SALMONELLA VACCINATION STUDIES IN BREEDERS AND THEIR PROGENY: RELATING HUMORAL AND MUCOSAL IMMUNITY WITH RESISTANCE TO CHALLENGE

by

ARIEL ROLON

(Under the Direction of Joseph Stan Bailey)

ABSTRACT

Salmonella are ubiquitous enteric bacteria and potential pathogens, some affecting a wide host species range. Eradication of poultry-specific *Salmonella* from commercial flocks resulted in increased prevalence of serovars with a wider host range in poultry and consequently increased potential of human food-borne disease due to consumption of poultry products. Regulatory Hazard Analysis Critical Control Points has been implemented with the goal of reducing *Salmonella* prevalence in poultry meat. Management intervention strategies to curb the occurrence of *Salmonella* have begun to look at competitive exclusion, killed and live *Salmonella* vaccines during production. Our studies focused on establishing how combined vaccination programs protect breeders through rearing and production, and if maternal antibody and competitive exclusion is protective for the progeny. Antibody response was measured at the systemic and mucosal humoral level and an infection model established to relate antibody response to actual bacterial prevalence. Day-of-age breeder vaccination increased intestinal IgG at 3 but not 10 weeks of age. Crop IgA and IgG, as well as gut and serum IgG peaks were observed after killed vaccines delivered at 11 and 17 weeks of age. An approximate 0.8 Log

reduction in *Salmonella* counts due to live 1 and 21-day vaccine applications were obtained at 3 and 6, but not 11 weeks of age. By week 22 all vaccination programs reduced *Salmonella* counts by approximately 1.3 Log. High maternal antibody throughout production passed to the progeny failed to reduce *Salmonella* counts, whereas competitive exclusion consistently reduced *Salmonella* counts by approximately 1.4 Log. Maternal intestinal IgG transferred to the progeny was observed up to 13 days, but no interference of maternal antibody on the effectiveness of day-of-age live vaccination was detected. Day-of-age live vaccine reduced *Salmonella* counts at 3 and 13 but not 34 days of age, indicating that more than one live vaccine is necessary for prolonged protection during rearing. Live vaccine protection is probably a combined effect of humoral, cell-mediated intestinal immunity, and a competitive exclusion effect. Competitive exclusion and vaccination programs will reduce but not eliminate the incidence of *Salmonella*, and therefore should constitute complementary and not substitutive tools to integral biosecurity programs.

INDEX WORDS: *Salmonella*, Challenge, Vaccine, Humoral Immunity, Mucosal Immunity, Maternal Immunity.

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DEDICATION

A mis padres, Erick y Martha

A mis hermanos, Erick Jr y Katia

Por darme el regalo más maravilloso: la vida y una buena familia.

A mi compañera eterna y amada: mi Maria Elena

Eres la mejor compañera que Dios pudo darme para viajar por la vida.

A mis amado hijos, Natalia, Camila y Santiago

Porque en ustedes se resume lo mas bello y sagrado del universo.

A Dios Nuestro Señor

Gracias Señor, Tus bendiciones son infinitas!

A todos ustedes,

Porque hoy no sería quien soy, sin vuestro amor.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Salmonella microorganisms have played a role in the development of the poultry industry from its beginning up to present times. During the early years of intensive poultry farming, two *Salmonella* serovars that constitute primary pathogens of poultry, *Salmonella enterica* serovar Pullorum and *Salmonella enterica* serovar Gallinarum, were common cause of high mortality and heavy losses. High-density houses were a major factor in favoring the spread of these diseases. Scarce or nonexistent biosecurity practices, convalescent birds becoming lifetime carriers, and vertical transmission from positive breeder flocks to their progeny favored the prevalence of these microorganisms across new flocks.

After initial efforts for monitoring flocks for *S.* Pullorum and *S.* Gallinarum in the early 1920's, the National Poultry Improvement Plan was developed as a response to this situation. Its objective was to eradicate *S.* Pullorum and *S.* Gallinarum from poultry flocks, and establish a permanent monitoring system to eliminate all carriers as soon as detected, in order to ensure permanent eradication of the disease. The development of the rapid polyvalent antiserum blood agglutination plate assay was a crucial instrument in this program, enabling on-site detection and elimination of positive birds. Today, these two *Salmonella* serovars have been successfully eradicated from American poultry flocks, although they still pose a significant disease in poultry premises of other parts of the world.

Eradication of *S.* Pullorum and *S.* Gallinarum from poultry flocks possibly left an open niche that tends to be promptly occupied by other *Salmonella* serovars. One of these serovars,

Salmonella enterica serovar Enteritidis, is a common agent associated with gastroenteritis in humans.

Thus, *Salmonella* serovars that caused disease in flocks were controlled, but other serovars that are potential human pathogens are of concern to today's modern poultry industry. Potential sources of *Salmonella* infections from poultry products are numerous. *Salmonella enteritidis* transmitted through table eggs is one possible source. Human *Salmonella* disease episodes linked to poultry meat products is generally due to a wider range of *Salmonella* serovars. All salmonellae are promptly killed by adequate cooking temperatures, and therefore adequate handling and cooking practices should practically eliminate the risk of *Salmonella* transmission from poultry products to the consumer. Flaws in cooking, but especially in handling, as well as cross contamination of other food products in the kitchen (i.e. cross-contamination of vegetables on kitchen counters), are probably the main factors involved in human *Salmonella* episodes.

As one of the efforts to minimize the risk of foodborne transmission of *Salmonella* to the consumer, the USDA established the implementation of Hazard Analysis Critical Control Points (HACCP) in poultry processing plants, through the HACCP systems final rule (United States Department of Agriculture, 1996). In short, HACCP is an assurance system that establishes permitted standards at defined control points considered to be critical during a given production process, and measures and ensures that these set standards are maintained. During poultry processing, a maximum of twenty percent positive carcasses to *Salmonella* measured by whole body carcass rinses are allowed.

Although the bulk of *Salmonella*-positive carcasses are probably generated by horizontal cross contamination during processing from a few positive birds, the poultry industry has

included vaccination of commercial broiler flocks with live *Salmonella* vaccines, as an additional practice in its effort to curb potential problems. Broiler breeder flocks are also frequently vaccinated with inactivated vaccines and live vaccines, in an effort to protect the breeder as well as elicit maternal antibody titers that may be passed to the progeny. The effectiveness of these approaches, however, is dependent on the dynamics between the vaccination strategies in breeder and commercial flocks, and the effect that these vaccines elicit on the bird's immune system. The effect of maternal antibody in conferring effective protection against early *Salmonella* challenge, and potential interference of maternal antibody on early vaccination is not well established.

Three chapters of this dissertation focus on establishing a better understanding of vaccination and protection of broiler and broiler breeders: Chapter three studies the broiler breeder's humoral and mucosal immune response to different vaccination protocols during rearing and production. Chapter four studies the effect of these vaccination protocols on actual resistance to challenge under a multiple marker-strain model. Finally, chapter five studies the effect of early vaccination on layer chicks with and without maternal antibody, as measured by mucosal antibody production and early resistance to challenge. The aim of these studies was to establish a better understanding of the following:

1. How different vaccination programs protect broiler breeder flocks through rearing.
2. How long during production do these programs sustain effective protection to the breeder and sustain maternal antibody in the egg.
3. How effectively does measured antibody response relate to actual *Salmonella* challenge.
4. How effective is maternal antibody in conferring protection against challenge.
5. Establish if maternal antibody will interfere with early live vaccination.
6. How effective is early live vaccination on protecting against early challenge.

A summarized discussion of these issues is presented in chapter six.

CHAPTER 2

LITERATURE REVIEW

2.1 Classification of *Salmonella*

Salmonella are gram-negative, facultative anaerobic, non spore-forming enteric bacilli, within a size range of 0.2 to 1.0 by 1 to 6 μm, belonging to the family *Enterobacteriaceae*. Most *Salmonella* are motile bacteria, with peritrichous flagella, although the two serovars that constitute specific poultry pathogens, *S. enterica* serovar Pullorum, and *S. enterica* serovar Gallinarum, are non-motile bacilli that lack flagella. These non-motile serovars are distinguished biochemically by serovar Gallinarum's inability to ferment dulcitol (Waltman *et al*, 1998). Most *Salmonella* are typically oxidase negative, lactose negative, H_2S positive, gas formation positive microorganisms. Exceptions within these patterns are common, with greater variation in H_2S production and gas formation patterns. Lactose fermenters are uncommon, and generally slow (i.e. Arizona serovars, 7-10 d).

The *Enterobacteriaceae* family includes two *Salmonella* species, *Salmonella enterica* and *Salmonella bongori*, with eighteen serovars within *Salmonella bongori*, and six subspecies within *Salmonella enterica.* Subspecies are referred to by name or number: (*enterica,* I or 1*; salamae,* II or 2; *arizonae,* IIIa or 3a; *diarizonae,* IIIb or 3b; *houtenae,* IV or 4; and *indica,* VI or 6), each subspecies containing numerous serovars, and more than two thousand serovars described within this family (McWhorter-Murlin *et al,* 1994).

Classification schemes changed through time, since initial isolation of the first *Salmonella* by Graffky (LeMinor, 1994) from a patient dying of typhoid fever during the late 1880's. Initial classification schemes were based on biochemical characteristics of the isolates,

but it was soon noted that classification of different Salmonellae based solely on their biochemical fermentative characteristics was difficult. White (1926) proposed a classification scheme based on surface (O) and flagellar (H1, H2) antigens, which was later modified by Kauffman (1975). Within this classification, greater than 2000 serovars were described. An additional virulence antigen (Vi) was introduced in the scheme, which corresponds to the production of capsular protective polysaccharide that may be found in a few serovars (Typhi, Paratiphi C and Dublin). Modifications to the initial scheme have been revised and updated (McWhorter-Mullin *et al*, 1994). Under this modified scheme, serovars should be referred by family and species, a particular subfamily reference optional. Individual serovars must not be italicized. As an example, a common serovar referred as *Salmonella typhimurium* in the past, must be referred to as *Salmonella enterica* subsp. e*nterica* ser. Typhimurium, as *Salmonella enterica* serovar Typhimurium or more commonly, as *Salmonella* serovar Typhimurium.

The Kauffman-White scheme for classification of *Salmonella* relies on the presence of somatic, flagellar and virulence antigens. The somatic or O antigen is composed of variable repeating oligosaccharides located on the outermost region of the lipopolysaccharide, and attached to the inner core and less variable region of the lipopolysaccharide, composed by repeating units of lipid A (Whitfield *et al,* 2003). Variability in the O antigen is attributed to variations in oligosaccharide composition and structure and possibly aid *Salmonella* adapt and evade the host's immune response (Schnaitman *et al*, 1993). The flagellar antigens are composed of flagellin, small protein subunits which confer motility to the bacteria. Since flagellar structures are conspicuous on the bacteria's surface, they are ideal structures for antigen recognition (Kanto *et al*, 1991). Variability in flagellar structures exists, and some serovars will alternatively repress expression of the primary structure, and express a second flagellar structure.

This phase variation possibly enhances the bacteria's capacity to evade the host's immune response (Macnab, 1987). *Salmonella* serovars Pullorum and Gallinarum, the primary poultry serovars, will typically not exhibit motility in semisolid media. However, a serovar Pullorum grown under high motility medium has been induced to express flagella and motility (Chaubal *et al*, 1999). Electron microscopy of this serovar has shown the presence of a single polar flagellum. Motility of the induced bacteria was inhibited by type D antiserum, but not by type J antiserum. Although these serovars will not normally express any flagellar proteins, PCR amplicons to primers targeting regions coding for g and m flagella in the FliC gene have consistently amplified, showing that the genes coding for these flagella are present in these serovars, but not expressed (Kwon *et al,* 2000, Hong *et al,* 2003).

DNA hybridization studies have also been proposed as a means to classify bacteria into different species, a homology of seventy percent or greater being the criterion used to establish if two Salmonellae belong to the same species or not (Wayne *et al*, 1987). Separation of *Salmonella enterica* and *Salmonella bongori* as two different species is based on this approach. In practice, some combinations of biochemical carbohydrate fermentation patterns and serogrouping by whole cell plate agglutination of somatic (O), flagellar (H1, H2), and virulence (Vi) antigen is often used in poultry diagnostic settings. Specific serotyping is more cumbersome, and requires the availability of surface and flagellar antigens. In the United States, only a few reference laboratories will routinely characterize a given *Salmonella* isolate by complete serotyping.

2.2. Isolation and Identification of *Salmonella*

Isolation and identification approaches may vary according to a particular setting: human diagnostics, veterinary diagnostics, or food safety monitoring. Within a human diagnostic

setting, isolation of enteric pathogens are commonly initially attempted on a selective media for E*nterobacteriaceae*, which usually contain certain substances for inhibition of non-enteric bacteria such as bile salts; a pH indicator which aids in determining if the isolates are lactose fermenters; and may or may not contain some indicator of sulfide production. Some whole-cell suspect *Salmonella* colonies from this general approach may be subject to poly-somatic (poly-O) antigen agglutination to see if the suspect colonies are indeed Salmonellae. Antibiotic sensitivity tests along with further serologic/carbohydrate metabolic schemes may be conducted to identify candidate antibiotics for treatment, as well as the specific serovar isolated. Multiple carbohydrate tests are commercially available, such as the Enterotube ® or API ® kits.

Common media used in isolating *Salmonella* include MacConkey agar, brilliant green sulpha with and without novobiocin (BGS), xylose lysine tergitol (XLT4), xylose lysine desoxycholate, modified lysine-iron agar (MLIA) and Rappaport Vassiliadis. Each agar has found preferred use under different conditions. MacConkey is a more general medium, which permits growth of other enteric bacteria such as *Escherichia coli, Proteus sp,* etc. Media such as BGS and XLT4 will partially inhibit other enteric bacteria. Rappaport Vassiliadis has been reported to be very sensitive for screening of food, feed and other samples where very low or no levels of enteric bacteria in general are expected (June *et al*, 1996). However, other investigators have found that the efficiency of *Salmonella* recovery may be more affected by the nature of the material being sampled than by the culture media used (Pangloli *et al*, 2003). In studies involving poultry samples, the use of BGS with novobiocin and MLIA have shown to be a good compromise between sensitivity and inhibitory potential of non-*Salmonella* enteric bacteria and are commonly used under this setting (Bailey *et al*, 1988). BGS has been shown to be a good alternative in sampling for *Salmonella* from poultry environmental samples (Gast *et al*, 2004).

PCR-based methods for identification of *Salmonella* have been developed. Some automated protocols permit rapid identification of *Salmonella*-contaminated samples from meat products in short time, and comparable sensitivity to traditional methods (Bailey et al, 2003). Recently, a multiplex PCR capable of discriminating among a significative number of *Salmonella enterica* serovars commonly isolated from poultry on the basis of differences in genes coding for O and H antigens has been developed (Hong *et al,* 2002). These investigators have developed a PCR protocol based on the amplification of flagellar genes followed by RFLP that discriminates among flagellar serotypes (Hong *et al,* 2003). In the future, these types of molecular approaches will probably find an increasing practical application under diagnostic settings. It's interesting to note, however, that under a diagnostic setting, it is desirable to periodically validate serotyping by PCR/RFLP with traditional serotyping, since some closely-related serovars have the potential to show similar RFLP patterns, and changes in the prevalence of a particular serovar might be otherwise overlooked. In the United States, for example, the most current prevalent serogroup D_1 isolate is *S.* Enteritidis. However, other closely related serovars, such as *Salmonella* serovars, Gallinarum and Dublin, would show similar amplicon products and RFLP patterns when following Hong's methodology, and be undistinguishable solely by the proposed PCR-serotyping scheme. Further development of primer and restriction enzyme schemes capable of discriminating among these serovars would enhance current serotyping by PCR.

2.3. *Salmonella* as an Avian Pathogen

Traditionally, two serovars of *Salmonella,* Pullorum and Gallinarum, were considered primary pathogens of poultry. Debate on whether these two organisms are members of the same serovar, being different biotypes, or are two different serovars, still exists. These avian pathogens

are considered different biotypes of the single serovar *Salmonella enterica* serotype Gallinarum by some investigators (Ryll *et al*, 1996; Kwon *et al,*2000). Pullorum disease (also referred to as bacillary white diarrhea) and fowl typhoid affect a wide species of poultry including chickens, turkeys, quail, pheasants and ducks (Shivrapasad, 2003). These serovars are highly host-adapted, and cause clinical disease mainly in chickens and turkeys.

Although this disease has been virtually eradicated from the United States and some European countries, it still is widely prevalent in many regions of the world. The lack of testing and eradication programs for positive flocks, as well as deficient infrastructure and biosecurity, along with the vertical (dam to hatchling) transmission potential of the disease accounts for its wide prevalence. Even though these serovars will cause disease mainly in poultry, rats have been identified as a major source of horizontal transmission. Studies with Norwegian rats have shown these will act as vectors and fomites in *Salmonella* infections, harboring *Salmonella* Gallinarum in their digestive tracts without the vector showing any clinical signs (Bali *et al*, 1992). In another study, of 39 *Salmonella*-positive rats of a total 601 rats sampled, 24 harbored *S.* Enteritidis, and 12 harbored *S.* Typhimurium. Serovars Montevideo and Derby were also isolated in this study (McKiel *et al*, 1970).

Fowl typhoid is more commonly associated with disease in older chickens, whereas pullorum disease is more commonly associated with disease in poults and young chicks. Vertical transmission will cause increased mortality during hatch and prevailing throughout the first three wks of life. Common clinical signs include anorexia, whitish-diarrhea, depression and dehydration (Burns-Keliher *et al*, 1998; Erbeck *et al*, 1993). Hepatomegaly, fibrinous myocarditis, sinovitis salpingitis, pneumonia and generalized septicemia may also be observed (Mayahi *et al*, 1995; Johnson *et al*, 1992; Salem *et al*, 1992; Ferguson *et al*, 1962). A high

percentage of carriers among the surviving birds is expected. Intermittent recurrence of mortality peaks is common, especially in poults (Christensen *et al*, 1997). Adult carriers may show few signs of disease, although acute cases will cause drops in feed consumption and egg production along with increased mortality, which has been reported to be greater than 90% in young chicks (Hall, 1949).

Surveillance and eradication programs for these pathogens have been possible thanks to the development of serologic tests which permit separation and elimination of suspect carriers. (Shivaprasad, 2003). These include the macroscopic tube agglutination test, rapid serum test, stained antigen whole blood test, and microagglutination test, using tetrazolium-stained antigens (Gast, 1997; Williams *et al*, 1971). Antigens usually contain standard strains of *S.* Pullorum, expressing flagellar antigens 1, 9 and 12. These antigens will detect carriers of both *S.* Pullorum and *S.* Gallinarum, and some have the ability of cross-reacting with antibodies to other group D serovars, such as *S.* Enteritidis. Reported sporadic outbreaks of *S.* Pullorum led some investigators to think that genetic mutation might have rendered traditional antigens used in monitoring less sensitive. Comparison of different commercially-available antigens with traditional and variant *S.* Pullorum strains showed good efficacy of these antigens in detecting positive birds (Gast, 1997).

Disease in chickens caused by motile Salmonellae is infrequent, but when it occurs, the disease is generally referred to as paratyphoid infection or paratyphosis (Gast, 2003). Pathological findings include hepatomegaly with necrotic foci, unabsorbed yolk sac remnants and pericarditis (Evans *et al*, 1999). *Salmonella* serovar Enteritidis has been reported to cause mortality rates of up to 6% during the first wk of life of the chicken (McIlroy *et al,* 1989). Other motile serovars might cause mortality in young chicks (i.e. *S.* Typhimurium). Age and infective

dose affects the degree of signs observed (Gast *et al,* 1989). Potential routes of infection include oral, intracloacal, intratracheal, navel, eye and aerosol routes (Cox *et al*, 1996). Disease in adult birds caused by paratyphoid Salmonellae is rare. Oral doses given to adult birds cause only mild diarrhea, although systemic invasion and dissemination to internal organs may occur, especially with a few serovars: Enteritidis, Typhimurium, Heidelberg, Infantis, etc. (Gast, 1999). Virulent strains of *S.* Enteritidis (phage type 4) have been associated to increased mortality in one year-old hens (Humphrey *et al*, 1991).

2.4. *Salmonella* as a Food Safety Concern Linked to Poultry Products

With the eradication of *S.* Pullorum and *S. G*allinarum from commercial poultry, other motile (paratyphoid) Salmonellae have increased their prevalence in poultry. A retrospective epidemiological study of prevalence of *S.* Enteritidis in humans has led Rabsch and coworkers (2000) to postulate that *S.* Enteritidis was competitively-excluded by serovar Gallinarum prior to eradication of the latter in Germany, and possibly in other countries (England, Wales, and the United States). The wide host range of some of the paratyphoid serovars and therefore the potential of these being transmitted to the final consumer and causing disease is a matter of major concern.

2.4.1 Epidemiology of *Salmonella*

In the United States, data collected and analyzed by the Centers for Disease Control and Prevention show changing trends of the prevalence of a particular serovar with time. The three most prevalent serovars in the United States from 1972 to 1996 were serovars Typhimurium, Enteritidis and Heidelberg. Isolation rates from human sources increased 47% during this 26-year period, possibly due to increased surveillance through time. The three more prevalent serovars increased by 46%, 36% and 459% (serovars Typhimurium, Heidelberg and Enteritidis

respectively), whereas all other serovars increased by only 18% (Angulo and Swerdlov, 1999). More recent data show that *S.* Heidelberg is now the fifth prevalent serovar, with serovars Newport and Javiana increasing their prevalence. The isolation rates for *Salmonella* linked to foodborne illness for the year 2002 were 19.6%, 16.8%, 11.9%, 5.3% and 4.2% for serovars Typhimurium, Enteritidis, Newport, Javiana and Heidelberg respectively. These data show that the increasing trend of *S.* Enteritidis during the 1972-1996 study has leveled off by 2002, with S. Typhimurium being 3% more prevalent than *S.* Enteritidis for this year. Other serovars account for 41.2% of total *Salmonella* isolates (Centers for Disease Control and Prevention, 2004). In some European countries (Scotland, France, The Netherlands), *S.* Enteritidis is reported as the most prevalent, usually followed by *S.* Typhimurium (Munro *et al*, 1999; Grimont *et al*, 1999; Van de Giessen *et al,* 1999).

Serovars causing foodborne-illness in a given area are usually related to the prevalence of that serovar in poultry populations. Emergence of *S.* Enteritidis in 1993 was most prevalent in the Pacific region, with a peak rate in 1996 of more than 25 percent of serovar *S.* Enteritidis isolated in the United States, corresponding to California. In contrast, fewer cases of *S.* Enteritidis isolates were obtained from the southeastern United States (Angulo and Swerdlow, 1999). The ten most prevalent *Salmonella* serovars isolated at the University of Georgia's Poultry Diagnostic and Research Center Laboratory are listed in table 2.1, for samples obtained from chicken, turkey and feed origin.

Although *S.* Typhimurium and *S.* Enteritidis, part of the top 5 serovars found in human isolates, are also in the top ten isolates from poultry origin, serovars Javiana and Newport were not isolated from these samples. Non-poultry sources of foodborne disease other than poultry are

possibly responsible for their prevalence. Serovar Newport, for example, is not associated to poultry samples, but has been linked to contamination of fresh produce (Kenney, 2004).

Table 2.1 Top ten Salmonella serovars isolated at the University of Georgia's Poultry Disease and Diagnostic Research Center

Rank	Chicken	(%)	Turkey	(%)	Feed	(%)
1	Heidelberg	30%	Bredeney	34%	Montevideo	44%
2	Enteritidis	18%	Senftenberg	15%	Litchfield	22%
3	Mbandaka	11%	Hadar	6%	Senftenberg	11%
4	Kentucky	10%	Typhimurium	9%	Drypool	11%
5	Senftenberg	5%	Heidelberg	5%	Livingstone	11%
6	Typhimurium	5%	Agona	4%	---	0%
7	Infantis	3%	Kentucky	4%	---	0%
8	Ohio	2%	Muenster	3%	---	0%
9	Montevideo	2%	Reading	2%	---	0%
10	Agona	2%	Montevideo	2%	---	0%
Total:		87%		84%		100%

Total samples serotyped: Chicken: 736; Turkey=576; Feed =9.
(Source: Dr Stephan G. Thayer, PDRC, Athens, GA 30605).

2.4.2. Poultry Meat as a Source of *Salmonella*

Surveillance of poultry products in recent years has shown that contaminated poultry meat and eggs are among the most frequently implicated sources of *Salmonella* outbreaks. The Centers for Disease Control and Prevention (2005a) reported 5601 confirmed clinical non-human *Salmonella* isolates from chickens and turkeys, of a total of 7589 isolates making 53.4% of total clinical non-human *Salmonella* isolates for the year 2001. An overall incidence of 16.10 cases per 100,000 for the year 2002 of a total 46.27 bacterial foodborne-illness cases per 100,000 were reported (Centers for Disease Control and Prevention, 2005b). The true incidence of *Salmonella*-related disease is estimated to be much higher (estimates 38 times the number of cases confirmed by isolation; Mead et al, 1999), since in many cases the disease is self-limiting and not all cases are confirmed by bacterial isolation. Poultry meat-associated *Salmonella* serovars are diverse, and generally related to the most prominent serovars present in a particular area. A recent study has identified chicken consumption as a risk factor in foodborne *S.* Enteritidis infections (Kimura

et al, 2004). The risk was related to consumption of chicken prepared outside the home and not related to chicken meat *per se*. Cross-contamination during food preparation remains an important risk factor in all *Salmonella* outbreaks.

2.4.3. Eggs as a source of *Salmonella*

Eggs are the dominant source of *S.* Enteritidis infections in humans (St. Hogue *et al,* 1997). Consumption of raw or undercooked eggs was identified as the major risk factor in acquiring *S.* Enteritidis infections (Passaro et al, 1996; Cowden *et al,* 1989). A model for estimating infection related to consumption of *S.* Enteritidis contaminated eggs predicted that between 81,535 and 276,500 cases occurred during 2002, 42 times more than the confirmed cases by bacterial isolation (Schroeder *et al,* 2005).

Despite the predominance of S. Enteritidis in eggs, other serovars may also be related to egg contamination and subsequent human infection (Chittik *et al*, 2004). *S.* Heidelberg has shown comparable potential to invade and colonize the intestinal and reproductive tracts (Gast *et al*, 2004). However, tissue colonization and egg contamination are not always directly correlated (Barrow *et al*, 1991).

2.5 Virulence of *Salmonella*

Fimbriae, flagella, plasmids and toxins are among the most studied factors involved in Salmonellae virulence (Van Asten *et al,* 2005). After the bacteria enter the digestive tract, they must adhere to the enterocytes to establish colonization. There is a general consensus that fimbriae and flagella are important mediators of adherence in the paratyphoid Salmonellae (Allen-Vercoe *et al* 1999; Thiagarajan *et al*, 1996); although certain exceptions do exist.

Fimbriae are filamentous structures coded for by an estimated 8 to 11 genes clustered in 7 to 9 Kb operon (Clouthier *et al*, 1994). Van Asten et al (2005) have summarized the different

fimbrial operons present in several *Salmonella* serovars. In one investigation, neither fimbria nor flagella were found to be required for *S.* Enteritidis to colonize the intestinal tract (Rajashekara *et al,* 2000). Mutations in the *fimH* gene coding for fimbriae in *S.* Typhimurium result in a non-fimbriate and non-adhesive phenotype (Hancox *et al,* 1997). Plasmid-encoded genes for fimbrial structural components increase the virulence of a *S.* Typhimurium isolate in mice (Kinsey *et al*, 1993).

Motile Salmonellae express 5 to 10 peritrichous flagella that confers them motility. Non-motile serovars Pullorum and Gallinarum exhibit a *fliC* gene (Li *et al*, 1993; Dauga *et al*, 1998) responsible for encoding phase 1 flagella in other subgroup D Salmonellae (i.e. serovars Enteritidis, Berta, Dublin). The phase two *fljB* gene in contrast, is not present (Dauga *et al*, 1998). Two point mutations in the sequence of the FliC gene were detected in serovars Pullorum and Gallinarum. However, these investigators hypothesize that lack of flagellar expression is probably not the result of these mutations, but of other mechanisms involved in regulation of expression. Lack of motility (or of flagellar antigen expression) may confer these host-adapted serovars an adaptive advantage over others. Flagella have been reported as essential (Lockman *et al,* 1990) and non-essential (Van Asten, 2004) for cellular invasion, and therefore the role they play in virulence might be serovar-specific. Adherence and invasiveness of a particular serovar might be separately regulated. Mutations affecting intestinal colonization in chicks after oral infection, did not affect virulence after intraperitoneal inoculation (Porter *et al*, 1997).

Plasmids associated to Salmonellae may confer virulence to their hosts, and many serotype-specific plasmids have been studied. Virulence may be partially correlated to the presence of some plasmids (Chu *et al,* 1999). Among plasmid-encoded genes affecting virulence,

are those associated with invasiveness, intracellular survival, macrophage lysis, immunosuppression and drug resistance (Gast, 2003).

Endotoxin, enterotoxin, and cytotoxin are three classes of toxins identified in Salmonellae. Endotoxin is related to the Lipid-A portion of lipopolysaccharide, and when released (generally due to cell lysis) will cause fever in the host. Cytotoxin is a heat-stable toxin that interferes with the host cell's normal protein synthesis and allows calcium ions to escape (Madigan *et al,* 2003). Enterotoxin is a heat-labile toxin present on some serovars which will cause enterocyte fluid loss and diarrhea (McDonough *et al,* 1989).

Recent intensive research has focused on studying chromosomal genes coding for different factors which ultimately enhance host invasion, macrophage colonization and bacterial reproduction. Most genes coding for these characteristics are clustered on pathogenicity islands (Marcus *et al*, 2000). Five pathogenicity islands (PI) have been characterized on the *Salmonella* chromosome (Van Asten *et al*, 2005). Among the most important are: PI-1, which codes for a type III secretion system involved in transport of bacterial proteins (SptP, SopE) into the host's cytosol, which leads to uptake of the bacterium by the cell (Hayward *et al*, 2002). Bacterial endocytosis is mediated by changes in the actin cytoskeleton (Guiney *et al*, 2004). Genes in the PI-2 inhibit the recruitment of NADPH and inducible nitric oxide synthase to Salmonellae-containing vesicles in macrophages, thereby inhibiting lysis of phagosome and conferring the bacteria a safe niche where to replicate (Fang *et al*, 2002). A type III secretion system enhances bacterial survival in epithelial cells and macrophages. Ten open reading frames have been identified in the PI-3, among which gene *mgt*C is found, responsible for survival under limited magnesium. *Salmonella* PI-4 carries a type I secretion system involved in toxin secretion (Blanc-

Potard *et al,* 1997). Genes within PI-4 also code for a type I secretion system which possibly also enhances bacterial intra-macrophage survival (Wong *et al*, 1998).

2.6. *Salmonella* Interaction With the Host's Immune System

After the onset of infection, interaction with the host's immune system initiates a non-specific immune response closely followed by B and T cell specific responses. Heterophils play an important role in phagocytizing and killing bacteria. Macrophages initially ingesting bacteria will present antigen for activation of B and T cell responses, with consequent antibody production and cell-mediated lysis of infected cells. *Salmonella* will generally elicit a strong humoral immune response after live antigen delivery to immune-competent birds (Gast, 2003).

The gut-associated lymphoid tissue (GALT) consisting of the bursa of Fabricius, cecal tonsils, and focal lymphocytic aggregates within the mucosal epithelium and lamina propia, acts as the first line of defense against enteropathogens (Lillehoj *et al*, 2004). Existence of an esophageal tonsil as a discrete unit of lymphoid tissue has recently been proposed (Nagy *et al,* 2003). After exposure to antigen, all three major classes of antibodies, IgM, IgA and IgG are produced. IgG is normally transferred through the egg yolk, and possibly confers a degree of maternal immunity (Lillehoj *et al*, 2004; Schat *et al*, 1991). IgA and IgM in contrast, are not present in egg yolk but may be found in the egg white, amniotic fluid, and in the intestine of the embryo following drinking of the amniotic fluid (Schat *et al,* 1991).

In young birds, the immune system is immature, and both innate and adaptive responses are functionally diminished before one wk of age. T and B cell areas and germinal centers in cecal tonsils and cecal patches of 5 d-old SPF birds have been described, with minor presence of lymphocytes in the proventriculus and ventriculus (Jeurissen *et al*, 1989). However, other

investigators (Bar-Shira et al, 2003) demonstrated that these GALT lymphocytes are functionally immature during the first 2 wks post-hatch, with degree of maturation increasing with age. Innate response is also functionally immature, as shown by a diminished phagocytic index of heterophils in chicks during the first wk of age (Wells *et al*, 1998; Kodama *et al*, 1976).

The GALT might provide a degree of compartmentalization of the immune response. A crop lavage procedure (Holt *et al,* 2002) can be used to sample ingluvial antibodies. Presence of IgA up to 24 d post challenge with *S.* Enteritidis by this sampling method along with presence of ingluvial lymphocytic aggregates has been demonstrated (Seo *et al*, 2003; Seo *et al,* 2002). These techniques will permit further understanding of mucosal immunity in the future.

2.7. Control Measures for *Salmonella* in the Production Environment

2.7.1. General Management Intervention Strategies

Being a ubiquitous microorganism, the control of *Salmonella* in the production environment becomes a management-related disease (Hofacre, 1998). Emphasis starts at the top of the production pyramid and is applied throughout the production process (Elites → Grandparents → parents → commercial birds). Implementation of biosecurity programs start with acquisition of *Salmonella*-free birds (or the gradual elimination of positive-flocks); adequate downtime, disinfection of premises and equipment (including litter) used in rearing and production, and feed and water devoid of Salmonellae. If all these elements have been adequately controlled to begin with, control of movement of potential vectors (including personnel) and fomites (including equipment used in moving feed/birds/ etc) is necessary to minimize the probability of contamination. Adequate rodent control programs should include not only a regular baiting program, but adequate maintenance of houses and surroundings to

minimize potential rodent-harboring and mating areas. In large integrations, convergence of eggs from multiple flocks at the hatchery increases probability of cross-contamination among chicks from *Salmonella*-positive to *Salmonella*-negative and positive flocks. Unless this problem is addressed, it is unlikely that the incidence of *Salmonella* positive birds would diminish. Utilization of competitive exclusion and vaccination may form part of an integral program, especially in situations where elimination of the bacteria is unfeasible.

The cost of controlling *Salmonella* in broiler and layer flocks, however, can be overwhelming and unrealistic under a particular economic background. In the case of layers, elimination of *S.* Enteritidis-positive flocks entail big losses, especially if no pullets are immediately available (increased opportunity cost of idle capital). Diverting production to egg-breakers would also have a high cost, considering that an average 5-8 cents per dozen eggs are lost. If the producer has contracts to honor, purchasing of eggs in the open market may entail an additional cost (Morales and McDowell, 1999). Although a final economic analysis is still ongoing, economic feasibility for a *Salmonella* eradication program in the United States broiler industry is unlikely (Bailey *et al*, 2004).

2.7.2. Competitive Exclusion

Competitive exclusion in broiler chickens dates back to 1973, when intestinal contents of healthy adult birds were orally inoculated to hatchlings to reduce the incidence of a *S.* Infantis in Finland (Nurmi *et al*, 1973; Rantala *et al*, 1973). The modes of action of competitive exclusion cultures include competition for adhesion sites on the mucosal epithelium, competition for essential nutrients, and production of antibacterial substances such as bacteriocin and short-chain fatty acids (Corrier and Nisbet, 1999). Defined competitive exclusion flora has been difficult to develop, due to the presence of a large number of facultative and obligate anaerobes, as well as

the complex interrelationships between these bacteria. First attempts to use defined flora as competitive exclusion include the use of *Lactobacillus*, or mixtures of a few species of bacteria, and their protective efficacy was generally inconsistent (Stavric et al, 1993). An approximate 1 log reduction in *Salmonella* colonization by use of a strain of *Lactobacillus reutieri* used in and ex-ovo has been reported (Parkhurst *et al*, 1997). More recent work show promising results using mixtures of defined species of facultative anaerobes (Bielke *et al*, 2003). Development of a mucosal competitive exclusion culture and its use by coarse spray delivery and in the first drinking water resulted in significant reductions of litter, feathered skin and cecal Salmonellae, as well as reduced prevalence in processed carcass rinses (Blankenship *et al*, 1993).

Commercial undefined competitive exclusion products are available and in use outside the United States. A study with three of these products, Aviguard, MSC and Avifree, showed comparable efficacy in reducing *Salmonella* by Aviguard and MSC, with the MSC treatment yielding the numerically smallest mean cecal counts, and the smallest proportion of *Salmonella*-positive birds (Ferreira *et al*, 2003).

Combination of enrofloxacin followed by competitive exclusion treatment has shown to be efficacious in treating adult birds, and has been used as an alternative to elimination of positive breeder flocks (Edel, 2002). Shedding of *S.* Enteritidis was decreased from 33 and 25% in untreated molted birds, to 4 and 0% by the combined treatment (Seo, *et al*, 2000).

2.7.3. Vaccines for control of *Salmonella*.

Live, inactivated and recombinant vaccines for *Salmonella* have been developed. Studies with inactivated vaccines show good humoral immune responses. Inactivated vaccines may elicit acceptable levels of humoral immunity but are unable to induce cell-mediated immunity and consequent T-cell cytolytic responses (Nagaraja *et al*, 1998). Reduced organ colonization

resulted after inactivated vaccines were given twice at four wk intervals to mature birds and subsequently challenged with *S.* Enteritidis. However, a good portion of the birds still shed the bacteria 1 wk after challenge (Gast *et al*, 1993). Autogenous bacterins for breeders combining the most prevalent serovars in a given area or operation are currently used by the industry.

Although whole cell antigen in traditional oil emulsions are the most common preparations used in autogenous vaccines, other alternatives for killed antigen types and antigen delivery systems are currently being explored. Subunit vaccines using outer membrane proteins (OMPs) have shown to provide immunity and some degree of cross protection (Charles *et. al*, 1993; Bouzoubaa *et. al*, 1987; Nagaraja *et al.*, 1988). When OMPs are presented in novel immune-stimulating complexes as adjuvants, these have shown slightly higher humoral immune response compared to OMPs presented in a traditional mineral oil emulsion. However, comparative studies using traditional and outer membrane protein (OMP) inactivated vaccines show no significant differences in decreasing SE counts between the vaccine types (Charles *et al.*, 1994). Although OMP vaccination with subsequent homologous serovar challenge was shown to render adequate protective efficacy, the degree of protection under heterologous challenge was less efficient. Traditional autogenous bacterins containing whole cell antigen of the most common field isolates encountered by a particular operation, probably result in adequate protection against these field serovars. Outer membrane protein vaccines have not found significant use to date. Comparable efficacies of protection and possibly lower cost of production would explain preference for autogenous whole cell preparations.

Strain attenuation strategies used in the development of live *Salmonella* vaccines have been diverse. Although not licensed for use in the United States, a *S.* Gallinarum rough strain, lacking lipopolysaccharide (LPS) has probably been the most globally used live *Salmonella*

vaccine, and is an important tool in areas of the world where fowl typhoid remains endemic. Lack of LPS in this vaccine has the advantage of not eliciting anti-LPS antibodies, and therefore not interfering in serum or whole blood plate agglutination tests that may be used in routine monitoring programs.

A Δcrp and Δcya ST was developed and tested specifically in poultry (Hassan and Curtis, 1994), and a licensed vaccine is commercially available (Babu *et al,* 2003). Live auxotroph mutants recently developed and licensed for marketing in the United States include an Aro-A *S.* Typhimurium mutant. A similar double-deletion Aro-A mutant against *S.* Enteritidis is currently licensed and in use in Europe with promising results. Use of these attenuated live vaccines is promising, especially due to the potential of eliciting a more effective cell-mediated immune response. The efficacy of live attenuated vaccines in eliciting adequate immune responses and consequent protection, alone or in combination with killed bacterins requires further assessment, and constitute the focus of our investigations.

CHAPTER 3

SALMONELLA VACCINATION PROGRAMS IN BROILER BREEDERS I. HUMORAL AND MUCOSAL HUMORAL IMMUNE RESPONSE[1]

[1] Rolón, a., J. S. Bailey, P.S. Holt, C. L. Hofacre, J. L. Wilson and N. A. Cox. To be submitted to Poultry Science.

ABSTRACT

Although vaccination against *Salmonella* has been used more frequently in broiler breeders in recent years, there is a paucity of information in the literature demonstrating the immunological response of combinations of live and killed whole cell vaccines. The present research assesses the immunological response generated by three different vaccination protocols. Treatment vaccines consisted of a live Aro-A mutant commercial *Salmonella* Typhimurium (ST) vaccine (Fort Dodge Animal Health) and an autogenous commercially prepared killed vaccine consisting of a pool of *Salmonella* serovars Berta (D_1), Heidelberg (B), and Kentucky (C_2). Three vaccination treatments using live, killed or a live-killed combination plus a non-vaccinated control were evaluated. Serum (SER), crop lavage (CL), gut lavage (GL), hatchling serum and egg yolk were tested for specific IgA and IgG anti *Salmonella* Enteritidis (SE) or *Salmonella* Typhimurium lipopolysaccharide (SELPS or STLPS, respectively) antigen by indirect ELISA. Immunological response was stronger on STLPS than SELPS. IgA of SER and CL were short-lived peaks after the first killed vaccine, with optical densities (OD) greater than 1.000. A short-lived peak of IgG of CL on STLPS (OD>1.500) was also observed. Strong GL IgG after first live and both killed vaccine events were observed (OD>1.000), with the response to the killed preparation enduring longer. SER IgG responses observed after killed vaccination lasted throughout 40 wks of age with no demonstrable differences between treatments. Hatchling serum and egg yolk IgA were negligible, and IgG was comparable among all treatments throughout time. Results confirm that killed antigen is vital in eliciting adequate IgG in serum

and gut. Live vaccination with Aro-A mutant ST vaccine enhances gut IgG and possibly aids in conferring adequate immunity during the breeder's first wks of life.

(*Key words*: *Salmonella* challenge; mucosal-humoral; mucosal; immune response; immunity)

Abbreviation Key: 2K = two killed vaccines; 2L2K = two live and two killed vaccines; 3L1K = three live and 1 killed vaccine; CL = crop lavage; GL = gut lavage; OD = optical density; SELPS = *Salmonella enteritidis* lipopolysaccharide; SER = serum; ST = *Salmonella typhimurium*; STLPS = *Salmonella typhimurium* lipopolysaccharide

INTRODUCTION

Mandatory implementation of Hazard Analysis and Critical Control Points (HACCP) as the primary tool for pathogen reduction in the processing plant has increased pressure on poultry processors to minimize any potential source of *Salmonella* coming into the plant (USDA, 1996). Risk analysis for the processing plant has shown water, environment, live haul transportation and fomites in general, as well as carrier birds, to be the main sources of *Salmonella* contamination. Of these factors, live transport equipment and carrier birds are possibly the major culprits (Mc Capes and Riemann, 1998).

Salmonella vaccination studies resulted in the development of live vaccines as well as killed bacterins, which are both commonly used in the field for layer, breeder and commercial broilers. The bacterin type used in commercial layer operations is generally restricted to SE bacterins, since egg transmission of this potential human pathogen is the primary concern in layer flocks. In contrast, the most widely used bacterins in broiler breeder settings are traditional water-in-oil autogenous emulsions, generally manufactured by a commercial vaccine manufacturer for a particular customer and using a blend of two or three of the most prevalent serovars commonly encountered in the field by the customer. The goal of vaccination in broiler

breeder operations is to curb the incidence of vertical transmission of field *Salmonella* to the progeny. Reduction of vertical transmission may have some effect on overall broiler performance depending on the serovar's degree of virulence and host adaptation, but more importantly, may help reduce the incidence of *Salmonella* carried into the processing plant.

Gene deletion (Δ) used as a tool for attenuation of vaccine strain candidates has seen diverse approaches. A licensed ST live vaccine for poultry was developed by deletion of the aro-A gene, which encodes 5-enolpyruvylshikimate-3-phosphate synthase, an enzyme involved in synthesis of the aromatic amino acid precursor chorismate (Hosieth and Stocker, 1981; Dougan *et. al,* 1987; Dougan *et. al,* 1988). Other gene deletion mutants (Aro-C and Aro-D, encoding for chorismate synthase and 3-dehydroquinase) involved in chorismate synthesis, and double and triple-deletion combinations have been developed in ST and *Salmonella* serovar typhi, the causative agent of human typhoid (Chatfield *et al*, 1992; Hone *et al*, 1991). Double deletion of genes coding for receptor protein of cAMP and adenylate cyclase (Δcrp and Δcya) yielded a severely attenuated ST. Deletion of these genes affects carbohydrate metabolism, affecting expression of fimbriae and flagella (Curtiss *et al*, 1988).

Although cell-mediated immunity is widely recognized as an important mechanism in the bird's response to *Salmonella* challenge (Arnold and Holt, 1995), specific aspects of this response are still largely unknown (Zhang-Barber *et al*, 1999; Lillehoj and Okamura, 2003), and no practical test for cell-mediated immunity in the field exists. Measurement of antibody as an indicator of humoral immune response by ELISA is still the most widely used tool to monitor a flock's immune status. Cell-mediated responses may better reflect an animal's potential resistance to challenge compared to humoral response (Lee *et al*, 1983). However, a genetic line to antibody production correlation, as well as, greater antibody production to decreased

Salmonella colonization correlation have been demonstrated (Kaiser and Lamont, 2001; Kaiser *et al*, 2002), showing that antibody monitoring is a practical and valuable tool for relating antibody response to resistance to challenge. Commercially-available kits and research-type protocols exist for measuring anti-*Salmonella* immunoglobulin in serum, with commercial ELISA assays measuring IgG on flagellin-coated plates and research ELISA assays capable of measuring IgA or IgG on LPS or flagellin-coated plates (Holt and Porter, 1993, Idexx, 2004).

Few long-term studies focusing on live *Salmonella* vaccination and effects on the chicken's immune response have been conducted (Hassan and Curtiss, 1997), and to our knowledge, no reports using protocols combining live and killed vaccines with commercial breeds under industry-type vaccine delivery and rearing conditions exist. The few long-term studies have used ΔcyaΔcrp mutants using direct oral gavaging of the vaccine, and assessed protection to homologous serovar Typhimurium and heterologous serovar Enteritidis (Hassan and Curtiss, 1997). Although a degree of cross-protection of live vaccines on subsequent challenge with heterologous serotypes has been demonstrated (Hassan and Curtiss, 1994; Hassan and Curtiss, 1997), efficacy of protection is affected by the particular vaccine and challenge strains (Zhang-Barber *et al.*, 1999). Efficacy of protection would be expected to decrease as antigenic differences between vaccine and challenge strains increase.

The gut-associated lymphoid tissues are the secondary lymphoid tissues located in the alimentary tract and intestinal mucosa, and classically associated with intestinal Peyer's patches and cecal tonsils (McGhee et al, 1992; Schat and Myers, 1991). More recent studies have focused attention on the crop as a possible site for mucosal immunity. A procedure for harvesting immunoglobulins from chicken's crops was developed (Holt *et al*, 2002) and production of crop anti-SE IgA following infection has been demonstrated (Seo *et al*, 2002, Seo *et al,* 2003a). The

crop-lavage technique provides a useful tool in studying humoral mucosal responses at the alimentary tract level, and similar lavage procedures may be used in obtaining samples for intestinal antibody monitoring. In this case however, euthanization of the chicken to be sampled is necessary prior to the intestinal lavage procedure. Studying differences in serum and humoral mucosal antibody dynamics may provide further insight to the bird's response to *Salmonella* vaccination and challenge.

Primary airborne exposure in hatching cabinets (Cason *et al,* 1994) or in the houses can precede intestinal *Salmonella* colonization of healthy chickens. Although *Salmonella* exposure in commercial broiler and breeder flocks requires colonization of the intestinal tract, environment reduction of S*almonella* by use of an electrostatically charged apparatus resulted in decreased incidence of infection, demonstrating the importance of airborne *Salmonella* transmission in broiler breeder houses (Richardson et al, 2003a; Richardson et al, 2003b). Commercially-available live *Salmonella* vaccines are massively aerosolized at the hatchery or on arrival to the farm, and sometimes a second application is given by aerosol or drinking water. Our studies therefore, focused on profiling humoral and gut mucosal IgG and IgA responses of broiler breeders subjected to 3 different vaccination protocols under vaccination and rearing conditions closely resembling today's industry practices.

MATERIALS AND METHODS

Chickens and Premises

One thousand female and one hundred and fifty male day-old Cobb x Cobb broiler breeder parents were obtained from a major commercial broiler breeder supplier, and placed at the University of Georgia's Poultry Science Research facilities. Females came from a 57 wk-old, and males from a 34 wk-old grandparent stock, respectively. After randomization, chicks were

placed in four separate units consisting of identical environmentally-controlled rooms each having independent mechanical trough feeding systems and nipple drinkers. Rooms were negatively ventilated; force air heated or evaporatively cooled; and these systems were electronically controlled. Air inlets and exhausts were fitted with light traps. Light was provided by high pressure sodium and fluorescent bulbs. Each room was 9.1m wide x 7.3m deep and 3.05m high. All rooms and equipment were washed, and foam-disinfected with BioSentry 904®[1] according to the manufacturer's specifications. Approximately 3 inches of fresh pine shavings were placed on the previously cleaned premises, and formalin allowed to react with potassium dichromate at an approximate concentration of 10g of formalin per cubic meter of the premise. Drag swabs of equipment and premises 4 d after sanitation were cultured for *Salmonella* by direct plating on Brilliant Green-Sulfa agar[2], or pre-enriched and delayed pre-enriched on tetrathionate broth base[2] before plating, yielding negative results. On arrival of chicks to the farm, chick box liners were cultured for *Salmonella*, and $1m^2$ live paper liners placed wkly under feeder troughs, and cultured for *Salmonella* monitoring on d 7, 21, 42, 77, 98 and 119 of age.

Vaccines

On arrival to the farm, female chicks were randomized into four treatments, consisting of a non-vaccinated control, a two-live/two-killed (2L2K), a three-live/one-killed (3L1K), and a two-killed (2K) group. Live vaccine was Poulvac-ST ®[3], an Aro-A serovar Typhimurium mutant. The live vaccine was given as coarse spray while inside chick boxes at day of age, or via drinking water at d 21 or 77 of age. Killed vaccine was a water-in-oil emulsion of an autogenous blend of serovars Heidelberg (group B), Kentucky (group C2) and Berta (group D1),

[1] DuPont Animal Health Inc, Chilton Industrial Estate, Sudbury, Suffolk, UK
[2] Becton, Dickinson and Company, 1 Becton Drive, Franklin Lakes, NJ 07417-1880

commercially prepared[4] for a major broiler grower in the southeast. Killed vaccines were given subcutaneously on wks 11 or 17 of age. Vaccination treatments and days of delivery are shown in Table 1. Males were raised in a separate identical unit. Pullets were fed *ad libitum* for the first four wks, and entered a skip-a-day feed restriction program until moved to the production units. Amounts of feed delivered were calculated weekly based on weekly body weights. Lighting was 24 hr for the first day, and was reduced to 8 h at 4 wks, followed by light stimulation once pullets were 21 wk of age. Feeding and lighting programs closely resembled current broiler breeder husbandry practices.

At 18 wks of age, pullets were moved to almost identical rooms equipped with nests on laterally placed slats on 2/3 of the total floor area and a central non-slatted mating/scratch area. Mechanical feeding chain troughs, automatic nipples and belt-conveyed nests resembled a typical broiler breeder house. Males were introduced a few days after the females.

Humoral and Mucosal Samples

Blood, crop lavage and gut lavage samples were collected periodically to profile immunoglobulin concentrations on each sample type through time. Blood samples were obtained from the brachial vein of chickens, except for the day-of-age samples which were obtained from the jugular vein. Crop lavage samples were taken according to Holt *et al* (2002). Briefly, lavage solution consisting of a 1M Tris/glycine buffer with 0.25% Tween20 was flushed into the crop and then gently massaged, and the solution aspirated back into the syringe. Five ml of lavage solution was administered using 3/16 inch outer diameter Tygon™ tubing when sampling birds 6 wks or older, but only 2.5-5ml of lavage solution using a 1/8 inch tubing was used for younger birds. Gut lavage samples were obtained after euthanizing a subset of chicks. The small intestine

[3] Fort Dodge Animal Health Inc, Overland Park, KS.
[4] Lohmann Animal Health International, 1146 Airport Pkwy, Gainesville, GA 30501.

was carefully excised at the ventriculo-duodenal and at the ileo-cecal junctions. The section was removed and flushed with 10ml of lavage solution by inserting a feeding needle[5] through the ileal extreme, and collecting flushed material through the duodenal extreme into 15ml centrifuge tubes. Samples were kept on ice until reaching the laboratory, where they were immediately centrifuged at 2,500g for 10 min. The supernatant was frozen at -70C until the ELISA assay procedure. Once in production, egg yolk and hatchling serum samples were taken. Immunoglobulin was extracted using the oily-acid protocol of Seo *et al,* 2003b. Table 2 summarizes samples taken at each bird age.

ELISA Assays

Indirect ELISA assays were conducted according to the method of Holt and Porter (1993). Antigens used for coating plates were SE LPS[6] or ST LPS[6] at a concentration of 10µl/ml, incubated overnight. Serum samples were diluted at 1:250, and crop and gut lavage samples were diluted at a 1:2 ratio.

Plates were blocked with 0.1*M* PBS plus 0.5ml Tween 20 plus 1% Bovine Serum Albumin[6] BSA for one h to minimize non-specific binding. Previously diluted samples (Serum at a 1:250 ratio, and gut or crop lavages at a 1:2 ratio) were added to the microplates along with positive and negative controls, and incubated for 90 minutes. Plates were washed two to three times between steps with 0.1M PBS plus 0.l5ml Tween 20. All incubation steps were done at room temperature, and plates placed on mechanical mixer during incubation. Primary antibodies used were mouse anti-chicken IgA[7] diluted 1:1000 or mouse anti-chicken IgG kindly provided by Dr. Peter Holt, and diluted 1:40. Primary antibodies were incubated for one h. A secondary

[5] Oxoid Inc, Ogdensburg, NY.
[6] Sigma, Saint Louis, MO.
[7] Southern Biotech, Birmingham, AL.

goat anti-mouse IgG heavy and light chain specific antibody[8], at a 1:2000 dilution was added and incubated for one h. Para-nitro-phenyl phosphate chromogen[6] diluted at 1mg/ml in diethanolamine[6] was added and incubation allowed to proceed for 20-30 minutes under dark conditions. Plates were read at 405nm absorbance with an Ascent[9] microplate reader.

Statistical Analysis

A Log_{10} transformation of OD data was performed and a Completely Randomized Design was used to analyze transformed data, using the General Linear Model procedure of SAS[10]. Data was analyzed independently within each sampling event (day of breeder age). Means were discriminated using Duncan's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

Salmonella monitoring of chick liners and paper pad results are shown in Table 3. Box liners from female birds were positive for group B *Salmonella* (serovar Heidelberg), indicating hatchery contamination of the females but not the males. The same group B *Salmonella* was isolated from premises housing the 2K group at d 7, 21 and 42 of age, but no more positive isolates were obtained at d 77, 98 and 119. Increased age-related resistance as well as increased susceptibility of day-old chicks to *Salmonella* intra-cloacal colonization has been well documented (Cox et al, 1990). Low levels of *Salmonella* coming from the hatchery cultured for up to six wks from the environment indicate that these *Salmonella* may have colonized part of the 2K group initially, and were probably cleared with time. Although no isolates from the other groups were obtained, birds in this treatment group were possibly subject to a low level exposure of this field isolate.

[8] Calbiochem, La Jolla, CA.
[9] Ascent Lab systems, Helsinki, Finland.

Crop IgA

IgA data are summarized in Figure 1. Although significant differences were observed between groups during wks 14, 17, 22, 27, 34 and 40, the greatest effect of vaccination treatments on Crop IgA was observed for the 2K group on wk 14. Optical densities for this group were 1.700 and 1.136 on ST and SE LPS plates, respectively. Although no significant differences were observed between the 2L2K and 3L1K groups with respect to controls at 14 wks, ODs were numerically higher for these treatments, with ODs of 0.940 and 0.613 observed for the 2L2K and 3L1K groups.

There are no previous reports demonstrating crop IgA responses following one subcutaneous dose of killed antigen, as we observed in this study (samples taken at 14 wks of age). Crop IgA response to oral live antigen exposure has been demonstrated (Seo *et* al, 2002; Seo *et* al, 2003). However, as these investigators noted, no specific antibody-producing cells within the crop have yet been identified, and the origin of crop IgA needs to be characterized. Intestinal mucosal IgA peak at 14-40 d post vaccination when chicks are exposed to orally-administered single dose of killed antigen in microspheres, but not when exposed to these microspheres intramuscularly (Liu *et al*, 2001). Although no positive *Salmonella* isolates were obtained after 6 wks of age, it is also possible that the IgA response obtained may actually be a result of late exposure to low levels of the hatchery isolate, for a longer time than was demonstrable by environmental sampling. This would explain a peak crop IgA response at 14 wks after IM administration of a killed dose 3 wks earlier. If this is the case, our observations would confirm that monitoring crop IgA is a good indicator of recent and low-level exposure to *Salmonella*, as hypothesized earlier (Seo *et al*, 2002). Although every effort was made to avoid bruising and subsequent inadvertent contamination with blood content during crop lavaging and

[10] SAS Institute Inc., Carry, NC

no samples with visible blood in the crop lavage were assayed, high crop IgA may also be due to trace contamination of the lavage sample with blood.

Slightly higher crop IgA at wk 22 (5 wks post second killed vaccination) indicates a weak response to killed vaccine delivery at 17 wks when measured on SELPS, but not when measured on STLPS. Mean ODs on STLPS at this time were higher than mean ODs on SELPS, but lack of significance of 22 wks STLPS crop IgA data was due to higher within group variability. Within group variability was the result of only a fraction of the breeders responding with high crop IgA to the 17 wks killed vaccination. This variability may have to do with differences in the degree of previous exposure to live antigen between treatment birds.

Crop IgG

Crop IgG measured on ST LPS and SE LPS are shown (Figure 2). For ST LPS, after a first dose of killed antigen at 11 wks, a faster rise in crop IgG of the 2K compared to the 2L2K treatment at wk 14 was observed, but both peaked by 17 wks. The faster rise of crop IgG for the 2K treatment is explained if these birds were previously exposed to field antigen orally, as previously discussed. A second dose of killed vaccine at 17 wks did not elicit a similar crop IgG response (by 22 wks and onward, ODs linger below 0.5). When measured on SE LPS (Fig 1b), Crop IgG reached a short-lived peak at 14 wks, with no other mean OD's being over 0.500 after 17 wks. These findings seem to indicate that crop IgA and IgG are short lived in time when compared with serum IgA and IgG levels, and that oral exposure to antigen is a requirement for raising these antibody's concentrations. The differences in OD's between SE LPS and ST LPS assays would indicate that responses were primed by a (live) *Salmonella* serovar Typhimurium (which is the live vaccine strain), or a closely related (the hatchery-associated group B) serovar.

Gut IgA

Gut IgA was measured on SE LPS only at day of age (wk 0), due to the small amount of lavage sample available (Figure 3). No differences among treatments throughout time were noted on gut IgA measured on SE LPS, although OD's were slightly higher for all treatments on wks 3 and 17. On these wks, ODs of all vaccinated groups were numerically higher than the controls. Higher ODs for gut IgA would be expected following exposure to oral (live) antigen. When measured on ST LPS, gut IgA ODs for the 3L1K and 2K treatments were higher on wk 17 compared to the control. The 2L2K treatment had a numerically higher OD than the control, but was not statistically different from either the control or the other vaccinated groups.

Gut IgG

Differences among treatments were noted only on measurements on ST LPS (Figure 4). Peak ODs were observed on wks 3, 17 and 22. Chicks receiving the live vaccine had higher gut IgG by wk 3, but these concentrations dropped to control levels by wk 11, regardless of a second live vaccine given at wk 6. A killed vaccine given at wk 11 was capable of raising gut IgG levels by wk 17, regardless of previous live priming, as seen by higher gut IgG for the 2K and 2L2K treatments. In contrast, birds receiving only live vaccines were not able to sustain a high gut IgG response by wk 17, as seen by the low 3L1K OD. By wk 22, all vaccinated groups had received at least 1 killed vaccine by wk 17 and consequently showed higher gut IgGs. These findings indicate that gut oral live vaccine elicits a short-lived gut IgG response.

Serum IgA

Although differences for serum IgA were obtained on wks 14, 27, 34 and 40 (Figure 5), the numerically highest was obtained by wk 14, for the 2K and 2L2K groups receiving a killed vaccine previously (wk 11). This peak was detected 3 wks post vaccination, only when measured

on ST LPS, and no comparable peak was observed thereafter. No peaking ODs were observed when sampling at wk 22, 5 wks after the second killed vaccine (wk17). Although other investigators have reported serum IgA peaking up to 6 wks post vaccination (Liu *et* al, 2001), we were unable to show a comparable long-lasting serum IgA response. All vaccinated treatments showed slightly higher ODs when compared to controls at varying times throughout wks 27 to 40, but none was consistently higher, and numerical differences though statistically significant, were relatively small in magnitude.

Serum IgG

Optical densities for serum IgG are depicted in figure 6. Initial high titers of serum IgG at day of age were detected and were maternally derived, since the 57 wk-old female parent stock had been vaccinated twice with an autogenous bacterin, containing groups B and D1 Salmonellae. These titers waned as expected by 3 wks of age. Killed but not live vaccination elicited the highest serum IgG responses as seen on wk 14 for the 2K and 2L2K groups, and wks 22 and after, for all vaccinated groups. Response to only one killed vaccine diminished faster than for 2 killed vaccines, as seen by the decline in ODs by wk 17 of 2K and 2L2K treatments, and by a numerically (not statistically) faster decline of the 3L1K group by wks 22, 34, and 40. Although all treatments were different from controls throughout wk 40, the rate of decline in ODs seems to suggest that IgG titers would not last throughout a normal 65-wk production period.

Yolk and Hatchling Serum Antibodies

Only yolk IgG (Figure 7) but no yolk IgA was detected (data not shown). These results were expected, since IgG is deposited in the hen's maturing follicle, whereas IgA is deposited in the amniotic fluid. Egg yolk IgG was higher for all vaccinated groups throughout all wks

sampled. Hatchling Serum IgA (data not shown) and IgG (Figure 8) followed egg yolk trends, with no detectable IgA and higher IgG for hatchlings from vaccinated treatments throughout all sampling periods. IgG levels in yolk and hatchling sera were maintained through time.

Finally, ELISA responses are clearly dependent on the antigen type used, as can be seen in general differences in profiles when using ST or SE LPS. When adapting a particular ELISA procedure for field monitoring, it would be best to choose an LPS group-compatible with the most common serovar encountered in the field.

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TABLE 3.1 Vaccination treatments at different breeder ages

	Breeder Age (d)			
Treatments	1	21	77	119
C				
2L2K	**L**	**L**	**K**	**K**
3L1K	**L**	**L**	**L**	**K**
2K			**K**	**K**

C = non-vaccinated controls. 2K = Killed vaccines given on wk 11 and 17; 2L2K = live vaccines given on d 1 and 21 and killed vaccines given on wk 11 and 17; 3L1K = live vaccines on d 1, 21 and wk 11, and 1 killed vaccine given on wk 17. **L**=live vaccine; **K**=killed vaccine

TABLE 3.2 Age of chickens and samples taken for antibody assays

	Sample				
Breeder Age (d)	Breeder Serum	Crop Lavage	Gut Lavage	Egg Yolk	Hatchling Serum
1	**S**	**S**	**S**		
21	**S**	**S**			
42	**S**	**S**	**S**		
77	**S**	**S**	**S**		
98	**S**	**S**			
119	**S**	**S**	**S**		
154	**S**	**S**	**S**		
189	**S**	**S**	**S**	**S**	**S**
238	**S**	**S**	**S**	**S**	**S**
280	**S**	**S**	**S**	**S**	**S**

S=Sampled

TABLE 3.3 Breeder box liner and paper pad monitoring for Salmonella

	Treatment Group				
Age (d)	2K	2L2K	3L1K	C	MALES
7	+	-	-	-	-
21	+	-	-	-	-
42	+	-	-	-	-
77	-	-	-	-	-
98	-	-	-	-	-
119	-	-	-	-	-

2K = Killed vaccines given on wks 11 and 17; 2L2K = live vaccines given on d 1 and 21 and killed vaccines given on wks 11 and 17; 3L1K = live vaccines on d 1, 21 and wk 11, and 1 killed vaccine given on wk 17. C = non-vaccinated controls; + = positive isolations; - = negative isolations.

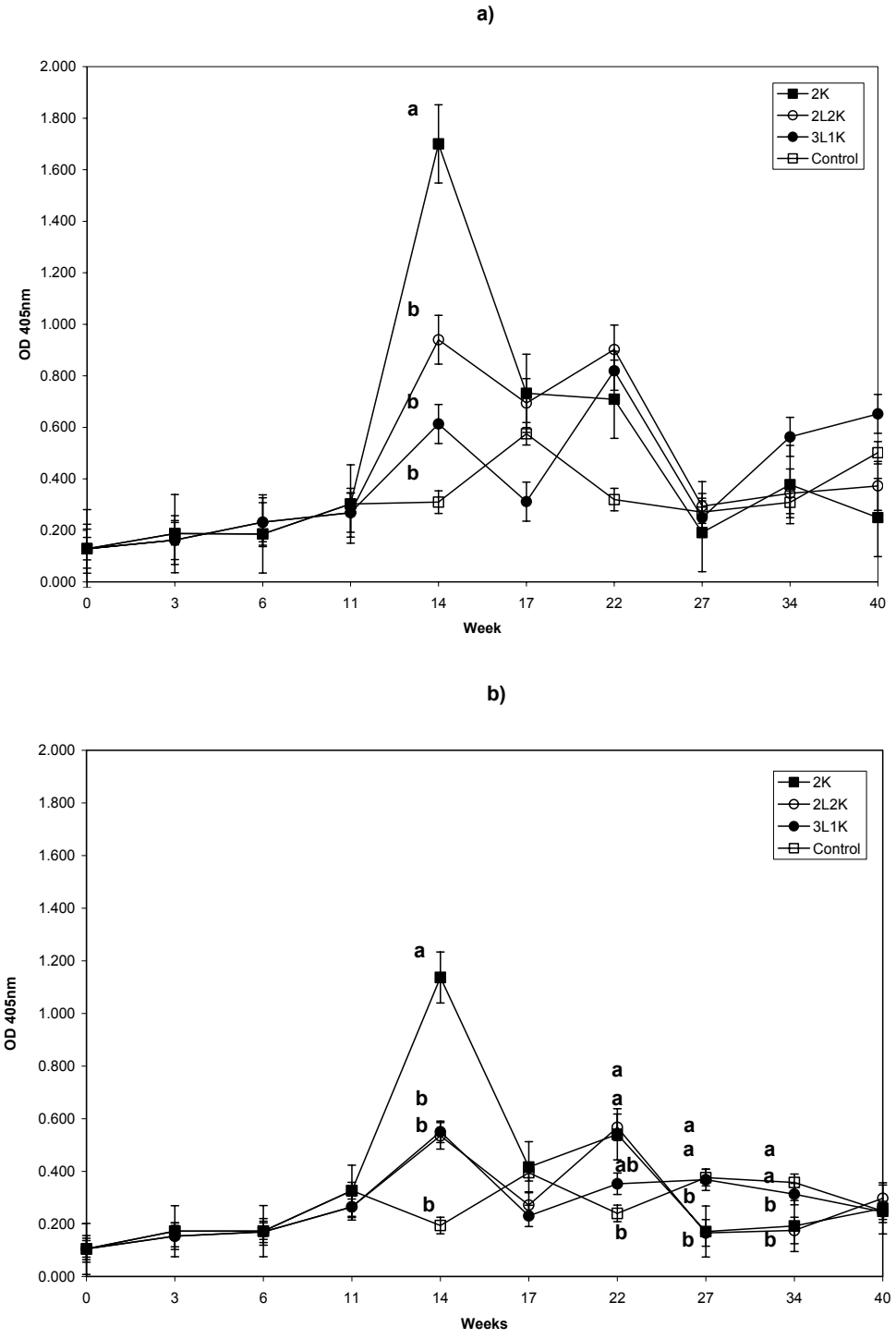


FIGURE 3.1 Optical densities (405nm) of crop IgA assayed on *Salmonella* serovar Typhimurium (a) or *Salmonella* serovar Enteritidis (b) lipopolysaccharide. 2K = Killed vaccines given on wks 11 and 17; 2L2K = live vaccines given on d 1 and 21 and killed vaccines given on wks 11 and 17; 3L1K = live vaccines on d 1, 21 and wk 11, and 1 killed vaccine given on wk 17; C = non-vaccinated controls.

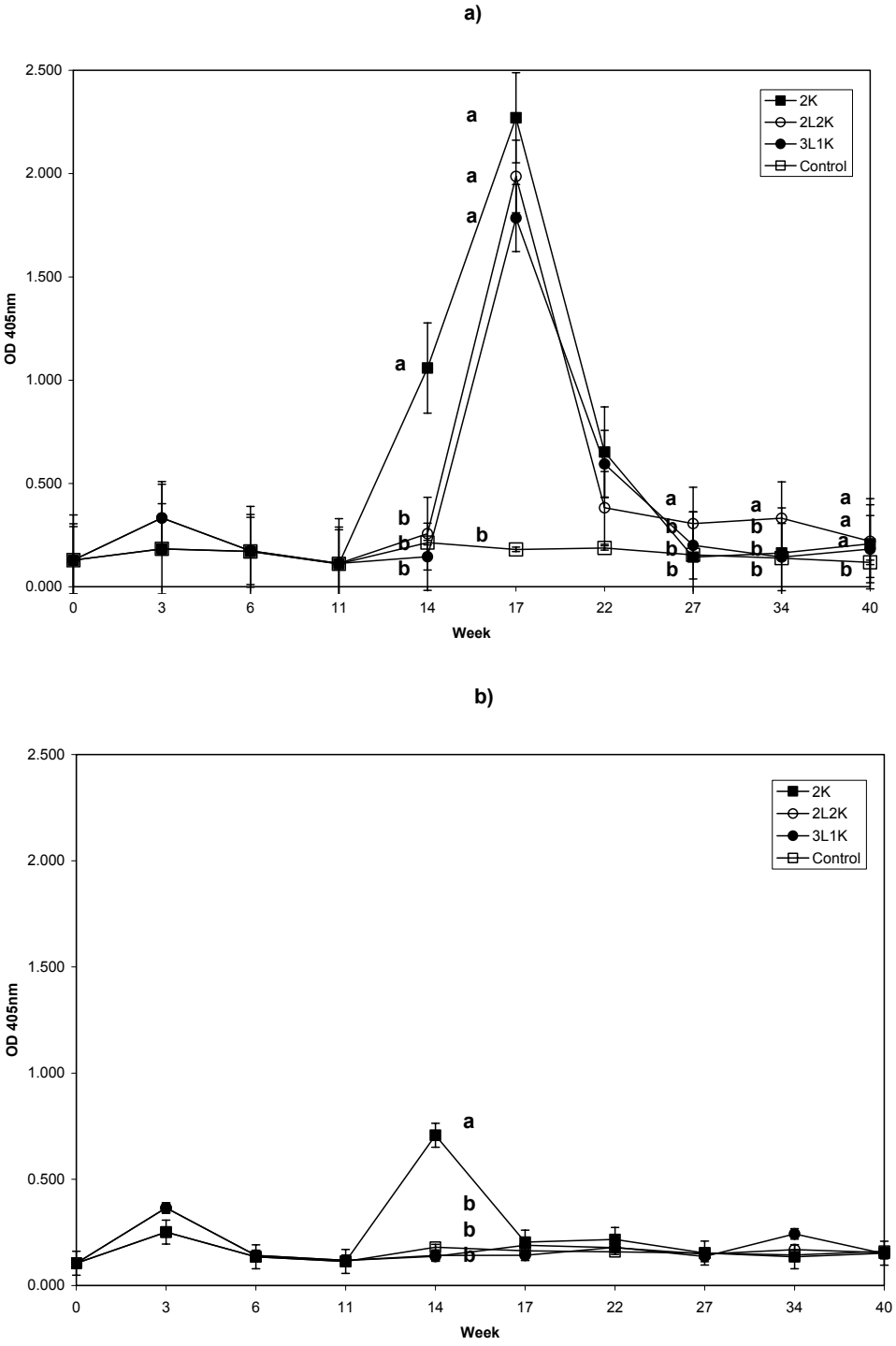


FIGURE 3.2 Optical densities (405nm) of crop IgG assayed on *Salmonella* serovar Typhimurium (a) or *Salmonella* serovar Enteritidis (b) lipopolysaccharide. 2K = Killed vaccines given on wks 11 and 17; 2L2K = live vaccines given on d 1 and 21 and killed vaccines given on wks 11 and 17; 3L1K = live vaccines on d 1, 21 and wk 11, and 1 killed vaccine given on wk 17; C = non-vaccinated controls.

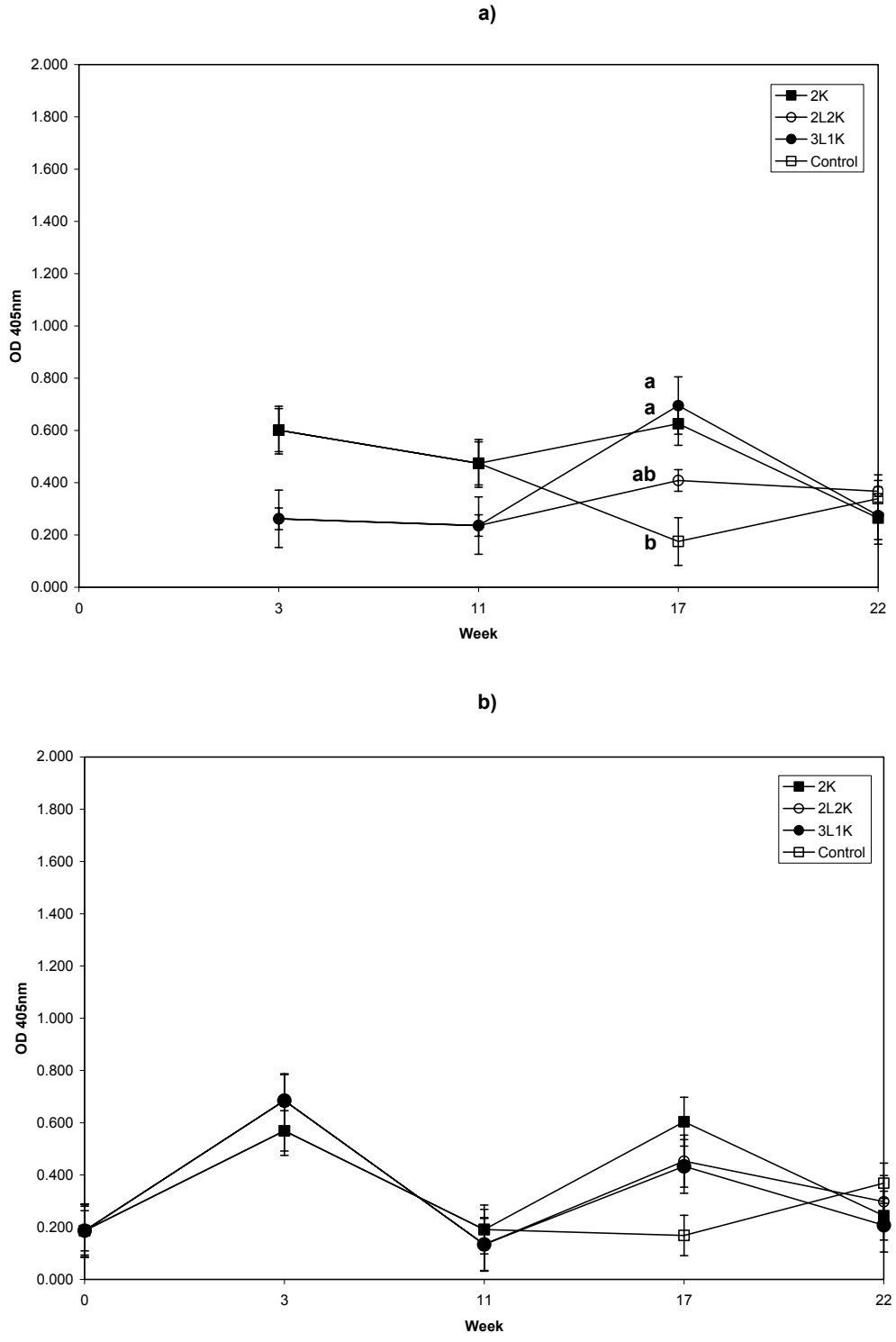


FIGURE 3.3 Optical densities (405nm) of gut IgA assayed on *Salmonella* serovar Typhimurium (a) or *Salmonella* serovar Enteritidis (b) lipopolysaccharide. 2K = Killed vaccines given on wks 11 and 17; 2L2K = live vaccines given on d 1 and 21 and killed vaccines given on wks 11 and 17; 3L1K = live vaccines on d 1, 21 and wk 11, and 1 killed vaccine given on wk 17; C = non-vaccinated controls.

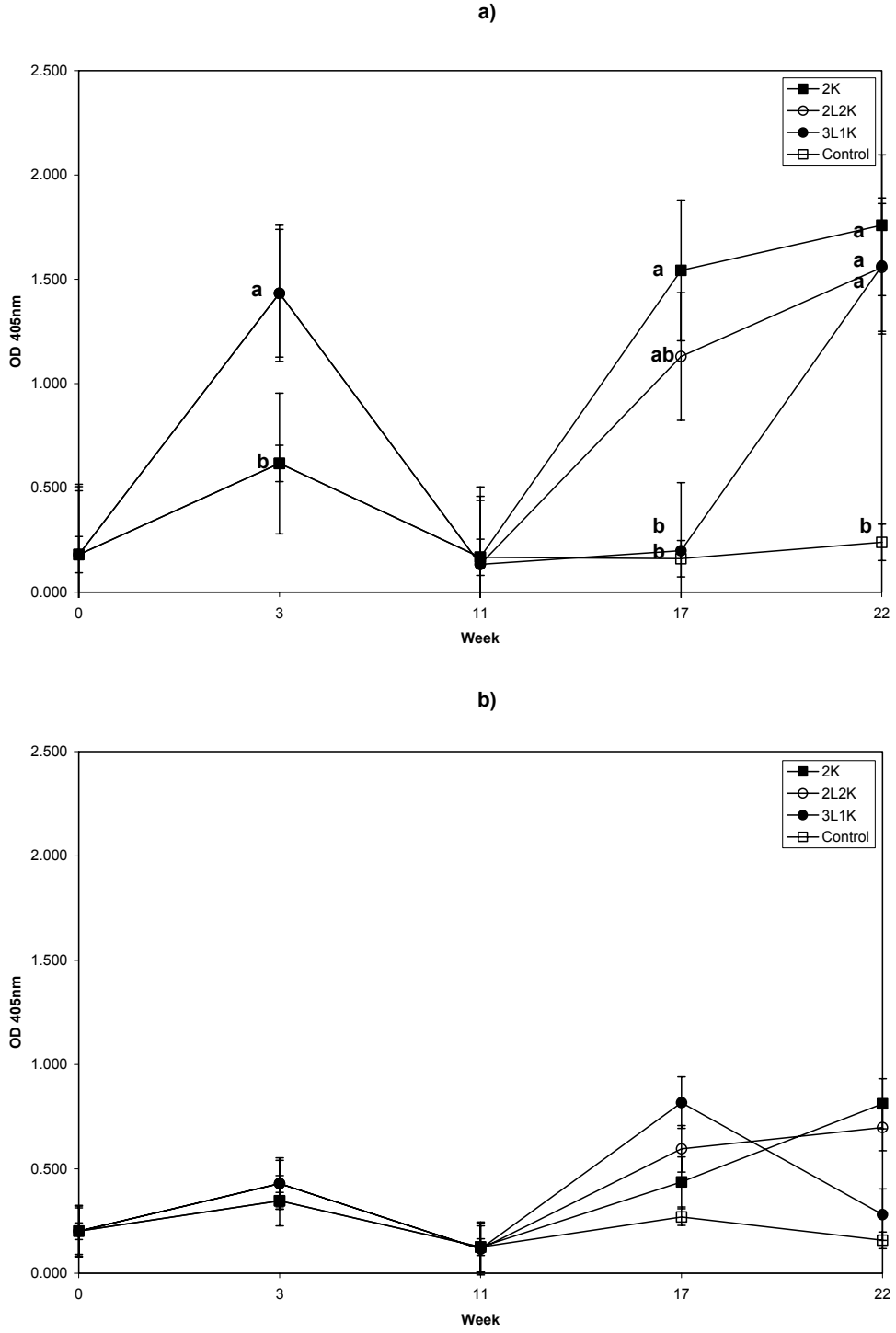
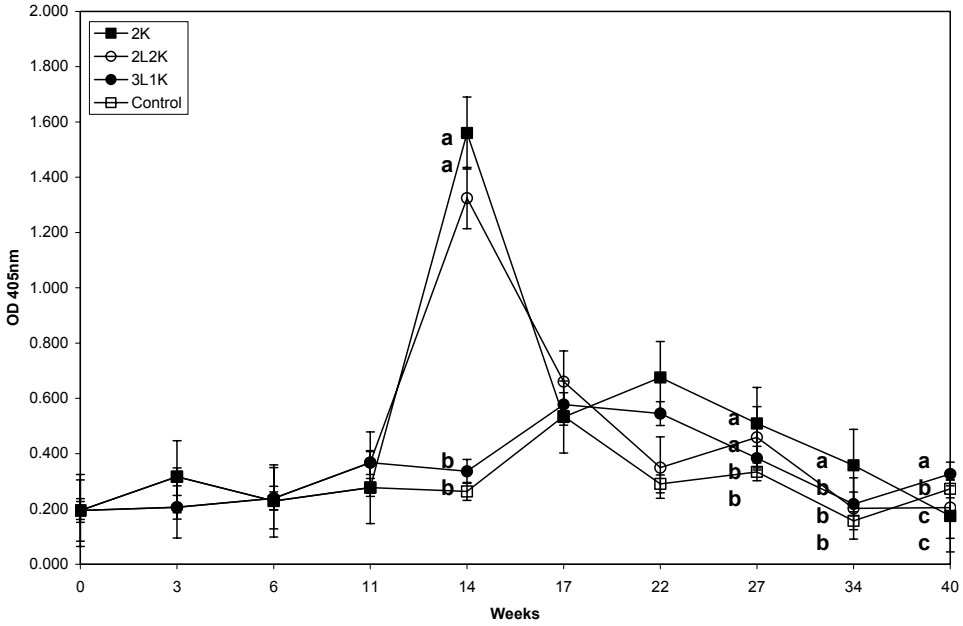


FIGURE 3.4 Optical densities (405nm) of gut IgG assayed on *Salmonella* serovar Typhimurium (a) or *Salmonella* serovar Enteritidis (b) lipopolysaccharide. 2K = Killed vaccines given on wks 11 and 17; 2L2K = live vaccines given on d 1 and 21 and killed vaccines given on wks 11 and 17; 3L1K = live vaccines on d 1, 21 and wk 11, and 1 killed vaccine given on wk 17; C = non-vaccinated controls.

a)



b)

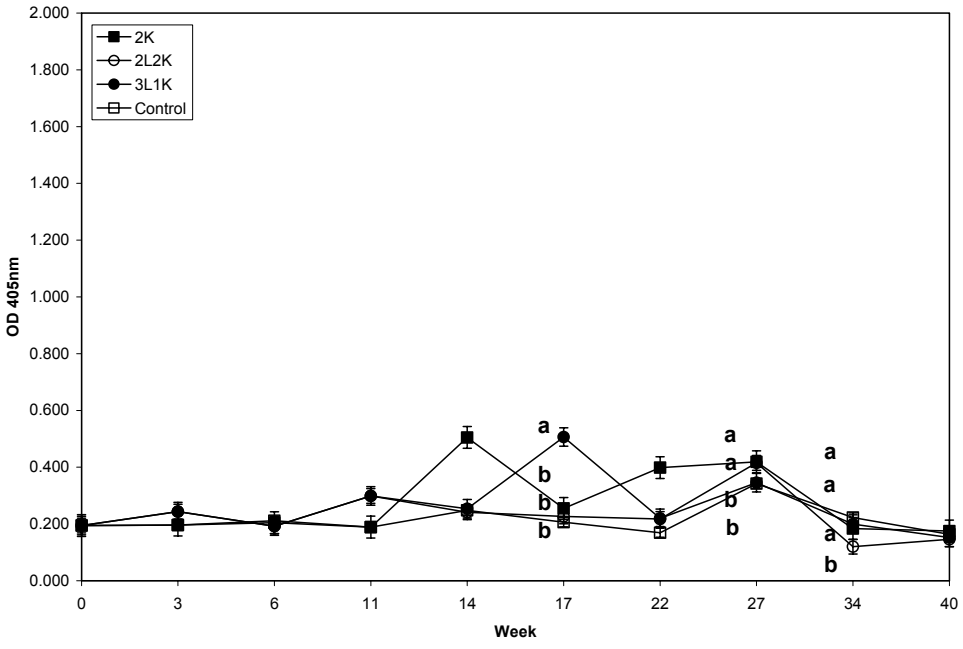


FIGURE 3.5 Optical densities (405nm) of serum IgA assayed on *Salmonella* serovar Typhimurium (a) or *Salmonella* serovar Enteritidis (b) lipopolysaccharide. 2K = Killed vaccines given on wks 11 and 17; 2L2K = live vaccines given on d 1 and 21 and killed vaccines given on wks 11 and 17; 3L1K = live vaccines on d 1, 21 and wk 11, and 1 killed vaccine given on wk 17; C = non-vaccinated controls.

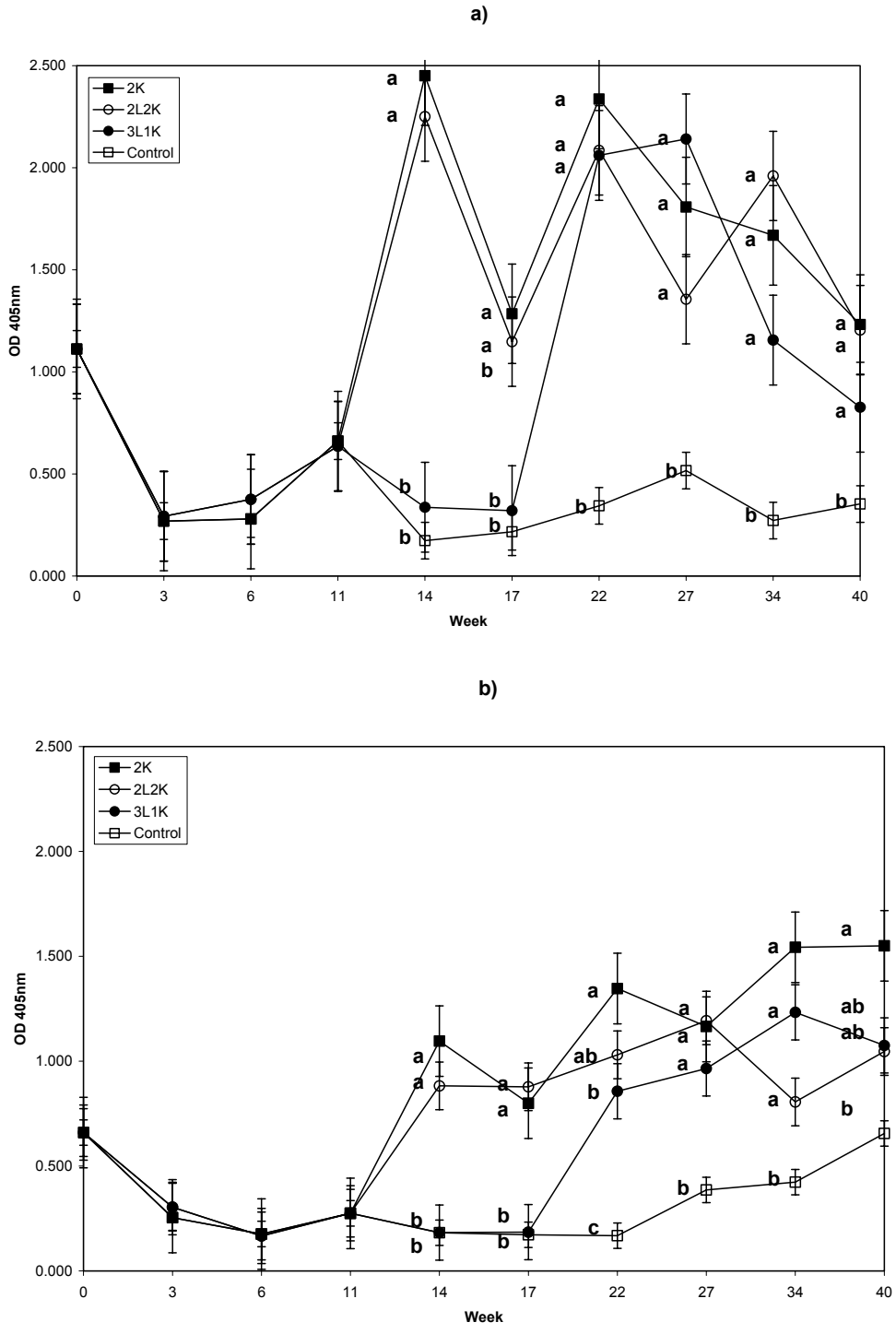


FIGURE 3.6 Optical densities (405nm) of serum IgG assayed on *Salmonella* serovar Typhimurium (a) or *Salmonella* serovar Enteritidis (b) lipopolysaccharide. 2K = Killed vaccines given on wks 11 and 17; 2L2K = live vaccines given on d 1 and 21 and killed vaccines given on wks 11 and 17; 3L1K = live vaccines on d 1, 21 and wk 11, and 1 killed vaccine given on wk 17; C = non-vaccinated controls.

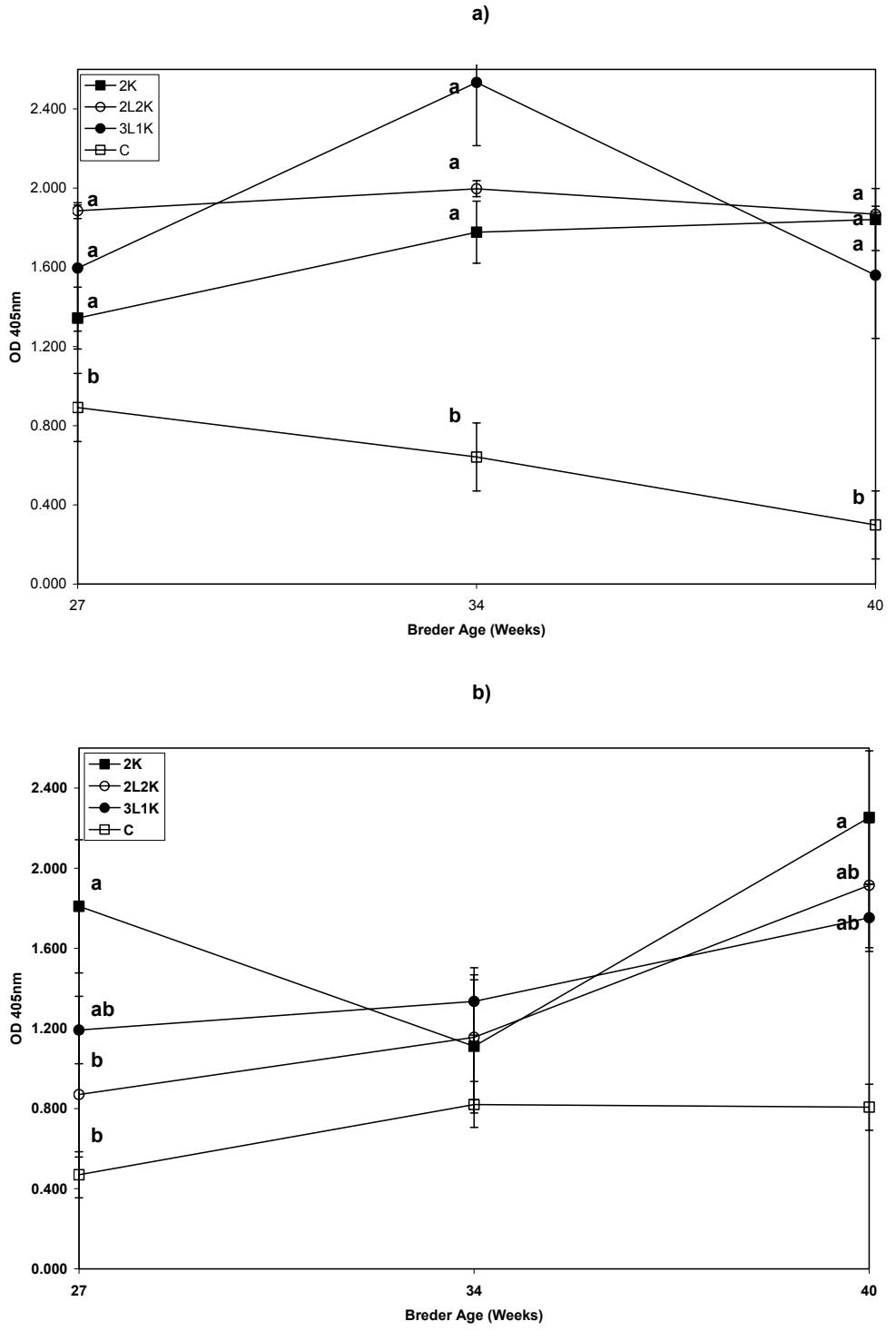


FIGURE 3.7 Optical densities (405nm) of yolk IgG assayed on *Salmonella* serovar Typhimurium (a) or *Salmonella* serovar Enteritidis (b) lipopolysaccharide. 2K = Killed vaccines given on wks 11 and 17; 2L2K = live vaccines given on d 1 and 21 and killed vaccines given on wks 11 and 17; 3L1K = live vaccines on d 1, 21 and wk 11, and 1 killed vaccine given on wk 17; C = non-vaccinated controls.

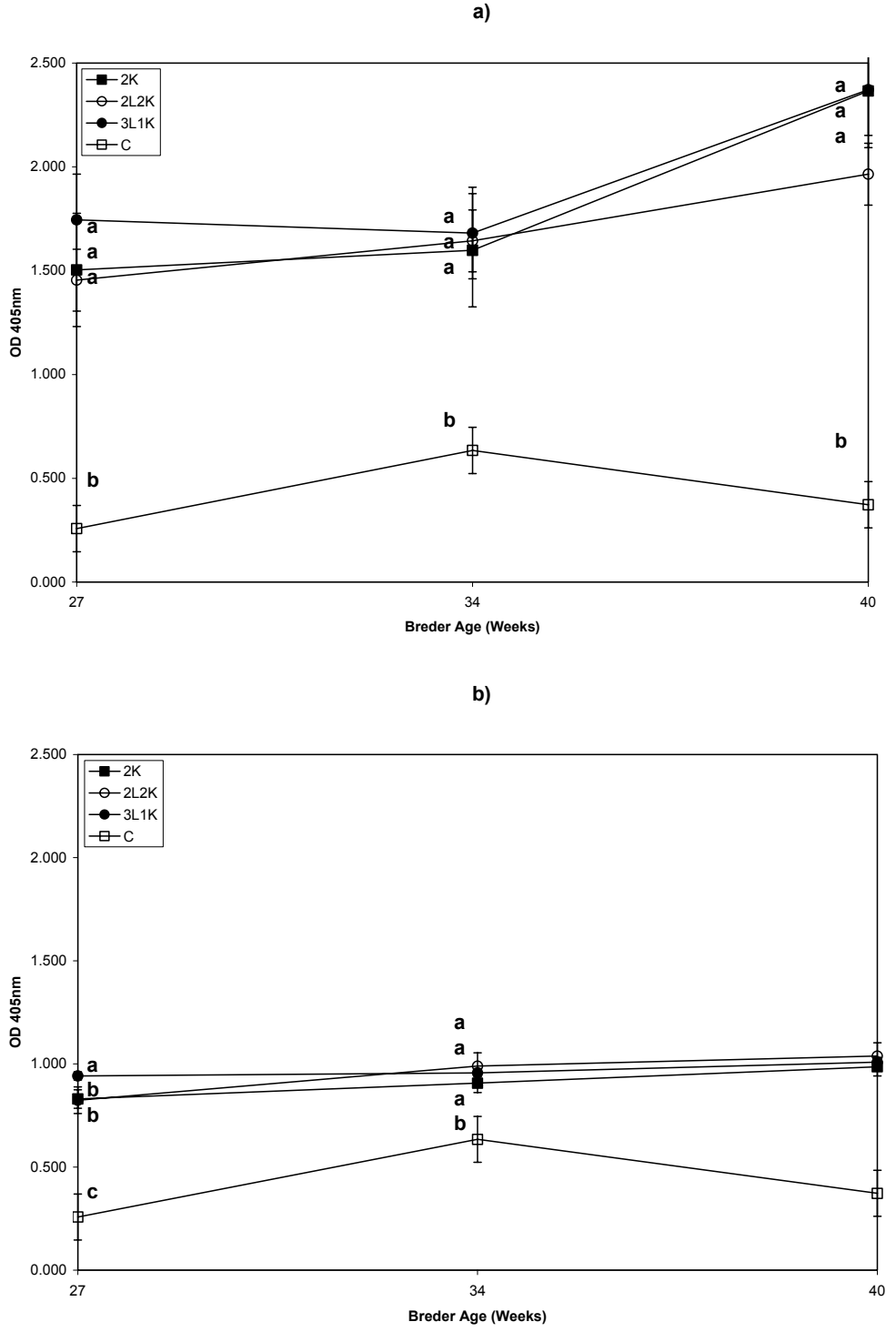


FIGURE 3.8 Optical densities (405nm) of hatchling serum IgG assayed on *Salmonella* serovar Typhimurium (a) or *Salmonella* serovar Enteritidis (b) lipopolysaccharide. 2K = Killed vaccines given on wks 11 and 17; 2L2K = live vaccines given on d 1 and 21 and killed vaccines given on wks 11 and 17; 3L1K = live vaccines on d 1, 21 and wk 11, and 1 killed vaccine given on wk 17; C = non-vaccinated controls.

CHAPTER 4

SALMONELLA VACCINATION PROGRAMS IN BROILER BREEDERS II. RESISTANCE TO CHALLENGE OF BREEDERS AND THEIR PROGENY WITH AND WITHOUT COMPETITIVE EXCLUSION[1]

[1] Rolón, A., J. S. Bailey, C. L. Hofacre, P. S. Holt, J. L. Wilson, and N. A. Cox. To be submitted to Poultry Science.

ABSTRACT

Resistance to *Salmonella* challenge of breeders under three vaccination programs and of their chicks with and without mucosal competitive exclusion (CE) (CHR Hansen) treatment was assessed. Vaccine treatments combined a live Aro-A *Salmonella* Typhimurium (ST) vaccine and an autogenous commercially prepared (Lohmann Animal Health) trivalent killed vaccine (serogroups B, C_2 and D_1). Treatments combined: 2 live and 2 killed doses or 3 live and 1 killed dose delivered at 1, 21, 77 and 126 d of age; or 2 killed doses delivered at 77 and 126 of age; and a non-vaccinated control (C). At 3, 6, 11, 17 and 22 wks of age, a portion of breeder pullets was removed and challenged *per os* with 10^7 cells of a 3-strain mixture of antibiotic-resistant Salmonellae. Chicks from eggs laid at 29, 34 and 40 wks of age were challenged at 1 d of age with and without CE pre-treatment, with 10^7 cells of a 2-strain mixture of antibiotic-resistant Salmonellae and kept in isolation units for one and two wks. Ceca and Liver-Heart-Spleen (LHS) samples were cultured for each strain on BGS + antibiotic plates and colonies enumerated. Log_{10} data were analyzed under factorial designs. Breeder *Salmonella* counts showed significant reductions between (live) vaccinates and non-vaccinates at 3 (0.82 log) and 6 wks (0.85 log) challenges. By 11 wks, there were no differences in *Salmonella* levels between vaccinates and controls, indicating that 1-d and 3-wk live vaccine protection had diminished with time. All vaccination treatments reduced breeder cecal counts (1.15-1.30 log) by wk 22. Passive immunity from breeder vaccination treatments was not effective in diminishing chick cecal counts as shown by comparable susceptibility of chicks from vaccinated and control breeders, regardless of breeder age. Chick CE treatment consistently diminished cecal (1.41 log) and LHS (0.306 log)

counts. These results show that live Aro-A ST vaccination decreases counts during the first 6 wks of age, as do all programs by 22 wks of age, and that competitive exclusion is the most effective treatment in reducing hatchling *Salmonella* counts.

(*Key Words: Salmonella* challenge, vaccine, competitive exclusion, broiler breeders)

Abbreviation Key: 2K = 2 killed vaccines; 2L2K = 2 live and 2 killed vaccines; 3L1K = 3 live and 1 killed vaccine; Amp-STH = Ampicillin-resistant *Salmonella Thompson*; BGS = brilliant green sulpha agar; CE = competitive exclusion; DOA = 1 d of age; LHS = liver-heart-spleen; MSC = mucosal starter culture; Nal-SE = nalidixic acid-resistant *Salmonella enteritidis*; PF = protection factor; Rif-ST = rifampicin-resistant *Salmonella typhimurium*; SE = *Salmonella enteritidis*; SG = *Salmonella gallinarum*; ST = *Salmonella typhimurium*; WOA = wk-of-age

INTRODUCTION

Exposure to *Salmonella* and subsequent enumeration of cecal and other organ samples, as well as measurements of indicators of humoral or cell-mediated immunity are the most common methods to assess chicken's resistance to *Salmonella* challenge. Early studies show the protective effect of autogenous preparations of live and killed vaccines to subsequent homologous serovar Typhimurium (ST) challenge, with best protection obtained when priming with live and boosting with killed oil-emulsion vaccine (Suphabphant *et al*, 1983). An attenuated ST strain by double deletion of genes coding for receptor protein of cAMP and adenylate cyclase (Δcrp and Δcya) was extensively studied. Application of the vaccine at 1 and 14 d of age (DOA) prevented colonization of the small intestine, and reduced cecal and rectal counts when birds were challenged with a different ST strain at 21 or 28 DOA (Hassan and Curtiss, 1990). In a subsequent experiment, cecal colonization was prevented, with vaccine doses of 10^7 or 10^8 cfu/ml (Hassan *et al*, 1993). When protection to heterologous serovars (serogroups C1, C2, C3,

D and E) was assessed, varying degrees of cross-protection to spleen, ovary, bursa, ileum, feces or cecal samples were observed, with a general tendency of better protection to (homologous) group B strains, and limited protection to heterologous (C2, C3, E) strains. Even within serogroups, protection profiles varied: Challenge with a (group D) serovar Enteritidis (SE) strain showed bursal, fecal and cecal counts similar to controls, whereas fewer cfu/ml of fecal and cecal samples of birds challenged with serovar Panama (also group D) were observed (Hassan and Curtiss, 1994). A long-term study evaluating the protective effect of vaccination at 2 and 4 wks by challenge and culture with ST and SE at 3, 6, 9 and 12 months of age, showed that vaccination completely eliminated colonization of spleen, liver, ileum, ceca, ovary and reproductive tract samples, except for one positive SE isolation obtained from a magnum at 6 months of age (Hassan and Curtiss, 1997).

The use of *Salmonella* attenuated strains by deletion of the Aro-A gene (essential for the synthesis of chorismate) as potential vaccine candidates has also been studied. An AroA ST mutant initially reduced fecal excretion of an ST challenge strain on 4 DOA vaccinates, but the effect did not persist. Aro-A SE provided little protection either by oral or intramuscular administration at 20 and 22 wks of age (WOA), of birds challenged with SE at 24 wks. In contrast, a similarly-vaccinated group with the attenuated serovar gallinarum mutant strain (R9) reduced liver, spleen, ovary and gut colonization by the challenge SE strain (Barrow *et al*, 1990). A similar experiment showed reduced cfu/ml organ re-isolations from birds vaccinated with 9R but not with Aro-A SE and challenged with an SE phage type 4 strain (Barrow *et al*, 1991). An Aro-A serovar Gallinarum (SG) was compared with the 9R ST vaccine, and shown to protect if given intramuscularly (single dose at 2wks) but not orally against wild-type SG challenge. Mortality was reduced from 63 to 30% and from 3 to 12% for Aro-A and 9R vaccinated birds,

respectively (Griffin and Barrow, 1993). In contrast to these investigations reporting limited protection by Aro-A mutants, other investigators found that Aro-A mutants provide adequate protection to challenge. A minimum of $10^{1.3}$ count reduction in feces, and greater than 10^2 reductions in liver and cecal counts were obtained when birds were vaccinated with the Aro-A mutant at 1 and 14, or 1, 7, 14,and 21 d of age and challenged at 40 d of age (Cooper *et al*, 1990). Further studies by these authors showed DOA single-dose vaccination and challenge at 14 DOA using a seeder bird model protected the vaccinated group from colonization, but protection did not persist when challenged at 56 DOA. Birds vaccinated at 1 DOA and 2 wks, or at 1 DOA and 2, 16, and 18 wks and challenged at 23 wks showed similar reductions in organ counts, with greatest reductions shown by birds vaccinated four times (Cooper *et al*, 1993). A 1 DOA and 16 wks vaccination program with 10^6 and 10^9 showed similar reductions in spleen, liver, ovary and cecal counts when birds challenged at 23 wks with SE. Only the higher vaccine dose reduced intestinal shedding. However, when birds were challenged with ST, organ counts were similar to controls, indicating limited protection to heterologous challenge (Cooper *et al*, 1994). Intramuscular Aro-A ST vaccination at 3 DOA and intramuscular challenge with virulent ST at 7 DOA showed complete protection of vaccinates in contrast to controls, which did not survive challenge. The vaccine strain under a challenge model was shown to be shed for 5 d, but was eliminated by 14 d. A second experiment with oral DOA vaccination and challenge with varying virulent ST doses (10^4, 10^6, 10^8), showed that vaccinated birds stopped shedding by 35 DOA, whereas controls still had a 33% shedding frequency. (Alderton *et al*, 1991).

Studies reported in the literature describe delivery of live vaccines intramuscularly or by oral gavaging, but to our knowledge, no studies using modern commercial broiler breeders and administering vaccines by standard industry methods (aerosol at 1 DOA and in drinking water

after DOA) have been reported. Our previous work (in press) profiled the humoral and gut mucosal IgA and IgG responses to vaccination programs using a licensed Aro-A ST vaccine alone or combined with an autogenous killed bacterin. The present study complemented our previous work, and attempted to evaluate the protective efficacy of the different vaccination programs to challenge using a multiple antibiotic-resistant strain challenge model throughout rearing and on 1 d old progeny of the breeders.

MATERIALS AND METHODS

Chickens, premises and vaccines

Chickens, premises and vaccines were fully described previously (Rolón *et al*, submitted). Briefly, Cobb x Cobb broiler breeders from a commercial broiler supplier were placed at the University of Georgia's Poultry Science Research facilities. Chicks were randomly placed in disinfected premises, consisting of four environmentally-controlled industry-type rooms, with chain feeders and nipple drinkers, forced-air furnaces, negative ventilation systems, and fresh pine shavings as litter material.

Vaccination treatments consisted of combinations of live Aro-A SE (Poulvac -ST®[11]) and a commercially-prepared[12] oil-in-water emulsion containing serovars Heidelberg (group B), Kentucky (group C_2) and Berta (group D_1). The vaccines were administered in three different treatment combinations: a non-vaccinated control, a two-live/two-killed (2L2K), a three-live/one-killed (3L1K), and a two-killed (2K) group. Vaccine delivery resembled commercial delivery practices, with live vaccine given as a coarse spray while inside chick boxes at a of age, or via drinking water at 21 or 77 DOA. Killed vaccines were delivered by neck subcutaneous injection at 77 and 126 DOA. Lighting and feeding programs followed commercial broiler

[11] Fort Dodge Animal Health Inc, Overland Park, KS.
[12] Lohmann Animal Health International, 1146 Airport Pkwy, Gainesville, GA 30501.

breeder husbandry practices. At 18 WOA pullets were housed in three separate rooms equipped with manually belt-conveyed nests, 2/3 slats and a central 1/3 mating/scratch area with softwood shavings, and males introduced for mating.

Monitoring for Environmental *Salmonella*

On arrival of chicks to the farm, chick box liners were cultured for *Salmonella*, and $1m^2$ paper liners placed weekly under feeder troughs, and cultured for *Salmonella* monitoring on d 7, 21, 42, 77, 98 and 119 of age. Chick box liners were cut, placed in a large stomacher bag with 250ml of buffered peptone, and contents manually mixed. A 10^{-1} dilution of buffered peptone was selectively enriched in tetrathionate-brilliant green broth by incubation for 24h at 41°C , and three replicates of a 0.1ml of the enriched sample were spread-plated on BGS agar and incubated for 24 and 48 hr at 37°C.

Bacterial Challenge Strains and Growth Media

A mixture of 3 different antibiotic-resistant *Salmonella* serovars was used for all challenge studies: A rifampicin-resistant serovar Typhimurium (Rif-ST), a nalidixic acid-resistant serovar Enteritidis (Nal-SE), and an ampicillin-resistant serovar Thompson (Amp-STH), corresponding to serogroups B, D_1 and C_2, respectively. A pre-trial study with mixtures of $10^6 – 10^7$ cfu/dose *per os* to 1 DOA broilers proved that all three isolates could be recovered one wk post-challenge and serovars segregated effectively on antibiotic-containing media. Media used for isolation was Bacto ® brilliant green sulpha (BGS) agar[13] prepared in our laboratory with 200ppm of antibiotic (Rifampicin, Ampicillin or Nalidixic Acid) and 15ppm of Novobiocin added after autoclaving and just prior to plating.

[13] Becton Dickinson Diagnostics, Franklin Lakes, NJ

Breeder Challenge and Bacterial Enumeration:

At time of challenge, a subgroup of 10 breeders per treatment were taken to the USDA's Poultry Microbiological Research Unit's research facilities in Watkinsville, GA, and placed on pen isolation units, equipped with nipple drinkers, bell-type feeders and fresh pine shavings. Challenge strains were grown for 24h at 37°C earlier on BGS with corresponding antibiotic, and cells suspended in 0.85% sterile saline. Each strain suspension was adjusted to 0.120 optical density at 540nm, and equal aliquots mixed for gavaging. The mixture was plated on BGS added with the corresponding antibiotic and colonies counted to confirm they were within a 10^8 to 10^9 cfu/ml. Breeders were gavaged with 0.1ml of the mixture, to deliver 10^7 to 10^8 cfu/ml per breeder. One wk post-challenge, the breeders were euthanized. Each breeder's left liver lobule, heart, and spleen were pooled and placed on sterile stomacher bags with filter. Both ceca were removed and placed in a separate stomacher bag with filter. All samples were kept in ice until reaching our laboratory (less than 1h). Samples were weighed and peptone broth corresponding to 3 times sample weight added. Samples were stomached thoroughly and 500 µL of suspension placed on sterile eppendorf tubes, plated on BGS plus corresponding antibiotic using a Spiraltech®[14] plater and incubated at 37°C for 24h and read using a Spiraltech® [3] reader. Two colonies from one fifth of all plates were serogrouped to confirm that colonies on each antibiotic-added plate corresponded to the expected serogroup.

Hatchling Challenge, Competitive Exclusion Delivery and Bacterial Enumeration:

Eggs from treatment breeders were collected at 29, 34 and 40 wks of breeder age, and incubated. Immediately after hatch, 40 chicks per treatment were randomized into 4 subgroups, of which two subgroups were gavaged with Mucosal Starter Culture (MSC)[15], an undefined flora

[14] Spiraltech, Rockville, MD.
[15] CHR Hansen Inc., Milwaukee, WI.

competitive exclusion culture. Three to four h after MSC treatment, all chicks were challenged with a blend of Rif-ST and Nal-SE containing at least 10^7 cfu/ml of each strain. Eight chicks per subgroup were challenged and sampled as described earlier, one wk post challenge for breeder age 29, and 1 and 2 wks post-challenge for breeder ages 34 and 40. Bacterial enumeration for chick challenges was done following the swab-plate method of Bailey *et al*, (1988). Two colonies from one fifth of all plates were serogrouped to confirm that colonies on each antibiotic-added plate corresponded to the expected serogroup.

Statistical Analysis

For each challenge event, data was transformed (Log_{10}) and analyzed under factorial designs. Main effects were Vaccination Treatment and Serovar for breeder challenges, and Vaccination Treatment, Serovar and Competitive Exclusion Treatment for progeny challenges. Data was analyzed using SAS®[16] software and mean differences discriminated using Student-Newman-Keuls' Multiple Range Test.

RESULTS AND DISCUSSION

Environmental Sampling

Environmental sampling yielded positive samples for *Salmonella* serovar Heidelberg on chick paper liners of female breeders on arrival, which was recovered at d 7, 21 and 42 but no longer at d 77, 98 and 119. This serovar was linked to a serovar commonly encountered at the hatchery, and was apparently cleared by 42 DOA. No *Salmonella* was detected in the male breeder population. The *Salmonella* serovar Heidelberg found in environmental samples was present at very low counts, and sensitive to low levels of all three antibiotics used during challenge trials. No growth of the field isolate was obtained in plates containing 100 ppm (1/2 the dose) of any of the three antibiotics used in the challenge model.

Breeder counts by vaccination treatment:

All colonies that were serogrouped from antibiotic-added plates corresponded to expected serogroups, showing that reliable counts for each particular serovar in the multiple-strain model could be obtained from the same sample by plating on media containing the antibiotic to which each marker strain was resistant.

Day of age vaccination with live Aro-A ST vaccine resulted in an average reduction of 0.82 log at 3 wks and a 0.85 log reduction at 6 wks of all serovar counts (Table 1). The live vaccine's protective effect waned by 11 wks. All vaccination treatments at wk 18 had numerically smaller counts compared to controls, but only the 2K treatment was statistically significant. Week 18 counts indicate that neither two live (delivered at d 1 and wk 3) and one killed vaccine (delivered at wk 11) program, nor a three live (delivered at d 1, wk 3 and wk 11) program, were better in reducing wk 18 cecal colonization than just a single killed vaccine delivered at wk 11. Contrary to our expectations, live vaccination at d 1 and wk 3 protected against early challenge, but had no booster effect measurable at wk 18. Challenge at wk 22 showed reduced counts for all vaccinates, indicating that all vaccination programs were equally efficient in reducing cecal colonization by this time. No differences for LHS counts due to vaccination treatments were observed.

Protection factors (PF), defined as the ratio of *Salmonella* counts of treated groups to *Salmonella* counts of controls (Bailey *et* al, 1983) and calculated for all treatments showed live vaccination conferred 1.5 and 1.7 PF for 3 and 6 wk counts. Values between 1.8 and 2.0 PF were obtained for 22 wk challenges. Protection factor values show that although reductions due to vaccination treatments were statistically significative, actual bacterial counts of controls versus

[16] SAS Institute Inc., Carry, NC

vaccinates were between 1.5 and twice as great as non-vaccinated controls. These data show that vaccination helps in reducing overall counts but does not preclude *Salmonella* colonization.

Breeder Counts by serovars:

Counts obtained from composite LHS samples are an indicator of invasiveness. No consistency of relative serovar colonization through time was observed (Table 2). Serovar Thompson was more prevalent on 3 (87.5%) and 18 wk (85.3%) challenges; serovar Enteritidis was more prevalent on 6 (96.4%) and 10 wk (87.8%) challenges, and serovar Thompson was slightly more prevalent on wk 22 (48.5%) challenge. Factors affecting intestinal microbial ecology (i.e. age of the birds, gut microflora composition), which are independent of vaccination treatments but vary through time, are probably responsible for this lack of serovar consistency between challenge events. However, in most challenge events, a particular serovar was more successful in establishing itself over the other two, as observed by the tendency for a particular serovar to be present at a higher concentration (% composition) at each challenge event. All LHS counts were substantially lower than corresponding cecal counts, and in some cases no *Salmonella* was recovered from these samples. Although counts were numerically somewhat higher on young birds (wk 3 challenge), no differences among treatments were observed.

Progeny counts by treatment

Progeny of vaccinated breeders and challenged at 1 d of age showed no effect of maternal antibody on cecal counts, except for progeny from 40 wk-old breeders, sampled one wk post-challenge (Table 3). At this time, progeny from the 2K treatment had higher cecal counts than 2L2K (0.95 log) and Controls (0.91 log). This higher count was transient and counts were comparable to controls when progeny was sampled a wk later (2 wks post challenge). Similarly, LHS samples showed no differences except between progeny of all vaccinated treatments and

controls sampled 2 wks post challenge, at 34 wks of breeder age. At this time, a mean 0.43 log reduction in vaccinates compared to controls was observed at this time. Serum maternal antibody passed through the yolk in these treatments was mainly IgG (Rolón *et al*, submitted), with negligible IgA. Immunoglobulin G levels through time were consistently high, and the slight differences in *Salmonella* counts observed at 34 wk cecal counts sampled 1 wk post challenge, and 34 wk LHS counts sampled 2 wk post challenge cannot be directly related to differences in yolk IgG content. Immunoglobulin A passed through the egg (not measured) has been found to be more concentrated in the albumen (Kimijama *et al,* 1990), and might play a greater role in initial protection against *Salmonella* challenge. The dynamics of albumen IgA as a response to vaccination of the dams may be different than the dynamics of IgG. Although we have not related albumen IgA concentrations to actual challenge, this is an area worth pursuing in future studies.

Progeny counts by serovar

Cecal and LHS counts were higher for the Rif-ST serovar on all progeny challenge events, except for LHS counts of progeny of 34 wk-old breeders, sampled 2 weeks post challenge (Table 4). This particular sample point showed the lowest overall *Salmonella* counts, which would explain the lack of differences between serovar counts.

Progeny counts by competitive exclusion

Delivery of MSC reduced *Salmonella* counts on all progeny challenge events, except for progeny of 29 wk-old breeders, where a numerical (not statistical) reduction was observed and overall counts were extremely small. Competitive exclusion was more effective than vaccination of breeders in reducing *Salmonella* hatchling colonization, as shown by a consistent reduction (1.35 to 1.55 log) of cecal *Salmonella* counts of CE-treated chicks compared to controls (Table

4). Liver-Heart-Spleen counts also showed consistent reductions (0.02 to 0.35 log), although these were lower in magnitude, as were LHS counts compared to cecal counts.

Throughout the 5 challenge events and for higher (cecal) counts, vaccination had a mean protection factor of 1, whereas competitive exclusion had a mean protection factor of 2.9. With lower (LHS) counts, differences in protection factors were much smaller, with a mean protection factor of 1.4 for vaccination treatments, and 1.7 for competitive exclusion treatments. The higher protection factor values of CE-treated birds (Table 3) compared to vaccinated birds (Table 1) show that passive immunity obtained by the tested vaccination programs against *Salmonella* did not diminish counts as did the competitive exclusion treatment. These results highlight the importance of establishing beneficial gut microflora early in the life of the chick as an effective tool in curbing potential field challenges.

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Table 4.1 Breeder Salmonella counts by vaccination treatment

	Cecal Counts						
	2 Killed		2Live-2Killed		3Live-1Killed		Control
Week	Log cfu/ml	PF	Log cfu/ml	PF	Log cfu/ml	PF	Log cfu/ml
3	2.572 [a]		1.752 [b]	1.5	1.752 [b]	1.5	2.572 [a]
6	1.994 [a]		1.141 [b]	1.7	1.141 [b]	1.7	1.994 [a]
11	1.816 [a]		1.700 [a]	1.1	1.700 [a]	1.1	1.816 [a]
17	0.157 [b]	6.2	0.485 [ab]	2	0.675 [ab]	1.4	0.975 [a]
22	1.380 [b]	1.9	1.304 [b]	2	1.403 [b]	1.8	2.558 [a]
	Liver-Heart-Spleen Counts						
	2 Killed		2Live-2Killed		3Live-1Killed		Control
Week	Log cfu/ml	PF	Log cfu/ml	PF	Log cfu/ml	PF	Log cfu/ml
3	0.359 [a]		0.278 [a]	1.3	0.278 [a]	1.3	0.360 [a]
6	0.088 [a]		0.044 [a]	2	0.044 [a]	2	0.088 [a]
11	0.219 [a]		0.000 [a]	Total	0.000 [a]	Total	0.220 [a]
17	0.000 [a]	Total	0.000 [a]	Total	0.000 [a]	Total	0.000 [a]
22	0.044 [a]	7.9	0.198 [a]	1.8	0.121 [a]	2.9	0.349 [a]

Vaccination Treatments: 2Killed = 2 killed vaccines given at 11 and 17 wks of age; 2Live2Killed = 2 live vaccines given at d 1 and 21, and 2 killed vaccines given at wks 11 and 17 of age; 3Live-1Killed = 3 live vaccines given at d 1, 21 and 77, and 1 killed vaccine given at 17 wks of age; C = non-vaccinated controls. PF (Protection Factor) = Log cfu/ml of non-vaccinated controls / Log cfu/ml of vaccinated treatments. Means with different subscripts within rows are statistically significant ($P<0.05$).

Table 4.2 Breeder Salmonella counts by serovar

	Cecal Counts					
	Salmonella enteritidis		Salmonella thompson		Salmonella typhimurium	
Week:	Log cfu/ml	% Comp.	Log cfu/ml	% Comp.	Log cfu/ml	% Comp.
3	2.112 [b]	10.7%	3.025 [a]	87.5%	1.348 [b]	1.8%
6	2.860 [a]	96.4%	0.454 [c]	0.4%	1.389 [b]	3.3%
11	2.841 [a]	87.8%	0.464 [c]	0.4%	1.969 [b]	11.8%
17	0.279 [b]	8.4%	1.286 [a]	85.3%	0.155 [b]	6.3%
22	1.691 [a]	33.1%	1.437 [b]	18.4%	1.857 [a]	48.5%
	Liver-Heart-Spleen Counts					
	Salmonella enteritidis		Salmonella thompson		Salmonella typhimurium	
Week:	Log cfu/ml	% Comp.	Log cfu/ml	% Comp.	Log cfu/ml	% Comp.
3	0.749 [a]	68.4%	0.188 [ab]	18.8%	0.020 [c]	12.8%
6	0.198 [a]	44.1%	0.000 [a]	27.9%	0.000 [a]	27.9%
11	0.095 [a]	31.5%	0.000 [a]	25.3%	0.234 [a]	43.3%
17	0.000 [a]	---	0.000 [a]	---	0.000 [a]	---
22	0.066 [a]	25.2%	0.298 [a]	43.0%	0.169 [a]	31.9%

% Comp. = Percent serovar composition of total *Sallmonella* isolated. Means with different subscripts within rows are statistically significant ($P<0.05$).

Table 4.3 Salmonella counts from offspring of vaccinated breeders

		Cecal Counts						
Breeder	Wks Post-	2Killed		3Live-1Killed		2Live-2Killed		Control
Age (Wk)	Challenge	Log cfu/ml	PF	Log cfu/ml	PF	Log cfu/ml	PF	Log cfu/ml
20	1	1.088 [a]	1.4	2.056 [a]	0.8	1.308 [a]	1.2	1.556 [a]
34	1	2.638 [a]	1.1	2.303 [a]	1.3	3.380 [a]	0.9	2.952 [a]
34	2	1.497 [a]	1.2	1.502 [a]	1.2	1.250 [a]	1.5	1.848 [a]
40	1	2.091 [a]	0.5	1.503 [ab]	0.7	1.145 [b]	1.0	1.108 [b]
40	2	1.289 [a]	0.8	1.086 [a]	1.0	0.998 [a]	1.1	1.081 [a]
		Liver-Heart-Spleen Counts						
Breeder	Wks Post-	2Killed		3Live-1Killed		2Live-2Killed		Control
Age (Wk)	Challenge	Log cfu/ml	PF	Log cfu/ml	PF	Log cfu/ml	PF	Log cfu/ml
20	1	0.575 [a]	1.4	0.700 [a]	1.1	0.469 [a]	1.7	0.788 [a]
34	1	0.983 [a]	1.1	0.864 [a]	1.3	1.077 [a]	1.0	1.098 [a]
34	2	0.000 [b]	Total	0.211 [b]	2.6	0.127 [b]	4.3	0.548 [a]
40	1	0.352 [a]	0.7	0.352 [a]	0.7	0.386 [a]	0.7	0.258 [a]
40	2	0.539 [a]	1.0	0.534 [a]	1.0	0.455 [a]	1.2	0.539 [a]

Vaccination Treatments: 2Killed = 2 killed vaccines given at 11 and 17 wks of age; 2Live2Killed = 2 live vaccines given at d 1 and 21, and 2 killed vaccines given at wks 11 and 17 of age; 3Live-1Killed = 3 live vaccines given at d 1, 21 and 77, and 1 killed vaccine given at 17 wks of age; C = non-vaccinated controls. PF (Protection Factor) = Log cfu/ml of non-vaccinated controls / Log cfu/ml of vaccinated treatments. Means with different subscripts within rows are statistically significant ($P<0.05$).

Table 4. 4 Hatchling Salmonella counts by competitive exclusion and serovar

Cecal Counts

		Competitive Exclusion			Serovar			
Breeder	Wks Post-	MSC		Control	Salmonella enteritidis		Salmonella typhimurium	
Age (Wk)	Challenge	Log cfu/ml	PF	Log cfu/ml	Log cfu/ml	% Comp	Log cfu/ml	% Comp
20	1	0.686 [a]	3.3	2.231 [a]	0.503 [b]	1.2%	2.414 [a]	98.8%
34	1	2.067 [b]	1.7	3.569 [a]	1.239 [b]	0.1%	4.397 [a]	99.9%
34	2	0.957 [b]	2.2	2.091 [a]	0.245 [b]	0.3%	2.804 [a]	99.7%
40	1	0.702 [b]	3.2	2.221 [a]	0.295 [b]	0.5%	2.629 [a]	99.5%
40	2	0.435 [b]	4.1	1.792 [a]	0.085 [b]	0.9%	2.142 [a]	99.1%

Liver-Heart-Spleen Counts

		Competitive Exclusion			Serovar			
Breeder	Wks Post-	MSC		Control	Salmonella enteritidis		Salmonella typhimurium	
Age (Wk)	Challenge	Log cfu/ml	PF	Log cfu/ml	Log cfu/ml	% Comp	Log cfu/ml	% Comp
20	1	0.469 [b]	1.7	0.813 [a]	0.091 [b]	7.4%	1.191 [a]	92.6%
34	1	0.701 [b]	1.9	1.310 [a]	0.291 [b]	3.6%	1.720 [a]	96.4%
34	2	0.211 [a]	1.1	0.232 [a]	0.148 [a]	41.6%	0.295 [a]	58.4%
40	1	0.234 [b]	1.9	0.439 [a]	0.000 [b]	17.5%	0.673 [a]	82.5%
40	2	0.340 [b]	2.0	0.694 [a]	0.012 [b]	9.0%	1.014 [a]	91.0%

MSC = Mucosal Starter Culture undefined flora competitive exclusion treatment; % Comp. = Percent composition of serovar of all *Salmonella* isolated. PF (Protection Factor) = Log cfu/ml of non-vaccinated controls / Log cfu/ml of vaccinated treatments. Means with different subscripts within rows are statistically significant ($P<0.05$).

CHAPTER 5

INTESTINAL HUMORAL IMMUNE RESPONSE AND RESISTANCE TO SALMONELLA CHALLENGE OF PROGENY FROM BREEDERS VACCINATED WITH KILLED ANTIGEN[1]

[1] Rolón, A., J. S. Bailey, C. L. Hofacre, P. S. Holt, and J. L. Wilson. To be submitted to Avian Diseases.

SUMMARY

Salmonella vaccination programs using killed bacterins in breeders and live auxotrophic-strain vaccines early in the life of their progeny have gained popularity in today's poultry industry. In this study we evaluated the intestinal humoral immune response to a live auxotrophic vaccine used on hatchlings with and without maternal antibody, and related this response to challenge with a blend of two antibiotic-resistant *Salmonella* marker strains. Forty wk-old ISA Brown® (Institute de Selection Animale, France) breeders from a *Salmonella*-free flock were vaccinated twice at a three wk interval with commercially-prepared autogenous trivalent bacterin, serogroups B, C and D1 (Lohmann Animal Health International, Gainesville, GA), or a serovar Enteritidis bacterin (Fort Dodge Animal Health Inc, Overland Park, KS). Half of the progeny from these treatments (hatched from eggs layed 3 wks after second bacterin dose) were given a live *Salmonella* serovar Typhimurium (LiveST) mutant vaccine (Fort Dodge Animal Health Inc, Overland Park, KS), by coarse spray on arrival to the brooding premises. On d 3, 13 and 34, intestinal Immunoglobulins (Ig) A and G were sampled and measured on enzyme-linked immunosorbent assay plates coated with *Salmonella* serovars Enteritidis (SELPS) or Typhimurium (STLPS) Lipopolysaccharide. On the same days, a second group of birds was challenged with a blend of antibiotic-resistant serovars Enteritidis and Typhimurium strains. Cecal and composite liver-heart-spleen samples obtained 7 d post-challenge were cultured and colonies enumerated. Maternal IgG observed up to 13 d had no effect on subsequent LiveST-stimulated antibody production. No protective effect of maternal antibody was demonstrated, except when combined with LiveST given to the progeny. Killed vaccines delivered to the

breeders combined with a live vaccine delivered to the progeny resulted in reduced invasiveness after challenge, as shown by a reduction in liver-heart-spleen *Salmonella* counts. One dose of LiveST enhanced intestinal IgG (Optical Densities (OD) >0.576) up to 34 d when measured on STLPS, but only to 13 d when measured on SELPS, with titers decreasing with age. Increased IgA was observed only at 13 d. Three and 13 but not 34 day bacterial counts were decreased by the live ST vaccine treatment, for both cecal (1.05 and 1.09 log) and liver-heart-spleen (0.32 and 0.06 log) samples, indicating that a second dose might be necessary for prolonged protection. The protective effect of the live vaccine, but not of maternal IgG, leads us to hypothesize that protection might be due to stimulation of cell-mediated intestinal immunity, and/or a competitive exclusion effect of the LiveST vaccine. Reduction but not elimination of *Salmonella* colonization by vaccination highlights the importance of vaccines as complementary tools, and not substitutes of integral biosecurity programs to control *Salmonella* in poultry.

Key Words: *Salmonella*, challenge, immune response, mucosal immunity, passive immunity, vaccine, breeders

Abbreviations: Ig= Immunoglobulin; IgA = Immunoglobulin A; IgG = Immunoglobulin G; LiveST = Live Aro-A Mutant *Salmonella* serovar Typhimurium vaccine; SEBAC = *Salmonella* serovar Enteritidis bacterin; SELPS = *Salmonella* serovar Enteritidis lipopolysaccharide; STLPS = *Salmonella* serovar Typhimurium lipopolysaccharide; TRIBAC = trivalent autogenous bacterin, serogroups B, C_2 and D_1; OD = Optical Density; d = day; wk=week

INTRODUCTION

Although the cell-mediated and humoral intestinal immune responses are recognized as the primary mechanisms in defense against enteric bacterial pathogens, the intestinal immunoglobulin dynamics of progeny vaccinated with live auxotrophs shortly after hatch, and

its correlation to actual resistance to challenge in progeny has not been extensively studied. Vaccination of breeder flocks with autogenous *Salmonella* vaccines, as well as, vaccination of newly-hatched chicks with live vaccines has gained popularity in the poultry industry. However, there exists potential interference of maternal immunoglobulin in neutralizing live vaccines given early in life.

Early studies with turkey poults originating from breeders with a history of a *Salmonella* serovar Typhimurium field outbreak early in life, and vaccinated multiple (4 to 6) times with an autogenous aluminum hydroxide-adjuvanted bacterin showed decreased overall poult mortality when challenged after hatch, compared with hatchlings from unvaccinated dams (14). Turkey breeders vaccinated twice with a *Salmonella* serovar Hadar bacterin at 41 and 45 wks and hatched from eggs layed at 51 wks, showed a decrease in the number of positive serovar Hadar isolates compared to turkey poults derived from non-vaccinated dams up to 39 d (17). A reduction in *Salmonella* colonization of progeny from breeders vaccinated at 16 and 18 wks with a live Δcya Δcrp vaccine strain has been previously reported (6). The authors also observed an interference of maternal antibody on early vaccination, since day-of-age delivery of a live serovar Typhimurium Δcya Δcrp vaccine strain to progeny with maternal antibody would clear the vaccine strain by two wks, compared to a persistence of the vaccine strain for at least 21 d in SPF birds. In their study, maternally-derived immunity reduced efficacy of a 1 and 3 wk, but not a 2 and 4 wk vaccination program.

The present study was set to evaluate the effectiveness of early vaccination with a live *Salmonella* vaccine and in the presence of maternal antibody, in protecting hatchlings against *Salmonella* challenge at d 3, 13 and 34. Intestinal humoral (IgA and IgG) immune response and *Salmonella* counts seven days post-challenge were the parameters evaluated.

MATERIALS AND METHODS

Breeders, Vaccination and Incubation:

Forty wk-old ISA Brown® breeders (Institute de Selection Animale, France) in a small commercial operation were used as the parent stock. Breeders were housed on a 13.5 x 34 m open house with six 6.5 x 9.5 m compartments, three on each wing with a central corridor and transverse corridors separating each compartment. Each compartment housed approximately 380 female breeders. Litter material consisted of eucalyptus shavings, and equipment consisted of manual bell feeders, automatic bell drinkers and metal nests with eucalyptus shavings as nest material. Nest shavings were changed on a bi-weekly basis, with 15g of paraformaldehyde added to each nest weekly. As part of the farm's established monitoring programs, routine bi-monthly environmental sampling for *Salmonella* during grow-out and production were carried out using drag swab, feed, litter and water samples. A 1% sample of the breeders were plate-agglutination tested with a polyvalent *Salmonella* antigen (Intervet International BV, Boxmeer, Holland) at start of production, and 0.5-1% again after peak production, with any suspect bird being separated, and fecal and/or cecal samples taken after euthanization and cultured for *Salmonella*. Samples were pre-enriched on tetrathionate-brilliant green broth for 24hrs at 40°C before culturing on brilliant green sulpha agar.

Two of the six compartments were randomly chosen for vaccination treatments, and a third chosen as a non-vaccinated control. Breeder vaccine treatments consisted of a monovalent serovar Enteritidis bacterin (SEBAC), POULVAC-SE (Fort Dodge Animal Health Inc., Overland Park, KS), or a commercially-prepared (Lohmann Animal Health International, Gainesville, GA) autogenous trivalent bacterin (TRIBAC) comprising serovars Heidelberg

(serogroup B) Kentucky (serogroup C_2) and Berta (serogroupD_1). Breeders were vaccinated at 40 and 43 wks, and progeny obtained from eggs layed at 46 wks.

Eggs were manually collected three to four times per day from outside the compartments, with nests placed on the perimeter of each compartment. Eggs were disinfected immediately after collection by submersion in a 0.5% solution of warm (37-45°C x 0.5-2min) Virkon-S (DuPont Animal Health Solutions, Wilmington, DE) disinfectant. Eggs were stored for 3-4 d at 14°C until incubation. The eggs were incubated in a single stage NSS-10 (Natureform Hatchery Systems Inc, Jacksonville, FL) incubator and hatched in separate trays, in a H-152 (Natureform Hatchery Systems Inc, Jacksonville, FL) hatcher along with eggs from the same breeder flock only. Samples of dead-in-shell embryos and chick box liners were also cultured for *Salmonella*. Chicks were vaccinated against Marek's disease (strains FC126/SB1/CVI988, Fort Dodge Animal Health Inc, Overland Park, KS) at hatch.

Chick Treatments:

On arrival to the farm, 396 chicks were randomized into 3 x 2 treatments (Breeder Bacterin x Live auxotroph vaccine), each treatment wingbanded with different colored bands, with a total of 33 chicks per treatment. While in the chick boxes, treatments receiving POULVAC-ST (Fort Dodge Animal Health Inc, Overland Park, KS) ® (LiveST), a live Aro-A auxotroph deletion mutant serovar Typhimurium strain, were coarse-spray vaccinated and placed in the same room as treatments not receiving the LiveST, but in a different brooding pen. Each brooding pen was equipped with an infrared gas heater, two manual brooding feed pans and waterers, gradually replaced by one automatic bell drinker and manual tube feeder between 7 and 14 d. Rooms with brooding pens were previously disinfected by sublimation of 10 g of

paraformaldehyde per m^3 of room volume 48 hrs prior to chick placement and with eucalyptus shavings and brooding equipment in place.

Intestinal Ig Sampling:

On d 3, 13 and 34, 10 chicks per treatment were sampled for intestinal Ig. Chicks were removed from feed 16 hrs prior to sampling, euthanized, and the small intestine excised at the ventriculo-duodenal and ileo-cecal junctions. Five ml of a lavage solution, consisting of 1M Tris/glycerine buffer and 0.25% Tween 20, as used by Holt *et al.* (9) was flushed by inserting a feeding needle (Oxoid Inc, Ogdensburg, NY) through the ileal end and collecting flushed material through the duodenal end and into 15 ml centrifuge tubes. Samples were kept on ice until centrifuged at 2,500g for 10 min. The supernatant was frozen at -8°C until the enzyme-linked immunosorbent assay procedure was performed.

ELISA Assays:

The ELISA protocol was completed on each sample as previously described (7). Briefly, the assays were conducted using 96-well Immulon ® plates (Dynex Technologies, Inc., Chantilly, VA) coated with Lipopolysaccharide from serovars Typhimurium (STLPS) or Enteritidis (SELPS) (Sigma, Saint Louis, MO), at a concentration of 10µL/ml, incubated overnight, and blocked with a solution consisting of 1% bovine serum albumin, 1% phosphate buffered saline and 0.5ml/L Tween 20 (Sigma, Saint Louis MO). The blocking step solution was added to the plates for one hour. All plates were washed between steps twice with a 1% phosphate buffered saline plus 0.5ml/L Tween 20, for 2 to 3 minutes. Intestinal lavage samples were diluted 1:2 and added to the microplates together with controls, and incubated for 90 minutes. Primary antibodies were mouse anti-chicken IgA or mouse anti-chicken IgG (Southern Biotech, Birmingham, AL) and diluted 1:1000. Primary antibodies were incubated for one hour.

Secondary antibody was a goat anti-mouse heavy and light chain-specific IgG (Calbiochem, La Jolla, CA), used at a 1:2000 dilution and incubated for one hour. Para-nitro-phenyl phosphate chromogen (Sigma, Saint Louis, MO) at a 1mg/mL concentration diluted in diethanolamine (Sigma, Saint Louis, MO) was added, incubated for 20-30 minutes under dark conditions, and plates read at 405nm.

Bacterial Challenge:

On the same d (3, 13 and 34), 10 chicks per treatment were challenged with a blend of $10^7 – 10^8$ CFU/ml of a nalidixic acid resistant Serovar Enteritidis, and a Rifampicin resistant serovar Typhimurium strains. Strains were grown 24hrs earlier on brilliant green sulpha agar with 200 ppm of corresponding antibiotic (rifampicin or nalidixic acid), and cells suspended in 0.85% saline. Cell suspensions were adjusted to 0.120 OD at 540nm, and equal aliquots mixed prior to gavaging. The gavaging mix was plated and colonies counted to confirm that the mixed colony concentration was within the $10^8 – 10^9$ cfu/ml range. Each chick was gavaged with 0.1ml of challenge mixture. Challenged chicks of all treatments were placed together in one brooding pen, in a separate room from non-challenged chicks. Seven d after challenge, six chicks per treatment were euthanized and sampled for bacterial enumeration. The left liver lobule, heart and spleen were pooled, weighed and placed in a stomacher filter bag (Fischer Scientific International Inc, Hampton, NH) with filter. Both ceca were removed and placed in a second stomacher bag with filter. All samples were kept in ice until processing (less than 2 h). Samples were weighed, and peptone broth corresponding to 3 times sample weight added. Samples were stomached thoroughly and plated for enumeration as reported earlier (2), with some modifications. Briefly, four BGS plates per sample, two with rifampicin and two with nalidixic acid added at 200 ppm during preparation, were used. For each antibiotic-added plate, one plate

was spread-plated with 100 µL of each stomached sample, and a second with 100 µL of a 1:100 dilution of each sample. Hence, two plates with final dilutions of 10^{-1} and 10^{-3} per sample and per antibiotic were obtained. Plates were incubated at 37°C for 24 hrs, and colonies counted. From each dilution (10^{-1} and $10^{-3)}$, plates with counts within or closest to a 30-300 colony per plate range were separated and enumerated for data analysis.

Statistical Analysis:

In order to contrast differences between treatments (vaccination regimens), ELISA data were analyzed under a completely randomized design and treatment differences contrasted using Duncan's Multiple Range Test. Additionally, to visualize the vaccination effects on ELISA profiles and *Salmonella* counts, transformed data (Log_{10}) were analyzed using the GLM procedure (SAS Institute, Cary, NY) under a factorial design (Breeder Killed Bacterin x Live Progeny Vaccine), and mean differences discriminated using Duncan's Multiple Range Test.

RESULTS

Intestinal IgA profiles showed no measurable maternally-derived IgA, as revealed by similar OD of all treatments by day 3 (Figure 1). Live vaccination elicited a small increase in intestinal IgA by 13 d, the largest increase observed was on STLPS. The increased IgA was temporary (OD > 0.65), and not present by 34 d. In contrast, treatment IgG profiles shown (Figure 2) for chicks from vaccinated breeders were higher than for chicks from non-vaccinated breeders at 3 d (OD >0.75). IgG titers decreased by 13 d and were no different from controls by 34 d. Live vaccination increased IgG by 13 d (OD >0.75), but titers showed a declining trend by day 34. Titers were higher for live-vaccinated chicks by day 34 when measured on STLPS, but not on SELPS.

Analysis of the main effects (vaccines) on IgA and IgG (Table 1) show that LiveST caused a temporary small but significative rise in intestinal IgA by 13 d, but this effect wasn't measurable by 34 d. Although significative interactions for IgA when measured on STLPS on day 13 were observed, IgA titers between treatments not receiving LiveST were very similar to the control, with a slightly higher titer for non-vaccinated controls compared to breeder-vaccinated treatments. LiveST caused an increase in IgG for d 13 when measured on SELPS. This increased effect was greater and lasted longer when measured on STLPS, as seen by increased titers for d 13 (mean OD = 0.934) and 34 (mean OD = 0.576). Maternally-derived IgG was higher for the TRIBAC treatment at 3 d when measured on SELPS, and titers were higher from non-vaccinated controls throughout all sampling d when measured on STLPS. Although higher IgG was observed by day 34 for TRIBAC chicks, there was a consistent decrease in titers through time, as would be expected, with treatment means between TRIBAC and No-TRIBAC effects being very similar by day 34 (0.418 vs. 0.507 OD for No-TRIBAC and TRIBAC groups respectively). The same decrease in titers with time was observed with IgG measured on SELPS, but significant differences were observed only for d 3 and 13. A significative interaction for 13 day IgG measured on SELPS between LiveST and SEBAC shows a smaller rate of titer increment for SEBAC chicks that received LiveST than for non-SEBAC chicks that received LiveST for day 13 samples. Analysis of this interaction shows that non-SEBAC chicks receiving Live ST increased mean OD from 0.408 to 0.624, whereas SEBAC chicks receiving Live ST showed only a slight increase of mean OD, from 0.590 to 0.621. These data show that LiveST will result in similar end titers (OD of 0.621 and 0.624) regardless of the presence or not of maternally-derived antibody due to SEBAC treatment. Although an interaction for 34 day IgG on STLPS was detected, breakdown of data show OD increments of 0.262 and 0.247 for no-SEBAC

and SEBAC birds when receiving LiveST respectively. These values can be considered equivalent from a biological standpoint.

Total *Salmonella* counts showed no differences between treatments for cecal samples, but some differences were observed for liver-heart-spleen samples (Figure 3). No *Salmonella* was recovered from liver-heart-spleen cultures from all treatments including breeder killed bacterin and live progeny vaccination, except for day 13 LiveST + SEBAC treatment. These differences were significant for d 3 and 34, but not for day 13. Although the LiveST treatment when contrasted to the other treatments did not show a decrease in liver-heart-spleen *Salmonella* counts, a factorial analysis of main effects (Table 2) reveals differences for LiveST for d 3 and 13, but not for day 34. Cecal sample data showed *Salmonella* count reductions of 1.05 and 1.09 Log, and liver-heart-spleen sample data showed reductions of 0.319 and 0.125 log for 3 and 13 day samples.

DISCUSSION

The different IgG and IgA profiles at 3 d in chicks from vaccinated breeders show IgG is the most prevalent maternally-derived immunoglobulin type. Although we did not measure IgG and IgA levels in the breeders after vaccination, it appears that two doses of killed antigen delivered to the breeders induced IgG but not IgA passive immunity to the progeny. Maternal IgG is deposited in the egg yolk, and IgA is deposited in the amniotic fluid, which is swallowed by the embryo prior to hatching (16). Passive yolk-derived IgG is parenterally transferred to the embryo through the vitelline vessels, or enters the intestinal lumen via the omphaloenteric duct, and can be detected as early as three d prior to hatch (11,13). Maternally derived IgG in serum is reported to be highest after hatch, and decreases after 2 to 5 wks post hatch (12). In our study, intestinal IgG followed a similar trend. We were unable to clearly demonstrate interference of

maternally-derived immunoglobulins on the LiveST vaccination treatment's ability to elicit increased IgG and IgA titers in the offspring of the vaccinated breeders. However, treatments combining breeder vaccination and progeny LiveST were the most effective in eliciting higher IgG titers for d 13 and 34, and IgA titers for day 13. This effect is more evident when reviewing the ELISA data using STLPS antigen. Higher titers on STLPS would be expected when the birds were vaccinated with a serogroup B (LiveST). LiveST is a serovar Typhimurium strain, and serovar Heidelberg, contained in the TRIBAC bacterin, is a group B serovar, therefore they share common somatic antigens 4, 12, and possibly 5 with serovar Typhimurium (1). We also observed, as expected, marginally lower IgG titers for the SEBAC treatment when measured on STLPS, and marginally higher titers when measured on SELPS. In general, ELISAS using LPS as capture antigen tend to be more sensitive as somatic antigen homology with the challenge/vaccine strain eliciting the immune response increases.

IgA priming by the liveST was shorter lived than IgG. Previous research has shown serum IgA and IgG titers after six wk-old prime-infection with an invasive serovar Typhimurium strain to persist up to 10 wks post challenge (4). However, immunoglobulin responses on younger birds elicited by LiveST appear to be shorter lasting. This may be explained in part by the lack of invasive nature of the auxotroph mutant, as well as, by the hyporesponsiveness of young birds to early infection (8). Although LiveST and TRIBAC yielded higher IgG responses throughout 34 d (Table 1), IgG titers consistently decreased after day 13, approaching control values by day 34. A second dose of LiveST is probably necessary if longer-lasting Ig titers are desired, such as for birds with a longer lifespan than commercial broilers (i.e. breeders and layers).

The lack of treatment differences for total *Salmonella* cecal counts despite differences in Ig profiles (Figures 1 and 2) show that increased intestinal IgG and IgA were not sufficient to effectively reduce *Salmonella* incidence. It is interesting to note, however, that combining maternal immunity with LiveST vaccination diminished invasiveness of challenge strains at all sampling events, except for day 13 SEBac x LiveST treated birds (Figure 3b). Innate and cell-mediated immunity as well as adequate humoral responses are important components of immunity against bacterial pathogens. Ontogenic studies of the gut-associatied lypmphoid tissue have shown the presence of IgG and IgA B cells as early as 5 d post-hatch, and IgA and IgG plasma cells by 14 d post-hatch in the intestinal lamina propia (10). However, a recent study using chicken Interferon γ mRNA as a marker of T-cell effector functionality showed reduced Interferon γ expression of intestinal T cells, leading the authors to conclude that gut resident T cells are functionally immature during the first 2 wks of life (3). Similarly, suboptimal functional activity of the heterophil in young chicks as measured by decreased phagocytic indices on 1 and 4 day-old chicks compared to 7 day-old chicks show that innate immunity also undergoes maturation with time (18).

The beneficial effect of LiveST in young chicks may be explained not only by an increment in intestinal Immunoglobulins, but by a colonization and hence exclusion effect of the vaccine strain. The potential competitive exclusion effect of vaccine strains against *Salmonella* challenge has been previously hypothesized (5). Studies combining day-of-age vaccine or homologous strain delivery followed shortly (as early as 2 d post vaccination) by challenge show a decrease in colonization of birds previously vaccinated/colonized (15). The short time lapse between vaccination and challenge probably is not enough for an adequate immune response, but a decrease in colonization apparently points towards an initial competitive-exclusion effect of

the vaccine, complemented by immune-priming as times goes by. Although LiveST vaccination decreases overall bacterial load, *Salmonella* still present in the ceca at considerable numbers highlight the importance of vaccine programs in breeders and newly hatched chicks as a complementary tool in controlling *Salmonella* in poultry. However, this work by no means may substitute implementation of adequate biosecurity programs throughout breeding, production and processing.

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Table 5.1 IgA and IgG profiles[A] as affected by a day-of-age live *Salmonella* serovar Typhimurium vaccine, or maternally-derived from breeders vaccinated with a killed *Salmonella* serovar Enteritidis bacterin, or a trivalent (*Salmonella* serovars Heidelberg, Kentucky and Berta) bacterin.

	IgA Optical Density on Serovar Enteritidis Lipopolysaccharide												Interaction P Value			
Day of Age	No LiveST		LiveST		No TriBac		TriBac		No SEBac		SEBac		LiveSTxTriBac		LiveSTxSEBac	
3	0.268	a	0.268	a	0.278	a	0.249	a	0.262	a	0.280	a	-		-	
13	0.318	a	0.439	b	0.355	a	0.426	a	0.399	a	0.337	a	0.566		0.140	
34	0.347	a	0.418	a	0.387	a	0.374	a	0.384	a	0.379	a	0.539		0.646	
	IgA Optical Density on Serovar Typhimurium Lipopolysaccharide												Interaction P Value			
Day of Age	No LiveST		LiveST		No TriBac		TriBac		No SEBac		SEBac		LiveSTxTriBac		LiveSTxSEBac	
3	0.261	a	0.261	a	0.244	a	0.294	a	0.281	a	0.220	a	-		-	
13	0.351	a	0.794	b	0.567	a	0.582	a	0.591	a	0.220	a	0.029	*	0.012	*
34	0.401	a	0.488	a	0.451	a	0.430	a	0.427	a	0.220	a	0.905		0.798	
	IgG Optical Density on Serovar Enteritidis Lipopolysaccharide												Interaction P Value			
Day of Age	No LiveST		LiveST		No TriBac		TriBac		No SEBac		SEBac		LiveSTxTriBac		LiveSTxSEBac	
3	0.646	a	0.646	a	0.590	a	0.758	b	0.531	a	0.876	b	-		-	
13	0.469	a	0.623	b	0.542	a	0.554	a	0.516	a	0.606	a	0.065		0.041	*
34	0.375	a	0.434	a	0.404	a	0.405	a	0.406	a	0.402	a	0.550		0.746	
	IgG Optical Density on Serovar Typhimurium Lipopolysaccharide												Interaction P Value			
Day of Age	No LiveST		LiveST		No TriBac		TriBac		No SEBac		SEBac		LiveSTxTriBac		LiveSTxSEBac	
3	0.852	a	0.852	a	0.660	a	1.237	b	0.752	a	1.053	b	-		-	
13	0.645	a	0.934	b	0.729	a	0.911	b	0.694	a	0.981	b	0.844		0.285	
34	0.319	a	0.576	b	0.418	a	0.507	b	0.457	a	0.428	a	0.057		<.0001	**

[A]Optical Densities at 405nm; LiveST = Live *Salmonella* serovar Tyhphimurium vaccine; TriBac = Trivalent *Salmonella* serovars Heidelberg, Kentucky and Berta bacterin; SEBac = *Salmonella* serovar Enteritidis bacterin; [ab]Means with different superscripts within each main effect are statistically significant (P<0.05); Significant interactions are depicted at 0.05 (*) and 0.01 (**) P values.

Table 5.2 Cecal and liver-heart-spleen total *Salmonella* counts as affected by day-of-age live *Salmonella* serovar Typhimurium vaccine, or by dam vaccination with a killed *Salmonella* serovar Enteritidis bacterin, or a trivalent (*Salmonella* serovars Heidelberg, Kentucky and Berta) bacterin.

	Liver-Heart-Spleen Total *Salmonella* Counts [A]												Interaction P Value	
Day of Age	No LiveST		LiveST		No TriBac		TriBac		No SEBac		SEBac		LiveSTxTriBac	LiveSTxSEBac
3	0.367	a	0.048	b	0.236	a	0.150	a	0.237	a	0.149	a	0.845	0.840
13	0.215	a	0.160	b	0.206	a	0.150	a	0.212	a	0.139	a	0.639	0.092
34	0.151	a	0.090	a	0.150	a	0.062	a	0.145	a	0.072	a	0.209	0.167
	Cecal Total *Salmonella* Counts [A]												Interaction P Value	
Day of Age	No LiveST		LiveST		No TriBac		TriBac		No SEBac		SEBac		LiveSTxTriBac	LiveSTxSEBac
3	2.284	a	1.230	b	1.788	a	1.696	a	1.799	a	1.673	a	0.918	0.553
13	1.749	a	0.659	b	1.151	a	1.310	a	1.249	a	1.115	a	0.830	0.525
34	0.806	a	0.509	a	0.817	a	0.337	a	0.731	a	0.510	a	0.636	0.448

[A] Log_{10} CFU/mL; LiveST = live *Salmonella* serovar Typhimurium vaccine; TriBac = *Salmonella* trivalent serovars Heidelberg, Kentucky and Berta bacterin; SEBac = *Salmonella* serovar Enteritidis bacterin; [ab]Means with different superscripts within each main effect are statistically significant (P<0.05); Significant interactions are depicted at 0.05 (*) and 0.01 (**) P values.

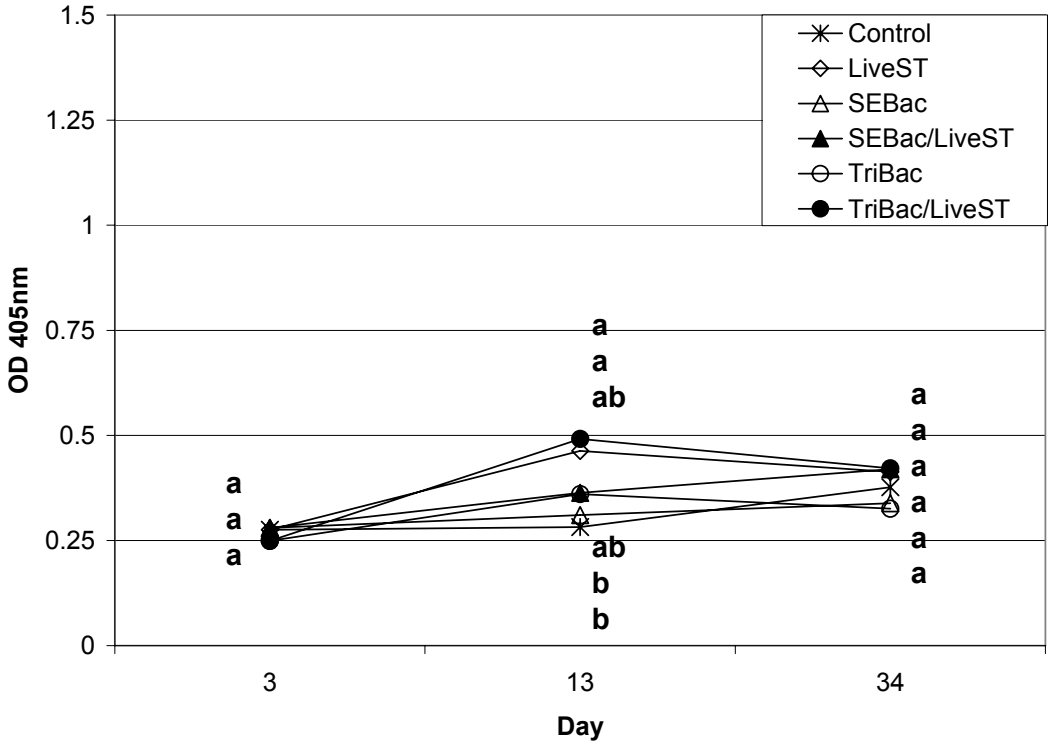
Fig. 5.1. *Intestinal* IgA optical densities of ELISAS with *Salmonella* serovars Enteritidis a) or Typhimurium b) lipopolysaccharide-coated plates; LiveST=live *Salmonella* serovar Typhimurium vaccine; TriBac = trivalent *Salmonella* serovars Heidelberg, Kentucky and Berta bacterin; SEBac = *Salmonella* serovar Enteritidis bacterin; Bacterins were delivered twice to breeders, and live vaccine coarse-sprayed at day of age to their chicks. Intestinal lavage samples were taken on days 3, 13, and 34 of age. Means with different superscripts within each sampling day are statistically significant ($P<0.05$).

Fig. 5.2. Intestinal IgG optical densities of ELISAS with *Salmonella* serovars Enteritidis a) or Typhimurium b) lipopolysaccharide-coated plates; LiveST=live *Salmonella* serovar Typhimurium vaccine; TriBac = trivalent *Salmonella* serovars Heidelberg, Kentucky and Berta bacterin; SEBac = *Salmonella* serovar Enteritidis bacterin; Bacterins were delivered twice to breeders, and live vaccine coarse-sprayed at day of age to their chicks. Intestinal lavage samples were taken on days 3, 13, and 34 of age. Means with different superscripts within each sampling day are statistically significant ($P<0.05$).

Fig. 5.3. Total *Salmonella* counts of a) cecal and b) pooled liver-heart-spleen samples; LiveST=live *Salmonella* serovar Typhimurium vaccine; SEBac = *Salmonella* serovar Enteritidis bacterin; SEBac/LiveST = *Salmonella* serovar Enteritidis bacterin and live *Salmonella* serovar typhimurium vaccine; TriBac = trivalent *Salmonella* serovars Heidelberg, Kentucky and Berta bacterin; SEBac/LiveST = *Salmonella* serovar Enteritidis bacterin and live *Salmonella* serovar typhimurium vaccine; Bacterins were delivered twice to breeders, and live vaccine coarse-sprayed at day of age to their chicks. Chicks were challenged with mixed *Salmonella* serovars Enteritidis and Typhimurium strains on days 3, 13, and 34 of age, and bacterial counts assessed

one week post-challenge. Means with different superscripts within each sampling day are statistically significant ($P<0.05$).

a)


1.5
1.25
1
0.75
0.5
0.25
0
OD 405nm
3
13
34
Day
Control
LiveST
SEBac
SEBac/LiveST
TriBac
TriBac/LiveST
a
a
a
a
a
ab
ab
b
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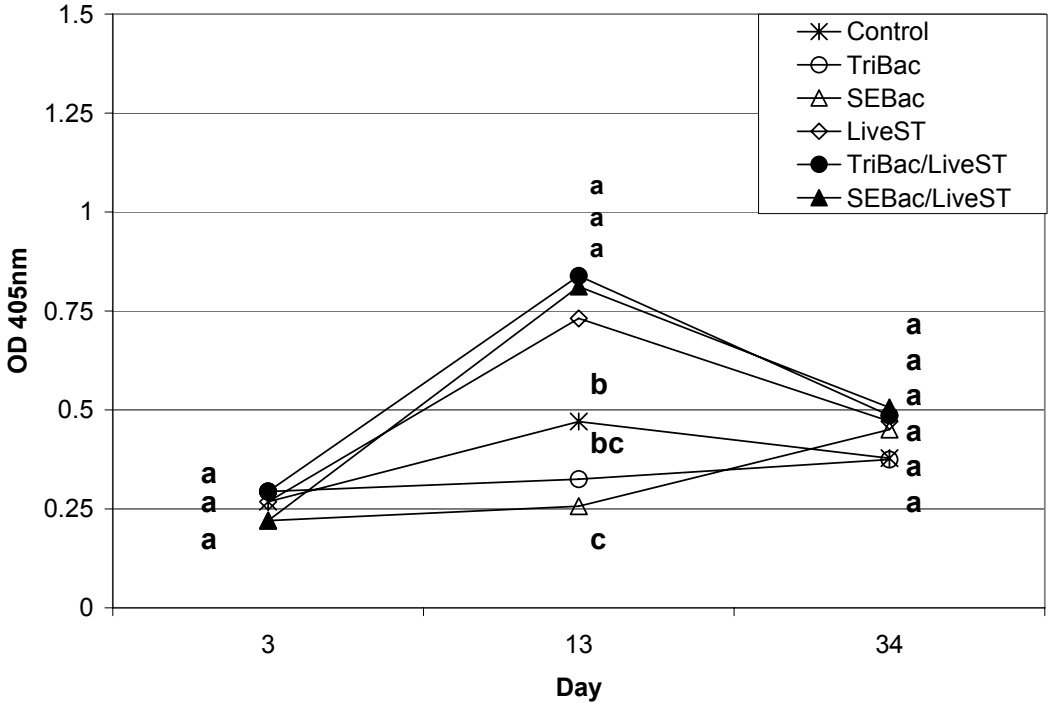
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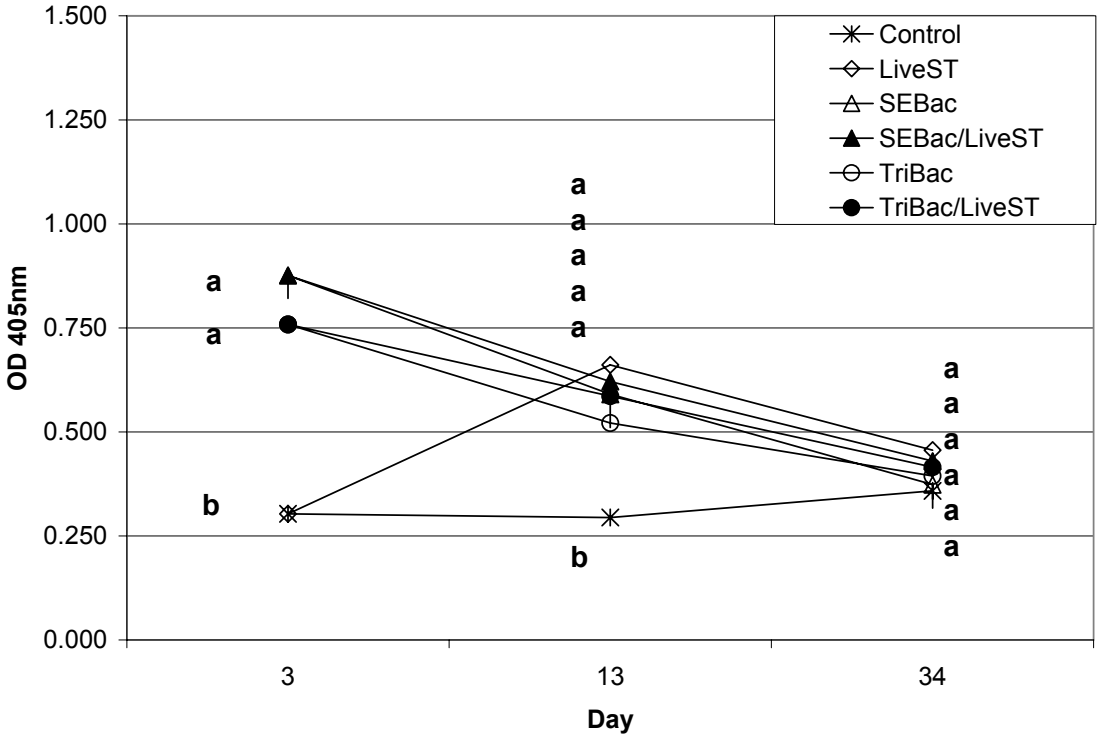
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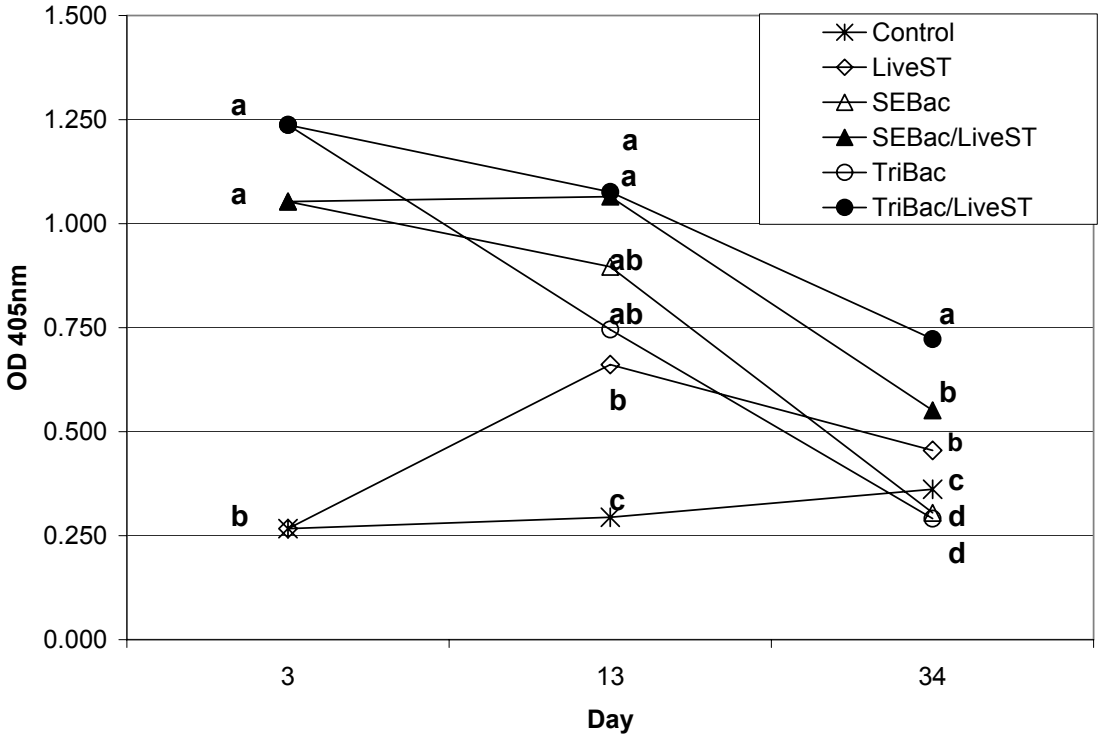
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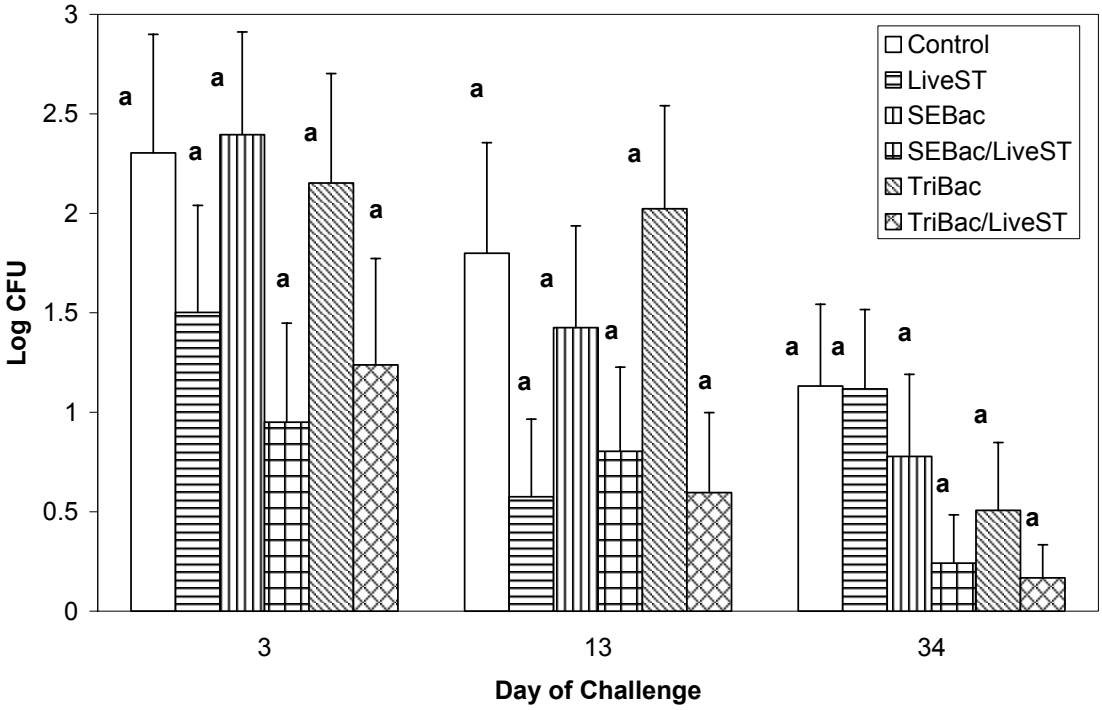
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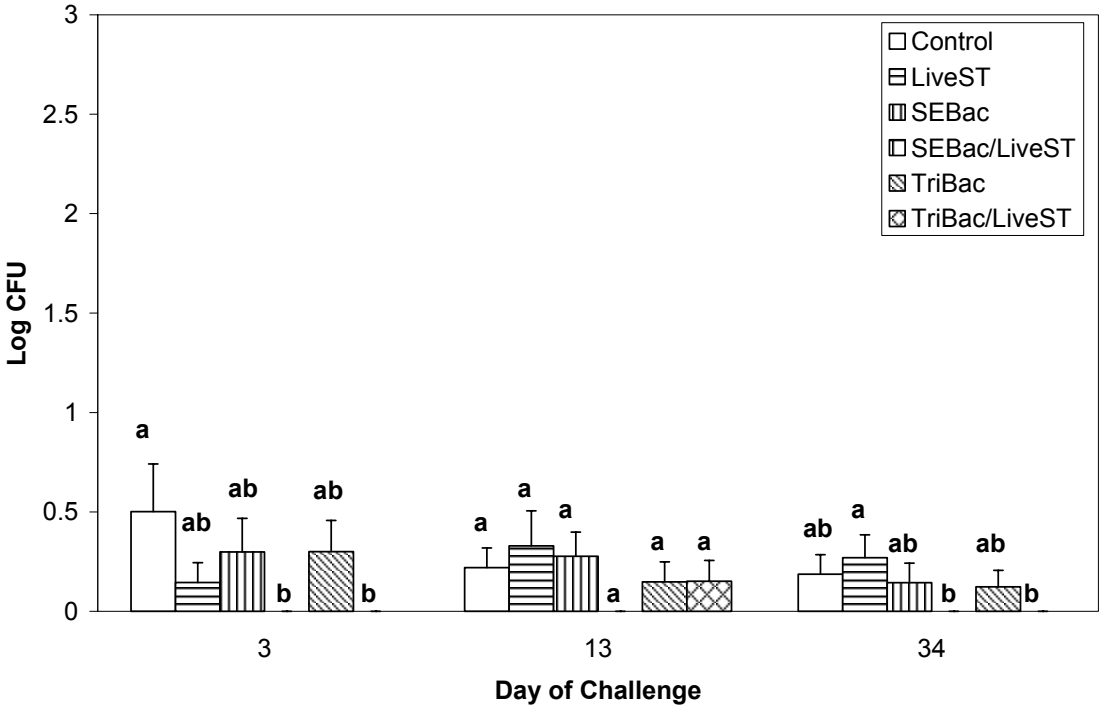
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CHAPTER 6

SUMMARY AND CONCLUSIONS

ELISA is an effective tool for monitoring *Salmonella* intestinal and serum humoral immune response. Optical densities were consistently higher when using plates coated with *Salmonella* serovar Typhimurium lipopolysaccharide (STLPS) than when using plates coated with *Salmonella* serovar Enteritidis lipopolysaccharide (SELPS).

In the first study, serum, crop and gut IgA rose after an 11-wk killed vaccine, but the trend was short lived with optical densities below 0.500 by wk 27 regardless of vaccination treatment. Expected maternal IgG was detected in 1 d of age breeder serum, since parent stock had been vaccinated with an inactivated vaccine. No IgG was detected on breeder 1 d of age crop or intestinal samples. Live vaccine elicited a strong gut and crop IgG response. A small numerical (not statistical) increase in crop IgG after the first live vaccine was also observed. By ten wks IgG was comparable to controls. Killed vaccines at 11 and 17 wks increased crop, gut and serum IgG. Serum but not crop IgG persisted throughout production up to 40 wks of age. Breeder *Salmonella* counts showed significant differences between (live) vaccinates and non-vaccinates at 3 and 6 wk challenges, showing that the commercially available Aro-A vaccine conferred adequate early protection. By 11 wks, comparable levels of *Salmonella* between vaccinates and controls indicated that the protective effect of the live vaccine had diminished. All vaccination programs reduced *Salmonella* counts by 22 wks.

Chick serum and egg yolk IgA were negligible, and IgG comparable among all breeder treatments throughout production. Chick *Salmonella* counts were variable and no clear

differences due to breeder vaccine treatments were observed, indicating that passive immunity obtained by the breeder vaccination programs did not diminish prevalence of *Salmonella* in the progeny. In contrast, delivery of undefined mucosal competitive exclusion (MSC) consistently diminished chick's *Salmonella* counts, and was not affected by breeder vaccination treatments or by breeder age. Reduction of *Salmonella* by vaccination at 3 and 6 wks was accompanied by increased gut IgG at 3 wks and serum IgG and partially explains the protective effect of the live vaccine. No gut IgG was measured at 6 wks in this study, and the importance of gut IgG was further assessed in the next study.

In the second study, two killed immunizations of *Salmonella*-free breeders at 3 wk intervals resulted in high chick gut IgG up to 13 d of age, for breeders receiving a killed serovar Enteritidis bacterin, and up to 34 d of age, for chicks from breeders receiving a killed trivalent (serovars Heidelberg (B) Kentucky (C_2), and Berta (D_1)) bacterin. A small IgA response at 13 d due to live vaccination was observed. Live vaccination also enhanced gut IgG up to 34 d when measured on STLPS, but only up to 13 d when measured on SELPS. No interference of maternal antibody on the live vaccine's ability to stimulate Immunoglobulin was demonstrable, an observation having practical implications in the use of live vaccines in the field, since differences in maternal antibody status of 1 d of age breeders are commonplace. Three and 13 but not 34 d bacterial counts were decreased by the live ST vaccine treatment, for both cecal (1.05 and 1.09 log) and liver-heart-spleen (0.32 and 0.06 log) samples, indicating that a second dose is necessary for prolonged protection. Approximate 1 Log cecal and 0.3 Log liver-heart-spleen *Salmonella* count reductions can be obtained by the combined effects of live vaccine and maternal antibody. Lack of adequate protection at 34 d indicates the need for a second vaccination to sustain adequate protection at this age.

Our studies show that combinations of killed vaccine programs in breeders and live vaccines during the early stages of life of the progeny will decrease *Salmonella* prevalence and invasiveness. Choice of autogenous preparations may be more effective if serovars normally encountered by a particular operation are included in the killed vaccines. However, only reductions in *Salmonella* counts are obtained, the bacteria prevailing at lower counts. Although vaccines decrease overall bacterial load, *Salmonella* was still present at considerable numbers highlights the importance of vaccines as complementary tools in controlling *Salmonella* in poultry and not a substitute for effective biosecurity programs.

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