiple lines of defense in the bronchial airway include airway epithelial cells, which are ciliated through the proximal end of the secondary bronchi with a variable number of mucus producing goblet cells, and hence illustrating its role in mucociliary clearance [180].

Aerosols in the Upper Respiratory Tract

In conventional poultry production, birds are exposed to high loads of aerosolized particles, and considering the high probability of pathogens in these bioaerosols, the bird's upper respiratory tract would be highly vulnerable to infection. The respiratory immune system has developed strategies to remove inhaled particles and to adequately respond to those microorganisms that cross the epithelial barrier in order to maintain integrity and function. The respiratory mucosal system is covered by epithelial cells, which make a barrier to separate the rapidly changing exterior environment from the highly stable internal environment of the body. Inhaled bioaerosol particles are eliminated from the respiratory system by multiple independent mechanisms including aerodynamic filtration, mucociliary clearance, and

phagocytosis [185] [189].

Mucus is a nonhomogeneous viscoelastic fluid containing glycoproteins, proteins, and lipids in a watery matrix. Airway mucus is the secretory product of mucous cells, the goblet cells of the pseudostratified columnar surface layer of the airway epithelium and the mucous cells of submucosal glands. Mucous secretions, along with serous fluid, form the airway surface fluid (ASF), which provides a protective milieu for the airways [195]. Secretory mucins are stored in secretory granules and released at the apical surface in response to mucin secretagogues, while membrane-tethered mucins are integrated into the cell membrane [196]. ASF consists of two phases: a superficial gel or mucous layer and a liquid or periciliary fluid layer that bathes the epithelial cilia; these two layers are probably separated by a thin layer of surfactant. In normal healthy states, the mucous layer in the trachea is about 2 to 5 μm thick and extends from bronchial sections to the upper airway [195] [63].

The composition and physical characteristics of ASF allow for normal ciliary activity and airway hygiene. Mucus provides a protective barrier against pathogens and toxins and contributes to the innate defensive system in mucosal immunology. Mucin glycoproteins are the major macromolecular constituents of epithelial mucus and have long been implicated in health and disease [195] [196]. Mucus plays a crucial role in protecting the conducting airways from inhaled bacterial, fungal, and viral pathogens, as well as from noxious particles and gases that we inhale. Some of this protection is physical, as mucus forms a three dimensional, cross-linked network that inhibits the migration of pathogens. Some of this protection is dilutional, especially if inhalation stimulates secretory mechanisms, and further protection is provided by chemical or immunologic components with a vast array of antigenically active oligosaccharides that serve as recognition sites for surface receptors on bacteria and viruses. Indeed, mucus has likely evolved as a universal soluble receptor to manage a variety of invaders, and enhancement of soluble receptor capacity is a possible strategy to combat viral infection [197] [195] [196].

Mucus is transported from the lower respiratory tract into the pharynx by mucociliary clearance and airflow. Ciliary beating moves a layer of mucus on the tracheo-bronchial mucosa toward the larynx,

thereby removing inhaled particles and microorganisms from airways. This physio-mechanical particle clearance mechanism, termed mucociliary clearance, has been extensively studied [195 112 198]. Inhaled foreign particles are initially removed by nasal mucociliary action through the mucociliary escalator mechanisms of the trachea, primary bronchi, and secondary bronchi in a concerted effort to clear particulate matter. Filtration of inspired air begins at the nasal cavity, which is well designed to heat, humidify, and filter inspired air. The nasal cavity and trachea are lined mostly with ciliated columnar epithelial cells and intermittent mucus secreting goblet cells. Cilia appear to move the overlying mucous layer in an oral direction at a speed of about 10 mm/ minute [197 199]. Humidified particles become trapped in these expanded, mucous-covered epithelial surfaces possessing cilia, and are quickly carried in an oral direction along with the mucous sheet. Here the inspired particulate material maybe moved to the pharynx where it may be swallowed and enter the GI tract. [197 195 196]

Mensah and Brain (1982) exposed chickens for 30 to 40 minutes to bioaerosol particles with a median aerodynamic diameter of 0.45 μm containing 99mTc (technetium) sulfur colloid and reported that although not much radioactivity was found in the trachea immediately after exposure, most was removed within 12 h. Likewise, a large fraction of the radioactivity had been removed from the lungs within 1 h after exposure and a large accumulation of radioactivity had occurred in the GI tract. This study indicated a rapid-phase clearance of insoluble technetium from the trachea and lungs to the feces. There was essentially no radioactivity in the heart, kidneys, ovaries, or liver, indicating the technetium had not entered the blood [173].

Mucociliary escalators in the primary and secondary bronchi act in a similar fashion. Primary bronchi and roots of the secondary bronchi are lined mostly with ciliated columnar epithelial cells, which are longitudinally folded and ciliated. However, much of the remaining epithelium of the secondary bronchi is nonciliated cuboidal or squamous in structure, hence, mucociliary movement is restricted only up to the proximal end of the secondary bronchus [173 116]. Thus, clearance mechanisms in the upper respiratory tract through the proximal end of the secondary bronchi primarily rely on particles becoming trapped in mucus, its oral mucociliary transport, and expectoration or swallowing of trapped material. However, a

detailed study by Hayter and Besch showed particle size critically affects the site of deposition within the respiratory tract [200]. This portion of the respiratory system forms the first line of defense against large inspired particles up to 4 - 7 µm, but does not entrap many smaller particles. Thus, it appears that trapped particles can be rapidly removed from the upper respiratory tract however, the relationship between size and absence of ciliated epithelium in latter parts of the secondary bronchi, raises uncertainty about associated effectiveness of this site in preventing microorganisms in the air stream from entering lower parts of the respiratory system [200].

Dose Estimation for Respiratory Route

Considerable evidence regarding generation and survivability of *Salmonella* within bioaerosols raises the question of exposure to infectious doses via respiratory inoculation. It is generally accepted that for paratyphoid *Salmonella,* the infectious dose via oral route is around 10^5 to 10^6 organisms [16]. Hence, while considering the relevance of levels of *Salmonella* in air, it is important to include recognition of differences in infectivity of various serovars. In turn, invasiveness and composition of the pathogen, even at the serovar level, has to be considered.

Factors determining, or at least predictive of, the dose for respiratory inoculation include volume of air respired by a bird over a period of time, size of the aerosol containing *Salmonella*, and amount of bioaerosols inhaled and deposited. It has long been estimated and considered standard that approximately 1 m^3 of air is inspired by a chicken per day under commercial poultry production conditions (Green et al., Animal Anesthesia, 1979). Many studies reporting air particle analysis, to determine the relationship between particles in the air have a standard of estimation procedure of determining this count per m^3 of air [14 132 201]. This direct correlation between total respirable particles and size to that of a dose of *Salmonella* can be drawn using such air particle analysis. Exposure assessment shows that the concentration of airborne microorganisms in livestock stable air can reach values up to 10^{10} cells/ m^3 of air [202]. However, the concentration of pathogenic bacteria within this may vary. Of further consideration is the fact that only particulate matter having a size of 3 µm can reach below the primary bronchi and may be

involved in infection. Aerosols of about this size should be able to carry *Salmonella* considering its typical diameters of around 0.7 to 1.5 μm and lengths from 2 to 4 μm.

A study by Harbaugh et al. (2006) hypothesized the possibility of *Salmonella* contamination in turkeys and in general poultry holding areas prior to slaughter on the prevalence of airborne *Salmonella* and suggested that these waiting areas outside of processing facilities can, in fact, be a source of contamination. The concentration of *S.* Typhimurium in the air during this experiment was not determined, however, air particle analysis was performed to determine the relationship between size and number of particles in the air to the number of infected birds. Based on the air particle data collected, an approximate number of total respirable particles that could contain *Salmonella* were calculated. At the time fans were turned on, there were approximately 8×10^4 particles in the air and of those particles of dust, 7.8×10^4 were 1 μm or less in size, and therefore, were too small to carry *Salmonella*. Thus, approximately 2×10^3 of the remaining particles could potentially carry *Salmonella* and hence were estimated to be the dose that may have been involved in airborne infection, if 100% of appropriate-sized particles carried the pathogen [203].

In a study by Fallschissel et al. (2010) in Germany between January and September 2007, samples were taken at different working places in a conventional farmed duck fattening facility and two broiler chicken houses, each of which accommodated flocks of 26, 000 animals. Birds were allowed to move freely on sawdust litter and samples were collected at different locations at different operation times - (i) in the duck stables: (a) during egg collecting and (b) during general stable work consisting of straw dispersing and cleaning; (ii) in the slaughterhouse: during shackling and hanging; (iii) in the duck hatchery: during packaging of ducklings; (iv) outdoors in an upwind direction; and (v) in the office at desk work. Depending on the examined working area, average concentrations ranged between 2.5×10^1 and 3×10^6 of bacteria per m^3 of air, of which *Salmonella* counted concentrations were $3.3 \times 10^2 – 1.2 \times 10^4$ CFU/ m^3 of air [152], [151].

Another study by Lever et al. (1996), undertaken to establish the number of *S.* Enteritidis within air of

rooms housing chicks orally infected with *S.* Enteritidis related these numbers to airborne infection of uninfected control birds. In brief, two groups of birds were randomly allocated to two rooms, with each room having a group of 20 and a group of 10 birds that were housed in separate pens. Two separate rooms were used to house the chickens which acted as replicates. Temperatures and relative humidity in room A ranged between 21-24°C and 35-45%, respectively, and in room B between 24-28°C and 41-45%, respectively. In both rooms, chicks of the orally infected control group were inoculated by direct gavage into the crop with approximately 1 x 10^7 CFU of *S.* Enteritidis PT4. Cloacal swabs were performed daily on five of the infected group and all control birds. All orally-infected chicks showed positive cloacal swabs 1 d post-infection. Within the first 3 d following oral infection, the number of uninfected chicks, in a separated pen, with positive cloacal swabs increased. This increase was directly correlated to the increasing quantities of airborne *S.* Enteritidis. Particle sampling counts and *Salmonella* counts were performed at the minimum sizes of 0.5, 1.0, 2.0, 5.0 and 10 µm. Over a period of 14 d the mean particle size profile of the two rooms were approximately 2 µm and in both rooms the numbers of small particles were higher than in an equivalent sized empty room. This study showed that in well-ventilated rooms containing 30 chicks housed in similar conditions to a poultry house, *S.* Enteritidis can be found in the air and can be related to airborne infection of uninfected control birds. Increasing numbers of airborne *Salmonella* correlated with increasing colonization of control chicks in both rooms [132 168]. Interestingly, in every m^3 of air, the researchers detected approximately 10 CFU of *Salmonella*, suggesting that each bird would, on average, inhale 10 CFU every 24 h, which may suggest the unlikelihood of infection. However, given that exposure would be continuous and that no other route of transmission was possible through the experimental setup, it seems likely that these quantities of airborne *Salmonella* were sufficient to cause infection of control birds. The conditions in which chicks were housed produced high numbers of small particles, creating a potential vector for transmission to chicks by the respiratory route.

Further, sentinel mice housed in both rooms had no *Salmonella* in their liver, spleen or blood after 14 d, which made sure that airborne transmission of *Salmonella*, was the only mechanism of cross contamination. This evidence further suggests that mice were not susceptible to infection by these numbers of airborne *Salmonella*. The minimum infectious dose of *S.* Enteritidis PT4 by aerosol for mice is

not known, however, as few as 150 CFU of *S.* Typhimurium are required to produce disease in mice when given as an aerosol [16]. Given the low levels of airborne *Salmonella* in this experiment, chicks were clearly more susceptible to *Salmonella* infection by the aerosol route than mice. This report is well supported by studies showing high susceptibility of chickens to infection by *Salmonella* Enteritidis when administered as a small particle aerosol [132,204].

Further, the impact of poultry house pollutants on such particulate clearance from the respiratory system of birds remains largely unknown. Any substance that reduces ciliary motility or disrupts the ciliated epithelium could be expected to adversely affect the resistance of birds to microorganisms that normally enter their bodies via the respiratory system. Interestingly, in an earlier study involving 8 separate trials Weinack et al. (1984) considered this assumption and evaluated any effects of respiratory tract impairment on the gut microflora and gut pathogens, with astounding conclusions. Chickens that were exposed through aerosolized *Mycoplasma gallisepticum* (MG) and / or Infectious Bronchitis Virus (IBV) began shedding higher doses of *S.* Typhimurium and *E. coli*. This observation was linked to a decrease in intestinal microflora due to respiratory stress and they suggested the possibility of infection via aerosol route for these organisms could not be ruled out [205] [116].

As a result of the chemical decomposition of uric acid in droppings by certain bacteria in the litter, poultry houses are susceptible to high levels of ammonia in the environment and are recorded to be particularly high in houses where the same litter is used for successive flocks [206]. Hence, the main factors involving generation of high amounts of atmospheric ammonia concentration in poultry houses are litter conditions, air movement, moisture content, pH and temperature of the litter, which in concert influence degradation of uric acid [206]. Poor ventilation, loose droppings and faulty, over-filled or low positioned drinkers are common causes of wet litter, which have additional effects on ammonia gas production in poultry houses [207] [208] [170].

Ammonia gas, with a characteristic pungent odor, is irritating to mucous membranes of the respiratory tract and the conjunctivae and corneas at high concentrations. The effect of ammonia gas on mucosal

surfaces of the trachea ranges from paralysis of cilia, to deciliation of epithelial cells, to necrosis of the mucosal epithelium itself [209] [199] [210]. Considering the importance of cilia and mucous membrane in pathogen defense, as explained earlier, these effects of ammonia may seriously attenuate mucociliary clearance. Attenuation of the mucosal epithelium, with loss of cilia and increased numbers of goblet cells, are common lesions of aerial ammonia toxicity seen in tracheas of affected birds. Damage to mucous membranes of the respiratory system increases the susceptibility of birds to bacterial respiratory infection, especially *E. coli* infection [209]. Devitalisation of tracheal mucosa may explain, in part, the high incidence of airsacculitis, pneumonia and septicemia caused by *E. coli* and now *Salmonella* in poultry flocks that have been exposed to high atmospheric ammonia levels. High levels also have a negative impact on overall livability, weight gain, feed conversion, condemnation rate at processing and the immune system of the birds [207208170].

However, the type and extent of damage depends on the concentration of ammonia in the air and the duration of exposure. The National Institute of Occupational Safety and Health (NIOSH) has set the maximum levels of ammonia in poultry houses to be ~25 ppm and 50 ppm by the Occupational Safety and Health Administration (OSHA), for the safety of human workers in the production houses, with a maximum limit of 8 h of exposure [208] [152]. OSHA's studies set the level of 50 ppm based on the lowest level of ammonia to cause irritation to eyes, nose and throat of sensitive individuals working in these poultry farms [51]. However the effects of such quantities of ammonia have not been studied recently. Experimentally, broiler chickens kept in an environment with ammonia concentrations of 50 ppm and 75 ppm, which is the set value by above mentioned bodies, were shown to have reductions in body weight of 17% and 20%, respectively, at 7 weeks of age compared to broiler chickens kept in an environment with near zero ammonia concentration. In a review on the effects of ammonia on the health of poultry growers, Whyte et al. reported an increase in susceptibility of the respiratory system to airborne pathogens when combined with ammonia concentrations below the occupational exposure limit of 25 ppm [207]. In a more recent dose-response study of poultry workers by Donham et al. results suggest that respiratory function may be impaired above concentrations of 12 ppm [208]. Ammonia, in combination with other respiratory hazards, such as dust and bio-aerosols may contribute to numerous health concerns [170].

Considering, these intrinsic characteristics of poultry production, many studies to date performing air particle analysis to determine the relationship between the particles in air, both size and number and dose of *Salmonella* can be considered an underestimation. There may not be a direct correlation of total respirable particles and their size to that of dose of *Salmonella*, and larger particles assumed to be cleared by the mucociliary clearance, may also contribute to the dose involved through the respiratory route. Thus, particles that are not cleared by mucociliary mechanisms must face the cellular and immunological mechanisms of the bronchial airway.

Immune System of the Bronchial Airway

Entrances to the secondary bronchi, with the exception of the medioventral bronchial openings, show a modified mucosa with a non-ciliated flat zone, the structure of which is deduced by scanning electron microscopic studies [211] [212] [186]. This zone was found to be the primary location of the strategically placed mucosal and bronchial associated lymphoid tissue (BALT) in avian lungs and has been exclusively studied in chickens and turkeys by Fagerland et al. [213] [214].

Most avian species, including chickens, lack draining lymph nodes (dLN), but have a system of BALT structures referred to as lymphoid nodules [91]. In general, BALT structures of chickens and turkeys are located at the junctions of primary and secondary bronchi, as well as at the ostia to the air sacs and are found regularly in birds raised under conventional conditions [116]. In chickens they are confined to the openings of most caudal secondary bronchi, and also mediodorsal, lateroventral and laterodorsal secondary bronchi, while a wider distribution pattern including regions of longitudinal foldings of the mucosa has been observed in turkeys [193] [214]. Inspired air containing foreign materials bypasses the medioventral secondary bronchi opening, facilitating deposition of heavier foreign materials in the epithelium. It is not until after passing filtering mechanism of the lungs and air sacs that air passes through the medioventral openings which do not have BALT. Also, BALT is constitutive structures in lungs of specific pathogen free (SPF) and conventionally reared birds. This is in contrast to the situation of healthy humans and similar to rabbits and rats, where they are referred to as induced BALT (iBALT) [84].

Development of BALT depends on age and on environmental stimulation. In day old chickens and turkeys, no or very few infiltrating lymphocytes are seen in the primary bronchi region. In chickens, within the first week after hatch CD45+ leucocytes start to migrate into the primary bronchus and small lymphocytic infiltrates are found consistently at openings of the secondary bronchi [215 216]. Lymphoid nodules develop at these locations within the next 3–4 weeks and IgM, IgG and IgA producing cells can be detected, and CD4+ and CD8+ T cells are found at the same time localized in distinctive patterns. In the absence of GC, CD4+ cells form single clusters while CD8+ lymphocytes are distributed throughout the nodules [193 189 91]. Germinal centers can first be detected in 2 to 4-week-old birds. In addition, IgM and IgG bearing cells with long cytoplasmic processes resembling follicular dendritic cells are present in GC. In these BALT structures CD4+ T cells form a large parafollicular caps around the GC while CD8+ cells are scattered throughout the tissue. The number of IgG, IgA or IgM-producing cells continues to increase during the following weeks and BALT nodules can be found surrounding the entire opening to the secondary bronchi in 6 - 8 week old chickens [189].

Whether an antigenic stimulus is essential for the induction of BALT formation in chickens is unknown. Using a *Bordetella avium* infection model in turkeys, Van Alstine et al. showed that the number of BALT nodules significantly increased in infected birds. Studies demonstrating lymphocytic infiltrates in the trachea and the HG of germ free chickens indicate that BALT structures might also be induced without microbial stimulation [194]. However, the presence of inflammatory substances such as endotoxins in inhaled air cannot be excluded and may play a role in BALT formation. It is therefore conceivable that antigenic stimulation is required for the induction of BALT formation and for development into fully mature structures in the first few weeks after hatch [194].

Mature BALT structures consist of lymphocyte aggregates which are covered by a distinct layer of epithelial cells called the lymphoepithelium or FAE, harboring considerable numbers of lymphocytes. Light and transmission electron microscopy revealed a similar cellular composition of the lymphoid nodules in chickens and turkeys. In 6 to 8 week-old birds almost every bronchial opening was surrounded by raised ring of lymphoid tissue and germinal centers are found in most nodules and occasionally

plasma cells are seen under the FAE [186 91]. Macrophages, cells with dendritic cell morphology, and heterophils are distributed in considerable numbers throughout BALT nodules. Immunohistological studies on the composition of BALT structures, carried out with the availability of an extended set of leukocyte specific monoclonal antibodies in the chicken system, has confirmed the presence of large numbers of B cells, with IgM^+ cells in excess of IgG^+ cells, and these again are in excess of IgA+ lymphocytes [215]. In cases where GC is not present in BALT, B cells are confined to the edge of lymphoid tissue, whereas lymphoid nodules are composed of aggregates of T cells in the center surrounded by B cells [186].

The role of Microfold cells (M cells) in *Salmonella* infection has been extensively studied and discussed above. In the alimentary tract, ileal M cells function as a portal for the entrance of *Salmonella* Typhimurium and *Salmonella* Typhi. Alternatively, *Salmonella* Pullorum preferentially invaded an organized lymphoid tissue, the bursa of Fabricius, instead of targeting intestinal epithelium, but *Salmonella* Gallinarum did not show any preference for the bursa of Fabricius over jejunum and caecal tonsils [212]. For chickens, the phenotype and function of M cells on gut-associated lymphoid tissue (GALT) has been observed to be less distinguishable from epithelial cells than is seen in mammals [212]. Further, epithelial cells in chicken BALT have been shown to lack M cells. However, recent research has described four epithelial cell types in the FAE, depending on age, including cells which have irregular microvilli on their luminal surface and close contact to lymphocytes and therefore suggested to display some features of M cells. However, particle uptake by these cells has not been observed and characteristic organelles such as apical vesicles are absent. It has been suggested that these cells may not be involved in antigen uptake and processing, but participate in tissue repair [189]. In a study, that successfully reproduced fowl typhoid via intratracheal *Salmonella* Gallinarum challenge, Bahadur et al., suggested that *Salmonella* Gallinarum is likely to penetrate both M cells and epithelial cells on GALT and epithelial cells on BALT to infect lymphoid cells.

The FAE is made up of ciliated and non-ciliated cells, the relative numbers of which differ significantly at different stages of BALT development. The various stages of development of these epithelial cells are

best described in turkeys by Fagerland et al. [214] who extensively studied development of BALT structures in turkeys, over a period of 18 weeks, which essentially is the duration of one production cycle. They reported that overall, BALT was similar to the structure described for chickens and was made up of a population of lymphocytes covered by a specialized epithelium different from typical pseudostratified ciliated columnar bronchial epithelium. They clearly observed a distinct change in epithelial structure with respect to age. The primary cell type of BALT epithelium between day of hatch and 2 weeks was non ciliated cuboidal in shape, while in 2 week old turkeys it was squamous and in turkeys older than 4 weeks the epithelium was primarily ciliated columnar. Further, between 1 - 4 weeks of development they drew large numbers of intraepithelial lymphocytes disrupting normal organization of the epithelium. Cell processes of these lymphocytes and macrophages, extended between basal epithelial cells, displaced them from their normal and apical cytoplasm of the epithelium was attenuated, due to which the epithelial barrier appeared to be disrupted over lymphoid nodules [191 213 217 214].

The large numbers of heterophils in BALT of both chickens and turkeys may be functionally significant. Phagocytes, particularly heterophils, are an important element of avian respiratory defense systems as birds lack a large pulmonary macrophage population. Studies have suggested that macrophages and granulocytes are capable of transporting materials captured in the bronchial lumen to sites where specific immune responses may occur [193]. Hence, to aid this process, at least 2 means of handling foreign particles in avian BALT are recognized, the first of which is passage between disrupted epithelial cells directly to phagocytic cells. On the other hand, FAE cells are believed to mediate antigen contacts with lymphocytes through endocytosis [213 214]. Endocytosis has been demonstrated taking place in epithelial cells within BALT with the use of live and ultraviolet-killed *Bordetella avium* and ferritin. Ferritin and *B. avium* were taken up by both ciliated and non-ciliated cells of the epithelium overlying BALT. Ferritin was found in organelles associated with endocytosis and was apparently transported across epithelial cells, since it was also found in intercellular spaces. Bacteria, in this study, *B. avium*, were found in vacuoles within BALT epithelial cells, but not free in intercellular spaces [186].

The appearance of disrupted epithelial barrier over lymphoid nodules, supported by described endocytosis taking place in epithelial cells, might suggest that antigens would be readily available to

lymphocytes and phagocytes in BALT. These demonstrations of highly developed and constitutively present BALT structures in chickens and turkeys have encouraged some investigators to suggest that these mucosal associated lymphoid structures may functionally compensate for the lack of lymph nodes in birds [112 84 116 193]. However, it remains to be shown if these structures specifically respond to antigenic stimulation by the generation of memory B and T lymphocytes. The relationship between development of these immune structures and age is an important detail that needs attention. As discussed initially, NALT and CALT, which are at the forefront of defense, are fully functional with GC and plasmatic cells, only at the age of 4 weeks. It is now evident that BALT also requires a fair amount of time to develop its maximum potential, which is about 6 weeks, in both chickens and turkeys, which approaches the age of the broilers at the end of a typical production cycle [186]. Having assessed the high risk of contamination of birds, through the aerosol route as early as in the hatcheries, these structures seem to have a very minute role to play in preventing these infections. Overall, entrapped particles may not necessarily be eliminated through the bronchial airway mucociliary escalator system, and the immune structure might either be underdeveloped or even indirectly encouraging *Salmonella* to infect the host successfully, which needs further evaluation. Particulate matter and pathogens that could not be efficiently cleared by the upper respiratory tract and bronchial airway may enter the parabronchial region or ventilating air sacs.

Parabronchial Region and Avian Air Sacs

Avian Lung

Avian lungs have specific structural attributes that relate strongly to function. The lungs lie in the dorsal part of the coelomic cavity where they are firmly affixed to the vertebral ribs. As gas exchangers, they are situated separate from the mechanical ventilators, air sacs. Approximately one-quarter of the lung volume is contained between the ribs. Overall, the lungs are therefore compact, fixed, and practically rigid. They are homogeneous at the gas exchange level, where air capillaries (ACs) and blood capillaries (BCs) form approximately 90% of exchange tissue [180 91 189]. The ACs is generally strong due to meshwork created between them and the BCs, and mechanical compression of the lungs does not cause them to collapse. The lungs are ventilated unidirectionally and continuously in a caudocranial direction by synchronized action of air sacs and parabronchial structures.

It is remarkable that the two great classes of vertebrates capable of sustained high oxygen consumption, mammals and birds, have significantly different lung architecture. In brief, the avian respiratory system has separate structures for ventilation and gas exchange comprised of air sacs that act as bellows and rigid lungs attached to the ventral aspect of the vertebral column. The microstructure of respiratory anatomy in the lung parenchyma is likewise organized differently in birds when compared to that of mammals. The arrangement of the bronchial airway system of mammalian lungs displays repeating dichotomous bifurcations which terminate in to blind-ended air conduits, described as bronchioalveolar lungs [189]. While avian lungs have a highly intricate anastomotic joining of branches of tubular structures so as to make or become continuous a system called the parabronchial systems. Hence, the functional unit of avian lungs is the parabronchus, in which terminal air spaces called ACs are heavily intertwined with BCs for oxygen exchange [180].

Parabronchi (Tertiary Bronchi)

Most of the parabronchi are organized as a parallel series of several hundred tubes connecting caudal groups of secondary bronchi to the single cranial secondary bronchi. Four groups of secondary bronchi - medioventral, lateroventral, mediodorsal and laterodorsal - originate from the primary bronchus. The parabronchi connect 3 caudal secondary bronchi - lateroventral, mediodorsal and laterodorsal - in a hoop-like pattern to the cranial medioventral secondary bronchi, forming a contiguous loop of air canals. Such parabronchi are referred to as paleopulmonic parabronchi and together, with the secondary bronchi, they compose the simplest scheme of bronchial branching in avian lungs. In most avian species, additional parabronchi have been observed, called neopulmonic parabronchi, which are known to connect another set of laterodorsal secondary bronchi to caudal air sacs [218] [177]. These are not organized as regular parallel stacks and exhibit irregular branching patterns. In general, these gas exchanging tubes are several millimeters long and have a diameter ranging from 0.5 to 2.0mm, depending on the size and age of the bird [116]. The parabronchi are separated from each other along their length by a boundary of connective tissue and larger pulmonary blood vessels. The parabronchial lumen is lined by a meshwork of smooth muscle, which outlines the entrances to atria radiating from the parabronchial lumen [180] [116].

Atria, Infundubulae, and Air capillaries

Gas exchange takes place in the parabronchial tissue mantle, which is the fundamental functional part of avian lungs. The parabronchi and sizeable parts of the secondary bronchi comprise a centrally located parabronchial lumen surrounded by an exchange tissue mantle. The thickness of exchange tissue mantle ranges from 200 to 500 μm in different species [180] [116]. From the parabronchial lumen, the exchange tissue mantle is penetrated by the atria, which are bordered by interatrial septa that consist of thick bundles of smooth muscle and connective tissue. Depending on the species, atria measure about 60–100 μm X 120–130 μm in diameter, while small energetic species, like Galliforms, have shallow atrial projections [177] [219]. Four to eight infundibulae originate from the floor of each atrium and have a diameter of 25–40 μm, and extend into the exchange tissue mantle to a depth of approximately 100–150 μm [219] [180]. In turn, the

infundibulae give rise to ACs, which range in diameter from 3 to 20 μm [220] [219]. These ACs are much smaller in diameter than alveoli of mammals, and are estimated to be a fraction of the size of the mammalian alveoli [174]. This results in an increase in the surface area over which gas exchange occurs and aids to increase the diffusing capacity of lungs. It is important to note at this point that any particles or pathogens in aerosols surpassing secondary bronchi or the mucociliary escalator can potentially end up within the parabronchial region. Further, the parabronchi are lined by unciliated cuboidal and squamous epithelium and, therefore, do not possess a means of moving inhaled foreign particles in the cranial/oral direction [174].

The ACs anastomose profusely and intertwine closely with BCs. Functionally, BCs in avian lungs constitute a multicapillary serial arterialization system and the general arrangement of avian pulmonary circulation is similar to that of mammals, with respect to the pulmonary arteries, capillaries, and pulmonary veins [181]. It has been proposed that the support of BCs by the surrounding ACs contributes to the strength of capillary walls [174] [176].

Interparabronchial blood vessels are located between exchange tissue mantles of adjacent parabronchi. In avian lungs, the terms ‘air capillary’ and ‘blood capillary’ were derived from observation that the exchange tissue mantle consisted of a network of small air and vascular units. Woodward & Maina (2005), showed ACs to be small, rather rotund respiratory units that connect through short, narrow passageways, while BCs comprise short, tubular segments of relatively regular dimensions that are coupled and spread out in three-dimensional space. Here, BCs and ACs are intricately intertwined and form a meshwork of parenchyma tissue separated by numerous atria in the parabronchi. Geometrically, the bulk air flow in the parabronchial lumen, in a longitudinal direction, and the flow of deoxygenated blood from the periphery, in a centripetal direction, are perpendicularly arranged to produce a cross-current relationship. The amount of oxygen and carbon dioxide exchanged arises from many modest transactions that occur where air and BCs interface, called the blood-gas barrier, along the parabronchial lengths, an additive process that greatly enhances respiratory efficiency [177].

Blood-Gas Barrier

The blood-gas barrier (BGB) comprises of endothelial cells that border BCs, an intermediate extracellular matrix space called the interstitium, and epithelial cells that front air spaces. Endothelial cells contain numerous micropinocytotic vesicles while epithelial cells are remarkably thin and have few organelles [221]. On average, the tissue thickness of avian BGB is known to be thinner than that of mammals, and various data show that this holds true even for weak flyers such as domestic chickens [221 222 223].

Watson et al. morphometrically analyzed the respiratory BGB in chickens [178] and as an extension to previous studies by Maina and King [222], documented the following conclusions. Endothelial cells, extracellular matrix, and epithelial cells comprise approximately 67%, 21%, and 12%, respectively, of the volume of the blood-gas barrier of avian lungs. Thicknesses of endothelium, interstitium, and epithelium layers in the chicken lung averaged 0.135 - 0.06, 0.045 - 0.02, and 0.086 - 0.05 μm, respectively. All individual layers of tissue were significantly thinner in chickens compared with that of horses, dogs, and rabbits. The average total thickness of the BGB as a whole in chicken lungs was found to be 0.266 to 0.09 μm, which was, as expected, significantly thinner than the total thickness of BGB of rabbits, dogs, and horses (0.593 - 0.40, 0.796 - 0.86, and 0.921 - 1.00 μm, respectively), thus BGB in chicken lungs was 54% thinner than BGB in the rabbit, 66% thinner than that of the dog, and 70% thinner than that of the horse. West et al. (2010) also drew similar conclusions after analysis of data from 34 species of birds and 37 species of mammals, noting that the mean harmonic thickness of the barrier was 0.19 and 0.47 μm respectively, a 2.5 fold difference [176].

West and co-workers also noted that the interstitial layer was found to be strikingly thin in the chicken lung at about 86% thinner than dogs and horses, and 75% thinner than rabbits, which was hence a paradox because the strength of the BGB is believed to come from the interstitium. In addition, thickness of the interstitium was remarkably uniform, unlike the mammalian interstitium. Uniformity of the interstitial layer in the chicken is attributable to a lack of the supportive type I collagen cable that is found in mammalian alveolar lungs. Overall, interstitial layers possess an especially high proportion (~72%) of thin

measurements (~0.02 μm) in chicken lungs compared with mammalian lungs. Collectively, based on data collected on the BGB of various species, it can be concluded that chicken lungs have a higher proportion of thin measurements in all layers compared with that of mammals and the interstitium layer of chickens has a much higher percentage of very thin measurements in very few thickness categories [224] [223] [225] [226].

Structural Failures in the Chicken Lung

Functionally, this uniformly thin barrier translates into a higher proportion of surface area that is maximized for gas exchange compared with mammals. One of the most confounding properties of the functional design of the avian respiratory system concerns stability on these fine structures [176]. On one hand, they are considered to be remarkably stable and do not yield to stress. Based upon research on the microanatomy of avian lungs, it has been proposed that support of BCs by surrounding ACs contributes to the strength of capillary walls and thus allows a very thin and stable BGB [174] [176]. Further, morphologically, lungs are deeply anchored to ribs and vertebrae on the dorsal lateral aspect and are ventrally attached to the horizontal septum, a tough membranous connective band that peripherally firmly attaches onto vertebral ribs [227]. These results in as much as one-fifth to one-third of its volume being sandwiched between the ribs, and these attachments render the lungs practically rigid. During a respiratory cycle, avian lungs changes in volume by a mere 1.4% [228]. Its rigidity means that surface tension is not a severely limiting factor to the degree of internal subdivision of the gas-exchange tissue, parenchyma [183]. A third striking difference is that in birds, capillaries are essentially rigid when compared to mammals, which show a remarkable degree of recruitment and distention during breathing [176]. However, these properties appear to have conflicting roles because thinness is essential for efficient flux of oxygen by passive diffusion, and strength is crucial for maintaining structural integrity [179]. The extreme thinness of the barrier is paradoxical because flying, even with light fliers like galliforms, is very energetic, and thinness of the barrier predisposes it to stress failure. On account of the fact that how thin these BGBs are, the indisputable strengths of BGBs cannot be sufficiently explained by their structural features [183]

Most recently, research in this regard has been described by Maina et al., in which aspects involving failure of the structural integrity of BGBs in chicken lungs with respect to arterial blood pressures were compared [175]. Like mammals, birds are endothermic homeotherms in that they operate at relatively high body temperatures (40–42˚C). Oxygen is acquired by a structurally complex and functionally efficient respiratory system, the lung and air sac system. To support an energetic lifestyle, large hearts, as compared to relative body mass, with large cardiac outputs supply tissues with oxygen. Compared to mammals, arterial blood pressures in birds are much higher [229]. For example, compared to that of 95 mmHg (12.64 kPa) in a mammal such as cynomologus monkey, *Macaca fascularis,* with a body mass of 4.60 kg, turkeys, *Meleagris gallopavo*, with a similar body mass of 4.77 kg has a higher systolic blood pressure of 150 mmHg (19.95 kPa). Turkeys can have exceptionally high systolic blood pressures of as much as 400 mmHg (53.3kPa), and sudden deaths have been attributed to aortic rupture, a condition termed dissecting aneurism, or turkey heart attack. Fast growing, healthy turkeys, usually males between 7 to 24 weeks of age, die suddenly of bleeding after aortic rupture which is caused by high blood pressure. Birds like fast growing broiler chickens and turkeys frequently experience hypertensive problems that lead to high myocardial stress, resulting in complications such as aortic aneurysm and ascites. Hence, it has been hypothesized that with their relatively high systolic and arterial blood pressures, birds possess a BGB which is ~36 times thinner than that of mammals of equivalent body mass [175 174 230].

This hypothesis led to studies of structural failure of BGB and epithelial–epithelial cell connections (EECCs) in different vascular regions of exchange tissue in lungs of rested and exercised chickens [175]. Blood was sampled to determine blood lactate levels (BLLs) to ensure chickens reached their highest physiologically tolerable level and metabolically pushed close to their exercise endurance limit. The number of red blood cells (nRBCs) was counted as an indicator of BGB failure, and protein concentration (PC) was measured after lavaging the respiratory system. The numbers of complete BGB breaks (nBGBBs) and those of the EECCs (nEECCBs) were counted in different vascular territories of the lungs. For exercised chickens, the pre-exercise BLL values were comparable while post-exercise birds increased gradually with increasing workload. A small but consistent difference could have ensued from

incidental distress of the chickens prior to taking the first measurement. The same can explain the small differences between the resting groups of birds in different exercise regimens. Between rest and running speed of 0.66 m/sec, post-exercise BLL rose 2.5-fold, compared to 4-fold between those run at the fastest treadmill speed of 2.95 m/sec. This shows that under the exercise regimen, BLL approached or reached the highest physiologically tolerable level and that metabolically, the birds were pushed close to their exercise endurance limit. In both rested and exercised chickens, RBCs were observed in the lavaged fluid, which was indicative of BGB failure. The nRBCs increased as treadmill speed increased to a speed of 1.97 m/sec after which the level of RBCs stopped rising. Also, presence of RBCs on the respiratory surface of normal birds has been previously reported in chickens [231] [185].

Moderate structural failure of the BGB was found to be a common occurrence, even in lungs of resting unstressed birds. In all cases of experimental exercise, the nEECCBs exceeded nBGBBs. The greater nEECCBs compared to those of BGB, pointed to the fact that the former sites are structurally weaker than the latter [175]. The EECCs are areas of exchange within avian lung, where thin strands of epithelial cells lay back-to-back, separating ACs and connecting BCs. The increase in the number of breaks with increasing exercise can be attributed to an increase in pulmonary capillary blood pressure (PCBP) from faster heart rates and higher cardiac outputs [175].

Mechanical failure of the BGB, in turn due to its thinness, is an important but overlooked factor [226] [225]. Increases in the PCBP are probably the foremost direct challenge to mechanical integrity of the blood capillary wall, especially the BGB, and indirectly the EECCs. For that, avian lungs should be more susceptible to pulmonary mechanical injuries and hence pathogenic diseases. Given these vulnerable conditions, the avian respiratory system is expected to be equipped with a strong and robust immune system. Conversely, the avian lung immune system is exclusively investigated to be inadequate and incompetent. Here, we explore the reasons and consequences of paucity of such an immune system with respect to airborne infections of *Salmonella* in poultry.

At first consideration, lung parenchyma appears to be well equipped with resident phagocytes and

antigen presenting cells (APC) in the interstitial tissue and in parabronchial wall. Immunohistological staining for a pan-leukocyte marker revealed the presence of diffusely distributed leukocytes in the interstitial tissue of lungs [192]. The majority of these cells were identified as monocyte/macrophage lineage and dendritic cell type because of their expression of major histocompatibility complex (MHC) class II molecules [232]. Lymphocytes were also seen widely distributed in the tissue. While, IgM-expressing cells appear in 1-week-old chicks, IgA and IgY cells were found at later time points. B cells and T cells were frequently observed at later stages of development, most of which showed the classical cytotoxic T lymphocyte phenotype with expression of CD8 and α/β TCR molecules. $CD4^+$ cells and γ/δT cells were less frequent [233]. Surprisingly, even with these observations, it is believed that very little is known about the structure and functional relevance of the immune system in the avian lung parenchyma [116]. Most of the query regarding inadequacy and incompetence of functional aspects rises from comparison with mammalian counterparts, most of which appear reasonable.

Respiratory Cellular Defenses

Phagocytes, both resident and free, constitute the first line of defense against infectious agents in the respiratory system. Microscopic techniques have shown that macrophages are absent on the surface of ACs but present on the epithelial lining of the atria [177 234]. Interestingly, macrophages were also seen in connective tissue below this epithelium on the floor of the atria [235 236] and in the interatrial septa [189], indicating that phagocytic cells are strategically located at the start of the gas exchange area to clear the air of inhaled particles before it reached thin and vulnerable ACs [116]. Further, using aerosolized inert iron particles with a diameter of 0.18 μm, Stearns et al. (1987) showed that particles were phagocytosed in the gas exchange area and endocytosed by epithelial cells lining the atria and the proximal parts of the infundibula. Iron particles were also found inside tissue macrophages residing underneath the atrial epithelium [237].

However, reports of extremely low numbers of residing macrophages in the normal, steady-state respiratory tract of chickens are available [237], an observation confirmed subsequently by other

laboratories [91,187,211]. The average number was estimated to be between 2 x 10^5 to 3 x 10^5 macrophages, with a range of 1 x 10^4 to 2 x 10^6 when compared to greater than 10^9 cells in mammalian systems. To understand the extent of scarcity, it would be interesting to know that the highest number of free avian respiratory macrophages (FARMs) obtained, from the largest bird used, which were retired breeders weighing 3, 800 g, with 10 replica lavages and a total volume of 500 ml was 2 X 10^6 [187]. Compared to the number of phagocytes obtained from rats (up to 5.4 X 10^6) and guinea pigs (up to 2.5 X 10^7), animals whose body weights were near or below the lower limits of the birds [187]. These observations were even more surprising, because the ratio between respiratory epithelium and the volume of lungs is higher in birds than in mammals. Thus, if anything, one would have expected a higher yield of phagocytes residing on the respiratory epithelium of poultry instead of up to 10 times lower than obtained from small mammals. Thus, it clearly appears that poultry are lacking resident phagocytes, not only in the respiratory system, but also in various other body cavities. In totality, to defend against invading infectious agents, instead of relying on the resident population of phagocytes, they depend rather on the influx of these cells.

Considering the importance of macrophage mediation of heterophil chemotaxis and activation, it is theorized that defense of the respiratory tract of poultry may be twice handicapped. First, the lack of a significant resident macrophage population in the normal steady-state lungs and air sacs denies the front-line defense provided by macrophages against invading infectious agents. This further renders the avian respiratory system to heavily depend on the influx of phagocytes as mentioned earlier. Second, the minimal number of normal, residing macrophages may not be able to produce and release sufficient phagocytic chemotactic activating factors, and thus the second-line defense provided by surface FARMs will also be impaired. Thus, the direct defense roles that FARMs may perform remains ambiguous, and paucity and even lack of FARMs have been reported in the avian respiratory system [185].

The conclusions regarding scarcity of the cellular defenses come from a number of research observations. While in mammals alveolar macrophages can easily be harvested by pulmonary lavage, in birds, more difficult and unsuccessful experiences attempting to collect FARMs have been reported [187 211]

[238] [239] [240] [241] [242] [234] [231]. Phagocytic cells were estimated to be 20-fold less frequent in avian lavage samples than in those obtained from mice or rats [211], or even considered to be entirely absent [243] [244] [236]. Due to which, studies on blood monocytes, splenic macrophages and peritoneal exudate macrophages have been extrapolated to apply to an understanding of the function of respiratory macrophages [243243–245]. Studies trying various means of inducing avian phagocytes to migrate to the respiratory tract yielded no significant, consistent increases in the number of FARMs observed. The birds were elicited by a mixture of *Escherichia coli* lipopolysaccharide, *Saccharomyces cerevisiae,* glucan, and Freund's incomplete adjuvant (IFA) in a water-in-oil-in-water emulsion administered intravenously, subcutaneously, or by administration of aerosolized thioglycolate and proteose-peptone [211]. These results compel the questions of whether FARMs occur on the surface of the lung-air sac system, and are FARMs qualitatively and/or quantitatively deficient or functionally incompetent [177].

Stearns et al. (1987) proposed that the narrow diameters of ACs might be partly or wholly responsible for blocking the presence of large, motile phagocytic cells, a possible explanation for the paucity of FARMs in the parabronchus of avian lungs [237]. The diameter of ACs range from 3 to 20 µm [188] [246]. Recent three-dimensional studies [219] further showed that passageways connecting globular parts of ACs range in diameter from a mere 0.5 µm to 2 µm, thus, FARMs would obstruct ACs, hindering gas exchange.

Two contrary beliefs exist based on various observations and studies, the first being a large resident population of surface macrophages may not be necessary if vascular phagocytes can be quickly translocated to the respiratory surface, which seems acceptable based on the demonstrations discussed below [211] [187] [244] [247] [189] [116] [185] [186] [91], and both resident and few infiltrated FARMs may exhibit exceptionally efficient phagocytotic activity such that lower numbers are sufficient to protect the lung adequately.

With regard to the first observation, several studies reported about mobilization of phagocytes to the respiratory tract. Injection of a suspension of IFA into the abdominal air sacs [248], intratracheal inoculation of heat-killed *Escherichia coli* [211], and live, apathogenic *Pasteurella multocida* vaccine [240] evoked a dramatic increase in FARMs. It was possible to induce these phagocytes to enter the air sacs by injecting

foreign substances such as Freund's complete adjuvant or Sephadex G-100, disease producing organisms, and *Aspergillus fumigatus* spores into their lumen [173]. Direct inoculation of the abdominal air sacs of turkeys with IFA 1:4 diluted in PBS, elicited macrophages to migrate to the lungs and air sacs in relatively high numbers at 5.47X 10^6 cells. These cells were highly adhesive onto glass surfaces, actively phagocytic and cytotoxic for *Escherichia coli* as analyzed by *in vitro* assays [242]. Comparable results were obtained in a *Pasteurella multocida* model, where intratracheal, but not oral administration of an apathogenic vaccine strain efficiently activated the nonspecific cellular defense system of the respiratory tract. The most significant and rapid influx of phagocytes was seen when a low virulence live *E. coli* was inoculated intratracheally, by 3 h post inoculation (hpi) a statistically significant influx raised the group average number above 7 $\log_{10}$ and, by 24 hpi, the group average number of FARMs were about 50-100 times higher than the number in non-stimulated control chickens [240 241].

Despite the proposed paucity, migration of phagocytes to the respiratory tract of a stimulated chicken was impressive, possibly because of the replicative nature of the stimulant. In this regard, Karnowsky and Lazdins, defined activated cells as those having been exposed to infectious agents, and elicited cells as those having been obtained from animals inoculated with inert mitogenic or chemotactic substances [249]. It was also important to note that none of the birds yielding high numbers of FARMs in any of these trials were reported to have signs of respiratory disease like pneumonitis and airsacculitis. These findings pointed to an important characteristic of the avian respiratory system: although it was unresponsive or deficient in responding to attempts of elicitation, it reacted extremely rapidly to activation such as invasion by a live, replicating infectious agent [186].

A mutant of *S.* Typhimurium, lacking adenylate cyclase and cyclic AMP protein was found to be avirulent yet immunogenic [250] and holds a separate importance in context of this review, an effort to understand and support the possibility of infection of poultry via the respiratory route. Furthermore, fusaric acid-resistant derivatives of these mutants, with or without the virulence plasmid pStSR100, possessed wild-type ability to attach, invade and persist in GALT of mice for up to two weeks, this strain was evaluated for its ability to activate FARMs in chickens [238]. Intratracheal inoculation with 1.9 X 10^9 viable mutant *S.*

Typhimurium organisms stimulated chickens and approximately 53-fold more FARMs were infiltrated and had significantly higher phagocytic proportion and phagocytic capacity, than mock-inoculated control chickens. An interesting observation was that between 26 hpi and 8 days pi, the number of FARMs was not significantly higher in stimulated chickens when compared to controls. This demonstrated an acute response by the respiratory tract, one similar to those observed in chickens inoculated with *E. coli* or *P. multocida*. It also suggested, though did not prove, that the *S.* Typhimurium strain did not colonize pulmonary tissues of chickens for an extended time. It may also explain why none of the birds yielding high numbers of FARMs in any of the above demonstrations were reported to have signs of respiratory disease like pneumonitis and airsacculitis [250].

Researchers have argued that scarcity of FARMs should not, by default, mean that the pulmonary defenses are compromised [234 231 185] and both resident and infiltrated FARMs, even though low in number, may exhibit exceptionally efficient phagocytotic activity such that lower numbers are sufficient to protect the lungs adequately. Unresponsive or deficiency in responding to attempts of elicitation by FARMs was described to be an important characteristic of the avian respiratory system, although, it was infiltrated extremely rapidly to activation, by invasion of a live, replicating infectious agent [186]. However, from structural and functional considerations, it is impractical for FARMs to exist if they do not operate effectively in ACs. In support of this, radioactive technetium particles exposed to chickens were cleared from the lungs to the intestines within 1 h of exposure [251]. Additionally, a bacterial load introduced intratracheally was cleared from the avian respiratory system within 24–48 h [206]. In order to prove, or at the least support, this hypothesis, a comparative study on the phagocytic capacities of respiratory cells of domestic fowl and rats was performed under similar experimental conditions by *in vitro* exposure to polystyrene particles [185]. The researchers harvested chicken FARMs and erythrocytes by lung lavage and airway epithelial cells by exfoliation from the trachea, secondary bronchi, and/or ostia to test their ability to phagocytose the polystyrene particles. Ultrastructurally, chicken and rat respiratory FARMs were similar. Typically, they had filopodial extensions, variably electron dense vesicular cytoplasmic organelles presumed to be lysosomes, mitochondria, and sparsely distributed rough endoplasmic reticulum. Morphometric observations of the mean diameter of chicken FARMs (8.5 ± 0.90 μ m SD) was not

significantly different from that of rat cells (9.0 ± 0.57 μ m SD). The number of polystyrene particles phagocytized by FARMs from chickens increased with exposure time. After exposing the cells to polystyrene particles for a period of 1.5 h, using time-lapse photography, it was noted that chicken FARMs engulfed the polystyrene particles more efficiently, compared to those of rats. This was assessed qualitatively from progressive darkening of the FARMs as the cells took up the polystyrene particles, the lytic enzymes acted on the particles, and the cells died. In some areas, the cells appeared to form a continuum of phagosomes. Quantitative estimation of the loading of FARMs with polystyrene particles showed that the chicken cells formed a volume density of 22.5% of the volume of the cell and took up significantly more particles, compared to those of rats, of which the volume density of the particles formed only 5.3%. The study also refuted several claims involving enzymatic deficiencies of FARMs in oxidative metabolism [252], and differences in enzymatic systems of avian lung tissue itself [253].

Well-endowed with lytic enzymes, epithelial cells exfoliated and desquamated from the trachea, secondary bronchi, and/or ostia, were seen to avidly phagocytose the polystyrene particles [231]. Under *in vivo* conditions, these cells may play the significant role of removing and preventing harmful particulates and pathogenic microorganisms. When ducks were exposed to aerosols with an aerodynamic mass mean diameter of 0.18 μm containing iron oxide particles, these particles were found trapped within the trilaminar substance coating the atria and infundibuli in the parabronchi. The iron oxide particles were seen entering epithelial cells, in phagosomes within these cells, passing from the epithelial cells into the interstitium, and in interstitial macrophages [237]. These observations may explain why parabronchial macrophages are not usually seen in avian lungs – each epithelial cell in the region of atria and parts of the infundibula is found functioning as a macrophage and removes foreign material that becomes embedded in the trilaminar substance overlaying this region. Such a function would act to protect ACs from contamination. Also, it is possible to explain the observations that the caudal groups of air sacs are those most prone to infections while the cranial groups of air sacs are less often affected. All of the gas must pass through paleopulmonic parabronchi prior to reaching the cranial air sacs, resulting in trapping and removal of most foreign particles. On the other hand, gas that enters the caudal group of air sacs passes only through ventilated neopulmonic parabronchi [254] or directly into these air sacs and, thereby, is

not filtered to the same extent as gas reaching the cranial group of air sacs.

Another interesting, rather unusual observation in this study was that the chicken lung-lavaged erythrocytes readily phagocytized the 1.5 μm diameter polystyrene particles compared to rat erythrocytes which could not. Twenty-two percent of the erythrocyte cells engulfed one to six particles after 5 h co-incubation. The number of erythrocytes containing particles after 24 h could not, however, be determined, as most cells had died on extended incubation. Little is known about such interactions, but the phagocytic activity by these chicken erythrocytes is attributed to nucleated properties. Inherently, phagocytic erythrocytes may play a role in defense of the pulmonary system and the body in general needs to be confirmed with further studies. For now, it is supposed that avian erythrocytes may clear particles from the lung and deliver them to organs like the liver and spleen where they may be destroyed or sequestered [185].

Avian Air Sacs

Avian air sacs are thin membranous structures connected to the primary and secondary bronchi. All the air sacs are capacious, transparent structures that attach to avian lungs at ostia. They are the ventilating system, comprising most of the volume of the respiratory system [255 256 91 116]. They have an extremely thin wall, do not contribute to gas exchange with blood, and occupy every available space in the body coelom not occupied by other viscera. The locations, sizes, interconnections, and extensions of air sacs into and between the immediate structures differ among species [255 116 218]. The two types of connections are direct when air sacs are coupled to lungs by the primary bronchus and secondary bronchi while indirect ones are formed entirely by parabronchi. Most of the air sacs have one or two direct and several indirect connections. Most birds, including chickens, have nine air sacs - paired cervical air sacs, clavicular air sac that is connected to each lung, paired cranial thoracic air sacs, paired caudal thoracic air sacs, and paired abdominal air sacs. The cervical, clavicular, and cranial thoracic air sacs arise from the first set of secondary bronchi leaving the intrapulmonary primary bronchus (medioventral secondary bronchi). They are often considered as a group called the cranial air sacs because of the similarity in oxygen and carbon dioxide concentrations within them. The caudal thoracic and abdominal air sacs, often collectively

referred to as the caudal air sacs, arise from a second and third set of secondary bronchi (lateroventral and mediodorsal secondary bronchi) and from the continuation of the intrapulmonary primary bronchus. The oxygen concentration is higher and carbon dioxide concentration is lower in caudal air sacs than in the cranial air sac group [173 255 256 257 189].

The walls of air sacs consist largely of a simple epithelium that is supported by a thin layer of connective tissue, and the epithelium consists of squamous cells, however, near ostia, ciliated cuboidal and columnar cells exist. In *Gallus gallus*, a broad band of pseudostratified ciliated columnar epithelium with goblet cells extends from the primary bronchus into the abdominal air sac [218]. Epithelial cells of air sacs of chickens are joined by junctional complexes at the luminal aspect and laterally by interdigitations. Microvilli project into the luminal space and electron-dense lysosome-like granules were found in the cytoplasm [257256255].

The predominant sites of respiratory infections are the caudal air sacs, while cranial air sacs are the most devoid of infections compared to other parts of the respiratory system. Part of the reason may be that air sacs variably extend well beyond the limits of the coelomic cavity, with many bones being extensively pneumatized [257 258]. Some of the air sac diverticulae lie very close to the skin, which makes the respiratory system highly vulnerable to trauma and the lungs susceptible to diffusion of air-borne pathogens after damage to the extensions of the air sacs, such as air sacculitis [177]. A key explanation regarding the susceptibility of caudal air sacs lies in the gas flow pathway and mechanisms present in the parabronchi for particle removal. Due to parabronchial arrangement, avian lungs have a flow through system of breathing, in complete contrast to tidally ventilated mammalian respiratory systems. Fresh inhaled air is mixed with residual stale air in respiratory airways of mammalian system, while inspired air completely bypasses the cranial lying openings of the medioventral secondary bronchi, a process, which is termed inspiratory aerodynamic valving (IAV), of avian systems [189 180 173]. During the inspiratory phase, as well as the expiratory phase, air flows in the mediodorsal and lateroventral secondary bronchi and this flow pattern results in a continuous ventilation of the parabronchial lung in a caudocranial direction [189 174 176 177 178 180].

Due to this unidirectional flow, inhaled aerosol particles that could not be eliminated by several independent mechanisms including aerodynamic filtration, mucociliary clearance and phagocytosis in the upper respiratory tract end up in caudal air sacs. A detailed study by Hayter and Besch, showed that the deposition of particles critically depends on their size. Large particles, of up to 7 μm in diameter are removed in the nasal cavities and the proximal trachea, while smaller particles are deposited throughout the respiratory system [200]. Midsize particles of 1.1 μm are trapped primarily in the lungs and cranial air sacs while smaller particles of approximately ~0.1 μm pass through the entire lung and are finally trapped in the abdominal air sacs. However, as discussed above, pathogen clearance mechanisms are not as efficient as has been presumed, which results in deposition of particles of larger size and higher dose. Further, thin air sac walls are primarily covered by squamous and cuboidal epithelial cells. In contrast, the epithelium of the parabronchial atria particle uptake by the air sac epithelium has not been described [185]. In addition, a mucociliary escalator system was only found in the most proximal part of air sacs, right next to bronchial openings. Thus, particle clearance is largely accomplished by phagocytic cells and is shown to be significantly lower than in the parabronchial region [189].

Air sacs, because of their fragility, are very difficult to work with and therefore little is known about immunity within their structures. However, most studies regarding the scarcity of phagocytes, their demonstrated infiltration, and phagocytic activity seen associated with the parabronchial region, hold relevant for avian air sacs [257] [256] [255]. Also, various other evaluations have been carried out with the air sacs. Briefly, the epithelium lining the thoracic air sac varies greatly from area to area and from bird to bird in SPF layers and the epithelial layer rests on a thin basal lamina, supported by a stroma [257]. Small nodules are occasionally present in air sacs of turkeys, however these were not characterized further [217] [213]. Blood vessels were present in thicker parts of the air sacs, but absent in thinner regions. These blood vessels are important in the recruitment of phagocytes to air sacs. The cell types collected from air sacs depended on age of the animals, and also on rearing conditions. In day-old turkeys, the predominant cell type was macrophages, while heterophils predominated in older birds. Numbers of cells present in air sacs depended on the environment, as air sac washes from turkeys raised under commercial conditions contained a higher proportion of macrophages than washes from birds reared in isolation [91]. Occasional

migrating leukocytes were observed between columnar epithelial cells in control birds and in the stroma heterophils, the functional homologues of mammalian neutrophils, lymphocytes, plasma cells, monocytes, mast cells and macrophages were present. These cells were generally located in the vicinity of blood vessels [257].

In broiler chickens vaccinated intranasally with *Escherichia coli* and challenged 10 days later via intra-air sac, IgY, IgA and IgM directed against *E. coli* could be measured in air sac washes at 20 dpi, with IgY being the most prominent isotype [259]. Leukocytes were present on the surface of air sacs. Granulocytes make up the majority of these cells followed by macrophages, while lymphocytes were relatively rare [256]. As in the lung parenchyma, mononuclear phagocytes were detected in connective tissue of the air sacs. Clearance of particles occurred within 24-48 h and not within 1 h as in the lungs; however, the mechanisms by which foreign particles were removed from the lungs after being engulfed by interstitial macrophages are unknown [235]. Overall, the air sacs are deemed very vulnerable because of their location, their thin walls and low numbers of resident immune cells.

It is absolutely necessary to elucidate the exact mechanisms involved in each of the aspects described above, be it conclusions regarding the paucity of resident macrophages, speculations about the infiltration of phagocytes, unresponsive or deficiency in responding to attempts of pathogen invasion. Having said this, none of these properties of the avian respiratory system give the impression of a major challenge to highly adapted *Salmonella*. Scarcity of respiratory immune cells is clearly an advantage to *Salmonella* and any pathogen invading the respiratory system. All these studies demonstrating the infiltration and subsequent phagocytosis of pathogens, to an extent greater than seen in mammals, might essentially be of advantage to *Salmonella*, which already has an established intracellular survival mechanism. Taking into account the predisposing poultry production conditions leading to *Salmonella* bioaerosols and inherent incongruity of the immune avian immune system, defenses may become overwhelmed and the avian respiratory system is likely to be susceptible to *Salmonella* infections via the respiratory route.

Proposed *Salmonella* Infection Process via the Avian Respiratory Route

Introduction

The perceived susceptibility of poultry to respiratory diseases is proposed to stem from various human interventions that includes extreme genetic manipulation and intensive management for maximum productivity [244] [116] [185]. Since the domestication of wild jungle fowl, *Gallus gallus* of South East Asia, through intense genetic breeding, the domestic fowl has been selected for efficient weight gain and egg production. While better husbandry and management may be partly responsible for present performance of broilers, which reach a body mass of 2500 g in less than 40 days when compared to the late 1940s when broilers took approximately 90 days to reach a slaughter body mass of 1800 g [260]. Most of this progress is said to begin from an increased growth rate during the first two weeks post hatch. During such "directed, rather enforced, evolutionary selection" for maximal productivity, structural-functional disequilibria is bound to occur and has been evident [177] [185].

For example, in an above mentioned study by Maina et al. evaluated the structural failure of BGB and EECCs in different vascular regions of exchange tissue of the lungs in rested and exercised chickens [175]. During treadmill exercise, male chickens collapsed before they could attain maximum oxygen consumption. With regard to this, they suggested that the growth rate and functional performance of chickens were approaching physiological limits. The remarkable breeding progress in domestic birds is now sometimes associated with a mortality of up to 10% per flock because of various metabolic diseases.

In battery production schemes, commercial birds are kept in crowded conditions and may well be placed on a strict feeding regimen that entails force-feeding. Stress under such unnatural conditions may compromise the immunological robustness and cellular body defenses. This has led to an inference that birds are exceptionally susceptible to respiratory diseases, because the organs and organ systems of the body, particularly the immunological and cellular defenses may not have had time to adjust and to adapt to the environmental and behavioral changes [175] [260] [185].

Depending on the etiological agent and specific defects in host defenses, a respiratory infection can be overwhelming and result in severe consequences. We proposed *Salmonella*, an established enteropathogen, can infect poultry via the respiratory route and cause clinical manifestations. Current knowledge of *Salmonella* bioaerosol generation, its fate, transport, and survivability, as a means to assess the transport of bioaerosols and subsequent risk of exposure and infection of poultry was discussed previously. Furthermore, extensive analyses of poultry anatomy, physiology and immunology have enabled an understanding of the inherent possibilities of *Salmonella* infection via the respiratory route. At this point a meaningful understanding of how, where, and when respiratory defenses are breached and the process of *Salmonella* infection and subsequent dissemination is essential and is addressed in this section of the review.

Survival in the Upper Respiratory Tract

Physiologically, *Salmonella* has to overcome many hurdles while transiting through the GI tract and the role of numerous innate immune functions of the host in *Salmonella* pathogenesis is well understood and discussed above. Briefly, upon ingestion, in order to reach their sites of colonization, *Salmonella* must be able to survive various antimicrobial properties of the proventriculus, including low pH and the presence of many organic acids. The surviving population reaches the beginning of the intestinal lumen where they are exposed to bile, the role of which in *Salmonella*e pathogenesis is well recognized [61], as described above in more detail.

Salmonella transiting through the respiratory tract, evidently, does not face any of the above hurdles, at least not to the extent seen under GI conditions. Above, we have discussed the inefficacy of mucociliary clearance of the upper respiratory tract and the airway bronchial system, at least under poultry rearing conditions, the consequences of which are multiple. *Salmonella* in bioaerosols can theoretically reach the parabronchial and caudal air sac regions, potentially the most vulnerable areas for infection. Due to impaired mucociliary clearance, the dose at which it infects the parabronchi is potentially much lower than the dose required for oral infection. Furthermore, bacteria are not forced to repress their invasive capacity

in the respiratory lumen by various host driven processes. Also, considering NALT and CALT, which are at the forefront of defense, are fully functional with GC and plasmatic cells by the age of 4 weeks, it is theoretically a straightforward process for *Salmonella* infection in the early stages of bird life [186 91].

Bacterial Adhesion and Invasion

At later stages, in the GIT, *Salmonella* adhesion to host epithelial cells is the most crucial step of infection which enables bacteria to colonize the host intestine and these steps are the result of coordination, in time and space, of expression of many virulence genes. As an enteric pathogen, the most formidable barrier that *Salmonella* has to overcome is the intestinal epithelial lining. Several epithelial cell types are shown be invaded by *Salmonella*, and Peyer's patches represent the main portal of entry in early *Salmonella* infection, as described above [65].

Importantly, the process of invasion and subsequent infection is widely accepted to be pathogen-driven and host cells have a minor or no role to play. While in the respiratory system, age related developmental differences in the BALT structures of the bronchial airway may have functional consequences with respect to *Salmonella* pathogenesis. The disrupted epithelial barrier and previously described endocytosis could be a relatively easy portal of entry for *Salmonella* [217 189]. Thinning of the epithelium, reduced numbers of the ciliated cells, and absence of mucus producing goblet cells, may ultimately be to the benefit of the pathogen, facilitating a relatively open and localized interaction for *Salmonella*, which is known to infect its host through similar structures.

In the parabronchial region, the scarcity of respiratory immune cells is clearly a potential advantage to *Salmonella* and any pathogen invading the respiratory system. All these studies demonstrating the infiltration and subsequent phagocytosis of pathogens, to an extent greater than seen in mammals, might essentially be of advantage to *Salmonella*, which already has an established intracellular survival mechanism [98123109]. Illustration of respiratory epithelial cells lining the vast surface area, well endowed with lytic enzymes and phagocytizing the particulate matter, might potentially provide *Salmonella* with an

area as vast as the parabronchial region. It is interesting to note that a rapid change in virulence gene expression occurs during the transition from the intestinal lumen into tissue which promotes systemic dissemination of *Salmonella* under enteric conditions [110]. These rapid changes are considered to be due to various stimuli in the intestinal tract, such as bile, quorum sensing molecules, and other signals, as discussed above. Because of these findings, one might suggest that *Salmonella* transit through the respiratory airway, which essentially lacks these signals, might not result in the expression of virulence genes and any infection that is caused may solely be due to host driven mechanisms described above. Nevertheless, these suggestions require methodical evaluation and experimental evidence.

Intracellular Lifestyle and Infection

Salmonella must overcome the defense mechanisms of the enteric immune system through development of an intracellular lifestyle [67], which may also be true for infection through the respiratory route. Macrophages were seen in connective tissue below the parabronchial epithelium on the floor of the atria [236 235 234] and in the interatrial septa [189], indicating that phagocytic cells are strategically located at the start of the gas exchange area to clear the air of inhaled particles [116]. Further, a study by Stearns et al. showed that aerosolized inert iron particles with a diameter of 0.18 µm were phagocytosed in the gas exchange area, endocytosed by epithelial cells lining the atria and the proximal parts of the infundibula, resulting in transport to tissue macrophages underneath the atrial epithelium [237]. This indicates that if *Salmonella* invasion occurs, these pathogens, surely must adapt to an intracellular life style, although this specifically has not been described.

Following successful infection in the GI tract *Salmonella* either remains generally within the intestines, in the case of non-typhoidal strains, or systemically infects the host, as seen in typhoidal *Salmonella*, as described above. However, theoretically it is unlikely that *Salmonella* will colonize the respiratory tract and develop an intra-bronchial or intra-parabronchial infection. One of the fundamental reasons for this might be unavailability of diverse nutrients essentially required for pathogen growth.

In a recent study evaluating the diversity of host nutrients available for *Salmonella* in the intestinal tract, Steeb et al. found that at least 31 different host nutrients were accessed by *Salmonella* during its intestinal infection [261]. Furthermore, growth of *Salmonella* is aided by the very inflammatory responses aimed at controlling luminal bacteria. As reviewed above, the majority of intestinal microbiota is strictly anaerobic bacteria belonging to the classes Bacteroidetes and Clostridiales that rely on fermentation of amino acids and complex polysaccharides. One fermentation end product generated by the microbiota is hydrogen sulfide (H_2S), a cytotoxic compound that is converted to thiosulfate ($S_2O_3^{2-}$) by the colonic epithelium. During inflammation, neutrophils that transmigrate into the intestinal lumen release reactive oxygen species (ROS) in an attempt to kill bacteria. A by-product of releasing ROS is the oxidation of thiosulfate to tetrathionate ($S_4O_6^{2-}$). In contrast to fermenting microbiota, *S.* Typhimurium can use tetrathionate as a terminal electron acceptor to support its growth by anaerobic respiration, which is more efficient for energy production than fermentation. Also anaerobic tetrathionate respiration facilitates growth on poorly fermentable carbon sources. All these very demonstrations imply that the respiratory environment is totally different from that of the GI tract and hence intra-bronchial or intra-parabronchial persistence is highly unlikely [101].

A partial support to this view was provided in a study where a mutant of *S.* Typhimurium, lacking adenylate cyclase and cyclic AMP protein, was found to be avirulent yet immunogenic [250]. Furthermore, fusaric acid resistant derivatives of these mutants, with or without the virulence plasmid pStSR100, possessed wild-type ability to attach, invade and persist in GALT of mice for up to two weeks. This strain was evaluated for its ability to activate FARMs in chickens [262]. Intratracheal inoculation with 1.9 X 10^9 viable, mutant *S.* Typhimurium organisms stimulated chicken FARMS at a level approximately 53-fold higher and had significantly higher phagocytic proportion and phagocytic capacity, than mock-inoculated control chickens. An interesting observation was that, between 26 hpi and 8 dpi, the number of FARMs was not significantly higher in stimulated chickens as compared to controls. This demonstrated an acute response by the respiratory tract, one similar to those observed in chickens inoculated with *E. coli*, or with *P. multocida*. It also suggested, though did not prove, that the *S.* Typhimurium strain did not colonize lung tissues of chickens for an extended time. It may also explain why none of the birds yielding high numbers

of FARMs in any of the demonstrations were reported to have signs of respiratory disease such as pneumonitis or air sacculitis.

Taking into consideration that *Salmonella* would not colonize respiratory tissue, it is necessary to recognize its subsequent fate, which unfortunately is unknown and needs elucidation. Under GI conditions, *Salmonella* invasion causes a heavy heterophil influx, a stereotypical yet effective response. Ingested *Salmonella* from the basolateral surface of the intestinal epithelium will then be returned back into the gut lumen by epithelial transmigration of these heterophils, anticipating clearance of *Salmonella* via fecal route. Stearns et al. (1987) trying to reason the paucity of FARMs in the parabronchus of avian lungs, proposed that phagocytes would have to travel extremely long distances to deliver ingested particles to the ciliated parts of the lung for onward transmission to the trachea and larynx for eventual clearance. This indirectly suggested a way, more innate, through which *Salmonella* could be cleared by the respiratory system.

In mammals, phagocytes migrate over a relatively short distance of 200–300 μm to reach the terminal bronchioles from where the cells and engulfed particles are mechanically transported by the mucociliary escalator system to the pharynx and expectorated. Considering that the parabronchial lumen is lined by a non-secretory, non-stratified, non-ciliated epithelium, FARMs would have to travel over a distance of about 10,000 μm to reach the nearest parts of the lungs, the secondary bronchi, which have a ciliated epithelium [185]. To survive such a distance, cells would have to be exceptionally motile and resilient.

Further, in mammalian lungs, macrophage overloading causes impairment in migration of bacteria [263]. This suggests that macrophages can only phagocytose up to a certain maximum volume of particles [264] [263]. Morrow (1988) noted that alveolar macrophages function began to be impaired when an average of 6% of its volume was filled by phagocytized particles. Chen & Morrow (1989) noted that a macrophage maintained its mobility up to a certain particle burden beyond which the mobility was drastically compromised [265]. In the above mentioned study by Kiama et al., for phagocytes of comparable diameters and by extrapolation cell volumes, chicken FARMs phagocytized substantially more particles, four times

more, 22.5% of the cell volume in chickens compared to 5.3% of that of rat cells [185]. Moreover, chicken FARMs engulfed particles at a faster rate. This may imply whether the apparently more phagocytic avian FARMs are agile enough to travel to the ciliated parts for pathogen clearance.

On a different note, let us presume an efficient phagocytic system exists, which can travel the great distance and efflux these bacteria into the ciliary compartment. Considering the previously described inefficacy of the mucociliary clearance due to poultry practices, and other reasons, unidirectional airflow, would potentially lead these bacteria back into the parabronchial region or caudal air sacs. This eventually might cause a cyclical re-infection of the respiratory tract under prevailing poultry rearing conditions. This is especially possible in case of *Salmonella*, and potentially other pathogens, which can survive and replicate in these phagocytes. Care should be taken to realize that no such process has been reported as of yet and needs empirical evidence is needed to consider this as an anomaly of the innate immune system, or even a pathogen exploited process.

Another mode by which *Salmonella* could potentially infect and systemically spread is by entering into blood directly and causing bacteremia. This mode is quite conceivable, considering the disrupted epithelial barrier of developing BALT structures, described endocytosis by epithelial cells, and further transport to the interstitial matrix and high prevalence of BGBBs and EECCs described above. Conversely, continued survival of these bacteria entering blood is thought to be unlikely. Reports suggest that blood-borne bacteria are rapidly cleared from circulation and the clearance rate is enhanced significantly in the presence of opsonic factors in the blood [244]. Further, passively immunized turkey poults, when challenged with *E. coli*, rapidly reduced the bacterial load by approximately 5 $\log_{10}$ within 10 minutes post-intravenous challenge, in contrast to the non-immunized poults [266]. In a previously reported study by, erythrocytes collected from lavaged chicken lungs readily phagocytized 1.5 µm diameter polystyrene particles compared to rat erythrocytes which could not, 22% of chicken erythrocyte cells engulfed one to six particles after 5 h co-incubation [185]. However, little is known about such interactions but phagocytic activity by these chicken erythrocytes is attributed to nucleated properties. Roles, if any, regarding the inherently phagocytic properties of erythrocytes regarding defense of the pulmonary system

and body in general requires further elucidation. As described above, it is currently supposed that avian erythrocytes may clear particles from lungs and deliver them to organs like the liver and spleen where they may be destroyed or sequestered.

Alternatively, birds may present processed antigens locally to mount antigen-specific immune responses, either in the constitutively present BALT, or in organized lymphoid nodules which are inducible in many avian tissues by antigen inoculation [173] [116] [186]. However, functional studies on antigen sampling properties, the transfer and processing of antigens and the stimulation of naïve T and B lymphocytes has not been published to date. Information regarding mucosal effector sites in avian lungs is also limited. The presence of intraepithelial and interstitial lymphocytes has been described. More recently, the isolation and functional characterization of CD8+ effector T cells expressing IFN-γ from lung tissue of influenza virus infected birds was described [267] [268]. Unfortunately, this work did not specify whether these cells were obtained from mucosal inducer or effector sides of lung tissue. Similarly, a recent study in an infectious bronchitis virus model in chickens applied microarray analysis to investigate the host response within lungs but did not discriminate between BALT structures and the interstitial immune system as the source of RNA [269]. With the chicken genome sequence available [270], future work on host-pathogen interaction will increasingly apply this approach of more targeted gene expression analysis to lung tissue [271]. Such work should take the distinct anatomical structures of the lung-associated immune system into account which may very well show strikingly different phenotypic properties and functional activities.

Systemic Dissemination and Cecal Colonization

Theoretically, *Salmonella* thriving and replicating in FARMs, could be transported from lungs to secondary lymphoid organs such as the spleen, and systemically spread to liver and potentially other organs. Systemic dissemination of *Salmonella*, departing the respiratory organ, might well follow the same mechanisms observed in the oral-fecal route. Similar to the established process of oral infection, macrophages are major effecter cells eliciting innate immunity, which also transport *Salmonella* to host tissues [272], primarily to organs of the reticulo-endothelial system, such as the liver and spleen along with

kidney, blood, or reproductive tract [123]. Macrophages provide a protected site for intracellular bacterial replication and further act as a vehicle of dissemination and the process involved has been discussed above. In short, the overall process results in release of *Salmonella* from macrophages into the location or more specifically into the organ the macrophages have migrated to, leading to systemic infection. Colonization of the gall bladder by *Salmonella* is of special importance, since the organ is recognized to be involved in the chronic carrier state [61]. Eventually, *Salmonella* could end up in the intestinal tract via bile secretion of the gall bladder and begin its intestinal life cycle for further fecal dissemination.

Even though this may be the most conceivable mode of systemic spread of *Salmonella*, there can be other possible channels through which *Salmonella* can spread systemically. A growing body of evidence is demonstrating that the mucosa behaves as a system-wide network, suggesting that it be viewed from a holistic viewpoint as a global organ. Recent research evaluating the existence of communication between various mucosal sites, indicate unappreciated levels of cross-talk between mucosal compartments, influencing mucosal immunity [273]. Reports discussed below have demonstrated that stimulation in one compartment of the mucosal immune system can lead to changes in distal areas. Intranasal immunization resulted in vaginal protection against genital infection with herpes simplex virus type 2 and demonstrated that high innate immune kinetics and distribution of adaptive response induced in the nasal mucosa appears to be key factors in generating protective memory responses against HSV [274]. Clinical studies of patients infected with human immunodeficiency virus (HIV) have shown that high concentrations of HIV–specific IgA are found in various mucosal secretions, including vaginal secretions, nasal washes, saliva and endocervical secretions [275]. The authors of this review support these observations based on unpublished data, where oral administration of a proprietary vaccine against an enteric pathogen could induce IgA secretions in trachea. Furthermore, the use of antibiotics in neonates has been associated with a greater risk of developing asthma [276], suggesting that alterations in microflora of the gut can have an effect on lungs and highlights the potential for an undetermined link between mucosal immune compartments. Further, the impact of poultry house pollutants on particulate clearance from the respiratory system of birds was discussed above. Any substance that reduces ciliary motility or disrupts the ciliated epithelium could be expected to adversely affect the resistance of birds to microorganisms

that normally enter via the respiratory system. Interestingly, an earlier study involving 8 separate trials, Weinack et al. (1984), considered this assumption and evaluated any effects of respiratory tract impairment on the gut microflora and gut pathogens, with astounding conclusions. Chickens exposed through aerosolized *Mycoplasma gallisepticum* (MG) and / or Infectious Bronchitis Virus (IBV), started shedding higher doses of *Salmonella* Typhimurium and *Escherichia coli* more quickly than via oral challenge. They linked this observation to a decrease in intestinal microflora due to respiratory stress, which is additional support to the study involving use of antibiotics in neonates associated with a greater risk of developing asthma [276]. However, they certainly suggested the possibility of infection via aerosol inoculation for these organisms should not be ruled out.

Collectively, such studies project that the mucosal immune system is essentially one large interconnected network and that the individual components are very efficient at sharing information distally. When we extrapolate this concept in context of *Salmonella* pathogenesis and hypothesize those macrophages, as a critical part of the immune system with regard to *Salmonella* migrating capabilities, it is possible that macrophages can actually carry ingested *Salmonella* from the respiratory system to cecal tissue. Experimental understanding of this sort of communication between mucosal sites is fundamental to the next phase of disease characterization, and perhaps vaccine development. One way we might suggest to provide evidence for such a cross talk is through use of molecular markers. Alveolar macrophages (AM) in mammalian systems are the most abundant antigen-presenting cells in the lungs, and play a critical role in regulating pulmonary immune responses to inhaled pathogens and allergens. In an attempt to fully define the unique nature of AM, Guth et al. (2009) compared the phenotype and function of AM with the phenotype and function of resident peritoneal lavage-derived macrophages (PLM). They found striking phenotypic differences between AM and PLM, particularly with regard to CD11c expression. They proposed and investigated the role of the local airway environment in generation of such an unusual phenotype of AM. Cell transfer experiments were carried out to compare macrophage differentiation in airways with that in the peritoneal cavity. They observed significant up regulation of CD11c expression on bone marrow macrophages and peritoneal macrophages which were adoptively transferred into the

airways. Identification of same or similar markers in the avian system would prove beneficial and might be employed to prove the migration of respiratory macrophages from airway regions to cecal tissue.

References

1. Wright R. *Agricultural uses of municipal, animal, and industrial byproducts*. US Department of Agriculture, Agricultural Research Service; 1998.

2. Gerba CP, Smith JE. Sources of pathogenic microorganisms and their fate during land application of wastes. *Journal of environmental quality*. 2005;34(1):42–48.

3. Venglovsky J, Sasakova N, Placha I. Pathogens and antibiotic residues in animal manures and hygienic and ecological risks related to subsequent land application. *Bioresource technology*. 2009;100(22):5386–5391.

4. Heederik D, Sigsgaard T, Thorne PS, et al. Health effects of airborne exposures from concentrated animal feeding operations. *Environmental health perspectives*. 2007;115(2):298.

5. Risse L, Cabrera M, Franzluebbers A, et al. Land application of manure for beneficial reuse. 2006.

6. Ritter WF, Power J, Dick W, et al. Potential impact of land application of by-products on ground and surface water quality. *Land application of agricultural, industrial, and municipal by-products*. 2000:263–287.

7. Chang C, Chung H, Huang C-F, Su H-JJ. Exposure of workers to airborne microorganisms in open-air swine houses. *Applied and Environmental Microbiology*. 2001;67(1):155–161.

8. Wilson S, Morrow-Tesch J, Straus D, et al. Airborne microbial flora in a cattle feedlot. *Applied and Environmental Microbiology*. 2002;68(7):3238–3242.

9. Cole N, Todd R, Auvermann B, Parker D. Auditing and assessing air quality in concentrated feeding operations. *The Professional Animal Scientist*. 2008;24(1):1–22.

10. Dungan RS, Leytem AB. Airborne endotoxin concentrations at a large open-lot dairy in southern Idaho. *Journal of environmental quality*. 2009;38(5):1919–1923.

11. Chinivasagam H, Tran T, Maddock L, Gale A, Blackall P. Mechanically ventilated broiler sheds: a possible source of aerosolized *Salmonella*, Campylobacter, and Escherichia coli. *Applied and environmental microbiology*. 2009;75(23):7417–7425.

12. Dungan R*S*. BOARD-INVITED REVIEW: Fate and transport of bioaerosols associated with livestock operations and manures. *Journal of animal science*. 2010;88(11):3693–3706.

13. López FE, de las Mercedes Pescaretti M, Morero R, Delgado MA. *Salmonella* Typhimurium general virulence factors: A battle of David against Goliath? *Food Research International*. 2012;45(2):842–851.

14. Harbaugh E, Trampel D, Wesley I, et al. Rapid aerosol transmission of *Salmonella* among turkeys in a simulated holding-shed environment. *Poultry science*. 2006;85(10):1693–1699.

15. Oliveira C, Carvalho L, Garcia T, others. Experimental airborne transmission of *Salmonella* Agona and *Salmonella* Typhimurium in weaned pigs. *Epidemiology and infection*. 2006;134(1):199.

16. Wathes C, Zaidan W, Pearson G, Hinton M, Todd N. Aerosol infection of calves and mice with *Salmonella* Typhimurium. *Veterinary Record*. 1988;123(23):590–594.

17. Hermann J, Muñoz-Zanzi C, Zimmerman J. A method to provide improved dose–response estimates for airborne pathogens in animals: An example using porcine reproductive and respiratory syndrome virus. *Veterinary microbiology*. 2009;133(3):297–302.

18. Douwes J, Thorne P, Pearce N, Heederik D. Bioaerosol health effects and exposure assessment: progress and prospects. *Annals of Occupational Hygiene*. 2003;47(3):187–200.

19. Pillai SD, Ricke SC. Review/Synthèse Bioaerosols from municipal and animal wastes: background and contemporary issues. *Canadian journal of microbiology*. 2002;48(8):681–696.

20. Brenner F, Villar R, Angulo F, Tauxe R, Swaminathan B. *Salmonella* nomenclature. *Journal of Clinical Microbiology*. 2000;38(7):2465–2467.

21. Fricke WF, Mammel MK, McDermott PF, et al. Comparative genomics of 28 *Salmonella* enterica isolates: evidence for CRISPR-mediated adaptive sublineage evolution. *Journal of bacteriology*. 2011;193(14):3556–3568.

22. Thomson NR, Clayton DJ, Windhorst D, et al. Comparative genome analysis of *Salmonella* Enteritidis PT4 and *Salmonella* Gallinarum 287/91 provides insights into evolutionary and host adaptation pathways. *Genome research*. 2008;18(10):1624–1637.

23. Thorns C, others. Bacterial food-borne zoonoses. *Revue scientifique et technique (International Office of Epizootics)*. 2000;19(1):226.

24. Lahiri A, Lahiri A, Iyer N, Das P, Chakravortty D. Visiting the cell biology of< i> *Salmonella*</i> infection. *Microbes and infection*. 2010;12(11):809–818.

25. Santos R, Tsolis R, Bäumler A, Adams L. Pathogenesis of *Salmonella*-induced enteritis. *Brazilian journal of medical and biological research*. 2003;36(1):03–12.

26. Foley S, Lynne A. Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. *Journal of animal science*. 2008;86(14 suppl):E173–E187.

27. Chinivasagam H, Tran T, Blackall P. Impact of the Australian litter re-use practice on< i> *Salmonella*</i> in the broiler farming environment. *Food Research International*. 2012;45(2):891–896.

28. Zhang S, Kingsley RA, Santos RL, et al. Molecular pathogenesis of *Salmonella* enterica serotype Typhimurium-induced diarrhea. *Infection and immunity*. 2003;71(1):1–12.

29. Crump JA, Mintz ED. Global trends in typhoid and paratyphoid fever. *Clinical infectious diseases*. 2010;50(2):241–246.

30. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. Invasive non-typhoidal *Salmonella* disease: an emerging and neglected tropical disease in Africa. *The Lancet*. 2012.

31. Majowicz SE, Musto J, Scallan E, et al. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clinical Infectious Diseases*. 2010;50(6):882–889.

32. Woodward DL, Khakhria R, Johnson WM. Human salmonellosis associated with exotic pets. *Journal of clinical microbiology*. 1997;35(11):2786–2790.

33. Jarquin R, Hanning I, Ahn S, Ricke SC. Development of rapid detection and genetic characterization of *Salmonella* in poultry breeder feeds. *Sensors*. 2009;9(7):5308–5323.

34. Cox J, Pavic A. Advances in enteropathogen control in poultry production. *Journal of applied microbiology*. 2010;108(3):745–755.

35. Betancor L, Pereira M, Martinez A, et al. Prevalence of *Salmonella* enterica in poultry and eggs in Uruguay during an epidemic due to *Salmonella* enterica serovar Enteritidis. *Journal of clinical microbiology*. 2010;48(7):2413–2423.

36. Bone A, Noel H, Hello SL, et al. Nationwide outbreak of *Salmonella* enterica serotype 4, 12: i:- infections in France, linked to dried pork sausage, March-May 2010. *Zoonotic diseases*. 2010:44.

37. Braden CR. *Salmonella* enterica serotype Enteritidis and eggs: a national epidemic in the United States. *Clinical Infectious Diseases*. 2006;43(4):512–517.

38. Callaway T, Edrington T, Anderson R, Byrd J, Nisbet D. Gastrointestinal microbial ecology and the safety of our food supply as related to *Salmonella*. *Journal of animal science*. 2008;86(14 suppl):E163–E172.

39. Control C for D, Prevention C. Reports of Selected *Salmonella* Outbreak Investigations. 2013;2013(07/12).

40. Milner KC, Shaffer MF. Bacteriologic Stupies of Experimental *Salmonella* Infections in Chicks. *Journal of Infectious Diseases*. 1952;90(1):81–96.

41. Mitchell B, Buhr R, Berrang M, Bailey J, Cox N. Reducing airborne pathogens, dust and *Salmonella* transmission in experimental hatching cabinets using an electrostatic space charge system. *Poultry science*. 2002;81(1):49–55.

42. Jones F, Axtell R, Rives D, et al. A survey of *Salmonella* contamination in modern broiler production. *Journal of food protection*. 1991;54.

43. Davies R, Wray C. Persistence of *Salmonella* Enteritidis in poultry units and poultry food. *British poultry science*. 1996;37(3):589–596.

44. Ramirez G, Sarlin L, Caldwell D, et al. Effect of feed withdrawal on the incidence of *Salmonella* in the crops and ceca of market age broiler chickens. *Poultry science*. 1997;76(4):654–656.

45. Corrier D, Byrd J, Hargis B, et al. Presence of *Salmonella* in the crop and ceca of broiler chickens before and after preslaughter feed withdrawal. *Poultry science*. 1999;78(1):45–49.

46. Cox N, Stern N, Craven S, Berrang M, Musgrove M. Prevalence of Campylobacter and *Salmonella* in the cecal droppings of turkeys during production. *The Journal of Applied Poultry Research*. 2000;9(4):542–545.

47. Clouser C, Doores S, Mast M, Knabel *S.* The role of defeathering in the contamination of turkey skin by *Salmonella* species and Listeria monocytogenes. *Poultry science*. 1995;74(4):723–731.

48. McCapes R, Osburn B, Riemann H. Safety of foods of animal origin: model for elimination of *Salmonella* contamination of turkey meat. *Journal of the American Veterinary Medical Association*. 1991;199(7):875–882.

49. Luber P. Cross-contamination versus undercooking of poultry meat or eggs—which risks need to be managed first? *International journal of food microbiology*. 2009;134(1):21–28.

50. White P, Baker A, James W. Strategies to control *Salmonella* and Campylobacter in raw poultry products. *Revue scientifique et technique (International Office of Epizootics)*. 1997;16(2):525.

51. Series MRA. Risk assessments of *Salmonella* in eggs and broiler chickens.

52. Brynestad S, Braute L, Luber P, Bartelt E. Quantitative microbiological risk assessment of campylobacteriosis cases in the German population due to consumption of chicken prepared in homes. *International Journal of Risk Assessment and Management*. 2008;8(3):194–213.

53. Tessari ENC, Kanashiro AMI, Stoppa GF, et al. Important Aspects of *Salmonella* in the Poultry Industry and in Public Health.

54. Velge P, Cloeckaert A, Barrow P. Emergence of *Salmonella* epidemics: the problems related to *Salmonella* enterica serotype Enteritidis and multiple antibiotic resistance in other major serotypes. *Veterinary research*. 2005;36(3):267–288.

55. Vought KJ, Tatini SR. *Salmonella* Enteritidis contamination of ice cream associated with a 1994 multistate outbreak. *Journal of Food Protection®*. 1998;61(1):5–10.

56. Blaser MJ, Newman L*S.* A review of human salmonellosis: I. Infective dose. *Review of infectious diseases*. 1982;4(6):1096–1106.

57. Gast RK. Paratyphoid infections. *Diseases of poultry*. 1997;10:97–121.

58. Todar K. *Salmonella* and salmonellosis. *Todar's Online Textbook of Bacteriology*. 2005.

59. Bearson SM, Bearson BL, Rasmussen MA. Identification of *Salmonella* enterica serovar Typhimurium genes important for survival in the swine gastric environment. *Applied and Environmental Microbiology*. 2006;72(4):2829–2836.

60. Bearson S, Bearson B, Foster JW. Acid stress responses in enterobacteria. *FEMS microbiology letters*. 1997;147(2):173–180.

61. Begley M, Gahan CG, Hill C. The interaction between bacteria and bile. *FEMS microbiology reviews*. 2006;29(4):625–651.

62. Steele-Mortimer O, Méresse S, Gorvel J-P, Toh B-H, Finlay BB. Biogenesis of *Salmonella* Typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cellular microbiology*. 1999;1(1):33–49.

63. Maniatis NA, Chernaya O, Shinin V, Minshall RD. Caveolins and Lung Function. *Caveolins and Caveolae*. 2012:157–179.

64. de Jong HK, Parry CM, van der Poll T, Wiersinga WJ. Host-Pathogen Interaction in Invasive Salmonellosis. *PLoS Pathogens*. 2012;8(10):e1002933.

65. Schauser K, Olsen JE, Larsson L-I. Immunocytochemical studies of *Salmonella* Typhimurium invasion of porcine jejunal epithelial cells. *Journal of medical microbiology*. 2004;53(7):691–695.

66. Jepson MA, Clark M. The role of M cells in< i> *Salmonella*</i> infection. *Microbes and infection*. 2001;3(14):1183–1190.

67. Dorman C. Global regulators and environmental adaptation in Gram-negative pathogens. *Clinical Microbiology and Infection*. 2009;15(s1):47–50.

68. Mills DM, Bajaj V, Lee CA. A 40 kb chromosomal fragment encoding *Salmonella* Typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome. *Molecular microbiology*. 1995;15(4):749–759.

69. Haneda T, Ishii Y, Danbara H, Okada N. Genome-wide identification of novel genomic islands that contribute to *Salmonella* virulence in mouse systemic infection. *FEMS microbiology letters*. 2009;297(2):241–249.

70. Saroj SD, Shashidhar R, Karani M, Bandekar JR. Distribution of *Salmonella* pathogenicity island (SPI)-8 and SPI-10 among different serotypes of *Salmonella*. *Journal of medical microbiology*. 2008;57(4):424–427.

71. Shah DH, Lee M, Park J, et al. Identification of *Salmonella* gallinarum virulence genes in a chicken infection model using PCR-based signature-tagged mutagenesis. *Microbiology*. 2005;151(12):3957–3968.

72. Tahoun A, Mahajan S, Paxton E, et al. *Salmonella* transforms follicle-associated epithelial cells into M cells to promote intestinal invasion. *Cell host & microbe*. 2012;12(5):645–656.

73. Marchetti M, Sirard JC, Sansonetti P, Pringault E, Kernéis *S.* Interaction of pathogenic bacteria with rabbit appendix M cells: bacterial motility is a key feature in vivo. *Microbes and Infection*. 2004;6(6):521–528.

74. Wagner C, Hensel M. Adhesive mechanisms of *Salmonella* enterica. In: Springer; 2011:17–34.

75. Stecher B, Barthel M, Schlumberger MC, et al. Motility allows *S.* Typhimurium to benefit from the mucosal defence. *Cellular microbiology*. 2008;10(5):1166–1180.

76. Ly KT, Casanova JE. Mechanisms of *Salmonella* entry into host cells. *Cellular microbiology*. 2007;9(9):2103–2111.

77. Steele-Mortimer O. The< i> *Salmonella*</i>-containing vacuole—Moving with the times. *Current opinion in microbiology*. 2008;11(1):38–45.

78. McGhie EJ, Brawn LC, Hume PJ, Humphreys D, Koronakis V. *Salmonella* takes control: effector-driven manipulation of the host. *Current opinion in microbiology*. 2009;12(1):117–124.

79. Mukherjee S, Ghosh RN, Maxfield FR. Endocytosis. *Physiological Reviews*. 1997;77(3):759–803.

80. Ghosh RN, Gelman DL, Maxfield FR. Quantification of low density lipoprotein and transferrin endocytic sorting HEp2 cells using confocal microscopy. *Journal of cell science*. 1994;107(8):2177–2189.

81. Eckmann L, Kagnoff MF. Cytokines in host defense against< i> *Salmonella*</i>. *Microbes and infection*. 2001;3(14):1191–1200.

82. Dwivedy A, Aich P. Importance of innate mucosal immunity and the promises it holds. *International journal of general medicine*. 2011;4:299.

83. Gordon S, Read R. Macrophage defences against respiratory tract infections The immunology of childhood respiratory infections. *British medical bulletin*. 2002;61(1):45–61.

84. Haley PJ. Species differences in the structure and function of the immune system. *Toxicology*. 2003;188(1):49–71.

85. Raffatellu M, Wilson RP, Winter SE, Baumler AJ. Clinical pathogenesis of typhoid fever. *The Journal of Infection in Developing Countries*. 2008;2(04):260–266.

86. Boumart Z, Roche SM, Lalande F, et al. Heterogeneity of persistence of *Salmonella* enterica serotype Senftenberg strains could explain the emergence of this serotype in poultry flocks. *PloS one*. 2012;7(4):e35782.

87. Thiennimitr P, Winter SE, Bäumler AJ. < i> *Salmonella*</i>, the host and its microbiota. *Current opinion in microbiology*. 2012;15(1):108–114.

88. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-γ: an overview of signals, mechanisms and functions. *Journal of leukocyte biology*. 2004;75(2):163–189.

89. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *The Journal of Immunology*. 2001;167(4):1882–1885.

90. Vance RE, Isberg RR, Portnoy DA. Patterns of pathogenesis: discrimination of pathogenic and nonpathogenic microbes by the innate immune system. *Cell host & microbe*. 2009;6(1):10–21.

91. de Geus E. Respiratory immune responses in the chicken: Towards development of mucosal avian influenza virus vaccines. 2012.

92. Guo R-F, Ward PA. Role of C5a in inflammatory responses. *Annu.Rev.Immunol.* 2005;23:821–852.

93. Winter SE, Keestra AM, Tsolis RM, Bäumler AJ. The blessings and curses of intestinal inflammation. *Cell host \& microbe*. 2010;8(1):36–43.

94. Kamada N, Hisamatsu T, Okamoto S, et al. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-γ axis. *The Journal of clinical investigation*. 2008;118(6):2269.

95. Goto M, Murakawa M, Kadoshima-Yamaoka K, et al. Murine NKT cells produce Th17 cytokine interleukin-22. *Cellular immunology*. 2009;254(2):81–84.

96. Godinez I, Raffatellu M, Chu H, et al. Interleukin-23 orchestrates mucosal responses to *Salmonella* enterica serotype Typhimurium in the intestine. *Infection and immunity*. 2009;77(1):387–398.

97. Raffatellu M, Santos RL, Verhoeven DE, et al. Simian immunodeficiency virus–induced mucosal interleukin-17 deficiency promotes *Salmonella* dissemination from the gut. *Nature medicine*. 2008;14(4):421–428.

98. Ohl ME, Miller SI. *Salmonella*: a model for bacterial pathogenesis. *Annual review of medicine*. 2001;52(1):259–274.

99. Donnenberg M*S.* Pathogenic strategies of enteric bacteria. *Nature*. 2000;406(6797):768–774.

100. Tsolis R, Adams L, Ficht T. Ba «umler, AJ (1999) Contribution of *Salmonella* Typhimurium virulence factors to diarrheal disease in calves. *Infect.Immun*. 67:4879–4885.

101. Winter SE, Thiennimitr P, Winter MG, et al. Gut inflammation provides a respiratory electron acceptor for *Salmonella*. *Nature*. 2010;467(7314):426–429.

102. Lawley TD, Bouley DM, Hoy YE, et al. Host transmission of *Salmonella* enterica serovar Typhimurium is controlled by virulence factors and indigenous intestinal microbiota. *Infection and immunity*. 2008;76(1):403–416.

103. Salzman NH, Hung K, Haribhai D, et al. Enteric defensins are essential regulators of intestinal microbial ecology. *Nature immunology*. 2009;11(1):76–82.

104. Ackermann M, Stecher B, Freed NE, et al. Self-destructive cooperation mediated by phenotypic noise. *Nature*. 2008;454(7207):987–990.

105. Stecher B, Robbiani R, Walker AW, et al. *Salmonella* enterica serovar Typhimurium exploits inflammation to compete with the intestinal microbiota. *PLoS biology*. 2007;5(10):e244.

106. Sakamoto K, Hirose H, Onizuka A, et al. Quantitative study of changes in intestinal morphology and mucus gel on total parenteral nutrition in rats. *Journal of Surgical Research*. 2000;94(2):99–106.

107. Dougan G, John V, Palmer S, Mastroeni P. Immunity to salmonellosis. *Immunological reviews*. 2011;240(1):196–210.

108. Andrews-Polymenis HL, Bäumler AJ, McCormick BA, Fang FC. Taming the elephant: *Salmonella* biology, pathogenesis, and prevention. *Infection and immunity*. 2010;78(6):2356–2369.

109. Setta A, Barrow PA, Kaiser P, Jones MA. Immune dynamics following infection of avian macrophages and epithelial cells with typhoidal and non-typhoidal< i> *Salmonella* enterica</i> serovars; bacterial invasion and persistence, nitric oxide and oxygen production, differential host gene expression, NF-κB signalling and cell cytotoxicity. *Veterinary immunology and immunopathology*. 2012.

110. Winter SE, Winter MG, Godinez I, et al. A rapid change in virulence gene expression during the transition from the intestinal lumen into tissue promotes systemic dissemination of *Salmonella*. *PLoS pathogens*. 2010;6(8):e1001060.

111. Lalmanach A-C, Lantier F. Host cytokine response and resistance to< i> *Salmonella*</i> infection. *Microbes and infection*. 1999;1(9):719–726.

112. Sato S, Kiyono H. The mucosal immune system of the respiratory tract. *Current Opinion in Virology*. 2012.

113. Wick MJ. The role of dendritic cells during< i> *Salmonella*</i> infection. *Current opinion in immunology*. 2002;14(4):437–443.

114. Coburn B, Grassl GA, Finlay B. *Salmonella*, the host and disease: a brief review. *Immunology and cell biology*. 2006;85(2):112–118.

115. Berndt A, Wilhelm A, Jugert C, et al. Chicken cecum immune response to *Salmonella* enterica serovars of different levels of invasiveness. *Infection and immunity*. 2007;75(12):5993–6007.

116. Davison TF, Kaspers B, Schat KA. *Avian immunology*. Academic Press; 2008.

117. Wick MJ. Living in the danger zone: innate immunity to< i> *Salmonella*</i>. *Current opinion in microbiology*. 2004;7(1):51–57.

118. Lee I-T, Shih R-H, Lin C-C, et al. Role of TLR4/NADPH oxidase/ROS-activated p38 MAPK in VCAM-1 expression induced by lipopolysaccharide in human renal mesangial cells. *Cell Communication and Signaling*. 2012;10(1):33.

119. Bijlsma JJ, Groisman EA. The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella* enterica. *Molecular microbiology*. 2005;57(1):85–96.

120. Sabbagh SC, Forest CG, Lepage C, Leclerc J, Daigle F. So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella* enterica serovars Typhimurium and Typhi. *FEMS microbiology letters*. 2010;305(1):1–13.

121. Deiwick J, Nikolaus T, Erdogan S, Hensel M. Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. *Molecular microbiology*. 1999;31(6):1759–1773.

122. Vazquez-Torres A, Stevanin T, Jones-Carson J, et al. Analysis of Nitric Oxide-Dependent Antimicrobial Actions in Macrophages and Mice. *Methods in enzymology*. 2008;437:521–538.

123. Okamura M, Lillehoj HS, Raybourne RB, et al. Differential responses of macrophages to< i> *Salmonella*</i>< i> enterica</i> serovars Enteritidis and Typhimurium. *Veterinary immunology and immunopathology*. 2005;107(3):327–335.

124. Knodler LA, Finlay BB, Steele-Mortimer O. The *Salmonella* effector protein SopB protects epithelial cells from apoptosis by sustained activation of Akt. *Journal of Biological Chemistry*. 2005;280(10):9058–9064.

125. Layton AN, Galyov EE. *Salmonella*-induced enteritis: molecular pathogenesis and therapeutic implications. *Expert Rev Mol Med*. 2007;9(18):1–17.

126. Shah DH, Zhou X, Addwebi T, et al. Cell invasion of poultry-associated *Salmonella* enterica serovar Enteritidis isolates is associated with pathogenicity, motility and proteins secreted by the type III secretion system. *Microbiology*. 2011;157(5):1428–1445.

127. Sano G, Takada Y, Goto S, et al. Flagella facilitate escape of *Salmonella* from oncotic macrophages. *Journal of bacteriology*. 2007;189(22):8224–8232.

128. Liljebjelke KA, Hofacre CL, Liu T, et al. Vertical and horizontal transmission of *Salmonella* within integrated broiler production system. *Foodbourne Pathogens & Disease*. 2005;2(1):90–102.

129. Davies R, Wray C. An approach to reduction of *Salmonella* infection in broiler chicken flocks through intensive sampling and identification of cross-contamination hazards in commercial hatcheries. *International journal of food microbiology*. 1994;24(1):147–160.

130. Byrd J, DeLoach J, Corrier D, Nisbet D, Stanker L. Evaluation of *Salmonella* serotype distributions from commercial broiler hatcheries and grower houses. *Avian Diseases*. 1999:39–47.

131. Berrang M, Cox N, Bailey J. Measuring air-borne microbial contamination of broiler hatching cabinets. *The Journal of Applied Poultry Research*. 1995;4(1):83–87.

132. Lever M, Williams A. Cross-infection of chicks by airborne transmission of *Salmonella* Enteritidis PT4. *Letters in applied microbiology*. 1996;23(5):347–349.

133. Wathes C, Howard K, Webster A. Survival of Escherichia coli in an aerosol at air temperatures of 15 and 30 C and a range of humidities. *Journal of hygiene*. 1986;97:489–496.

134. Choct M. Managing gut health through nutrition. *British poultry science*. 2009;50(1):9–15.

135. Henderson S, Vicente J, Pixley C, Hargis B, Tellez G. Effect of an early nutritional supplement on broiler performance. *Int. J. Poult. Sci*. 2008;7:211–214.

136. Noy Y, Sklan D. Metabolic responses to early nutrition. *The Journal of Applied Poultry Research*. 1998;7(4):437.

137. Sklan D, Noy Y. Hydrolysis and absorption in the small intestines of posthatch chicks. *Poultry Science*. 2000;79(9):1306–1310.

138. Casteel E, Wilson J, Buhr R, Sander J. The influence of extended posthatch holding time and placement density on broiler performance. *Poultry science*. 1994;73(11):1679–1684.

139. Careghi C, Tona K, Onagbesan O, et al. The effects of the spread of hatch and interaction with delayed feed access after hatch on broiler performance until seven days of age. *Poultry science*. 2005;84(8):1314–1320.

140. Yi G, Allee G, Knight C, Dibner J. Impact of glutamine and oasis hatchling supplement on growth performance, small intestinal morphology, and immune response of broilers vaccinated and challenged with Eimeria maxima. *Poultry Science*. 2005;84(2):283–293.

141. Uni Z, Smirnov A, Sklan D. Pre-and posthatch development of goblet cells in the broiler small intestine: effect of delayed access to feed. *Poultry science*. 2003;82(2):320–327.

142. Noy Y, Geyra A, Sklan D. The effect of early feeding on growth and small intestinal development in the posthatch poult. *Poultry Science*. 2001;80(7):912–919.

143. Juul-Madsen HR, Su G, Sørensen P. Influence of early or late start of first feeding on growth and immune phenotype of broilers. *British poultry science*. 2004;45(2):210–222.

144. Dibner J, Knight C, Kitchell M, et al. Early feeding and development of the immune system in neonatal poultry. *The Journal of Applied Poultry Research*. 1998;7(4):425–436.

145. Hooshmand M. Effect of early feeding programs on broiler performance. *Int. J. Poult. Sci*. 2006;5(12):1140–1143.

146. Halevy O, Geyra A, Barak M, Uni Z, Sklan D. Early posthatch starvation decreases satellite cell proliferation and skeletal muscle growth in chicks. *The Journal of nutrition*. 2000;130(4):858–864.

147. Santos F, Sheldon B, Santos A, Ferket P. Influence of housing system, grain type, and particle size on *Salmonella* colonization and shedding of broilers fed triticale or corn-soybean meal diets. *Poultry*

science. 2008;87(3):405–420.

148. Ivanov I. Treatment of broiler litter with organic acids. *Research in veterinary science*. 2001;70(2):169–173.

149. Rothrock M, Cook K, Warren J, Sistani K. The effect of alum addition on microbial communities in poultry litter. *Poultry science*. 2008;87(8):1493–1503.

150. Vicente J, Higgins S, Hargis B, Tellez G. Effect of poultry guard litter amendment on horizontal transmission of *Salmonella* Enteritidis in broiler chicks. *International Journal of Poultry Science*. 2007;6(5):314–317.

151. Fallschissel K, Kämpfer P, Jäckel U. Direct detection of *Salmonella* cells in the air of livestock stables by real-time PCR. *Annals of Occupational Hygiene*. 2009;53(8):859–868.

152. Fallschissel K, Klug K, Kämpfer P, Jäckel U. Detection of airborne bacteria in a German turkey house by cultivation-based and molecular methods. *Annals of Occupational Hygiene*. 2010;54(8):934–943.

153. Baykov B, Stoyanov M. Microbial air pollution caused by intensive broiler chicken breeding. *FEMS microbiology ecology*. 1999;29(4):389–392.

154. Gast RK, Mitchell BW, Holt P*S*. Detection of airborne *Salmonella* Enteritidis in the environment of experimentally infected laying hens by an electrostatic sampling device. *Avian Diseases*. 2004;48(1):148–154.

155. Heber AJ, Peugh MW, Lutgring KR, Zimmerman NJ, Linton RH. Poultry slaughtering plants: concentrations of microbial aerosols in poultry slaughtering and processing plants. *ASHRAE Transactions*. 2006:644–655.

156. Bull S, Allen V, Domingue G, et al. Sources of Campylobacter spp. colonizing housed broiler flocks during rearing. *Applied and Environmental Microbiology*. 2006;72(1):645–652.

157. Kwon Y, Woodward C, Pillai S, et al. Litter and aerosol sampling of chicken houses for rapid detection of *Salmonella* Typhimurium contamination using gene amplification. *Journal of industrial microbiology \& biotechnology*. 2000;24(6):379–382.

158. Corry J, Allen V, Hudson W, Breslin M, Davies R. Sources of *Salmonella* on broiler carcasses during transportation and processing: modes of contamination and methods of control. *Journal of applied microbiology*. 2002;92(3):424–432.

159. Berrang M, Northcutt J. Use of water spray and extended drying time to lower bacterial numbers on soiled flooring from broiler transport coops. *Poultry science*. 2005;84(11):1797–1801.

160. Vieira-Pinto M, Tenreiro R, Martins C. Unveiling contamination sources and dissemination routes of< i> *Salmonella*</i> sp. in pigs at a Portuguese slaughterhouse through macrorestriction profiling by pulsed-field gel electrophoresis. *International journal of food microbiology*. 2006;110(1):77–84.

161. Berends B, Urlings H, Snijders J, Knapen FV. Identification and quantification of risk factors in animal management and transport regarding< i> *Salmonella*</i> spp. in pigs. *International journal of food microbiology*. 1996;30(1):37–53.

162. Gray JT, Fedorka-Cray PJ. Survival and infectivity of *Salmonella* choleraesuis in swine feces. *Journal of Food Protection®*. 2001;64(7):945–949.

163. Fedorka-Cray PJ, Kelley LC, Stabel TJ, Gray JT, Laufer JA. Alternate routes of invasion may affect pathogenesis of *Salmonella* Typhimurium in swine. *Infection and immunity*. 1995;63(7):2658–2664.

164. Berrang M, Meinersmann R, Buhr R, et al. Presence of Campylobacter inthe respiratory tract of broiler carcasses before and after commercial scalding. *Poultry science*. 2003;82(12):1995–1999.

165. Suresh T, Hatha A, Sreenivasan D, Sangeetha N, Lashmanaperumalsamy P. Prevalence and antimicrobial resistance of< i> *Salmonella*</i> Enteritidis and other *Salmonella*s in the eggs and egg-storing trays from retails markets of Coimbatore, South India. *Food Microbiology*. 2006;23(3):294–299.

166. Adesiyun A, Offiah N, Seepersadsingh N, et al. Microbial health risk posed by table eggs in Trinidad. *Epidemiology and infection*. 2005;133(6):1049–1056.

167. Lahellec C, Colin P, Bennejean G, et al. Influence of resident *Salmonella* on contamination of broiler flocks. *Poultry science*. 1986;65(11):2034–2039.

168. McDermid A, Lever M. Survival of *Salmonella* Enteritidis PT4 and Salm. Typhimurium Swindon in aerosols. *Letters in applied microbiology*. 1996;23(2):107–109.

169. Donnison A, Ross C, Noonan M, Fisher G, Waller J. Bacterial survival and dispersal in spray irrigation aerosols. *New Zealand Journal of Agricultural Research*. 2004;47(4):575–585.

170. Brooks J, McLaughlin M, Scheffler B, Miles D. Microbial and antibiotic resistant constituents associated with biological aerosols and poultry litter within a commercial poultry house. *Science of the total environment*. 2010;408(20):4770–4777.

171. Stersky A, Heldman D, Hedrick T. Viability of airborne *Salmonella* newbrunswick under various conditions. *Journal of dairy science*. 1972;55(1):14–18.

172. Pedersen TB, Olsen JE, Bisgaard M. Persistence of *Salmonella* Senftenberg in poultry production environments and investigation of its resistance to desiccation. *Avian Pathology*. 2008;37(4):421–427.

173. Fedde M. Relationship of structure and function of the avian respiratory system to disease susceptibility. *Poultry science*. 1998;77(8):1130–1138.

174. Watson RR, Fu Z, West JB. Morphometry of the extremely thin pulmonary blood-gas barrier in the chicken lung. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. 2007;292(3):L769–L777.

175. Maina JN, Jimoh SA. Structural failures of the blood-gas barrier and the epithelial-epithelial cell connections in the different vascular regions of the lung of the domestic fowl, Gallus gallus variant

domesticus, at rest and during exercise. *Biology Open*. 2013;2(3):267–276.

176. West JB, Fu Z, Deerinck TJ, et al. Structure-function studies of blood and air capillaries in chicken lung using 3D electron microscopy. *Respiratory physiology \& neurobiology*. 2010;170(2):202–209.

177. Maina J. Some recent advances on the study and understanding of the functional design of the avian lung: morphological and morphometric perspectives. *Biological Reviews*. 2002;77(1):97–152.

178. Watson RR, Fu Z, West JB. Minimal distensibility of pulmonary capillaries in avian lungs compared with mammalian lungs. *Respiratory physiology \& neurobiology*. 2008;160(2):208–214.

179. Maina JN, West JB. Thin and strong! The bioengineering dilemma in the structural and functional design of the blood-gas barrier. *Physiological reviews*. 2005;85(3):811–844.

180. Maina JN. Development, structure, and function of a novel respiratory organ, the lung-air sac system of birds: to go where no other vertebrate has gone. *Biological Reviews*. 2006;81(4):545–579.

181. West JB, Watson RR, Fu Z. Major differences in the pulmonary circulation between birds and mammals. *Respiratory physiology \& neurobiology*. 2007;157(2):382–390.

182. Kuethe DO. Fluid mechanical valving of air flow in bird lungs. *Journal of experimental biology*. 1988;136(1):1–12.

183. Maina JN, Jimoh SA, Hosie M. Implicit mechanistic role of the collagen, smooth muscle, and elastic tissue components in strengthening the air and blood capillaries of the avian lung. *Journal of Anatomy*. 2010;217(5):597–608.

184. Mitchell B, Buhr R, Berrang M, Bailey J, Cox N. Reducing airborne pathogens, dust and *Salmonella* transmission in experimental hatching cabinets using an electrostatic space charge system. *Poultry science*. 2002;81(1):49–55.

185. Kiama S, Adekunle J, Maina J. Comparative in vitro study of interactions between particles and respiratory surface macrophages, erythrocytes, and epithelial cells of the chicken and the rat. *Journal of Anatomy*. 2008;213(4):452–463.

186. Smialek M, Tykalowski B, Stenzel T, Koncicki A. Local immunity of the respiratory mucosal system in chickens and turkeys. *Polish Journal of Veterinary Sciences*. 2011;14(2):291–297.

187. Toth TE. Nonspecific cellular defense of the avian respiratory system: a review. *Developmental \& Comparative Immunology*. 2000;24(2):121–139.

188. Duncker H-R. Structure of the avian respiratory tract. *Respiration physiology*. 1974;22(1):1–19.

189. Reese S, Dalamani G, Kaspers B. The avian lung-associated immune system: a review. *Veterinary research*. 2006;37(3):311–324.

190. Ohshima K, Hiramatsu K. Distribution of T-cell subsets and immunoglobulin-containing cells in nasal-associated lymphoid tissue (NALT) of chickens. *Histology and histopathology*. 2000;15(3):713–720.

191. Fagerland JA, Arp LH. A morphologic study of bronchus-associated lymphoid tissue in turkeys. *American Journal of Anatomy*. 1990;189(1):24–34.

192. Jeurissen SH, Janse EM, Koch G, Boer GFD. Postnatal development of mucosa-associated lymphoid tissues in chickens. *Cell and tissue research*. 1989;258(1):119–124.

193. Bienenstock J, McDermott MR. Bronchus-and nasal-associated lymphoid tissues. *Immunological reviews*. 2005;206(1):22–31.

194. Alstine WGV, Arp LH. Histologic evaluation of lung and bronchus-associated lymphoid tissue in young turkeys infected with Bordetella avium. *American Journal of Veterinary Research*. 1988;49(6):835–839.

195. King M. Mucus and its role in airway clearance and cytoprotection. *Physiologic basis of respiratory disease*. 2005:409–416.

196. Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiological reviews*. 2006;86(1):245–278.

197. Hingley S, Hastie A, Kueppers F, et al. Effect of ciliostatic factors from Pseudomonas aeruginosa on rabbit respiratory cilia. *Infection and immunity*. 1986;51(1):254–262.

198. Henning A, Schneider M, Bur M, et al. Embryonic chicken trachea as a new in vitro model for the investigation of mucociliary particle clearance in the airways. *AAPS PharmSciTech*. 2008;9(2):521–527.

199. Gabridge MG, Meccoli RA. Cytotoxicity and ciliostasis in tracheal explants exposed to cadmium salts. *Environmental health perspectives*. 1982;44:189.

200. Hayter R, Besch E. Airborne-particle deposition in the respiratory tract of chickens. *Poultry science*. 1974;53(4):1507–1511.

201. Albrecht A, Redmann T, Nuchter H, et al. Airborne microorganisms in a rearing henhouse for layers during vaccination. *DTW.Deutsche tierarztliche Wochenschrift*. 2003;110(12):487–493.

202. Radon K, Danuser B, Iversen M, et al. Air contaminants in different European farming environments. *Annals of Agricultural and Environmental Medicine*. 2002;9(1):41–48.

203. Harbaugh E, Trampel D, Wesley I, et al. Rapid aerosol transmission of *Salmonella* among turkeys in a simulated holding-shed environment. *Poultry science*. 2006;85(10):1693–1699.

204. Baskerville A, Humphrey T, Fitzgeorge R, et al. Airborne infection of laying hens with *Salmonella* Enteritidis phage type 4. *Veterinary Record*. 1992;130(18):395–398.

205. Weinack OM, Snoeyenbos G, Smyser C, Soerjadi-Liem A. Influence of Mycoplasma gallisepticum, infectious bronchitis, and cyclophosphamide on chickens protected by native intestinal microflora against

Salmonella Typhimurium or Escherichia coli. *Avian Diseases*. 1984:416–425.

206. Nagaraja KV, Emery DA, Jordan KA, et al. Effect of ammonia on the quantitative clearance of Escherichia coli from lungs, air sacs, and livers of turkeys aerosol vaccinated against Escherichia coli. *American Journal of Veterinary Research*. 1984;45(2):392–395.

207. Whyte RT. Aerial pollutants and the health of poultry farmers. *World's poultry science journal*. 1993;49:139–153.

208. Donham K, Blake J, Patterson P. Occupational health hazards and recommended exposure limits for workers in poultry buildings. In: *Proceedings, 2000 National Poultry Waste Management Symposium, Ocean City, MD, USA, 16-18 October 2000.* National Poultry Waste Management Symposium Committee; 2000:92–109.

209. Dwars RM, Matthijs MGR, Daemen AJJM, Vervelde L, Landman WJM. Progression of lesions in the respiratory tract of broilers after single infection with Escherichia coli compared to superinfection with E. coli after infection with infectious bronchitis virus. *The pathogenesis of colibacillosis in broilers infected with virulent or vaccine strains of infectious bronchitis virus*. 2009:71.

210. Popy N, Asaduzzaman M, Miah M, et al. Pathological study on the upper respiratory tract infection of chickens and isolation, identification of causal bacteria. *Bangladesh Veterinarian*. 2012;28(2):60–69.

211. Toth T, Siegel P, Veit H. Cellular defense of the avian respiratory system. Influx of phagocytes: elicitation versus activation. *Avian diseases*. 1987:861–867.

212. Bahadur Basnet H, Kwon HJ, Cho SH, et al. Reproduction of fowl typhoid by respiratory challenge with *Salmonella* Gallinarum. *Avian diseases*. 2008;52(1):156–159.

213. Fagerland JA, Arp LH. Structure and development of bronchus-associated lymphoid tissue in conventionally reared broiler chickens. *Avian diseases*. 1993:10–18.

214. Fagerland JA, Arp LH. A morphologic study of bronchus-associated lymphoid tissue in turkeys. *American Journal of Anatomy*. 2005;189(1):24–34.

215. Fagerland JA, Arp LH. Distribution and quantitation of plasma cells, T lymphocyte subsets, and B lymphocytes in bronchus-associated lymphoid tissue of chickens: age-related differences. *Regional immunology*. 1993;5(1):28–36.

216. Bienenstock J. Bronchus-associated lymphoid tissue and the source of immunoglobulin-containing cells in the mucosa. *Environmental health perspectives*. 1980;35:39.

217. Fagerland JA, Myers RK, Arp LH. Uptake of ferritin and< i> Bordetella avium</i> in bronchus-associated lymphoid tissue of turkeys. *Veterinary immunology and immunopathology*. 1994;40(4):367–377.

218. Orosz SE, Lichtenberger M. Avian Respiratory Distress: Etiology, Diagnosis, and Treatment. *Veterinary Clinics of North America-ExoticAnimal Practice*. 2011;14(2):241.

219. Woodward J, Maina J. Study of the structure of the air and blood capillaries of the gas exchange tissue of the avian lung by serial section three-dimensional reconstruction. *Journal of microscopy*. 2008;230(1):84–93.

220. Maina JN, Nathaniel C. A qualitative and quantitative study of the lung of an ostrich, Struthio camelus. *Journal of Experimental Biology*. 2001;204(13):2313–2330.

221. Watson RR, Fu Z, West JB. Morphometry of the extremely thin pulmonary blood-gas barrier in the chicken lung. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. 2007;292(3):L769–L777.

222. Maina J, King A. The thickness of avian blood-gas barrier: qualitative and quantitative observations. *Journal of anatomy*. 1982;134(Pt 3):553.

223. West JB. Comparative physiology of the pulmonary blood-gas barrier: the unique avian solution. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 2009;297(6):R1625–R1634.

224. Marteyn B, West NP, Browning DF, et al. Modulation of Shigella virulence in response to available oxygen in vivo. *Nature*. 2010;465(7296):355–358.

225. West J, Mathieu-Costello O. Structure, strength, failure, and remodeling of the pulmonary blood-gas barrier. *Annual Review of Physiology*. 1999;61(1):543–572.

226. West JB, Mathieu-Costello O. Stress failure of pulmonary capillaries as a limiting factor for maximal exercise. *European journal of applied physiology and occupational physiology*. 1995;70(2):99–108.

227. Maina JN. *The lung-air sac system of birds: development, structure, and function*. Springer Verlag; 2005.

228. Jones F, Axtell R, Rives D, et al. A survey of *Salmonella* contamination in modern broiler production. *Journal of food protection*. 1991;54.

229. Lichtenberger M. Determination of indirect blood pressure in the companion bird. In: *Seminars in Avian and Exotic Pet Medicine*.Vol 14. Elsevier; 2005:149–152.

230. West JB, Watson RR, Fu Z. The honeycomb-like structure of the bird lung allows a uniquely thin blood-gas barrier. *Respiratory physiology \& neurobiology*. 2006;152(1):115–118.

231. Nganpiep L, Maina J. Composite cellular defence stratagem in the avian respiratory system: functional morphology of the free (surface) macrophages and specialized pulmonary epithelia. *Journal of anatomy*. 2002;200(5):499–516.

232. Jeurissen SH, Janse EM, Kok GL, Boer GFD. Distribution and function of non-lymphoid cells positive for monoclonal antibody CVI-ChNL-68.2 in healthy chickens and those infected with Marek's disease virus. *Veterinary immunology and immunopathology*. 1989;22(2):123–133.

233. Jeurissen S, Vervelde L, Janse M. Structure and function of lymphoid tissues of the chicken. *Poultry science reviews*. 1993;5(3):183–207.

234. Maina JN, Cowley HM. Ultrastructural characterization of the pulmonary cellular defences in the lung of a bird, the rock dove, Columba livia. *Proceedings of the Royal Society of London.Series B: Biological Sciences*. 1998;265(1405):1567–1572.

235. Klika E, Scheuermann DW, Groodt-Lasseel D, Bazantova I, Switka A. Pulmonary macrophages in birds (barn owl, Tyto tyto alba), domestic fowl (Gallus gallus f. domestica), quail (Coturnix coturnix), and pigeons (Columbia livia). *The Anatomical Record*. 1996;246(1):87–97.

236. Klika E, Scheuermann DW, Bazantova I, Switka A. Anchoring and support system of pulmonary gas-exchange tissue in four bird species. *Cells Tissues Organs*. 2008;159(1):30–41.

237. Stearns RC, Barnas G, Walski M, Brain J. Deposition and phagocytosis of inhaled particles in the gas exchange region of the duck,< i> Anas platyrhynchos</i>. *Respiration physiology*. 1987;67(1):23–36.

238. Toth TE, Curtiss III R, Veit H, Pyle R, Siegel P. Reaction of the avian respiratory system to intratracheally administered avirulent *Salmonella* Typhimurium. *Avian diseases*. 1992:24–29.

239. Toth T, Siegel P. Cellular defense of the avian respiratory tract: paucity of free-residing macrophages in the normal chicken. *Avian Diseases*. 1986:67–75.

240. Toth T, Veit H, Gross W, Siegel P. Cellular defense of the avian respiratory system: protection against Escherichia coli airsacculitis by Pasteurella multocida-activated respiratory phagocytes. *Avian Diseases*. 1988:681–687.

241. Toth TE, Siegel P. Cellular defense of the avian respiratory system: dose-response relationship and duration of response in intratracheal stimulation of avian respiratory phagocytes by a Pasteurella multocida bacterin. *Avian Diseases*. 1993:756–762.

242. Ficken MD, Edwards JF, Lay JC. Induction, collection, and partial characterization of induced respiratory macrophages of the turkey. *Avian Diseases*. 1986:766–771.

243. Qureshi M, Marsh J, Dietert R, et al. Profiles of chicken macrophage effector functions. *Poultry science*. 1994;73(7):1027–1034.

244. Qureshi MA, Heggen CL, Hussain I. Avian macrophage: effector functions in health and disease. *Developmental \& Comparative Immunology*. 2000;24(2):103–119.

245. Qureshi M, Miller L. Comparison of macrophage function in several commercial broiler genetic lines. *Poultry science*. 1991;70(10):2094–2101.

246. Duncker HR. The lung air sac system of birds. A contribution to the functional anatomy of the respiratory apparatus. *Ergebnisse der Anatomie und Entwicklungsgeschichte*. 1971;45(6):7–171.

247. Nakamura M, Nagamine N, Takahashi T, et al. Intratracheal infection of chickens with *Salmonella* Enteritidis and the effect of feed and water deprivation. *Avian diseases*. 1995:853–858.

248. Ficken MD, Edwards JF, Lay JC. Induction, collection, and partial characterization of induced respiratory macrophages of the turkey. *Avian Diseases*. 1986:766–771.

249. Karnovsky ML, Lazdins JK. Biochemical criteria for activated macrophages. *The Journal of Immunology*. 1978;121(3):809–813.

250. Curtiss R, Kelly SM. *Salmonella* Typhimurium deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. *Infection and immunity*. 1987;55(12):3035–3043.

251. Mensah GA, Brain JD. Deposition and clearance of inhaled aerosol in the respiratory tract of chickens. *Journal of applied physiology*. 1982;53(6):1423–1428.

252. Penniall R, Spitznagel JK. Chicken neutrophils: oxidative metabolism in phagocytic cells devoid of myeloperoxidase. *Proceedings of the National Academy of Sciences*. 1975;72(12):5012–5015.

253. Buckpitt AR, Boyd MR. Metabolic activation of 4-ipomeanol by avian tissue microsomes. *Toxicology and applied pharmacology*. 1982;65(1):53–62.

254. Scheid P. Mechanisms of gas exchange in bird lungs. In: Springer; 1979:137–186.

255. Farmer C. On the origin of avian air sacs. *Respiratory physiology \& neurobiology*. 2006;154(1):89–106.

256. Crespo R, Yamashiro S, Hunter DB. Development of the thoracic air sacs of turkeys with age and rearing conditions. *Avian Diseases*. 1998:35–44.

257. Bezuidenhout A. Light and electron microscopic study of the thoracic respiratory air sacs of the fowl. *Anatomia, Histologia, Embryologia*. 2005;34(3):185–191.

258. Bezuidenhout AJ, Groenewald HB, Soley JT. An anatomical study of the respiratory air sacs in ostriches. 1999.

259. Kariyawasam S, Wilkie B, Hunter D, Gyles C. Systemic and mucosal antibody responses to selected cell surface antigens of avian pathogenic Escherichia coli in experimentally infected chickens. *Avian Diseases*. 2002;46(3):668–678.

260. Manual R. ROSS broiler management manual. *Aviagen Ltd., UK*. 2002.

261. Steeb B, Claudi B, Burton NA, et al. Parallel exploitation of diverse host nutrients enhances *Salmonella* virulence. *PLoS pathogens*. 2013;9(4):e1003301.

262. Toth TE, III RC, Veit H, Pyle R, Siegel P. Reaction of the avian respiratory system to intratracheally administered avirulent *Salmonella* Typhimurium. *Avian Diseases*. 1992:24–29.

263. Oberdörster G, Ferin J, Lehnert BE. Correlation between particle size, in vivo particle persistence, and lung injury. *Environmental health perspectives*. 1994;102(Suppl 5):173.

264. Morrow P. Possible mechanisms to explain dust overloading of the lungs. *Fundamental and applied toxicology*. 1988;10(3):369–384.

265. Yu C, Chen Y, Morrow P. An analysis of alveolar macrophage mobility kinetics at dust overloading of the lungs. *Fundamental and Applied Toxicology*. 1989;13(3):452–459.

266. Arp L. Effect of passive immunization on phagocytosis of blood-borne Escherichia coli in spleen and liver of turkeys. *American Journal of Veterinary Research*. 1982;43(6):1034.

267. Seo SH, Peiris M, Webster RG. Protective cross-reactive cellular immunity to lethal A/Goose/Guangdong/1/96-like H5N1 influenza virus is correlated with the proportion of pulmonary CD8 T cells expressing gamma interferon. *Journal of virology*. 2002;76(10):4886–4890.

268. Seo SH, Webster RG. Cross-reactive, cell-mediated immunity and protection of chickens from lethal H5N1 influenza virus infection in Hong Kong poultry markets. *Journal of virology*. 2001;75(6):2516–2525.

269. Dar A, Munir S, Vishwanathan S, et al. Transcriptional analysis of avian embryonic tissues following infection with avian infectious bronchitis virus. *Virus research*. 2005;110(1):41–55.

270. Hillier LW, Miller W, Birney E, et al. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature*. 2004;432(7018):695–716.

271. Rothwell L, Young JR, Zoorob R, et al. Cloning and characterization of chicken IL-10 and its role in the immune response to Eimeria maxima. *The Journal of Immunology*. 2004;173(4):2675–2682.

272. Vazquez-Torres A, Xu Y, Jones-Carson J, et al. *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science*. 2000;287(5458):1655.

273. Gill N, Wlodarska M, Finlay BB. The future of mucosal immunology: studying an integrated system-wide organ. *Nature immunology*. 2010;11(7):558–560.

274. Toka FN, Gierynska M, Rouse BT. Codelivery of CCR7 ligands as molecular adjuvants enhances the protective immune response against herpes simplex virus type 1. *Journal of virology*. 2003;77(23):12742–12752.

275. Mascola JR, Stiegler G, VanCott TC, et al. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nature medicine*. 2000;6(2):207–210.

276. Sobko T, Schiött J, Ehlin A, et al. Neonatal sepsis, antibiotic therapy and later risk of asthma and allergy. *Paediatric and perinatal epidemiology*. 2010;24(1):88–92.

Chapter 3

Evaluation of Recovery of *Salmonella* from Trachea and Ceca in Commercial Poultry

For Publication in the Journal Entitled "*Poultry Science*"

Summary

Unpublished data from our laboratory suggest that the respiratory tract may be a viable portal of entry for *Salmonella* infection. Further, field reports have indicated that tracheal sampling can be a sensitive tool for monitoring for *Salmonella* infection in commercial flocks. In the present study, we conducted a series of field trials in North and South America to evaluate the association between cecal and tracheal recovery of *Salmonella* in chickens and turkeys from commercial flocks. In all trials, *Salmonella* was recovered from tracheal samples. Three of four trials in which both trachea and ceca were sampled, incidence of recovery was higher in tracheal samples. Though *Salmonella* was not recovered from ceca in trial 2, 5% of liver and spleen samples indicated infection. Environmental conditions were not associated with incidence of *Salmonella* recovery. These data suggest that tracheal contamination can be a good indicator of *Salmonella* infection under commercial conditions.

Introduction

Presently, our laboratory has hypothesized that tracheal sampling may be a viable method for detecting *Salmonella* contamination in poultry. Very recent research from our laboratory has suggested that tracheal inoculation is indeed possible, and that low levels of *Salmonella* administered directly into the trachea can cause systemic infection [1] [2]. Thus, if infection does occur through respiratory inoculation, *Salmonella* should be able to be isolated from the trachea and may be a reliable organ for detection purposes. The purpose of this study was to compare frequency of isolation of *Salmonella* between tracheal, cecal, and liver and spleen samples under a variety of environmental and commercial conditions in several countries in the Americas. Environmental humidity and temperature were measured to evaluate their effects on frequency of isolation from the organs.

Materials and Methods

Culture Methodologies

Conventional methodologies were used for isolation and enrichment of salmonellae in the laboratories from each of these trials. Trachea, ceca, and liver/spleen samples were aseptically collected for determination of *Salmonella* incidence. In trials 2, 3, and 5 samples were enriched with Tetrathionate enrichment broth (Tet, Catalog no. 210420, Becton Dickinson, Sparks, MD). Samples were pre-enriched in peptone water (Catalog no: 70179, Sigma) for 8 h, then enriched with double strength Tet in trials 1 and 4. Detection agars were McConkey agar (Catalog no: 221172, Becton Dickinson, Sparks, MD) in trial 1, Brilliant Green Agar (BGA) (Catalog no. 70134, Sigma) with 25µg/mL novobiocin (NO, catalog no. N-1628, Sigma) in trial 2; Xylose Lysine Deoxycholate agar (XLD, Catalog no: 221192, Becton Dickinson, Sparks, MD) with NO in trials 3 and 4; or Xylose Lysine Tergitol-4 agar (XLT4, Catalog no. 223419, BD Difco™) in trial 5. The presence or absence of typical lactose-negative colonies of *Salmonella* was determined after enrichment incubation at 37 °C, followed by selective plating on agar. In trials 1, 3 and 5, *Salmonella* serogroup was confirmed with poly-O *Salmonella*-specific antiserum (Catalog no: 226631, Becton Dickinson, Sparks, MD).

Field Trail 1

A diagnostic laboratory for a poultry company in Bucaramanga, Santander, Colombia participated in this study. Tracheas were sampled from broiler chickens of various ages with respiratory signs over a period of 16 months ranging from January 2012 to April 2013, involving conventional commercial broiler farms associated with the company. Average maximum and minimum temperature for the area was recorded to be in the range of 25.3 °C to 18.9 °C and the average air humidity was recorded to be 82% for the monitored period.

Field Trail 2

A commercial poultry company from Buenos Aires, Argentina participated in this study during December 2012, and involved four conventional commercial broiler farms housing 80,000 broiler chickens. Average maximum and minimum temperature for the area was recorded to be in the range of 28 °C to 21 °C and the average air humidity was recorded to be 63% for the mentioned period. Chickens were screened via meconium sampling in the hatchery for *Salmonella* and were deemed negative. On days 3 and 13 all farms were treated with a proprietary water applied nutritional supplement with potential to reduce *Salmonella*, increase flock health and increase production parameters. At 28 days of age, five chicks from each farm were cultured for *Salmonella* recovery in trachea, ceca, and liver/spleen.

Field Trail 3

A commercial broiler farm from Guanajuato, Mexico participated in this trial which was performed in the month of July 2012. Average maximum and minimum temperature for the area was recorded to be in the range of 18 °C and 28 °C and the average air humidity was recorded to be 65%. One-hundred 49 day old broiler chicks from the farm under study were cultured for enumeration of *Salmonella* incidence in ceca and trachea. Environmental samples including litter drag swabs and fan duct swabs were also collected for enumeration of *Salmonella* incidence. Standard drag swab technique was employed for the house, with subsequent enrichment and selective plating as described above. Drag swabs were assembled as previously described by Caldwell et al. 1994.[3] Standard sterile cotton swabs were used for taking drag

swabs of each fan duct in the house, with subsequent enrichment and selective plating, as described above.

Field Trail 4

A commercial turkey farm from Arkansas, USA was sampled in December 2011. Average minimum and maximum temperature for the area was recorded to be in the range of -2.89 °C and 4.88 °C, and the average air humidity was recorded to be 67 %. Sixteen week old female turkeys were cultured for *Salmonella* incidence in trachea and ceca from 100 turkeys.

Field Trail 5

A commercial broiler company from Arkansas USA participated in this study. The trial was performed during the months of January and February 2013 and involved six conventional commercial broiler farms. Average minimum and maximum temperature for the area was recorded to be in the range of -2.7 °C and 7.7 °C, and the average air humidity was recorded to at 69%. Chickens were screened in the hatchery for *Salmonella* and were deemed negative. At approximately 24 days of age, 25 chicks from each farm were cultured for *Salmonella* recovery in trachea, ceca, and liver/spleen.

Results and Discussion

Aerosolization is a traumatic process for most microorganisms, and survival can be dependent on the mechanisms of aerosolization, the climate into which these organisms are launched, the distance they are travelling, and time involved in the whole process. *Salmonella* has proven to be viable in laboratory-generated aerosols for more than 2 h [4]. As well, it has been shown that the death rate of *Salmonella* was influenced by the protective nature of the media during aerosolization, along with overall prevailing relative humidity and temperature of the air [5]. Environmental temperature and humidity, and temperatures within litter may have a role in supporting the continued survival of the organism in dust and aerosol generated in the poultry facility. In fact, humidity is known to play a major role in survivability of *Salmonella* in aerosols and dependence on humidity has known to be the characteristic of many Gram-negative organisms [6]. The average minimum and maximum temperatures and average humidity was

recorded for the location in which the field studies reported herein were conducted.

In the present study, five field trails were conducted to determine the recovery rate of *Salmonella* from tracheas of commercial poultry. Ceca and liver/spleen samples were also collected for comparison to traditionally accepted sampling methods of detection, as well as temperature and humidity measurements to determine the effects these parameters may have on the ability to detect *Salmonella* contamination in poultry under commercial conditions. All data for this report are represented in Table 1. In trial 1, conducted with a diagnostic laboratory of a poultry company from Bucaramanga, Colombia, a total of 1061 cases of broiler chickens with respiratory signs were tested for presence of *Salmonella*, of which 96/1061 (9.04%) of enriched tracheal samples were found to be positive. Serogroup determination showed 89/96 (92.7%) of the samples belonged to group B, confirmed to be *S.* Heidelberg, and 7/96 (7.3%) belonged to group A (data not shown). Trials 2 and 3 evaluated the presence of *Salmonella* in environmental samples, with enumeration of CFU/g of litter and incidence of *Salmonella* in litter through drag swabs, and approximately 10^4 CFU of *Salmonella* was estimated to be present per gram of litter in trial 2, along with 7/8 (87.5%) litter drag swabs and 5/10 (50%) fan duct swab samples being positive for *Salmonella* in trail 3 (data not shown). This correlated with the incidence of recovery of *Salmonella* from trachea of birds at 8/20 (40%) in trial 2 and 28/100 (28%) in trial 3. These data support previously described studies demonstrating the presence of *Salmonella* in aerosols and dust inhaled by poultry in grow-out barns [7 3 8 9 10 5 11]. Despite a low sample number in trial 2, relatively high incidence of 8/20 (40%) tracheal samples were positive for *Salmonella* which was similar to levels of 28/100 (28%) and 34/100 (34%) reported in trials 3 and 4, respectively. Tracheal recovery was low in trial 5, 3/150 (2%), despite ceca and liver/spleen incidence similar to other trials, suggesting that even low levels of tracheal contamination can be an indicator of infection. Additionally, despite previous reports of an association between high environmental temperature and humidity and increased *Salmonella* incidence [5 12 13 14], such a correlation was not noted in the present study. The highest temperatures and humidity, 18.9 – 25.3 °C and 82%, were recorded in trial 1, which had only 9.04% tracheal recovery of *Salmonella*. Additionally, trials 2, 3, and 4 each reported similar and the highest levels of *Salmonella* recovery, despite wide variations in temperature and humidity. In summary, in all trials evaluated in the present study, *Salmonella* was recovered from tracheal samples. These data support the hypothesis that tracheal

samples can be an indicator of *Salmonella* contamination, and such contamination is likely an indication of infection, as was evidenced by positive recovery in gastrointestinal and liver/spleen sampling.

References

1. Tellez G. Technologies for Salmonella Control in Live Production. In: ; 2013. Available at: http://www.thepoultryfederation.com/annual-events/salmonella-summit/speaker-presentations.

2. Tellez G. Detection of Salmonella from trachea in commercial poultry as an epidemiological tool. In: ; 2013. Available at: http://www.southernpoultrysciencesociety.org/businessmeeting.html.

3. Caldwell D, Hargis B, Corrier D, et al. Predictive value of multiple drag-swab sampling for the detection of Salmonella from occupied or vacant poultry houses. *Avian diseases*. 1994:461–466.

4. McDermid A, Lever M. Survival of Salmonella enteritidis PT4 and Salm. typhimurium Swindon in aerosols. *Letters in applied microbiology*. 1996;23(2):107–109.

5. Dungan RS. BOARD-INVITED REVIEW: Fate and transport of bioaerosols associated with livestock operations and manures. *Journal of animal science*. 2010;88(11):3693–3706.

6. Berrang M, Cox N, Bailey J. Measuring air-borne microbial contamination of broiler hatching cabinets. *The Journal of Applied Poultry Research*. 1995;4(1):83–87.

7. Venter P, Lues J, Theron H. Quantification of bioaerosols in automated chicken egg production plants. *Poultry science*. 2004;83(7):1226–1231.

8. Chinivasagam H, Tran T, Blackall P. Impact of the Australian litter re-use practice on< i> Salmonella</i> in the broiler farming environment. *Food Research International*. 2012;45(2):891–896.

9. Millner PD. Bioaerosols associated with animal production operations. *Bioresource technology*. 2009;100(22):5379–5385.

10. Fallschissel K, Klug K, Kämpfer P, Jäckel U. Detection of airborne bacteria in a German turkey house by cultivation-based and molecular methods. *Annals of Occupational Hygiene*. 2010;54(8):934–943.

11. Harbaugh E, Trampel D, Wesley I, et al. Rapid aerosol transmission of Salmonella among turkeys in a simulated holding-shed environment. *Poultry science*. 2006;85(10):1693–1699.

12. Chinivasagam H, Tran T, Maddock L, Gale A, Blackall P. Mechanically ventilated broiler sheds: a possible source of aerosolized Salmonella, Campylobacter, and Escherichia coli. *Applied and environmental microbiology*. 2009;75(23):7417–7425.

13. Wathes C, Howard K, Webster A. Survival of Escherichia coli in an aerosol at air temperatures of 15 and 30 C and a range of humidities. *Journal of hygiene*. 1986;97:489–496.

14. Hayter R, Besch E. Airborne-particle deposition in the respiratory tract of chickens. *Poultry science*. 1974;53(4):1507–1511.

Table 1: Evaluation of recovery of *Salmonella* in trachea, ceca, and liver/spleen in commercial chickens and turkeys

Field Trial and Location	Bird type	Average Temperature (°C)		Average % Humidity	*Salmonella* Recovery +/total (%)		
		Min	Max		Trachea	Ceca	Liver & Spleen
Trial 1 Bucaramanga, Colombia	Broiler	18.9	25.3	82	96/1061 (9.04%)	ND[1]	ND
Trial 2 Buenos Aires, Argentina	Broiler	21	28	63	8/20 (40%)	0/20 (0%)	2/20 (5%)
Trial 3 Guanajuato, Mexico	Broiler	18	28	65	28/100 (28%)	10/100 (10%)	ND
Trial 4 Arkansas, USA	Turkey	-2.9	4.9	67	34/100 (34%)	17/100 (17%)	ND
Trial 5 Arkansas, USA	Broiler	-2.7	7.7	69	3/150 (2%)	27/150 (18%)	11/150 (7.33%)

[1]ND = Not Determined

Appendix I – IACUC Approval

MEMORANDUM

TO: Lisa R. Bielke

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: June 9, 2011

SUBJECT: IACUC PROTOCOL **APPROVAL**
Expiration date **: June 2, 2014**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#11047- "EVALUATION OF DIRECT FED MICROBIALS AND PREBIOTICS FOR SALMONELLA CONTROL IN POULTRY**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes in the protocol during the research, please notify the IACUC in writing [Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **06-02-2014,** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

Appendix II – IACUC Approval

UNIVERSITY OF ARKANSAS

Office of Research Compliance

October 31, 2012

MEMORANDUM

TO:	Dr. Billy Hargis
FROM:	W. Roy Penney Institutional BioSafety Committee
RE:	IBC Protocol Approval
IBC Protocol #:	10012
Protocol Title:	"Experimental studies to evaluate probiotic cultures as alternatives"
Approved Project Period:	Start Date: September 24, 2012 Expiration Date: September 13, 2015

The Institutional Biosafety Committee (IBC) has approved the renewal of Protocol 10012, "Experimental studies to evaluate probiotic cultures as alternatives". 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.

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

Chapter 4

Evaluation of the Respiratory Route as a Viable Portal of Entry for *Salmonella* in Poultry via Intratracheal Challenge of *Salmonella* Senftenberg

For Publication in the Journal Entitled "*Poultry Science*"

Summary

In Experiment 1, a challenge-dose-related recovery incidence of *S.* Senftenberg (SS) from the ceca + cecal tonsils (CCT) and enumeration from cecal contents (CC) was observed 24h after intratracheal (IT) administration in 7-day chicks, with 33.33 % recovery with a low (10^4 CFU) challenge and 91.66 % with a high (3 x 10^6) CFU challenge. However, identical challenge levels through oral gavage (OR) produced no recovery of SS from the CCT after selective enrichment. Low levels of recovery from selectively enriched liver+spleen (LS) samples were observed only after high dose administration by either route. These results suggested that SS was far more likely to be recovered from CCT when IT-administered as compared to OR. In Experiment 2, considering that mucus coating of the SS through mucociliary clearance and subsequent ingestion was protective for the SS during upper gastrointestinal tract passage, we compared low (2 x 10^4 CFU), middle (4 x 10^6 CFU), or high (2 x 10^8 CFU) dose SS challenge by IT or OR administration, and administered the OR challenge with or without 1% porcine stomach mucin. Similar incidence and enumeration from CCT and CC was observed from within challenge dose groups by any route in this experiment, with the exception of lower recovery of SS from the CCT with the middle challenge group by OR+mucin. However, a significantly ($P<0.05$) higher recovery of SS from LS samples after selective enrichment was observed from chicks challenged by IT administration with the middle and high dose challenge groups, as compared to OR or OR+mucin administration at identical dosages. In this study, selective enrichment of lung tissue resulted in 41 to 92% recovery of SS in a challenge-dose-related manner when administered IT, whereas no SS was recovered from lung tissue in any group receiving SS challenge by OR or OR+mucin administration. These results suggest that IT administration is as, or more, effective at achieving colonization of the lower gastrointestinal tract of 7d chicks than OR or OR+mucin administration. There was a tendency to see more frequent SS recovery from LS 24h after IT administration for this SS isolate with relatively low invasiveness. Recovery from lung tissue 24h after IT administration suggests that IT administration was capable of delivering at least some of the challenge to the lower respiratory tract. Taken together, these data may suggest respiratory invasion and systemic transport of IT administered SS to the lower intestinal tract of 7d broilers.

Introduction

The frequent recovery of *Salmonella* from dust and bioaerosols from infected poultry is well documented [1] [2] [3] [4] [5], and bioaerosolized *Salmonella* of very small droplet size, capable of theoretically reaching the lower respiratory tract of chicks has been reported [6] [7] [8]. Published studies have also suggested that airborne transmission of *Salmonella* in poultry is possible [9] [10] [2]. While the usual assumption regarding airborne *Salmonella* transmission is that it would ultimately involve oral ingestion, the unique architecture of the avian lung, involving only phagocytosis of particles reaching the non-ciliated portions just below the proximal end of the secondary bronchi , and the propensity of *Salmonella* for epithelial translocation and systemic macrophage dissemination [11] [12] [13] [14] [15] [16] might suggest that the respiratory tract is an overlooked potential portal of entry for *Salmonella*e. In laboratory studies, relatively high dosages of *Salmonella* are usually required for chicks by one week of age and older, and yet efficient spread of *Salmonella* under field conditions sometimes suggests that the infectious dose must be smaller. We recently hypothesized that the respiratory tract might be an overlooked portal of entry for *Salmonella* infections in poultry. Presently, we have evaluated the ability of a relatively low-invasive isolate of *S.* Senftenberg (SS) [17] [18] for ability to colonize the ceca following oral *versus* intratracheal administration, as well as recovery from liver+spleen (LS) samples. We also evaluated recovery of this isolate from lung tissue 24h after challenge.

Materials and Methods

Experimental Animals

Day-of-hatch, off-sex broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR, USA) and were placed in isolators, in a controlled age-appropriate environment. Chickens were provided *ad libitum* access to water and a balanced unmedicated corn-soybean diet meeting the nutrition requirements of poultry recommended by National Research Council (NRC, 1994). All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas. A small number of chickens (n=12), for each trial, were humanely killed upon arrival, CCT, LS and trachea were

aseptically removed individually, cultured in tetrathionate (Tet) enrichment broth (Catalog no. 210420, Becton Dickinson, Sparks, MD) and confirmed negative for *Salmonella* by plating the samples on to Xylose Lysine Tergitol-4 (XLT4) (Catalog no. 223410, BD Difco™) selective media as previously described [19].

Salmonella Cultures

The SS challenge isolate was obtained from a field case submitted to our laboratory. For the present studies, 100µL of SS, from a frozen aliquot were added to 10 mL of Tryptic Soy Broth (TSB) (Catalog no. 211822, Becton Dickinson, Sparks, MD) and incubated at 37°C for 8 h. This was followed by three passages every 8 h into fresh TSB, for a total of 24 h, to ensure culture was in log phase. Post incubation, bacterial cells were washed 3 times in sterile 0.9% saline by centrifugation (1,864 × *g,* 4 °C, 15 min), quantified with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific) at 625nm and diluted in sterile 0.9% saline as per required concentrations (CFU/mL) for the trials. Concentrations of SS were determined retrospectively by serial dilution and further plating on to XLT4 selective media for enumeration of actual CFU/ml used for challenge, as reported below.

In experiment 2, one SS inoculum was prepared in a 1% suspension of mucin from porcine stomach, type II (Catalog no. M2378, Sigma) in 0.9% saline and the viscosity was measured with Brookfield cone and plate digital viscometer (Model - DVI Prime, Brookfield Engineering Laboratories, INC. MA USA), according to manufacturer's instructions, with an average viscosity of 2.1 Centipoises.

Challenge

All groups were challenged with *Salmonella* using sterile gavage needles with a 22 gauge 304 stainless steel assembly of tubing and ball (1.25 mm diameter), in a volume of 0.25 mL. Oral (OR) challenge was through crop gavage (0.25 mL). Care was taken, while challenging chickens intratracheally (IT), to accurately insert the gavage needle into the trachea as deep as possible and discharge the challenge near the bifurcation of the trachea.

Experiment 1

60 - Day of hatch, off-sex broiler chicks were obtained and randomly assigned to 4 groups (N=15 chickens) and placed in isolators by group with unrestricted access to feed and water. On day 7, treatment groups were challenged OR with a dose of 1 X 10^4 and 3 X 10^6 CFU of SS / chick, or IT with the same doses (Table 1). 24 h post-challenge, all chickens were humanely killed and 12 chicks / group were cultured for *Salmonella* recovery in selectively enriched CCT after and enumeration sampling from cecal contents as previously described (reference). The limit of detection employed was calculated to be 500 CFUs. If no colonies were recovered and the matched sample was positive for *Salmonella* post-enrichment the CFU count was recorded to be 2.7 Log_{10}.

Experiment 2

A similar experimental design was employed for Experiment 2, with minor variations. A total of 135, day of hatch off-sex broiler chicks were randomly assigned to 9 groups (N=15 chickens) and placed in individual isolators according to groups as described above (Table 2). On day 7, chicks were challenged with a 0.25 mL suspension of SS by OR administration (with or without mucin) at 2 X 10^4, 4 X 10^6 and 2 X 10^8 CFU of SS / chick, or by IT administration at the same doses. 24 h post challenge (day 8), all chicks were humanely killed and 12 chicks / group were cultured for *Salmonella* recovery. Cecal CFU enumeration followed by incidence of *Salmonella* for enriched CCT content, LS, as well as dissected lung samples were enriched for detection of recoverable SS as described above.

Data and Statistical Analysis

Data from all the trials were subjected to analysis of variance of Log_{10} CFU values (SS/g cecal content) (ANOVA, SAS, Cary, NC) using Duncan's multiple range test to separate significantly ($p \leq 0.05$) different means. Enrichment data were expressed as positive/total chickens (%) and the percent recovery of *Salmonella* was compared using the chi-squared test of independence, testing all possible combinations, to determine significant ($p \leq 0.05$) differences, described by Zar et. al. (1984).

Results and Discussion

In Experiment 1, a dose response relationship was observed in the rate of SS recovered from CCT post-enrichment, for groups that were challenged IT with SS (33.33 to 91.66%) as compared to 0 % recovery from OR challenged groups, and significantly more SS were recovered from IT challenged groups in a dose-related manner (Table 1). Low levels of recovery from selectively enriched LS samples were observed only after high dose administration by either route, consistent with previous reports suggesting the non-invasive properties of SS [20]. These results suggested that SS was far more likely to be recovered from CCT when IT-administered as compared to OR.

The increased recovery and enumeration from ceca following IT administration suggested that challenge by the IT route might be more effective at colonization of 7d chicks. It is possible that SS, reaching the non-ciliated regions of the respiratory tract, was able to either translocate or be directly phagocytized through several documented mechanisms for removing particulate matter from the non-ciliated avian respiratory tract [21 13 22]. However, we considered that it might also be possible that mucus exposure from the ciliated trachea [14 7] might be protective as some of the challenge inoculum was likely removed through the mucociliary elevator, followed by expectoration and swallowing. For this reason, an additional treatment group was evaluated in Experiment 2, which involved suspension in 1% mucin prior to OR challenge.

However, in Experiment 2, similar incidence and enumeration from CCT and CC was observed within challenge dose groups by any route in this experiment, with the exception of lower recovery of SS from the CCT with the middle challenge group by OR+mucin (Table 1). Similar to the results of Experiment 1, a significantly ($P<0.05$) higher recovery of SS from LS samples after selective enrichment was observed from chicks challenged by IT administration with the middle and high dose challenge groups, as compared to OR or OR+mucin administration at identical dosages in Experiment 2. The challenge SS isolate was recovered following enrichment of lung tissue only in the groups receiving IT challenge, suggesting that IT administration was capable of delivering at least some of the challenge to the lower respiratory tract where either cellular translocation or phagocytic removal are the only possibilities for

particulate removal [11 12 13 14 15 16], thus providing a potential mechanism for systemic infection with this relatively low invasive SS challenge. In support of this hypothesis, numerically or significantly ($P<0.05$) more frequent SS recovery from LS 24h after IT administration was observed after IT administration (Table 2).

While the mucin suspension used in experiment 2 was a heterologous porcine-origin product, and might not be equivalent to the exposure conditions of the chick trachea, suspension in 1% mucin did not increase infectivity at any dose of SS when compared to OR administration without mucin (Table 2). Taken together, these data indicate that IT administration was either more effective or at least as effective at colonizing the ceca of 7d chickens. The present study does not prove that bioaerosol transmission under commercial conditions is possible, but suggests that the respiratory tract could be a potential portal of entry, even for a relatively low-invasive serovar of *Salmonella*.

References

1. Lues J, Theron M, Venter P, Rasephei M. Microbial composition in bioaerosols of a high-throughput chicken-slaughtering facility. *Poultry science*. 2007;86(1):142–149.

2. Mitchell B, Buhr R, Berrang M, Bailey J, Cox N. Reducing airborne pathogens, dust and Salmonella transmission in experimental hatching cabinets using an electrostatic space charge system. *Poultry science*. 2002;81(1):49–55.

3. Dungan RS. BOARD-INVITED REVIEW: Fate and transport of bioaerosols associated with livestock operations and manures. *Journal of animal science*. 2010;88(11):3693–3706.

4. Fallschissel K, Klug K, Kämpfer P, Jäckel U. Detection of airborne bacteria in a German turkey house by cultivation-based and molecular methods. *Annals of Occupational Hygiene*. 2010;54(8):934–943.

5. Chinivasagam H, Tran T, Maddock L, Gale A, Blackall P. Mechanically ventilated broiler sheds: a possible source of aerosolized Salmonella, Campylobacter, and Escherichia coli. *Applied and environmental microbiology*. 2009;75(23):7417–7425.

6. Cox J, Pavic A. Advances in enteropathogen control in poultry production. *Journal of applied microbiology*. 2010;108(3):745–755.

7. Mensah GA, Brain JD. Deposition and clearance of inhaled aerosol in the respiratory tract of chickens. *Journal of applied physiology*. 1982;53(6):1423–1428.

8. Hayter R, Besch E. Airborne-particle deposition in the respiratory tract of chickens. *Poultry science*. 1974;53(4):1507–1511.

9. Harbaugh E, Trampel D, Wesley I, et al. Rapid aerosol transmission of Salmonella among turkeys in a simulated holding-shed environment. *Poultry science*. 2006;85(10):1693–1699.

10. Albrecht A, Redmann T, Nuchter H, et al. Airborne microorganisms in a rearing henhouse for layers during vaccination. *DTW.Deutsche tierarztliche Wochenschrift*. 2003;110(12):487–493.

11. Maina JN. Development, structure, and function of a novel respiratory organ, the lung-air sac system of birds: to go where no other vertebrate has gone. *Biological Reviews*. 2006;81(4):545–579.

12. Maina J. Some recent advances on the study and understanding of the functional design of the avian lung: morphological and morphometric perspectives. *Biological Reviews*. 2002;77(1):97–152.

13. Fedde M. Relationship of structure and function of the avian respiratory system to disease susceptibility. *Poultry science*. 1998;77(8):1130–1138.

14. Toth TE. Nonspecific cellular defense of the avian respiratory system: a review. *Developmental \& Comparative Immunology*. 2000;24(2):121–139.

15. Smialek M, Tykalowski B, Stenzel T, Koncicki A. Local immunity of the respiratory mucosal system in chickens and turkeys. *Polish Journal of Veterinary Sciences*. 2011;14(2):291–297.

16. Reese S, Dalamani G, Kaspers B. The avian lung-associated immune system: a review. *Veterinary research*. 2006;37(3):311–324.

17. Pedersen TB, Olsen JE, Bisgaard M. Persistence of Salmonella Senftenberg in poultry production environments and investigation of its resistance to desiccation. *Avian Pathology*. 2008;37(4):421–427.

18. Boumart Z, Roche SM, Lalande F, et al. Heterogeneity of persistence of Salmonella enterica serotype Senftenberg strains could explain the emergence of this serotype in poultry flocks. *PloS one*. 2012;7(4):e35782.

19. Chen C-Y, Nace GW, Irwin PL. A 6 X 6 drop plate method for simultaneous colony counting and MPN enumeration of< i> Campylobacter jejuni</i>,< i> Listeria monocytogenes</i>, and< i> Escherichia coli</i>. *Journal of Microbiological Methods*. 2003;55(2):475–479.

20. He L, Genovese K, Swaggerty C, Nisbet D, Kogut M. A comparative study on invasion, survival, modulation of oxidative burst and nitric oxide responses of macrophages (HD-11), and systemic infection of chickens by prevalent poultry Salmonella serovars. *Foodborne Pathogens and Disease*.

21. Fagerland JA, Arp LH. Structure and development of bronchus-associated lymphoid tissue in conventionally reared broiler chickens. *Avian diseases*. 1993:10–18.

22. Fagerland JA, Arp LH. Distribution and quantitation of plasma cells, T lymphocyte subsets, and B lymphocytes in bronchus-associated lymphoid tissue of chickens: age-related differences. *Regional immunology*. 1993;5(1):28–36.

Table1. Experiment 1 - Evaluation of intratracheal challenge of chickens with *Salmonella* Senftenberg

Dose	Route of Challenge	Log_{10} SS / gram of ceca content	Ceca - Cecal Tonsil	Liver and Spleen
$1 X 10^4$	OR	0.000 ± 0.000^{c}	$0/12 (0\%)^{d}$	$0/12 (0\%)^{b}$
	IT	0.900 ± 0.384^{b}	$4/12 (33.33\%)^{c}$	$0/12 (0\%)^{b}$
$3 X 10^6$	OR	0.000 ± 0.000^{c}	$0/12 (0\%)^{d}$	$1/12 (8.33\%)^{ab}$
	IT	2.474 ± 0.225^{a}	$11/12 (91.66\%)^{a}$	$2/12 (16.66\%)^{ab}$

Experiment 1: Chicks were challenged with *Salmonella* senftenberg (SS) on day 7 either intratracheally (IT) or orally (OR). 24 hrs post-challenge, ceca+cecal tonsils were enumerated to determine Log_{10} SS/ gram recovery and selectively enriched for recovery. Enumeration data are expressed as mean ± SE. Recovery incidence data are expressed as positive/total for each tissue sampled (%). Different superscripts within columns indicate significant differences $P<0.05$, N=12 / group

Table2. Experiment 2 - Evaluation of intratracheal versus oral challenge of *Salmonella* Senftenberg in chicks

Dose	Route of Challenge	Log_{10} SS / gram of ceca content	Ceca - Cecal Tonsil	Liver and Spleen	Lung
$2 X 10^4$	OR	0.675 ± 0.352 ^{abc}	3/12(25%) ^{abc}	0/12(0%) ^{c}	0/12(0%) ^{c}
	IT	0.675 ± 0.352 ^{abc}	3/12(25%) ^{abc}	1/12(8.33%) ^{c}	5/12(41.67%) ^{b}
	OR + M	0.225 ± 0.225 ^{bc}	1/12(8.33%) ^{b}	0/12(0%) ^{c}	0/12(0%) ^{c}
$4 X 10^6$	OR	0.675 ± 0.352 ^{abc}	3/12(25%) ^{abc}	2/12(16.67%) ^{bc}	0/12(0%) ^{c}
	IT	0.900 ± 0.384 ^{abc}	4/12(33.33%) ^{ab}	6/12(50%) ^{b}	8/12(66.67%) ^{ab}
	OR + M	0.000 ± 0.000 ^{c}	0/12(0%) ^{c}	0/12(0%) ^{c}	0/12(0%) ^{c}
$2 X 10^8$	OR	1.574 ± 0.401 ^{a}	7/12(58.33%) ^{a}	2/12(16.67%) ^{bc}	0/12(0%) ^{c}
	IT	1.125 ± 0.401 ^{ab}	5/12(41.67%) ^{ab}	11/12(91.67%) ^{a}	11/12(91.67) ^{a}
	OR + M	1.574 ± 0.401 ^{a}	7/12(58.33%) ^{a}	1/12(8.33%) ^{c}	0/12(0%) ^{c}

Experiment 2: Chicks were challenged with *Salmonella* senftenberg (SS) on day 7 either intratracheally (IT), orally (OR), or OR in a 0.1% mucin suspension. 24 hrs post-challenge, ceca+cecal tonsils were enumerated to determine Log_{10} SS/ gram recovery and selectively enriched for recovery. Enumeration data are expressed as mean ± SE. Recovery incidence data are expressed as positive/total for each tissue sampled (%). Different superscripts within columns indicate significant differences $P<0.05$, N=12/group

Appendix I – IACUC Approval

MEMORANDUM

TO: Lisa R. Bielke

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: June 9, 2011

SUBJECT: IACUC PROTOCOL **APPROVAL**
Expiration date **: June 2, 2014**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#11047- "EVALUATION OF DIRECT FED MICROBIALS AND PREBIOTICS FOR SALMONELLA CONTROL IN POULTRY**". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes in the protocol during the research, please notify the IACUC in writing [Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **06-02-2014,** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

Appendix II – IACUC Approval

UNIVERSITY OF ARKANSAS

Office of Research Compliance

October 31, 2012

MEMORANDUM

TO:	Dr. Billy Hargis
FROM:	W. Roy Penney Institutional BioSafety Committee
RE:	IBC Protocol Approval
IBC Protocol #:	10012
Protocol Title:	"Experimental studies to evaluate probiotic cultures as alternatives"
Approved Project Period:	Start Date: September 24, 2012 Expiration Date: September 13, 2015

The Institutional Biosafety Committee (IBC) has approved the renewal of Protocol 10012, "Experimental studies to evaluate probiotic cultures as alternatives". 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.

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

Chapter 5

Evaluation of Respiratory Route as a Viable Portal of Entry for *Salmonella* in Poultry via Intratracheal Challenge of *Salmonella* Enteritidis and *Salmonella* Typhimurium

For Publication in the Journal Entitled, "*Poultry Science*"

Summary

Experimental and epidemiological evidence suggests that primary infection of *Salmonella* is by oral fecal route for poultry. However, the airborne transmission of *Salmonella* and similar enteric zoonotic pathogens has been historically neglected. Studies involving *Salmonella* bioaerosol production in combination with suggestions of vulnerable avian respiratory architecture indicate the respiratory route as a possible portal of entry for *Salmonella* for poultry. Presently, we evaluated this hypothesis through intratracheal (IT) administration of SE and ST, in comparison with oral administration, in a total of 5 separate trials, with subsequent enumeration of colony forming units (CFU) recovery in ceca-cecal tonsils (CCT) and recovery incidence in liver and spleen (LS). In all trials, IT administration of both SE and ST, colonized cecae to a similar or greater extent than oral administration at identical challenge levels. Chickens cultured for CFU enumeration from IT-challenged chicks at same dose as orally challenged, resulted in an increase of 1.5 log higher SE from CCT. Further, a much lower dose IT of SE could colonize ceca to the same extent than a higher oral-challenge. This trend of increased cecal colonization due to IT challenge was observed with all trails involving week-old birds, which are widely considered to be more difficult to infect via the oral route. Liver-spleen incidence data revealed 33% of LS positive samples when SE was administered IT at 10^6 CFU / Chick, as compared to 0% when administered OR. Collectively, these data may suggest that the respiratory tract may be a largely-overlooked portal of entry for *Salmonella* infections in chickens.

Introduction

There is good experimental and epidemiological evidence that primary infection of *Salmonella* is by the oral fecal route, along with an established infectious prerequisite dose [1 2 3]. Although the common route of transmission for many zoonotic pathogens such as *Salmonella* is direct ingestion, the inhalation of infectious particles should also be considered[4]. Overall, the potential airborne transmission of zoonotic enteric pathogens such as *Salmonella* has been largely neglected, even though several studies have suggested that airborne transmission of *Salmonella* is possible [5 6 7].

Current knowledge of *Salmonella* bioaerosol generation, fate and transport, at various stages of commercial poultry production have been studied with an emphasis on survivability of *Salmonella* in these bioaerosols as a means to assess the transport and subsequent risk of exposure and infection of poultry [8 9 10 11 12 13]. Reports have demonstrated that fan driven spread of *Salmonella* within the hatching cabinet and hatchery incubator to be possible [14].

Modern poultry production with densely stocked and enclosed production buildings is often accompanied by movement of large volumes of air through the house, by negative pressure, to improve ventilation and environmental conditions for broiler growth, and can clearly carry pathogens such as *Salmonella* in the form of bioaerosols and or contaminated dust [15 16 17 18 19 20 21].

Salmonella contaminated or infected live poultry entering the processing plant are the primary source of processing-plant contamination, which may affect the microbial quality of the end product [22 23 24]. In this regard, Harbaugh et al. hypothesized that rapid airborne *Salmonella* infection (less than 4 h) occurs in turkeys and prior to slaughter, suggesting the prevalence of airborne *Salmonella* in commercial poultry [5]. They predicted multiple points of increased exposure prior to slaughter, may occur when birds are taken off feed for 3 to 6 h before loading. This can increase the flock's contact with *Salmonella* through increased ingestion of feces or through increased aerosolization due to scratching of the litter in the barn in search of food [25 26]. Thus, increased *Salmonella*-laden dust in the air may increase the risk of infection due to inhalation of *Salmonella* bioaerosols. Similarly, large amounts of dust generated during the load-out process and improper cleaning and disinfection of crates are also likely to contaminate chickens [25].

Finally, once turkeys arrive at the slaughter plant, they are kept in cooling sheds until shackled. Large fans blow air on the turkeys to keep them cool during the summer months. Turkeys arriving at the cooling sheds may harbor *Salmonella* and air flow may transfer the organism to the environment and to uninfected bird. These events occur in the last few hours, and all increase exposure to *Salmonella* laden dust [5].

Survivability and bioaerosol size may be of importance, as is the dose which is actually delivered to the respiratory tract. *Salmonella* has shown to be viable in laboratory-generated aerosols for more than 2 - 4 h depending on overall prevailing relative humidity and temperature of the air, strain of the bacteria [27 6 14 11]. A detailed study by Hayter and Besch [28] showed that the site of deposition of particles critically depends on their size. Large inspired particles with a size of 4 - 7 µm gets trapped and removed by the mucociliary ladder, but particles smaller than this is deposited at deeper areas. Aerosols of size < 4 µm should be able to carry *Salmonella*, considering its typical diameters of around 0.7 to 1.5 µm and lengths from 2 to 4 µm, hence would not face the mucociliary challenge. In this regard, several studies have examined particle analysis, to determine the relationship between the particles in the air, both size and number, and dose of *Salmonella,* with estimates ranging from 10 CFU to 1.2 X 10^4 cfu of *Salmonella* per m^3 of air, depending on study conditions [29 5 30 31 27 6 32].

Mucociliary clearance is the predominant physio-mechanical apparatus involved in the pathogen clearance, up to the proximal end of the secondary bronchi [33 34 35 36 37]. However, a number of factors during poultry production can affect mucociliary clearance, including ammonia exposure, *Mycoplasma gallisepticum* (MG) / Infectious Bronchitis Virus (IBV) / *Bordetella* infections, resulting in more inspired particles/pathogens reaching the lower respiratory tract [38 39 40 13].

In this regard, our laboratory has recently hypothesized that transmission by the fecal-respiratory route may be a viable portal of entry for *Salmonella* and could explain some clinical impressions of relatively low-dose infectivity under field conditions in relation to the requisite high oral challenge dose that is typically required for infection of poultry through the oral route in laboratory studies. Presently, we compared intratracheal (IT) with oral administration of two pathogenic *Salmonella* serovars, *Salmonella*

Enteritidis (SE) and *Salmonella* Typhimurium (ST), with relatively different invasiveness, on ability to colonize the ceca and liver and spleen.

Materials and Methods

Experimental Animals

Day-of-hatch, off-sex broiler chickens were obtained from Cobb-Vantress (Siloam Springs, AR, USA) and placed in isolators, in a controlled age-appropriate environment. Chickens were provided *ad libitum* access to water and a balanced unmedicated corn-soybean diet meeting the nutrition requirements of poultry recommended by National Research Council (NRC, 1994). All animal handling procedures were in compliance with Institutional Animal Care and Use Committee at the University of Arkansas. Twelve chickens for each trial were humanely killed upon arrival at the laboratory, and ceca-cecal tonsils, liver & spleen and trachea were aseptically removed, cultured in tetrathionate (Tet) enrichment broth (Catalog no. 210420, Becton Dickinson, Sparks, MD) and confirmed negative for *Salmonella* by plating the samples on to selective Brilliant Green Agar (BGA) (Catalog no. 70134, Sigma) with novobiocin (NO) (25µg/mL, catalog no.N-1628, Sigma).

Salmonella Cultures

SE and ST served as challenge pathogens, both originally obtained as primary poultry isolates from the USDA National Veterinary Services Laboratory (Ames, Iowa). These isolates were resistant to novobiocin and were selected for resistance to naladixic acid (NA) (20µg/mL, catalog no.N-4382, Sigma). For the present studies, 100µL of SE or ST (depending on the trial) from a frozen aliquot were added to 10 mL of Tryptic Soy Broth (TSB) (Catalog no. 211822, Becton Dickinson, Sparks, MD) and incubated at 37°C for 8 h. This was followed by three passages every 8 h into fresh TSB, for a total of 24 h, to ensure log phase growth. Post incubation, bacterial cells were washed 3 times in sterile 0.9% saline by centrifugation (1,864 × *g,* 4 °C, 15 min), quantified with a spectrophotometer (Spectronic 20D+, Spectronic Instruments Thermo Scientific) at 625nm using an established concentration curve, and diluted in sterile 0.9% saline as per required concentrations (CFU/mL) for the trials. Concentrations of SE and ST were also

determined retrospectively by serial dilution and further plating on to BGA NO-NA for enumeration of actual CFU/ml used for challenge, as reported below.

Challenge

All groups were challenged with *Salmonella* using sterile gavage needles with a 22 gauge 304 stainless steel assembly of tubing and smooth terminal ball (1.25 mm diameter), in a volume of 0.25 mL. Oral (OR) challenge was through crop gavage. Care was taken, while challenging chickens intratracheally (IT), to accurately insert the gavage needle into the trachea as deep as possible and then discharge desired volume of challenge near the primary bronchi.

Experimental Design

A set of 3 experiments were carried out with two different *Salmonella* serovars, varying in their invasiveness, to evaluate respiratory route as a viable portal of entry in comparison with the established oral route. A highly invasive SE bacteriophage type 13A was used for experiment 1 (single trial) and experiment 2 (total of 3 trials). For experiment 3 (single trial), a relatively less invasive ST was used as the challenge pathogen. Chickens were challenged either OR or IT followed by enumeration of CFU in ceca and incidence of *Salmonella* in trachea and liver + spleen samples as described below.

Experiment 1

For this preliminary trial, 45 - day of hatch, off-sex broiler chicks were obtained, randomly selected, divided into 3 groups (N=15 chickens) and placed in individual isolators. Group 1 was challenged orally OR with a dose of 3 X 10^4 CFU of SE / chick, group 2 and 3 were challenged IT with 1.5 X 10^2 and 3 X 10^4 CFU of SE / chick. A total of 0.25 ml was used for both IT and OR administration. All chickens were placed in individual isolators according to groups with unrestricted access to feed and water. 24 h post challenge, all chickens were humanely killed and 12 chicks / group were cultured for *Salmonella* recovery in cecae. Briefly, CCT were aseptically removed, collected in sterile bags, homogenized, weighed and 1:4 w/v dilutions were made with sterile 0.9% saline. Tenfold dilutions of these samples were plated on BGA with NO and NA, incubated at 37 °C for 24 h to enumerate total *Salmonella* colony forming units (CFU's).

Further, the cecal samples were enriched in a double strength tetrathionate (Tet) broth and incubated for 24 h at 37 °C. Also, liver-spleen and tracheal samples were aseptically collected in 10 mL of Tet broth for enrichment and incubated at 37 °C for 24 h. Following this, Cecal, liver-spleen (LS) and tracheal enrichment samples were plated on BGA with NO and NA plates and incubated at 37°C for 24 h to confirm presence/absence of typical lactose-negative colonies of *Salmonella.*

Experiment 2

A total of 3 trials were carried out in the second experiment with a similar experimental design as of the preliminary trial. For trial 1, a total of 60, day of hatch off-sex broiler chicks were obtained, randomly selected and divided into 4 groups (N=15 chickens). All chickens were placed in individual isolators according to groups with unrestricted access to feed and water. On day 7, all groups were challenged with a 0.25 mL suspension of SE, with group 1 and 3 challenged OR with a dose of 1×10^4 and 1.5×10^6 CFU of SE / chick; group 2 and 4 challenged IT with 1×10^4 and 1.5×10^6 CFU of SE / chick. 24 h post challenge (day 8), all chickens were humanely killed and 12 chicks / group were cultured for only incidence of *Salmonella* for cecae and liver-spleen as described above.

For trial 2, a total of 90, day of hatch off-sex broiler chicks were obtained, randomly selected and divided into 6 groups (N=15 chickens). All chicks were placed in individual isolators according to groups, with unrestricted access to feed and water. On day 7, all groups were challenged with a 0.25 mL suspension of SE, with group 1, 3 and 5 challenged OR with a dose of 1.5×10^4 and 2×10^6 and 1×10^8 CFU of SE / chick, group 2, 4 and 6 were challenged IT at same doses, respectively. 24 h post challenge (day 8), all chickens were humanely killed and cultured for *Salmonella* recovery in cecae. Cecal CFU enumeration and incidence of *Salmonella* for cecae, liver-spleen and tracheal samples were determined in the same manner as that of experiment 1.

For trial 3, exactly the same procedure was followed, however the doses used to challenge were 2×10^4 for group 1 & 4, 1×10^6 for groups 2 & 5 and 2×10^8 CFU of SE / chick for groups 3 & 6 respectively, given OR or IT.

Experiment 3

For experiment 3, a relatively less invasive ST served as the challenge isolate. A total of 90, day of hatch off-sex broiler chicks were obtained, randomly selected and divided into 6 groups (N=15 chickens). On day 7, all groups were challenged with a 0.25 mL suspension of ST, with group 1, 3 and 5 challenged OR with a dose of 1.5×10^4 and 2.5×10^6 and 1×10^8 CFU of ST / chick, group 2, 4 and 6 were challenged IT at same doses respectively. 24 h post challenge (day 8), all chickens were humanely killed and cultured for *Salmonella* recovery. Enumeration of CFU for cecae was not performed for this trial, while the incidence of *Salmonella* for cecae, liver-spleen and tracheal samples were determined in manner described above.

Data and Statistical Analysis

Numerical data from all the trials were subjected to analysis of variance (ANOVA, SAS, Cary, NC). Log_{10} CFU values of *Salmonella* / gram of ceca were expressed as mean ± standard error of mean and deemed significant if $p \leq 0.05$. The data were also subjected to mean separation using Duncan's multiple range test significance. The enrichment data were expressed as positive/total chickens (%) and the percent recovery of *Salmonella* was compared using the chi-squared test of independence, testing all possible combinations to determine the significance ($p \leq 0.05$) for these studies (Zar et. al., 1984)

Results and Discussion

Data of Experiment 1 are presented in table 1. Chicks challenged IT could colonize CCT within 24 h of challenge, indicating the respiratory route is as viable as that of the oral route under these experimental conditions. Although there were no significant differences in colonization rates in this experiment, CFU enumeration from group 3 challenged IT at same dose (3×10^4 CFU / Chick) as that of group 1 (OR), resulted in numerical 1.5 log - higher recovery of SE from CCT, with values 7.76 ± 0.23 and 6.25 ± 0.76 respectively for IT and OR. Further, group 2, which was challenged with a much lower dose IT (1.2×10^2 CFU / Chick) of SE could colonize ceca to the same (or to a numerically higher) extent than that of group

1 challenged at a higher dose OR (3 X 10^4 CFU / Chick), with values 6.88 ± 0.41 and 6.25 ± 0.76 respectively for IT and OR.

Chickens at a younger age, especially hatchlings, are very susceptible to *Salmonella* infection [41 42 43 44 45 46 47 48 49 50], however, this trial shows that they can be infected via the respiratory route with around 100 cells. Further, given at such a low dose, the cecal colonization was still equivalent to that recovered from a higher OR challenge. These results may be important, considering a report [14] demonstrating fan driven spread of *Salmonella* within the hatching cabinet and hatchery incubator, which conventionally would be considered to settle on dust, fluff and water droplets, which then ingested by these birds would lead to oral infection. Considering hatchlings will spend up to 48 hrs before placement in commercial brooding houses, respiratory transmission may be a possibility.

Experiment 2 - trial 1 was performed to extend the evaluation with relatively older chickens, the results of which are provided in table 2. The incidence of *Salmonella* infection was compared using two routes of challenge in week old chicks. At a lower dose of approximately 10^4, the incidence of cecal colonization and positive LS were only numerically higher as compared to zero positive samples in OR challenged chicks. At the higher dose of 10^6, positive CCT samples were higher (10/12 - 83.33%) for OR route of challenge compared to that of IT (6/12 – 50%)

Experiment 2 - trial 2 and 3 was an extension of trial 1, evaluating the same objective at three different doses, in week old birds, and the data are shown in table 3 and 4. In trial 2, the ceca CFU recovery of *Salmonella* at lowest (10^4) and highest dose (10^8) were similar, if not higher. However, it was interesting to note a significantly higher cecal recovery for the intermediate dose (10^6) given IT, with values 1.86 ± 0.40 CFU / g for OR and 3.20 ± 0.17 for IT. Overall, we observed a clear dose response curve with the IT groups as compared to groups challenged OR. The CFU recovery data for trial 3 was similar to trial 2, with numerically lower CFUs recovered and there was no significant difference in recovery at the intermediate dose as seen before. When incidence of recovery from cecal tonsils were compared, there was significantly greater recovery of SE from the CCT following enrichment in the group receiving the lowest challenge of SE (10^4 CFU) IT as compared to OR. Similarly, a significantly higher recovery of SE

was observed following enrichment from LS samples at the intermediate and highest challenge doses administered by IT as compared to OR. As might be expected, tracheal recovery at each challenge level was significantly higher following IT challenge as compared to OR. However, it is interesting to note that SE was recovered from some tracheal samples following OR challenge with each of the challenge levels.

Historically, we have experienced difficulty consistently infecting older chickens with low doses of *Salmonella* in our laboratory. While not proven by these experiments, the possibility that respiratory transmission is apparently more effective might explain the relative difficulty in consistently infecting chicks via oral administration under laboratory conditions, as opposed to the apparent ease of transmission under commercial conditions, is an intriguing concept. The ability of *Salmonella* to infect chickens at lower doses (as low as 100 cells) via the respiratory route needs to be further investigated, nevertheless, this could support the studies (discussed above), establishing the relationship between the particles, size and number of particles relative to the concentration of *Salmonella* generated in the air. These studies estimated the concentration of airborne *Salmonella* to be up to $3.3 \times 10^2 - 1.2 \times 10^4$ cfu m^3 of air [32 30 31 27 6]. Thus, it is conceivable that under field conditions airborne *Salmonella* concentrations are able to infect poultry by this route of infection. The minimum infectious dose of *Salmonella* to infect chickens via the respiratory route in our study was similar to that found to produce disease in sentinel mice (150 CFU of *S. Typhimurium*) [6].

Furthermore, previous studies to date have not described the subsequent fate of *Salmonella* infecting the respiratory system, while evaluating the infection via the respiratory route [51 52 5 6 7]. In this regard, both the cecal CFU recovery data and LS incidence data from the present experiments provided evidence that *Salmonella* administered IT could colonize ceca, potentially involving a systemic route, as suggested by the increased recovery from liver and spleen samples. In Experiment 2 - trial 1, 33% of LS samples were positive for SE given IT as compared to 0% given OR. The incidence data for trial 2 and 3 displayed a similar trend as that of the preliminary trials. Significantly higher positive LS were observed at all 3 IT challenged doses, with 6/12 (50%), 10/12 (83.33%) and 11/12 (91.66%) positive LS samples as compared to OR challenged groups with 0/12 (0%), 2/12 (16.66%) and 1/11 (9.09%) positive samples, for respective doses, all together indicating systemic organ invasion of *Salmonella* post IT challenge. Taken

together, the results from this study may suggest that systemic infection, leading to biliary clearance, was responsible for the intestinal infections from the IT-challenged chicks, although other mechanisms are possible.

One mode by which *Salmonella* could infect and systemically spread can be by entering the blood directly and causing bacteremia. This mode is quite conceivable considering previous studies proposing disrupted epithelial barrier of the developing BALT structure, endocytosis by epithelial cells and further transport to the interstitial matrix and the high prevalence of BGBBs and EECCs [53 54 51 55]. Alternatively, various reports have described the presence of an efficient phagocytic system in chickens, involving phagocytic epithelial lining, interstitial macrophages in the BGB and the infiltrating free avian respiratory macrophages (FARMs) [36 56 51 55 57 58 59 60]. *Salmonella,* having an established intracellular lifestyle and known to systemically disseminate via macrophages when given orally [61 62], could potentially infect chickens via a similar pathway following respiratory exposure.

Further, there have been speculations that *Salmonella* infecting via the respiratory route might acquire increased virulence traits and hence infect chickens efficiently at low doses, when compared to oral route. For example, Leach et al. observed a marked increase in reported cases of human Salmonellosis caused by a multi-antibiotic-resistant strain of ST definitive type (DT) 104, in England and Wales over a period of 1995 to 1998 [63]. They hypothesized and successfully tested the infection via the aerosol route and were able to reproduce desired infection, where in the frequency of *Salmonella* isolation from eggs rose from 2.1% following oral challenge to 14% to 25%, and the frequency of isolation from muscle rose from 0% to 27% following aerosol infection. Based on their observations, they suggested a greater virulence of the pathogen when given by aerosol. Even though this may suggest increased virulence, it is entirely possible that higher rates of egg and muscle tissue contamination is more to do with the respiratory route being a much simpler and more vulnerable route for *Salmonella* entry rather than necessarily involving virulence. Experiment 3, employing a relatively less enteroinvasive ST as the challenge pathogen, provided support in this regard. Although comparing between experiments, cecal and LS incidence at all doses given IT, were numerically lower for ST, as compared to SE. Nevertheless, IT administration was equally effective for infecting the CCT of chickens as compared to OR, at all challenge doses (Table 5).

Overall, our data suggests that the respiratory route might be a viable portal of entry for *Salmonella* in poultry. Ongoing studies are aimed at further evaluation of this hypothesis using bioaerosol administration. Clarification of the potential importance of the respiratory tract for *Salmonella* transmission under field conditions may be of critical importance as efforts to develop intervention strategies to reduce transmission of these pathogens in poultry.

References

1. Galanis E, Wong DMLF, Patrick ME, et al. Web-based surveillance and global Salmonella distribution, 2000-2002. *Emerging infectious diseases*. 2006;12(3):381.

2. White P, Baker A, James W. Strategies to control Salmonella and Campylobacter in raw poultry products. *Revue scientifique et technique (International Office of Epizootics)*. 1997;16(2):525.

3. Blaser MJ, Newman LS. A review of human salmonellosis: I. Infective dose. *Review of infectious diseases*. 1982;4(6):1096–1106.

4. López FE, de las Mercedes Pescaretti M, Morero R, Delgado MA. Salmonella Typhimurium general virulence factors: A battle of David against Goliath? *Food Research International*. 2012;45(2):842–851.

5. Harbaugh E, Trampel D, Wesley I, et al. Rapid aerosol transmission of Salmonella among turkeys in a simulated holding-shed environment. *Poultry science*. 2006;85(10):1693–1699.

6. Wathes C, Zaidan W, Pearson G, Hinton M, Todd N. Aerosol infection of calves and mice with Salmonella typhimurium. *Veterinary Record*. 1988;123(23):590–594.

7. Oliveira C, Carvalho L, Garcia T, others. Experimental airborne transmission of Salmonella Agona and Salmonella Typhimurium in weaned pigs. *Epidemiology and infection*. 2006;134(1):199.

8. Cox J, Pavic A. Advances in enteropathogen control in poultry production. *Journal of applied microbiology*. 2010;108(3):745–755.

9. Jarquin R, Hanning I, Ahn S, Ricke SC. Development of rapid detection and genetic characterization of Salmonella in poultry breeder feeds. *Sensors*. 2009;9(7):5308–5323.

10. Fallschissel K, Klug K, Kämpfer P, Jäckel U. Detection of airborne bacteria in a German turkey house by cultivation-based and molecular methods. *Annals of Occupational Hygiene*. 2010;54(8):934–943.

11. Dungan RS. BOARD-INVITED REVIEW: Fate and transport of bioaerosols associated with livestock operations and manures. *Journal of animal science*. 2010;88(11):3693–3706.

12. Douwes J, Thorne P, Pearce N, Heederik D. Bioaerosol health effects and exposure assessment: progress and prospects. *Annals of Occupational Hygiene*. 2003;47(3):187–200.

13. Brooks J, McLaughlin M, Scheffler B, Miles D. Microbial and antibiotic resistant constituents associated with biological aerosols and poultry litter within a commercial poultry house. *Science of the total environment*. 2010;408(20):4770–4777.

14. Berrang M, Cox N, Bailey J. Measuring air-borne microbial contamination of broiler hatching cabinets. *The Journal of Applied Poultry Research*. 1995;4(1):83–87.

15. Baykov B, Stoyanov M. Microbial air pollution caused by intensive broiler chicken breeding. *FEMS microbiology ecology*. 1999;29(4):389–392.

16. Gast RK, Mitchell BW, Holt PS. Detection of airborne Salmonella enteritidis in the environment of experimentally infected laying hens by an electrostatic sampling device. *Avian Diseases*. 2004;48(1):148–154.

17. Heber AJ, Peugh MW, Lutgring KR, Zimmerman NJ, Linton RH. Poultry slaughtering plants: concentrations of microbial aerosols in poultry slaughtering and processing plants. *ASHRAE Transactions*. 2006:644–655.

18. Davies R, Wray C. An approach to reduction of Salmonella infection in broiler chicken flocks through intensive sampling and identification of cross-contamination hazards in commercial hatcheries. *International journal of food microbiology*. 1994;24(1):147–160.

19. Bull S, Allen V, Domingue G, et al. Sources of Campylobacter spp. colonizing housed broiler flocks during rearing. *Applied and Environmental Microbiology*. 2006;72(1):645–652.

20. Byrd J, DeLoach J, Corrier D, Nisbet D, Stanker L. Evaluation of Salmonella serotype distributions from commercial broiler hatcheries and grower houses. *Avian Diseases*. 1999:39–47.

21. Chinivasagam H, Tran T, Maddock L, Gale A, Blackall P. Mechanically ventilated broiler sheds: a possible source of aerosolized Salmonella, Campylobacter, and Escherichia coli. *Applied and environmental microbiology*. 2009;75(23):7417–7425.

22. McCapes R, Osburn B, Riemann H. Safety of foods of animal origin: model for elimination of Salmonella contamination of turkey meat. *Journal of the American Veterinary Medical Association*. 1991;199(7):875–882.

23. Clouser C, Doores S, Mast M, Knabel S. The role of defeathering in the contamination of turkey skin by Salmonella species and Listeria monocytogenes. *Poultry science*. 1995;74(4):723–731.

24. Mitchell B, Buhr R, Berrang M, Bailey J, Cox N. Reducing airborne pathogens, dust and Salmonella transmission in experimental hatching cabinets using an electrostatic space charge system. *Poultry science*. 2002;81(1):49–55.

25. Corrier D, Byrd J, Hargis B, et al. Presence of Salmonella in the crop and ceca of broiler chickens before and after preslaughter feed withdrawal. *Poultry science*. 1999;78(1):45–49.

26. Ramirez G, Sarlin L, Caldwell D, et al. Effect of feed withdrawal on the incidence of Salmonella in the crops and ceca of market age broiler chickens. *Poultry science*. 1997;76(4):654–656.

27. McDermid A, Lever M. Survival of Salmonella enteritidis PT4 and Salm. typhimurium Swindon in aerosols. *Letters in applied microbiology*. 1996;23(2):107–109.

28. Hayter R, Besch E. Airborne-particle deposition in the respiratory tract of chickens. *Poultry science*. 1974;53(4):1507–1511.

29. Radon K, Danuser B, Iversen M, et al. Air contaminants in different European farming environments. *Annals of Agricultural and Environmental Medicine*. 2002;9(1):41–48.

30. Fallschissel K, Kämpfer P, Jäckel U. Direct detection of salmonella cells in the air of livestock stables by real-time PCR. *Annals of Occupational Hygiene*. 2009;53(8):859–868.

31. Lever M, Williams A. Cross-infection of chicks by airborne transmission of Salmonella enteritidis PT4. *Letters in applied microbiology*. 1996;23(5):347–349.

32. Baskerville A, Humphrey T, Fitzgeorge R, et al. Airborne infection of laying hens with Salmonella enteritidis phage type 4. *Veterinary Record*. 1992;130(18):395–398.

33. Rose MC, Voynow JA. Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiological reviews*. 2006;86(1):245–278.

34. Hingley S, Hastie A, Kueppers F, et al. Effect of ciliostatic factors from Pseudomonas aeruginosa on rabbit respiratory cilia. *Infection and immunity*. 1986;51(1):254–262.

35. King M. Mucus and its role in airway clearance and cytoprotection. *Physiologic basis of respiratory disease*. 2005:409–416.

36. Mensah GA, Brain JD. Deposition and clearance of inhaled aerosol in the respiratory tract of chickens. *Journal of applied physiology*. 1982;53(6):1423–1428.

37. Fedde M. Relationship of structure and function of the avian respiratory system to disease susceptibility. *Poultry science*. 1998;77(8):1130–1138.

38. Weinack OM, Snoeyenbos G, Smyser C, Soerjadi-Liem A. Influence of Mycoplasma gallisepticum, infectious bronchitis, and cyclophosphamide on chickens protected by native intestinal microflora against Salmonella typhimurium or Escherichia coli. *Avian Diseases*. 1984:416–425.

39. Whyte RT. Aerial pollutants and the health of poultry farmers. *World's poultry science journal*. 1993;49:139–153.

40. Donham K, Blake J, Patterson P. Occupational health hazards and recommended exposure limits for workers in poultry buildings. In: *Proceedings, 2000 National Poultry Waste Management Symposium, Ocean City, MD, USA, 16-18 October 2000.* National Poultry Waste Management Symposium Committee; 2000:92–109.

41. Noy Y, Sklan D. Metabolic responses to early nutrition. *The Journal of Applied Poultry Research*. 1998;7(4):437.

42. Sklan D, Noy Y. Hydrolysis and absorption in the small intestines of posthatch chicks. *Poultry Science*. 2000;79(9):1306–1310.

43. Yi G, Allee G, Knight C, Dibner J. Impact of glutamine and oasis hatchling supplement on growth performance, small intestinal morphology, and immune response of broilers vaccinated and challenged with Eimeria maxima. *Poultry Science*. 2005;84(2):283–293.

44. Uni Z, Smirnov A, Sklan D. Pre-and posthatch development of goblet cells in the broiler small intestine: effect of delayed access to feed. *Poultry science*. 2003;82(2):320–327.

45. Noy Y, Geyra A, Sklan D. The effect of early feeding on growth and small intestinal development in the posthatch poult. *Poultry Science*. 2001;80(7):912–919.

46. Juul-Madsen HR, Su G, Sørensen P. Influence of early or late start of first feeding on growth and immune phenotype of broilers. *British poultry science*. 2004;45(2):210–222.

47. Dibner J, Knight C, Kitchell M, et al. Early feeding and development of the immune system in neonatal poultry. *The Journal of Applied Poultry Research*. 1998;7(4):425–436.

48. Henderson S, Vicente J, Pixley C, Hargis B, Tellez G. Effect of an early nutritional supplement on broiler performance. *Int. J. Poult. Sci*. 2008;7:211–214.

49. Hooshmand M. Effect of early feeding programs on broiler performance. *Int. J. Poult. Sci*. 2006;5(12):1140–1143.

50. Halevy O, Geyra A, Barak M, Uni Z, Sklan D. Early posthatch starvation decreases satellite cell proliferation and skeletal muscle growth in chicks. *The Journal of nutrition*. 2000;130(4):858–864.

51. Nganpiep L, Maina J. Composite cellular defence stratagem in the avian respiratory system: functional morphology of the free (surface) macrophages and specialized pulmonary epithelia. *Journal of anatomy*. 2002;200(5):499–516.

52. Stearns RC, Barnas G, Walski M, Brain J. Deposition and phagocytosis of inhaled particles in the gas exchange region of the duck,< i> Anas platyrhynchos</i>. *Respiration physiology*. 1987;67(1):23–36.

53. West JB, Mathieu-Costello O. Stress failure of pulmonary capillaries as a limiting factor for maximal exercise. *European journal of applied physiology and occupational physiology*. 1995;70(2):99–108.

54. West J, Mathieu-Costello O. Structure, strength, failure, and remodeling of the pulmonary blood-gas barrier. *Annual Review of Physiology*. 1999;61(1):543–572.

55. Kiama S, Adekunle J, Maina J. Comparative in vitro study of interactions between particles and respiratory surface macrophages, erythrocytes, and epithelial cells of the chicken and the rat. *Journal of Anatomy*. 2008;213(4):452–463.

56. Maina JN, Cowley HM. Ultrastructural characterization of the pulmonary cellular defences in the lung of a bird, the rock dove, Columba livia. *Proceedings of the Royal Society of London.Series B: Biological Sciences*. 1998;265(1405):1567–1572.

57. Toth TE, Curtiss III R, Veit H, Pyle R, Siegel P. Reaction of the avian respiratory system to intratracheally administered avirulent Salmonella typhimurium. *Avian diseases*. 1992:24–29.

58. Curtiss R, Kelly SM. Salmonella typhimurium deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. *Infection and immunity*. 1987;55(12):3035–3043.

59. Karnovsky ML, Lazdins JK. Biochemical criteria for activated macrophages. *The Journal of Immunology*. 1978;121(3):809–813.

60. Nagaraja KV, Emery DA, Jordan KA, et al. Effect of ammonia on the quantitative clearance of Escherichia coli from lungs, air sacs, and livers of turkeys aerosol vaccinated against Escherichia coli. *American Journal of Veterinary Research*. 1984;45(2):392–395.

61. Vazquez-Torres A, Xu Y, Jones-Carson J, et al. Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science*. 2000;287(5458):1655.

62. Okamura M, Lillehoj HS, Raybourne RB, et al. Differential responses of macrophages to< i> Salmonella</i>< i> enterica</i> serovars Enteritidis and Typhimurium. *Veterinary immunology and immunopathology*. 2005;107(3):327–335.

63. Leach SA, Williams A, Davies AC, et al. Aerosol route enhances the contamination of intact eggs and muscle of experimentally infected laying hens by Salmonella typhimurium DT104. *FEMS microbiology letters*. 1999;171(2):203–207.

Table1. Experiment 1 - Evaluation of intratracheal infection of chickens with *Salmonella* Enteritidis

Group no.	Dose CFU of SE per Chick	Route of Challenge	Log_{10} CFU of SE / gram of ceca content	Ceca - Cecal Tonsil	Liver and Spleen	Trachea
Group 1	3×10^4	OR	6.2547 ± 0.7578	11/11 (100%)	11/11 (100%)	11/11 (100%)
Group 2	1.5×10^2	IT	6.8833 ± 0.4089	12/12 (100%)	12/12 (100%)	12/12 (100%)
Group 3	3×10^4	IT	7.7568 ± 0.2264	12/12 (100%)	12/12 (100%)	12/12 (100%)

Chicks were challenged with *Salmonella* Enteritidis (SE) – on day of hatch – Intratracheally (IT) or orally (OR) at concentrations 1.5×10^2 or 3×10^4 CFU/chick. 24 hrs post challenge, ceca-cecal tonsils were cultured to enumerate Log_{10} SE/ gram of ceca content and the data were expressed as mean ± standard error of mean. Ceca - cecal tonsils, trachea and liver & spleen enrichment data were expressed as positive/total chickens for each tissue sampled (%). No significant differences ($p < 0.05$, N=12 / group) were found between the groups.

Table2. Experiment 2 – Trial 1 - Evaluation of intratracheal infection of chickens with *Salmonella* Enteritidis

Group no.	Dose CFU of SE per Chick	Route of Challenge	Ceca - Cecal Tonsil	Liver and Spleen
Group 1	1×10^4	OR	0/12 (0%) [c]	0/12 (0%) [b]
Group 2	1×10^4	IT	2/12 (16.66%) [bc]	1/12 (8.33%) [ab]
Group 3	1.5×10^6	OR	10/12 (83.33%) [a]	0/12 (0%) [b]
Group 4	1.5×10^6	IT	6/12 (50%) [ab]	4/12 (33.33%) [a]

Chicks were challenged with *Salmonella* Enteritidis (SE) – on day 7 – Intratracheally (IT) or orally (OR) at concentrations 1×10^4 or 1.5×10^6 CFU/chick. 24 hrs post challenge, and cecal tonsils and liver & spleen were cultured in an enrichment broth. The enrichment data were expressed as positive/total chickens for each tissue sampled (%). Different superscripts within columns indicate significant differences $p < 0.05$, N=12 / group.

Table3. Experiment 2- Trial 2 - Evaluation of intratracheal infection of chickens with *Salmonella* Enteritidis

Group no.	Dose CFU of SE per Chick	Route of Challenge	Log_{10} CFU of SE / gram of ceca content	Ceca - Cecal Tonsil	Liver and Spleen	Trachea
Group 1	1.5×10^4	OR	1.799 ± 0.384 ^{c}	5/12 (41.66%) ^{b}	0/12 (0%)^{d}	1/12 (8.33%) ^{d}
Group 2		IT	1.125 ± 0.401 ^{c}	8/12 (66.66%) ^{b}	6/12 (50%) ^{bc}	8/12 (66.66%) ^{bc}
Group 3	2×10^6	OR	1.858 ± 0.400 ^{c}	8/12 (66.66%) ^{b}	2/12 (16.66%) ^{cd}	3/12 (25%) ^{d}
Group 4		IT	3.195 ± 0.166 ^{b}	12/12 (100%) ^{a}	10/12 (83.33%) ^{ab}	11/12 (91.66%) ^{ab}
Group 5	1×10^8	OR	5.989 ± 0.512 ^{a}	11/11 (100%) ^{a}	1/11 (9.09%) ^{d}	5/11 (45.45%) ^{cd}
Group 6		IT	5.114 ± 0.472 ^{a}	12/12 (100%) ^{a}	11/12 (91.66%) ^{a}	12/12 (100%) ^{a}

Chicks were challenged with *Salmonella* Enteritidis (SE) – on day 7 – Intratracheally (IT) or orally (OR) at concentrations 1.5×10^4 or 2×10^6 or 1×10^8 CFU/chick. 24 hrs post challenge, ceca-cecal tonsils were cultured to enumerate Log_{10} SE/ gram of ceca content and the data were expressed as mean ± standard error of mean. Ceca - cecal tonsils, trachea and liver & spleen enrichment data were expressed as positive/total chickens for each tissue sampled (%). Different superscripts within columns indicate significant differences $p < 0.05$, N=12 / group.

Table4. Experiment 2 – Trial 3 - Evaluation of intratracheal infection of chickens with *Salmonella* Enteritidis

Group no.	Dose CFU of SE per Chick	Route of Challenge	Log_{10} CFU of SE / gram of ceca content	Ceca - Cecal Tonsil	Liver and Spleen	Trachea
Group 1	2×10^4	OR	1.189 ± 0.530 ^{d}	3/12 (25%) ^{b}	0/12 (0%) ^{b}	1/12 (8.33%) ^{d}
Group 2		IT	2.204 ± 0.352 ^{cd}	9/12 (75%) ^{ab}	2/12 (16.66%) ^{b}	8/12 (66.66%) ^{ab}
Group 3	1×10^6	OR	2.941 ± 0.190 ^{bc}	12/12 (100%) ^{a}	0/12 (0%) ^{b}	2/12 (16.66%) ^{cd}
Group 4		IT	2.922 ± 0.127 ^{bc}	12/12 (100%) ^{a}	7/12 (58.33%) ^{a}	10/12 (83.33%) ^{a}
Group 5	2×10^8	OR	4.289 ± 0.443 ^{a}	12/12 (100%) ^{a}	2/12 (16.66%) ^{b}	6/12 (50%) ^{bc}
Group 6		IT	3.332 ± 0.242 ^{ab}	12/12 (100%) ^{a}	9/12 (75%) ^{a}	11/12 (91.66%) ^{a}

Chicks were challenged with *Salmonella* Enteritidis (SE) – on day 7 – Intratracheally (IT) or orally (OR) at concentrations 2×10^4 or 1×10^6 or 2×10^8 CFU/chick. 24 hrs post challenge, ceca-cecal tonsils were cultured to enumerate Log_{10} SE/ gram of ceca content and the data were expressed as mean ± standard error of mean. Ceca - cecal tonsils, trachea and liver & spleen enrichment data were expressed as positive/total chickens for each tissue sampled (%). Different superscripts within columns indicate significant differences $p < 0.05$, N=12 / group.

Table5. Experiment 3 - Evaluation of intratracheal infection of chickens with *Salmonella* Typhimurium

Group no.	Dose CFU of ST per Chick	Route of Challenge	Ceca - Cecal Tonsil	Liver and Spleen	Trachea
Group 1	1.5×10^4	OR	4/12 (33.33%) [b]	0/12 (0%) [b]	0/12 (0%) [c]
Group 2		IT	3/12 (25%) [b]	1/12 (8.33%) [b]	8/12 (66.66%) [b]
Group 3	2.5×10^6	OR	11/12 (91.66%) [a]	0/12 (0%) [b]	0/12 (0%) [c]
Group 4		IT	9/12 (75%) [a]	1/12 (8.33%) [b]	12/12 (100%) [a]
Group 5	1×10^8	OR	12/12 (100%) [a]	2/12 (16.66%) [b]	2/12 (16.66%) [c]
Group 6		IT	10/12 (83.33%) [a]	9/12 (75%) [a]	12/12 (100%) [a]

Chicks were challenged with *Salmonella* Typhimurium (ST) – on day 7 – Intratracheally (IT) or orally (OR) at concentrations 1.5×10^4 or 2.5×10^6 or 1×10^8 CFU/chick. 24 hrs post challenge, humanely killed and ceca - cecal tonsils, trachea and liver & spleen were cultured in an enrichment broth. The enrichment data were expressed as positive/total chickens for each tissue sampled (%). Different superscripts within columns indicate significant differences $p < 0.05$, N=12 / group.

Appendix II – IACUC Approval

MEMORANDUM

TO: Lisa R. Bielke

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: June 9, 2011

SUBJECT: IACUC PROTOCOL **APPROVAL**
Expiration date **: June 2, 2014**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#11047- "EVALUATION OF DIRECT FED MICROBIALS AND PREBIOTICS FOR SALMONELLA CONTROL IN POULTRY** ". You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes in the protocol during the research, please notify the IACUC in writing [Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **06-02-2014,** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

Appendix II – IACUC Approval

UNIVERSITY OF ARKANSAS

Office of Research Compliance

October 31, 2012

MEMORANDUM

TO:	Dr. Billy Hargis
FROM:	W. Roy Penney Institutional BioSafety Committee
RE:	IBC Protocol Approval
IBC Protocol #:	10012
Protocol Title:	"Experimental studies to evaluate probiotic cultures as alternatives"
Approved Project Period:	Start Date: September 24, 2012 Expiration Date: September 13, 2015

The Institutional Biosafety Committee (IBC) has approved the renewal of Protocol 10012, "Experimental studies to evaluate probiotic cultures as alternatives". 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.

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

Chapter 6

Conclusions

Conclusions

The present studies evaluated the hypothesis that transmission by the fecal-respiratory route may be a viable portal of entry for *Salmonella* in poultry.

Firstly, we updated the current knowledge of *Salmonella* bioaerosol generation, its transport and fate, at various stages of commercial poultry production, with further emphasis on survivability of *Salmonella* in these bioaerosols, as a means to assess the transport and subsequent risk of exposure and infection of poultry. Additionally, the anatomical structure, physiological functions and immunological defense were reviewed for each anatomical section of the avian respiratory system, to provide an understanding of the possibilities for *Salmonella* entering the respiratory system. Further descriptions emphasized the potential weaknesses inherent to each component of the respiratory system that could potentially support our hypothesis regarding the respiratory route as a possible portal of entry for *Salmonella* in poultry.

Secondly, we conducted a series of field trials in North and South America to evaluate the association between cecal and tracheal recovery of *Salmonella* in chickens and turkeys from commercial flocks. *Salmonella* recovered from tracheal samples from all field studies indicated that tracheal sampling can be a sensitive tool for monitoring *Salmonella* infection in commercial flocks. Additionally, despite previous reports of an association between high environmental temperature and humidity and increased *Salmonella* incidence, such a correlation was not noted in the present study. Overall these field data suggested that tracheal contamination is likely an indication of infection under commercial conditions, as was evidenced by positive recovery in gastrointestinal and liver/spleen sampling.

Thirdly, the frequent documented recovery of *Salmonella* from dust and bioaerosols from infected poultry, further supported by our field trails demonstrating tracheal recovery of *Salmonella*, together suggested that bioaerosolized *Salmonella* of very small droplet sizes are capable of theoretically reaching the lower respiratory tract of chicks. However, a usual assumption regarding airborne *Salmonella* reaching the upper respiratory tract, would ultimately involve oral ingestion, due to the presence of the mucociliary clearance was evaluated. Suspension in 1% mucin failed to increase the infectivity at any dose of *Salmonella* when compared to OR administration without mucin. We also evaluated the ability of a

relatively low-invasive isolate of *S.* Senftenberg (SS) for ability to colonize the ceca following oral *versus* intratracheal administration, as well as recovery from liver+spleen (LS) samples. IT challenge was more effective or at least as effective at colonizing the ceca of 7d chickens further suggested that the respiratory tract is an overlooked potential portal of entry for *Salmonella*e. There was a strong relationship of more frequent SS recovery from LS samples 24h after IT administration for this SS isolate with relatively low invasiveness, as compared to oral administration. Recovery of SS from lung tissue 24h after IT administration suggested that IT administration was capable of delivering at least some of the challenge to the lower respiratory tract

The unique architecture of the avian lung, involving only phagocytosis of particles reaching the non-ciliated portions just below the proximal end of the secondary bronchi, and the propensity of *Salmonella* for epithelial translocation, together suggested the possibility of systemic macrophage dissemination. We evaluated this hypothesis through IT administration of SE and ST, in comparison with oral administration, in a total of 5 separate trials, with subsequent enumeration of colony forming units (CFU) recovery in ceca-cecal tonsils (CCT) and recovery incidence from LS samples. A significantly higher or equivalent cecal recovery of *Salmonella*, with a clear dose response curve, with the IT groups as compared to groups challenged OR and LS incidence data from these experiments provided evidence for the subsequent fate of *Salmonella* infecting the respiratory system, potentially involving a systemic route to the gastrointestinal tract.

While not proven by these experiments, the possibility that respiratory inoculation is apparently more effective at causing systemic and enteric infection might explain the relative difficulty in consistently infecting chicks via oral administration under laboratory conditions, as opposed to the apparent ease of transmission under commercial conditions. The ability of *Salmonella* to infect chickens at lower doses (as low as 100 cells) via the respiratory route needs to be further investigated. Nevertheless, this could support previous studies which have established the relationship between the size and number of particles relative to the concentration of *Salmonella* generated in the air, under field conditions, estimated to be up to $3.3 \times 10^2 - 1.2 \times 10^4$ cfu m^3 of air. Thus, it is conceivable that under field conditions airborne *Salmonella* are able to infect poultry by this route of infection and further clarification of the respiratory

tract for *Salmonella* transmission under field conditions may be of critical importance to develop intervention strategies to reduce transmission of these pathogens in poultry.