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*Review Article*

# Application of Molecular Approaches for Understanding Foodborne *Salmonella* Establishment in Poultry Production

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Salmonellosis in the United States is one of the most costly foodborne diseases. Given that *Salmonella* can originate from a wide variety of environments, reduction of this organism at all stages of poultry production is critical. *Salmonella* species can encounter various environmental stress conditions which can dramatically influence their survival and colonization. Current knowledge of *Salmonella* species metabolism and physiology in relation to colonization is traditionally based on studies conducted primarily with tissue culture and animal infection models. Consequently, while there is some information about environmental signals that control *Salmonella* growth and colonization, much still remains unknown. Genetic tools for comprehensive functional genomic analysis of *Salmonella* offer new opportunities for not only achieving a better understanding of *Salmonella* pathogens but also designing more effective intervention strategies. Now the function(s) of each single gene in the *Salmonella* genome can be directly assessed and previously unknown genetic factors that are required for *Salmonella* growth and survival in the poultry production cycle can be elucidated. In particular, delineating the host-pathogen relationships involving *Salmonella* is becoming very helpful for identifying optimal targeted gene mutagenesis strategies to generate improved vaccine strains. This represents an opportunity for development of novel vaccine approaches for limiting *Salmonella* establishment in early phases of poultry production. In this review, an overview of *Salmonella* issues in poultry, a general description of functional genomic technologies, and their specific application to poultry vaccine developments are discussed.

## 1. Introduction

*Salmonella* induced gastroenteritis is the result of consumption of contaminated food and subsequent local infection in the gastrointestinal tract leading to symptoms of diarrhea, abdominal pain, and fever within 12 to 72 hours that can persist up to a week and in rare cases become fatal [1]. *Salmonella* infections are the leading cause of hospitalizations and deaths due to foodborne illness [2, 3]. Consequently, foodborne disease resulting from exposure to serovars of *Salmonella* continues to be a major human health concern resulting in billions of dollars in annual costs [3]. *Salmonella* comes in contact with food production and preparation from a wide range of sources including animal and pet feed, during food processing, at retail establishments and in the home during meal preparation [1, 4–12]. Salmonellosis occurs worldwide and can originate from a wide range of raw food vehicles including most animal red meat, swine, poultry, eggs, vegetables, and produce as well as ready-to-eat foods [7, 12–21]. Of these food sources, raw poultry products and eggs are considered a primary contributor to human exposure to foodborne *Salmonella* serovars with numerous salmonellosis cases attributed to these sources [2, 17, 18, 20–22].

Enteric or typhoid fever in humans occurs from waterborne exposure to serovars *S.* Typhi and Paratyphi, while several of the other *S. enterica* serovars are responsible for foodborne nontyphoidal salmonellosis [1]. Several *Salmonella* serovars have been associated with nontyphoidal human illness, but *Salmonella enterica* serovar Typhimurium along with *S.* Enteritidis, *S.* Newport, and *S.* Heidelberg have been the most commonly associated serovars with documented outbreaks [18, 20]. In outbreaks directly linked to poultry *S.* Typhimurium and *S.* Enteritidis have been the most commonly identified serovars [20].

*Salmonella* serovars are able to survive under various niches ranging from diverse animal hosts, fruits, and vegetables to various livestock and food production environments; this ability has made the control of *Salmonella* colonization and transmission difficult [7–9, 14]. The important characteristics of these serovars are that they can be harbored subclinically in livestock and poultry as asymptomatic commensal microorganisms, persist in the environment for long periods of time, and are thus difficult to control in the absence of a detailed knowledge of these organisms in that particular niche [7, 17, 18, 23]. A wide variety of experimental techniques and strategies have been applied to develop the level of understanding of the ability of *Salmonella* to survive and cross contaminate multiple environments. Bacterial characteristics required for being able to exist in these environments have been studied extensively with a combination of laboratory and animal infection models. However, the availability of molecular technologies including sequencing and targeted mutagenesis has enabled the development of comprehensive functional genomics-based approaches to delineate the genetic requirements for *Salmonella* colonization, infection, and survival under environmental conditions that the microorganism is exposed to. The development and application of such novel functional genomics tools may reveal previously unknown vulnerabilities that can be explored to develop novel interventions and break the *Salmonella* transmission chain [24]. The objectives of this review are to discuss *Salmonella* occurrence in poultry production and the wide range of experimental methods that have been historically applied to understanding how *Salmonella* persists in poultry. The remainder of the review will be focused on more recent developments in functional genomics and their potential for general application to interventions and specific vaccine construction strategies to limit *Salmonella* establishment in poultry production.

## 2. *Salmonella* in Broiler and Egg Production

*2.1. Postharvest Contamination Routes: Broiler Meat.* *Salmonella* contamination in poultry production continues to be an ongoing problem both in laying hens and in broiler production [1, 20–22]. In broiler meat processing, multiple mechanical steps are involved in converting a live bird into a chicken carcass including scalding and subsequent removal of feathers, evisceration of intestinal organs, and eventual immersion in a chiller tank. There are a variety of opportunities for introduction and subsequent cross contamination of *Salmonella* spp. during poultry processing and these have been discussed in detail in previous comprehensive reviews [10, 19]. However, a number of factors contribute to the difficulties associated with establishing consistent patterns of frequency and prevalence of *Salmonella* in these environments and on the carcasses themselves. For example, there may not only be differences in the frequency of *Salmonella* on carcasses but attachment sites on the carcass may also be variable which in turn could influence recovery when carcasses are rinsed for microbial analyses [25–27]. Early work suggested that prolonged immersion of chicken carcasses tended to enhance attachment of *Salmonella* [28–30]. In followup studies, Lillard [30] demonstrated that prolonged water immersion of chicken skin enhanced transfer of *Salmonella* from the surface film outside the skin into the skin. Specific mechanisms had been proposed as being involved in bacterial attachment such as the presence of flagella and fimbrae, electrostatic attraction, or uptake of water but over time these were ruled out as primary mechanisms for *Salmonella* attachment [27–29, 31–34]. Based on several studies utilizing electron microscopy to examine chicken carcass tissue topography [32, 34–36] it was concluded by Lillard [27, 34] that some *Salmonella* may in fact become lodged in the crevices and ridges of poultry skin and thus are not only protected to some extent from application of disinfectants but less recoverable for detection based on carcass rinse sampling methods [25–27]. In addition, after conducting multiple carcass rinses during various stages of processing, Lillard [26, 27] concluded that bacteria become firmly attached to carcasses prior to the birds being processed. More recently, approaches such as online reprocessing involving inside-outside bird washers have been shown to be very efficient in removing pathogens from carcasses and have been incorporated into U.S. Food Safety and Inspection Service policy [20, 37].

Based on the difficulty of removal of *Salmonella* from carcass surfaces, Lillard [27] suggested that while processing interventions can reduce cross contamination, further reduction of *Salmonella* carcass contamination in individual birds may require interventions during live bird production. However, part of the problem associated with assessment of postharvest contribution to contamination is that the preharvest origins of *Salmonella* can be fairly varied with primary sources being the fecal and alimentary tract contents (crop, cecal, and intestines) which in turn can contaminate the carcass during processing, particularly if one of the organs ruptures at some point [38–40]. Implementation of feed withdrawal prior to transportation to the processing plant was initiated as a means to decrease the total volume of the alimentary tract including contents thus reduce fecal contamination on carcasses, but this can lead to increases of *Salmonella* in the crops of chickens and potential for increased contamination at the processing plant [38–40]. This has been difficult to establish for a variety of reasons. First of all, most of the data that are generated from these types of samples are more along the lines of prevalence rather than quantitative estimations of resident populations [10]. In addition, the linkages between farm contaminations and processing plant levels are not well established and only recently have in-depth enumeration studies been conducted that have established a positive correlation between farm environmental levels of *Salmonella* and contamination at the processing plant [41]. However, as these authors [41] pointed out, this relationship decreased significantly as carcasses progressed through the processing plant with only 2.4% of the postchill carcasses positive for *Salmonella*. Such studies suggest that there is clearly a need to construct larger comprehensive data sets that rely not only on quantitation of *Salmonella* populations during the various phases of processing but more detailed characterization of the serovars involved. This will become more critical as regulatory and economic pressures continue to build towards lowering

acceptable levels of *Salmonella* on chicken products [42]. Although improvements in molecular detection technologies have certainly benefited from advances in *Salmonella* genomics and sequence information, fundamental problems of cost, sampling, sensitivity, and practicality for routine analysis remain as barriers for implementation [10, 16, 27, 43–46]. However, if more precise salmonellosis attributable to poultry or any other food source is to be achieved these barriers will need to be overcome [10, 16].

*2.2. Postharvest Contamination Routes: Eggs.* While routes for *S.* Enteritidis contamination of table eggs have also been extensively documented in numerous reviews there is evidence that other serovars are associated with eggs as well [21, 23, 47–50]. Likewise, the majority of the focus on *Salmonella* contamination in eggs has focused on transovarian vertical infection mostly by serovar Enteritidis of laying hens during egg production [9, 22, 23, 50–56] and there has been less emphasis in recent times on external shell horizontal contamination during egg processing. This is in part due to the advancements in understanding the mechanisms for external contamination by spoilage bacteria and *Salmonella* in hatching and table eggs from fundamental research on both types of eggs along with the subsequent improvements made in table egg processing over the past half century in the U.S. and elsewhere [21–23, 57–65].

Messens et al. [66] classified the factors associated with *Salmonella* penetration through the egg shell as either intrinsic (presence of cuticle on egg, shell quality, and membrane characteristics) or extrinsic (exposure to bacterial levels and types, and physical treatment of eggs such as temperature, moisture, and storage conditions). Certainly the presence of egg shell defects and cracks that occur either during processing or as a result of exposure to egg washing chemicals is conducive to egg contamination and the associated risks have been documented accordingly [65, 67–69]. However, the necessity for an intact cuticle which can be easily damaged and at least partially removed during egg processing is less clear cut although this may change now that proteomic analysis has revealed the presence of several antibacterial proteins in the cuticle [66, 70]. As more has become known on the impact of environmental conditions, several suggestions have been made to design egg washing approaches that limit available iron, maintain alkaline pH, and administer wash waters held within a certain temperature range above the egg temperature at all times followed by cold storage of eggs in retail markets [22, 58, 63, 66, 71–75]. These conditions appear to be critical in commercial operations as *Salmonella* has been isolated on eggs from egg processing plants when pH of the egg wash water was less alkaline [63, 76]. However, both egg washing and subsequent refrigeration of stored eggs are not practiced uniformly worldwide and remain controversial in some countries [22, 63].

Risk of *Salmonella* external contamination of eggs during egg processing does still exist in the U.S. and other developed countries. In-line egg processing facilities where the egg processing facilities are physically connected to the egg layer operation, off-line operations where they are not physically connected, and mixed-line operations that include a combination of in-line processing as well as externally shipped in eggs have been shown in several studies to be a source of *Salmonella* contamination with or without egg washing [76–78]. In addition, numerous opportunities for general microbial cross contamination occur in these types of operations as the eggs move through the processing facilities [79, 80]. In a series of studies on mixed-line and off-line egg processing facilities, Musgrove and coworkers [78, 81] demonstrated that metal carts with unpainted plywood shelves, commonly referred to as nest run carts could serve as sources of microbial cross contamination.

Cross contamination can apparently occur from the farm to the egg packing plant as well. Characterization of *Salmonella* cross contamination of eggs transported from farms to whole sale and retail markets located in North India recovered *S.* Typhimurium as the predominant serovar with a higher incidence occurring in eggs collected from markets compared to fresh eggs from the farms [82]. This led them to conclude that surface contamination must have occurred during handling, storage, and transportation of the eggs from the farms to the market [82]. This was supported by earlier work [83] where *Salmonella* spp. were recovered from egg shells, egg contents, and egg trays in South India retail markets and these authors concluded that reused egg trays were a potential risk for exposure to *Salmonella*. A comprehensive study on *Salmonella* frequency in samples collected from eggshells, egg contents, reusable egg trays, and associated environments in Thailand egg farms and markets suggested reusable egg trays as a potential source of horizontal transmission [84]. It appears that further *Salmonella* cross contamination can occur from conveyors, rollers, and associated equipment in egg packing plants such as those in the UK where egg washing is not practiced which would represent additional risk to egg retail markets [77].

A final consideration is the changing nature of egg retail markets. In a review of the current literature Holt et al. [85] pointed out that a movement away from caged laying house systems to enriched cage or cage free systems must be evaluated for their impact on food safety. This would be true for any alternative egg production systems such as free range (hut-paddock system), cage-free, and organic based operations that have been described in previous studies [86, 87]. Likewise with more commercial emphasis on local egg marketing in the U.S. some of these issues will need to be reexamined with the restrictions unique to these systems.

*2.3. Preharvest Contamination Routes: Broilers.* Numerous factors contribute to continued exposure of birds to *Salmonella* from the environment during the production cycle [8, 10, 88, 89]. Carriers for *Salmonella* during the conventional poultry production cycle can include a wide variety of sources including rodents and cats as well as insects which when present in poultry houses can serve as infected or mechanical carriers and potential sources of this pathogen to susceptible birds [5, 8, 90, 91]. Less conventional poultry production facilities such as organic, pasture, or free range raised poultry where birds have more outdoor exposure represent potential contact with an even wider range of vectors and environmental sources [92, 93]. In addition to *Salmonella* vectors, environmental

transmission routes (aerosols, water) and reservoirs such as litter and feed play a key role in this dissemination. Aerosols in particular have been identified in several studies as a transmission route not only in broiler and layer houses but in hatcheries as well [94–98]. Part of the issue with these sources is the ability of *Salmonella* to survive for fairly long periods of times in some of the reservoirs that vectors and other carriers such as aerosols carrying *Salmonella* come in contact with. For example, *Salmonella* spp. have been demonstrated to survive several weeks in low water activity poultry feeds and litter [4, 6, 99–104]. As a result there is a multitude of sustained opportunities for birds to encounter *Salmonella* over fairly long time periods and some sources such as contaminated feed and litter could impact poultry throughout the production cycle including the breeder flocks [6, 8, 105].

*2.4. Preharvest Contamination Routes: Layer Hens.* While in broilers several serovars have been isolated, *S.* Enteritidis has been the serovar most commonly associated with shell egg production and products containing eggs as a food ingredient [20, 21, 49, 64, 106, 107]. *S.* Enteritidis has continued to persist with periodic increases and decreases in frequency as a primary table egg associated serovar identified with foodborne salmonellosis outbreaks [49, 108, 109]. Although other serovars such as *S.* Typhimurium and *S.* Heidelberg have been identified with egg contamination, *S.* Enteritidis has received most of the research attention because it appears to possess unique traits that make it particularly trophic towards physiological processes and reproductive tissues associated with the generation of the shell egg in the laying hen [21, 22, 48, 49, 53, 56, 64]. Consequently considerable effort has been made to document and describe the role that preharvest egg production plays in the generation of contaminated eggs and how this impacts commercial egg production.

A brief assessment of the routes of exposure of laying hens to *S.* Enteritidis during egg production is warranted to help understand the apparent association between this particular serovar and table eggs. While laying hen susceptibility to *S.* Enteritidis varies with age and physiological status, there are numerous opportunities for these birds to become exposed to *S.* Enteritidis not only during the egg laying cycle, but also prior to that during the growth and maturing of the laying hen from hatch onwards [48, 50–56]. This is in part due to ongoing environmental exposure, housing, and presence of numerous vectors well known for carrying *S.* Enteritidis such as insects, mice, rats, and other persistent pests in laying houses [8, 22, 85, 110–115]. In addition, poultry feed has been speculated over the years to be an important source for *Salmonella* spp. exposure to chickens at various stages of live production including breeder flocks [4, 6, 105–121]. Consequently, interest in more rapid detection methods specifically suitable for poultry feed matrices that can not only detect *Salmonella* spp. but potentially quantitate population levels have continued to be pursued over a number of years [24, 46, 105, 122–127].

Nutritional management of laying hens during egg production can also be a major contributor to risk of exposure of susceptible birds to *S.* Enteritidis. Although it varies somewhat depending upon the balance between the price of eggs and the costs associated with egg production, egg laying flocks in the U.S. typically are maintained for more than one egg laying cycle [50, 128–132]. In order to initiate an additional egg laying cycle, laying hen egg production is terminated by physiologically inducing a molt onto the hens giving the reproductive tract a resting period where eggs are no longer produced and the reproductive tract is essentially dormant followed by a restart of egg production [129, 130]. These physiological changes that occur during molt are manifested not only in the reproductive tract but also in feathers as a changeover, alteration of bone metabolism due to fluctuations in calcium demand, and shifts in metabolism and immune responses that have been described in comprehensive detail in several reviews and will not be discussed further here [50, 130–134]. To achieve management controlled egg production by physiological manipulation of the reproductive tract in laying hens is usually done via alteration of the nutritional status and lighting schedule of the bird to cause overall physiological and hormonal shifts that signal a need to rest egg synthesis [130, 133, 135, 136].

Historically, molting was induced by simply removing all feed from the laying hen for a period of time until egg production was stopped for several days and subsequently egg production was restarted by bringing feed back to the hens [129, 130, 137]. Although this approach was a very effective means for providing the appropriate signals to the bird to physiologically respond and cease egg production, the emptying of the gastrointestinal tract in these birds was demonstrated in experimental challenge studies to cause them to be susceptible to colonization and infection by *S.* Enteritidis [54–56]. In this series of experimental challenge trials it was well documented that birds undergoing feed withdrawal after just a few days were easily colonized by *S.* Enteritidis and extensive organ invasion occurred including the ovaries [9, 56, 138]. Further investigation revealed that the conditions of the empty gastrointestinal tract and ceca were conducive to increased expression of *S.* Enteritidis virulence genes which aligned with increased host invasion and decreased fermentation activity and in some cases reduced levels of indigenous gastrointestinal tract bacteria [9, 56, 138–140]. Due to animal welfare concerns, feed withdrawal molting is not allowed in the UK and Europe [137]. More recently in the USA, the United Egg Producers (UEP) no longer permit feed withdrawal molting and the UEP has led the effort to develop alternative molting approaches that do not involve complete removal of feed [137]. This will be discussed in a later section.

## 3. Interventions to Limit *Salmonella* Establishment in Poultry Production

*3.1. Postharvest Interventions.* Historically, much of the emphasis for limiting *Salmonella* contamination in poultry production resided in identification and management of poultry processing plant sites where microbial contamination was most likely to occur as well as application of interventions during the various stages of processing. Hazard Analysis of

Critical Control Points (HACCP) programs were designed and implemented to systematically identify critical control points where microbial contamination occurs during processing and subsequently prevent and control that contamination to reduce foodborne pathogen risk associated with raw poultry product [141]. Since that time, considerable effort has been continued to refine hazard identification in the context of risk assessment of likelihood of exposure from consumption of food product and level of disease attributable to the foodborne pathogen [11, 142]. Specific targets identified as important contributors to risk have included the level of fecal contamination which in turn led to concerted regulatory efforts to encourage and implement approaches for reducing the level of fecal contamination on carcasses [37].

During postharvest poultry production there is potential for variable survival and physiological responses of *Salmonella* spp. to environmental conditions that are both inherent with the processing plant as well as via various antimicrobial interventions applied during processing. Certain antimicrobials such as short-chained organic acid based compounds are known to elicit tolerance mechanisms in *Salmonella* spp. if they have been exposed to sublethal concentrations [143, 144]. Organic acid resistance in *Salmonella* spp. can also lead to cross protection and subsequent resistance to other antimicrobial compounds [145]. This becomes more of a challenge in organic poultry processing where only a limited range of antimicrobials are approved for use [146, 147]. Consequently, systems incorporating multiple antimicrobials also known as "multiple hurdle" approaches have been proposed to prevent buildup of resistance in pathogens to one particular antimicrobial [148–150]. Theoretically, this has been believed to be an optimal approach but it remains unclear how to routinely design such approaches to work in a multitude of environments. Part of the problem is assessing what levels of antimicrobial exposure actually occurs in the environment. Therefore, even though numerous laboratory-based studies have demonstrated the potential for resistance development, it is much more difficult to determine how much development of certain antimicrobial resistances occurs under processing plant conditions.

It is anticipated that as more molecular profiling of *Salmonella* responses are conducted experimentally there will be opportunities to further elucidate mechanisms that contribute to the ability of *Salmonella* to persist in processed poultry and poultry processing environments [24, 150]. For example, transcriptomic work involving *S.* Typhimurium microarrays has revealed that combinations of thermal and organic acids are synergistic in inhibiting *Salmonella* and may involve membrane damage followed by the inability of *Salmonella* to mount a heat shock response [151, 152]. Chemical composition of the food matrix may also be important. Chalova et al. [153] demonstrated that the formation of specific Maillard products in a simulated low water activity environment generated a transcriptomic response in *S.* Typhimurium that reflected bacterial cells experiencing starvation conditions. However, using molecular genomic screening methodology directly on surfaces such as chicken carcass surfaces remains problematic due to the difficulty of extracting enough nucleic acid material for conducting the analyses [154].

For postharvest interventions on shell egg production, efforts have mostly been focused on elimination of microbial and *Salmonella* contamination on the shell surfaces of eggs. Interventions have typically involved the application either as a spray, dip or in some cases as a foam in combination with some type of chemical sanitizer candidate that is known to possess antimicrobial properties. While there is considerable overlap between the choices of sanitizers used for hatching eggs versus table shell eggs, the evaluation criteria for acceptability differ with impact on mortality and hatchability being critical for hatching eggs, whereas table eggs are destined for human consumption and therefore any sanitizer used for these types of eggs must meet those standards. Historically, a wide range of chemical-based sanitizers have been screened for efficacy as potential control measures to limit *Salmonella* and microbial contamination of both table and hatching egg shell surfaces [21, 155, 156]. Although an incomplete list, sanitizer chemicals that have been screened as egg disinfectants over the years have included such compounds as cetylpyridinium chloride, chlorine, chlorine dioxide, hydrogen peroxide, n-alkyl dimethyl benzyl ammonium chloride, peroxyacetic acid, phenol, polyhexamethylenebiguanide hydrochloride, sodium hypochlorite, quaternary ammonium compounds, and trisodium phosphate just to name a few that have been experimentally tested [21, 22, 63, 67, 155–160].

As additional emphasis has been placed on limiting microbial and pathogen contamination on shell eggs, research efforts have become focused on potential alternatives that may offer unique mechanisms to achieve efficacy. More novel sanitizer approaches that may serve as potential control agents for reducing microbial and *Salmonella* populations on shell and hatching eggs include electrolyzed oxidized water solutions, peroxidase catalyzed sanitizers that generate bactericidal levels of iodine, chitosan-based egg shell coatings, and application of plant-derived antimicrobials [161–172]. Nonchemical-based interventions have focused on approaches such as exposure of intact table and hatching eggs to ultraviolet light irradiation, ozone, pulsed light, and gas plasma [22, 173–180]. Interest has also grown in applying biologically active agents that can, not only directly and specifically interact with the target pathogen such as *Salmonella*, but also completely eliminate the organism. Bacteriophages, historically, were considered an effective antimicrobial against most pathogenic bacteria and renewed interest has emerged as conventional antimicrobials have fallen out of favor in the public health sector. Bacteriophages are essentially viral particles capable of attaching and inserting their nucleic acid information into the bacterial cell via a series of proteins specifically designed to accomplish this multistep process. Once the viral genetic material has entered the bacterial cells they insert phage DNA into the bacterial chromosome ultimately using the bacterial replication mechanisms to produce multiple copies of the phage for further replication, subsequent lysis of the bacterial cell, and infection of neighboring bacterial cells. Obviously this ability of an antimicrobial agent to replicate and ultimately destroy a bacterial population offers numerous opportunities for practical application in the food industry. For use in eggs to limit *Salmonella* contamination, lytic

*S.* Enteritidis bacteriophage single isolates or cocktails of several isolates have been mostly applied to fresh table eggs to eliminate the presence of the pathogen or administered in the gastrointestinal tract of young chicks to reduce colonization by this pathogen but some researchers have also examined the possibility of using bacteriophage to reduce *S.* Enteritidis in fertile eggs [181–186]. However, limits to this approach remain including the specificity of the bacteriophage to a fairly narrow genetic range of serovars and the inherent ability of the host bacterial cell to develop resistance to a respective phage which can become problematic with continued use.

Ultimately, developing more effective postharvest control measures for egg processing and *Salmonella* contamination will depend on achieving a better understanding of the interactions between *Salmonella* spp. and their association with the egg structure and composition. A few limited studies have been conducted to identify potential *Salmonella* genes that might be directly involved in shell egg contamination. Kwon et al. [187] using a transposon Tn5-based footprinting was able to identify several *S.* Typhimurium mutants that were unable to survive on the surface of shell eggs after incubation at 37°C for 24 hr. Lu et al. [188] screened a genomic *S.* Enteritidis library and isolated the *yafD* gene which appeared to impart enhanced survivability in the egg albumen. Although unable to define *yafD*'s function they speculated that its putative protein sequence similarity to known exonuclease-endonuclease-phosphatase enzyme families may enable *S.* Enteritidis to repair DNA after exposure to the egg albumen nucleases. More recently, Shah et al. [189] screened a *S.* Enteritidis transposon insertion library and noted that mutants that had reduced invasiveness in Caco-2 mammalian cells also exhibited diminished survivability in egg albumen.

Clearly, more studies need to be done to develop a more complete picture of not only the mechanisms involved in *S.* Enteritidis penetration and survival in shell eggs but to establish the sequence of events that occur during this process. Now that proteomics-based methods are being used to study components of the egg such as the cuticle as discussed earlier, the possibility to expand this type of approach to changes that occur during exposure of shell eggs to bacterial pathogens may reveal unique interactions that occur between the egg and the respective pathogen. This may help to develop better control measures as well as predicting when contamination is more likely to occur. This could be important for further assessing risk. Much of the risk modeling that has been done, thus far, has identified terminal egg product processes such as rapid cooling and liquid whole egg pasteurization as having a major impact on substantially reducing the outbreaks associated with *S.* Enteritidis contaminated eggs [190, 191]. However, as more detailed biological information is obtained, the possibility exists of deriving more comprehensive risk models that can be used to predict and factor in when and how much *Salmonella* contamination may occur during egg production and processing.

*3.2. Preharvest Interventions.* The exposure of chickens during egg production and broiler grower cycles to *Salmonella* is considered to be quite complex since the susceptibility of birds to *Salmonella* infection varies over the course of the production cycle thus altering the infectious dose of *Salmonella* in exposed birds. More specifically, it is fairly well established that young newly hatched birds that have minimally developed gastrointestinal tract microflora are highly susceptible to colonization and infection by *Salmonella* in low numbers [192–195]. Given the vulnerability of young chicks, it is not surprising that many of the preharvest intervention approaches have focused on minimizing their exposure. For example, numerous control measures have been suggested for poultry feeds over the years including acids and other antimicrobial additives that can be directly added into the feed as well as physical processing such as irradiation and thermal exposure [6, 118, 119, 144, 196–198]. Ensuring *Salmonella* free-feed would be considered a fairly expensive avenue in general broiler and layer flock populations but might make economic sense at more critical junctures of vertically integrated poultry production such as the breeder and parent stocks [105].

Interventions administered to chicks soon after hatch have also been used as a rationale for introduction of probiotics and competitive exclusion cultures to accelerate development of gastrointestinal tract microflora that might serve as a barrier to *Salmonella* establishment [199–208]. Probiotic cultures have been examined in numerous studies for control and prevention of *Salmonella* establishment in poultry and the development of these cultures has been reviewed extensively elsewhere and will only be briefly discussed here [204, 206–210]. As expected, most of the focus in poultry was historically on administration of cultures in young newly hatched broiler chickens to limit *S.* Typhimurium [201, 203, 211–214]. In general, probiotic cultures appear to be effective for *S.* Typhimurium although other serovars have not been examined nearly as extensively. However, some of these same types of cultures and approaches do appear to be effective in limiting *S.* Enteritidis colonization in either broiler or layer chicks [202, 215–219]. As probiotic cultures have evolved, the trend has moved away from the more complex multibacterial species based probiotic cultures to simpler single species-based approaches that have been shown to be easier to administer commercially and more robust for practical management purposes. In particular, *Bacillus* spore-based probiotic cultures have recently become attractive due to their potential ability to withstand heat processing associated with feed preparation [208].

However, under certain circumstances adult birds can become easily infected as well. For example, as discussed earlier, when egg laying hens were induced to molt via complete removal of feed over several days, this was eventually demonstrated to be problematic for enhancing colonization of *S.* Enteritidis [56]. The concept that the resulting changes in the layer hen gastrointestinal tract from feed removal altered the microbial gastrointestinal tract ecology sufficiently that the birds were more systemically infected by *S.* Enteritidis intuitively led to the idea that retaining some sort of fermentable matrix in the gut lumen might prevent this [56, 131, 138, 220]. Consequently, a series of studies demonstrated that decreased colonization occurs only when some sort of molt feed mixture or dietary supplement

was provided to sufficiently support the fermentative gastrointestinal tract population [9, 56, 131, 135–140, 221–225]. It was concluded that formulating a nutritional balance in the laying hen diet between providing sufficient substrates for the gastrointestinal microflora but still physiologically shift the hen into a reproductive resting state was necessary. This, in turn, led to the development of a multitude of dietary strategies that would still induce an effective molt but retain sufficient gastrointestinal microbial activity [50, 56, 131]. Dietary approaches that limited *S.* Enteritidis and induced molt have been described and discussed extensively elsewhere but typically included manipulation of nutrient content, alterations in mineral content such as addition of zinc, or increasing the level of fiber in the diet using ingredients such as alfalfa alone or in some cases combined with prebiotics or lactose [50, 55, 56, 130–132, 135, 136, 139, 140, 220, 222, 224–230]. Ultimately, these dietary strategies were attempts to target the hen physiologically while avoiding extensive disruption of the gastrointestinal microflora.

In summary, there are a variety of strategies and approaches either currently employed or potentially available to limit *Salmonella* preharvest establishment in egg laying and broiler flocks. As with most food animal production systems implementation of multiple interventions and management choices would appear to provide the best opportunities for limiting dissemination of *Salmonella* both among birds within a flock as well as across multiple flocks. Likewise, early prevention of *Salmonella* establishment in breeder flocks prior to hatching of chicks and during early stages of chick growout for either broilers or layer hen replacement would represent a highly effective means to limit vertical dissemination of this pathogen in commercial poultry flocks. Strategies have been explored in attempts to either prevent establishment of *Salmonella* in laying hens at various stages of the production cycle or eliminate already established *Salmonella*. Attempts have been made to protect birds against *Salmonella* using a variety of live and attenuated vaccines that were specifically designed to limit *Salmonella* colonization and infection in young birds via resistance immunologically [231–233]. As more emphasis is being placed on preharvest prevention of *Salmonella* colonization there will be further need to develop and expand preventative interventions such as vaccines. However, this will require not only a better understanding of the mechanisms involved in *Salmonella* infection of birds but also more precise experimental approaches for defining host pathogen relationships. The following sections address current experimental methods for assessing *Salmonella* infection of poultry, followed by the use of functional genomics to delineate phenotype responses in *Salmonella* and finally the specific application of functional genomics and targeted mutagenesis to the evolution of vaccine development.

## 4. Assessment of *Salmonella* Establishment in Poultry Production

Assessing effective control measures and impact of exposure to *Salmonella* in poultry is further complicated due to the complex nature of *Salmonella* pathogenesis. *In vivo Salmonella* infection models involving the use of a marker strain that carries gene encoding an easily selectable phenotype such as a unique antibiotic resistance profile have been routinely used in both laying hens as well as broilers for a wide range of applications [8, 9, 138, 139, 201, 203, 220, 222–225, 229, 230, 234]. Essentially the use of such genetically modified strains of *Salmonella* allow for ease of recovery in selective media against a highly complex background of non-*Salmonella* microflora by simply incorporating the corresponding antibiotics in the selective media. This is critical if quantitation of the *Salmonella* strain recovered from the infected bird is needed to estimate colonization impact and assess the effectiveness of potential interventions. However there are limitations with such studies not only in terms of availability of marker strains for different serotypes but also a more general concern as to whether antibiotic resistance could influence or alter the marker strain's infectivity compared to the corresponding wildtype isolate. To circumvent some of these limitations numerous attempts have been made over the years to create appropriate *in vitro* experimental models to simulate *in vivo Salmonella* pathogenesis. Anaerobic growth cultures that use either a batch or continuous incubation system have been used to assess *Salmonella* response to the presence of poultry cecal organisms but some type of marker strain is needed to estimate the *Salmonella* populations from these mixed culture backgrounds [212, 213, 235]. Pure culture studies involving either batch or continuous culture systems have also been conducted with *Salmonella* to delineate certain physiological factors but these do not precisely replicate the background matrices that *Salmonella* would be exposed to in the gastrointestinal tract [9, 236–242]. Tissue culture has been historically useful to identify key virulence genes associated with attachment, invasion, and intracellular pathways as well as factors that influence these properties. However, it is difficult to screen a large number of variables and there remains some question as to whether tissue culture studies can serve as exact models for *in vivo* conditions [243–250].

As genetic techniques have developed, more detailed answers to *Salmonella* pathogenesis from both *in vitro* and *in vivo* systems have emerged. The ability to construct gene reporter fusion systems by combining key regulatory genes with structural genes encoding $\beta$-galactosidase, luciferase, or fluorescent green proteins has allowed for quantitative expression assessment when *Salmonella* strains carrying the reporter-fusion gene were exposed to different external and environmental conditions [251–261]. While there are some constraints with $\beta$-galactosidase fusion strains when used in certain backgrounds where non-*Salmonella* bacteria may also possess $\beta$-galactosidase genes, the luciferase and fluorescent green protein-based fusion *Salmonella* strains are usually detectable in most environmental settings. However, in general all of these strains are limited by the availability of fusion constructs that represent the spectrum of possible genetic elements involved in the virulence process. More recently quantitative polymerase chain reaction (PCR) assays have greatly expanded the ability to assess a wider range of genes *in vivo* and have been used in several poultry-based studies to determine virulence gene expression [126, 139, 262].

This information still represents a limited idea of overall *Salmonella* genetic capacity to respond to differing conditions in poultry production. For instance, during the poultry production cycle, *Salmonella* could encounter osmotic stress, temperature and pH fluctuations, desiccation, and starvation as well as exposure to high pressure and chemical decontaminants such as organic acids [6, 10, 88, 144, 146–149]. Considering overlapping stress signals that exist in both host environments and food production systems, there should be common pathways in bacterial cells that are essential for survival in both food environments and human hosts. However, factors uniquely associated with food environments more than likely also exist. Developing a detailed understanding of the unique genetic pathways essential for survival of *Salmonella* and other foodborne pathogenic bacteria in all phases of food production systems has the potential to provide the basis for the development of target-specific and more effective preventative and intervention strategies.

Traditionally, the development of intervention agents has been based mainly on screening for natural compounds exhibiting antibacterial properties. However, such broad cell-based screening usually does not provide information on the respective biochemical targets, which in turn hinders efforts to optimize each compound on the basis of structure-activity relationships [263]. To overcome this obstacle, the pharmaceutical industry redirected its antibacterial discovery approach in the early 1980's from screening for antibacterial activity to inhibiting specific biochemical targets [264]. The molecular targets of most antibiotics developed by whole cell-based screening are the essential gene products, which still remain important targets for development of novel antibiotics. Inhibition of those essential functions usually leads to eradication of the bacteria in the infected hosts. However, the bactericidal activity in those antibiotics is also a major factor in the emergence of antibiotic resistance bacteria in recent years [264]. In an effort to reduce the rate of resistance development, the bacterial genes that are not essential for *in vitro* growth but required for survival in infected host tissues have been suggested as alternative targets. Such *in vivo* essential gene products are more likely to provide a relatively narrow therapeutic range, thereby decreasing the likelihood of selection for broad antibiotic resistance.

In addition, there are not only different serotypes that can emerge but even subtle strain and serovar differences in stress response and virulence gene expression which in turn could cause shifts in *Salmonella* prevalence and subsequent emergence of new isolates [17, 18, 265–268]. Not only is this an issue from a detection and surveillance standpoint but it may also represent sufficient changes in virulence and physiological properties that alter risk, impede traceability, and negatively influence intervention strategies. Sorting out and perhaps predicting evolution of such isolates requires a much more comprehensive analysis of the respective genetic sequences of many more strains and isolates [269]. For *Salmonella* this remains somewhat of a challenge because as Becker et al. [270] have pointed out *Salmonella* is difficult to target for antibiotic therapy due to the high degree of redundancy in metabolic pathways in conjunction with its infection cycle occurring in nutrient rich host environments. This redundancy may in turn allow for multiple parallel pathways for the pathogen to simultaneously disseminate in an infected animal via different host tissues [271]. Consequently, identifying unique genetic factors in *Salmonella* essential for counteracting stresses at different stages of poultry production might be needed to overcome this metabolic resiliency. However, this would involve designing more precise intervention strategies that employ antimicrobial agents or combinations of agents that target specific cellular components that render the organism highly vulnerable and less likely to recover. Functional genomics offers up a potential means to accomplish this as Rohmer et al. [272] have suggested that genome analysis focus could be more directed toward metabolic genes associated with host-pathogen relationships and inhibition of virulence factors could serve as possible therapeutic targets to avoid selection for resistance.

This paradigm is similar to current human clinical research strategies involving the development of new antibiotics for emerging pathogens by focusing on extensive searching for novel ecological sources for natural antimicrobial products as well as redesigning/modifying core structures of known antibiotics [273, 274]. Continued advances in a variety of genetic functional screening techniques coupled with high throughput sequencing approaches have now made it possible to contemplate a much more comprehensive resolution to understanding the complex nature of biological systems such as *Salmonella*-host relationships [272, 275–278]. These approaches should help uncover some of the more subtle nuances in pathogenesis mechanisms and in turn achieve a better understanding of how *Salmonella* interacts with its poultry host [278, 279]. The following sections will discuss these developments in general terms and how they might be used for understanding foodborne *Salmonella* functionality and subsequent application in vaccine development.

## 5. Functional Genomics and Applications in *Salmonella*

*5.1. General Concepts.* Genomic expression analyses can identify genes expressed under certain conditions but cannot define and/or identify genes that are required for survival in that particular niche. Therefore, a more direct way of finding the required genes is the use of genetic mutagenesis-based approaches that directly screen the genome to identify functionality with specific open reading frames on the chromosome. This type of functional genomics can be pursued by either forward or reverse genetic approaches [280]. Forward genetics essentially involves generation of random mutations in the organism of interest by a mutagenesis agent or biological mutagenic entity such as transposons, profiling the resulting mutants for detectable phenotype changes and identifying the gene(s) encoding the phenotype via location of the mutation [280]. Reverse genetics takes advantage of the increasing availability of large scale sequences of bacterial genomes to provide a platform for targeting a specific genetic change at a particular site in the genome via gene inactivation or deletion and assessing potential phenotype responses [280, 281].

Many of the more recent molecular applications in *Salmonella* associated with poultry have involved use of targeting specific genes to generate mutants that lack certain key functions related to their ability to establish in birds. Much of this effort has been directed specifically towards designing *Salmonella* vaccine strains which can successfully initiate an optimal immune response resulting in an immunized bird that resists future infections by nonvaccine *Salmonella* isolates. Historically, there have been a number of gene candidates that were believed to be appropriate targets, but as functional genomics and targeted mutagenesis methods have become more sophisticated better targets have been identified. These efforts have become more critical as the preharvest control measures have been identified as one of the avenues to be emphasized by the U.S. Food Safety and Inspection Service agency for overall control of *Salmonella* in poultry [282]. Of the preharvest approaches that have been characterized in the United Kingdom, O'Brien [283] concluded that the mass poultry *Salmonella* vaccination programs appeared to be the greatest contributor to the impressive fall in the number of cases occurring in that country since the late 1990's. Consequently, there is renewed commercial interest in the further development of more effective *Salmonella* vaccine strains. Some of these developments and the application of targeted mutagenesis will be described in the following sections.

*5.2. Targeted Mutagenesis and Salmonella Vaccine Development in Poultry.* In addition to conducting fundamental reverse genetic studies to achieve a better understanding of the specific phenotype response that an organism such as *Salmonella* might elicit after targeting a particular region of the chromosome more specific applications such as optimizing vaccine strain construction are possible. In poultry, *Salmonella* vaccination programs to limit the dissemination of foodborne serotypes have been developed over a number of years using either inactivated (killed) *Salmonella* or attenuated *Salmonella* strains [233, 284, 285]. Barrow et al. [285] summarized the properties of an ideal *Salmonella* vaccine that should include among other characteristics: (1) prevention of establishment of *Salmonella*, (2) minimization of persistence of *Salmonella* excretion and avoiding carriers (3) possession of ease of administration; (4) stimulation of immunity against target *Salmonella* and related serovars and the resulting immune resistance be passed onto the progeny; (5) exhibition of genetic stability of the live attenuated vaccines, and (6) no deleterious side impact on the host. Historically, heat killed *Salmonella* vaccines have been shown to provide protection but with somewhat variable results [285]. This may in part be due to their inability to trigger a cell-mediated immune response by the host as well as the destruction of some of the more fragile antigens during the heating process [233, 285].

Live *Salmonella* vaccine preparations have enjoyed widespread use and possess several advantages over their dead counterparts including their ability to elicit cellular and humoral immune responses in the host and when administered orally, engage mucosal immunity similar to what the host encounters with the corresponding foodborne pathogen [233, 286–288]. The key to optimizing live vaccine effectiveness in the respective host is to reduce (attenuate) virulence while retaining the immunogenicity by constructing a vaccine strain with decreased ability to grow in the host but still replicate sufficiently to persist long enough to trigger protective immunity [289]. Attenuation is typically achieved by mutagenesis of metabolic biosynthetic pathways such as those encoding synthesis of amino acids and purines or deletion of genes encoding specific virulence factors that limit the ability of the resulting mutant to survive for sustained periods of time but remain capable of inducing a sufficient immune response from the host animal to perpetuate immunity [233, 286, 289–291]. Historically, chemical or ultraviolet light induced mutagenesis approaches were used to generate these types of mutants but much more precise biological recombinant mutagenesis techniques are now employed to target specific gene sites [286, 289].

More recently Bohez et al. [292, 293] used the lambda Red approach described by Datsenko and Wanner [294] to promote recombination and generation of *S.* Enteritidis deletion mutants in *hilA* and *sipA* genes, respectively in *Salmonella* Pathogenicity Island (SPI-1) and the *ssrA* gene in SPI-2. Bohez et al. [293] selected these genes for potential vaccine development based on their decreased ability to colonize host tissues. This fits with their functionality in virulence expression as HilA is one of the important transcriptional activators responsible for coordinating regulation of invasion by environmental and regulatory factors, SipA is an actin-binding protein which stabilizes actin polymers at the interface with host cells, and SsrA is the sensor of the two-component secretion regulator protein for SPI-2 [251, 276, 295–297]. When young chickens were administered *sipA* and *ssrA* mutants, less internal organ colonization occurred versus wildtype while gut colonization remained consistent throughout the trials, and prevention of cecal colonization and internal organ infection after a wildtype *S.* Enteritidis challenge was maintained over the six-week period. Bohez et al. [293] concluded that the inability of these mutants to be cleared would be impractical for commercial application. However, the *hilA* mutant showed more promise as it was cleared from the gut after 4 weeks and although protection from gut colonization by the challenge strain was maintained for only 9 days, prevention of internal organ infection occurred over the entire 6-week period [293]. In a followup trial, long term (6 weeks) reduction in pathogen transmission among seeder birds (5 birds inoculated with the challenge wildtype *S.* Enteritidis) and contact birds (20 birds not inoculated with the challenge wildtype *S.* Enteritidis) was also demonstrated after birds had initially received the *hilA* mutant strain [298].

Karasova et al. [299] also used a PCR inactivation approach to conduct a comparative analysis of *S.* Enteritidis mutants generated for all 5 SPIs for vaccine potential using mice and concluded that all SPI-1 mutants generated (*hilA, sipA, sipB*, and *avrA*) as well as other mutants identified with epithelial cell invasion (*sopB* and *sopA*) remained virulent in mice, while *htrA* appeared to be an optimal candidate due to its balance between attenuation and immunogenicity. This approach was taken a step further in chickens, where *S.* Enteritidis mutants were generated, each missing one of

the 5 SPIs, as well as a mutant missing all 5 SPIs [300]. They demonstrated that even in the absence of all 5 major SPIs the resulting mutant could still become established in the ceca but systemic infection of internal organs (liver and spleen) required both SPI-1 and SPI-2. Dieye et al. [301] generated similar deletion mutants for *S.* Typhimurium SPI-1 and 2 and infected one-week-old chickens with either a mixture of the two mutants or mixtures of the respective mutant and the wildtype strain. They [301] concluded that that while SPI-1 could be identified with colonization of the cecum and spleen, SPI-2 was only associated with spleen infection and not cecal colonization. They also noted that, in the absence of SPI-1, SPI-2 inhibited cecal colonization and that SPI-1 appeared to be more of a contributor to spleen infection, which, as they also pointed out, differed from mice studies where SPI-2 is considered to be more critical for systemic infection. Based on these results and previous research by others Dieye et al. [301] speculated that these differences in hosts may be related to the severity of the disease and the extent of systemic infection versus infections that only result in asymptomatic carriage.

It is conceivable that both serovar and host are important contributors to the resulting respective virulence gene involvement. Evidence of this has been generated from both *in vivo* and *in vitro* studies. Karasova et al. [302] reported that when genes *sodCl* and *spvBC,* associated with highly invasive *Salmonella* serotypes, were deleted in *S.* Enteritidis, virulence of the resulting mutants were reduced in mice but not chickens. They confirmed that this was consistent with serotypes *S.* Infantis, *S.* Hadar, and *S.* Agona which do not possess genes *sodCl* and *spvBC*. Using porcine cell lines Volf et al. [303] demonstrated that incubation of cells with either wildtype *S.* Typhimurium or a SPI-2 *ssrA* mutant induced cytokine response when quantified by real time (RT)-PCR, but the cytokine was not induced for the SPI-1 *hilA* mutant. When examining chicken macrophage cells, He et al. [304] observed suppression of nitric oxide production by *S.* Typhimurium and *S.* Enteritidis but not *S.* Heidelberg, *S.* Kentucky, and *S.* Senftenberg even though all 5 serotypes are commonly isolated from poultry. Strain and isolate differences in virulence properties have also been noted for both *S.* Typhimurium and *S.* Enteritidis serovars [305–307]. When these studies are considered collectively, there are implications for further refinement in vaccine development as the respective animal models used will probably continue to reveal further nuances both in the host defense mechanisms as well as the complexity of *Salmonella* virulence counterresponses and suggest additional potential vaccine targets [308–311].

*5.3. Targeted Mutagenesis and Salmonella Carrier Vaccine Development in Poultry.* As an understanding of *Salmonella* pathogenesis and host immune responses evolved and vaccines specifically targeting *Salmonella* achieved more sophistication it became clear that multivalent vaccines could be genetically constructed to generate host immunity not only to *Salmonella* antigens but also to non-*Salmonella* antigens [286]. The ability of *Salmonella* to colonize the gastrointestinal tract, cross gastrointestinal tract epithelial mucosal barriers, and interact intracellularly in host cells makes it an ideal carrier for presentation of heterologous antigens to the target host's immune system [312–316]. Many of the early *Salmonella* heterologous vaccine constructs involved use of *Salmonella* biosynthetic gene mutants transformed with external genetic elements such as antibiotic resistant plasmids carrying genes encoding a myriad of non-*Salmonella* antigens that were of interest for targeting the immune system of the host [317–320]. However, several problems emerged with these approaches for vaccine administration. The primary concern was the use of antibiotic resistance as a means to retain the plasmid under these conditions where it may be a risk for dissemination of antibiotic resistant bacteria in the environment and thus would be considered a regulatory issue [284, 320–323]. In response to this concern, nonantibiotic markers have been developed for *Salmonella* vaccine strains carrying plasmids as well as balanced lethal plasmid stabilization by constructing a plasmid containing not only the heterologous antigen encoding gene(s) but also a biosynthetic gene that complements an auxotrophic gene on the chromosomal gene [324–329]. To optimize heterologous antigen expression in *Salmonella* vaccine strains, varying the copy number of the expression plasmid has been employed [330, 331]. However, it has been pointed that a vaccine strain carrying plasmids is not only vulnerable due to the metabolic burden/reduced fitness of the organism when introduced to a relatively hostile gastrointestinal tract environment but this may in turn serve as selective pressure to enhance the likely loss of the plasmid [321, 322, 330, 332].

To avoid plasmid vehicles for heterologous antigen expression altogether requires insertion directly into the chromosome in such a fashion that the resulting gene encoding the heterologous antigen is expressed optimally and stably to ensure a sufficient host immune response. Strugnell et al. [333] demonstrated that an *E. coli* cloning vector containing a *S.* Typhimurium *aroC* gene fragment with several unique restriction sites allowed expression of heterologous antigen encoding genes once inserted into the *S.* Typhimurium chromosome via homologous recombination. Over a decade later, significant improvements on this approach occurred when the bacteriophage-encoded recombinase systems were developed for directing homologous recombination and subsequently were utilized for a variety of mutational manipulations and genetic analyses of *Salmonella* [334, 335]. Husseiny and Hensel [328, 336] further modified the Red recombinase approach initially developed by Datsenko and Wanner [294] to construct functional expression cassettes that included either constitutive or *in vivo* activated promoters to direct heterologous protein encoding genes after simulataneous insertion in the *Salmonella* chromosome and simultaneous deletion of chromosomal genes. However, Husseiny and Hensel [336] concluded that constitutive expression of heterologous antigen could cause stability issues with the respective *Salmonella* carrier vaccine as well as overattenuation leading to an overall poor host immune response. Instead, they proposed development of promoters that controlled synthesis of the heterologous antigen only after induction at the target cell. The concept of enhancing host immune response to *in vivo* induced foreign antigens had been previously shown to

increase IgG antibody levels only in mice immunized with a *S.* Typhimurium mutant carrying an inducible Pag-C alkaline phosphatase fusion protein encoding gene in the chromosome but not the constitutive carrying mutant [322, 337]. Husseiny and Hensel, [336] focused on the SPI-2 *sse* genes which encode proteins that are part of the type III secretion system responsible for survival of intracellular bacteria in the surrounding host cell [296]. They immunized mice and observed that the intracellular *sseA* promoter activated mutant strain when compared to its constitutive counterparts enhanced secreted and serum antibodies, increased T-cell proliferation as well as providing protection against *Listeria monocytogenes* infection. Stratford et al. [322] also reported increased antigen specific IgG responses in mice inoculated with an *ssaG* promoter *S.* Typhi mutant but not when this promoter was replaced with a constitutive promoter.

As more is understood on the complexity of the immune system it is becoming clear that there is a need for more precisely defined heterologous antigen *Salmonella* vaccine carriers and that multiple antigens may need to be targeted to optimize and sustain the host immune response. Likewise accurate expression of foreign protein antigens during the immune response is critical if immunization is to be successful against the respective targeted infective agent. When Datsenko and Wanner [294] developed the Red recombinase system to disrupt and insert DNA into target host's chromosome the subsequent removal of the antibiotic resistance or FRT sequences leaves a residual sequence or "scar" in the target sequence. They pointed out that such remaining sequences would be problematic because of unintentional consequences such as recombination of a new PCR fragment with the FRT scar site rather than the newly targeted gene could occur given the limited requirement for homology. These residual or scar sequences represent other potential issues as well such as intereference with gene expression under certain conditions [338]. Several scarless strategies have been developed to resolve this including using marked cassettes for positive or negative selection which can be counter selected with markerless linear DNA via a second round of recombination [339–342]. Alternatively, a counter-selection cassette containing an inducible gene encoding I *Sce*-I endonuclease and its recognition site along with an antibiotic gene can be used in the initial recombination event with the chromosomal target site, followed by induction of the endonuclease and generation of a double stranded break that serves as the site for the second round of recombination with a markerless homologous PCR fragment [338, 343–345].

Cox et al. [346] adapted these approaches to develop a scarless, site directed mutagenesis method for *S.* Enteritidis where longer sequence homologies of 100 to 1000 bp appear to be required for efficient recombination versus 36 to 50 bp for *E. coli* [188, 294]. They combined an overlapping extension PCR, the Red recombinase system, along with the intermediary insertion of the I-*Sce*I endonuclease as a counter selection marker to mediate the recombination of linear DNA with long flanking homology (200 to 300 bp) to *S.* Enteritidis genome sequences. The *S.* Enteritidis mutation by this system was a two-step process with homologous recombination of the linear DNA cassette containing the I-*Sce*I site and kanamycin resistance gene undertaken in the first step. This was followed in the second step by replacement of this inserted fragment with a PCR fragment with a targeted insertion sequence along with a chloramphenicol resistant containing plasmid encoding the I-*Sce*I endonuclease to cut the chromosome at the I-*Sce*I and final selection of *S.* Enteritidis recombinant isolates that were chloramphenicol resistant and kanamycin sensitive. This method was applied to a series of studies designed to develop effective *Salmonella* foreign antigen carrier vaccines for limiting avian influenza and to serve as a polyvalent vaccine for multiple *Salmonella* serotypes in chickens and turkeys. Layton et al. [347] constructed a recombinant *S.* Enteritidis strain expressing the avian influenza M2e membrane protein and the CD154 peptide found on the surface of T cells that are involved in activation of antigen-presenting immune cells and used these to orally immunize day old chicks on day of hatch followed by a booster on day 21. Vaccinated chickens produced higher levels of M2e specific IgG that were increased further when also exposed to the expressed CD-154 peptide but were only protected when challenged with a low pathogenic avian influenza strain but not a highly pathogenic strain.

O'Meara et al. [348] reported that by day 21, turkeys immunized with various *S.* Enteritidis M2e/CD-154 combinations (CD154 peptides: turkey, human, and chicken) exhibited a higher M2e specific antibody level than negative (sterile saline) and positive (*S.* Enteritidis wildtype) control birds but no M2e antibody differences were observed among the vaccine strains expressing the different CD154 peptides. Wolfendren et al. [349] reported that a *S.* Enteritidis recombinant expressing the CD154 peptide antigen along with a *Salmonella* flagellar filament protein (fliC) did not protect against a heterologous *S.* Typhimurium challenge in chickens. When M2e-fliC carrier vaccines were used to immunize turkeys, increases in M2e antibodies compared to the negative and positive controls occurred, but no differences among the *S.* Enteritidis vaccine treatments were observed [350].

Although numerous *Salmonella*-based vaccines developed for administration to poultry have been generated, consistent effectiveness remains somewhat elusive. Several issues remain including the ongoing need for multivalent *Salmonella* vaccines that are effective against a multitude of *Salmonella* serotypes [351]. This is somewhat difficult since serotypes continue to emerge and become predominant only to be replaced by another serotype. Consequently, achieving cross protection among serotypes remains an elusive target [233, 351]. However, perhaps a better strategy may be to use the host animal as a means to screen for immune signals that are common signatures to systemic infection of *Salmonella* in chickens as has been done recently in murine and human *Salmonella* systemic infections [352, 353]. Improvements in design of *Salmonella* carrier vaccines are also emerging that induce greater immune responses by using regulated *in vivo* attenuation as well as regulated delayed *in vivo* antigen synthesis [323].

## 6. Conclusions

Substantial advances have been made in the understanding of *Salmonella* spp. establishment and cross contamination in food system environments including poultry [5, 8, 19, 354]. Likewise stresses occurring in the gastrointestinal tract that *Salmonella* is known to respond to have been identified [9, 246, 272, 314, 354, 355] and the concept that pathogenesis may be driven by nutrient scavenging may help to understand additional gastrointestinal cues [272]. However, as genetic analyses has become more sophisticated a better fundamental understanding has emerged on different aspects of the physiological ability of *Salmonella* to respond to environmental stresses during its entire life cycle [297, 315, 316, 356, 357]. For example, it is now becoming apparent that even fairly well characterized stress responses such as acid tolerance may in fact be further complicated as more pathways for adaptation have been identified, responses to different acids may differ, and not all *Salmonella* serovars may be either equally acid resistant and/or mount similar virulence responses in the presence of different acids [265–268, 358–364]. It is likely that such nuances and complexities will probably come to light for nonacid stress responses as more details become known about *Salmonella* physiology and functional genomics.

*Salmonella* can also use its own metabolism to manipulate its surrounding environment. *Salmonella* has been shown to change the environment in the gastrointestinal tract in an attempt to overcome colonization barriers by literally inducing inflammation to generate metabolities that alter energy metabolism in such a way that it can outcompete the fermentative gastrointestinal microbial consortium [365–368]. Likewise, the ability of *Salmonella* to form biofilms complicates the development of intervention strategies since the biofilm matrix can be protective against environmental stresses as well as antimicrobials [19, 369]. Formation of biofilms by *Salmonella* is believed to be mediated by chemical compounds which function as quorum sensing signal molecules that serve as communication signals among bacterial populations by being released by some cells and subsequently internalized by other cells [370–372]. Biofilms can form on a multitude of hard surfaces such as glass and stainless steel [369]. It is unclear how important quorum sensing and biofilm formation are in food systems although several compounds have been isolated that are antagonistic to quorum sensing compounds [373]. For example, poultry meat-derived fatty acids have been identified as being inhibitory to a quorum sensor autoinducer [374]. The prevalence in food matrices of these inhibitors and their subsequent impact in biofilm formation remain to be determined. However, *Salmonella* biofilm formation does appear to be involved in adhesion on epithelial cells and is mediated by exopolysaccharide composition [369, 375, 376]. Conversely, the presence of the autoinducer AI-2 actually decreased *S.* Typhimurium *in vitro* mouse macrophage invasiveness [377]. A further complication may be the possible *Salmonella* strain variability influence on biofilm formation [378]. As more becomes known about the factors that influence biofilm formation, the role(s) these formations play in surviving environmental stresses as well as initial colonization in various niches will become clearer.

In poultry, more is now known about what factors influence the ability of *Salmonella* to colonize the bird's gastrointstinal tract and trigger systemic invasion [17, 18, 22, 354]. More recently, the interactions between the host's immune system and pathogens such as *Salmonella* are becoming better understood and thus predictable [278, 281]. As more has become known regarding these different aspects of the dynamic *Salmonella*-host interface interactions, development of more effective intervention measures has become possible. However, despite these technological advances foodborne *Salmonella* occurring in poultry continues to be a potential source of foodborne disease in the public sector. Although a wide range of interventions have been developed, limitations remain in their effectiveness. Certainly some of this is attributable to the ability of *Salmonella* spp. to express resistance mechanisms and overcome individual intervention hurdles. However, there also appears to be differences in response to certain antimicrobials and environmental conditions, among not only serovars but also strains and isolates within serovars as well. In addition, historically it appears that different *Salmonella* serovars have emerged to become predominant and that this evolution of serovars may be an ongoing process in poultry production. This not only presents a challenge for development of detection technologies, but also makes it more difficult to design interventions that will broadly limit most *Salmonella* spp.

Advances in molecular-based research should provide some insight to differences at the genomic level that are responsible for *Salmonella* isolates and strains that are subtly distinguishable at the phenotype level. The number of *Salmonella* isolates that have been sequenced continues to grow and adding this information to the genomic data base for individual *Salmonella* serovars will certainly be helpful for resolving detection and intervention issues when only subtle differences exist among strains and isolates. However, sequencing a wide range of *Salmonella* genomes is only the beginning. Assessing genomic functionality beyond just the sequence is becoming more important to not only understand factors responsible for the complex interaction between the chicken host and the invading *Salmonella* but also to delineate what host characterstics trigger initiation of *Salmonella* pathogenesis and how *Salmonella* overcomes and/or circumvents host defenses. Such approaches represent a much more comprehensive strategy for not only studying *Salmonella* overall responses but also readily exploring complex genetic mechanisms of this important foodborne pathogen to survive and persist in poultry production as well as human food chains in general. These approaches also have utility for designing interventions. This becomes particularly critical when constructing more advanced vaccine strategies where a balance between maximum stimulation of the immune system and consistent removal of the candidate vaccine strain is required. High-resolution functional screening of the respective *Salmonella* genomes offers the opportunity to identify distinct genetic components responsible for different virulence mechanisms involved in the pathogenic pathway and precisely identify individual genes for targeted mutagenesis. Such approaches are now being more routinely employed and it is anticipated that understanding the genetic factors

and survival mechanisms will continue to provide valuable insights for development of effective strategies to reduce *Salmonella* in poultry and poultry products.

## Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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