

**Enterobactin-based Immune Interventions against
Gram-negative Bacterial Infections**

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DEDICATION

This dissertation is dedicated to my beloved family and friends who have been unconditionally supporting me along the journey of pursuing my Ph.D. degree.

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ABSTRACT

Enterobactin (Ent)-mediated iron acquisition is critical for many Gram-negative bacteria to survive in the host. Given the bacteriostatic effect of lipocalin-2 resulting from its potent Ent-binding ability, immune interventions directly targeting Ent is a promising antimicrobial strategy against Gram-negative bacterial infections. Moreover, hyperimmune egg yolk antibody is an emerging passive immune agent for the control of bacterial infections. Oral administration of anti-Ent egg yolk antibody may confer passive immune protection against Ent-dependent enteric pathogens. In this dissertation study, multidisciplinary approaches in conjunction with different model systems (*in vitro*, *in vivo*, and *ex vivo*) were used to examine protective efficacies of the Ent-based immune interventions. Anti-Ent antibodies induced by an Ent conjugate vaccine were demonstrated to exert *in vitro* bacteriostatic effect on Ent-dependent Gram-negative bacterial pathogens, including diverse *Escherichia coli* and *Salmonella enterica* strains with different siderophore production profiles as well as *Campylobacter* spp. In the subsequent chicken trial aimed to assess active immune protection of the Ent conjugate vaccine against avian pathogenic *E. coli* (APEC) infection, the vaccine induced high titers of anti-Ent IgY in the serum as well as specific IgY in the intestine. As a result, the vaccination significantly alleviated the organ lesions caused by a highly virulent APEC and affected the chicken intestinal microbiota. Regarding the passive immunization, when a large quantity of anti-Ent hyperimmune egg yolk was orally administered as a feed additive to chickens, it failed to suppress *C. jejuni* colonization in the chicken intestine, consistent with the finding that the egg yolk antibody was not detected in gastrointestinal tracts and sera of the chickens. Further *ex vivo* evaluation of egg yolk antibody stability revealed that the antibody was efficiently degraded when entering chicken gizzard. Lastly, an epidemiological survey of *E. albertii* in broilers revealed chickens as an important reservoir for this emerging zoonotic pathogen. The *in vitro* growth assay also showed significant inhibitory effect of the anti-Ent antibodies on *E. albertii*. Collectively, this dissertation study demonstrated significant potential of the Ent-based immune interventions in controlling different Gram-negative bacterial infections.

Keywords: enterobactin, immune intervention, Gram-negative bacteria, egg yolk antibody

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Introduction

Gram-negative bacterial pathogens, such as *Escherichia coli*, *Salmonella enterica*, and *Campylobacter* spp., cause a wide range of infectious diseases in humans and animals. Infections caused by these microorganisms, such as foodborne illness and sepsis, have posed serious threats to human and animal health worldwide (1). With the increasing emergence of antibiotic resistance and the dearth of new antibiotics in the pharmaceutical pipeline (2), it is imperative to develop alternatives to antibiotics for the control of Gram-negative pathogens. Over the past decades, immense efforts have been devoted to the studies of Gram-negative bacterial pathogenesis and metabolism, which paves the way for developing innovative intervention strategies (3). Of the many strategies, immune interventions have been historically demonstrated as a promising approach for the prevention and control of infectious diseases (4).

Iron is an essential nutrient for nearly all bacterial pathogens to survive and colonize the host. However, iron is primarily bound to hemoglobin, ferritin, and transferrin in vertebrate hosts, rendering the concentration of freely available iron much lower than the requirement for bacterial growth (5). To obtain sufficient iron for survival and multiplication *in vivo*, bacteria have evolved multiple and complex systems for iron uptake. Of these systems, the high-affinity iron acquisition mediated through iron-chelating siderophores is a highly efficient and conserved strategy in Gram-negative bacteria and thus has been a promising target for iron-dependent pathogen control (6). Among different siderophores, enterobactin has the highest affinity for ferric iron (Fe^{3+}) and is an archetype for iron acquisition in Gram-negative bacteria (7). Thus, the enterobactin system has been targeted for developing antimicrobial strategies over the past few decades, such as inhibitors for Ent biosynthesis and vaccines against outer-membrane ferric Ent receptors (8, 9). However, these intervention strategies have only gained limited success primarily due to the complexity of Ent synthesis and redundancy of Ent utilization systems (10).

During bacterial infection, the host secretes an acute-phase protein named lipocalin-2 that can sequester the ferric Ent complex and consequently suppress bacterial growth (11). We were inspired by the unique antibacterial function of lipocalin-2 and proposed to directly target the Ent molecule to develop immune intervention strategies. To this end, we have recently prepared an Ent conjugate vaccine that induced high titers of anti-Ent antibodies in the immunized rabbit

serum. Moreover, the anti-Ent antibodies were demonstrated to have broad binding spectrum towards Ent-associated siderophores and exert *in vitro* bacteriostatic effects on representative *E. coli* and *Campylobacter* strains (10).

In addition, egg yolk antibody-based passive immunization has drawn increasing research interest due to the cost-effective production of large quantities of hyperimmune egg yolk that can provide instant immune protection against pathogens (12, 13). We hypothesized that oral administration of anti-Ent egg yolk antibody would confer passive immune protection against enteric pathogens dependent on Ent. Collectively, to achieve our long-term goal of developing Ent-based active and passive immune interventions against Gram-negative bacterial infections, the following objectives were pursued in this dissertation study:

- 1) Conduct *in vitro* growth assays to examine antimicrobial efficacy of the anti-Ent antibodies against different Gram-negative bacterial pathogens.
- 2) Perform a chicken trial to assess active immune protection of the Ent conjugate vaccine against systemic infection of avian pathogenic *E. coli* (APEC).
- 3) Carry out a chicken trial to evaluate passive immune protection of orally administered anti-Ent hyperimmune egg yolk against intestinal colonization of *C. jejuni*.
- 4) Use an *ex vivo* model to examine stability of the hyperimmune egg yolk antibody in the chicken gastrointestinal tract.
- 5) Isolate and characterize *Escherichia albertii*, an emerging zoonotic pathogen, from broilers, and assess antibacterial potential of the anti-Ent antibodies against this pathogen.

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Chapter I: Review of Literature

1.1. Siderophore-mediated iron acquisition in bacteria

Virtually all bacteria need iron as a cofactor in enzymes to catalyze redox reactions involved in fundamental cellular processes such as respiration, DNA synthesis, and protection from reactive oxygen species (1, 2). Since iron usually exists in its oxidized ferric state (Fe^{3+}) and is largely insoluble at neutral and basic pH levels, the concentration of free ferric iron ranges from 10^{-8} to 10^{-9} M. In vertebrate hosts, the bioavailable ferric iron is further lowered by iron-binding proteins (about 10^{-24} M in serum), much below the requirement (10^{-6} M) for bacterial growth (3, 4). To counteract the iron restriction, bacterial pathogens have evolved various strategies to obtain adequate iron, in which a siderophore-based high-affinity iron acquisition system is the major and most efficient approach (5).

1.1.1. General features of siderophores

Siderophores are of low molecular mass ($<1,000$ Da) and characterized by their high specificity and affinity towards Fe^{3+} . Based on the moieties conferring iron-binding capacity, siderophores are divided into four main types: carboxylate, phenolate, catecholate, and hydroxamate. In addition, a panel of siderophores have different types of moieties within a single molecule, resulting in mixed-type siderophores (6). The biosynthesis of siderophores in bacterial pathogens is induced by intracellular iron deficiency. When inside a host, bacteria secrete the siderophores to capture iron from proteins such as hemoglobin, transferrin, and lactoferrin. Subsequently, iron-loaded siderophores are transported into the bacterial cell, where the iron is released either by hydrolysis of the siderophores or by reduction of Fe^{3+} to Fe^{2+} (7). Many bacteria can utilize multiple siderophores for iron acquisition, including xenosiderophores that are not synthesized by the bacteria but can be internalized via specific receptors into the bacteria to strip iron (8). The importance of siderophores to bacterial virulence has been demonstrated by the decreased fitness of siderophore-defective strains in animal models of infection (9).

Regulation of siderophore biosynthesis and utilization is mediated at the transcriptional level by ferric uptake repressor (Fur) or diphtheria toxin regulator (DtxR) (4). Fur is the global iron regulator in many Gram-negative and low GC-content Gram-positive bacteria (*e.g.*, *Bacillus* spp.), while DtxR plays a comparable role in Gram-positive bacteria with high GC-content (*e.g.*, *Corynebacterium diphtheriae*, *Mycobacteria tuberculosis*, and *Staphylococcus aureus*) (4). As a

transcriptional repressor, Fur-Fe²⁺ homodimer directly binds to the regulatory element of a gene and consequently impedes the binding of RNA polymerase (10). In addition, there is mounting evidence showing that Fur can also indirectly suppress siderophore synthesis (11). For example, Fur represses the highly conserved small non-coding RNA RyhB which activates several genes involved in siderophore biosynthesis (*i.e.*, enterobactin, salmochelins, and aerobactin) in the uropathogenic *Escherichia coli* strain CFT073 or confers increased stability of the polycistronic mRNA involved in vibriobactin siderophore biosynthesis in *Vibrio parahaemolyticus* (12, 13). In addition to the classic gene repression described above, Fur can also act as an activator to regulate complex genes responsible for key metabolic pathways, enzymes associated with defense against ROS, and various virulence factors (10). For instance, Fur induces the expression of HilA in *Salmonella enterica*, which activates the type III secretion system enabling the invasion into host epithelial cells (14). Thus, the regulation of siderophore biosynthesis not only is critical for maintaining iron homeostasis but also plays a multifaceted role in bacterial pathogenicity and stress responses.

1.1.2. Enterobactin-mediated iron acquisition

Enterobactin is a catecholate siderophore that has the highest affinity for Fe³⁺ of all natural siderophore compounds and is an archetype for iron acquisition in Gram-negative bacteria (15). Many species of Enterobacteriaceae, such as *Escherichia* spp., *Salmonella* spp., *Shigella* spp., and *Klebsiella* spp., harbor a 24-kb gene cluster encoding 13 proteins that collaborate to synthesize, transport, and process enterobactin (4). Following synthesis in the cytoplasm, enterobactin is exported through the inner membrane by EntS, a major facilitator superfamily exporter, and through the outer membrane by a TolC-dependent mechanism. Once secreted outside of bacterial cell, enterobactin can successfully compete for Fe³⁺ and the ferric enterobactin (FeEnt) complex is then imported through the outer-membrane enterobactin-specific receptors. Subsequently, the FeEnt complex is escorted through the periplasm by FepB and pumped through the two-protein inner-membrane channel FepDG into the cytoplasm by FepC-catalyzed ATP hydrolysis (16). Lastly, liberation of Fe³⁺ from the FeEnt complex requires enzymatic degradation of its trilactone scaffold by the esterase Fes (17).

The battle for iron between host and bacterial pathogens can be regarded as a scenario of coevolution (4). Enterobactin-mediated iron acquisition is efficiently impaired by the 24-kDa host acute-phase protein lipocalin 2 (also named siderocalin or neutrophil gelatinase-associated lipocalin [NGAL]). Lipocalin 2 has potent binding affinity for the FeEnt complex and thus exerts its antimicrobial effect by sequestering FeEnt from uptake system of bacterial pathogens (18). However, bacteria have evolved to express stealth siderophores that can evade sequestration by lipocalin 2. In many Gram-negative bacterial pathogens, such as *E. coli* and *Salmonella* spp., the *iroA* gene cluster is found to glycosylate Ent into salmochelins, which cannot be captured by lipocalin 2 due to steric hindrance from the glucose groups (19).

1.1.3. Ent-based antimicrobial strategies

Due to its critical role in Gram-negative bacterial pathogenesis, Ent-mediated iron acquisition has been targeted for developing intervention strategies against Gram-negative bacterial infections. EntE, an early-stage biosynthesis enzyme for enterobactin, has been targeted for generating competitive inhibitors. These chemical compounds showed potent inhibitory activity against EntE and were promising candidates for the development of novel antimicrobial agents (20, 21). Using a “Trojan horse” strategy, Zheng *et al.* harnessed Ent as a scaffold to conjugate antibiotics, which could facilitate delivery of the antimicrobial cargo across the outer membrane permeability barrier of Gram-negative pathogens utilizing Ent for iron acquisition (22, 23). Under iron-limited condition, the Ent-modified antibiotics exerted enhanced antibacterial activity against pathogenic *E. coli* compared to their parent β -lactams (23). In addition, FepA is an iron-regulated outer-membrane receptor in *E. coli* and other Enterobacteriaceae species that specifically recognizes and transports FeEnt into the periplasm (4). Lin *et al.* developed polyclonal and monoclonal antibodies directed against FepA, both of which significantly inhibited the growth of *E. coli* and *K. pneumoniae* under iron-restricted condition (24, 25).

Recently, an immunization strategy directly targeting Ent, inspired by the bacteriostatic action of lipocalin 2, was developed to sequester Ent from bacterial uptake. Sassone-Corsi *et al.* chemically synthesized a conjugate of Ent with an immunogenic carrier. The Ent conjugate vaccine elicited anti-Ent antibodies in immunized mice and protected them from *Salmonella* infection (26). In another independent study, Wang *et al.* utilized a more simplified and efficient

conjugation method to generate another Ent conjugate vaccine, which induced high titers of anti-Ent antibodies in rabbits (27). The anti-Ent antibodies had a broad binding spectrum towards not only Ent but also Ent-derived siderophores including salmochelins. This suggested the superiority of anti-Ent antibodies to lipocalin 2 in inhibiting a variety of Gram-negative pathogens producing additional salmochelins. As expected, the anti-Ent antibodies significantly suppressed Ent-dependent *in vitro* growth of *E. coli* and *Campylobacter* spp. (27).

1.2. Vaccination strategies against avian pathogenic *E. coli*

Avian pathogenic *E. coli* (APEC) is the etiological agent of colibacillosis, one of the most commonly occurring and economically devastating infectious diseases in poultry worldwide. The economic losses caused by colibacillosis arise from increased mortality and carcass condemnation at processing, and decreased growth rate and productivity (28). After entry through oral, nasal, or cloacal routes, APEC colonizes the mucosal sites of gastrointestinal (GI), respiratory, and reproductive tracts without causing diseases in poultry. In the presence of concurrent viral or mycoplasma infection, or under environmental stresses, APEC invades the mucosal layers and reaches extraintestinal organs (*e.g.*, heart, liver, lung, spleen, kidney, and reproductive organs), resulting in multi-systemic infections (28). APEC can affect all types of poultry in all types of production systems, whose infection forms include septicemia, airsacculitis, omphalitis/yolk sac infection, peritonitis, and cellulitis, with the systemic infection occurring most often (28). APEC utilizes an arsenal of virulence factors to cause diseases in poultry, primarily adhesins, invasins, protectins, iron acquisition systems, and toxins. Intriguingly, these factors do not occur universally among APEC, suggesting multiple alternative mechanisms involved in the pathogenicity (29, 30). Multiple APEC serotypes have been associated with colibacillosis causes in the field outbreaks, but three major serotypes (*i.e.*, O1, O2, and O78) account for more than 80% of the cases. (28).

Besides the negative impact on poultry health, APEC is also suggested to cause human diseases, such as urinary tract infection, neonatal meningitis, and sepsis, and considered as a major source for spreading antimicrobial resistance to other bacterial pathogens (31, 32). Therefore, control of APEC is of great significance not only for poultry industry but also for public health. Antibiotics such as tetracyclines, sulfonamides, and aminoglycosides are frequently used to control

colibacillosis in chickens (33). However, resistance to multiple antibiotics, especially those of medical importance, has been reported in APEC; antibiotic usage is also undergoing unprecedented scrutiny in poultry production (28). Thus, alternatives to antibiotics against APEC are in urgent need in poultry industry. Vaccines represent an effective and evolving way to curb infectious diseases and is a promising approach to combat APEC (31). Generally, developing an ideal APEC vaccine needs to address several key challenges. First, the vaccine has to be able to induce cross protection against various APEC serogroups. Second, the vaccine should be deliverable *via* a mass immunization method, such as in drinking water or feed, *in ovo*, and spray, to immunize a large number of poultry on a farm. Third, the vaccine has to induce protective immunity at a young age when the birds are most vulnerable to APEC infection (31). To date, various vaccine candidates have been investigated for the protective efficacy against APEC infection and a few vaccines are commercially available.

Early attempts for developing APEC vaccine started with inactivated APEC strains several decades ago (31). Different serotypes of APEC strains were killed by formalin and formulated into injectable vaccines. The bacterins provided different extents of immune protection against homologous challenge of APEC, but they had limited effectiveness against heterologous APEC challenges (34-36). To overcome the lack of cross-protection, a subsequent study combined multiple prevalent serotypes (O1, O2, and O78) of APEC in a single vaccine formula. This bacterin induced significant protection against the challenge with all three serogroups of APEC (31). Recently, new forms of inactivated vaccine are emerging for APEC control, such as outer-membrane vesicle and bacterial ghost vaccines. Outer-membrane vesicle is released from the outer-membrane of Gram-negative bacteria and enriched with many bioactive molecules of the parental bacteria, while bacterial ghost is an empty and intact cell wall of Gram-negative bacteria discharged of all the cytoplasmic contents (37, 38). Both types of vaccine can resemble the properties of live bacteria, such as pathogen-associated molecular patterns, and thus are considered as promising vaccine candidates for APEC control. In several independent studies, outer-membrane vesicles and bacterial ghost vaccines were prepared from different serotypes of APEC and used for immunization of chickens. The vaccinations exerted immune protection against homologous APEC infections (37-40).

With better understanding of APEC pathogenesis, live attenuated and subunit vaccines have been increasingly explored for APEC control in recent years. Many conserved APEC components, such as outer-membrane proteins and fimbria that are essential for APEC pathogenicity, have been targeted for developing subunit vaccines. For example, Van Goor *et al.* generated a recombinant subunit vaccine by combining four outer-membrane proteins involved in APEC adhesion and serum resistance. This vaccine elicited strong immune responses and conferred protection against different serotypes of APEC (41). As for live attenuated vaccines, they are feasible for mass immunization *via* spray or oral route, making them more attractive to the broiler industry than other vaccines requiring individual administration. For instance, La Ragione *et al.* generated an avirulent mutant (Δ aroA) from a virulent APEC O78 field strain. Immunization of young chicks with this live attenuated vaccine *via* spray and drinking water provided immune protection against homologous and heterologous APEC infections (42). Additionally, live attenuated bacteria can also serve as a vehicle for the delivery of specific antigens, which can maximize the invasion into lymphoid tissue and the induction of humoral and cellular immunities. For example, Han *et al.* constructed a recombinant attenuated *Salmonella* vaccine expressing lipopolysaccharide O-antigens of APEC O1 and O2 strains. The vaccine induced specific serum IgG and mucosal IgA and promoted opsonization, consequently protecting chickens from APEC O1 and O2 infections (43).

1.3. Immune interventions against *Campylobacter* in poultry

Campylobacter is a zoonotic pathogen and a leading bacterial cause of gastroenteritis worldwide. Although usually causing self-limited diarrhea in humans, *Campylobacter* may induce severe or systemic infections in immunocompromised, young, or elderly patients. *Campylobacter* is widely distributed in food animals and is transmitted to humans primarily through the foodborne route (44). While highly prevalent and commensal in poultry's intestine, *Campylobacter* of poultry origin is responsible for vast majority of campylobacteriosis in humans (45). Thus, control of *Campylobacter* in the poultry reservoir will reduce the risk of human exposure to this pathogen and have a significant impact on food safety and public health. It has been well established that infection of *C. jejuni* in chicken can induce protective immunity against reinfection of *Campylobacter*; the wide presence of maternal anti-*Campylobacter* antibodies in young chickens is partly responsible for the absence of *Campylobacter* in the intestine during the

first two weeks of life (46). These findings demonstrate the protective nature of anti-*Campylobacter* antibodies and support the feasibility of developing immune interventions to control *Campylobacter* infection in poultry.

In general, two immune intervention approaches have been pursued to reduce *Campylobacter* burden in poultry: active vaccination and passive immunization. Regarding the active vaccination, three types of vaccines have been extensively investigated for *Campylobacter* control in poultry, *i.e.*, subunit, inactivated, and live attenuated vaccines. For the subunit vaccine, identification of immunogenic and protective antigens in *Campylobacter* is critical for the development of effective vaccines. Various candidates have been identified and investigated in previous studies, most of which are outer-membrane proteins required for *Campylobacter* pathobiology (47). For example, intramuscular injections of chickens with each of the recombinant *C. jejuni* adhesins CadF and FlaA, and flagellar protein FlpA reduced the cecal colonization of *C. jejuni*, with the largest reduction from the FlpA vaccine (48). In addition, oral or intranasal immunization of the subunit vaccines may better induce mucosal immunity against *Campylobacter* colonization, which can be achieved *via* different delivery systems, such as avirulent *Salmonella*, *Lactobacillus*, and *Eimeria* vectors, and nanoparticle encapsulation (49). For instance, when chickens were orally administered with transgenic *Eimeria* or *Salmonella* constitutively expressing *C. jejuni* antigen A (CjaA), the vaccination significantly reduced cecal colonization of *C. jejuni* (50, 51). Inactivated vaccine can induce high protective immunity without the concern of potential pathogenicity to the host and has been explored for *Campylobacter* control (49). For example, Taha-Abdelaziz *et al.* orally administered chickens with *C. jejuni* lysate, which elicited specific immune responses and reduced intestinal colonization of *C. jejuni* by over 100 fold (52). Similarly, live-attenuated vaccine can also be effective in reducing *Campylobacter* colonization in poultry. Jeon *et al.* prepared an live-attenuated *C. jejuni* vaccine by mutating the *aphC* gene that is responsible for oxidative stress defense (53). Oral inoculation of this vaccine reduced *C. jejuni* shedding in feces by over 1,000 fold. Notably, this vaccine strain was removed from the chicken intestine within 42 days and could not survive after shed in aerobic environments due to the defect in colonization and lack of aerotolerance (53).

The short life span of broiler chickens (about 6 weeks) and the need to induce a protective immunity in the intestine have posed a significant challenge for development of immune interventions against *Campylobacter* in chickens. This challenge may be tackled by passive immunization. Oral administration of hyperimmune egg yolk antibodies may lead to instant passive immunity in the intestine against *Campylobacter* colonization (44). Detailed information about the egg yolk antibody-based passive immunization is reviewed in the last section of this chapter. Here we briefly summarize several studies exploring the feasibility of passive immunization against *Campylobacter* in chickens. In these studies, hyperimmune egg yolk were produced *via* vaccination of laying hens with *Campylobacter* whole-cell lysate or subunits. Then the hyperimmune egg yolk was supplemented in chicken feed and exerted prophylactic or therapeutic effect against intestinal colonization of *Campylobacter* in chickens (54, 55). Meanwhile, in a study by Paul *et al.*, when the hyperimmune egg yolk against colonization-associated subunits of *Campylobacter* were supplemented in chicken feed, it failed to confer passive immune protection against *Campylobacter* colonization (56). The discrepancies in passive immune protection may result from different antigens used in these studies that generated varied levels of protective antibodies. Overall, these studies demonstrate the potential of passive immunization against *Campylobacter* colonization in chickens and suggest that antigen selection is especially important considering *Campylobacter* strains are antigenically diverse (44).

1.4. *Escherichia albertii*, the emerging zoonotic pathogen

E. albertii is an emerging foodborne enteropathogen under Enterobacteriaceae and can cause characteristic attaching and effacing (A/E) lesions, like enteropathogenic *E. coli* (EPEC) or enterohemorrhagic *E. coli* (EHEC) (57). Due to the close genetic relationship and shared traits of *E. albertii* with EPEC and EHEC, multiple reports have demonstrated that many provisional EPEC and EHEC isolates incriminated in disease outbreaks were actually *E. albertii* (57). Although the clinical importance of *E. albertii* has been increasingly recognized worldwide, the epidemiology, transmissibility, prevalence, and incidence of this pathogen remain unexplored currently (58, 59). In addition to humans, *E. albertii* has been reported to infect wild and domestic birds in different countries (60). In particular, the isolation of *E. albertii* in poultry flocks indicates that chicken represents an important reservoir for *E. albertii* (61). Besides

chicken, *E. albertii* has also been isolated from other meat products, including duck, mutton, and pork (62). Interestingly, a recent study shows that raccoon might be another reservoirs for *E. albertii* at least in Japan (63).

Humans infected with *E. albertii* typically exhibit symptoms associated with gastroenteritis (64). Moreover, *E. albertii* has also been reported to cause bacteremia in a patient (65). *E. albertii* possesses an array of candidate virulence factors, some of which are shared with other pathovars of *E. coli* (57). Type III secretion system (T3SS) is expressed in many *E. albertii* strains that can form A/E lesions both *in vivo* and *in vitro* (66). The repertoire of effectors translocated by the T3SS of *E. albertii* remains uncharacterized except for Tir that is essential for the invasion of epithelial cells and the formation of A/E lesions (57, 66). In addition, a subset of *E. albertii* strains express Shiga toxin (*i.e.*, Stx2a and Stx2f), suggesting its potential to cause severe diseases such as hemorrhagic colitis and hemolytic uremic syndrome in humans (63). Last but not least, cytolethal distending toxin (CDT), a genotoxin resulting in irreversible cell cycle arrest and apoptosis, is synthesized by several GI bacteria including *E. albertii* (57). A recent study suggested that the presence of *cdtB-II* subtype is a more reliable biomarker for *E. albertii* instead of *E. coli* (67).

Discrimination and isolation of *E. albertii* is challenging. To date, different protocols have been developed to discriminate and isolate *E. albertii*, but no commercial test is available yet to differentiate *E. albertii* from other Enterobacteriaceae species (68). Most *E. albertii* isolation protocols comprise an enrichment step using various broths, such as buffered peptone water, *E. coli* broth (ECB), antibiotics-supplemented ECB, tryptic soy broth (TSB), antibiotics-supplemented TSB, etc., at various temperatures ranging from 20 to 44.5 °C (68). To obtain a single isolate of *E. albertii*, the culture needs to be plated on selective agar plates after enrichment. Recently, deoxycholate hydrogen sulfide lactose (DHL) and MacConkey agars supplemented with 1% xylose, 1% rhamnose, and/or 1% melibiose have been developed for differential isolation of *E. albertii* (69, 70). Most *E. albertii* isolates described to date cannot ferment the abovementioned sugars and thus grow as uncolored colonies, which can be isolated from colored *E. coli* colonies (70). Nevertheless, a small portion of *E. albertii* strains have been reported to be able to ferment xylose, rhamnose, and melibiose (68). Thus, current selective agar

plates still need to be optimized to improve the isolation capacity. Polymerase chain reaction (PCR) is frequently used for detection and identification of *E. albertii*. Currently, the DNA-binding transcriptional activator of the cysteine biosynthesis gene (*EAKF1_ch4033*) and the *cdtB* gene seem to be the most highly specific PCR targets for identification of *E. albertii* (71, 72). Muchaamba *et al.* used the two distinct pairs of primers to perform an *in-silico* PCR against 319 publicly available *E. albertii* whole genome sequences and found that both PCR assays had over 96% of accuracy (68). 16S rRNA gene, though commonly used for identification of bacterial species, cannot effectively distinguish between *E. coli*, *E. fergusonii*, *E. albertii*, and *Shigella* spp. (68). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) is an accurate, cost-effective, and efficient method for identification and typing of microorganisms. Recently, Hatanaka *et al.* developed an MALDI-TOF-MS-based highly specific *E. albertii* identification protocol and mass spectra library that can be used to accurately distinguish *E. albertii* from *E. coli* (73). Although, MALDI-TOF-MS is a promising tool for differentiating *E. albertii* from other Enterobacteriaceae species, the application is hindered by the high initial investment and is not feasible for most laboratories. Moreover, *E. albertii*-specific database libraries need to be created locally to effectively distinguish this emerging pathogen (68).

1.5. Hyperimmune egg yolk antibody for passive immunization

The chicken has three classes of immunoglobulins (IgY, IgA, and IgM), of which IgY is considered as functionally equivalent to mammalian IgG and is predominant in both serum and egg yolk (74). IgY has several unique characteristics when compared to IgG (75). For example, IgY has four constant regions on the heavy chain while IgG has three, resulting in the larger overall molecular mass of IgY (76). In addition, IgY is less flexible than IgG in structure due to the absence of a hinge region and the presence of glycine and proline residues on IgY heavy chain (77). During egg formation, IgY in maternal serum is selectively transferred to the yolk, consequently conferring passive immunity for embryos and hatchlings (78). Each egg yolk contains a large quantity of IgY (50–100 mg) with a high proportion of antigen-specific antibodies (up to 10%) (79, 80). Therefore, immunization of laying hens with a particular antigen can yield high-level hyperimmune IgY in the egg yolk within a short time (76).

Passive immunization refers to the process of providing host specific antibodies for prevention and/or therapeutic treatment of microbial infections. The protection conferred by passive immunization is rapid but short-term in nature (81). Therefore, continuous administration of antibodies is usually needed and large quantities of specific antibodies are required for passive immunization (78). Clearly, the easy and efficient production of hyperimmune egg yolk IgY makes egg yolk-based passive immune intervention strategies promising in both humans and animals. It has been estimated that one hen can produce more than 20 g of egg yolk IgY per year, which is equivalent to the production of serum IgG from over four rabbits in the same period (82). In addition, the well-established system in industry for hen immunization and egg collection/processing further makes mass production of egg yolk IgY feasible and cost-effective (78).

Therefore, development of hyperimmune egg yolk IgY for passive immunization has drawn considerable research interest in the past three decades. Numerous studies have been conducted worldwide to develop hyperimmune egg yolk IgY to combat various microbial infections (76). The egg yolk IgY was administered to humans or animals *via* different routes, depending on the infection sites of targeted pathogens, with varied extents of success (76, 83). Despite the potential of using egg yolk IgY as an antimicrobial agent, the significant challenges of this technology have been rarely recognized and discussed in previous research and review articles. Therefore, in addition to briefly summarizing the status of egg yolk IgY-based passive immunization approaches, we comprehensively reviewed several significant challenges associated with this technology and discussed potential solutions to address these issues.

1.5.1. Current status of egg yolk IgY as a passive immune agent

Infectious diseases in humans. Numerous studies have been conducted to develop hyperimmune egg yolk IgY against diverse human pathogens (84). However, most *in vivo* efficacy studies are still limited to animal model systems; only a few clinical trials have been reported and no product has been approved for application in human medicine to date (85). Various important viral pathogens, such as SARS-CoV-2 (86-88), influenza virus (89-92), mosquito-borne dengue and Zika viruses (93, 94), Ebola virus (95), and hantavirus (96), have been targeted for developing prophylactic/therapeutic egg yolk IgY. Taking influenza virus as an example, due to

high frequency of antigenic drift in the hemagglutinin, influenza vaccine antigens should be updated regularly and annual vaccination is highly recommended. However, yearly influenza vaccination not only is inconvenient but also may lead to diminished B cell responses, lower postvaccination antibody titers, and reduced antibody affinity maturation (97). To overcome these drawbacks, inclusion of influenza virus-specific egg yolk IgY in intranasal spray has been developed and proved to be effective in mouse models (89-92). Hyperimmune egg yolk IgY also has been developed to control bacterial and fungal infections in humans, such as those caused by *Pseudomonas aeruginosa* (98, 99), *Helicobacter pylori* (100-102), *Clostridium difficile* (103-106), and *Candida albicans* (107, 108). The egg yolk antibodies specific to *H. pylori* and *C. albicans* have been evaluated in humans with promising protective outcomes (102, 108).

Emerging coronaviruses. Two highly pathogenic coronaviruses, the severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), emerged and caused fatal respiratory illness in humans in 2002 and 2012, respectively. Recently, a novel and highly transmissible coronavirus, designated as SARS-CoV-2, has led to an extraordinary health emergency globally (109). Development of antiviral drugs and vaccines is highly warranted, but these approaches are inherently lengthy and costly (110, 111). Although the vaccines against SARS-CoV-2 have been developed, produced, and deployed at an unprecedented speed, there are still some major limitations and hurdles for the vaccine strategy against SARS-CoV-2 (112). For instance, the vaccines may not provide effective protection for immunodeficient people (113). Ramping up vaccine manufacturing is limited by a global shortage of essential materials, which especially delays the vaccine availability to developing countries (84). Vaccine hesitancy is prevalent worldwide due to various concerns or misinformation (112). Considering the high infectivity and morbidity of the emerging coronaviruses, inclusion of coronavirus-specific egg yolk IgY in a nasal spray to neutralize the virus upon the entry into human body is likely a feasible and effective approach to control the transmission and severity of the disease (84).

Development of the egg yolk IgY directed against the emerging coronaviruses has been reported in several studies (84). Yu *et al.* developed anti-SARS-CoV egg yolk IgY by immunizing hens with the inactivated virus. The extracted IgY effectively neutralized SARS-CoV in VERO cells

and showed excellent thermal and acidic stability in lyophilized form (114). Similarly, Abbas *et al.* generated egg yolk IgY directed against spike protein of MERS-CoV. The purified IgY neutralized MERS-CoV *in vitro* and mitigated the virus-induced lung inflammation in a mouse model (115). Since the outbreak of SARS-CoV-2 in 2020, specific egg yolk antibodies have been developed targeting the spike protein, nucleocapsid protein, or the inactivated virus (86, 87, 116-118). Notably, Shen *et al.* showed the anti-SARS-CoV-2 IgY, through nasal drip or oral spray, was able to persist in the upper airway of mice for several hours (87), further supporting the feasibility of using hyperimmune egg yolk IgY to prevent coronavirus infections.

The egg yolk IgY technology has several advantages concerning the aforementioned drawbacks of SARS-CoV-2 vaccines. First, active vaccination requires an adequate immune system to induce immune protection, while the action of egg yolk IgY for passive immunization is almost independent of host immune system (83). As a result, the IgY may provide protection to the widest range of population, especially immunocompromised patients, the elderly, and young children. Second, manufacturing of vaccines is costly and the capacity is limited by complex and high-standard production lines (119, 120). The frequent emergence of SARS-CoV-2 variants may intensify the necessity of additional booster vaccinations (121). In contrast, production of egg yolk IgY is simple and cost-effective, particularly in lower-income areas. The turnover time of generating the hyperimmune IgY against new SARS-CoV-2 variants is also potentially short. Finally, egg yolk IgY may have fewer adverse events due to the lack of interaction with mammalian complement system (84). This safety feature may increase public acceptance of the effective egg yolk IgY for coronavirus control, particularly for those who have concerns on the safety of injectable vaccines.

Infectious diseases in livestock. Hyperimmune egg yolk IgY has been widely developed to control different bacterial, viral, and parasitic infections in food animals (76). Currently, several egg yolk IgY products have been made available on market for disease control in livestock (78). In livestock, egg yolk IgY is primarily administered into host *via* oral route and recognized as a promising non-antibiotic feed additive against various enteric pathogens, such as *E. coli* (122-124), *S. enterica* (125, 126), *Campylobacter* spp. (54, 55), rotavirus (127-129), *Cryptosporidium parvum* (130, 131), and *Eimeria* spp. (132, 133). Nevertheless, all but one (125) of these studies

did not specifically examine the stability of orally administered egg yolk IgY in GI tract, a critical issue for assessing the efficacy against enteric pathogens. In addition to oral supplementation, egg yolk IgY also has been subjected to purification and the purified IgY was injected into animal hosts *via* different routes (*e.g.*, intramuscular, subcutaneous, and intraperitoneal) for the control of systemic infections, such as those caused by avian pathogenic *E. coli* (134), infectious bursal disease virus (135, 136), and *Trypanosoma evansi* (137). The hyperimmune egg yolk IgY has been shown to confer livestock passive immune protection against various microbial infections by suppressing the pathogen colonization, alleviating the morbidity or mortality, or improving growth performance of the animals. The different extents of protection on farm animals by egg yolk IgY have been comprehensively summarized in the previous review articles (78, 83, 138).

1.5.2. Challenges of IgY-based passive immunization

Despite promising features of egg yolk IgY as a passive immune agent and the long-term effort on developing IgY-based passive immunization, to date, there is still no product approved for treating infectious diseases in humans and only limited egg yolk IgY products are commercially available for disease control in livestock. Several challenging issues of the IgY-based passive immune intervention, which were barely addressed in previous publications, have impeded development of consistent and effective egg yolk IgY products used in humans and animals. The major challenges associated with IgY technology are briefly reviewed and discussed below.

Stability. Egg yolk IgY could encounter various stress factors during processing (*e.g.*, purification and spray drying) and passive immunization that may potentially affect IgY stability and functionality (84). Major factors of concern are high temperature, extreme pH, and proteolytic enzymes given the protein nature of IgY. In particular, upon oral administration, IgY confronts different proteases in GI tract, including gastric enzyme pepsin and those in the intestine (*e.g.*, trypsin, chymotrypsin, and elastase) (139). It has been well recognized that IgY is quite stable over a wide pH range (3.5-11) and under high temperature (up to 70°C) (76, 140). Regarding protease-mediated degradation, this significant issue was barely addressed in previous *in vivo* studies when assessing the antimicrobial efficacy of egg yolk IgY. In fact, an early *in vitro* study has shown that neutralizing activity of specific IgY was totally lost in a pepsin

solution but largely retained in a trypsin solution, suggesting significant degradation of egg yolk IgY in stomach (141). Therefore, to develop a successful and sustainable IgY-based passive immunization approach to control enteric pathogens, the *in vivo* stability and bioavailability of egg yolk IgY in the GI tract is a critical issue that needs to be addressed.

IgY purification. Passive immunization of host with IgY *via* parenteral routes (*e.g.*, intraperitoneal, intramuscular, and subcutaneous routes) requires high purity of IgY extracted from egg yolk (84). The protein A or protein G affinity chromatography is widely and successfully used for efficient purification of mammalian IgG; however, such chromatography approach does not work for IgY purification due to lack of affinity of the Fc region of IgY to protein A or protein G (76). Some novel IgY ligands have been discovered and reported to be effective for efficient purification of IgY (142-144). For example, Dong *et al.* screened a synthetic ligand that had a high IgY-binding capacity (74.8 mg IgY per mL gel) and achieved an excellent purity and a high recovery rate of egg yolk IgY (143). This synthetic ligand was acclaimed to be easily regenerated for large-scale purification of egg yolk IgY (143). However, to date, all the identified ligands (142-144) are still not commercially available. An alternative approach is to immobilize specific target antigen to a solid phase for affinity purification of the corresponding IgY from egg yolk. However, application of this approach has been impeded by several technical challenges, such as low stability and binding capacity of immobilized antigens (145, 146). Therefore, currently, differential precipitation is still the most commonly used approach to extract IgY from egg yolk for passive immunization of host with IgY *via* parenteral routes, which usually involves removal of lipids and lipoproteins followed by precipitation of proteins including IgY (78, 147). The IgY extract resulting from differential precipitation still contains other miscellaneous proteins with a purity ranging from 87% to 94% (147). Therefore, a decent amount of non-IgY proteins in crude IgY extract may elicit undesired immune responses in a passively immunized host, consequently increasing background noise for data interpretation and efficacy evaluation, and even causing safety issues.

Heterologous immunogenicity. IgY is a heterogenous protein to non-avian species and thus is likely to induce undesired anti-IgY immune responses in the host when administered parenterally. It was not surprising that typical anti-IgY IgM and IgG responses were observed in

the mice that were intravenously injected with the purified egg yolk IgY for passive immunization (148). Moreover, Aranda-Urbe *et al.* reported that subcutaneous injection of mice with the purified pigeon egg yolk IgY not only triggered production of anti-IgY IgG but also led to induction of anti-Fc γ IgG autoantibodies as well as the reduced proportion of regulatory T cells (149). Therefore, the heterologous immunogenicity issue related to parenteral administration of IgY may erode the efficacy of IgY-based passive immunization and raise concerns on the safety of this approach. To date, the heterologous immunogenicity of IgY is still a significantly understudied area. Thus, to ensure a safe and sustainable passive immune efficacy conferred by the parenterally administered egg yolk IgY, the heterologous immunogenicity issue needs to be considered and examined in future studies.

Repertoire diversity. Antibody repertoire refers to the entire set of unique antibodies produced by individual animal hosts, which is primarily determined by variable fragment sequences (150). Human IgG and chicken IgY undergo distinct genetic mechanisms to form their extremely diverse antibody repertoires (151). Therefore, there is a pronounced difference in the size of antibody repertoire between human IgG and chicken IgY (152, 153). It was estimated that the size of the IgG repertoire in humans is approximately 10^{15} (152). In contrast, chicken IgY displays lower diversity and the estimated repertoire size is only about 10^{11} (153). This estimate is consistent with the sizes of phage display antibody libraries generated from the human- and chicken-derived B cells, respectively. Specifically, the library sizes derived from human B cells are typically 10^{10} to 10^{11} (154-156), whereas the library sizes from chicken B cells are mostly 10^7 to 10^8 (77). Therefore, chicken IgY is likely to display limited binding spectrum toward various antigens compared to human IgG. This may become a significant pitfall when aiming to develop high-affinity egg yolk IgY with specific paratopes, the antigen-binding sites, that do not exist in the chicken IgY repertoire.

1.5.3. Potential solutions

To develop effective and sustainable IgY-based immune interventions, we should fully recognize the challenging issues discussed above. Due to low repertoire diversity of egg yolk IgY, it is not surprising that high-affinity egg yolk IgY directed against specific epitopes of certain antigen may not be generated. This intrinsic issue may be addressed with the advancement of genome-

editing technologies. Recently, Wang *et al.* developed a multiplexed CRISPR-Cas9 method to insert genes for variable regions of heavy and light chains from human germline immunoglobulin into their endogenous loci in mice (157). The B cells from the transgenic mice were fully functional as they could be primed by specific immunogen, secrete desired antibodies, and differentiate into memory B cells (157). This genome-editing approach may be applied to chickens to expand the repertoire diversity of egg yolk IgY, enabling production of highly desired IgY that is beyond the traditional repertoire of the chicken.

Regarding heterologous immunogenicity of IgY, we must take this issue into consideration when assessing the safety and efficacy of parenterally administered egg yolk IgY. Development of recombinant IgY equivalents, such as single-chain variable fragment (scFv), antigen-binding fragment (Fab), and chimeric antibody, using antibody engineering approach may help to minimize the heterologous immunogenicity (158). However, the recombinant IgY technology is still technically complicated and expensive, rendering the application in humans only and not practical for large-scale livestock production. With respect to the purity issue, a standard affinity chromatography system needs to be established for generation of high-purity egg yolk IgY for passive immunization *via* parenteral routes. To achieve this goal, more research should be placed on the discovery of innovative IgY ligands for developing a simple, efficient, and cost-effective affinity chromatography purification technology.

To prevent orally administered egg yolk IgY from gastric degradation, encapsulation of IgY with different protective materials has been investigated and demonstrated as a promising strategy. In the past years, various coating materials and approaches have been explored for microencapsulation of egg yolk IgY, such as chitosan-coated alginate beads (159), co-precipitated IgY-CaCO₃ solid particles coated with multilayers of alternating cationic poly(L-lysine) and anionic poly(L-glutamic acid) (160), granules with 1 part of IgY and 4 parts of starch coated with polyethylene glycol 6000 and methacrylic acid copolymer (161), or alginate/carrageenan composite beads (162). These approaches, to different extents, prevented IgY from activity loss and achieved controlled release of IgY in the simulated GI fluid matrix (159-162). For example, methacrylic acid copolymer is commonly used to coat capsules and tablets for enteric delivery of drugs because it is insoluble in acidic medium but begins to

dissolve when the pH is above 5.5 (161). Conceivably, egg yolk IgY encapsulated with this polymer would gain resistance to gastric conditions and be released in the nearly neutral small intestine. Kovacs-Nolan *et al.* showed that encapsulation of egg yolk with this polymer led to controlled release of IgY in simulated intestinal fluid and significantly protected IgY from activity loss in the pig's stomach when compared to non-encapsulated IgY (161). However, the practicality of these encapsulation systems is questionable due to the cost of highly purified IgY, the low loading capacity of IgY, scalability and cost of encapsulation processes (*e.g.*, extrusion to prepare alginate beads), cost and regulatory status of encapsulating materials, and the uncertainty of capsules surviving thermal conditions in food/feed manufacturing.

Conceptually, partially purified egg yolk powder can be used in encapsulation by adopting different strategies. Emulsification is commonly used in the food industry to produce micrometer-sized oil droplets. To produce micrometer-sized capsules, the egg yolk powder can be dispersed in lipids that are then emulsified a protein emulsifier and subsequently coated with an anionic polysaccharide at acidic conditions to resist gastric digestion, resulting in solid-in-oil-in-water (S/O/W) emulsions. This concept was demonstrated for the powder of an acid-instable and heat-labile probiotic bacterium *Lactobacillus salivarius* NRRL B-30514 (163, 164) and enzyme β -glucosidase (165, 166). Encapsulation of the powder in S/O/W emulsions reduced the deactivation by thermal treatments and gastric digestion, and the gradual digestion of the protein emulsifier in the intestinal conditions resulted in the gradual release of the encapsulated bacterium and enzyme. To produce millimeter-sized capsules, the egg yolk powder can be granularized before coating with a layer of food grade molecules with features for enteric delivery, which can be done in commercial granulation and spray coating unit operations. In the lab scale, this was demonstrated recently for *L. salivarius* NRRL B-30514 powder pellets after dip-coating with a composite shellac-rice protein layer that is insoluble at gastric pH but soluble at neutral pH (167).

In addition, in most of the studies, only simulated GI fluid, *i.e.*, buffers supplemented with digestive enzymes, was used to evaluate the efficacy of encapsulation in IgY protection and release (159, 161, 162). This *in vitro* system cannot fully reflect the complexity of *in vivo* GI conditions. Kovacs-Nolan *et al.* showed that the activity of encapsulated IgY was largely

retained for hours in the simulated *in vitro* GI fluid but was substantially undermined in pig's GI tract despite significant protection conferred by encapsulation (161). Thus, even if some previous studies (122, 123, 125) showed the encapsulated egg yolk IgY had enhanced antimicrobial effects on enteric pathogens in animal models when compared to non-encapsulated IgY, to assess protective efficacy of IgY encapsulation, it is imperative to specifically examine *in vivo* integrity and activity of encapsulated IgY in GI tracts.

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Chapter II: Enterobactin-specific Antibodies Inhibit *in vitro* Growth of Different Gram-negative Bacterial Pathogens

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Abstract

Enterobactin (Ent)-mediated high affinity iron acquisition is critically important for Gram-negative bacterial pathogens to survive and infect the host. Recently, we reported an efficient method to prepare novel Ent conjugate vaccines for inducing high level of Ent-specific antibodies, which displayed similar bacteriostatic feature as lipocalins, the host innate immune effectors with potent Ent-binding ability. The Ent-specific antibodies also showed a significant advantage over lipocalins by cross-reacting to various Ent derivatives including salmochelins, the glycosylated Ent that can help enteric pathogens evade the siderophore sequestration by host lipocalins. To demonstrate significant potential of the Ent conjugate vaccine for broader applications to prevent and control various Gram-negative infections in human and animal, in this study, we examined inhibitory effect of Ent-specific antibodies on the *in vitro* growth of three significant Gram-negative pathogens: *Escherichia coli* (n=27), *Salmonella enterica* (n=8), and *Campylobacter* spp. (n=6). The tested strains were diverse with respect to hosts, geographical origins, serotypes, infection sites, and siderophore productions. The Ent-specific antibodies significantly suppressed the growth of each tested strain under iron-restricted conditions. For example, the Ent-specific antibodies consistently exerted 2-5 log₁₀ units of growth reduction on most tested avian pathogenic *E. coli* (9 of 10 strains) isolated in five countries. Despite various dynamic growth responses observed, notably, the Ent-specific antibodies displayed significantly higher magnitude of growth reduction than lipocalin-2 (up to 5 log₁₀ units of difference) on the majority of tested *E. coli* and *S. enterica*, which is likely due to sequestration of other siderophores (*e.g.*, salmochelins) by the Ent-specific antibodies. Production of a variety of major siderophores by the tested *E. coli* and *S. enterica* strains was examined and confirmed by ultra-high performance liquid chromatography-high resolution mass spectrometry analysis. Collectively, this study provides critical and compelling *in vitro* evidence supporting the feasibility of Ent-based immune interventions against several Gram-negative pathogens.

Keywords: Gram-negative bacteria, conjugate vaccine, enterobactin, lipocalin, salmochelins

2.1. Introduction

The emergence of antibiotic resistance has outpaced the discovery of new antibiotics, posing a great challenge to global health (1). Therefore, development of effective alternative approaches to conventional antibiotics is highly warranted for controlling bacterial infections in humans and animals (2). Recently, many strategies have been explored, which include but are not limited to vaccination, microbiota modulation, enhancement of host innate resistance, phytochemicals, bacteriophage, and innovative enzymes (3-5). Among these strategies, vaccination has been historically demonstrated as a promising approach for the prevention and control of infectious diseases. However, effective vaccines against many pathogens, particularly Gram-negative bacterial pathogens, are still not available. This is primarily due to lack of in-depth understanding of complex host-microbe interactions and the challenges to trigger desired protective immunity in the host.

Iron is an essential nutrient for nearly all bacterial pathogens to survive and colonize the host. However, in vertebrate host, iron is usually bound to hemoglobin, ferritin, and transferrin, rendering the concentration of freely available iron much lower than that required for bacterial growth (6, 7). To obtain sufficient iron for survival and multiplication *in vivo*, bacteria have evolved complex and multiple systems for iron uptake. Of these systems, the high-affinity iron acquisition mediated through iron-chelating siderophores is a highly efficient and conserved strategy in Gram-negative bacteria and thus has been a promising target for iron-dependent pathogen control (6). However, the intervention measures targeting iron-acquisition, such as vaccination and siderophore pathway inhibitors, have had only partial success likely because of the redundancy of iron acquisition pathways and constantly evolving interactions between bacteria and the host (6, 7).

For example, enterobactin (Ent), a catecholate siderophore with the highest affinity for ferric iron among all natural siderophores, is an archetype for iron acquisition in Gram-negative bacteria (8). Ent-mediated iron acquisition is therefore regarded as an important virulence-associated factor during the infections of many Gram-negative pathogens, such as *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, and *Campylobacter* spp. (9-12). Nevertheless, existence of multiple Ent receptors in single cell, such as up to five receptors in *E. coli* (FepA,

Cir, Fiu, IroN, and Iha) (13), presents a significant challenge for vaccine development by targeting outer membrane proteins. In addition, some successful Gram-negative pathogens have evolved systems to produce modified Ent, named as salmochelins, to counteract Ent sequestration by lipocalins, the host acute-phase proteins produced and secreted to inflammatory sites during infection (6, 14). Lipocalins display high affinity for Ent, consequently limiting Ent-mediated iron acquisition and exerting a bacteriostatic effect against Gram-negative pathogens (14, 15). To win the “tug-of-war” for iron, some bacterial pathogens, such as *E. coli* and *S. enterica*, can produce salmochelins (mono-, di-, and tri-glucosyl Ent) that display low affinity for lipocalins (16). Therefore, production of salmochelins can confer an *in vivo* growth advantage to bacterial pathogens by evading lipocalin sequestration (17, 18).

Recently, a novel Ent-based immune intervention strategy was developed (19, 20). In these studies, Ent was conjugated to a carrier protein, creating a conjugate vaccine to induce Ent-specific antibodies that were expected to function similarly as lipocalins to sequester Ent. Sassone-Corsi *et al.* reported an Ent conjugate vaccine that was synthesized using a lengthy and complicated procedure; this Ent conjugate elicited weak mucosal response (*i.e.*, <4 fold increase in Ent-specific immunoglobulin A [IgA]) and no systemic response (IgG) in the immunized mice (20). In our recent study, we developed a new Ent conjugate vaccine with a simple and efficient protocol (19). This new vaccine could trigger strong systemic immune responses, resulting in up to 4,096 fold increase in the titer of Ent-specific IgG in serum. Remarkably, the Ent-specific antibodies generated by the vaccine also displayed high affinity for other Ent derivatives including salmochelin molecules (19). This finding strongly suggested that the Ent-specific antibodies may overcome a significant pitfall of lipocalins, which have low affinity for salmochelins (21, 22). Moreover, using representative *E. coli* and *Campylobacter* strains, we demonstrated lipocalin-like bacteriostatic effect of the Ent-specific antibodies, providing a direct and critical evidence for validating the concept of Ent conjugate vaccine (19).

Clearly, the findings from our recent study (19) showed significant potential of the Ent conjugate vaccine as a broad spectrum vaccine against different Gram-negative pathogens. In particular, the Ent-specific antibodies induced by this new conjugate vaccine (19) may have a significant advantage over host lipocalins in terms of sequestration of various catecholate siderophores

including salmochelins. To test this hypothesis, in this study, we conducted quantitative *in vitro* growth assays to assess bacteriostatic effect of the Ent-specific antibodies on 41 diverse clinical isolates that belong to three significant Gram-negative bacterial pathogens: *E. coli* (n=27), *S. enterica* (n=8), and *Campylobacter* spp. (n=6). The clinical strains selected for this study also have defined background for production of salmochelins and other siderophores. The findings from this study provide critical and compelling *in vitro* evidence supporting the feasibility of Ent-based immune interventions against a variety of Gram-negative pathogens.

2.2. Materials and methods

2.2.1. Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 2-1. *E. coli* and *S. enterica* strains were routinely grown in Luria-Bertani (LB) broth (Difco, Detroit, MI) with shaking (200 rpm) or on agar at 37 °C overnight. *Campylobacter* strains were routinely grown in Mueller-Hinton (MH) broth (Difco) or on agar at 42 °C in a microaerobic incubator (5% O₂, 10% CO₂, and 85% N₂) for 48 hours.

2.2.2. Purification of Ent

An Ent transport mutant of *E. coli* AN102 (JL122, Table 1) was generously provided by Dr. Sandra Armstrong (University of Minnesota) and used for Ent purification. The purification procedure was described previously (9, 23). The purified Ent was dissolved in dimethylformamide (DMF) and stored at -20 °C prior to use.

2.2.3. Purification of human lipocalin-2

His-tagged recombinant human lipocalin-2 was purified from JL1002 (Table 1), an Ent deficient strain (Δ entD) ensuring the purified recombinant lipocalin-2 was not bound by endogenous Ent. Purification procedure was described in a previous publication with slight modifications (24). Briefly, JL1002 was grown in LB broth supplemented with tetracycline (final concentration of 15 µg/ml) to mid-log phase and was then induced by isopropyl β -D-thiogalactopyranoside (IPTG, 0.5 mM) for 3 hours for production of recombinant lipocalin-2. Subsequently, the induced cells were harvested *via* centrifugation and incubated in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, 5 mM ATP, 5 mM MgCl₂, 1 mg/ml lysozyme, and 10 mM β -

mercaptoethanol, pH 7.0) at 0 °C for 1 hour. After sonication, soluble fraction of the lysate was mixed with pre-equilibrated Ni-NTA agarose (Thermo Fisher Scientific) and the mixture was loaded onto column. After wash of the column with washing buffer (50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 5mM ATP, 5mM MgCl₂, and 5 mM β-mercaptoethanol, pH 7.0), the recombinant lipocalin-2 was eluted from the column by elution buffer (50 mM sodium phosphate, 300 mM NaCl, 300 mM imidazole, 10% glycerol, and 5 mM β-mercaptoethanol). The eluted recombinant lipocalin-2 was dialyzed against PBS, sterilized by membrane filtration (0.22-μm-pore-size filter), aliquoted, and stored at -20 °C. High purity of the recombinant lipocalin-2 was confirmed by SDS-PAGE. Concentration of the purified human lipocalin-2 was determined using a BCA assay (Thermo Fisher Scientific).

2.2.4. *In vitro* growth assay of *E. coli* and *S. enterica*

Inhibitory effect of the Ent-specific antibodies on the growth of all *E. coli* and *S. enterica* strains under iron-restricted condition was evaluated using a microtiter plate growth assay as described in previous publications (19, 24). Both rabbit control and anti-Ent sera used in the assay were generated in our recent study (19). The purified recombinant human lipocalin-2, because of its high affinity for Ent and the demonstrated bacteriostatic feature (15), was used as positive control in growth assay.

Briefly, prior to the assay, both rabbit control and anti-Ent sera were heat-inactivated at 56 °C for 30 minutes to abolish complement activity, followed by dialysis against PBS and sterilization *via* membrane filtration (0.22-μm-pore-size filter). The sterile rabbit sera were stored at 4 °C. To perform *in vitro* growth assay, specific *E. coli* or *S. enterica* strain was inoculated into LB broth supplemented with 0.6 mM 2,2'-dipyridyl. The log-phase cells were washed twice with sterile PBS and subsequently diluted with RPMI medium (Gibco, Dublin, Ireland) to final concentrations of about 5×10⁵ CFU/ml. In each microplate well, 150 μl of RPMI medium was mixed with 50 μl of control serum, 50 μl of anti-Ent serum, or 50 μl of control serum plus 10 μl of stock human lipocalin-2 (0.8 mg/ml). Then 5 μl of the above bacterial inoculum was added to each well. The microplate was incubated at 37 °C for up to 24 hours. At different time points post-inoculation, 20 μl of culture was taken from each well, serially diluted in ice-cold RPMI medium, and plated onto LB agar plates. The colony forming units (CFUs) were enumerated

after 16 hours of growth and *in vitro* growth was expressed as log₁₀ CFU/ml. Each treatment was performed in duplicate. Notably, in this *in vitro* growth assay, the molar concentration of the Ent-specific antibodies (< 0.05 µM) was much lower than that of lipocalin-2 (1.6 µM), which is based on conservative estimation as justified in our previous publication (19).

2.2.5. *In vitro* growth assay of *Campylobacter* spp.

Campylobacter does not produce any siderophores but has the ability to utilize exogenous siderophores to grow under iron-limited conditions (9). Thus, in the growth assay of *Campylobacter*, a final concentration of 0.2 µM of Ent was supplemented to the iron-restricted medium to support the bacterial growth. *Campylobacter* strain was inoculated and grown in MEM-alpha medium (Gibco, Dublin, Ireland), which has been used as an iron-restricted medium to study iron-acquisition in *Campylobacter* (25). Following growth at 42 °C under microaerobic condition, the log-phase cells were washed twice and subsequently diluted with MEM-alpha medium to the concentration of about 5×10⁵ CFU/ml. In each microplate well, 150 µl of MEM-alpha medium, supplemented with Ent to the final concentration of 0.2 µM, was mixed with 50 µl of control serum, 50 µl of Ent antiserum, or 50 µl of control serum plus 10 µl of stock human lipocalin-2 (0.8 mg/ml). Subsequently, 5 µl of the above bacterial inoculum was added to each well. The microplate was incubated in the microaerobic incubator. At different time points post-inoculation, 20 µl of culture was taken from each well, serially diluted in ice-cold MEM-alpha medium, and plated onto MH agar plates. The CFUs were enumerated after 48-hour growth. Each treatment was performed in duplicate.

2.2.6. UHPLC-HRMS analysis for siderophore profiling

Ultra-high performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) analysis was performed to quantify specific siderophores produced by the tested *E. coli* or *S. enterica* strains. Bacterial strains were grown to mid-log phase in LB broth supplemented with 0.6 mM of 2,2'-dipyridyl in order to induce the production of siderophores. After centrifugation at 13,000 g for 2 min, the supernatant was collected and FeCl₃ was added to final concentration of 5 mM to keep the siderophores in iron-bound state. The mixture was filtered using a 0.22-µm-pore-size membrane filter and added with 5% (v/v) of formic acid prior to MS analysis as described previously (26). A total of 10 µl of each sample was loaded onto column

for UHPLC-HRMS analysis as described in a recent work (27) with modifications. Briefly, chromatographic separations were performed over 15 minutes using a Kinetex C18 reversed phase column (100 mm x 2.1 mm, 2.6 μ m, 100 Å, Phenomenex, Torrance, CA) and an UltiMate 3000 pump (Thermo Fisher). Subsequently, mass analysis was performed in parallel reaction monitoring (PRM) mode using a Q Exactive Orbitrap Mass Spectrometer (Thermo Scientific) with electrospray ionization (ESI) in both positive and negative modes. The ESI parameters were as follows: scan range of 120-1800 m/z , resolution of 140,000, AGC target of 3.0×10^6 , MaxIT of 200 ms, sheath gas flow rate of 25 units, aux gas flow rate of 10 units, spray voltage ± 4.0 kV, and capillary temperature at 350 °C. All solvents used were high-performance liquid chromatography (HPLC) grade (Fisher Scientific). Chromatographic peak area was integrated from the full scan of fragments from each isolation mass using Quan browser (Thermo Fisher). The specific transition ions monitored from pseudomolecular ions to daughter ions were as follows: Ent (670 > 224 m/z), Ent linear trimer ((DHBS)₃, 688 > 224 m/z), Ent dimer ((DHBS)₂, 465 > 224 m/z), Ent monomer (DHBS, 242 > 137 m/z), monoglucosyl-Ent (MGE, 832 > 224 m/z), linear MGE (850 > 224 m/z), diglucosyl-Ent (DGE, 994 > 224 m/z), linear DGE (1,012 > 224 m/z), triglucosyl-Ent (TGE, 1,156 > 266 m/z), salmochelin S1 (627 > 224 m/z), salmochelin S5 (789 > 386 m/z), salmochelin SX (404 > 299 m/z), aerobactin (565 > 205 m/z), yersiniabactin monomer (535 > 349 m/z) and dimer (1,068 > 882 m/z) (26, 28). The extracted high-purity Ent (19) was used to validate the standard UHPLC-HRMS protocol described above. The relative abundance of specific siderophore is expressed as log₁₀ transformed arbitrary unit of the corresponding isolated fragment peak.

2.2.7. Statistical analysis

Statistical analyses were conducted using One-way ANOVA. Data are presented as means $\pm$ standard deviations. A probability level of $P < 0.05$ was considered a statistically significant difference.

2.3. Results

2.3.1. Inhibition on the human uropathogenic *E. coli* (UPEC) with characterized siderophore production systems

We first tested a panel of human UPEC strains with known background concerning specific siderophores they produced under iron-restricted condition (Table 2-1). The strain UTI89 can produce Ent, salmochelins, and yersiniabactin for iron acquisition (29). As shown in Figure 2-1A, compared to control serum, anti-Ent serum significantly inhibited the growth of UTI89 at 8 hours (1.3 log₁₀ units of growth reduction, $P=0.02$) and 16 hours (2.2 log₁₀ units of growth reduction, $P<0.01$) of growth. The lipocalin-2 positive control also showed significant inhibition on UTI89 growth at 8 hours (1.0 log₁₀ units of growth reduction, $P=0.02$) and 16 hours (0.9 log₁₀ units of growth reduction, $P=0.02$) when compared to control serum. Notably, anti-Ent serum started to display stronger inhibitory effect on UTI89 growth than lipocalin-2 at 8 hours, and more significantly at 16 hours (1.3 log₁₀ units of difference, $P<0.01$). By 24 hours, the levels of bacterial growth under different treatments became comparable (Figure 2-1A).

The remaining tested UPEC strains only produce Ent siderophore under iron-restricted conditions (Figure 2-1B-E). Consistently, the presence of Ent antiserum in growth medium led to significant growth reduction for all strains (Figure 2-1B-E). However, with respect to different strains, the Ent antiserum and lipocalin-2 could display different magnitudes and patterns of growth inhibition (Figure 2-1B-E). For both ASB4 (Figure 2-1B) and acute2 (Figure 2-1C) strains, anti-Ent serum potently inhibited bacterial growth in comparison with control serum, with up to 6.4 log₁₀ units and 4.0 log₁₀ units of growth reduction at 24 hours for ASB4 and acute2, respectively (Figure 2-1B & C). Despite its robust growth inhibition on both ASB4 and acute2 strains, lipocalin-2 led to significantly smaller magnitude of growth reduction than Ent antiserum at 8, 16, and 24 hours of growth points. Regarding strains pyelo2 (Figure 2-1D) and rUTI1 (Figure 2-1E), the anti-Ent serum still greatly inhibited bacterial growth, with approximately 5 log₁₀ units of growth reduction at 16 hours and 24 hours of time points when compared to control serum (Figure 2-1D & E). Interestingly, unlike the scenario for ASB4 and acute2 (Figure 2-1B & C), the positive control lipocalin-2 exerted stronger inhibitory effect than anti-Ent serum on the growth of pyelo2 (Figure 2-1D) and rUTI1 (Figure 2-1E).

2.3.2. Inhibition on diverse avian pathogenic *E. coli* (APEC)

Subsequently, we selected ten APEC strains originated from five different countries (Table 2-1) and examined the inhibitory effect of Ent-specific antibodies on their growth (Figure 2-2). At 16 hours post-inoculation, compared to control serum, anti-Ent serum displayed significant inhibitory effect on the growth of all the ten APEC strains with growth reduction ranging from 0.8 to 5.1 log₁₀ units. However, lipocalin-2 barely had significant inhibitory effect on most of the tested APEC strains (8 out of 10); only the strain 492 and 517 showed 0.8 ($P=0.02$) and 3.1 ($P<0.01$) log₁₀ units of growth reduction, respectively, in response to lipocalin-2 treatment (Figure 2-2). Remarkably, in all APEC strains, the anti-Ent serum led to greater magnitude of growth reduction than lipocalin-2; the difference in the growth reduction between these two treatments was more than 1 log₁₀ unit for 9 out of 10 strains, with three strains (487, 492, and 474) displaying about 4 log₁₀ units of difference (Figure 2-2).

2.3.3. Inhibition on the *E. coli* isolates from swine and bovine.

We also selected four *E. coli* isolates causing diarrhea in swine and eight Shiga toxin-producing bovine *E. coli* isolates displaying diverse serotypes (Table 2-1) for growth assay. With respect to the four swine *E. coli*, the Ent antiserum dramatically inhibited the growth of all the strains when compared to control serum, leading to up to 5.4 log₁₀ units of growth reduction (Figure 2-3). Lipocalin-2 did not significantly inhibit the growth of the three tested swine *E. coli* isolates but exerted greater magnitude of growth reduction on JL1269 than the anti-Ent serum ($P<0.01$) (Figure 2-3).

For the eight bovine *E. coli* isolates, treatment of the anti-Ent serum consistently led to significant growth reduction ranging from 0.9 to 5.6 log₁₀ units when compared to control serum (Figure 2-3). Although the growth of most bovine strains (7 of 8) was also significantly inhibited by lipocalin-2 (up to 5.1 log₁₀ units of growth reduction), the anti-Ent serum could lead to additional significant growth reduction when compared to lipocalin-2 on strains NEc210, NEc236, NEc250, NEc261, and NEc271, which are 0.9 ($P<0.01$), 3.6 ($P<0.01$), 3.4 ($P<0.01$), 4.8 ($P<0.01$), and 3.6 ($P<0.01$) log₁₀ units, respectively (Figure 2-3).

2.3.4. Inhibition on *S. enterica* with different serotypes.

Initially, we chose *S. enterica* serovar Typhimurium ATCC14028 (Table 2-1) as a representative *Salmonella* strain for growth assay because this widely used model strain has a defined genetic background and is known to produce both Ent and salmochelins (30). As shown in Figure 2-4A, the growth of ATCC14028 strain was significantly inhibited in response to Ent antiserum as early as 8 hours of growth (1.2 log₁₀ units of reduction) and more pronounced at 16 hours (4.0 log₁₀ units of reduction) and 24 hours (4.5 log₁₀ units of reduction) when compared to control serum. Clearly, the lipocalin-2 displayed significantly weaker inhibitory effect on the growth of ATCC14028 than anti-Ent serum. Specifically, lipocalin-2 did not exert significant inhibitory effect at 8 hours of growth but caused up to 2.7 log₁₀ units of growth reduction at 24 hours of growth point (Figure 2-4A). With respect to another commonly used model organism *S. enterica* serovar Typhimurium LT2 (JL1559 in Figure 2-4B), both anti-Ent serum and lipocalin-2 exhibited similar inhibitory effect on bacterial growth, leading to approximately 4 log₁₀ units of growth reduction when compared to control serum.

The similar pattern of growth inhibition of *S. enterica* serovar Typhimurium ATCC14028 (Figure 2-4A) was also observed in different *S. enterica* serovars, which include Enteritidis (JL1553, JL1558, and JL1560), Heidelberg (JL1556 and JL1557), and Montevideo (JL1555) (Figure 2-4B). Specifically, all these strains with different serovars were significantly ($P < 0.01$) inhibited by anti-Ent serum with up to 3.9 log₁₀ units of growth reduction when compared to control serum (Figure 2-4B). However, lipocalin-2 either barely inhibited *Salmonella* growth (JL1553 and JL1560) when compared to control serum or led to significantly smaller magnitude of growth reduction than anti-Ent serum (Figure 2-4B).

2.3.5. Inhibition on the *Campylobacter* spp. of different host origins.

Campylobacter does not produce any siderophores but has the ability to utilize exogenous siderophores for growth and pathogenesis (9). To examine the inhibitory effect of anti-Ent serum on *Campylobacter* growth, 0.2 μM of Ent was supplemented in iron-restricted medium to support *Campylobacter* growth. Three *C. jejuni* and three *C. coli* strains of various origins (Table 2-1) were selected for *in vitro* growth assay. As expected, lipocalin-2 exhibited significant ($P < 0.01$) inhibitory effect on all tested *Campylobacter* strains with growth reduction ranging from

0.8 to 2.1 log₁₀ units when compared to control serum (Figure 2-5). The growth of all the *Campylobacter* strains was also significantly inhibited by anti-Ent serum; however, the magnitude of growth reduction in response to anti-Ent serum was smaller than that caused by lipocalin-2 in majority of tested strains (JL241, JL11, JL522, and JL170) (Figure 2-5). Only in JL105, a *C. jejuni* human clinical isolate, anti-Ent serum displayed stronger inhibitory effect on bacterial growth than lipocalin-2 (Figure 2-5).

2.3.6. Profiling of siderophore production in the tested strains

We performed UHPLC-HRMS analysis on the tested strains whose siderophore repertoires have not been elucidated (*E. coli*, n=22; *S. enterica*, n=7). For each strain, a total of 15 siderophore compounds, which include Ent and its hydrolysis products (n=4), salmochelins and their hydrolysis products (n=8), aerobactin (n=1), and yersiniabactins (n=2), were determined (Table 2-2). Figure 2-6 shows the structures of the siderophores examined in this study.

Ent, the fundamental and highly conserved catecholate siderophore in Enterobacteriaceae, was detected in all the strains except the swine *E. coli* JL1266 and *S. enterica* JL1556 (Table 2-2). However, detection of salmochelins MGE and DGE, the glycosylated derivatives of Ent, in JL1266 and JL1556 (Table 2-2) indicates that Ent was indeed produced by these two strains under iron-restricted condition. With respect to salmochelins and their derivatives, production of MGE, linear MGE, DGE, and salmochelin S5 were detected in all 29 strains; production of TGE, the triglucosyl-Ent, was detected in majority of the tested strains (23 out of 29) (Table 2-2). Production of aerobactin was also prevalent and detected in most of the tested strains (24 out of 29) (Table 2-2). Interestingly, production of yersiniabactin monomer was only detected in a very limited number of strains (6 out of 29) while yersiniabactin dimer was detected in many more strains (23 out of 29) (Table 2-2), which suggests that yersiniabactin was widely produced in the tested strains but tends to exist as non-monomer for chelating Fe³⁺.

The semi-quantitative UHPLC-HRMS analysis also provided relative production level of specific siderophore across the tested strains (Table 2-2). In general, the abundance of each siderophore varied greatly among the strains. For example, regarding Ent quantity, the difference of more than two log₁₀ transformed units (*e.g.*, 4.83 for APEC 492 vs. 7.01 for bovine *E. coli*

NEc241) was observed; majority of the tested strains (20 out of 29) displayed consistently high levels of Ent production reflected by units ranging from 6.56 to 7.01. With respect to major salmochelins (MGE, DGE, and TGE), similar pattern was also observed (Table 2-2). For several strains that produced low levels of Ent, which include APEC 492 and 510, swine *E. coli* JL1266, bovine *E. coli* NEc225 and NEc250, and *S. enterica* JL1556 and JL1557, the relative abundance of different salmochelins was also consistently lower (up to 100 fold) in these strains than in other strains.

2.4. Discussion

In a recent study (19), we have demonstrated the Ent-specific antibodies induced by a new Ent conjugate vaccine are able to bind to a panel of structurally similar catecholate siderophores, which include *apo* Ent, ferric-Ent complex, Ent linear trimer, and especially salmochelin molecules (*i.e.*, mono-, di-, and tri-glucosyl Ent). However, host lipocalins can only bind Ent with high affinity but not salmochelins (21, 22). Based on these findings, we speculated that the Ent-specific antibodies could be superior to host lipocalin-2 in sequestering an array of catecholate siderophores including salmochelins, consequently inhibiting bacterial growth more effectively. In this study, using defined salmochelin-producing organisms, we demonstrated that anti-Ent serum exerted significantly higher magnitude of bacteriostatic effect than lipocalin-2 under iron-restricted conditions, reflected by the growth assays for *E. coli* UTI89 (Figure 2-1A) and *S. enterica* Typhimurium strain ATCC14028 (Figure 2-4A). The *E. coli* UTI89 (Figure 2-1A) can produce Ent, salmochelins as well as yersiniabactin (29). Similar to salmochelins, yersiniabactin also has low affinity for lipocalin-2 (31). Given distinct molecular structures between Ent and yersiniabactin (8, 31, 32), it is unlikely that Ent-specific antibodies have high affinity for yersiniabactin. Despite functional compensation resulting from the production of yersiniabactin, the Ent-specific antibodies still displayed significantly greater magnitude of growth reduction than lipocalin-2 at 16 hours post-inoculation in *E. coli* UTI89 (Figure 2-1A). Notably, the Ent-specific antibodies exhibited significantly stronger inhibition than lipocalin-2 for vast majority of other diverse *E. coli* (18 of 22, Figures 2-2 & 2-3) and *S. enterica* (6 of 7, Figure 2-4B) strains examined in this study. Based on this finding and published information (16, 33), we speculated that under iron-restricted conditions these strains could effectively produce and utilize various Ent derivatives including salmochelins that can be sequestered by the Ent-

specific antibodies but not by lipocalin-2. This speculation was supported by the metabolomics analysis in this study (Table 2-2), which indicates that various salmochelins were widely produced in these strains.

The findings from this study provide further evidence showing catecholates-mediated high affinity iron acquisition plays a critical role in the survival of Gram-negative bacteria under iron-restricted conditions. In this study, the growth of all tested strains was significantly inhibited in response to lipocalin-2 and/or anti-Ent antibodies although other iron acquisition systems are also expected to be induced in these strains for utilization of different iron sources in growth medium used in this study, such as transferrin, ferritin and even heme that are carried in the supplemented serum (7); in other words, other iron acquisition systems cannot effectively and fully compensate the defect of catecholates-dependent iron acquisition. In this study, as expected, lipocalin-2 exhibited higher inhibitory effect than the Ent-specific antibodies for a portion of strains (5 of 27 *E. coli*, 1 of 8 *S. enterica*, and 4 of 6 *Campylobacter* strains). Based on conservative estimation, which is detailed in our previous publication (19), the molar concentration of lipocalin-2 used in growth assay was at least 32 times higher than that of the Ent-specific antibodies used in the assay. Thus, assuming lipocalin-2 and the Ent-specific antibodies have similar binding ability to the active motif of Ent, the higher molar concentration of lipocalin-2 added to growth medium may be the major explanation of the higher magnitude of growth inhibition on these tested bacterial strains, particularly for those not producing salmochelins, which include the 4 uropathogenic *E. coli* (Figure 2-1B-E) and the 4 *Campylobacter* strains (Figure 2-5).

Development of siderophore-based conjugate vaccine is an enticing concept for controlling Gram-negative infections. To date, only four siderophore conjugates were reported: two different types of Ent conjugate vaccines (19, 20), the yersiniabactin/aerobactin conjugate vaccines (34), and the vibriobactin conjugate (35). The paramount goal of a siderophore conjugate vaccine is to trigger high titer of specific and protective antibodies directed against the siderophore compound. Many factors, such as conjugation condition, choice of carrier protein, and the strategy to link siderophore to carrier protein, can influence the efficacy of the conjugate vaccine to induce high titer of specific antibodies. In the studies by Sassone-Corsi *et al.* and by Mike *et*

al., the siderophore conjugate vaccines either only induced subtle level of Ent-specific IgA in mucosa with no systemic response detected in mice (20), or failed to elicit any detectable siderophore-specific antibodies in mice (34). In addition, these recent studies (20, 34) did not provide any direct evidence demonstrating the bacteriostatic effect of the siderophore-specific antibodies, a critical issue that needs addressing to validate siderophore-based conjugate vaccine. Bergeron *et al.* synthesized a vibriobactin analogue and linked it to ovalbumin and bovine serum albumin carrier proteins by using complex synthetic procedure (35). Immunization of mice with the conjugates induced specific polyclonal and monoclonal IgG directed against vibriobactin (35); however, there are no follow-up studies for the vibriobactin conjugate vaccine with respect to functional characterization of vibriobactin-specific antibodies as well as protective efficacy against vibriobactin-producing pathogens. Compared to these studies (20, 34, 35), the new Ent conjugate vaccine in our recent study (19) showed several significant advantages, such as rapid and simple method for conjugate vaccine preparation, and induction of high level of Ent-specific IgG. More importantly, biochemical and microbiological characterization of the Ent-specific antibodies induced by the novel Ent conjugate vaccine demonstrated that the Ent-specific antibodies can bind to various Ent derivatives including salmochelins and display lipocalin-like bacteriostatic feature (19). Therefore, the new Ent conjugate vaccine has significant potential to prevent and control various Gram-negative infections in humans and animals. To test this, in this study, we examined diverse Gram-negative bacterial organisms, which represent three leading foodborne bacterial pathogens significant in food safety and public health, for their growth responses to the Ent-specific antibodies. The findings from this study clearly showed the efficacy of Ent-specific antibodies to inhibit different Gram-negative bacteria and demonstrate the feasibility of Ent-based immune interventions against a variety of Gram-negative pathogens.

Besides the active immunization approach discussed above, passive immunization using Ent-specific hyperimmune egg yolk antibodies is another promising strategy to prevent and control Gram-negative infections. Laying hen has been recognized as an efficient “bioreactor” to produce a large quantity of specific egg yolk antibodies (about 22.5 g antibodies per hen per year) (36). Passive immunization with specific egg yolk antibodies is a promising alternative approach to replace antibiotics for the prevention and control of various animal diseases (37-39). Thus, immunization of layers with the new Ent conjugate vaccine (19) may also effectively

induce Ent-specific egg yolk antibodies for practical applications, such as being used as a feed supplement to control *E. coli*, *Salmonella* and *Campylobacter* in animal production system for enhanced animal health and food safety. Recently, we have successfully optimized vaccination regimen to produce Ent-specific egg yolk antibodies (40). In the future, large quantities of Ent-specific egg yolk antibodies will be produced and assessed for protective efficacy against Gram-negative pathogens using both *in vitro* and *in vivo* systems.

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Appendix

Table 2-1. The strains used in this study

Strain	Description	Source or Reference
<i>E. coli</i>		
UTI89	Human uropathogenic isolate producing enterobactin, salmochelins, and yersiniabactin	(29)
ASB4	Human uropathogenic isolate producing enterobactin	(29)
acute2	Human uropathogenic isolate producing enterobactin	(29)
rUTI1	Human uropathogenic isolate producing enterobactin	(29)
pyelo2	Human uropathogenic isolate producing enterobactin	(29)
487	Avian pathogenic isolate from the U.S.	Dr. Catherine Logue ¹
492	Avian pathogenic isolate from the U.S.	Dr. Catherine Logue
474	Avian pathogenic isolate from Mexico	Dr. Catherine Logue
478	Avian pathogenic isolate from Mexico	Dr. Catherine Logue
456	Avian pathogenic isolate from Brazil	Dr. Catherine Logue
463	Avian pathogenic isolate from Brazil	Dr. Catherine Logue
498	Avian pathogenic isolate from China	Dr. Catherine Logue
502	Avian pathogenic isolate from China	Dr. Catherine Logue
517	Avian pathogenic isolate from Turkey	Dr. Catherine Logue
510	Avian pathogenic isolate from Turkey	Dr. Catherine Logue
NEc210	Bovine isolate, serotype O26:H11	Dr. Rodney Moxley ²
NEc225	Bovine isolate, serotype O26:H11	Dr. Rodney Moxley
NEc236	Bovine isolate, serotype O103:H2	Dr. Rodney Moxley
NEc241	Bovine isolate, serotype O103:H11	Dr. Rodney Moxley
NEc250	Bovine isolate, serotype O111:H8	Dr. Rodney Moxley
NEc261	Bovine isolate, serotype O157:H7	Dr. Rodney Moxley
NEc271	Bovine isolate, serotype O157:H7	Dr. Rodney Moxley
NEc294	Bovine isolate, serotype O69:H11	Dr. Rodney Moxley
JL1266	Swine isolate (diarrhea)	(41)
JL1269	Swine isolate (diarrhea)	(41)
JL1273	Swine isolate (diarrhea)	(41)
JL1279	Swine isolate (diarrhea)	(41)
JL122	AN102, Ent transport mutant for Ent purification	(23)
JL1002	Tuner, Δ entD derivative QT2908 producing His-tagged human lipocalin-2	(24)
<i>S. enterica</i>		
JL133	<i>S. enterica</i> serovar Typhimurium ATCC14028 producing enterobactin and salmochelins	(30)
JL1559	<i>S. enterica</i> serovar Typhimurium LT2 ATCC15277	(42)
JL1555	<i>S. enterica</i> serovar Montevideo ATCC8387	(43)
JL1556	<i>S. enterica</i> serovar Heidelberg FDA520	Dr. Young Min Kwon ³
JL1557	<i>S. enterica</i> serovar Heidelberg FDA5105	Dr. Young Min Kwon
JL1558	<i>S. enterica</i> serovar Enteritidis NCTC13349	(44)
JL1553	<i>S. enterica</i> serovar Enteritidis LK5	(45)
JL1560	<i>S. enterica</i> serovar Enteritidis	Dr. Young Min Kwon
<i>Campylobacter</i> spp.		
JL105	<i>C. jejuni</i> human isolate	(46)
JL241	<i>C. jejuni</i> NCTC11168 human isolate	(47)
JL11	<i>C. jejuni</i> ATCC33560 bovine isolate	(48)
JL522	<i>C. coli</i> human isolate	(46)
JL170	<i>C. coli</i> turkey isolate	(46)
QZ4	<i>C. coli</i> swine isolate	Dr. Qijing Zhang ⁴

¹Dr. Catherine Logue, University of Georgia; ²Dr. Rodney Moxley, University of Nebraska-Lincoln; ³Dr. Young Min Kwon, University of Arkansas; ⁴Dr. Qijing Zhang, Iowa State University.

Table 2-2. Production of various siderophores by the tested strains¹

	Strain ID	Ent ²	(DHBS) ₃	(DHBS) ₂	DHBS	MGE	MGE-L	DGE	DGE-L	TGE	Sal-S5	Sal-S1	Sal-SX	Aer	Ybt	(Ybt) ₂
APEC	498	6.88	ND	7.00	6.45	6.80	6.79	6.06	4.98	5.61	6.75	7.21	7.38	7.06	ND	5.96
	502	6.96	4.12	7.16	6.61	6.83	6.85	6.10	5.33	5.52	6.80	7.27	7.47	7.14	ND	6.00
	474	6.33	4.69	ND	5.75	6.33	6.21	5.51	ND	4.25	6.16	6.56	6.69	6.57	ND	4.80
	478	6.90	4.60	7.03	6.57	6.79	6.80	6.12	5.08	5.63	6.76	7.22	7.32	7.09	ND	5.97
	487	6.86	4.02	7.02	6.61	6.77	6.74	5.97	5.05	5.45	6.72	7.20	7.29	7.08	ND	5.91
	492	4.83	4.07	ND	ND	5.00	4.67	4.66	ND	ND	5.06	4.82	ND	ND	ND	ND
	456	6.94	ND	7.13	6.56	6.84	6.84	6.14	5.45	5.65	6.83	7.27	7.11	7.16	ND	5.99
	463	6.73	ND	6.86	6.18	6.69	6.66	5.91	4.84	5.33	6.61	7.07	7.15	6.96	ND	5.74
	517	6.89	ND	7.01	6.59	6.80	6.80	6.09	5.09	5.51	6.77	7.23	7.17	7.11	ND	5.94
	510	5.13	ND	ND	ND	5.43	5.29	4.77	ND	ND	5.40	4.78	ND	ND	ND	ND
Swine <i>E. coli</i>	JL1266	ND	ND	ND	ND	4.84	4.98	4.34	ND	ND	4.96	ND	ND	ND	ND	ND
	JL1269	6.88	ND	6.97	6.58	6.78	6.78	6.06	5.06	5.41	6.73	7.22	7.32	7.07	ND	5.92
	JL1273	6.89	4.78	6.92	6.71	6.74	6.79	6.12	5.21	5.73	6.72	7.20	7.26	7.10	ND	5.86
	JL1279	6.88	5.47	6.94	6.41	6.73	6.74	6.15	5.26	5.68	6.71	7.18	7.33	7.09	ND	5.90
Bovine <i>E. coli</i>	NEc210	6.56	4.06	6.67	6.23	6.53	6.43	5.75	4.57	5.12	6.39	6.84	7.01	6.76	ND	5.27
	NEc225	5.64	4.80	5.50	4.91	5.73	5.50	5.14	ND	3.99	5.33	5.89	6.03	5.77	ND	ND
	NEc236	6.92	6.63	7.02	6.50	6.81	6.81	6.11	5.20	5.63	6.75	7.21	7.32	7.13	ND	5.99
	NEc241	7.01	ND	7.05	6.95	6.86	6.88	6.13	5.74	5.74	6.83	7.29	7.44	7.19	5.84	6.07
	NEc250	5.83	ND	5.67	5.46	5.93	5.77	5.05	ND	ND	5.57	6.11	6.07	6.03	ND	4.13
	NEc261	6.79	ND	7.00	6.52	6.76	6.69	6.06	4.53	5.43	6.67	7.15	7.24	7.00	4.05	5.80
	NEc271	6.76	6.31	6.88	6.35	6.67	6.62	6.01	4.88	5.48	6.61	7.08	7.10	6.98	4.02	5.68
	NEc294	6.99	6.67	7.02	6.96	6.85	6.84	6.08	5.59	5.65	6.82	7.22	7.27	7.15	6.06	6.00
<i>S. enterica</i>	JL1553	6.85	4.48	6.96	6.37	6.80	6.78	6.10	5.16	5.51	6.74	7.20	7.28	7.06	ND	5.84
	JL1555	6.46	ND	6.50	5.76	6.43	6.35	5.71	4.58	5.08	6.32	6.72	6.91	6.66	ND	5.33
	JL1556	ND	5.09	ND	ND	5.18	5.00	4.18	ND	ND	4.73	4.66	ND	ND	ND	ND
	JL1557	4.97	4.04	ND	ND	5.22	5.01	4.17	ND	ND	5.01	4.68	ND	ND	ND	ND
	JL1558	6.77	4.19	6.90	6.25	6.73	6.70	6.02	4.70	5.38	6.65	7.15	7.11	6.97	4.02	5.78
	JL1559	6.91	4.36	7.12	6.67	6.81	6.78	6.11	5.19	5.55	6.74	7.23	7.36	7.04	ND	5.97
	JL1560	6.84	4.04	6.99	6.43	6.77	6.76	6.00	4.98	5.47	6.70	7.21	7.38	7.04	4.10	5.92

¹Production of specific siderophore in the tested strains was determined by UHPLC-HRMS analysis of bacterial culture (detailed in *Materials and Methods*). The relative level of specific siderophore is expressed as log₁₀ transformed arbitrary unit of corresponding MS peak, which can only be compared across different strains for the same siderophore.

²Abbreviation: Ent, enterobactin; DHBS, 2,3-dihydroxybenzoylserine, Ent monomer; (DHBS)₃, Ent linear trimer; (DHBS)₂, Ent dimer; MGE, monoglucosyl-Ent; MGE-L, linear MGE; DGE, diglucosyl-Ent; DGE-L, linear DGE, TGE, triglucosyl-Ent; Sal-S1, salmochelin S1, Sal-S5, salmochelin S5; Sal-SX, salmochelin SX; Aer, aerobactin, Ybt, yersiniabactin monomer; (Ybt)₂, yersiniabactin dimer; ND, not detected.

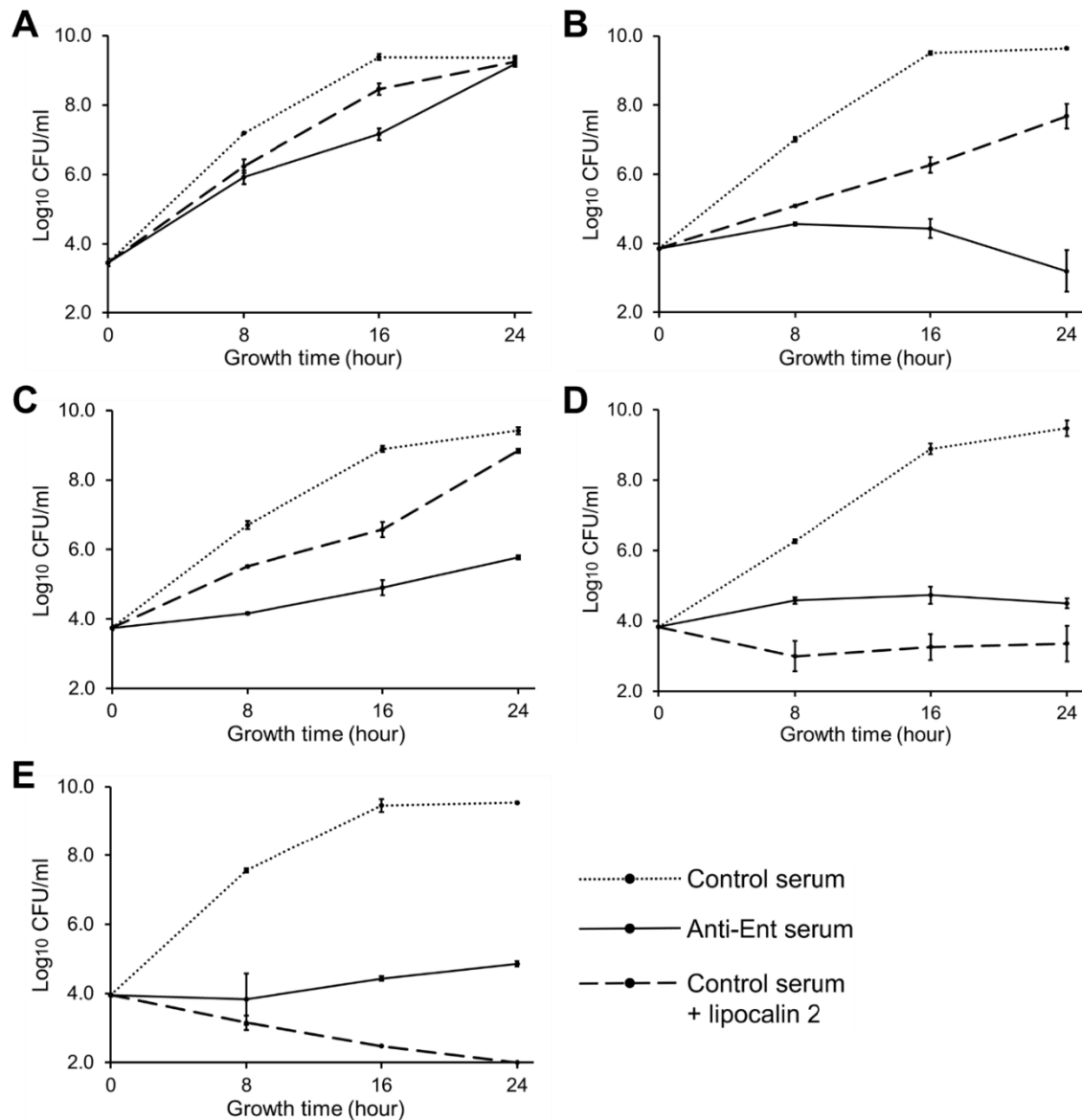


Figure 2-1. Inhibitory effect of anti-Ent serum on human uropathogenic *E. coli*. The *E. coli* cells were grown in the RPMI medium supplemented with rabbit control serum, anti-Ent serum, or the control serum plus lipocalin-2 (final concentration of 37 μ g/ml). Detailed procedure was described in *Materials and Methods*. Each data point represents mean $\pm$ standard deviation of log transformed CFU obtained from duplicate wells in microtiter plate. The tested clinical strains include UTI89 (A) that can produce three siderophores (enterobactin, salmochelins, and yersiniabactin) as well as the other four strains, ASB4 (B), acute2 (C), pyelo2 (D), and rUTI1 (E), that produce enterobactin only.

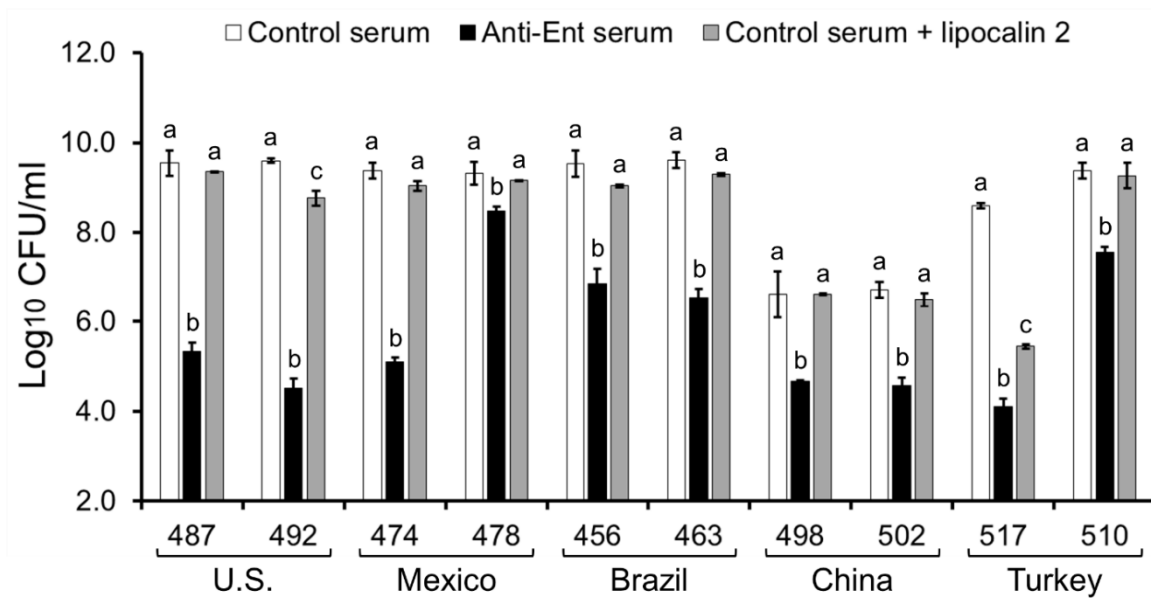


Figure 2-2. Inhibitory effect of anti-Ent serum on avian pathogenic *E. coli* isolates from different countries. The *E. coli* cells were grown in the RPMI medium supplemented with rabbit control serum, anti-Ent serum, or the control serum plus lipocalin-2 (final concentration of 37 µg/ml). Detailed procedure was described in *Materials and Methods*. Each bar represents mean ± standard deviation of log transformed CFU obtained from duplicate wells in microtiter plate at 16 hours post-inoculation. Different letters (a, b, or c) above the bars denote significant difference ($P < 0.05$) between treatments on the same *E. coli* strain. The country origin is indicated below strains.

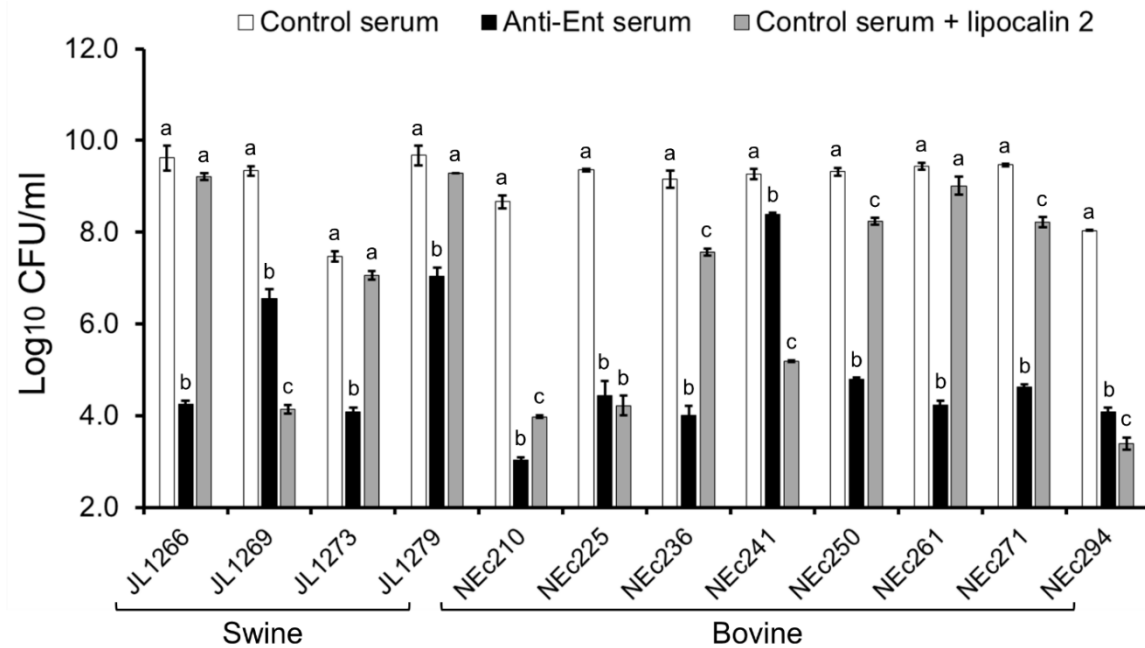


Figure 2-3. Inhibitory effect of anti-Ent serum on *E. coli* isolates from swine and bovine.

The *E. coli* cells were grown in the RPMI medium supplemented with rabbit control serum, anti-Ent serum, or the control serum plus lipocalin-2 (final concentration of 37 $\mu\text{g/ml}$). Detailed procedure was described in *Materials and Methods*. Each bar represents mean $\pm$ standard deviation of log transformed CFU obtained from duplicate wells in microtiter plate at 16 hours post-inoculation. Different letters (a, b, or c) above the bars denote significant difference ($P < 0.05$) between treatments on the same *E. coli* strain. The animal host origin is indicated below strains.

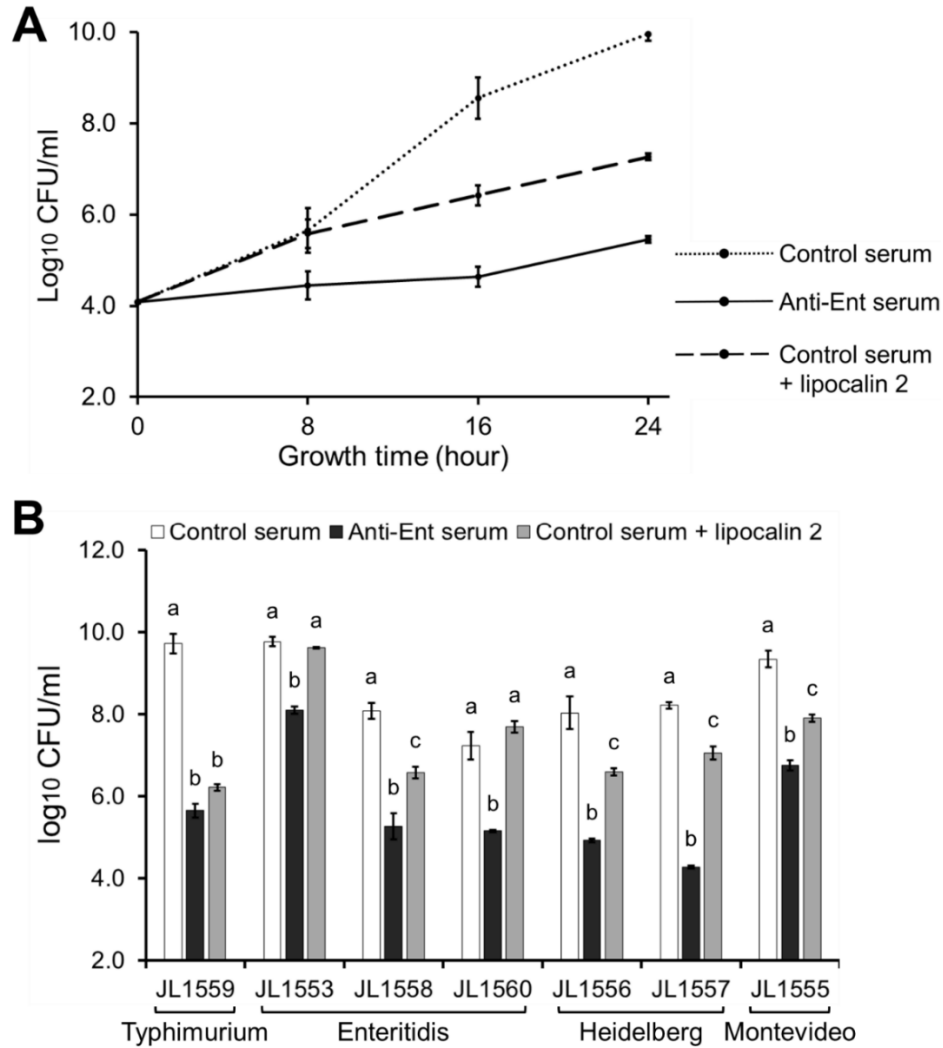


Figure 2-4. Inhibitory effect of anti-Ent serum on *Salmonella* spp. of different serotypes.

The *Salmonella* cells were grown in the RPMI medium supplemented with rabbit control serum, anti-Ent serum, or the control serum plus lipocalin-2 (final concentration of 37 μ g/ml). Detailed procedure was described in *Materials and Methods*. (A) Growth of *S. enterica* serovar Typhimurium ATCC14028 that can produce both enterobactin and salmochelins. Each data point represents mean $\pm$ standard deviation of log transformed CFU obtained from duplicate wells in microtiter plate. (B) Growth of different serotypes of *S. enterica* strains. Specific serovars are indicated below strain names. Each bar represents mean $\pm$ standard deviation of log transformed CFU obtained from duplicate wells in microtiter plate at 24 hours post-inoculation. Different letters (a, b, or c) above the bars denote significant difference ($P < 0.05$) between treatments on the same *S. enterica* strain.

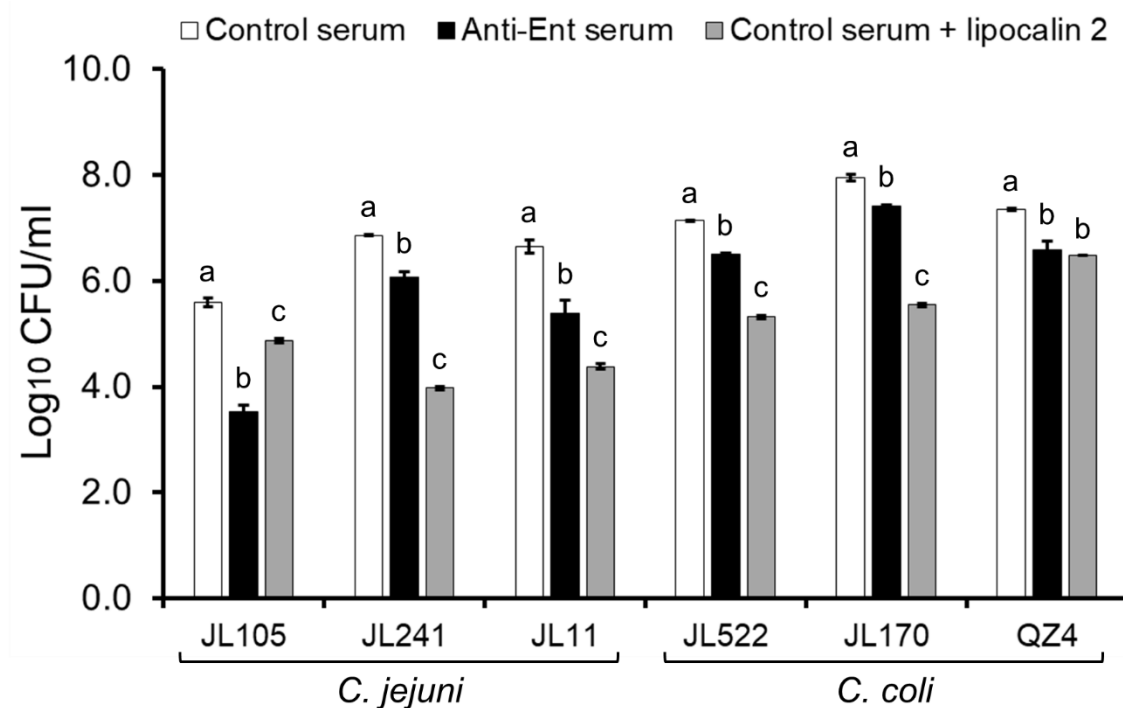


Figure 2-5. Inhibitory effect of anti-Ent serum on *Campylobacter* spp. of different origins.

The *Campylobacter* cells were grown in the enterobactin-containing MEM-alpha medium supplemented with rabbit control serum, anti-Ent serum, or the control serum plus lipocalin-2 (final concentration of 37 $\mu\text{g/ml}$). Detailed procedure was described in *Materials and Methods*. Each bar represents mean $\pm$ standard deviation of log transformed CFU obtained from duplicate wells in microtiter plate at 16 hours post-inoculation. Different letters (a, b, or c) above the bars denote significant difference ($P < 0.05$) between treatments on the same *Campylobacter* strain.

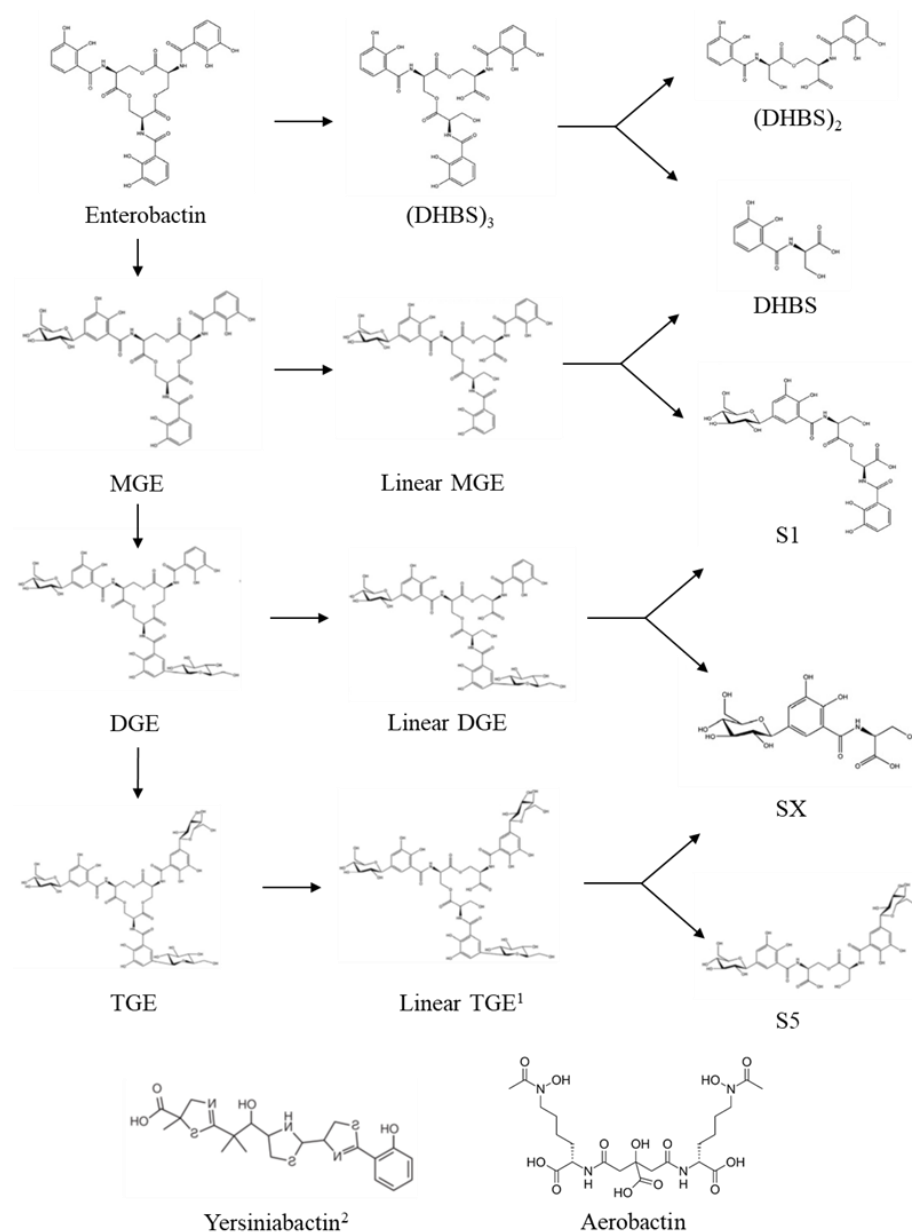


Figure 2-6. Structures of the siderophores determined in this study. The sequential synthesis and hydrolysis derivatives of enterobactin are indicated by arrows. DHBS, 2,3-dihydroxybenzoylserine, Ent monomer; (DHBS)₃, Ent linear trimer; (DHBS)₂, Ent dimer; MGE, monoglucosyl-Ent; DGE, diglucosyl-Ent; TGE, triglucosyl-Ent; S1, salmochelin S1; S5, salmochelin S5; SX, salmochelin SX. ¹Linear TGE was not determined in this study but is listed here to show synthesis pathways. ²Yersiniabactin was determined in both monomer and dimer forms.

Chapter III: Evaluation of Immunogenicity and Protective Efficacy of the Enterobactin Conjugate Vaccine in Protecting chickens from Colibacillosis

Abstract

Colibacillosis caused by avian pathogenic *Escherichia coli* (APEC) is one of the most commonly occurring and economically devastating infectious diseases in poultry worldwide. Innovative universal vaccines are urgently needed to protect chickens from colibacillosis caused by genetically diverse APEC. Enterobactin (Ent) is a highly conserved siderophore required for *E. coli* iron acquisition and pathogenesis. The Ent-specific antibodies induced by a novel Ent conjugate vaccine significantly inhibited the *in vitro* growth of diverse APEC strains. In this study, we evaluated immunogenicity and protective efficacy of the Ent conjugate vaccine in protecting chickens from colibacillosis. White Leghorn chickens were subjected to a crossed design with two variables, vaccination (with or without) and APEC challenge (O1, O78, or PBS control), leading to six groups (9 or 10 birds per group). The chickens were subcutaneously injected with the Ent conjugate vaccine (100 µg per bird) or PBS at 7 days old, followed by booster immunization at 21 days old. Subsequently, the chickens were intratracheally challenged with APEC strain (O1 or O78, 10⁸ CFU/bird) or PBS at 28 days old. At 5 days post-infection, all the chickens were euthanized to examine lesions and APEC colonization in major organs. Blood and intestinal samples were collected for immunological or microbiota analysis. Immunization of chickens with the Ent conjugate vaccine elicited strong immune responses with 64 fold increase in Ent-specific IgY in serum. The APEC O78 challenge led to extensive lesions in the lung, air sac, heart, liver, and spleen, with significantly alleviated overall lesions in vaccinated chickens. The APEC O1 infection only caused sporadic lesions in the organs and the vaccination did not display significant alleviation of the lesions. Interestingly, the vaccination did not significantly reduce APEC level in the examined organs. Microbiota analysis indicated that the vaccine altered the bacterial community in the ileum and cecum. Taken together, the findings from this study showed the Ent conjugate vaccine could trigger strong specific immune responses in chickens and has promise for conferring protection against APEC infection.

Keywords: enterobactin, vaccine, avian pathogenic *E. coli*, microbiota

3.1. Introduction

Avian pathogenic *Escherichia coli* (APEC) is the etiologic agent of colibacillosis in poultry, a complex of localized and systemic infections leading to early mortality, reduced productivity, and carcass condemnation (1). Recent studies suggest that APEC also has zoonotic potential to cause urinary tract infection, neonatal meningitis, and sepsis in humans (2). Thus, control of avian colibacillosis is important not only for the poultry industry but also for public health. Currently, different classes of antibiotics are commonly used to control APEC infections in poultry. However, resistance to multiple antibiotics, especially those of medical importance, has been reported in APEC; and antibiotic usage is undergoing unprecedented scrutiny in poultry production (3). Thus, alternatives to antibiotics are in urgent need to protect poultry from colibacillosis. Vaccines represent an effective and evolving way to curb infectious diseases and is a promising approach to combat APEC (3, 4). To date, various vaccine candidates have been investigated for the protective efficacy against APEC infections and two vaccines are commercially available (3). However, due to high genetic diversity of APEC, these vaccines fall short in protection against heterogenous APEC infections (4). Therefore, to develop a widely effective vaccine against colibacillosis, it is imperative to target essential bacterial components that are highly conserved in APEC.

The efficient iron acquisition system mediated by siderophores (iron chelators) is essential for bacterial pathogens to survive and infect hosts (5). Enterobactin (Ent) is an archetypical siderophore produced by multiple Enterobacteriaceae species including *E. coli* and is a universal and critical contributor to *E. coli* pathogenesis (6). Not surprisingly, the Ent system has been targeted for developing antimicrobial strategies in the past decades, such as inhibitors for Ent biosynthesis and vaccines against outer-membrane Ent receptors (5, 7). However, these approaches only gained limited success, primarily due to the complexity and redundancy of the systems for synthesizing and transporting Ent in *E. coli*. For instance, up to five Ent receptors (FepA, Cir, Fiu, IroN, and Iha) have been identified in different *E. coli* strains for uptake of Ent and the derived products (5, 8). These challenges have prompted us to switch the vaccine target to Ent itself, which was further supported by the Ent-sequestering effect of human lipocalin 2. Lipocalin 2 is a pleiotropic innate immune protein that can inhibit *E. coli* and other bacterial infections by tightly binding Ent and restricting iron availability for the bacteria (9). We

expected that Ent vaccine could induce specific antibodies that exerted lipocalin 2-like bacteriostatic effect on Ent-dependent bacterial pathogens including *E. coli*.

Recently, we have generated two novel Ent conjugate vaccines by biochemically coupling Ent with carrier protein keyhole lipid hemocyanin (KLH) or bovine serum albumin (BSA) (8). Immunization of rabbits and chickens with the KLH-Ent conjugate vaccine induced high titers of anti-Ent antibodies in the sera of both animals (8, 10). Further, the anti-Ent antibodies were demonstrated to significantly inhibit *in vitro* growth of different pathovars of *E. coli*, including various APEC strains with diverse siderophore production profiles (11). More importantly, immunization of chickens with the KLH-Ent conjugate vaccine suppressed intestinal colonization of *Campylobacter jejuni*, an Ent-dependent pathogen (12), by up to approximately 1,000 fold (13). The outcomes showed significant potential of the Ent conjugate vaccine in controlling different Ent-dependent bacterial pathogens. In this study, we conducted a chicken trial to assess immunogenicity and protective efficacy of the Ent conjugate vaccine against APEC infection in chickens.

3.2. Materials and methods

3.2.1. Preparation of Ent conjugate vaccines

An Ent transport mutant of *E. coli* AN102 (courtesy of Dr. Sandra K. Armstrong at the University of Minnesota) was used for Ent purification as described previously (14, 15). The purified Ent was coupled with BSA (Cat. No. PI77110, Fisher Scientific, USA) or KLH (Cat. No. PI77600, Fisher Scientific, USA) by following the method detailed in our previous publication (8). Then, the KLH-Ent and BSA-Ent conjugates were subjected to SDS-PAGE analysis to confirm the success of conjugation. Finally, the Ent conjugates (1 mg/ml in PBS) were stored at -20 °C prior to use.

3.2.2. Chicken trial for immunization and APEC challenge

A chicken trial was performed to evaluate immunogenicity and protective efficacy of the Ent conjugate vaccine against APEC infection. The chicken experiment was approved by Institutional Animal Care and Use Committee at the University of Georgia (IACUC No. A2021 02-008-R1). APEC O1 and O78, two major serogroups linked to colibacillosis worldwide, were

used to infect the chickens. APEC O1 is an O1:K1:H7 strain originally isolated from the lung of a chicken clinically diagnosed with colisepticemia (16). APEC O78 is an O78:K80:H9 strain (χ 7122, courtesy of Dr. Roy Curtiss III at the University of Florida) originally isolated from the liver of a diseased turkey (17). The APEC strains were routinely grown in Luria Bertani (LB) broth or on LB agar. Upon inoculation to chickens, the strains were washed and diluted in sterile PBS.

As shown in Table 3-1, the newly hatched specific-pathogen-free White Leghorn chicks were subjected to a crossed design with two factors, vaccination (with or without) and APEC challenge (APEC O1, O78, or PBS), leading to 6 groups (9 or 10 birds per group). At 7 days of age, the chicks were subcutaneously (neck) immunized with 100 μ L of KLH-Ent (100 μ g per bird) emulsified with Freund's complete adjuvant, followed by one booster immunization (100 μ g of KLH-Ent emulsified with Freund's incomplete adjuvant) at 21 days of age. Subsequently, the chickens were intratracheally challenged with 100 μ L of APEC (O1 or O78 strain, 10^8 CFU per bird) or PBS at 28 days of age. Five days after the challenge, all the chickens were euthanized. Necropsy was conducted to examine the lesions in lung, air sac, heart, liver, and spleen. Lesion scores were graded independently by at least two veterinarians according to a previous publication (18). To assess the APEC load in lung, liver, and spleen, the tissues were aseptically removed, weighed, and homogenized in sterile PBS. Serial dilutions of the homogenized tissue were then plated on MacConkey agar. After overnight incubation at 37 °C, the colonies on plates were enumerated and converted to bacterial load in tissue (CFU/g). At the abovementioned four time points (7, 21, 28, and 33 days of age), blood samples were collected from each chicken to monitor the specific immune response in serum. Upon euthanization, cecum and ileum contents of each chicken were collected, stored at -80 °C, and subjected to ELISA assay and 16S rRNA gene amplicon-based microbiota analysis as described below.

3.2.3. Enzyme-linked immunosorbent assay (ELISA)

The specific antibody level in chicken serum and intestine was measured by indirect ELISA according to our previous study (8). Briefly, microtiter plates (Nunc-MaxiSorp™ Plate, Thermo Fisher Scientific) were coated with 100 μ L of appropriate antigens (detailed below) in coating buffer (sodium bicarbonate/carbonate buffer, pH 9.6) overnight at room temperature.

Specifically, to determine the antibody level against the whole KLH-Ent conjugate, KLH-Ent conjugate (30 ng/well) was used to coat ELISA plates. To determine the antibody level against the molecule Ent, BSA-Ent (2 µg/well) was used to coat ELISA plates. The coated plates were blocked with blocking buffer (PBS containing 0.05% Tween 20 and 5% skim milk) for 1 h at room temperature. Subsequently, to evaluate the specific immunoglobulin Y (IgY) titer in chicken serum, each serum sample was 2-fold serially diluted in blocking buffer and incubated with the coated plates for 1 h at room temperature. To assess the specific IgY or IgA level in the chicken intestine, the cecum and ileum contents collected from the two groups of chickens challenged with PBS (Table 3-1) were 2-fold diluted with PBS, vortexed, and centrifuged at 5000 *g* for 5 min. The supernatants were diluted with an equal volume of blocking buffer and incubated with the coated plates for 1 h at room temperature.

Following washing four times with washing buffer (PBS containing 0.05% Tween 20), horseradish peroxidase-conjugated goat anti-chicken-IgY or anti-chicken-IgA secondary antibody (SeraCare, USA) with 2000-fold dilution in blocking buffer was added and the plates were incubated at room temperature for 1 hour. After washing four times with washing buffer, the plates were finally developed using an ABTS peroxidase substrate (SeraCare, USA). The reaction was stopped after 30 min by adding 100 µL of stop solution (1% SDS). The absorbance was measured at the optical density of 405 nm (OD₄₀₅) using a microplate reader (BioTek Instruments, USA) and data were collected using Gen5 software (BioTek Instruments). The wells without addition of the primary antibody served as background control. Endpoint titer was defined as the last dilution at which OD₄₀₅ of the sample wells exceeded the cutoff value (0.1). Duplicate measurements were performed for each sample.

3.2.4. Intestinal microbiota analysis

Intestinal microbiota analysis was conducted based on 16S rRNA gene amplicon sequencing as described in our previous publication (19). Briefly, genomic DNA was extracted from the cecum or ileum contents using Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, USA) according to the manufacturer's instructions. Quality and quantity of the purified genomic DNA were determined using NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA).

The genomic DNA was used as template for PCR amplification by targeting V4 region of the 16S rRNA gene and using barcoded primer pair of 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (20). The PCR was performed in a 25 μ L cocktail mix containing 12.5 μ L Phusion Flash PCR Master Mix (Thermo Scientific, USA), 8.5 μ L ultrapure water, 1 μ L forward primer (10 μ M), 1 μ L reverse primer (10 μ M) with barcode, and 2 μ L (100 to 150 ng) DNA template. The PCR program included initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 90 s, with a final extension at 72 °C for 10 min. After amplification, the PCR products were purified using DNA Clean-Up Kit (Zymo Research, USA) and quantified using NanoDrop 1000 Spectrophotometer. Subsequently, the purified products were pooled together in equal concentration and further quantified using KAPA Library Quantification Universal Kit (Roche, USA). Before loaded to a MiSeq Reagent Kit v3 (Illumina, USA), the library was denatured, diluted, and spiked with PhiX library as an internal control according to the protocol for 16S Metagenomic Sequencing Library Preparation (Illumina, USA). Sequencing of the amplicon libraries was performed using an Illumina MiSeq System.

After sequencing operations, the acquired amplicon sequence data were analyzed using the Microbial Genomics Module in the software CLC Genomics Workbench (QIAGEN, USA) with reference database SILVA v.138 (21). The raw sequence reads are publicly available in the NCBI Sequence Read Archive under BioProject PRJNA822438.

3.2.5. Statistical analysis

For binary comparison, unpaired Student's *t*-test was conducted in GraphPad Prism 9. For comparison of microbial structure, permutational multivariate analysis of variance (PERMANOVA) was conducted in R using the package *vegan*. For comparison of taxonomic abundance, Wilcoxon rank-sum (Mann-Whitney) test was performed in GraphPad Prism 9. A probability level of $p < 0.05$ was considered a statistically significant difference.

3.3. Results

3.3.1. Immune responses in the serum and intestine

Immunization of chickens with two doses of Ent conjugate vaccine induced high titers of specific IgY against KLH-Ent conjugate (Figure 3-1A) and Ent molecule (Figure 3-1B) in the sera. Specifically, one week after the second dose (*i.e.*, 28 days of age), anti-KLH-Ent IgY titers were dramatically boosted by an average of 9 log₂ units (512 fold) in the three vaccinated groups of chickens when compared to those in the three unvaccinated groups; the high titers persisted till the end of the trial (Figure 3-1A). Similarly, anti-Ent IgY titers in the three vaccinated groups were significantly elevated at one week following the second dose (*i.e.*, 28 days of age) by an average of 6 log₂ units (64 fold) relative to those in the three unvaccinated groups; the high titers also maintained to the end of the trial (Figure 3-1B).

To examine if the vaccine induced specific IgY and IgA in the intestine, cecum and ileum contents were collected upon termination of the trial from the two groups of chickens challenged with PBS and were subjected to ELISA analysis. The anti-KLH-Ent IgY was induced in the immunized chicken intestine. Specifically, for anti-KLH-Ent IgY (Figure 3-1C), OD₄₀₅ of ileum contents from the vaccinated chickens was significantly higher than that from the unvaccinated chickens. In contrast, OD₄₀₅ of cecum contents from the both groups was extremely low. The results indicated the vaccination led to a significant increase of specific IgY in the chicken ileum. As for the anti-KLH-Ent IgA (Figure 3-1D), there was no significant difference between the vaccinated and unvaccinated chickens with regard to OD₄₀₅ of cecum or ileum contents, meaning the vaccination did not induce a detectable level of specific IgA in the chicken intestine.

3.3.2. Organ lesions caused by APEC infection

As shown in Figure 3-2, challenge with APEC O1 or O78 strain resulted in different extents of lesions in major organs of the chickens; the vaccination significantly mitigated the overall lesions caused by O78 strain. Specifically, O1 strain caused sporadic lesions in the lung, air sac, heart, and liver (Figure 3-2A-D), and no lesions in the spleen (Figure 3-2E). Consequently, the vaccination did not show alleviation on the overall lesions resulting from APEC O1 challenge (Figure 3-1F). For the chickens challenged with O78 strain, the average lesion scores in lung, air sac, heart, and liver of the vaccinated group were clearly lower than those of the unvaccinated

group (Figure 3-2A-D); almost no lesion was found in spleen in both groups (Figure 3-2E). When the lesions in the five organs were accumulated, the vaccinated chickens displayed significantly alleviated lesions resulting from APEC O78 infection than the unvaccinated ones (Figure 3-2F). As expected, the chickens challenged with PBS had no lesions at all in the five organs (Figure 3-2A-E). Together, the vaccination significantly ameliorated the organ lesions caused by APEC O78 infection, but it did not for the chickens infected with APEC O1 due to mild lesions caused by this strain.

3.3.3. APEC burden in organs

As shown in Figure 3-3, the vaccination did not reduce the burden of APEC O1 or O78 strain in lung, spleen, and liver. Specifically, as expected, APEC was not detected in any organs of the chickens receiving PBS challenge (Figure 3-3). For O1 strain, its load in spleen and liver was comparably low between vaccinated and unvaccinated chickens (Figure 3-3B & C); however, the bacterial load in the lung of vaccinated chickens was significantly higher than that of unvaccinated chickens (Figure 3-3A). For O78 strain, there was no significant difference between the vaccinated and unvaccinated groups with regard to the bacterial load in lung, spleen or liver (Figure 3-3A-C).

3.3.4. Impact of the vaccination on intestinal microbiota

We examined impact of the vaccination on chicken intestinal microbiota by conducting 16S rRNA gene amplicon sequencing. To solely examine influence of the vaccination on intestinal microbiota and exclude confounding factor of APEC challenge, we compared the unvaccinated and vaccinated chickens in control group receiving PBS inoculum. Generally, the vaccination altered bacterial community of the chicken intestine. In specific, regarding Shannon diversity (Figure 3-4A & D), the microbiota in cecum or ileum exhibited similar diversity between the vaccinated and unvaccinated chickens within PBS group. To compare the microbiota structure among different samples, principal coordinate analysis was performed based on Bray-Curtis distance as shown in Figure 3-4B & E. Subsequent PERMANOVA analysis revealed that microbiota structures in both cecum and ileum were significantly different between the vaccinated and unvaccinated chickens within PBS group ($p < 0.05$). The specific taxonomic composition of cecum and ileum microbiota was also examined at family level (Figure 3-4C &

F). Specifically, Ruminococcaceae and Lachnospiraceae were predominant in cecum microbiota while Lacobactillaceae and Clostridiaceae were the majority in ileum microbiota (Figure 3-4C & F). In ceca of the chickens receiving PBS, the vaccination led to a significant shrink of Clostridiales vadin BB60 and Lactobacillaceae (Figure 3-4C). In ilea of the chickens receiving PBS, there was also a significant decrease of Lactobacillaceae caused by the vaccination (Figure 3-4F). Rest of the families remained comparable between the vaccinated and unvaccinated chickens in PBS group (Figure 3-4C & F).

Interestingly, when comparing the unvaccinated chickens receiving APEC challenge (O1 or O78) or PBS, we found significant alterations of intestinal microbiota caused by APEC infections. Specifically, Shannon diversities of cecum and ileum microbiota were significantly elevated by both APEC infections when compared to PBS (Figure 3-4A & D). In addition, the structures of cecum and ileum microbiota were also significantly altered by APEC O1 or O78 infection (Figure 3-4B & E). Notably, both APEC O1 and O78 infections, relative to PBS, consistently led to a significant reduction in the proportion of Enterobacteriaceae in ileum microbiota (Figure 3-4F).

3.4. Discussion

In this study, KLH-Ent conjugate vaccine triggered high titers of anti-Ent IgY in the immunized chicken sera (Figure 3-1B) and significantly alleviated overall organ lesions caused by APEC O78 infection (Figure 3-2F) despite the bacterial load in the organs being not changed (Figure 3-3). Meanwhile, bacterial load of APEC O1 in lung was surprisingly higher in the vaccinated chickens (Figure 3-3A) although the lesions in lung and other organs were comparably low between the vaccinated and unvaccinated chickens (Figure 3-2). According to our previous studies (8, 11), anti-Ent antibodies could suppress the growth of different APEC strains *in vitro* by capturing Ent and limiting the iron availability. In this study, iron availability for APEC was conceivably restrained by the vaccine-induced anti-Ent IgY, which could signal a complex regulatory network for iron homeostasis and virulence/fitness in APEC (22). For O78 strain, the lowered iron availability might directly or indirectly downregulate the virulence factors associated with formation of organ lesions. For example, ferric uptake regulator (Fur) is a global pleiotropic transcriptional regulator modulating iron homeostasis and other virulence/fitness

factors (22, 23). Iron depletion renders Fur lift the transcriptional repression of many genes, including a small regulatory RNA RyhB that represses virulence factors of pathogenic *E. coli* responsible for lesion formation (23, 24). Thus, iron scarcity caused by the vaccination could signal a downregulation of lesion-generating virulence factors of APEC O78 and consequently lead to less severe lesions in the vaccinated chickens. For O1 strain with different genetic background from O78 strain, its colonization/fitness displayed a niche-specific enhancement in the lung of vaccinated chickens. Since the presence of oxygen can loosen the transcriptional repression by Fur in *E. coli* (25), we speculate the interplay between bacterial exposure to oxygen in lung and restraint of iron availability caused by the anti-Ent IgY may result in the enhanced colonization/fitness of APEC O1 in the lung of vaccinated chickens. Further studies are needed to elucidate the mechanisms.

As noticed in Figures 3-2 & 3, 40% or more of the unvaccinated chickens receiving APEC O1 or O78 challenge had no detectable lesions or bacterial burden in the organs despite both O1 and O78 strains being highly pathogenic (26, 27). Although APEC is prevalent in all age groups of chickens, young chickens are most vulnerable to APEC infection (3). In fact, nearly all previous studies of APEC virulence used chickens under 3 weeks of age for APEC challenge to better exhibit the pathogenicity (18). However, given the immunization regimen in this study requires sufficient days to adequately induce anti-Ent IgY in the chickens, we can hardly optimize the APEC infection model by lowering the day age for APEC challenge. Thus, it is not surprising the 28-day-old chickens in this study were less vulnerable to O1 or O78 infection when compared to previous studies that used much younger chickens for O1 or O78 infection (26, 27). Since the APEC strains exerted moderate or mild pathogenicity on the chickens in this study, it may partly explain the Ent conjugate vaccine did not show significant suppression on bacterial burden of O1 and O78 as well as organ lesions caused by O1. In addition, from a practical standpoint, it is more important to use the Ent conjugate vaccine to protect younger chickens that are most vulnerable to APEC. Towards this goal, we can immunize hens with the Ent conjugate vaccine, and the induced maternal anti-Ent IgY can be transferred to the hatched progeny and consequently protect the chicks from APEC infection. This maternal passive immunization strategy has been extensively explored for the protection against different pathogens in chickens and gained varied extents of success (28-30). Currently, we are actively committed to evaluating

the efficacy of the Ent conjugate vaccine in inducing maternal immunity against APEC infection in hatchlings.

In addition to strong systemic immune responses and high titers of specific IgY in the serum (Figure 3-1B), the injectable KLH-Ent conjugate vaccine also induced specific IgY in the intestine of vaccinated chickens (Figure 3-1C). Therefore, the injectable Ent conjugate vaccine can be applied to control not only systemic infections but also enteric infections of bacterial pathogens that are dependent on Ent for iron acquisition. In fact, our recent study shows that intramuscular immunization of chickens with the KLH-Ent conjugate vaccine significantly reduced intestinal colonization of *C. jejuni* (13). Given that Ent-mediated iron acquisition is important and conserved in many Gram-negative bacterial pathogens (5), in future studies, we will expand the use of the Ent conjugate vaccine by exploiting different animal models of infections to assess the immune protection against other Ent-dependent pathogens, such as *Salmonella*, *Shigella*, *Klebsiella*, etc.

Intriguingly, when comparing the three groups of unvaccinated chickens that received APEC O1 or O78, or PBS inoculum, we found systemic infection of APEC resulted in significant changes in intestinal microbiota, reflected by elevated Shannon diversity and altered microbiota structure and composition (Figure 3-4). To our best knowledge, this is the first report of perturbation of intestinal microbiota caused by APEC infection without any antibiotic treatment. It has been increasingly recognized there is a complex interplay between intestinal microbiota and sepsis (31). Intestinal commensal microorganisms potentiate host immune cells against sepsis *via* multiple mechanisms, such as the production of short-chain fatty acids (32), while sepsis can induce dysbiosis of intestinal microbiota by modulating the host physiological and inflammatory status (31). Recently, Peng *et al.* showed that depletion of intestinal microbiota led to increased susceptibility of chickens to APEC infection, and dietary supplement of microbiota-derived acetate reduced APEC load in the lung (33). Thus, maintaining or restoring eubiosis of intestinal microbiota *via* probiotics, prebiotics, or fecal microbiota transplantation may foster host immune defense against APEC infection. Future studies are warranted to assess the feasibility of this antiseptic strategy.

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Appendix

Table 3-1. Experimental design of the chicken trial.

Group No.	No. of birds	KLH-Ent vaccination (age of 7 and 21 days)	Intratracheal challenge (age of 28 days)	Termination (age of 33 days)
1	10	Yes	APEC O1	Yes
2	10		APEC O78	
3	9		PBS	
4	10	No	APEC O1	
5	10		APEC O78	
6	9		PBS	

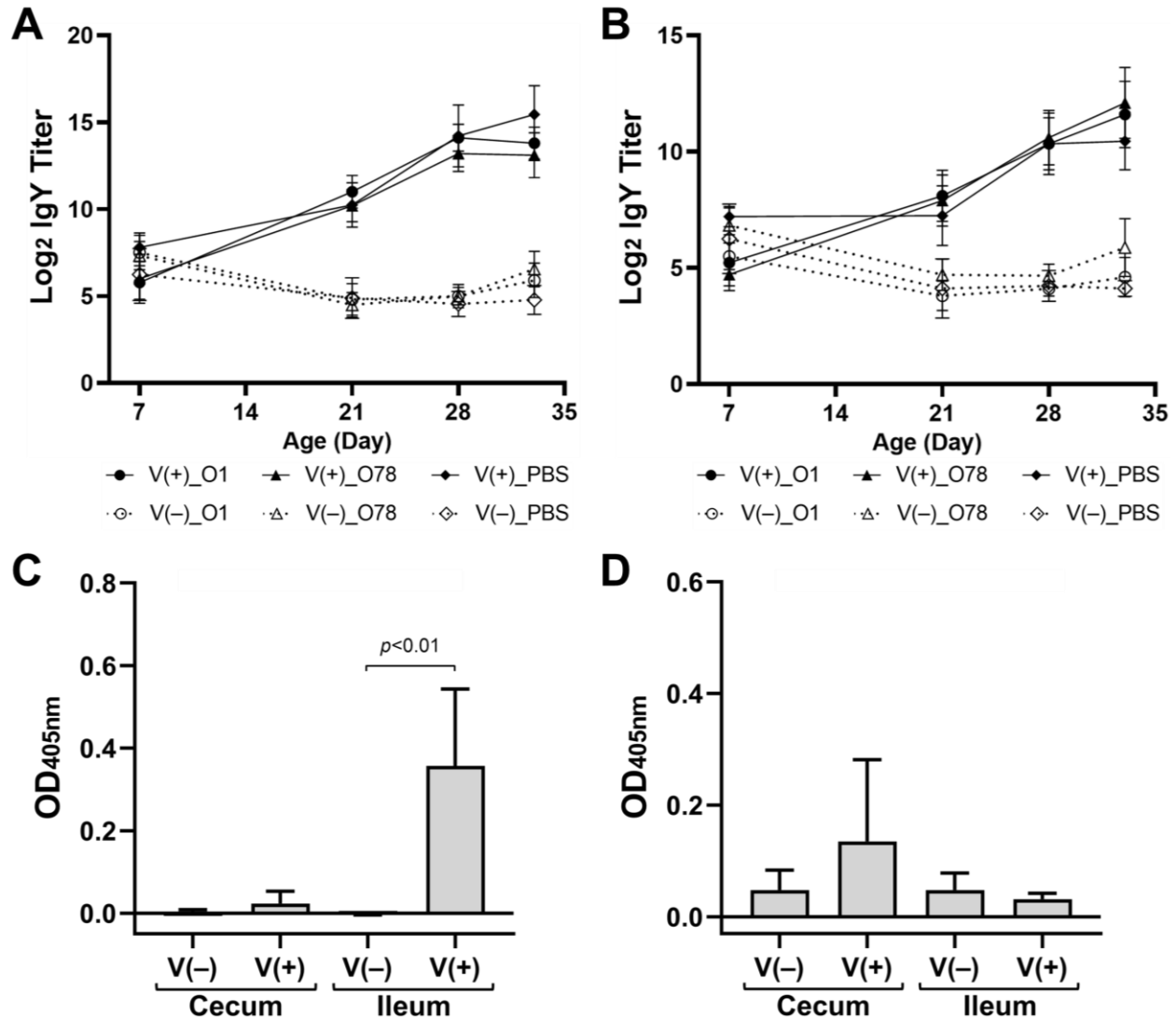


Figure 3-1. Immune responses of chickens upon subcutaneous immunization with the KLH-Ent conjugate vaccine. The titers of serum IgY directed against whole KLH-Ent conjugate vaccine (A) and Ent molecule (B) were measured using ELISA. The V(+) and V(-) indicated vaccinated and unvaccinated chickens, respectively, while O1, O78, and PBS denoted specific inoculum used for intratracheal challenge. The anti-KLH-Ent IgY (C) and IgA (D) were measured in the cecum and ileum of the chickens without APEC challenge. The ileum and cecum samples were collected upon termination of the trial (33 days of age). All the data were presented as mean $\pm$ SD.

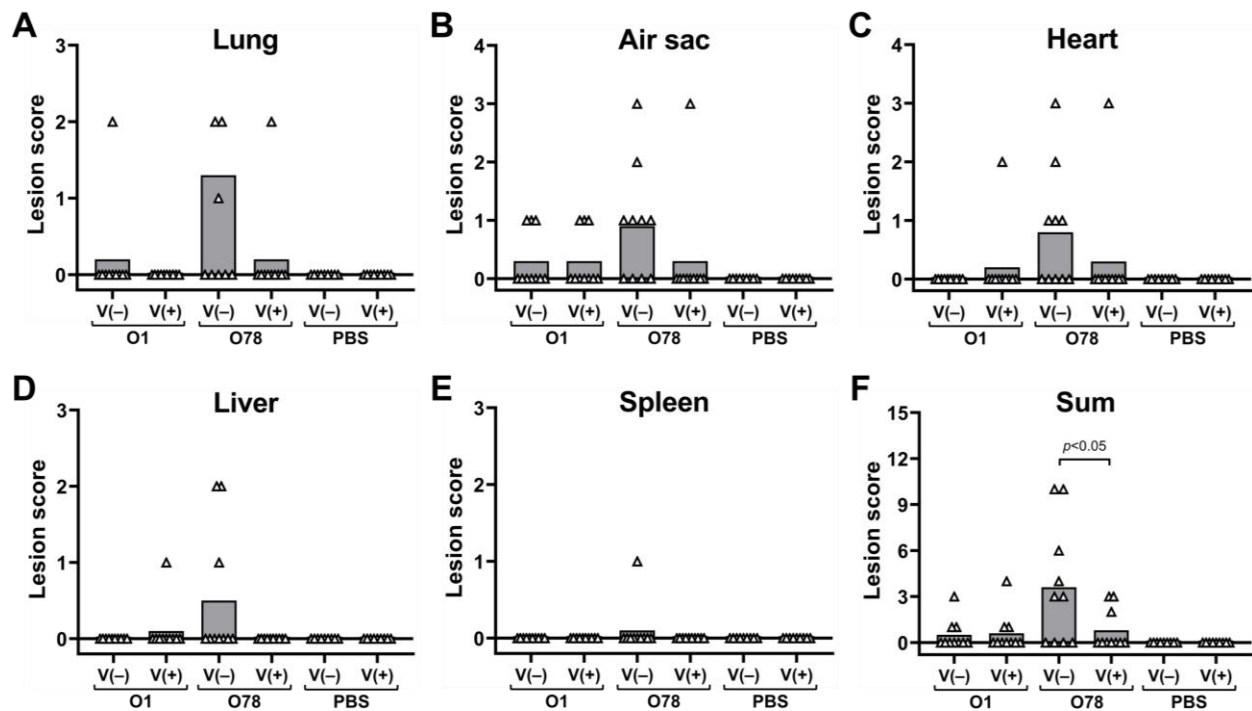


Figure 3-2. Lesions in major organs of the chickens. Lesion scores in lung (A), air sac (B), heart (C), liver (D), and spleen (E) of each individual chicken were graded. Overall lesion scores (F) were summed up from the above five organs in each chicken. The V(+) and V(-) indicated vaccinated and unvaccinated chickens, respectively, while O1, O78, and PBS denoted specific inoculum used for intratracheal challenge. Each dot represented an individual chicken and each bar represented mean of lesion scores within the group.

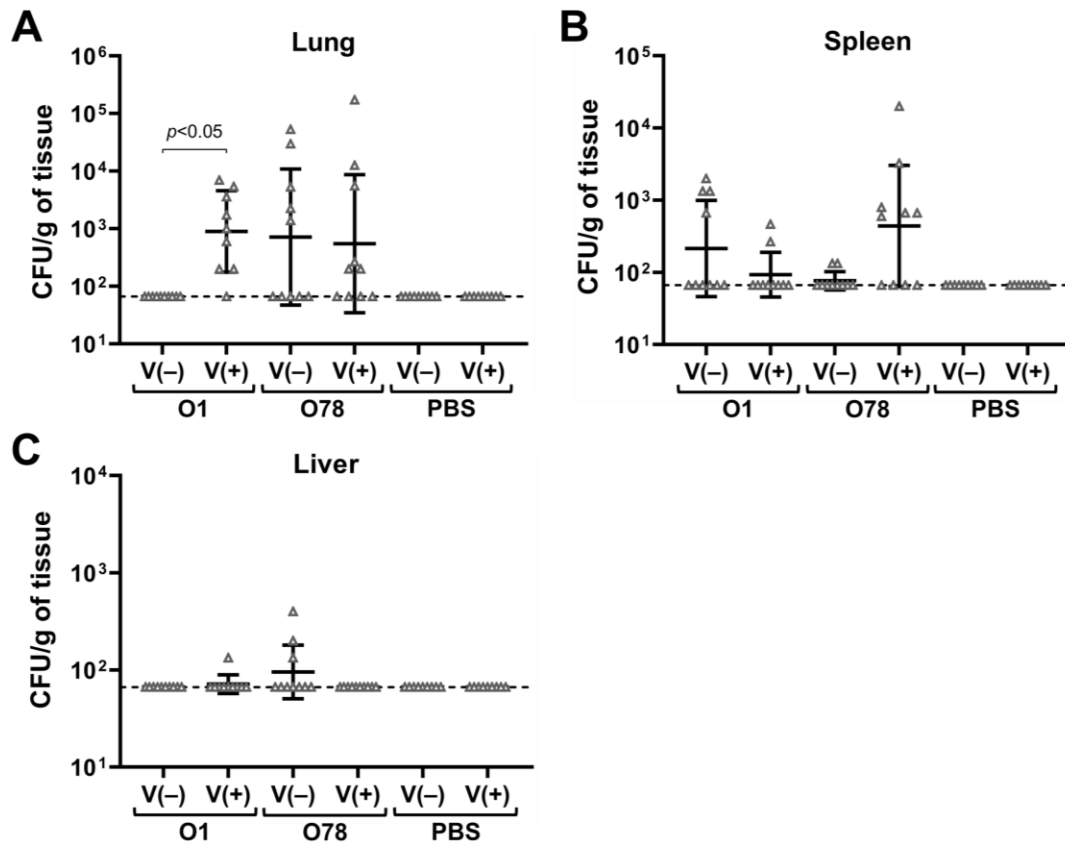


Figure 3-3. APEC load in major organs of the chickens. APEC load in lung (A), spleen (B), and liver (C) of each individual chicken was determined at 5 days post-infection. The detection limit for quantifying APEC load was 66.7 CFU/gram of tissue, which was denoted as the dashed line in each panel. The V(+) and V(-) indicated vaccinated and unvaccinated chickens, respectively, while O1, O78, and PBS denoted specific inoculum used for intratracheal challenge. Each dot represented an individual chicken and the data were presented as mean $\pm$ SD.

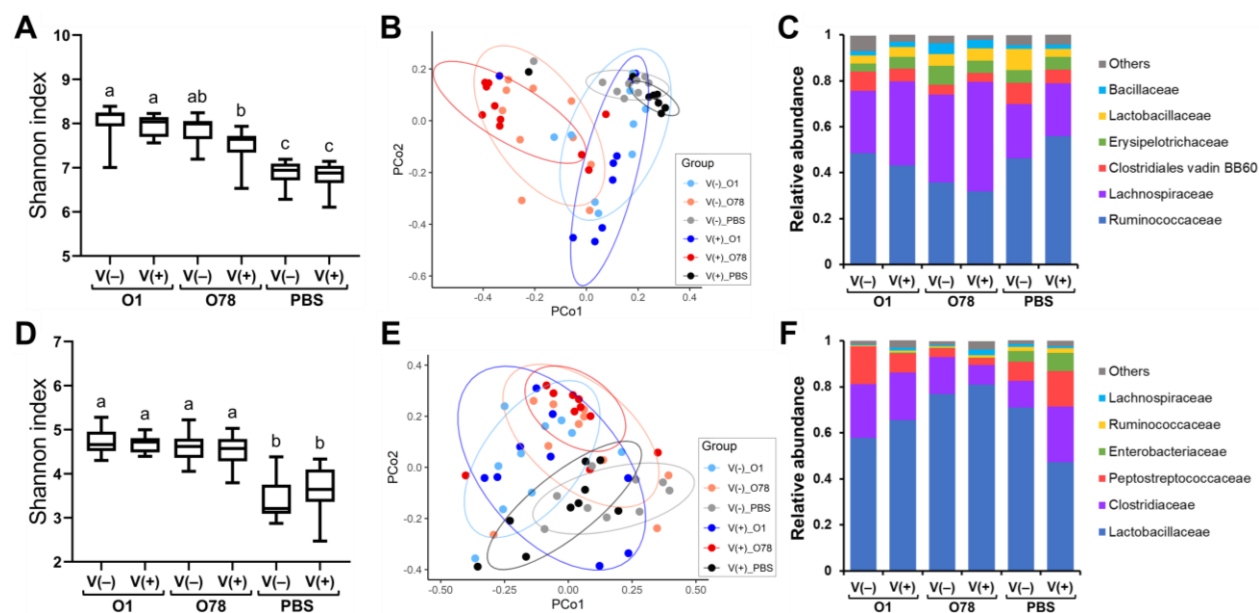


Figure 3-4. Analysis of the chicken intestinal microbiota using 16S rRNA gene amplicon sequencing. Shannon diversity of bacterial community in cecum (A) and ileum (D) of the six groups of chickens. Principal coordinate analysis, based on Bray-Curtis distance, on microbial structure in the cecum (B) and ileum (E). Relative abundance of specific families in the cecum (C) and ileum (F). Each bar represented averaged abundance of nice or ten individual samples within the group. The V(+) and V(-) indicated vaccinated and unvaccinated chickens, respectively, while O1, O78, and PBS denoted specific inoculum used for intratracheal challenge.

Chapter IV: Passive Immunization of Chickens with Anti-enterobactin Egg Yolk Powder for *Campylobacter* Control

A version of this chapter was originally published by Huiwen Wang, Ximin Zeng, Liu Cao, Qiang He, and Jun Lin on *Vaccine*, and by Ximin Zeng, Huiwen Wang, Canghai Huang, Catherine Logue, Nicole Barbieri, Lisa Nolan, and Jun Lin on *Frontiers in Immunology*.

Wang H, Zeng X, Cao L, He Q, and Lin J. 2021. Passive immunization of chickens with anti-enterobactin egg yolk powder for *Campylobacter* control. *Vaccines*. 9:569.

Zeng, X, Wang H, Huang C, Logue CM, Barbieri NL, Nolan LK, Lin J. 2021. Evaluation of the immunogenic response of a novel enterobactin conjugate vaccine in chickens for the production of enterobactin-specific egg yolk antibodies. *Frontiers in Immunology*. 12:629480.

Abstract

Enterobactin (Ent) is a highly conserved and important siderophore for the growth of many Gram-negative bacterial pathogens. Therefore, targeting Ent for developing innovative intervention strategies has attracted substantial research interests in recent years. Recently, we developed a novel Ent conjugate vaccine that has been demonstrated to be effective for controlling Gram-negative pathogens using both *in vitro* and *in vivo* systems. In particular, active immunization of chickens with the Ent conjugate vaccine elicited strong immune responses and significantly reduced intestinal colonization of *Campylobacter jejuni*, the leading foodborne bacterial pathogen. Given that hyperimmune egg yolk immunoglobulin Y (IgY) has been increasingly recognized as a promising and practical non-antibiotic approach for passive immune protection against pathogens in livestock, in this study, we assessed the efficacy of oral administration of broiler chickens with the anti-Ent hyperimmune egg yolk powder to control *C. jejuni* colonization in the intestine. However, supplementation of feed with 2% (w/w) of anti-Ent egg yolk powder failed to reduce *C. jejuni* colonization when compared to control group. Consistent with this finding, the ELISA titers of specific IgY in cecum, ileum, duodenum, gizzard, and serum were similar between the two groups throughout the trial. Neither was chicken intestinal microbiota changed in response to the egg yolk powder treatment. Subsequently, to examine *ex vivo* stability of egg yolk IgY, the chicken gizzard and duodenum contents from two independent sources were spiked with the egg yolk antibodies, incubated at 42 °C for different lengths of time, and subjected to ELISA analysis. The specific IgY titers were dramatically decreased in gizzard contents (up to 2,048 fold) but were not changed in duodenum contents. Collectively, oral administration of broiler chickens with the anti-Ent egg yolk powder failed to confer protection against intestinal colonization of *C. jejuni*, which was due to instability of IgY in gizzard as demonstrated by both *in vivo* and *ex vivo* evidence.

Keywords: anti-enterobactin egg yolk antibody, passive immunity, *Campylobacter jejuni*, chicken, food safety

4.1. Introduction

Hen's egg contains a large quantity of egg yolk antibodies (immunoglobulin Y, IgY), which are transferred from serum to yolk during egg formation. The vertically transferred antibodies can provide passive immune protection for the chick, either at embryonic or post-hatching stage, against pathogens (1). In recent decades, egg yolk IgY has drawn considerable interests as a passive immune agent and a promising alternative to antibiotics due to several unique features (2, 3). A hen can produce 15-30 g egg yolk IgY yearly with 2-10% being antigen-specific. Egg yolk IgY is fairly stable under wide pH range (3.5-11) and high temperature (up to 70 °C), making it feasible for storage and processing as feed supplement for food animals. Finally, large-scale maintenance of hens for mass production of desired egg yolk IgY is cost-effective, and collection of egg yolk IgY—a non-invasive practice—is favorable to animal welfare. Consequently, numerous studies have been conducted to develop antigen-specific egg yolk IgY for passive immune protection of food animals, aquaculture, and humans against diverse microbial pathogens, showing partial successes of this approach (1, 2). Therefore, developing pathogen-specific egg yolk IgY is a promising approach to prevent and control microbial infections in food animals for enhanced animal health and food safety (1, 3).

Campylobacter is the leading bacterial cause of human gastroenteritis worldwide.

Campylobacteriosis is responsible for 400-500 million cases of diarrhea each year; the mortality is estimated to be 24 deaths per 10,000 culture-confirmed cases (4). This organism is prevalent in food animals, especially in poultry, posing a significant risk of human infections from Farm to Fork (5). Therefore, effective control of *Campylobacter* in poultry, the primary animal reservoir, would reduce the risk of human exposure to this pathogen and have a significant impact on food safety and public health (6). *Campylobacter* colonization in poultry is determined by the factors involved in complex bacterium-host interaction and yet to be fully elucidated, which generally include bacterial metabolism, virulence, and stress response, as well as chicken immune system and intestinal microbiota (7). Over the past decades, the increasing antibiotic resistance due to extensive use of antibiotics in livestock has raised an urgent need of developing non-antibiotic approaches, such as vaccines and probiotics (5, 8), to control foodborne pathogens including *Campylobacter*. However, these efforts have only gained limited successes; developing novel

and practical alternative approaches for *Campylobacter* control in poultry is still highly warranted (5).

Recently, we developed a unique conjugate vaccine targeting enterobactin (Ent) (9), a small siderophore compound utilized by different Gram-negative pathogens, including *Campylobacter*, for *in vivo* growth and colonization (10, 11). The anti-Ent antibodies induced by the Ent conjugate vaccine inhibited *in vitro* growth of diverse *Campylobacter* strains (9, 12). More importantly, intramuscular immunization of chickens with the Ent conjugate vaccine elicited high levels of systemic anti-Ent IgY, leading to approximately 3-4 log₁₀ unit reduction of *C. jejuni* in the intestine, which was consistently observed in two independent trials (13). However, despite the significant protection of the Ent conjugate vaccine demonstrated in this proof-of-concept study (13), it was recognized that using injectable vaccine for *Campylobacter* control in poultry production system is not cost-effective. To address this issue, we proposed to produce a large quantity of anti-Ent hyperimmune egg yolk IgY by immunizing layers with the same Ent conjugate vaccine. Then supplementation of chicken feed with the anti-Ent egg yolk powder would control *C. jejuni* colonization in the chicken intestine. This was tested in this study using a well established chicken model of *C. jejuni* infection. Notably, this study, for the first time, assessed availability and stability of the hyperimmune egg yolk IgY in different segments of chicken gastrointestinal (GI) tract using both *in vivo* and *ex vivo* systems.

4.2. Materials and methods

4.2.1. Production of anti-Ent hyperimmune egg yolk

To produce a large quantity of anti-Ent hyperimmune egg yolk, fertilized specific-pathogen-free White Leghorn eggs were purchased from Charles River Laboratories (MA, USA) and hatched in the Poultry Diagnostic and Research Center at the University of Georgia ((IACUC No. A2018 01-014-Y1-A0). At the age of approximately 26 weeks and in full lay, ten layers were immunized subcutaneously in the neck with 100 µg of KLH-Ent conjugate that was emulsified with Freund's complete adjuvant. Two booster immunizations (100 µg of Ent conjugate emulsified with Freund's incomplete adjuvant) were performed at 3 and 5 weeks after the primary immunization, respectively. Blood samples were collected from the wing vein of each

chicken at different time points to monitor circulating IgY antibody levels. Eggs were collected every day from each chicken and stored at 4 °C prior to egg yolk processing and analysis.

4.2.2. Preparation of egg yolk-supplemented chicken feed

The hyperimmune egg yolks were lyophilized into powder using a freeze dryer (VirTis AdVantage Plus EL-85 Benchtop Lyophilizer, SP Scientific Inc., NY, USA) following a published protocol (14) with slight modifications. Briefly, the anti-Ent hyperimmune egg yolks were pooled, diluted with about half volume of sterile PBS, and homogenized using a sterilized egg whisk. Then the mixture was transferred into trays with about 500 ml per tray. The trays were wrapped by aluminum foil with several small pores for evaporation and put in -80 °C freezer overnight for complete solidification of egg yolks prior to lyophilization. Subsequently, the egg yolk mixture in trays was processed in lyophilizer for about 72 hours. Lastly, the lyophilized egg yolk was pulverized into rough powder and stored at -20 °C until use. Prior to chicken trial, the feed was supplemented with 2% (w/w) of the freeze-dried egg yolk powder; the dosage of egg yolk powder supplemented in feed (2%) was at upper range in most of previous animal trials (from 0.04% to 2%) (15-17). The unused egg yolk powder-supplemented feed was routinely stored at 4 °C in chicken facility.

4.2.3. Chicken trial for passive immunization

The chicken experiment was approved by Institutional Animal Care and Use Committee at The University of Tennessee, Knoxville with protocol number 1387-0219. Briefly, 30 newly hatched broiler chicks were assigned into two groups that received regular feed (control group, n=15) or the anti-Ent egg yolk powder-supplemented feed (treatment group, n=15) for 14 days. Fresh feed was replenished every 1-2 days. At 5 days of age, 5 chicks in each group were removed for blood sample collection and then euthanized for collecting GI contents (*i.e.*, cecum and ileum); the remaining chicks in each group were challenged with 1.5×10^6 colony forming units (CFU) of *C. jejuni* NCTC 11168 via oral gavage. At 9 and 14 days of age (*i.e.*, 4 and 9 days post-infection [DPI]), 5 chicks per group were subjected to collection of blood and various GI contents (*i.e.*, cecum, ileum, duodenum, and gizzard). Each of cecum and ileum contents was weighed and serially diluted in Mueller-Hinton (MH) broth (Difco, MD, USA). The diluted samples were plated on MH agar plates supplemented with *Campylobacter*-specific selective supplements

(Oxoid, UK) and the agar plates were incubated in a microaerobic chamber (5% O₂, 10% CO₂, and 85% N₂) at 42 °C for 2 days. Following incubation, the *C. jejuni* colonies were enumerated and the log₁₀-transformed CFU per gram of intestinal contents were calculated to reflect *C. jejuni* colonization level. The other samples were stored at -80 °C prior to immunological and molecular analyses as described below.

4.2.4. Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was conducted to determine specific IgY titer as described previously (9). Briefly, the ELISA plates (Nunc MaxiSorp, Thermo Fisher, USA) were coated with 100 µL of Ent conjugate (300 ng/mL) in coating buffer (bicarbonate/carbonate coating buffer, pH 9.6) overnight at room temperature. The coated plates were blocked with blocking buffer (PBS + 0.05% Tween 20 + 5% skim milk) for 1 hour at room temperature. Unless specified, each solid (feed and GI contents) or semisolid (egg yolk) sample was gently mixed with saline and supernatant was collected after centrifugation at 5,000 *g* for 2 minutes. The supernatant samples as well as blood samples were 2-fold serially diluted in blocking buffer and incubated with the coated plates for 1 hour at room temperature. Following washing procedure (PBS + 0.05% Tween 20), horse-radish peroxidase-conjugated goat anti-chicken IgY secondary antibody (SeraCare, MA, USA) was added (2,000 fold diluted in blocking buffer) and the plates were incubated at room temperature for 1 hour, followed by washing again. The plates were finally developed using ABTS Peroxidase Substrate Kit (SeraCare) and the reaction was stopped after 30 minutes by adding 100 µL of stop solution (1% SDS). The absorbance was measured at optical density of 405 nm (OD₄₀₅) using a microplate reader (BioTek Instruments, VT, USA) and data were collected using Gen5 software (BioTek Instruments). The wells without addition of primary antibody served as background control. Endpoint titer was defined as the last dilution at which OD₄₀₅ of sample wells exceeded the cutoff value (0.1). Duplicate measurements were performed for each sample.

4.2.5. Intestinal microbiota analysis

Genomic DNA was extracted from cecum or ileum content using QIAamp PowerFecal Pro DNA Kit (QIAGEN, USA). The purified genomic DNA was used as the template for PCR amplification targeting V4 region of the 16S rRNA gene and using the barcoded primer pair of

515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (18). The PCR was performed in a 25 µL cocktail mix containing 12.5 µL Phusion Flash PCR Master Mix (Thermo Scientific, USA), 8.5 µL ultra-pure water, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM), and 2 µL (100 to 150 ng) DNA template. The PCR program included initial denaturation at 94 °C for 3 minutes, followed by 35 cycles at 94 °C for 45 seconds, 55 °C for 60 seconds, and 72 °C for 90 seconds, with a final extension at 72 °C for 10 minutes. After amplification, the PCR products were purified using ChargeSwitch™ PCR Clean-Up Kit (Invitrogen, CA, USA) and quantified using NanoDrop spectrophotometer (Thermo Fisher, USA). Subsequently, the purified products were pooled together in equal concentration and further quantified using KAPA Library Quantification Universal Kit (Roche, USA). Before being loaded to MiSeq Reagent Kit v3 (Illumina, USA), the library was denatured, diluted, and spiked with PhiX library as an internal control according to the protocol for 16S Metagenomic Sequencing Library Preparation (Illumina, USA) (19). Sequencing of the amplicon libraries was performed with an Illumina MiSeq System.

After sequencing operations, the acquired amplicon sequence data were analyzed in QIIME 2 using DADA2 pipeline and scikit-learn classifier with reference database SILVA release 138 (20-22). Principal coordinate analysis (PCoA) on microbiota data was conducted and visualized using the R package phyloseq (23). The raw sequencing data are publicly available in the NCBI Sequence Read Archive with BioSample accession numbers from SAMN18699372 to SAMN18699431 under BioProject ID PRJNA721092 (<https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA721092>).

4.2.6. *Ex vivo* stability of egg yolk IgY

The GI contents obtained from two independent sources of broilers were used for evaluation of *ex vivo* stability of egg yolk IgY. The freeze-dried anti-Ent egg yolk powder was dissolved in saline at the ratio of 1:3 (w/w), followed by centrifugation at 13,000 g for 2 minutes. The supernatant containing specific egg yolk IgY was used to spike in various samples to evaluate *ex vivo* stability of specific IgY as described below.

In the first assay, duodenum and gizzard contents were obtained from the euthanized broilers (7 weeks old) in a slaughter plant in Mississippi (courtesy of Dr. Li Zhang, Mississippi State University); each sample was comprised of pooled contents from 5 randomly selected chickens. The samples were immediately packed with ice packs and shipped to our laboratory *via* overnight express carrier. Upon receiving the samples, the duodenum contents were gently mixed with 1/3 volume of sterile saline and centrifuged at 5,000 g for 5 minutes. The supernatant was spiked with the aforementioned egg yolk IgY solution at the ratio of 7:1 (v:v), gently mixed, and incubated at 42 °C (chicken body temperature) for 2.5 hours; the sample mixed with saline served as a control. The gizzard contents were gently mixed with the same volume of saline and centrifuged at 5,000 g for 5 minutes. The supernatant was collected and the pH of aliquot was adjusted to either 3.0 or 7.0 using less than 2% volume of HCl or NaOH, respectively. Subsequently, the pH-adjusted gizzard sample was mixed with the egg yolk IgY solution at the ratio of 7:1 (v:v) and the mixture was incubated at 42 °C for 1.5 hours; the sample mixed with saline served as a control. Upon completion of incubation, the mixtures were subjected to ELISA analysis for assessing specific IgY titer as described above.

In the second assay, fresh gizzard contents from 5 euthanized broilers at the age of 6 weeks were collected, pooled, and immediately used for stability assay. The fresh gizzard content sample was gently mixed with same volume of saline and centrifuged at 5,000 g for 5 minutes. The pH of supernatant was measured. Subsequently, the gizzard supernatant and HCl solution (1 mM, pH 3.0) were mixed with the egg yolk IgY solution at the ratio of 20:1 (v:v), respectively, and the mixture was incubated at 42 °C for 30 or 60 minutes; the sample mixed with saline served as a control. Upon completion of incubation, the mixtures were subjected to ELISA analysis for assessing specific IgY titer as described above.

4.2.7. Statistical analysis

Statistical analyses were conducted using Mann-Whitney test in GraphPad Prism 8. Data, unless specified, were presented as mean $\pm$ standard deviation (SD). A probability level of $P < 0.05$ was considered a statistically significant difference.

4.3. Results

4.3.1. The vaccine induced strong antibody responses in chicken serum and egg yolk

As shown in Figure 4-1A, the mean IgY titers directed against whole conjugate vaccine dramatically increased 3 weeks after primary immunization, continued to increase upon boost immunizations, and remained at high level at 9 weeks postimmunization in both serum and yolk. Notably, this trend was observed in each individual chicken with IgY titer increase in serum ranging from 64 to 128 fold and in yolk ranging from 512 to 8,192 fold at 9 weeks postimmunization (data not shown). The Ent-specific immune response was also significantly induced in all the vaccinated chickens (Figure 4-1B), showing similar titer increase pattern as that against the whole conjugate (Figure 4-1A). At 9 weeks postimmunization, the mean titer of Ent-specific IgY was increased by approximately 16 and 256 fold in serum and egg yolk, respectively (Figure 4-1B).

4.3.2. Specific egg yolk IgY was highly stable in chicken feed

ELISA was performed to examine the stability of the Ent conjugate vaccine-specific IgY in the daily supplied feed containing hyperimmune egg yolk powder. As shown in Figure 4-2A, the specific IgY titer in the newly prepared feed that was supplemented with 2% of egg yolk powder (0 d) was 14 log₂ units higher than that in control feed. After 2 days of exposure to room temperature at more than 32 °C in the chicken house, the specific IgY (2 d) still remained at a high level with more than 8,000 fold higher than that in control feed (Figure 4-2A).

4.3.3. The anti-Ent egg yolk failed to reduce intestinal colonization of *C. jejuni*

As shown in Figure 4-2B & C, supplementation of chicken feed with the anti-Ent egg yolk powder did not significantly reduce *C. jejuni* colonization in the chicken intestine when compared to control feed. Specifically, as expected, all the five randomly selected chickens in each group before *C. jejuni* challenge (0 DPI) were *C. jejuni* free in both cecum and ileum (below the detection limit of 100 CFU/g) (Figure 4-2B & C). At 4 days following *C. jejuni* challenge (4 DPI), the chickens in both groups displayed high level of *C. jejuni* colonization in the cecum; interestingly, the *C. jejuni* level in Ent group (9.38 log₁₀ CFU/g) was even higher ($P < 0.05$) than that in control group (8.46 log₁₀ CFU/g) (Figure 4-2B). By 9 DPI, anti-Ent egg yolk still did not exert significant inhibition on *C. jejuni* colonization in the cecum (Figure 4-2B). As

expected, for the chickens within the same group, the level of *C. jejuni* in the ileum was significantly lower ($P < 0.05$) than that in the counterpart cecum at 4 or 9 DPI (Figure 4-2B & C). Similar to the finding in cecum, there was no significant difference in *C. jejuni* colonization in ileum between control and Ent groups (Figure 4-2C).

4.3.4. Cecum and ileum microbiota was not dramatically affected by anti-Ent egg yolk

16S rRNA gene amplicon sequencing was conducted to examine the changes of intestinal microbiota in response to anti-Ent egg yolk treatment. Regarding Shannon diversity (Figure 4-3A), the microbiota exhibited similar diversity between control and Ent groups in both cecum ($P = 0.06$) and ileum ($P = 0.60$). To compare microbial structure among different samples, PCoA was performed based on Bray-Curtis distance. As shown in Figure 4-3B, the dots of cecum microbiota in control group were more clustered than those in Ent group (0.51 ± 0.16 vs. 0.58 ± 0.20), indicating significant dissimilarity in the structure of cecum microbiota between the two groups ($P < 0.01$). The dots of ileum microbiota in control or Ent groups were similarly scattered (0.57 ± 0.24 vs. 0.62 ± 0.31), meaning no significant difference in their microbiota structure ($P = 0.18$). We also looked at the specific taxonomic composition of the intestinal microbiota in each group (Figure 4-3C). The families of Lachnospiraceae and Enterococcaceae affiliated to the phylum Firmicutes were predominant in cecum and ileum, respectively. The second abundant phylum was Proteobacteria (mainly the family Enterobacteriaceae) (Figure 4-3C). Generally, there was no dramatic difference in taxonomic composition between control and Ent groups at different time points (Figure 4-3C). In addition, there was a notable expansion of Campylobacteraceae (the family *C. jejuni* belongs to) in both treatment groups at days 9 and 14 (*i.e.*, 4 and 9 DPI) in cecum but not ileum (Figure 4-3C). Taken together, microbiota analysis revealed there was no dramatic change in intestinal bacterial community caused by anti-Ent egg yolk.

4.3.5. The levels of specific egg yolk IgY were not significantly different between treatment groups

To properly interpret the findings resulting from oral administration of hyperimmune egg yolk powder, it was critically important to examine the level and bioavailability of specific IgY in different GI segments as well as the interlinked circulating system. Therefore, in this study,

ELISA was further performed to determine the titer of Ent conjugate-specific IgY in different GI contents (*i.e.*, cecum, ileum, duodenum, and gizzard) as well as serum samples collected from individual chickens. We hypothesized that the titer of specific IgY in the chickens receiving hyperimmune egg yolk powder-supplemented feed should be significantly higher than that in the chickens receiving control feed although the magnitude of difference might vary with respect to different GI segments.

However, despite the exceptionally high level of specific egg IgY in the treatment feed (Figure 4-2A), unexpectedly, the levels of specific egg yolk IgY in the cecum (Figure 4-4A), ileum (Figure 4-4B), duodenum (Figure 4-4C), and gizzard (Figure 4-4D) of Ent group were consistently low or below the detection limit at different time points, which were similar to those observed in the control group (Figure 4-4A-D). Although serum samples displayed high basal level of specific IgY titer, there was no significant difference in the titer of the specific egg yolk IgY in chicken serum between control and Ent groups at all three sampling days (Figure 4-4E). These surprising findings strongly suggested that the ingested specific egg yolk IgY was not stable when passing through GI tract, particularly gizzard.

4.3.6. *Ex vivo* assays revealed instability of egg yolk IgY in gizzard

To evaluate the stability of egg yolk IgY in gizzard and duodenum, we collected gizzard and duodenum contents from two independent sources of broilers (6-7 weeks old) and conducted *ex vivo* assays.

In the first assay, treatment of hyperimmune egg yolk IgY in duodenum content at 42 °C for 2.5 hours led to the same high titer of specific IgY as that in saline control, indicating that egg yolk IgY was stable in the duodenum content even after a long time of treatment (Figure 4-5A). Meanwhile, the duodenum content without addition of egg yolk IgY (negative control) displayed a very low basal level of the specific IgY (Figure 4-5A). For the *ex vivo* stability assay using gizzard content (Figure 4-5B), when compared to the saline control that displayed high level of IgY titer, the gizzard content with pH 3.0 showed dramatically reduced titer of the specific IgY that was below detection limit. However, when the pH of gizzard content was adjusted to 7.0, the IgY titer was as high as that observed in the saline control (Figure 4-5B). In addition, the titer of

specific IgY in the gizzard content (pH either 3.0 or 7.0) without addition of egg yolk IgY (negative controls) was below detection limit, indicating gizzard content had extremely low background noise for measuring the specific egg yolk IgY using ELISA (Figure 4-5B). Collectively, the findings from the first *ex vivo* assay indicated that egg yolk IgY was very unstable in chicken gizzard, which was dependent on the acidic environment of the gizzard.

The first assay had three potential limitations. First, the intestinal contents were obtained from a distant state *via* overnight shipping, which might affect sample quality for the *ex vivo* assay. Second, the pH of gizzard content extract was purposely adjusted to 3.0 to mimic average pH condition in chicken gizzard. However, using the original saline-extracted gizzard content (with slightly higher pH than 3.0) for stability assay would provide additional clue to explain instability of IgY in gizzard. Finally, although the instability of egg yolk IgY was dependent on low pH, it was still not clear this was due to direct impact (*i.e.*, denaturation of IgY) or indirect impact (*i.e.*, low pH-dependent activity of pepsin, a proteolytic enzyme in gizzard). To address these issues, another assay was performed using freshly prepared gizzard contents with additional controls and time points (Figure 4-5C). Consistent with the findings in the first assay (Figure 4-5B), incubation of egg yolk IgY in the gizzard content extract with natural pH 3.3 for as short as 30 minutes led to 32 fold reduction in the specific IgY titer when compared to saline control (Figure 4-5C). Notably, incubation of egg yolk IgY with hydrochloric acid solution (pH 3.0) for up to 60 minutes did not affect the titer of the specific IgY at all, indicating acidic denaturation did not contribute to the instability of IgY in gizzard content (Figure 4-5C).

4.4. Discussion

Passive immunization with specific egg yolk antibodies is emerging as a promising alternative to antibiotics for the treatment and prevention of various animal and human diseases (1, 2). In particular, hyperimmune egg yolk IgY has been commonly administrated orally through feed or drinking water to prevent and control enteric diseases in food animals (1, 15). Our recent study (13) demonstrated that immunization of chickens with the novel Ent conjugate vaccine significantly reduced *C. jejuni* colonization in the intestine. In this study, we produced a large quantity of anti-Ent hyperimmune egg yolk and examined the efficacy of oral administration of the anti-Ent egg yolk IgY for *C. jejuni* control in broiler chickens. Surprisingly, we did not

observe any protection conferred by the anti-Ent egg yolk IgY against *C. jejuni* colonization in the intestine. Subsequently, we were more surprised at the finding that the specific IgY was barely detected in different sections of GI tract including the upper digestive organ gizzard. Notably, to our best knowledge, none of previous animal trials evaluating the efficacy of egg yolk IgY has examined the level and bioavailability of egg yolk IgY in the GI tract following the ingestion. Given the harsh environment encountered by egg yolk IgY in the GI tract (*e.g.*, low pH and proteases), *in vivo* availability of the egg yolk IgY is a key issue that needs to be addressed for data interpretation and understanding the mechanism of passive immunity conferred by egg yolk IgY. This study, for the first time, utilized ELISA to directly quantitatively measure the specific egg yolk IgY level along different sites of GI tract as well as in systemic circulation (Figure 4-4). Examining the level of specific egg yolk IgY in serum can provide insights into the potential absorption of egg yolk IgY into systemic circulation through intestinal epithelia and justify oral administration of egg yolk IgY for passive immune protection against systemic infections.

To confirm the *in vivo* finding—instability of IgY in the GI tract—and to better understand the fate of egg yolk IgY in chicken's GI tract following the ingestion, we also performed *ex vivo* assays to examine the stability of egg yolk IgY in both gizzard and duodenum contents in this study. Gizzard and duodenum are two major digestive organs in chickens. In gizzard, the pepsin under the acidic condition maintained by secreted hydrochloric acid, together with grinding activity, play an important role in initial digestion of feed with about 60 minutes of retention (24). Duodenum, the upper digestive organ immediately after gizzard, contains digestive enzymes, such as the trypsin released from pancreas (25). Using a well controlled system plus different sources of samples, our *ex vivo* assays provide compelling evidence demonstrating that gizzard environment dramatically destabilized egg yolk IgY, which was likely mediated through potent enzymatic degradation by pepsin (Figure 4-5). Using hydrochloric acid solution (pH 3.0) as a control (Figure 4-5C), we have ruled out the possibility that acidic condition might denature egg yolk IgY and/or affect antigen-antibody interaction during ELISA assay, which might lead to the observed low IgY titer in gizzard content. It has been reported that chicken IgY was resistant to bovine trypsin and chymotrypsin treatment but could be degraded in swine pepsin (26). Notably, functionality of chicken pepsin strictly relies on low pH with optimal activity at pH 2.8

(27, 28), which may explain why magnitude of IgY titer reduction in original gizzard extract (pH 3.3) (Figure 4-5C) is lower than that in the extract adjusted to pH 3.0 (Figure 4-5B). At this stage, it is still unknown if chicken age can influence the instability of IgY in gizzard. In addition, direct evidence showing degradation of egg yolk IgY by the pepsin in chicken gizzard is still lacking. To address these issues, a comprehensive study using various individual samples in conjunction with molecular and biochemical approaches is highly warranted in the future.

Based on the findings from this study, potential use of hyperimmune egg yolk powder to control enteric diseases should be reevaluated in poultry and other food animals in the future. In particular, the *in vivo* stability of egg yolk IgY needs to be investigated in parallel to other phenotype assessments. In addition, to increase stability and bioavailability of administrated egg yolk IgY in GI tract, protective coating of the IgY is a feasible strategy. Previously, methacrylic acid copolymer, chitosan-alginate, and alginate/carrageenan hydrogels have been utilized for encapsulation of specific egg yolk IgY, which, to different extents, prevented activity loss of the IgY and achieved controlled releases of the IgY in a simulated GI fluid (29-31). In the future, cost-effective encapsulation methods should be developed for site-specific delivery and controlled release of innovative hyperimmune egg yolk IgY used in livestock, such as the anti-Ent egg yolk IgY investigated in this study.

Although passive immunization with the anti-Ent hyperimmune egg yolk IgY failed to reduce intestinal colonization of *C. jejuni*, we will continue to explore practical and cost-effective vaccination routes for the Ent conjugate vaccine to control Gram-negative pathogens in poultry. For instance, *in ovo* vaccination, especially with the adoption of automatic *in ovo* injection system (32), is an attractive approach for poultry industry to control infectious diseases (33). In addition, mucosal vaccination through oculo-nasal route or spray is a potentially convenient approach to induce desired local mucosal immunity (*e.g.*, respiratory or GI tract) in poultry and other food animals (34). Given that the pathogens targeted by the Ent conjugate vaccine are primarily involved in mucosal infections (*e.g.*, avian pathogenic *Escherichia coli* in the respiratory tract (35); *C. jejuni* and *Salmonella enterica* in the intestinal tract (36, 37)), this strategy, with proper adjuvants and delivery systems (38), would also expand the application of the Ent conjugate vaccine in food animals.

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Appendix

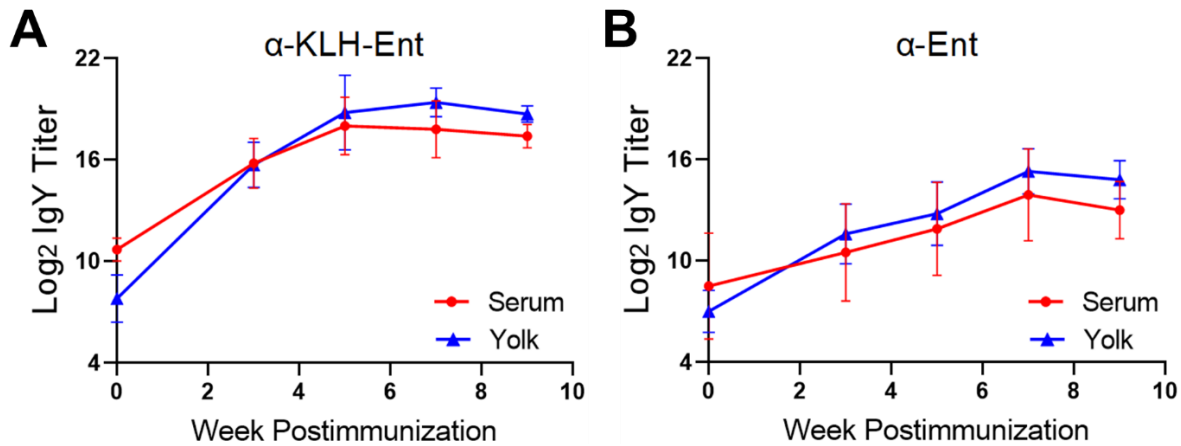


Figure 4-1. Immune responses of the layers immunized with the KLH-Ent conjugate. A total of ten White Leghorn laying hens received subcutaneous injections with the KLH-Ent conjugate three times. (A) Serum and egg yolk IgY titer against the KLH-Ent conjugate. Each point represents mean IgY titer $\pm$ standard deviation from ten layers with duplicate measurements within each chicken. (B) Serum and egg yolk IgY titer against Ent molecule. Each point represents mean IgY titer $\pm$ standard deviation from ten layers with duplicate measurements within each chicken.

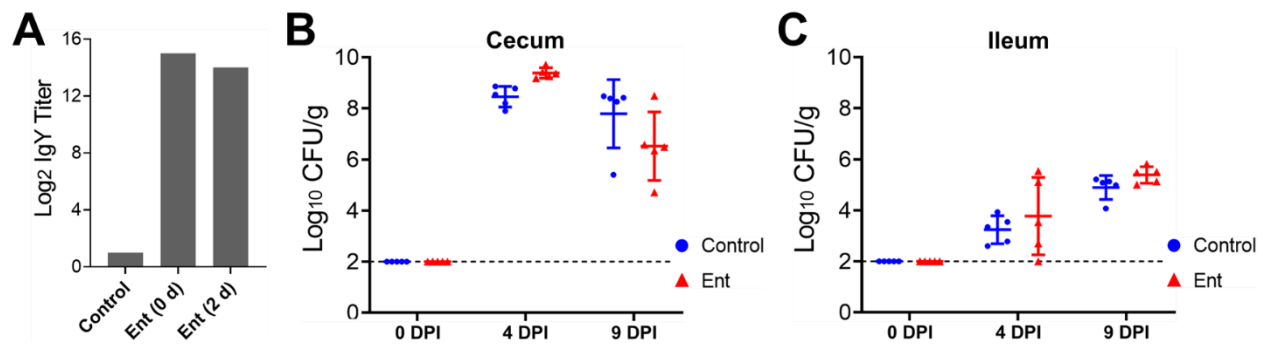


Figure 4-2. *C. jejuni* colonization levels in the chicken intestine upon anti-Ent egg yolk powder treatment. (A) Ent conjugate vaccine-specific IgY titer in chicken feed. ELISA was performed to determine the titer of Ent conjugate-specific egg yolk IgY in control feed (regular feed) or the feed supplemented with 2% (w/w) hyperimmune egg yolk powder (designated as “Ent”). With respect to the feed supplemented with egg yolk powder, both freshly prepared feed (0 d) and the feed with 2 days of exposure in chicken house (2 d) were subjected to ELISA analysis. Each bar represents duplicate measurements of the sample. (B) colonization of *C. jejuni* NCTC 11168 in the cecum. (C) Colonization of *C. jejuni* NCTC 11168 in the ileum. The intestinal samples were collected from 0, 4, and 9 DPI for *C. jejuni* enumeration. Each dot represents log₁₀-transformed CFU per gram of intestinal contents in an individual chicken. The bar represents mean±SD within each treatment group. The dashed line indicates the detection limit.

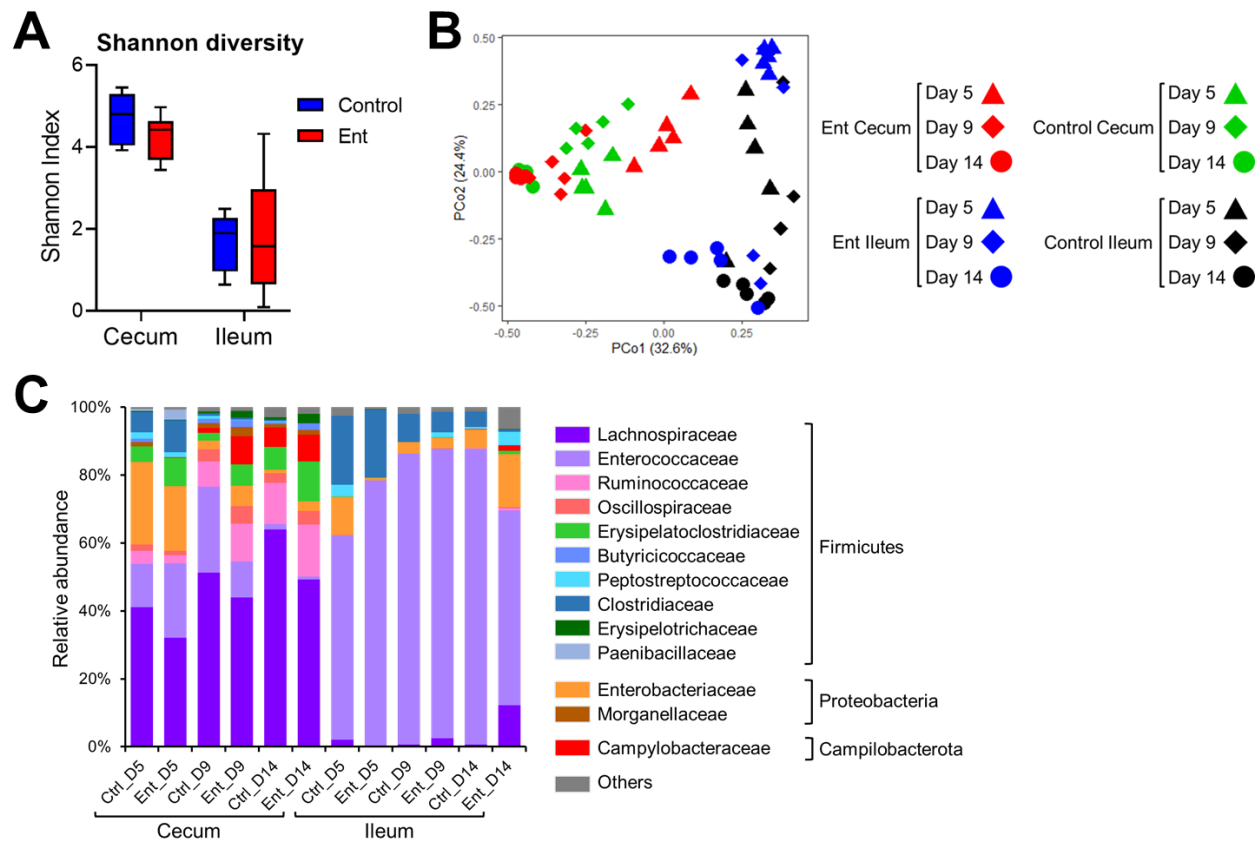


Figure 4-3. Analysis of intestinal microbiota in chickens using 16S rRNA gene amplicon sequencing. (A) Shannon diversity of bacterial community in the cecum and ileum. (B) Principal coordinate analysis, based on Bray-Curtis distance, on microbiota structure in cecum and ileum. (C) Relative abundance of specific family/phylum in the cecum or ileum of the chickens in two groups at different time points. Each bar represents the averaged abundance of 5 samples within each group.

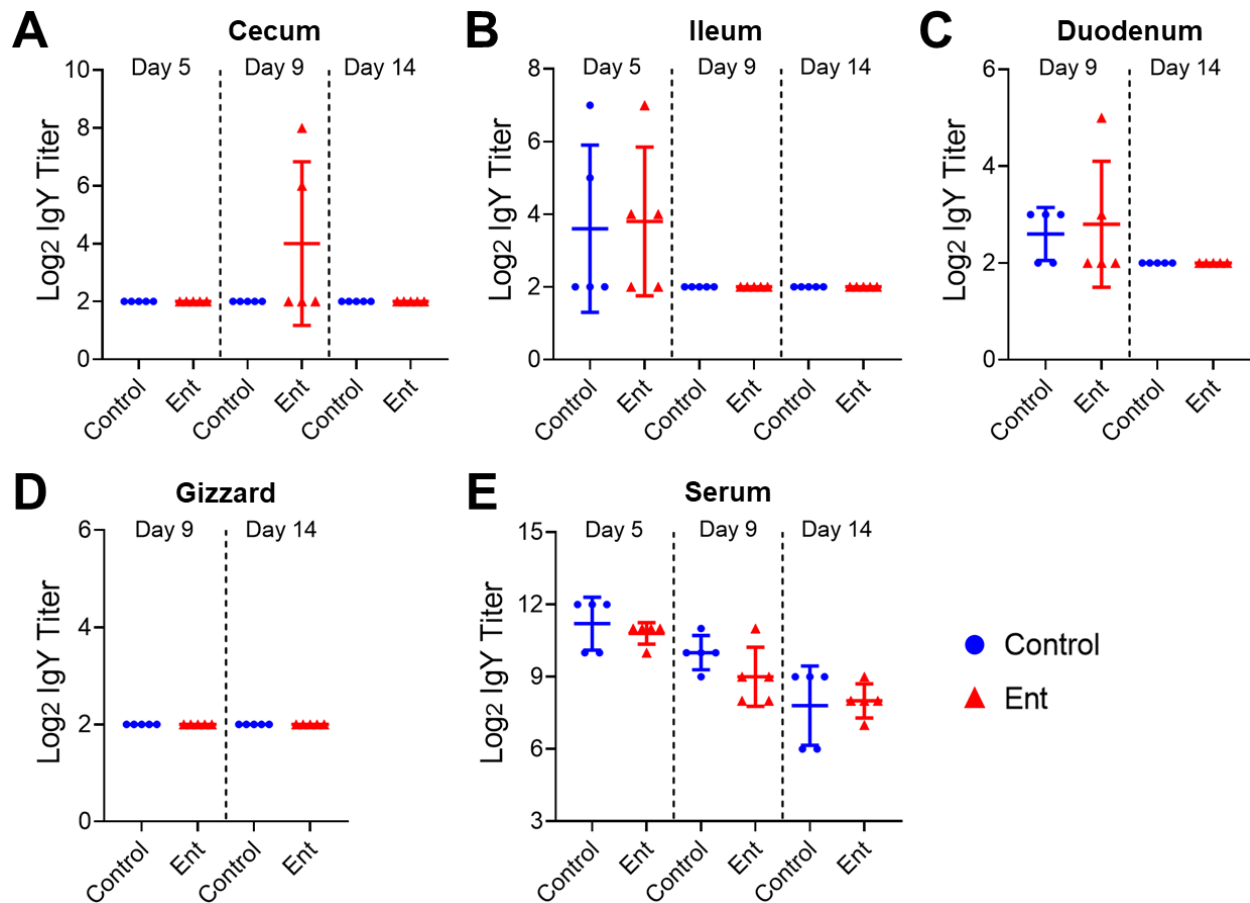


Figure 4-4. The levels of specific egg yolk IgY in different GI contents and serum. ELISA was performed to determine the titers of Ent conjugate-specific IgY in the (A) cecum, (B) ileum, (C) duodenum, (D) gizzard, and (E) serum collected from individual chickens fed with control or Ent feed at different time points. Each dot represents duplicate measurements of a specific sample. Each bar represents mean $\pm$ SD within each group.

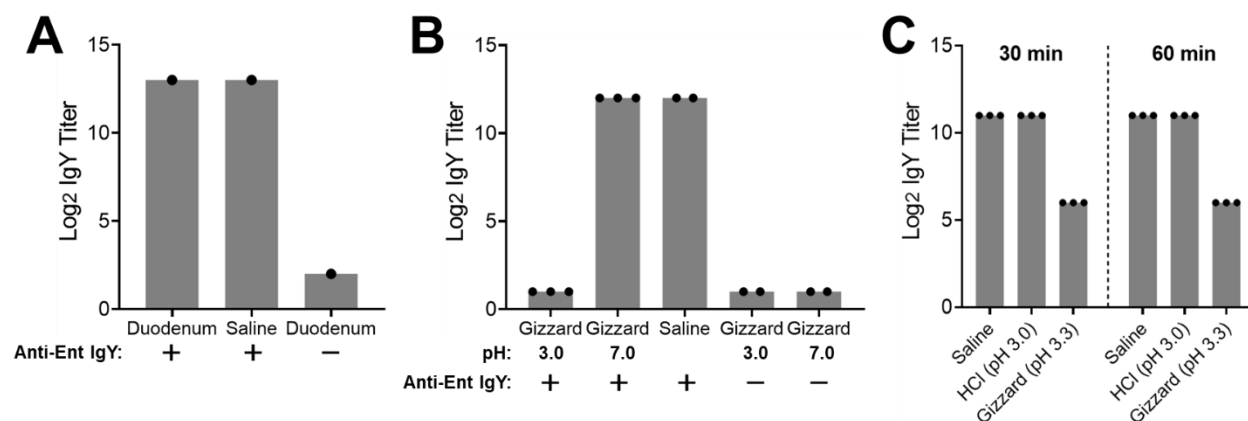


Figure 4-5. Ex vivo stability of the specific egg yolk IgY in duodenum and gizzard contents.

(A) *Ex vivo* stability of the specific egg yolk IgY in duodenum. The duodenum content was spiked with the hyperimmune egg yolk IgY and incubated at 42 °C for 2.5 hours, followed by ELISA analysis of the specific IgY titer. The saline spiked with the hyperimmune egg yolk IgY served as a positive control while the duodenum content without addition of egg yolk IgY served as a negative control (detailed in *Materials and Methods*). (B) *Ex vivo* stability of the specific egg yolk IgY in gizzard. The gizzard content (pH 3.0 or 7.0) was spiked with the hyperimmune egg yolk IgY and incubated at 42 °C for 1.5 hours, followed by ELISA analysis of the specific IgY titer. The saline spiked with the hyperimmune egg yolk IgY served as a positive control while the gizzard contents without addition of egg yolk IgY served as negative controls. (C) Effect of pH and incubation time on *ex vivo* stability of the specific egg yolk IgY in gizzard. Fresh gizzard content was collected and immediately subjected to *ex vivo* stability assay. The freshly prepared gizzard content extract (pH 3.3) was spiked with the hyperimmune egg yolk IgY and incubated at 42 °C for 30 or 60 minutes, followed by ELISA analysis of the specific IgY titer. The hydrochloric acid (pH 3.0) and saline that were spiked with egg yolk IgY served as controls. Each dot represents mean of duplicate measurements of an independent sample. Each bar represents mean $\pm$ SD of indicated number of samples.

Chapter V: *Ex vivo* Evaluation of Egg Yolk IgY Degradation in Chicken Gastrointestinal Tract

A version of this chapter was originally published by Huiwen Wang, Ximin Zeng, and Jun Lin.

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Abstract

Egg yolk antibody (immunoglobulin Y, IgY), due to its unique features (*e.g.*, cost-effectiveness for mass production), is emerging as a promising passive immune agent and alternative to antibiotics to combat infectious diseases, particularly in livestock. Oral administration of egg yolk IgY is the most common and convenient route that has been extensively investigated for controlling enteric pathogens. However, *in vivo* stability of egg yolk IgY in gastrointestinal (GI) tract, a critical issue for the success of this approach, still has not been clearly elucidated. Our recent study showed instability of orally administered egg yolk IgY in chicken GI tract, as demonstrated by both *in vivo* and *ex vivo* evidence. To better understand the magnitude and dynamics of instability of egg yolk IgY *in vivo*, in this study, we conducted comprehensive *ex vivo* analyses by spiking hyperimmune egg yolk IgY in fresh GI contents collected from five broilers at each sampling age (2, 4, or 6 weeks). The pH in gizzard slightly increased with age from 2.4 to 3.0 while the pH in small intestine was around 5.8. ELISA analysis indicated that a short time of treatment (30 or 60 min) of IgY with the gizzard contents from the chickens at 2, 4 and 6 weeks of age greatly reduced specific IgY titer by over 8, 6, and 5 log₂ units, respectively, when compared to the saline control. However, small intestine content only had a mild effect on egg yolk IgY, leading to 1 log₂ unit of reduction in IgY titer upon 30 min of treatment. Consistent with these findings, SDS-PAGE and immunoblotting analyses provided direct evidence demonstrating that egg yolk IgY could be drastically degraded to undetectable levels in gizzard content upon as short as 5 min of treatment; however, the IgY was only slightly degraded in small intestine content. Immunoblotting also showed that treatment of IgY with HCl (pH 3.0) for 60 min did not affect its integrity at all, further supporting the enzymatic degradation of IgY in gizzard. Collectively, egg yolk IgY could be substantially degraded in chicken gizzard, highly warranting development of effective approaches, such as encapsulation, for the controlled release and protection of orally administered egg yolk IgY in livestock.

Keywords: Egg yolk antibody, passive immunization, IgY stability, chicken gizzard, gastrointestinal digestion

5.1. Introduction

Chicken immunoglobulin Y (IgY) is the functional equivalent of IgG in mammals and can be transferred from serum to yolk during egg formation. Egg yolk contains a large quantity of IgY that can confer passive immunity for chicks against pathogens, either at embryonic or post-hatching stage (1). Immunization of hens with a particular antigen could yield a large amount of specific egg yolk IgY that has potential applications in human and veterinary medicine (2). In particular, as an emerging alternative to antibiotics, egg yolk IgY has drawn considerable research interest in recent decades due to several unique features (2). First, a laying hen is regarded as a cost-efficient “bioreactor” that can produce over 22.5 gram of egg yolk IgY yearly with 2-10% being antigen-specific (3). In addition, collecting egg yolk—a noninvasive practice—is convenient and also favorable from animal welfare perspective (3). Finally, egg yolk IgY is quite stable in a wide pH range (3.5-11) and under high temperatures (up to 70 °C), making it feasible for storage and processing as a feed supplement (2).

Due to these enticing features of egg yolk IgY, numerous studies have been conducted to develop specific egg yolk IgY for the control of microbial infections in humans and animals (1). In these studies, egg yolk IgY was administrated to animal hosts *via* different routes, depending on the infection sites of targeted pathogens. Oral administration through feed or drinking water is the most common and convenient approach, especially against enteric pathogens (1). However, the *in vivo* stability and bioavailability of administered egg yolk IgY in gastrointestinal (GI) tract have not been examined in most of previous studies and are still largely unknown to date. Addressing this issue is critical for appropriately assessing the efficacy of orally administrated egg yolk IgY and for developing effective egg yolk IgY-based passive immune intervention strategies. Limited *in vitro* data are available concerning the stability of IgY in response to pepsin (a gastric protease), trypsin (a protease in small intestine), and low pH alone (4-6). It has been reported that neutralizing activity of IgY against rotavirus was totally lost in a pepsin solution (pH 2) but largely retained in a trypsin solution (pH 8) (5). In addition, IgY was fairly stable under the pH ranging from 3.5 to 11 (6). However, these *in vitro* studies could not fully reflect *in vivo* conditions of GI tract. Therefore, a well-controlled *ex vivo* system to examine the fate of egg yolk IgY in GI tract is highly needed.

In a recent chicken trial (7), we have observed that orally administered hyperimmune egg yolk IgY was not stable in chicken GI tract, likely due to significant enzymatic degradation in chicken gizzard, which was supported by ELISA analysis of specific IgY in both *in vivo* and *ex vivo* samples. In this study, to better understand the magnitude and dynamics of instability of egg yolk IgY in GI tract and obtain direct evidence of IgY degradation, fresh GI contents were collected from five individual chickens at each sampling age (2, 4, or 6 weeks), slightly processed with saline, and subsequently subjected to comprehensive *ex vivo* analyses using multiple approaches (*i.e.*, ELISA, SDS-PAGE, and immunoblotting).

5.2. Materials and methods

5.2.1. Collection of chicken GI content samples

The chicken trial performed in this study was approved by Institutional Animal Care and Use Committee at The University of Tennessee, Knoxville with protocol number 1387-0219. Briefly, 15 newly hatched broiler chicks (Hubbard Efficiency Plus) were fed with regular feed for 6 weeks. At 2, 4, or 6 weeks of age, 5 chickens were randomly selected and euthanized. The pH in gizzard and small intestine (the junction site between duodenum and jejunum) was measured on site using a pH spear tester (Thermo Fisher, USA). Subsequently, the fresh GI contents (5 mL per sample) were collected using clean stainless steel laboratory scoops, transferred into 15 mL sterile plastic tubes, stored on ice, and immediately subjected to downstream work as detailed below.

5.2.2. *Ex vivo* IgY stability assays

The *ex vivo* IgY stability assays were performed as described previously (5) with modifications. Because supplementation of lyophilized egg yolk powder in feed is the most practical and widely adopted approach for oral administration of egg yolk IgY to control microbial infections in food animals (1), the lyophilized hyperimmune egg yolk powder produced in our recent study (7) was utilized for *ex vivo* assays to assess IgY stability in GI contents. The egg yolk powder was reconstituted in saline at the ratio of 1:3 (w/w), followed by centrifugation at 13,000 *g* for 2 min. The supernatant containing egg yolk IgY was used as stock to spike various GI samples. Each of fresh gizzard and small intestine content samples was gently mixed with same volume of sterile saline and centrifuged at 5,000 *g* for 5 min. The supernatant was kept and used as corresponding

GI content matrix system for *ex vivo* IgY stability assay. The supernatant was measured for pH and spiked with 5% (v/v) of the egg yolk IgY solution while saline or HCl (1 mM, pH 3.0) served as a control. The mixtures were incubated at 42 °C (chicken body temperature) for different lengths of time. Subsequently, the mixtures were subjected to ELISA or SDS-PAGE analysis. If needed, some mixtures containing gizzard content or HCl were neutralized to pH 7.0 using less than 2% volume of NaOH upon completion of treatment.

5.2.3. Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was conducted as described previously (8) to determine the titers of specific egg yolk IgY in the mixture after specific treatment. Briefly, the ELISA plates (Nunc MaxiSorp, Thermo Fisher, USA) were coated with 100 µL of immunogen [keyhole limpet hemocyanin-enterobactin conjugate (8), 300 ng/mL] in coating buffer (bicarbonate/carbonate, pH 9.6) overnight at room temperature. The coated plates were blocked with blocking buffer (PBS + 0.05% Tween 20 + 5% skim milk) at room temperature for 1 hr. Subsequently, the aforementioned samples under various treatments were 2-fold serially diluted in blocking buffer and incubated with the coated plates at room temperature for 1 hr. Following 4 times washing with PBS containing 0.05% of Tween 20, 100 µL of the horseradish peroxidase-conjugated goat anti-chicken IgY secondary antibody (SeraCare, MA, USA; 2,000 fold diluted in blocking buffer) was added in each well and the plates were incubated at room temperature for 1 hr. After 4 times washing with the washing buffer, the plates were finally developed using 100 µL of ABTS[®] One-component Peroxidase Substrate (SeraCare) and the reaction was stopped after 30 min by adding 100 µL of stopping solution (1% SDS). The absorbance was measured at optical density of 405 nm (OD₄₀₅) using a microplate reader (BioTek Instruments, VT, USA) and data were collected using Gen5 software (BioTek Instruments). The wells without addition of primary antibody served as a blank control. Endpoint titer was defined as the last dilution at which OD₄₀₅ of sample wells exceeded the cutoff value (0.1). Duplicate measurements were performed for each sample.

5.2.4. Purification of IgY from egg yolk

The hyperimmune egg yolk generated in our recent study (9) was used for IgY purification following a published protocol (10) with slight modifications. Briefly, 1 volume of egg yolk was

diluted with 4 volumes of PBS and then mixed with 3.5 % (w/v) of polyethylene glycol 6000 (PEG 6000, Fisher Scientific, USA) for lipid removal. After 20 min of incubation at room temperature, the mixture was centrifuged at 5,000 *g* for 20 min. Subsequently, the supernatant was added with 12% (w/v) of PEG 6000 for IgY precipitation and the mixture was incubated at room temperature for 10 min. Following centrifugation at 5,000 *g* for 20 min, the precipitate was dissolved in PBS and added with 12% (w/v) of PEG 6000 again for another round of IgY precipitation. After centrifugation, the precipitate was dissolved in a small volume of PBS, added with equal volume of precooled (-20 °C) ethanol, and centrifuged at 5,000 *g* and 4 °C for 20 min to remove PEG 6000. Lastly, the precipitate containing a high proportion of IgY was dissolved in a small volume of PBS and the solution was dialyzed against PBS. Initial egg yolk, the supernatant and precipitate fractions obtained from the centrifugation of 12% PEG 6000 mixture, and the final dialyzed IgY extract were subjected to SDS-PAGE analysis.

5.2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

All *ex vivo* assay samples were subjected to SDS-PAGE analysis (12% [w/v] polyacrylamide gel, Bio-Rad, USA) and stained with Coomassie blue R-250 dye (Thermo Fisher, USA). For immunoblotting, the proteins on polyacrylamide gel were electro-transferred to a nitrocellulose membrane, which was subsequently blocked in blocking buffer (PBS + 0.05% Tween 20 + 5% skim milk) at 4 °C overnight. Then the membrane was incubated with goat anti-chicken IgY-horseradish peroxidase (SeraCare, 5,000 fold diluted in blocking buffer) at room temperature for 1 hr. After washing procedure (PBS + 0.05% Tween 20), the membrane was developed using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher, USA).

5.2.6. Statistical analysis

Statistical analyses were conducted using Mann-Whitney test in GraphPad Prism 8. Data were presented as mean ± standard deviation (SD). A probability level of $P < 0.05$ was considered as a statistically significant difference.

5.3. Results

5.3.1. The pH in the chicken GI tract

As shown in Table 5-1, the pH in gizzard slightly increased with age, which was 2.42, 2.90, and 2.96 at 2, 4, and 6 weeks of age, respectively. There was no significant difference among the *in vivo* pH at these three time points. After dilution of the gizzard content using saline, the supernatant obtained for *ex vivo* assays had slightly elevated pH, which was approximately 0.57 higher than corresponding original gizzard content (Table 5-1). However, as expected, the small intestine had approximate pH of 5.84 at 6 weeks of age; the pH barely changed in the corresponding supernatant used for *ex vivo* assay upon slight dilution with saline (Table 5-1).

5.3.2. Drastic reduction of specific egg yolk IgY titer in gizzard content

With as short as 30 min of incubation with the gizzard content, the specific IgY titers in egg yolk were significantly reduced by over 8, 6, and 5 log₂ units at 2, 4, and 6 weeks of age, respectively, when compared to saline control (Figure 5-1A). After additional 30 min of treatment, the specific IgY titers at the three time points were further lowered by an average of 0.5 log₂ unit (Figure 5-1B). Because escalation of pH in gizzard with age (Table 5-1) appeared to be associated with the magnitude of reduction in specific IgY titers (Figures 5-1A & B), further correlation analysis was performed and revealed that there was a significant inverse correlation ($P < 0.01$) between *ex vivo* sample pH and the reduction of specific IgY titer in response to 30 min of treatment in gizzard content (Figure 5-1C).

5.3.3. IgY titer slightly decreased upon treatment with small intestine content

As shown in Figure 5-1D, incubation of egg yolk IgY with small intestine content obtained from the chickens at 6 weeks of age only led to 1 log₂ unit (2 fold) of reduction when compared to saline control.

5.3.4. Egg yolk IgY was greatly degraded in gizzard content

Above dynamic assessment of IgY stability in gizzard content confirmed our previous *in vivo* and *ex vivo* findings (7) and suggested enzymatic degradation of IgY in gizzard. To obtain direct evidence of IgY degradation, we subsequently conducted SDS-PAGE and immunoblotting assays on the egg yolk IgY upon different treatments. We first performed stepwise purification

of IgY from egg yolk powder to better understand the profile of IgY on SDS-PAGE. As shown in Figure 5-2A, the high-purity IgY extract was obtained after stepwise purification and displayed characteristic bands of heavy chain and light chain as reported previously (11).

To compare the effect of gizzard content or low pH on the integrity of egg yolk IgY, the egg yolk was spiked in a representative gizzard content sample (2 weeks of age, pH 3.0) or HCl solution (pH 3.0). The egg yolk treated in the matrixes of control saline, HCl for 60 min, and the gizzard content for 0 min consistently displayed multiple protein bands with similar pattern (Figure 5-2B). However, both 30 min and 60 min treatments of egg yolk in gizzard content led to all bands barely visualized (Figure 5-2B). Consistent with this finding, immunoblotting using specific anti-IgY antibodies clearly indicated that the IgY was undetected after 30 or 60 min of treatment in gizzard content while its integrity was still maintained well in the matrix of control saline or HCl upon 60 min of treatment (Figure 5-2C). These data are also consistent with previous ELISA analysis of IgY titer upon acid treatment (7) and provides further direct evidence demonstrating that acidic condition alone (*e.g.*, pH 3.0) did not alter the IgY integrity.

5.3.5. Potent but varied degradation of egg yolk IgY in individual gizzard content samples

To further examine the degradation capacity of individual gizzard contents collected from the chickens at different ages, each gizzard sample spiked with egg yolk was subjected to 30 min of incubation at 42 °C followed by SDS-PAGE and immunoblotting analyses (Figure 5-3).

Incubation of egg yolk with the five gizzard samples from the chicken at age of 2 weeks made all bands almost fade away when compared to similar multi-band pattern observed in control saline and 0 min of treatment (Figure 5-3A). Consistent with this SDS-PAGE result, immunoblotting using specific anti-IgY antibodies showed that the IgY was undetected after 30 min of treatment in each gizzard content when compared to the characteristic IgY bands in the matrix of control saline (Figure 5-3B). Similar finding—potent degradation of egg yolk IgY in gizzard content upon 30 min of treatment—was also observed in almost all the samples collected from the chickens at 4 weeks (Figures 5-3C & D) and 6 weeks of age (Figures 5-3E & F). However, incubation of egg yolk IgY in one gizzard content collected from a 6-week-old chicken (G3) for 30 min did not cause significant IgY degradation as shown by immunoblotting analysis (Figure 5-3F); notably, this gizzard content was an outlier because this *ex vivo* sample has much higher

pH (4.62) than other samples (Table 5-1) and only led to 1 log₂ unit of reduction in IgY titer when compared to saline control (the dot indicated by red arrow in Figure 5-1A).

5.3.6. Time course analysis of IgY degradation in gizzard content

To further determine the efficiency and capability of gizzard content to degrade egg yolk IgY, time course analysis was performed using a gizzard content sample at 6 weeks of age with incubation time ranging from 0 to 30 min. As shown in SDS-PAGE (Figure 5-4A), the pattern of protein bands at the beginning (0 min) of the gizzard treatment was very similar to that in saline with only subtle reduction in the intensity of most bands. Nevertheless, most bands were not visualized after only 5 min of treatment; there was no further notable change afterwards (*i.e.*, at 10, 20, and 30 min) (Figure 5-4A). Consistently, immunoblotting showed that the IgY bands were almost undetected after as short as 5 min of treatment while the characteristic IgY bands in both saline control and 0 min of sample were vividly detected by IgY-specific antibodies (Figure 5-4B).

5.3.7. Small intestine content displayed IgY-degrading capacity but with less magnitude than gizzard

In our recent study (7), the pooled small intestine content did not affect the titer of specific IgY with up to 2.5 hr treatment, suggesting weak or lack of IgY-degrading capability of small intestine content. In this study, using SDS-PAGE and immunoblotting analyses, we further evaluated the IgY-degrading capacity of each small intestine content from five 6-week-old chickens. Unlike what were observed for 30-min incubation of egg yolk IgY with gizzard content (Figures 5-2 & 5-3), 30-min incubation of egg yolk IgY with small intestine content led to moderate reduction in the intensity of various protein bands on SDS-PAGE (Figure 5-5A). Nevertheless, immunoblotting clearly indicated that majority of spiked IgY was degraded in the first three samples while much less was degraded in samples #4 and #5 (Figure 5-5B).

5.4. Discussion

In this study, we collected the contents from chicken gizzard and upper region of small intestine (the junction between duodenum and jejunum) for *ex vivo* analysis of egg yolk IgY stability based on following three major reasons. First, gizzard and small intestine are recognized as the

major digestive organs, which may directly degrade egg yolk IgY upon oral administration due to presence of various proteases (12). Pepsin, a gizzard protease whose function strictly relies on acidic condition resulting from secretion of hydrochloric acid (average pH 3.0), plays a critical role in the first line of feed digestion during the feed retention in poultry (12). Other digestive enzymes, trypsin in particular, are produced in pancreas and then released into upper region of small intestine for further feed digestion (13). Second, a panel of significant enteric pathogens, such as *Campylobacter jejuni* and *Salmonella enterica* serotype Typhimurium, colonize predominantly in lower region of intestine (*e.g.*, cecum) (14). Therefore, orally administered egg yolk IgY should take a long journey—up to 80 min of retention time in gizzard and approximately 30 min of retention time in upper region of small intestine—to reach desired target site for pathogen control (15, 16). During this long journey, egg yolk IgY may encounter a panel of harsh conditions that potentially affect its stability, such as low pH, proteases, and free radicals (12). Consequently, examination of stability of egg yolk IgY in upper GI tract is a critical issue for the success of using orally administrated IgY for passive immune intervention. Third, chicken crop, an organ prior to gizzard, is for transient storage and moisturization of ingested feed with internal pH above 6.0 (12). It is well recognized that crop does not have digestive functions and there are no secreted digestive enzymes in crop (12). Therefore, egg yolk IgY is expected to be stable in crop and we did not include crop content for *ex vivo* analysis here.

In this study, we have taken into careful consideration several critical parameters in *ex vivo* assay to better mimic *in vivo* conditions, with emphasis on reaction time and pH. Under physiological condition, the retention of feed in chicken gizzard ranges from 30 to 80 min while the retention time in upper small intestine is approximately 30 min (15, 16). Based on these facts, we examined the stability of egg yolk IgY in gizzard content for up to 60 min or in small intestine content for 30 min. Since titer reductions of the specific IgY in gizzard content were comparable between 30 and 60 min of treatments (Figures 5-1A & B), in the subsequent SDS-PAGE and immunoblotting analyses, we only examined the integrity of IgY upon 30 min of treatment with each of the 15 gizzard content samples. With respect to pH measurement, the pH meter with a thin probe enabled us to accurately determine the *in vivo* pH in GI tract. The GI content was slightly diluted with saline to minimize any artificial effects for the *ex vivo* assay. Despite slight increase of pH by an average of 0.57 in saline-processed gizzard contents when compared to

corresponding original *in vivo* contents (Table 5-1), pepsin in gizzard content should still remain over 50% of its maximal enzymatic activity because the pH of all but one saline-processed gizzard contents was still below 4.0 (17). The only outlier of gizzard content had pH 4.62 (described in Results), which may be the major factor leading to slight reduction (2 fold) in IgY titer (indicated by arrow in Figure 5-1A) and substantial residual IgY detected in immunoblotting (G3 in Figure 5-3F) upon 30 min of treatment in this gizzard content. Together, the slightly increased pH in the saline-processed gizzard contents used for *ex vivo* assays here might underestimate the capability of gizzard content to degrade IgY *in vivo* but would not affect the conclusion drawn from this study. Finally, despite compelling evidence here showing the instability of egg yolk IgY in the GI tract, we would like to mention that this study is still limited by using the broilers raised in animal facility. Examination of the GI contents collected from the birds raised in a poultry production setting with inter-flock replication is highly warranted in future studies.

Egg yolk IgY is increasingly recognized as a promising passive immune agent for the control of various microbial infections (1). To control enteric pathogens or toxins, egg yolk IgY has been commonly administrated through feed or drinking water to different animals (18). These studies have yielded varied successes, such as inhibiting microbial infections or improving growth performance (3, 18). However, the *in vivo* level and stability of orally administered egg yolk IgY have never been assessed in numerous studies conducted by other groups, which is surprising because egg yolk IgY may be undermined when passing through animal's GI tract, a destructive environment for proteins. To fill this significant knowledge gap, in this study, we performed delicate *ex vivo* assays to simulate the effect of GI tract on egg yolk IgY. The findings from this study, for the first time, provided direct and compelling evidence demonstrating that egg yolk IgY was dramatically degraded shortly after its exposure to gizzard content and could also be subjected to moderate degradation by small intestine content. However, it is interesting that numerous studies showed the efficacy of orally administered egg yolk IgY against different infectious agents (1, 2). Notably, none of previous studies from other research groups examined *in vivo* level and stability of the orally administrated egg yolk IgY when performing challenge trial, greatly impeding us to properly link the phenotype (*e.g.*, reduced pathogen load or clinical signs) to the antimicrobial effect resulting from orally administered egg yolk IgY. Thus, the

findings from previous studies need to be revisited and confirmed in conjunction with examination of the *in vivo* stability and level of the specific egg yolk IgY administered *via* oral route.

We expect the findings from this study can also be extrapolated to other animals and even humans. For monogastric organisms (*e.g.*, mouse, rabbit, swine, and human), pepsins are produced in their stomachs as the case in chicken gizzard. The low pH in these stomachs (1.5-4.0 in general) can fulfill the requirement of pepsins to exert degradation on various peptides and proteins (19-21). Ruminant (*e.g.*, bovine and ovine) abomasum, equivalent to the stomach of monogastric animals in anatomy and function, also has low pH environment (usually 1.5-2.5) and pepsins for protein digestion (22, 23). Therefore, to successfully utilize hyperimmune egg yolk as a passive immune agent to control enteric pathogens, it is critical to develop cost-effective strategies to prevent undesired degradation of the IgY in GI tract, particularly in stomach, and facilitate delivery of functional egg yolk IgY to specific GI site.

Encapsulation of egg yolk IgY is a feasible strategy for protecting the IgY from degradation in the GI tract of food-producing animals. Various coating strategies and materials have been assessed for microencapsulation of the IgY, such as alginate/chitosan, multilayer polypeptides, methacrylic acid copolymer, or alginate/carrageenan microgels (24-27). These approaches, to different extents, prevented IgY activity loss and achieved controlled release of IgY in the simulated GI fluid matrix system (24-27). However, these past studies have some limitations. First, the encapsulation procedures used in these studies were lengthy and complicated. For instance, encapsulation with alginate/chitosan involved purification of IgY from egg yolk, preparation of encapsulation medium, loading IgY into the microcapsules using professional facilities, as well as filtration, rinse, and lyophilization of the microcapsules (25). Second, some of the encapsulation materials, such as methacrylic acid copolymer (24) or multilayer polypeptides (26), were expensive, potentially impeding practical application of this strategy in livestock. Finally and more importantly, only *in vitro* system, *i.e.*, the buffer supplemented with digestive enzymes, was used to evaluate the efficacy of encapsulation in IgY protection and release in these studies (24-27). Since there are complex factors present in the GI tract, it is critically important to perform *ex vivo* as well as *in vivo* studies to evaluate encapsulation

strategy for protection and controlled release of IgY. Therefore, in future studies, cost-effectiveness, easiness, and *in vivo* assessment should be taken into special consideration when developing encapsulation methods for protection and controlled release of hyperimmune egg yolk IgY in the intestine of livestock.

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Appendix

Table 5-1. The pH in the chicken GI tract (*in vivo*) and corresponding *ex vivo* samples at different ages

	Gizzard ^a			Small intestine ^a
	Week 2	Week 4	Week 6	Week 6
<i>in vivo</i>	2.42 ± 0.28	2.90 ± 0.54	2.96 ± 0.79	5.84 ± 0.12
<i>ex vivo</i>	3.06 ± 0.28	3.23 ± 0.61	3.71 ± 0.56	5.75 ± 0.10

^aThe data are presented as mean ± SD of five individual chicken samples.

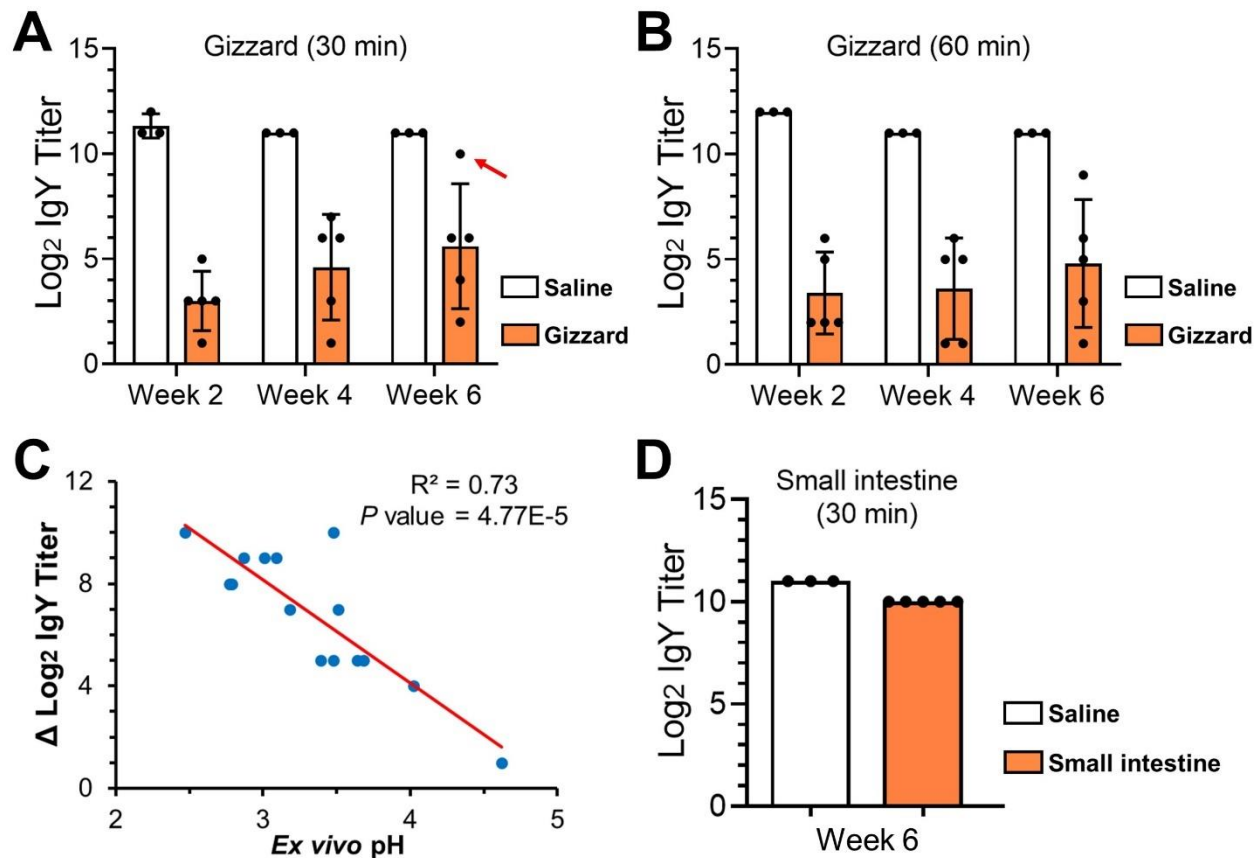


Figure 5-1. ELISA titers of specific egg yolk IgY in the GI samples collected from chickens at different ages. (A) Specific egg yolk IgY titers upon treatment in saline (control) or gizzard contents collected at 2, 4, and 6 weeks of age at 42 °C for 30 min. The dot indicated by red arrow is an outlier sample with the highest *ex vivo* pH of 4.62. (B) Specific egg yolk IgY titers upon treatment in saline (control) or gizzard contents at 2, 4, and 6 weeks of age at 42 °C for 60 min. (C) Scatter plot showing an inverse correlation between *ex vivo* pH in the assays and the reduction of specific IgY titer upon 30 min of treatment in gizzard content. The dots represent each of the 15 gizzard samples at different ages. *P* value of 4.77E-5 indicates a significant linear correlation between the two variables. (D) Specific egg yolk IgY titers upon treatment in saline (control) or small intestine contents at 6 weeks of age at 42 °C for 30 min. In the bar plots above, data are presented as mean $\pm$ SD of measurements from five chicken samples or three saline controls at each specific time point.

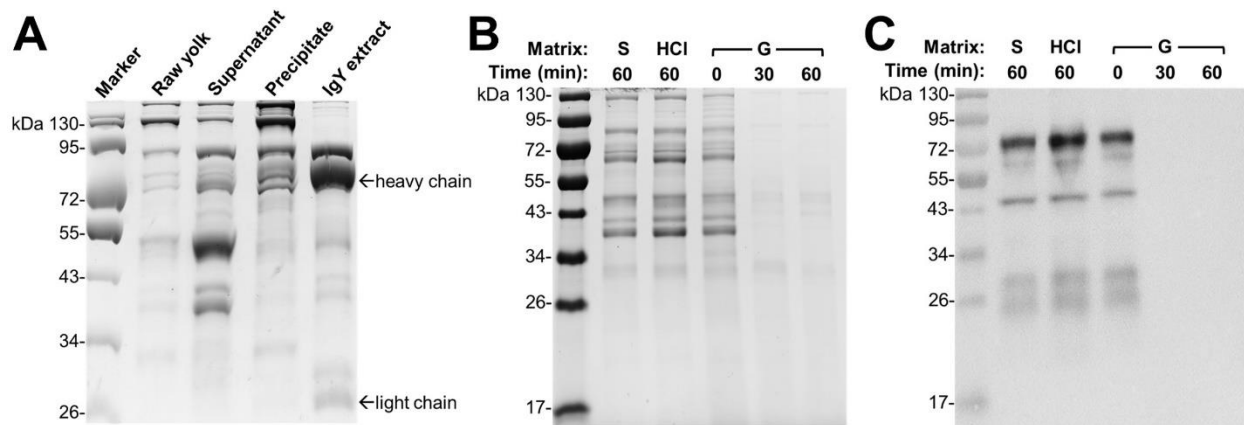
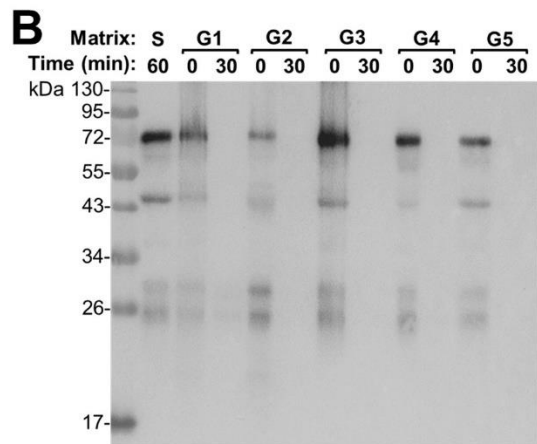
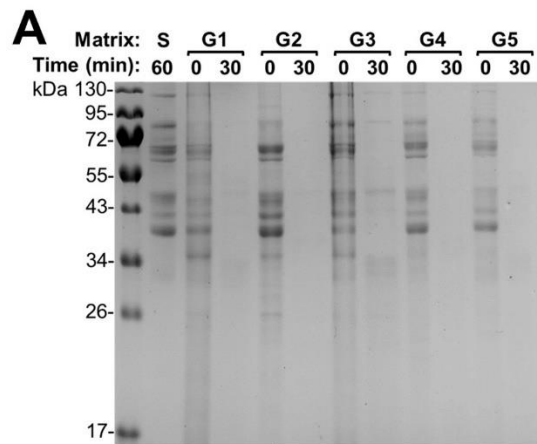
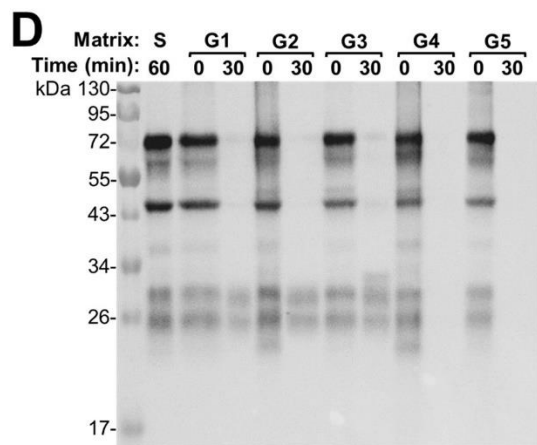
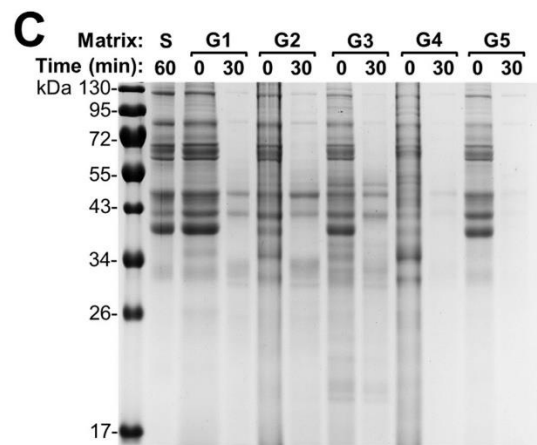


Figure 5-2. Integrity of egg yolk IgY in response to the treatment with HCl or gizzard content. (A) SDS-PAGE analysis of stepwise purification of IgY from egg yolk. Raw yolk: the egg yolk prior to IgY purification; Supernatant and Precipitate: the fractions obtained following centrifugation of 12% PEG 6000-treated egg yolk mixture; IgY extract: the purified IgY after final dialysis. (B) SDS-PAGE and (C) immunoblotting analyses of the integrity of egg yolk IgY in response to the treatment with HCl (1 mM, pH 3.0) or a representative gizzard content (pH 3.0) at 42 °C for the indicated length of time. The reconstituted hyperimmune egg yolk powder was used as stock to spike different matrixes as described in *Materials & Methods*. The far left lane is a protein marker. Abbreviation of the matrix system for treating egg yolk IgY: S, saline; G, gizzard content.

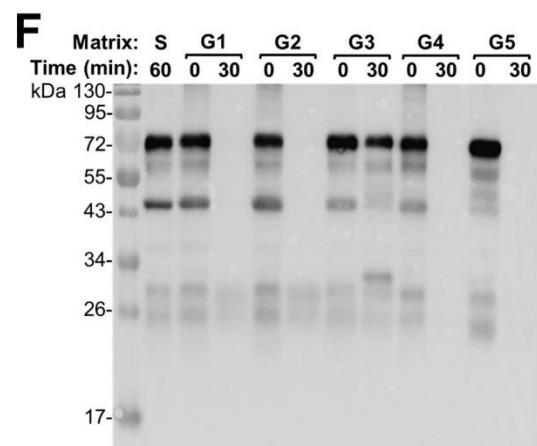
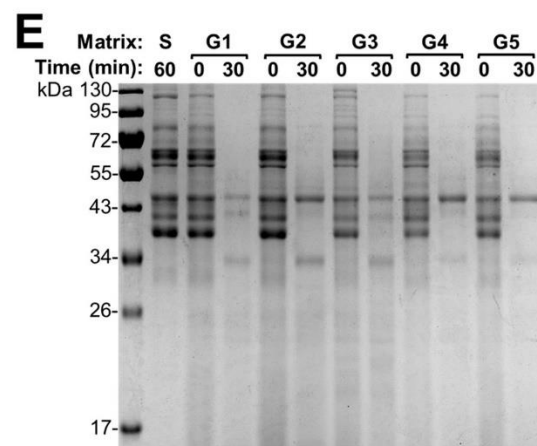
Figure 5-3. Integrity of egg yolk IgY in response to the treatment with the gizzard content collected from each individual chicken. (A) SDS-PAGE and (B) immunoblotting analyses of the integrity of egg yolk IgY in response to the treatment with each individual gizzard content collected at 2 weeks of age at 42 °C for 0 or 30 min. The reconstituted hyperimmune egg yolk powder was used as stock to spike the gizzard content matrixes as described in *Materials & Methods*. The far left lane is a protein marker. Abbreviation of the matrix system for treating egg yolk IgY: S, saline; G1-G5, gizzard contents of 5 individual chickens at 2 weeks of age (indicated on the right). Likewise, (C) SDS-PAGE and (D) immunoblotting analyses of the integrity of egg yolk IgY upon treatment in each individual gizzard content collected at 4 weeks of age; (E) SDS-PAGE and (F) immunoblotting analyses of the integrity of egg yolk IgY upon treatment in each individual gizzard content collected at 6 weeks of age.



Week 2



Week 4



Week 6

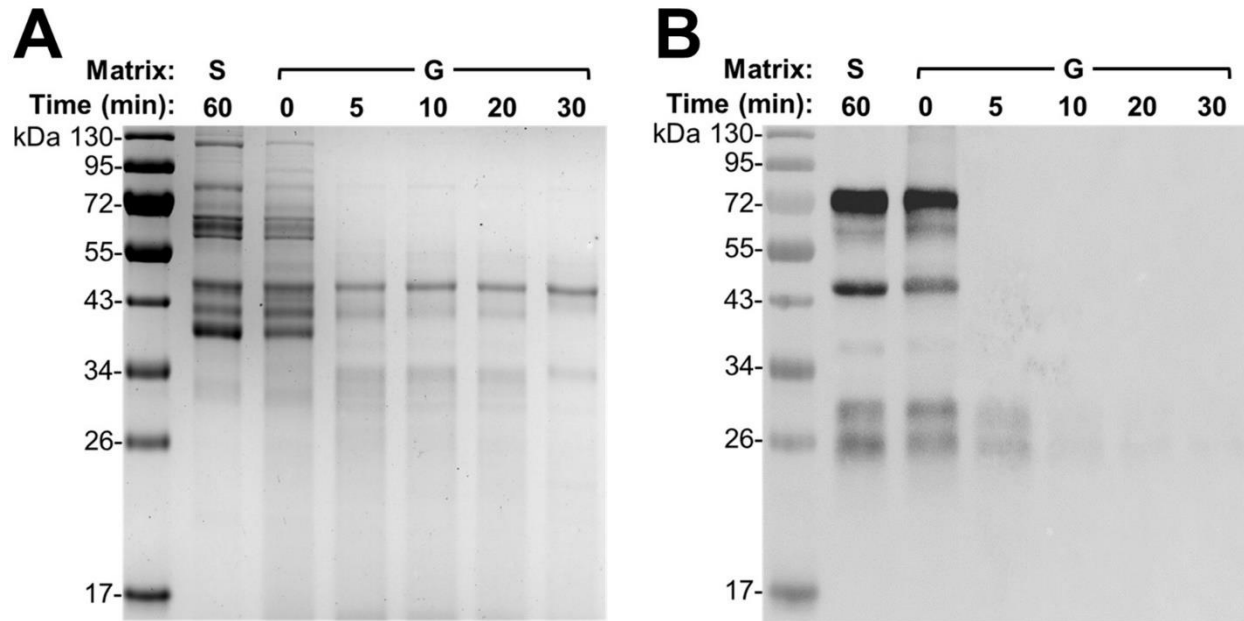


Figure 5-4. Degradation dynamics of egg yolk IgY in gizzard content. (A) SDS-PAGE and (B) immunoblotting analyses of the integrity of egg yolk IgY in response to the treatment with a representative gizzard content at 42 °C for the indicated length of time. The reconstituted hyperimmune egg yolk powder was used as stock to spike the gizzard content matrixes as described in *Materials & Methods*. The far left lane is a protein marker. Abbreviation of the matrix system for treating egg yolk IgY: S, saline; G, gizzard content.

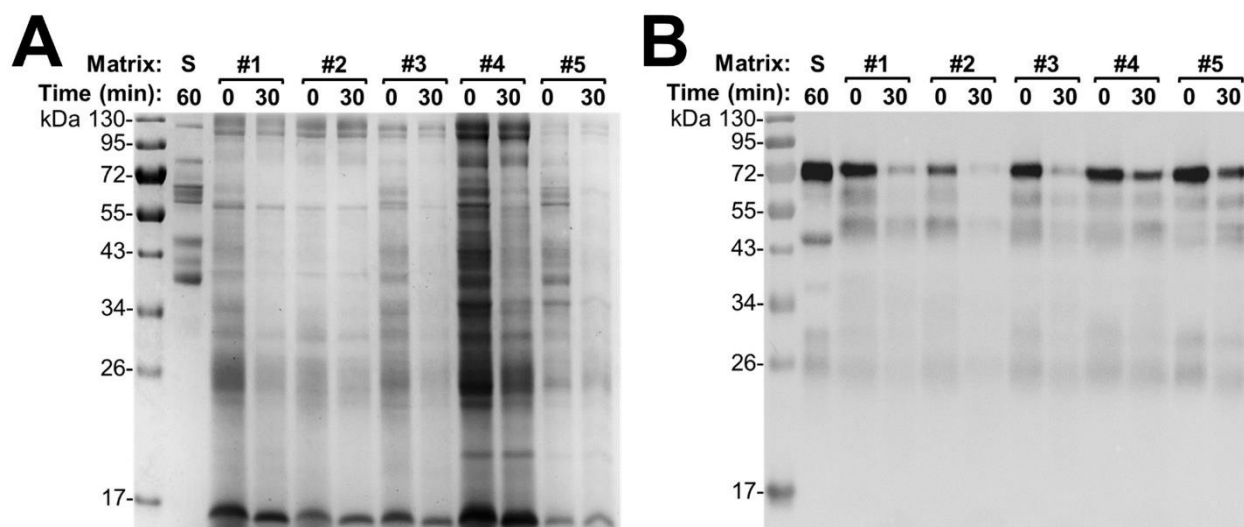


Figure 5-5. Integrity of egg yolk IgY in response to the treatment with small intestine content collected from each individual chicken at 6 weeks of age. (A) SDS-PAGE and (B) immunoblotting analyses of the integrity of egg yolk IgY in response to the treatment with each individual small intestine content collected at 6 weeks of age at 42 °C for 0 or 30 min. The reconstituted hyperimmune egg yolk powder was used as stock to spike the small intestine content matrixes as described in *Materials & Methods*. The far left lane is a protein marker. Abbreviation of the matrix system for treating egg yolk IgY: S, saline; #1-#5, small intestine contents of 5 individual chickens at 6 weeks of age.

**Chapter VI: Isolation and Characterization of *Escherichia albertii* Originated
from the Broiler Farms in Mississippi and Alabama.**

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Abstract

Escherichia albertii is an emerging foodborne enteropathogen with increasing outbreaks worldwide, particularly in Japan recently. However, major features of this zoonotic pathogen, such as prevalence, virulence, and antibiotic resistance (AR), still remain uncharacterized. In a recent pilot study, we reported isolation of *E. albertii* from a chicken farm in Tennessee, suggesting chicken is an important reservoir for *E. albertii*. In this large-scale study, we examined prevalence of *E. albertii* in 9 farms in Mississippi and Alabama. Of a total of 270 cloacal swabs (30 per farm), 43 were PCR positive and 12 *E. albertii* strains were isolated with different isolation rates in individual farms ranging from 0 to 23.3%. Both PFGE and whole genome analysis showed the *E. albertii* from different farms were phylogenetically distant, but those from the same farm displayed clonal relationships. Consistently, the antibiogram, AR gene profiles, and plasmid replicon types were similar across the strains in the same farm. Notably, 9 of the 12 *E. albertii* strains displayed multidrug resistance; one strain was even resistant to imipenem, a clinically important carbapenem antibiotic. In addition, comparative genomics analysis showed that two chicken *E. albertii* clusters displayed very close evolutionary relationships and similar virulence gene profiles to human *E. albertii* strains. *In vitro* growth assay demonstrated that the anti-enterobactin antibodies could dramatically inhibit the growth of two representative chicken *E. albertii*, supporting the feasibility of the novel enterobactin-based immune intervention for controlling this emerging pathogen. Taken together, the findings from this study further indicated chickens as an important reservoir for *E. albertii* in the U.S., highlighting the need to prevent and control *E. albertii* in poultry production.

Keywords: *Escherichia albertii*, poultry, epidemiology, genomics analysis

6.1. Introduction

Escherichia albertii is an emerging foodborne enteropathogen causing watery diarrhea, abdominal distention, vomiting, fever, and even bacteremia in humans (1). This bacterium has been frequently misidentified as enteropathogenic or enterohemorrhagic *E. coli* due to their similarity in genetic and phenotypic features (2). *E. albertii* possesses type III secretion system (T3SS) and intimin, encoded by the locus of enterocyte effacement (LEE) and responsible for characteristic attaching-effacing (A/E) lesions in host intestinal epithelium (1). In addition, this pathogen produces cytolethal distending toxin (CDT) and occasionally Shiga toxin that may lead to intoxication and death of epithelium (2). The *cdt* gene in *E. albertii* (*Eacdt*) has been targeted for PCR-based rapid and accurate identification of *E. albertii* (3). Other virulence factors, such as various fimbriae, Paa (porcine A/E-associated), and Ibe (invasion of brain endothelium) that are initially characterized in different pathovars of *E. coli* (4), are also present in *E. albertii* and may directly contribute to adherence and invasion of host epithelium (2).

E. albertii has caused many foodborne outbreaks in Japan and also has been reported in other countries including the U.S. (1). In addition to humans, *E. albertii* has been isolated from various animal sources, such as avian, racoon, swine, feline as well as raw meat and carcass rinse of poultry (1, 5-7). Despite the clinical importance of *E. albertii* being increasingly recognized worldwide (1), major features of this zoonotic pathogen, such as prevalence, transmission, pathogenicity, and antibiotic resistance (AR), remain largely unknown. In a recent pilot study, we isolated *E. albertii* from a broiler farm in Tennessee and further demonstrated that a human clinical *E. albertii* strain could colonize well in the chicken intestine, strongly suggesting chicken is one of the key vehicles for the transmission of *E. albertii* to humans (8). However, this pilot survey is limited to small sample size and lack of comprehensive genomics analysis. Thus, a large-scale survey together with in-depth characterization of *E. albertii* in chickens is highly warranted.

In this study, we investigated the prevalence of *E. albertii* in broilers from multiple states and farms. Multifaceted phenotypic and genetic characterizations of isolated chicken *E. albertii* provided insights into the epidemiology and evolution of *E. albertii* infection.

6.2. Materials and methods

6.2.1. Detection and isolation of *E. albertii* from broilers

The chicken sampling procedure was approved by the Institutional Animal Care Use Committee (IACUC) at Mississippi State University with protocol number 19-330. Cloacal swabs were directly collected from randomly selected broilers in 3 slaughter plants from July to September in 2020. The chickens subjected to sampling were from nine farms (30 chickens per farm) that were either in Mississippi (6 farms) or Alabama (3 farms). The cloacal swabs, collected using CultureSwab™ Cary-Blair Agar Transport System (BD BBL™, USA), were immediately packed with ice packs and shipped to the laboratory in Knoxville, Tennessee *via* overnight express carrier. Upon arrival, the swabs were subjected to *E. albertii* isolation procedure according to previous publications (3, 8, 9). Briefly, each cloacal swab was suspended in 1 mL sterile saline. An aliquot (300 µL) of the suspension was inoculated into 4 mL of Tryptic Soy Broth (TSB, BD™, USA) and grown for enrichment at 37 °C for 14 hr with vigorous shaking. Subsequently, 1 mL of enrichment culture was used for genomic DNA extraction using boiling method. The DNA template was subjected to PCR using the primer pair (forward, GCTTAACTGGATGATTCTTG; reverse, CTATTTCCCATCCAATAGTCT) targeting *Eacdt* gene (449 bp) that is highly specific for *E. albertii* (3). The PCR procedure was as follows: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation (95 °C for 0.5 min), annealing (50 °C for 0.5 min), and extension (72 °C for 1 min), with a final extension at 72 °C for 2 min.

Upon identification of a PCR-positive sample, the original swab suspension was 10 fold serially diluted with sterile saline and spread on XRM-MacConkey agar plates, a selective medium developed for *E. albertii* isolation (9). Following 24 hr of incubation at 37 °C, the suspected *E. albertii* colonies on agar plates were selected (up to 48 colonies per swab sample) and individually inoculated into 200 µL TSB broth. After the incubation at 37 °C for 3 hr, 1 µL of bacterial culture was directly used as template for the *Eacdt*-targeting PCR as described above. The PCR-positive culture was subsequently streaked on XRM-MacConkey agar plate and incubated overnight at 37 °C; a single colony was picked, inoculated into 3 mL of TSB broth, and grown at 37 °C with vigorous shaking for 4 hr. After confirmation of the culture using

another round of *Eacdt*-targeting PCR, the *E. albertii* culture was stored with 25% glycerol at -80 °C.

6.2.2. Pulsed-field gel electrophoresis (PFGE)

PFGE of the macrorestriction fragment patterns of genomic DNA using *Xba*I enzyme was performed by following the Centers for Disease Control and Prevention (CDC) standardized protocol for *E. coli* (10, 11) with slight modifications on the electrophoretic conditions. A *Salmonella* serotype Braenderup strain (H9812) was used as the universal size standard (12). The electrophoretic conditions were as follows: initial switch time, 2.16 s; final switch time, 35.07 s; run time, 12 hr; angle, 120°; gradient, 6.0 V/cm; temperature, 14 °C; ramping factor, linear. The PFGE image was analyzed in the software GelCompar II (Applied Maths, Belgium) by following its standard protocol.

6.2.3. Antimicrobial susceptibility test

Antimicrobial susceptibility assay was performed using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (M02-A12, 2015). The *E. albertii* strains were tested against 20 antimicrobial agents belonging to 14 classes while *E. coli* strain ATCC 25922 served as quality control. The antimicrobial disks were purchased from Becton, Dickinson and Company (USA). The sizes of growth inhibition zones were interpreted based on the criteria of CLSI M100 (ED30:2020) for the order Enterobacterales.

6.2.4. Whole genome sequencing

Isolated chicken *E. albertii* strains were sent to the Veterinary Diagnostic Laboratory at Iowa State University for whole genome sequencing using Illumina MiSeq platform. The procedures, including genomic DNA extraction, library preparation, sequencing, and *de novo* assembly of draft genome, were described in previous publications (8, 13).

5.2.5. Comparative genomics analyses

Before conducting genomics analysis, completeness and contamination of each assembled draft genome were assessed via CheckM (v1.0.18) (14) in KBase server (<https://www.kbase.us/>) (15). In addition, to further confirm the isolates were indeed *E. albertii*, complete genome sequence of

E. albertii strain KF1 (16) was used as a reference to calculate its average nucleotide identity (ANI) to the draft genome of each isolate via FastANI (v0.1.2) (17) in KBase server (15). If the ANI value exceeded 95%, the isolate could be confirmed as *E. albertii* species (17).

To identify AR genes and plasmid replicon types of the *E. albertii* strains, draft genome of each strain was uploaded to ResFinder (v4.1) (18) and of PlasmidFinder (v2.1) (19), respectively, in Center for Genomic Epidemiology (<http://www.genomicepidemiology.org/>). For the querying, minimum sequence identity and coverage were set as 90% and 80%, respectively. To further confirm the identified AR genes, draft genomes of the *E. albertii* strains were also uploaded to Resistance Gene Identifier (v5.2.0) (20) with minimum sequence identity as 95%.

Whole genomes of a total of 23 diverse *E. albertii* strains were subjected to phylogenetic analysis based on whole-genome single nucleotide polymorphism (wgSNP) as described in our recent publication (8). Draft genomes of the 12 chicken strains obtained from this study and the chicken strain TN-C1 from our recent study (8), together with complete genomes of other 10 diverse *E. albertii* from public database (9 human isolates and 1 bird isolate) (2), were uploaded to CSI Phylogeny (v1.4) (21) to perform wgSNP-based genome typing with *E. albertii* KF1 genome serving as a reference genome. The resulted phylogenetic tree was visualized using Interactive Tree of Life (<https://itol.embl.de/>) (22). The key information of the 11 diverse *E. albertii* strains that were not obtained from this study were briefly summarize in Table 6-1. All genomes except our draft genomes were retrieved from NCBI GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>).

To examine virulence factors present in above 23 *E. albertii* strains, draft or complete genome of each strain was uploaded to Virulence Factor Database (<http://www.mgc.ac.cn/VFs/>) (23); the major virulence factors were further selected based on previous publications (1, 2). In addition, enterobactin (Ent)-mediated high-affinity iron acquisition, an iron acquisition system important for the pathogenesis of Gram-negative bacterial pathogens, was identified by genome annotation. Briefly, draft or complete genomes of the 23 *E. albertii* strains were annotated using Prokka Annotation (v3.2.1) (24) and RAST (v1.8.1) (25) in KBase server (15). Then the gene clusters (*ent* and *fep*) involved in Ent synthesis and transport were queried for the presence of Ent system.

Virulence factor profile of each *E. albertii* strain was incorporated with the above wgSNP-based phylogenetic tree and visualized using Interactive Tree of Life (22).

6.2.5. *In vitro* growth assay

Inhibitory effect of anti-Ent antibodies on the growth of representative *E. albertii* strains was evaluated using a microtiter plate growth assay as described in a previous publication (26). The anti-Ent rabbit serum used in the assay was generated in our previous study (27). Prior to the assay, both anti-Ent and nonimmunized control sera were heat-inactivated at 56 °C for 30 min to abolish complement activity, followed by dialysis against PBS and sterilization *via* membrane filtration (0.22- μ m-pore-size filter). The sterile rabbit sera were stored at 4 °C. In addition, recombinant human lipocalin-2, due to its high affinity for Ent and the demonstrated bacteriostatic feature (28), was used as positive control in the growth assay. The recombinant human lipocalin-2 was purified from an Ent-deficient Tuner strain (Δ entD) (29) to ensure the purified lipocalin-2 was not bound by endogenous Ent. Purification procedure was described in our previous publication (26). High purity of the recombinant lipocalin-2 was confirmed by SDS-PAGE. Concentration of the purified lipocalin-2 was determined using a BCA assay kit (Thermo Scientific, USA).

To perform *in vitro* growth assay, *E. albertii* strain was inoculated into Luria-Bertani (LB) broth (Difco, USA) supplemented with 0.5 mM 2,2'-dipyridyl. The log-phase cells were washed twice with sterile PBS and subsequently diluted with RPMI medium (Gibco, Ireland) to a final concentration of 10^6 CFU/mL. In each microplate well, 150 μ L RPMI medium was mixed with 50 μ L control serum, 50 μ L anti-Ent serum, or 50 μ L control serum plus 10 μ L stock lipocalin-2 (0.8 mg/mL). Then 5 μ L of the above bacterial inoculum was added to each well. Each treatment was in duplicate. The microplate was incubated in a microplate spectrophotometer (Epoch™ 2, USA) at 37 °C for 24 hr. Optical density at 600 nm (OD600) of each well was automatically read in a 20-min interval and the data was analyzed using Gen5™ software (BioTek, USA) and GraphPad Prism 8 (GraphPad, USA).

6.2.7. Data availability

Genome assemblies of the twelve *E. albertii* chicken strains were deposited in NCBI Sequence Read Archive under BioProject PRJNA753873.

6.3. Results

6.3.1. Isolation of chicken *E. albertii* strains

As shown in Table 6-2, a total of 270 cloacal swabs were collected from the broilers originated from 9 different farms in Mississippi and Alabama. PCR screening of enrichment culture using *E. albertii*-specific primers identified 43 positive samples (15.9%). However, there was a substantial variation in PCR positive rates across different farms, ranging from 0 to 73.3% (Table 6-2). Due to the presence of abundant false positive *E. albertii* colonies in some PCR positive samples, particularly those from Farm 2, only 12 *E. albertii* strains (4.4%) were eventually isolated despite intensive efforts (Table 6-2).

6.3.2. PFGE analysis of chicken *E. albertii* strains

As shown in Figure 6-1, the four *E. albertii* isolates originated from Farm 1 in Mississippi displayed highly similar PFGE profile and formed a distinct phylogenetic cluster. The seven *E. albertii* isolates from Farm 4 in Alabama also showed a clonal relationship, resulting in another phylogenetic cluster (Figure 6-1). One strain (PS211) isolated from Farm 2 in Mississippi exhibited a unique pattern of bands and stood alone as a separate clade (Figure 6-1).

6.3.3. AR profile of chicken *E. albertii* strains

The 12 chicken *E. albertii* strains were examined for AR profile against 20 antibiotics belonging to 14 classes. As shown in Table 6-3, all strains were resistant to streptomycin, an aminoglycoside antibiotic. With respect to other two tested aminoglycosides, the eight strains from Farms 2 and 4 exhibited resistance to gentamicin while all strains, except for PS115 from Farm 1, displayed intermediate resistance to kanamycin. The only strain from Farm 2 (PS211) also had resistance to tetracycline. Regarding beta-lactam antibiotics, the eight gentamicin-resistant strains also displayed resistance to cephalothin; three strains (PT120, PT124, and PT125) had additional intermediate resistance to cefuroxime (Table 6-3). The PS114 strain from Farm 1 showed intermediate resistance to cephalothin and cefuroxime (Table 6-3). Notably, the

nine *E. albertii* strains from Farms 1 and 4 displayed intermediate resistant to imipenem and/or meropenem that belong to carbapenem, the antibiotics of critical clinical significance; in particular, one strain from Farm 1 (PS114) was even resistance to imipenem (Table 6-3).

Despite the high similarity in PFGE fingerprints that was observed for all the *E. albertii* strains isolated from the same farm (Figure 6-1), there were still noteworthy variations in AR profiles of the strains within a farm. Taking those from Farm 4 as an example, all the seven strains consistently showed resistance to cephalothin, streptomycin, and gentamycin, as well as intermediate resistance to kanamycin (Table 6-3). However, the seven strains displayed great variations in the resistance to carbapenem, with two strains (PT102 and PT125) susceptible to both carbapenem antibiotics, one strain (PT113) exhibiting intermediate resistance to the two tested carbapenem antibiotics, and other strains having intermediate resistance only to imipenem (PT106, PT120, and PT124) or only to meropenem (PT103) (Table 6-3). In addition, of the seven strains isolated from Farm 4, only three strains (PT120, PT124, and PT125) displayed intermediate resistance to cefuroxime, the second-generation cephalosporin. Together, these findings strongly suggested that the *E. albertii* strains isolated from the same farm still had significant differences with respect to specific genetic components, which prompted us to perform whole genome sequencing of each isolated strains for in-depth genomics analysis.

6.3.4. Whole genome sequencing and genomic analysis

Sequencing data were summarized in Table 6-4. For each assembled draft genome, completeness was 100% while contamination was below 0.4%, indicating high quality of draft genome. In addition, ANIs of the 12 draft genomes to reference genome of *E. albertii* KF1 were all above 98% (Table 6-4), further confirming the 12 isolates were indeed *E. albertii* species.

A panel of AR genes were identified from draft genome of each *E. albertii* strain using ResFinder and Resistance Gene Identifier. Consistent with PFGE typing (Figure 6-1), the strains originated from the same farm tended to have very similar distribution of AR genes (Table 6-5). AR genes of each strain were also generally in consistence with the AR phenotype (Table 6-5). For example, *aph(3'')-Ib* and *aph(6)-Id* were present in the 4 strains from Farm 1, conferring resistance to streptomycin. Meanwhile, *aadA1* and *aac(3)-Via* were prevalent in all the 8 strains

from Farms 2 and 4, consequently mediating resistance to streptomycin and gentamicin (Table 6-5). Moreover, *ampC* was the only beta-lactamase gene in the twelve *E. albertii* strains (Table 6-5), which could account for intermediate resistance and/or resistance to cephalosporin and/or carbapenem antibiotics (Table 6-3). Lastly, PS211 strain had additional *tet(A)* gene (Table 6-5), which was accordant to its phenotype of tetracycline resistance (Table 6-3). Nevertheless, there were still discrepancies between phenotype and genotype in the strains. For instance, *qnrB19* and *sul1* were reported to contribute to resistance to quinolone antibiotics and sulfamethoxazole, respectively, but no corresponding resistance phenotype was detected in the strains bearing relevant genes (*e.g.*, PS115 and PT102; Tables 6-3 and 6-5).

Plasmids play a critical role in the emergence and transmission of AR genes. Thus, we subsequently analyzed plasmid replicon types in each strain using PlasmidFinder. Plasmid replicon types were identical for the strains from the same farm (Table 6-5). In addition, IncFIB(AP001918) was a common plasmid replicon type in all strains. IncFII(29) was identified in all the 5 strains from Farms 1 and 2 while IncFII and IncI1-I(Alpha) were discovered in all the 8 strains from Farms 2 and 4 (Table 6-5). Lastly, for each of the 8 strains from Farms 2 and 4, the plasmid replicon IncI1-I(Alpha) and three resistance genes (*sul1*, *aac(3)-VIa*, and *aadA1*) were in the same contig (data not shown).

6.3.5. Comparative genomics analysis of virulence factors in diverse strains

To better understand evolution and virulence of the chicken *E. albertii* strains isolated in this study, we further performed in-depth comparative genomics analysis by including eleven previously reported *E. albertii* strains from different hosts in geographically diverse areas. To analyze the evolutionary relationship among different strains, a phylogenetic tree was constructed based on wgSNP of each strain (Figure 6-2). Regarding the 12 chicken *E. albertii* strains derived from this study, their phylogenetic relationship was similar to PFGE analysis (Figure 6-1), except for the single strain PS211 from Farm 2 that displayed high similarity to the 7 strains from Farm 4 (Figure 6-2). Remarkably, all the chicken *E. albertii* strains isolated in this study showed extremely close phylogenetic relationships to some human *E. albertii* pathogens (Figure 6-2). Specifically, the 4 strains from Farm 1 was evolutionarily very close to *E. albertii* KF1 that was isolated from stool sample of an 1-year-old child with acute diarrhea in Bangladesh

(16). The other 8 chicken strains (1 from Farm 2 and 7 from Farms 4) were highly close to another human strain 2014C-4356 isolated in the U.S. (Figure 6-2).

We also examined and compared major virulence factors that potentially contribute to *E. albertii* pathogenicity, such as those required for adherence, invasion and *in vivo* survival (Figure 6-2). All examined *E. albertii* strains possessed Ent-iron acquisition system except for strain PS114 from this study (Figure 6-2). Different adherence factors, including hemorrhagic coli pili (HCP), Type I fimbriae, Paa, and intimin, were highly prevalent in all the 23 strains. Colonization factor antigen I (CFA/I) fimbriae was only detected in 5 strains from human and bird while K88 fimbriae was exclusively present in the 8 chicken *E. albertii* strains from Farms 2 and 4 (Figure 6-2). With respect to the factors contributing to invasion, Ibe was common for all the strains while Tia/Hek was only identified in the strain TN-C1, a chicken *E. albertii* strain isolated in Tennessee in our recent study (8) (Figure 6-2). In terms of presence of toxins, CDT was present in all strains except the strain NCTC 9362; Shiga toxin was only identified in two human strains (Figure 6-2). All examined strains possessed T3SS that potentially contributed to bacterial pathogenicity and formation of A/E lesions (Figure 6-2). Consistent with high prevalence of T3SS, all the *E. albertii* strains also had various T3SS effectors for pathogenicity, such as EspB, EspG, Map, and NleA (data not shown). Number of the effectors varied greatly among the strains, ranging from 9 to 23 (Figure 6-2).

6.3.6. *In vitro* growth assay

The recently developed Ent-based immune intervention strategies are promising and effective to control various Gram-negative pathogens including *E. coli* (26, 27). Given that Ent-mediated iron acquisition system was also highly prevalent in *E. albertii* (Figure 6-2), we speculated that anti-Ent antibodies could also inhibit the growth of *E. albertii*. To test this, we selected two representative *E. albertii* strains, PS211 and PT102, for *in vitro* assays using a standard protocol. As shown in Figure 6-3, the anti-Ent antibodies exerted strong inhibitory effect on the growth of both tested strains. Specifically, for strain PS211, anti-Ent serum dramatically inhibited its growth with barely detected OD600 by 18 hr post-inoculation while the cells grew well to approximately 0.4 of OD600 in the presence of control serum (Figure 6-3A). Similarly, lipocalin-2, a positive control, also strongly inhibited cell growth by 18 hr post-inoculation

(Figure 6-3A). For strain PT102, both anti-Ent serum and lipocalin-2 displayed stronger inhibition on growth when compared to their effects on PS211 (Figure 6-3B), reflected by extremely low OD600 (approximating to 0 for both anti-Ent serum and lipocalin-2) throughout 24 hr.

6.4. Discussion

In this study, we conducted a large-scale survey to investigate the prevalence of *E. albertii* in broilers. There were dramatic variations in PCR positive rates of *E. albertii* across different farms, with the average rates being 9.4% and 28.9% in the farms in Mississippi and Alabama, respectively (Table 6-2). Meanwhile, our recent pilot survey, using the same isolation procedure, showed a higher PCR positive rate (35%) in a broiler farm in Tennessee (8). Based on these findings, *E. albertii* is likely widespread in poultry production of the U.S., but the prevalence rates may be greatly varied across different states, which needs to be investigated in future studies using more samples with high socioeconomic and geographic diversity. In addition, overall isolation rate of *E. albertii* (4.4%) was lower than the PCR positive rate (15.9%) in this study (Table 6-2), indicating the selective medium used in this study, the XRM-MacConkey agar, still needs to be optimized to improve the ability to isolate *E. albertii*.

Regarding AR of the 12 *E. albertii* strains, there are notable discrepancies between the phenotype and genotype (Table 6-5). Specifically, for the strain PS115, it carried a quinolone resistance gene *qnrB19* but did not show resistance to quinolone antibiotics nalidixic acid and ciprofloxacin (Tables 6-3 and 6-5). This inconsistency is likely due to low-level resistance to quinolone conferred by *qnrB19* (30) and has also been observed in the *E. coli* and *S. enterica* isolates harboring plasmid-borne *qnrB19* (31-33). Furthermore, when the plasmids carrying *qnrB19* were transferred to *E. coli* recipients, the MICs of the transconjugants for nalidixic acid and ciprofloxacin were elevated but still did not exceed the susceptibility breakpoints (31, 33). Therefore, it is not surprising that the strain PS115 that carried *qnrB19E* still displayed susceptible phenotype for nalidixic acid and ciprofloxacin as observed in this study. Nevertheless, *qnrB* genes-mediated low-level quinolone resistance should not be overlooked as it may provide a survival advantage to pathogens and facilitate selection of higher-level resistance, making the infections harder to treat (30).

In addition, for the 8 strains from Farms 2 and 4, the presence of *sul1* failed to confer resistance to trimethoprim-sulfamethoxazole (Tables 6-3 and 6-5). This discrepancy between the phenotype and genotype has also been observed in *E. coli* (34). The *sul1* in the examined *E. albertii* strains might not be expressed to the level required for conferring resistance to trimethoprim-sulfamethoxazole as proposed in a previous *E. coli* study (34). Lack of proper expression also may explain why wide presence of *ampC*, a gene contributing to beta-lactam resistance (35), led to different beta-lactam resistance profiles across the twelve *E. albertii* strains obtained from this study (Tables 6-3 and 6-5). However, other mechanisms, such as overexpression of efflux pumps and decreased permeability of cell membrane that are common in Enterobacteriaceae (36), may also contribute to the observed drastic variations in terms of beta-lactam resistance in these *E. albertii* isolates.

In this study, we utilized both PFGE fingerprints and whole genome sequences of chicken *E. albertii* strains to build phylogenetic trees. The strains originated from the same farms were evolutionally highly close to each other when using both approaches (Figures 6-1 and 6-2). However, the single strain PS211 from Farm 2 was comparably remote to the two clusters of Farms 1 and 4 in PFGE analysis while it fell into the cluster of Farm 4 in wgSNP analysis (Figures 6-1 and 6-2). A feasible explanation for this discordance is the mechanistic differences between PFGE and wgSNP analyses. The wgSNP analysis in this study is based on calling SNP in whole genome sequence against a reference genome (21), which will ignore lineage-specific regions that are absent in the reference genome (37, 38). In contrast, PFGE is more sensitive to these lineage-specific regions and consequently leads to higher magnitude of divergence among the strains (38). Although whole genome sequence is usually considered to provide much higher resolution than PFGE in discriminating strains, we should be prudent in selecting suitable algorithms for whole genome sequence-based epidemiological typing.

Strikingly, comparative genomics analysis reveals the 12 chicken *E. albertii* strains were phylogenetically very close to either KF1 or 2014C-4356, the two human pathogens; major virulence profiles of the chicken strains were also very similar to those of human origin (Figure 6-2). In a recent study, we demonstrated that a human clinical *E. albertii* strain could colonize

well in the chicken intestine (8). Therefore, the findings from comparative genomics analysis in this study further indicate chickens serve as an important reservoir of *E. albertii* for the emerging infections in humans.

Ent-mediated iron acquisition is highly conserved in various Gram-negative bacterial pathogens belonging to Enterobacteriaceae (39). As expected, Ent-mediated iron acquisition was present in almost all *E. albertii* examined in this study (Figure 6-2). The Ent-mediated high affinity iron acquisition plays a critical role in the survival and pathogenicity of many Gram-negative pathogens in various host niches, consequently serving as an attractive target for iron-dependent pathogen control in the past decades (39). Recently, we successfully developed a novel Ent conjugate vaccine that could elicit high titer of specific antibodies directed against Ent (27). The anti-Ent antibodies could sequester Ent and significantly inhibit the *in vitro* growth of *E. coli* and *S. enterica* strains with diverse origins and siderophore repertoires (26). In this study, we also demonstrated potent bacteriostatic effect of the anti-Ent antibodies on the representative *E. albertii* strains (Figure 6-3). Thus, the Ent-based immune intervention strategy could be a promising approach to prevent and control *E. albertii*, an emerging zoonotic pathogen.

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Appendix

Table 6-1. Summary of the diverse *E. albertii* strains from other sources and used for comparative genomics analysis

Strain ID	Host	Country	Accession no.	Reference
KF1	Human	Bangladesh	CP007025	DOI: 10.1128/genomeA.00004-14
1551-2	Human	Brazil	CP025317	DOI: 10.1128/genomeA.00075-18
2014C-4356	Human	USA	CP024282	DOI: 10.1128/genomeA.01384-17
NIAH_Bird_3	Bird	Japan	AP014855	DOI: 10.1093/gbe/evv211
EC06-170	Human	Japan	AP014857	DOI: 10.1093/gbe/evv211
CB9786	Human	Germany	AP014856	DOI: 10.1093/gbe/evv211
TN-C1	Chicken	USA	SAMN14781548	DOI: 10.1111/zph.12812
2012EL-1823B	Human	USA	CP030783	DOI: 10.1128/MRA.01663-18
2014C-4015	Human	USA	CP034166	DOI: 10.1128/MRA.01663-18
NCTC 9362	Human	Egypt	CP034213	DOI: 10.1128/MRA.01663-18
06-3542	Human	USA	CP034162	DOI: 10.1128/MRA.01663-18

Table 6-2. Isolation statistics of chicken *E. albertii* strains

Farm ID	Location	Sampling time	No. of swab	No. of PCR positive (%)	No. of <i>E. albertii</i> isolates (%)
1	Mississippi	2020 July	30	9 (33.3%)	4 (13.3%)
2	Mississippi	2020 July	30	7 (23.3%)	1 (3.3%)
3	Mississippi	2020 August	30	0	0
4	Alabama	2020 August	30	22 (73.3%)	7 (23.3%)
5	Alabama	2020 August	30	2 (6.7%)	0
6	Alabama	2020 August	30	2 (6.7%)	0
7	Mississippi	2020 September	30	0	0
8	Mississippi	2020 September	30	0	0
9	Mississippi	2020 September	30	1 (3.3%)	0
In total			270	43 (15.9%)	12 (4.4%)

Table 6-3. AR profile of chicken *E. albertii* strains

Class	Antimicrobial (µg)	Breakpoints (R, I, S)	Farm 1				Farm 2	Farm 4						
			PS107	PS114	PS115	PS128	PS211	PT102	PT103	PT106	PT113	PT120	PT124	PT125
1st generation cephalosporin	cephalothin (30)	14, 15-17, 18	18	17	18	18	13	13	13	12	13	12	12	13
2nd generation cephalosporin	cefuroxime (30)	14, 15-17, 18	20	17	19	21	18	18	19	19	20	17	17	17
3rd generation cephalosporin	cefotaxime (30)	22, 23-25, 26	31	34	33	34	31	33	36	35	33	32	33	33
	ceftazidime (30)	17, 18-20, 21	26	27	28	28	26	30	31	29	29	29	28	29
	ceftriaxone (30)	19, 20-22, 23	30	29	31	30	32	32	33	31	31	31	32	30
4th generation cephalosporin	cefepime (30)	18, 19-24, 25	30	29	32	32	33	34	34	34	32	33	33	32
Carbapenem	imipenem (10)	19, 20-22, 23	20	17	21	20	23	24	23	22	22	22	22	23
	meropenem (10)	19, 20-22, 23	21	21	23	22	23	25	21	25	20	23	23	24
Penicillin	ampicillin (10)	13, 14-16, 17	20	21	21	22	19	19	22	19	19	20	20	20
	piperacillin (100)	17, 18-20, 21	27	28	27	28	28	28	29	28	27	28	28	27
Monobactam	aztreonam (30)	17, 18-20, 21	33	31	32	34	35	37	35	33	32	32	32	34
Cepharmycin	cefoxitin (30)	14, 15-17, 18	24	23	26	26	24	24	26	25	24	24	25	24
Aminoglycoside	kanamycin (30)	13, 14-17, 18	15	16	18	17	16	17	15	16	16	16	15	16
	streptomycin (10)	11, 12-14, 15	6	6	6	6	6	6	6	6	6	6	6	6
	gentamicin (10)	12, 13-14, 15	17	16	17	19	8	8	8	8	8	8	8	10
Tetracycline	tetracycline (30)	11, 12-14, 15	22	21	22	22	6	20	19	19	21	21	19	19
New quinolone	ciprofloxacin (5)	21, 22-25, 26	35	36	33	35	39	37	36	35	36	37	37	33
Quinolone	nalidixic acid (30)	13, 14-18, 19	26	27	26	25	25	25	24	23	24	24	25	23
Metabolic inhibition	trimethoprim-sulfamethoxazole (1.25/23.75)	10, 11-15, 16	24	25	26	26	26	27	26	25	26	26	26	25
Chloramphenicol	chloramphenicol (30)	12, 13-17, 18	26	24	27	26	25	25	24	24	24	25	26	24

The antimicrobial susceptibility test was conducted using disk diffusion method on Mueller Hinton agar. The numbers in the cells with gray shading denote resistance while the numbers in bold denote intermediate resistance.

Table 6-4. Whole genome sequencing information of chicken *E. albertii* strains

Strain ID	Read pair counts	Coverage	No. of contigs	Total nt assembled	Average contig length	Longest contig length	N50	Genome completeness ^a	Contamination ^a	ANI to <i>E. albertii</i> KF1 ^b
PS107	665012	83X	257	4817771	18746	171699	42924	100%	0.39%	99.72%
PS114	590002	73X	229	4771519	20836	157082	50266	100%	0.39%	99.71%
PS115	645061	80X	260	4819827	18538	157134	39595	100%	0.39%	99.75%
PS128	832753	104X	248	4820267	19437	136789	44037	100%	0.39%	99.76%
PS211	663430	82X	192	5110925	26619	261219	86379	100%	0.23%	98.41%
PT102	1111499	115X	175	4907020	28040	222639	75913	100%	0.23%	98.41%
PT103	1325218	138X	164	4909787	29938	278061	95585	100%	0.23%	98.51%
PT106	1228581	127X	163	4901973	30073	278061	102999	100%	0.23%	98.42%
PT113	1184007	123X	134	4909773	36640	295440	128546	100%	0.23%	98.39%
PT120	1289232	134X	162	4904236	30273	344723	87618	100%	0.23%	98.46%
PT124	1422974	148X	112	4912282	43860	278137	140428	100%	0.23%	98.40%
PT125	779806	81X	140	4908511	35061	444287	128222	100%	0.23%	98.43%

^aCompleteness and contamination of each draft genome were assessed using CheckM (v1.0.18) in KBase server.

^bAverage nucleotide identity (ANI) of each *E. albertii* genome to the reference strain KF1 was calculated using FastANI (v0.1.2) in KBase server.

Table 6-5. AR phenotype and genetic analysis of chicken *E. albertii* strains

Farm ID	Strain ID	AR phenotype ^a	AR genotype ^b	Plasmid replicon ^c
Farm 1	PS107	STR	<i>aph(3'')-Ib, aph(6)-Id, ampC</i>	Col(pHAD28), IncFIB(AP001918), IncFIC(FII), IncFII(29), IncFII(pHN7A8), IncX1, pEC4115
	PS114	STR, IPM	<i>aph(3'')-Ib, aph(6)-Id, ampC</i>	Col(pHAD28), IncFIB(AP001918), IncFIC(FII), IncFII(29), IncFII(pHN7A8), IncX1, pEC4115
	PS115	STR	<i>aph(3'')-Ib, aph(6)-Id, ampC, qnrB19</i>	Col(pHAD28), IncFIB(AP001918), IncFIC(FII), IncFII(29), IncFII(pHN7A8), IncX1, pEC4115
	PS128	STR	<i>aph(3'')-Ib, aph(6)-Id, ampC</i>	Col(pHAD28), IncFIB(AP001918), IncFIC(FII), IncFII(29), IncFII(pHN7A8), IncX1, pEC4115
Farm 2	PS211	STR, CEF, GEN, TET	<i>sul1, aac(3)-VIa, aadA1, ampC, tet(A)</i>	Col(MG828), IncFIB(AP001918), IncFII, IncFII(29), IncI1-I(Alpha)
Farm 4	PT102	STR, CEF, GEN	<i>sul1, aac(3)-VIa, aadA1, ampC</i>	IncFIB(AP001918), IncFII, IncI1-I(Alpha)
	PT103	STR, CEF, GEN	<i>sul1, aac(3)-VIa, aadA1, ampC</i>	IncFIB(AP001918), IncFII, IncI1-I(Alpha)
	PT106	STR, CEF, GEN	<i>sul1, aac(3)-VIa, aadA1, ampC</i>	IncFIB(AP001918), IncFII, IncI1-I(Alpha)
	PT113	STR, CEF, GEN	<i>sul1, aac(3)-VIa, aadA1, ampC</i>	IncFIB(AP001918), IncFII, IncI1-I(Alpha)
	PT120	STR, CEF, GEN	<i>sul1, aac(3)-VIa, aadA1, ampC</i>	IncFIB(AP001918), IncFII, IncI1-I(Alpha)
	PT124	STR, CEF, GEN	<i>sul1, aac(3)-VIa, aadA1, ampC</i>	IncFIB(AP001918), IncFII, IncI1-I(Alpha)
	PT125	STR, CEF, GEN	<i>sul1, aac(3)-VIa, aadA1, ampC</i>	IncFIB(AP001918), IncFII, IncI1-I(Alpha)

Abbreviations: CEF, cephalothin; GEN, gentamicin; IPM, imipenem; STR, streptomycin; TET, tetracycline.

^aA total of 20 diverse antibiotics were used for susceptibility test using disk diffusion method on Mueller Hinton agar.

^bThe AR genes were identified from draft genome of each strain using ResFinder (v4.1) with nucleotide identity above 90% and length coverage above 80%. The AR gene *ampC* was additionally identified by Resistance Gene Identifier (v5.2.0) with nucleotide identity above 95%.

^cThe plasmid replicon types were identified from draft genome of each strain using PlasmidFinder (v2.1) with nucleotide identity above 90% and length coverage above 80%.

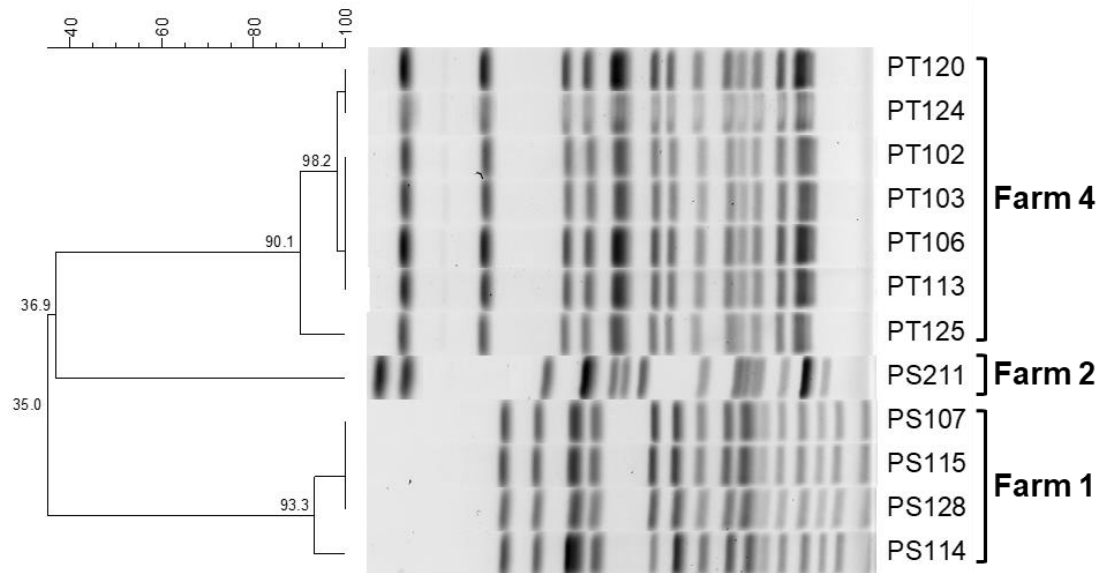


Figure 6-1. Dendrogram of PFGE analysis of chicken *E. albertii* strains. Genomic DNA of each *E. albertii* strain was digested with *Xba*I and separated by PFGE. Strain ID is on the right of each lane. Farm origin of each strain is denoted on the far right.

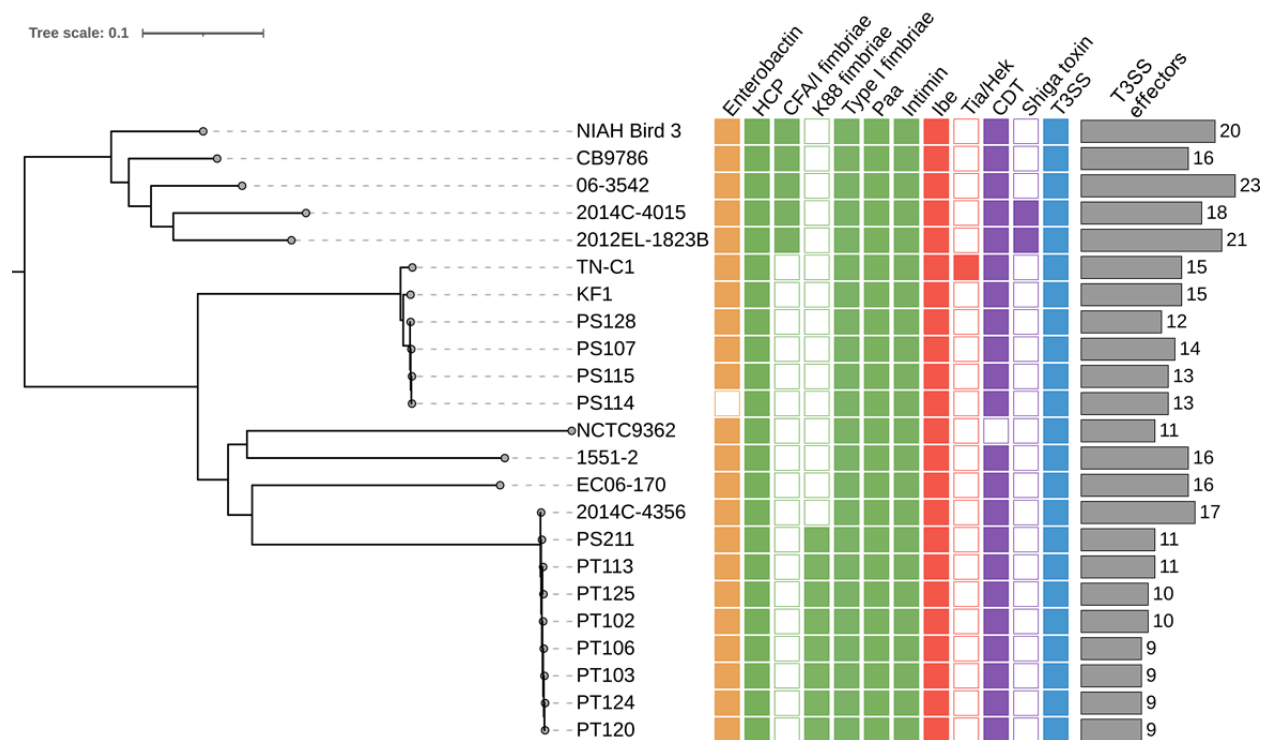


Figure 6-2. Comparative genomics analysis of *E. albertii* strains. Eleven *E. albertii* strains with diverse background were compared with the twelve chicken strains obtained from this study for genomics analysis. The phylogenetic tree was constructed using CSI Phylogeny (v1.4). Major virulence factors were identified using Virulence Factor Database and displayed on the right of each strain. Solid and open squares denote presence and absence of a virulence factor, respectively. The square colors yellow, green, red, and violet represent virulence categories iron acquisition, adherence, invasion, and toxin, respectively

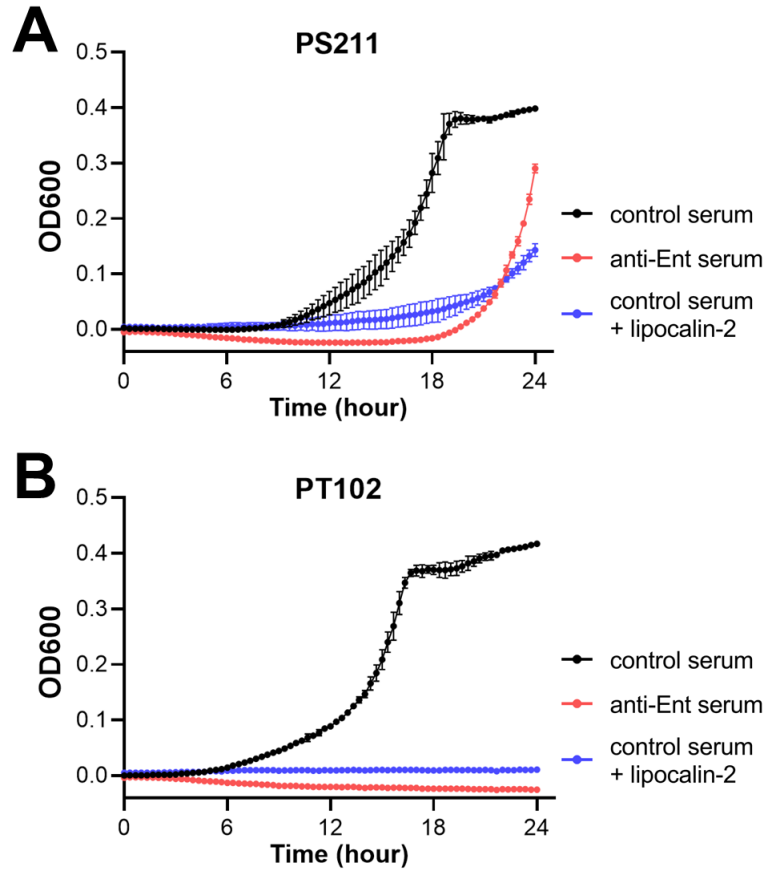


Figure 6-3. *In vitro* growth of representative chicken *E. albertii* strains in response to anti-Ent serum. The *E. albertii* cells were grown in the RPMI medium supplemented with rabbit nonimmunized control serum, anti-Ent serum, or the control serum plus lipocalin-2 (final concentration of 37 $\mu\text{g/mL}$). Detailed procedure was described in *Materials and Methods*. Each data point represents mean $\pm$ standard deviation of OD600 obtained from duplicate wells in a microtiter plate. The tested strains are PS211 (A) and PT102 (B) originated from Farms 2 and 4, respectively.

Conclusion

In this study, we have demonstrated *in vitro* bacteriostatic effect of anti-Ent antibodies on Ent-dependent Gram-negative bacterial pathogens, including different pathovars of *Escherichia coli* and *Salmonella enterica* that had varied siderophore production profiles as well as *Campylobacter* spp. Based on this, we further used a chicken model for avian pathogenic *E. coli* (APEC) infection and demonstrated that the vaccination induced high titers of anti-Ent IgY in the sera and consequently ameliorated the organ lesions caused by a highly virulent APEC despite the bacterial burden in the organs being not affected. We also detected the vaccine-induced specific IgY in the chicken intestine and found the vaccination affected the intestinal microbiota. Meanwhile, given the enticing features of egg yolk IgY as a passive immune agent, we assessed passive immune protection of the anti-Ent egg yolk IgY against intestinal colonization of *C. jejuni*. However, oral administration of anti-Ent hyperimmune egg yolk failed to confer protection against *C. jejuni* colonization, consistent with the finding of no presence of the egg yolk IgY in chicken gastrointestinal tract or serum. We further utilized an *ex vivo* model system to reveal that egg yolk IgY underwent substantial degradation when passing through chicken gizzard, highlighting the necessity of proper encapsulation of egg yolk IgY for controlling enteric pathogens. Lastly, an epidemiological survey of *E. albertii* in broilers revealed chicken as an important reservoir for this emerging zoonotic pathogen. We also demonstrated antimicrobial potential of the anti-Ent antibodies against *E. albertii*. Collectively, using multidisciplinary approaches and different model systems, this dissertation study demonstrated the feasibility of Ent-based immune interventions against different Gram-negative bacterial infections.

Vita

Huiwen Wang was born on December 27, 1991 in Gansu, China. He obtained a B.S. degree in Veterinary Medicine at China Agricultural University in 2015. Then he was enrolled into the Animal Science Program at the University of Tennessee, Knoxville in 2017 to pursue his graduate study. Under the mentorship of Dr. Jun Lin, he obtained a M.S. degree in Animal Science in 2019 and will soon get his Ph.D. degree in 2022.