Effect of synbiotic and organic acid plus phytochemical product supplementation on layer production performance and immune parameters

THESIS

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By

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Abstract

This project studied 1. Inhibitory effects of probiotic culture supernatant from four different probiotic strains against *Salmonella enterica* Enteritidis (*S. Enteritidis*) in vitro, 2. the effect of a synbiotic product on layer production parameters, gut bacterial profile, and immune parameters, pre- and post-*Salmonella* challenge, and 3. the effect of organic acid plus phytochemical supplement product on layer production parameters with and without *Salmonella* vaccine.

In the first objective, two experiments were conducted to study the inhibitory ability of culture supernatants from four different probiotic bacterial strains on *S. Enteritidis* growth in vitro. The first experiment studied the effect of probiotic culture supernatant on *Salmonella* inhibition in liquid media. Culture supernatants from the probiotics *L. reuteri*, *E. faecium*, *B. animalis*, or *P. acidilactici* probiotics inhibited (*P < 0.05*) the growth of *Salmonella*. The second experiment studied the effects of probiotic supernatants on *Salmonella* inhibition in solid media. The culture supernatant from *E. faecium* and *L. reuteri* separately inhibited *Salmonella* growth efficiently compared to that from *B. animalis* and *P. acidilactici*.

In the second objective, the effects of a synbiotic containing four live probiotic strains on layer production and local immune parameters following an experimental
Salmonella infection was studied. At 18 and 20 wk of age, birds fed synbiotics in both vaccinated and unvaccinated pullets had higher body weight (P < 0.05) than the non synbiotic control group. Birds fed synbiotics had 0.7%, 17.8%, 21.7%, 3%, and 4.2% higher (P < 0.05) hen day egg production (HDEP) at 19, 20, 21, and 23 wk of age, compared to the birds fed no synbiotics, respectively. At 24 wk of age, birds were challenged with S. enterica Enteritidis. Hens fed synbiotics had 3%, 6.7%, 4.3%, 12.5%, and 14.4% higher (P < 0.05) HDEP at 24, 25, 26, 27, and 28 wk of age, compared to the birds fed no synbiotics, respectively. Birds supplemented with synbiotics had higher (P < 0.05) number of cecal P. acidilacti and L. reuteri compared to the group with no synbiotic supplementation. Irrespective of the vaccination status, birds fed synbiotics and challenged with Salmonella had lower S. Enteritidis content (P < 0.05) compared to that in the no synbiotic supplemented and unvaccinated treatment group.

In the third objective, the effects of a blend of organic acid and phytochemical supplement on pullet growth and egg production parameters with and without Salmonella vaccination was studied. Supplementation of organic acid and phytochemical supplement increased body weight from day-of-hatch through 23 wk of age (P < 0.05). Birds which received diets supplemented with organic acid and phytochemical supplement had increased body weight between 14-23 wk of age (P < 0.05). Organic acid and phytochemical supplement increased weekly HDEP between 19-23 wk of age (P < 0.05).

In conclusion, this study quantified the inhibitory effects of four different probiotic strains on S. Enteritidis in vitro suggesting that these four probiotic strains can be fed to decrease Salmonella shedding in poultry. Supplementation of a synbiotic product to layer
diets increased production performance and protected against S. Enteritidis infection. Organic acid plus phytochemical supplementation to layer hens improved production performance in layer hens.
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Chapter 1: Introduction

Salmonella is the causative agent of Salmonellosis, one of the most common infectious diseases for both humans and animals. In humans, Salmonellosis is characterized by gastroenteritis, fever, nausea, and stomach cramps. In poultry, however, Salmonella is essentially asymptomatic, and can survive in the host indefinitely. Salmonellosis is an important public health problem and, due to the emergence of antibiotic resistant Salmonella, new ways of controlling Salmonella at the farm level are needed [1, 2]. Approximately half of all Salmonella infections are caused by three serovars, one of which is Salmonella enterica Enteritidis. S. Enteritidis is the most common serovar isolated in poultry [2]. S. Enteritidis causes Salmonellosis in humans and is linked to the consumption of contaminated eggs, egg products, and meat, and therefore control at this level is of increasing importance [3].

Many feed additives such as probiotics, enzymes, bacteriophages, organic acids, and essential oils have been identified to decrease Salmonella colonization in the chicken intestine [4]. Probiotics have become an increased interest in the agriculture world and have become acceptable alternatives to antibiotics, in broilers, and can potentially
minimize enteric pathogen diseases [5, 6] in both layers and broilers. Probiotics exert their actions through competitive exclusion [7] as well as modulation of the immune system [8] and altering the balance between pro- and anti-inflammatory cytokines [9, 10]. Control of *Salmonella* using probiotics is cited back to the first competitive exclusion study by Nurmi and Rantala in 1973 [7].

Organic acids include fatty acids, volatile fatty acids, or carboxylic acids, and are being studied for their potential antimicrobial properties as well as their effect on overall production performance improvement in poultry production [11]. It has been theorized that organic acids exert their antibacterial activities by disrupting the cytoplasmic membrane structure and uncoupling the electron transport chain, essentially hindering ATP production of the pathogen [12-14]. It has also been suggested that organic acids induces the accumulation of intracellular anions which would reduce the interior pH of the bacteria leading to decreased proliferation and death [15]. Previous studies have shown that supplemented organic acids in layer diets have been shown to reduce *Salmonella* infections [16, 17].

The objectives of the present studies are as follows:

Specific Aim 1

To determine the inhibitory potential of culture supernatant of four probiotic strains (*Lactobacillus reuteri, Pediococcus acidilacti, Bifidobacteria animalis, and Enterococcus facecium*) against *Salmonella*.

Specific Aim 2

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2
To determine the effects of synbiotic product supplementation on layer bird production performance, *Salmonella* colonization and immune response.

Specific Aim 3

To determine the effects of organic acid plus phytochemical product on pullet growth and layer production performance.
Chapter 2: Review of Literature

Salmonella

*Salmonella* bacteria are gram-negative bacilli that are typically anaerobic, non-capsulated, and non-sporulating. There are many different subspecies of *Salmonella* which can infect and cause various diseases in its host. *Salmonella* infection leads to illness and death in young poultry without developed immune systems and can be spread from poultry to humans [18]. *Salmonella enterica* is a gram-negative bacterial pathogen which includes more than 2500 different serovars and can infect a wide range of hosts [19]. *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium are typically the most frequently isolated serovars in chickens, although others are commonly detected [20]. Birds infected with *S. Enteritidis* are usually asymptomatic and can shed the bacteria in the feces for long periods of time, infecting the environment and other closely housed birds [21]. In addition, *S. Enteritidis* can also be found on layers’ egg shells and inside the eggs, as a consequence of bacterial infection of the reproductive tract [18]. This serovar is also associated with human infection and can lead to gastrointestinal disease, through the handling and consumption of poultry and poultry products. Infection with *S. Enteritidis* is usually self-limiting, but can become severe and require antibiotic intervention [22].
However, with the emergence of antibiotic resistant *Salmonella*, treatment is becoming more difficult and there has become a need for control of *Salmonella* in poultry flocks [1].

*Salmonellosis*

About 40,000 cases of salmonellosis are reported annually in the United States, although the unreported number is up to 30 fold higher [23]. Salmonellosis accounts for about half of food poisoning cases each year in the United States and results in about 2000 deaths annually and domestic poultry are the largest reservoir of *Salmonella* [18]. Human contact with *Salmonella* is typically through infected egg or meat products, however, egg contamination in the hatchery is low, only up to 10% of all 1-day old chicks are *Salmonella* positive due to contamination due to egg incubation[18]. Additionally, less than 10% of all chickens entering processing plants are *Salmonella* positive, but 30-50% of total carcasses become contaminated during processing. Control of *Salmonella* depends on pathogen reduction in the hatchery, farm, and processing plants. Although there are several measures to control *Salmonella* already in place, such as vaccinations and antibiotics, these measures are not as effective as they need to be and can cause environmental contamination and antibiotic resistance. It is because of this that there is a need for a better way of controlling the bacteria and many experimental measures are underway, such as competitive exclusion products [18].
**Gut Microflora**

The intestinal tract of newly hatched chicks contains very few microorganisms, if any, and over time is colonized and form stable microflora populations. We can facilitate in this process by providing various feed additives, such as probiotics [24].

Gut microflora gradually become established in young birds but in the first week of life there are many other important events because this is when transient gut inflammation is detected. During the first 3 days of life, the chicks’ cecum is protected by high levels of β-defensins until the normal gut immune system can develop through expression of pro-inflammatory cytokines. Because of this, newly hatched birds respond to *S. Enteritidis* via the Th1 type of immune response whereas adult birds respond via the Th17 type immune response [25].

Adult birds have a gastrointestinal tract populated with fungi, protozoa, and bacteria, with bacteria forming the largest population [26]. Of the microbes, there are both beneficial and pathogenic microbes that can inhabit the intestinal tract. Pathogenic microbes cause infections and can produce harmful secretions or molecules which can lead to the breakdown of gut integrity. Beneficial microbes, however, can produce products that are helpful to the host organism, such as vitamins, provide competitive exclusion advantages (nutrient and binding competition, acid production), and stimulate the immune system [27].
Course of Infection

Infection with *Salmonella* occurs through interactions between the environment and the host. This can include the hen house, the poultry plant facility with the carcasses, the hen reproductive tract with the egg, and the carcass or egg with the consumer. *Salmonella* is shed from the bird in the feces and is spread horizontally to other birds. During processing, *Salmonella* in feces or intestinal content can be spilled and can contaminate other carcasses, leading to human infection. Additionally, *Salmonella* can spread to the eggs of hens as a consequence of bacterial infection of the reproductive tract [28, 29].

*S. Enteritidis* and *S. Typhimurium* both have similar virulence factors and course of infections. Once ingested, the bacteria makes its way to the stomach of the host and activates an acid tolerance response, which allows the organism to protect itself from the acidity of the stomach. The acid tolerance response is a pH-homeostatic mechanism which enables the bacteria to maintain a higher intracellular pH than its surrounding environment [30]. The salmonellae are then able to adhere to the intestinal epithelial cells of the intestine and engage host cell signal pathways responsible for cytoskeletal rearrangement. This disruption of the epithelial brush border causes the formation of *Salmonella*-containing vacuoles (SCVs) wherein the *Salmonella* can survive and replicate. The SCVs are transported to the basolateral membrane and the salmonellae are released into the submucosa where they are engulfed by phagocytes which unknowingly transport the bacteria through the lymph and bloodstream and cause a systemic infection [23, 31].
Gene Regulation

Matulova et al. (2013) found gene expression in the chicken cecum after an *S.* Enteritidis infection. Of the genes downregulated after infection, they found that 15-hydroxyprostaglandin dehydrogenase (HPGD) was potentially involved in host defense against the bacteria. HPGD works to inactivate prostaglandin D2, however, of the genes that were upregulated after infection, prostaglandin D2 synthase, the enzyme responsible for synthesizing prostaglandin D2, was one of them. This suggests that prostaglandin D2 accumulates in the cecum following the infection and works as an inflammatory signal.

Of the genes upregulated after infection, those that were observed to have an induction rate of 100 fold or higher included matrix metalloproteinase-7 (MMP7), IgG, immunoreponsive 1 Homolog (IRG1), Serum amyloid A (SSA), Extracellular-FABP, IL-22, Thrombin Receptor Activator for Peptide 6 (TRAP6), Inos, IFNγ, IL-1β, Lysozyme G-Like 1 (LYG1), Interferon-Induced Protein With Tetratricopeptide Repeats 5 (IFIT5), IL-17, avidin, chemokine AH221, and SERPIN B. However, the genes which had the total highest levels of expression, not taking into account their fold increase included AVD, ExFABP, IgG, IgA, AH221 SAA, MMP7, and TRAP6. This shows that genes coding for cytokines were not among the genes with the highest expression levels.

The majority of the genes showing significant impacts from this study have not been previously linked with the chicken immune response to *Salmonella*, but have been linked with other inflammatory infections and cancer. The conclusion from the genes upregulated after the infection was that the response was primarily coordinated by epithelial cells, T and B-lymphocytes, macrophages, and heterophils [32].
**Maternal antibodies and passive transfer of immunity**

Passive immunization procedures have been done to examine the protective role of Salmonella-reactive maternal antibodies during Salmonella infections. In 1997, Methner and colleagues studied the effects of maternally transmitted Salmonella antibodies after the administration of an inactivated S. Enteritidis vaccine. They found that maternal antibodies that were transferred to the chicks did not significantly increase resistance to an oral Salmonella challenge at 1-day of age, although fewer bacteria were recovered from the internal organs and ceca [33]. In 1998, Yokoyama and colleagues studied the protective abilities of chicken egg yolk antibodies specific for S. Enteritidis outer membrane proteins, lipopolysaccharide, and flagella during both an S. Enteritidis and S. Thypimurium infection and identified that all three chicken antibodies were able to significantly protect against both infections, with the outer membrane protein antibody giving the highest level of protection [34].

**Chicken Immune Response**

The chicken immune response against intracellular bacteria includes several factors including pathogen associated molecular patterns (PAMPs) being detected by host pattern recognition receptors (PRRs), toll-like receptors, cytokine signaling, and a specific adaptive immune response [35]. The innate immune response is triggered by pro-inflammatory cytokines and monocytes, natural killer cells, macrophages, and heterophils, which are all important primary cells in the response against S. Enteritidis [36-39]. While
the innate immune response is important, the adaptive immune response provides specific immune responses against the pathogen [40].

**Innate Immune System**

The innate immune system is the first line of defense against pathogens and is considered to be fast and non-specific. This system works by recognizing PAMPs presented on foreign cells that are detected by PRRs from host cells. Toll-like receptors are an example of a PRR found on many host cells such as macrophages, B cells, dendritic cells, and certain T cells. PAMPs can be anything secreted or presented by foreign cells that host cells would not normally come across in ideal conditions, such as peptidoglycan and double stranded RNA. When immune cells bind to these PAMPs and are activated, adaptor proteins are recruited to the toll-like receptor, which then activates additional downstream signaling, resulting in production of pro-inflammatory cytokines and chemokines, as well as destruction by heterophils and macrophages through phagocytosis [41].

Pathogens specifically activate the innate immune response through modulins, which is a general term for proteins, lipoproteins, glycoproteins, carbohydrates, and lipids, produced by bacteria and used during infection as virulence factors [42]. Immune cells that are activated by the modulins produce pro-inflammatory cytokines.

The innate immune system activates the adaptive immune system. Antigen presenting cells, such as macrophages and dendritic cells, present antigens to naive CD4+ T cells, activating the adaptive immune response and the transformation of the naive CD4+
T cells into T helper cells or cytotoxic cells. T helper cells help other immune cells by releasing T cell cytokines such as IL-4 and IL-13, which help to regulate the immune response. Toll-like receptor activation typically results in more T helper cell differentiation and is an essential activator of an inflammatory response. Cytotoxic cells possess T cell receptors which can bind to specific antigens presented on foreign cells, allowing the cytotoxic cell to kill the foreign cell [41], [43].

**Adaptive Immune System**

The adaptive immune system provides a more pathogen-specific response to an infection than the innate immune system and primarily uses antigen-presenting cells and T and B lymphocytes to activate pathogen-specific immune responses. These responses generate memory for the adaptive immune cell during the first encounter with the antigen and when the cell encounters the same antigen again, they can respond more quickly [44].

There are two different populations of T cells: T helper cells (CD4+) and cytotoxic T cells (CD8+). These cells are differentiated by their surface proteins and recognize different antigens. T helper cells recognize antigens on MHC class II molecules and cytotoxic T cells recognize antigens on MHC class I molecules. T helper cells help other immune cells by releasing T cell cytokines such as IL-4 and IL-13, which help to regulate the immune response. Toll-like receptor activation typically results in increased T helper cell differentiation and is an essential activator of an inflammatory response. Cytotoxic cells possess T cell receptors which can bind to specific antigens presented on foreign cells, allowing the cytotoxic cell to kill the foreign cell [43]. Cells that are both CD4+ and CD25+
are considered T regulatory cells and specialize in immune suppression. They produce high levels of anti-inflammatory cytokines, such as IL-10, and other immune suppressing molecules such as TGF-B and CTLA-4. Avian T regulatory cells possess supersuppressive properties once inflammation starts to decrease and loses the suppressive properties when inflammation starts [45].

**Cytokines**

Cytokines are peptides that are involved in cell signaling. They affect the activity of other cells in the body via autocrine, paracrine, and endocrine pathways. Although cytokines can be produced by nearly all cells, T lymphocytes, endothelial cells, epithelial cells, and macrophages are especially active in producing cytokines. Cytokines, usually either pro or anti-inflammatory, are produced in response to a variety of stresses including pathogens, hormonal stimuli, and environmental factors [46, 47].

**IL-1**

Interleukin-1 is a low molecular weight protein cytokine produced primarily by macrophages and has an important role in the host immune response to infection through inflammatory events [48, 49]. IL-1 supports activation of T and B lymphocyte activation during immune reactions [50, 51]. Chicken IL-1 has been showed to have activity on fever, anorexia, skeletal muscle catabolism, acute phase changes in mineral metabolism, proteoglycan release from cartilage, and release of corticosterone [52, 53]. Both IL-1α and IL-1β bind to the IL-1 receptor which causes an accessory protein to form a heterodimeric
complex with the protein and receptor and this complex activates NF-kB. NF-kB is translocated into the nucleus after the adaptor protein MyD88 joins the complex. After NF-kB is translocated to the nucleus, transcription of inflammatory genes begins [54].

**IL-10**

Interleukin-10 is an anti-inflammatory cytokine that is produced by most nucleated cells. IL-10 targets monocytes and macrophages by reducing phagocytosis, additional cytokine production, and antigen presentation. IL-10 can bind to two receptors, IL-10 receptor 1 and IL-10 receptor 2. When IL-10 is bound to these receptors, it forms a transmembrane receptor complex, which activates Jak1 and Tyk2 kinases. These kinases phosphorylate IL-10 receptor 1, causing the STAT3 transcription factor to become active, which then inhibits the production of inflammatory molecules, such as IL-1, TNF-a, and IL-12 [55, 56]. Additionally, IL-10 can reduce inflammatory responses by inhibiting nitric oxide production and MHC class II expression [57].

**IL-6**

IL-6 is considered a multifunctional cytokine and has both pro and anti-inflammatory effects. IL-6 has a wide variety of functions and plays a central role in host defense mechanisms including acute-phase reactions, hematopoiesis, and immunity, and is an important frontline component of the host’s immune response against infection [58-60]. Additionally, IL-6 can modulate the Th1/Th2 response through upregulating suppression
of cytokine signaling [61]. IL-6 binds to the IL-6 receptor and results in the activation of both JAK/STAT and Ras/Raf signaling pathways [62].

TNF-α

TNF-α is a major inflammatory cytokine produced during parasitic, bacterial, and viral infections [63]. One of the most potent stimulators of TNF-α is lipopolysaccharide and is released by monocytes and macrophages [64]. A variety of regulatory factors drive transcription of the TNF-α gene and NF-kB is suggested to be necessary for TNF-α production by monocytes and macrophages. TNF-α is involved in many different cellular functions including inflammation, tissue repair, hematopoesis, immune response, and anti-tumor effects [65].

Salmonella Vaccines

There are various control measures, including vaccination, in place as part of a Salmonella control program in order to reduce consumer food borne infections [19, 20, 40]. There are three categories of Salmonella vaccines, live-attenuated, inactivated, and subunit. These vaccines are considered to be safe, induce cellular immunity, and are cross-protective [21, 40]. Although there is currently no law mandating the vaccination of poultry against Salmonella, many states and organizations are taking stands against non-vaccination chicken eggs [66]. Although there are effective vaccines out there which have shown positive results, there is the issue of administration large scale and costs [21, 67].
Due to these issues, there is a need for competitive exclusion products, such as probiotics, that can be supplemented to young chicks to provide protection against the bacteria.

**Live-attenuated**

Live-attenuated vaccines are those in which the bacteria itself is still alive, however the strain contains certain deletions or mutations which decreases its virulence, alters its metabolism, and essentially makes it unable to survive in the host organism. There are several advantages and disadvantages associated with live-attenuated *Salmonella* vaccines. An advantage of this vaccine is that it allows for administration orally to birds of any age, even young birds without fully developed immune systems, since the strain is made avirulent. Additionally, protective immunity is achieved through the activation of antibodies against the strain and through activation of cell mediated immune responses. Some disadvantages of a live vaccine include the fear of the strain reverting back to a virulent state as well as the live strain interfering with *Salmonella* testing. Another potential disadvantage is that because the strain is alive, it has the ability to live in the host for a prolonged period of time, and can potentially be transferred to the environment. In addition, the prolonged life in the host could become a threat to the human consumers. While this is a disadvantage of administering a live vaccine, it can also potentially be an advantage, because this prolonged life can aid in immunizing other birds through horizontal transfer, especially large scale when vaccinating each bird individually would be unrealistic [19, 40, 68].
**Killed vaccines:**

Killed vaccines consist of the entire bacteria that has been inactivated without deletions through methods such as heat or acetone. Usually, killed *Salmonella* vaccines must be administered at least twice via intramuscular (IM) or subcutaneous (SQ) injections. This results in some disadvantages regarding the route of administration, especially in a large scale operation. Injecting each bird individually, either IM or SQ, is not realistic and unlikely to be the most efficient route. This becomes even more unrealistic when you have to give at least two immunizations. Another disadvantage is that killed vaccines are eliminated from the host quickly, which doesn’t give the host’s immune system sufficient time to produce a strong immune response. Similarly, killed bacteria usually don’t express as many antigens as live strains. Killed vaccines typically require adjuvants to achieve the full benefits of the vaccine, which makes the vaccine more costly and harder to administer. Despite these disadvantages, a killed vaccine does provide some advantages, such as a lowered threat to both the environment and human health, as the bacteria is killed and cannot survive in the host or environment to cause additional damage [19, 40].

**Subunit Vaccines:**

Subunit vaccines are vaccines composed of the predominant antigen(s) from the *Salmonella* bacteria as opposed to the whole bacteria. These are antigens that are typically seen on the surface of the bacteria and typically have characteristics of virulence. Subunit vaccines have many of the same advantages and disadvantages that killed vaccines have; disadvantages including difficult route of administration (intramuscular or subcutaneous) as
well as the need to be combined with adjuvants to maximize immunological responses and advantages of not having a live bacteria to be transferred to the environment or to a human host [40, 69].

**Nucleic acid vaccines:**

A nucleic acid vaccine is one made of a bacterial plasmid. The plasmid encodes for specific antigens of the *Salmonella* and the expression of these antigens is enhanced by a eukaryotic promoter. This plasmid is administered through IM or SQ injections or mucosally. As with any vaccine, this newer vaccine method has various advantages and disadvantages. An advantage of this method of vaccination is the preservation of the antigen conformations as well as a better presentation of these antigens through MHC class I and class II molecules. Additionally, these plasmids are typically stable at high temperatures, rarely cause injection site reactions, and are easy to create. The biggest advantage of this vaccine is the ability to present many vaccine antigens through the same vaccine. This has the potential to be extremely beneficial to the poultry industry because the average bird receives 5-8 vaccines by 6 weeks of age and up to 12 vaccines by 18 weeks of age. The administration of this many vaccines is extremely difficult for large scale operations and is labor intensive and consistently increases the risk of environmental or human host harm (through live vaccines). DNA vaccines aid in the administration of multiple antigens from numerous vaccines through one injection. There are disadvantages, however, that include the protective capacity being limited to the antigens/proteins chosen
to be incorporated into the plasmid as well as the risk of the plasmid being incorporated into the chicken genome [70, 71].

In 2009, Nagarajan AG et al. tested the ability of a DNA vaccine against a *Salmonella enterica* serovar Typhimurium challenge. The vaccine consisted of a plasmid encoding a *Salmonella* SPI-1 effector protein, SopB. In conjunction with the live-attenuated *S. Typhimurium* strain vaccine, the SopB DNA vaccine produced reduced levels of the challenge strain in both the liver and spleen as compared to the live-attenuated vaccine alone in addition to increased serum IFN-gamma levels [72].

**Vectored vaccines:**

Vectored vaccines are similar to nucleic acid vaccines in that one or more antigens are administered to the bird through a single delivery. This is advantageous because antigens from several bacteria can be included and the bird can be vaccinated for several infections through one injection. *Salmonella* vector vaccines are attenuated and can carry the antigens on plasmids or the chromosome itself. Similar to nucleic acid vaccines, advantages include easy administration, induction of humoral, cell-mediated, and mucosal immunity, and the ability to administer without needles and without boosters [73, 74]. However, like nucleic acid vaccines, this includes the risk of interfering with *Salmonella* testing [19].

Live-attenuated *Salmonella* vectors are often used in various other situations as well to deliver antigens from several other pathogens including *Escherichia coli*, *Campylobacter jejuni*, and *Streptococcus pneumonia*, and HIV. These vectors not only
vaccinate the bird against the infections in which the antigen is selected for, but against *Salmonella* itself [75-81].

Jiang et al. (2010) performed an experiment testing the ability of a *S. Typhimurium* vector expressing a *Clostridium perfringens* antigen to protect the bird from both *C. perfringens* and *Salmonella* infections. They concluded that the chickens had lower levels of necrotizing enteritis often associated with *C. perfringens* as well as lower levels of colonization by both *S. Typhimurium* and *S. Enteritidis* [81].

**Routes of administration:**

The effectiveness of the previously described vaccines is not meaningful if the route of administration is not practical. Typically, *Salmonella* vaccines are delivered orally, subcutaneously, or intramuscularly. The vaccines typically used today are largely unsuccessful due to these administration limitations. Poultry producers are challenged with the high costs of the vaccination administration because mass immunization is not possible and each bird must be done separately with the SQ and IM methods and with the oral methods, immunity is usually not induced early enough to be 100% effective. In both cases, sometimes several boosters are needed as well (26, 38).

One possible solution to these issues is *in ovo* vaccines. Currently, *in ovo* vaccination is used against viral pathogens which cause Marek’s disease, bursal disease, and fowl pox. *In ovo* vaccines are typically given around day 18 of incubation through a needle and is injected directly into the egg. This method allows for mass immunization in
a very short amount of time (38). However, these vaccines do typically need boosters and/or adjuvants to work best (44, 45).

*Adjuvants and immune stimulants:*

Adjuvants and immune stimulants are typically needed to achieve optimal immunological potential. Subunit vaccines are typically less immunogenic than live-attenuated and killed vaccines (23).

For example, aluminum hydroxide (Alum), is one adjuvant formulated into vaccines to help induce strong humoral immune responses. In 2009, Ohta et al showed that a Newcastle disease *in ovo* vaccine alone did not produce as strong an antibody response as the same vaccine formulated with Alum. Additionally, the vaccine with the Alum was shown to be safer for the bird (44). In 2010, Lee *et al.* formulated an *Eimeria profilin in ovo* vaccine with a complex composed of Alum, among other things. They showed that the vaccine including the adjuvant resulted in higher levels of proinflammatory and cell-mediated cytokines as compared to the vaccine alone (45).

There have also been several studies that have used cytokines, such as IL-1, IL-2, IL-4, IL-8, and IFN-gamma, as the immune stimulant along with the vaccine. These studies have reported increased antibody responses and increased disease protection as compared to the vaccination alone (47-48).

Another immune stimulating addition to vaccines is oligodeoxynucleotides containing cytosine-phosphodiester-guanine (CpG-ODN) motifs. There have been studies
showing that injecting chicken eggs with CpG-ODNs activates the innate immune system and protects against *Salmonella, Escherichia coli*, and bronchitis (39-42).

There are some problems associated with adjuvants that need to be addressed during the formulation process. Most concerning is the risk that the adjuvant will inactivate the live-attenuated vaccine itself (14).

**Probiotics**

Measures for reducing *Salmonella* in poultry include vaccinations and strict hygienic standards. Although strict hygiene is important for the control of *Salmonella*, strict hygiene practices limit the vertical or horizontal transfer of good microbes to the chicks. Unlike other farm animals, chickens are hatched in a relatively sterile environment, and are never exposed to adult birds which would facilitate colonization with beneficial microflora. Therefore, colonization of the chicks’ mucosal surfaces is random and by coincidence; if a pathogen contaminates the environment and is introduced to the bird, it will colonize an empty and nutrient rich niche of the gut where it could multiply and colonize without restriction. This is dangerous because birds infected with *Salmonella* are virtually symptom free, thus allowing that bird to interact with the others can result in a mass infection of other birds as well as contamination of the environment [25]. Additionally, not being able to interact with adult birds at a young age and being denied the opportunity to establish a healthy microflora delays the maturation of the immune system, making the vaccinations given early in life less effective [33, 82].
For all these reasons, competitive exclusion products are popularly used in the poultry industry to facilitate rapid colonization with healthy gut microbiota. Probiotics are defined as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance” [83]. These healthy bacteria compete directly with pathogens for binding sites and nutrients, reducing the ability of pathogenic bacteria to multiply and colonize. Additionally, giving probiotics to chicks very early in life help stimulates gut and immune maturation, which allows for a better defense against pathogens as well as a more effective vaccination [33, 82].

*Lactic acid bacteria:*

Lactic acid bacteria have demonstrated antimicrobial effects such as the production of organic acids and bacteriocins [84]. The presence of enzymes such as peptidoglycan hydrolases have been reported, which can also be antibacterial [85-87]. Peptidoglycan hydrolases can hydrolyze glycosidic bonds located in the peptidoglycan, which is involved in cell structure, growth, division, and autolysis [88, 89]. Additionally, many lactic acid bacterial species have a competitive advantage over other gastrointestinal bacteria through their ability to tolerate acid and bile, antagonize pathogenic bacteria, and adhere to the intestinal epithelium [90].

*Lactobacillus reuteri*

*Lactobacillus reuteri* is a heterofermentative bacterium and is a true indigenous *Lactobacillus* species in mammals. *L. reuteri* is one of the most well documented probiotic
species and is one of the few endogenous \textit{Lactobacillus} species found in mammalian intestinal tracts, including pigs and humans and in avian intestinal tracts including chickens and turkeys [91]. \textit{L. reuteri} in chickens specifically has been shown to tolerate acid and bile salts, inhibit pathogenic bacteria, attach to intestinal epithelial cells, and improve overall growth and performance [92-94]. Not only is \textit{L. reuteri} capable of producing antimicrobial substances, such as lactic acid and hydrogen peroxide, which is harmful to pathogenic bacteria [95], but it has also been shown to have beneficial effects in the host, including production of vitamin B12 [96], L-lysine, and folic acid [97]. In chickens, \textit{L. reuteri} stimulates the immune system, increases phagocytosis of macrophages, and has various effects on expression of IL-10, interferon-gamma, and transforming growth factor-beta [98, 99]. \textit{L. reuteri} along with other \textit{Lactobacillus} species, has been shown to exhibit inhibitory effects against both \textit{S. Enteritidis} and \textit{S. Typhimurium} [99]. In addition to being able to survive the gastrointestinal tract via acid and bile resistance and having a competitive advantage over other bacterial species, \textit{L. reuteri} also survives due to having antibiotic resistant properties. Several studies have been performed to show that \textit{L. reuteri} is not susceptible to certain common antibiotics, including gentamicin, tetracycline, kanamycin, and streptomycin [100].

\textit{Enterococcus faecium}

Enterococci bacteria produce bacteriocins which are inhibitory against both Gram positive and Gram negative pathogenic bacteria [101]. \textit{E. faecium} has been shown to house the genes encoding enterocins which have an inhibitory zone against \textit{S. Enteriditis} of 4 to
6 mm [102]. The combined efforts of the produced bacteriocins and lactic acid from *E. faecium* have shown to be antagonistic against *Salmonella* Enteriditis growth, as well as other *Salmonella* serovars. Accumulation of the bacteriocins and lactic acid had bactericidal and bacteriostatic actions against *Salmonella* over time [103]. Viable cell counts of *Salmonella* Enteriditis in combination with *E. faecium* was observed to be significantly lower than those in pure cultures [102]. Overall, *E faecium* can be associated with decreased *Salmonella* colonization when in competition with each other [104, 105]. Additionally, *E faecium* has been tested for safety and has been considered safe. Its virulence determinants were tested and shown to be nontoxic. *E. faecium* has also been found to survive low pH, bile salts, and gastric and intestinal fluids *in vivo* [104].

*Bifidobacterium animalis*

*Bifidobacterium animalis* has been shown to be able to colonize and maintain high concentrations in the digestive tract and is considered safe in the sense that it has limited translocation ability and does not cause histological lesions [106]. *B. animalis* has an anti-inflammatory effect due to the reduced expression of proinflammatory transcription factor. It is likely that *B. animalis* can exert its attenuation of NFkB activation through recognition of the bacteria by dendritic cells and the following induction of CD25+ T cells [107]. *B. animalis* has also been shown to stimulate superoxide anion production and increased proliferation of leukocytes. Additionally, increased lysozyme and c-globulin levels, which are important for a cell mediated immune response, have been associated with *B. animalis* [108]. *B. animalis* has been shown to be antagonistic to *Salmonella* and has exhibited
bactericidal effects. The inhibition of *Salmonella* via *B. animalis* starts with inhibition of *Salmonella* growth before the end of the exponential phase, after which there is a decrease in live-cell numbers at the beginning of the stationary phase [109].

*Pediococcus acidilactici*

*Pediococcus acidilactici* has shown to have inhibitory activity against *Salmonella* and psychrotrophic characteristics associated with its use as a protective culture. *P. acidilactici* is considered safe in that it lacks the production of biogenic amines tyramine and histamine [110]. *P. acidilactici* is a bacteriocin producing strain and inhibits pathogenic microbes. Pediocins, the bacteriocins produced from *Pediococcus* strains, have been shown to have antimicrobial effects against both Gram positive and Gram negative pathogenic bacteria [111].

*Prebiotics*

Prebiotics are defined as, “a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” [112]. Typical prebiotic substances are fructooligosaccharides such as oligofructose and inulin but other substances such as glucooligosaccharides, raffinose, and glycooligosaccharides have also been shown to have beneficial effects as prebiotics [113-116]. Mannan oligosaccharides are also considered to be prebiotics, however, their mechanism is to bind and remove pathogens and to stimulate the immune system [117]. Prebiotics may also work indirectly. Fermentation of prebiotics provides nutrients for other
normal intestinal flora, increasing competitive exclusion, and can also produce antimicrobial metabolites [118].
Chapter 3: Effect of probiotic bacterial strain supernatant on inhibition of

*Salmonella enterica* Enteritidis proliferation *in vitro*

*Abstract*

Two experiments were conducted to study the inhibitory ability of culture supernatants from four different probiotic bacterial strains (*Lactobacillus reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis*, and *Pediococcus acidilactici*) on *Salmonella enterica* Enteritidis growth *in vitro*. The first experiment studied the effect of probiotic culture supernatant on *Salmonella* inhibition in liquid media. Supernatants from *L. reuteri*, *E. faecium*, *B. animalis*, or *P. acidilactici* at 10:1, 5:1, or 1:1 supernatant to *Salmonella* dilution were incubated with *Salmonella* for 12 hours in liquid media. Culture supernatants from all probiotics inhibited (P < 0.05) the growth of *Salmonella* at 10:1 dilution, compared to the group with 0 µl supernatant. *E. faecium*, *L. reuteri*, *B. animalis*, and *P. acidilactici* decreased the growth of *Salmonella* by 34%, 89%, 83%, and 45% when compared to the group with 0µl supernatant, respectively. The second experiment studied the effect of probiotic supernatant on *Salmonella* inhibition in solid media. Supernatants from *L. reuteri*, *E. faecium*, *B. animalis*, or *P. acidilactici* at 10:1, 5:1, or 1:1 supernatant to *Salmonella* dilution were incubated with *Salmonella* for 12 hours or supernatants from *L. reuteri* or *E. faecium* at 10:1, 5:1 supernatant to *Salmonella* dilution were incubated with
Salmonella for 24 hours in solid media. At 12 hours of incubation, 10:1 diluted supernatant from E. faecium and L. reuteri inhibited Salmonella growth efficiently compared to that from B. animalis and P. acidilactici. At 24 hours of coincubation, 10:1 diluted supernatant from E. faecium and L. reuteri did not significantly inhibit the Salmonella growth when compared to the 5:1 diluted group, respectively.

In conclusion, this study identified the inhibitory effects of four different probiotic strains on S. Enteritidis in vitro suggesting that these four probiotic strains can be fed to decrease Salmonella shedding in poultry.

Introduction

The use of probiotics, or direct-fed microbials, is becoming an increased interest in the poultry industry. The first established microbial populations after hatch are the precursors for the final microbial population which will persist throughout the bird’s life. The application of probiotics early in a bird’s life allows for the colonization of select beneficial bacteria early in life [119]. Additionally, poultry are raised under intensive management systems and experience many stressful conditions, such as overcrowding, transportation, vaccination, and overheating. Unfavorable or stressful conditions can create an imbalance of the natural microflora, which leaves the bird vulnerable to pathogen infections [120].

In 1973, both Tortuero and Nurmi and Rantala observed that supplementation with Lactobacilli or feces from adult birds have positive impacts on performance and Salmonella clearance, respectively [7, 121]. Since then, several studies have identified
beneficial effects of probiotics on poultry production. In layers, many studies have shown that supplementation with probiotics increases pullet feed consumption and body weight gain, layer body weight, egg production, egg size, mass, and weight, albumen quality, and feed conversion efficiency [122-125], as well as decreased yolk cholesterol concentration [126]. Some of the mechanism through which probiotics help layer birds are by stimulation of the immune system [127], competitive exclusion [128], production of digestive enzymes [129], and production of antibacterial substances [128].

One of the well-studied pathways through which probiotics help layer birds is by producing antimicrobial products that can inhibit pathogenic organisms, particularly Salmonella[130]. L. reuteri produces lactic acid and hydrogen peroxide and has been shown to be efficient against Salmonella Enteritidis and Salmonella Typhimurium [93, 95, 99]. Similarly, E. faecium and P. acidilacti produce enterocins and pediocins, respectively, which have been shown to be efficient against Salmonella and other Gram-negative pathogens [102, 111]. B. animalis produces lactic acid and other bactericidal substances which have been shown to inhibit Salmonella [108]

Salmonellosis is a zoonotic disease caused by the Gram-negative enteric bacteria Salmonella. They are not restricted to particular host species, with more than 2500 serotypes having been described, and their epidemiology can therefore be complex; most are able to colonize the gastrointestinal tract of animals without production of disease. More than 2500 serotypes have been described, mostly belonging to the species S. enterica [19]. The most prevalent serovars, Salmonella enterica serovar Typhimurium and serovar Enteritidis are major causes of zoonotic gastroenteritis in a wide range of host species.
worldwide. Both serovars have a broad host range able to infect humans, livestock, and birds [20]. *S. enterica* is very hard to eradicate from poultry flocks, as even thorough cleansing and disinfection fail to rid flocks of *S. enterica* once flocks are infected [131]. Use of vaccines has limitations because current vaccines are unable to eliminate bacteria shedding or to prohibit colonization of the gastrointestinal tract of chickens [132, 133]. Because vaccines are not effective in clearing the persistence of *S. enterica* infections of chickens, the commercial poultry production industry attempts to control *S. enterica* infections by supplementing vaccination programs with several other procedures like probiotics [134] or prebiotics [135] applications.

Identifying the correct probiotic that can specifically target *Salmonella* will benefit the poultry industry. Hence this experiment was conducted to study the ability of four selected probiotic culture supernatants in inhibiting *S. Enteritidis* growth *in vitro*.

**Materials and Methods**

**Bacteria culture**

A primary isolate of wild-type *S. enterica* serovar Enteritidis was grown. 1 X10⁶ CFUs of *S. Enteritidis* was inoculated into 15 ml of MRS media at 37 °C for 12 hours.

1 X 10⁷ CFU of either *L. reuteri* DSM16350, *E. faecium* DSM16211, *B. animalis* DSM16284, or *P. acidilactici* DSM16210 were inoculated in 15 ml of MRS medium and incubated at 37 °C. At 12 hours of incubation, the culture was centrifuged at 10,000 X g and the supernatant was collected.
Effect of probiotic supernatant on Salmonella inhibition in liquid media

10 µl of the *Salmonella* solution was added to each well of a 96-well plate and incubated with one of the four probiotic culture supernatant in a serial dilution of either supernatant: *Salmonella* ratio of 0:1, 10:1, 5:1, or 1:1 in 3 replications (n=3). The total volume of incubation was 110 µl. For supernatant: *Salmonella* ratio of 0:1 dilution, 100 µl of MRS media was incubated with 10 µl of the *Salmonella* culture. For supernatant: *Salmonella* ratio of 10:1 dilution, 100 µl of each probiotic culture supernatant was incubated with 10 µl of the *Salmonella* culture. For the 5:1 dilution, 50 µl of probiotic culture supernatant was mixed with 50 µl of MRS media and incubated with 10 µl of the *Salmonella* culture. For the 1:1 dilution, 10 µl of probiotic culture supernatant was mixed with 90 µl of MRS media and incubated with 10 µl of the *Salmonella* culture. The 96 well plate was then incubated at 37°C for 12 hours. The absorbance was measured at 600 nm and the effect of probiotic culture supernatant inhibition on *Salmonella* proliferation was reported as optical density (OD) values.

Effect of probiotic supernatant on Salmonella inhibition in solid media

10 µl of probiotic supernatant was diluted to either 1:1 or 1:2 or 1:10 dilution with MRS media and mixed with 15 µl of *Salmonella* culture and incubated in 15 ml of MRS medium for 12 hours in one (n=1) replications. At 12 hours of incubation, 10 µl of aliquot was plated on Agar and incubated at 37°C to determine the number of *Salmonella* in the co-incubated culture. At 24 hours of incubation, the number of CFU was counted.
The inhibition assay was repeated for *E. faecium* and *L. reuteri* culture supernatant at 1:1 and 1:2 dilutions in a procedure similar to described above, except that a second inoculation of probiotic supernatant at either 1:1 or 1:2 were added again at the end of first 12 h incubation, in two replications, and the incubation was continued for another one hour before plating the solution on agar to determine the concentration of *Salmonella*.

**Statistical Analysis**

A one-way ANOVA (JMP, SAS Institute INC., Cary, NC) was used to determine the effect of probiotic culture supernatant on *Salmonella* growth. When the main effect was significant (P < 0.05), differences between means were analyzed using Tukey’s least squares means comparison.

**Results**

*Effect of probiotic supernatant on Salmonella inhibition in liquid media*

Culture supernatants from all probiotics inhibited the growth of *Salmonella* at 10:1 dilution, compared to the group with 0 µl supernatant (P < 0.05) (Figure 1). *E. faecium, L. reuteri, B. animalis,* and *P. acidilacti* decreased the growth of *Salmonella* by 34%, 89%, 83%, and 45% when compared to the group with 0 µl supernatant, respectively. Culture supernatants from *L. reuteri, B. animalis,* and *P. acidilacti* inhibited the growth of *Salmonella* at 5:1 dilution, compared to the group with 0 µl supernatant (P < 0.05). At 1:1 dilution, the supernatants from all probiotic strains did not inhibit the growth of *Salmonella.*
Effect of probiotic supernatant on Salmonella inhibition in solid media

At 12 hours of incubation, 1:1 diluted supernatant from *E. faecium* and *L. reuteri* inhibited *Salmonella* growth efficiently compared to that from *B. animalis* and *P. acidilactici* (Table 1). At 24 of co-incubation, 1:1 diluted supernatant from *E. faecium* and *L. reuteri* did not significantly (P >0.05) inhibit the *Salmonella* growth when compared to the 1:2 diluted group.

**Discussion**

This study identified that all four probiotic strains studied had inhibitory effects against *S. Enteritidis* separately. Among the four strains studied, *L. reuteri* and *B. animalis* had the highest inhibitory potential against *S. Enteritidis*.

Our results are consistent with many previous studies. *B. animalis* has been shown to be antagonistic to *Salmonella* and has exhibited bactericidal effects. The inhibition of *Salmonella* by *B. animalis* starts with inhibition of *Salmonella* growth before the end of the exponential phase, after which there is a decrease in live-cell numbers at the beginning of the stationary phase [109]. *L. reuteri* is capable of producing antimicrobial substances, such as lactic acid and hydrogen peroxide [95]. *L. reuteri* as well as other *Lactobacillus* species have been shown to inhibit *S. Enteritidis* [99].

In conclusion, this study identified the inhibitory effects of four different probiotic strains on *S. Enteritidis in vitro* suggesting that these four probiotic strains can be fed to decrease *Salmonella* shedding in poultry.
Acknowledgements

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Chapter 4: Effect of synbiotic on production parameters, gut bacterial profile, and immune parameters

Abstract

This experiment studied the effects of a synbiotic (PoultryStar® me) containing four live strains isolated from adult chickens (Lactobacillus reuteri, Enterococcus faecium, Bifidobacterium animalis, and Pediococcus acidilactici) with prebiotic (Fructooligosaccharide) on layer production and local immune parameters following an experimental Salmonella infection. Birds were fed synbiotic product from day-of-hatch until wk 28. At 18 and 20 wk of age, birds fed synbiotics in both vaccinated and unvaccinated group had higher (P < 0.05) body weight than that in the no synbiotic fed group. Birds fed synbiotics had 0.7%, 17.8%, 21.7%, 3%, and 4.2% higher (P < 0.05) hen day egg production (HDEP) at 19, 20, 21, and 23 wk of age, compared to the birds fed no synbiotics, respectively. At 24 wk of age, birds were challenged with 250 µl of 1 X 10^9 CFU S. enterica Enteritidis. Birds fed synbiotics had 3%, 6.7%, 4.3%, 12.5%, and 14.4% higher (P < 0.05) HDEP at 24, 25, 26, 27, and 28 wk of age, compared to the birds fed no synbiotics, respectively. Birds supplemented with synbiotics had higher (P < 0.05) number of cecal P. acidilactici, and L. reuteri compared to the group with no synbiotic supplementation. Irrespective of the vaccination status, birds fed synbiotics and challenged with Salmonella had lower (P <
0.05) S. Enteritidis content compared to that in the no synbiotic supplemented and unvaccinated treatment group. Among the Salmonella challenged birds, birds in the synbiotic supplemented and unvaccinated treatment group had comparable S. Enteritidis content to the vaccinated group with no synbiotics. At 22 d post-Salmonella challenge, birds in synbiotic supplemented, vaccinated and challenged with Salmonella had the highest bile IgA content. At 8 d post-Salmonella challenge, birds fed synbiotic, vaccinated and challenged with Salmonella had the highest relative amounts of IL-10 mRNA in the cecal tonsils compared to all other treatment groups. At 8 and 10 d post-Salmonella challenge, synbiotic supplementation increased TNFα mRNA content in the cecal tonsils. The CD4+ and CD8+ cell percentages in the cecal tonsil were comparable between the group fed synbiotic but unvaccinated and the group un-supplemented with synbiotic but vaccinated. Among the birds challenged with Salmonella, the CD4+CD25+ cell percentages in the cecal tonsil was comparable between the group fed and not fed synbiotic. It can be concluded that supplementation of synbiotic product could be beneficial to layer diets as a growth promoter, performance enhancer, and for protection against S. Enteritidis infection.

**Introduction**

*Salmonella enterica* Enteritidis is one of the most isolated *Salmonella* serovars isolated in chickens, although over 200 serovars of *Salmonella* are able to colonize the gastrointestinal tract of poultry [19]. *Salmonella* typically are present in poultry as a sub-clinical infection and hence the bird provides a source of contamination for human infection [21]. Contaminated meat and eggs from S. Enteritidis-positive birds can cause
human food borne infections [136] which are usually self-limiting, but can require antibiotic intervention [22]. With the emergence of antibiotic resistant *Salmonella*, treatment is becoming more difficult and there has become a need for control of *Salmonella* in poultry flocks [1].

Interest in probiotic microorganisms and prebiotic oligosaccharides have been of increased interest in the poultry community due to the restriction of antibiotic growth promotors as well as the emergence of antibiotic resistant pathogens, such as *Salmonella* [137]. Manipulation of the gut microbiota by supplementations of prebiotics and probiotics have shown promising results in controlling bacterial infections in poultry [138]. Some health benefits associated with probiotics include stimulation of the immune response [127], competitive exclusion of pathogenic microbes [128], stimulation of digestive enzymes [129], and production of antibacterial substances [128]. Supplementation of prebiotics has been shown to modulate the gut microbiota by increasing specific beneficial bacteria as well as mimicking attachment sites of pathogens to reduce pathogenic bacteria adherence to the intestinal wall [139]. Providing a synbiotic product to the birds provides benefits of both prebiotics and probiotics and promotes the survival of existing beneficial bacteria as well as supplemented new strains [140].

Although *Salmonella* can cause severe infections in humans, poultry with *Salmonella* infections remain asymptomatic [21]. The question of how *Salmonella* persists in chickens sub-clinically over the span of their lives is still poorly understood. *Salmonella* infection activates the immune system; macrophages and heterophil infiltration as well as other innate immune cells such as granulocytes and dendritic cells [141]. The adaptive
immunity is also activated as observed by distinct changes in the T-cell populations [142], as well as increases in IgG and IgA antibodies [143].

Suppressor cells, such as T-regulatory cells, can suppress immune responses through producing anti-inflammatory cytokines, namely IL-10 and thereby suppress the host immune response against the pathogen and facilitates pathogen survival. S. Enteritidis lipopolysaccharide stimulates IL-10 and the single nucleotide polymorphism at the IL-10 locus is associated with S. Enteritidis burden [144].

The present study was carried out to determine the effects of synbiotic supplementation on immune responses, and production performances of layer birds challenged with S. Enteritidis. The synbiotic product (PoultryStar® Me, Biomin, San Antonio, TX) contained four live strains of *Lactobacillus reuteri* DSM16350, *Enterococcus faecium* DSM16211, *Bifidobacterium animalis* DSM16284, and *Pediococcus acidilactici* DSM16210 and contained the prebiotic, Fructooligo-saccharide.

**Materials and Methods**

**Birds**

One day old (n=384) White Leghorn chicks (Hy-Line North America; Johnstown, OH) were provided ad libitum intake of water and feed, housed in battery cages (pullet and layer), and raised using standard animal husbandry practices. The feed formulations are provided in Table 2. The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. The birds were housed in pullet
cages for the first 12 weeks after which they were transferred to layer cages. At week 18, the light hours were increased to 16 hours to bring them into production.

_Treatments_

The birds were fed a layer starter diet between 0-8 wk of age and a layer grower diet between 8-18 wk of age and a layer finisher diet between 19 to end of the experiment. A total of 384 one-day-old layer chicks were randomly distributed to one of the two treatment groups, control and synbiotic supplemented groups. Each treatment was replicated in 24 feeders of 8 chicks per replication (n=24). The basal diet was based on corn and soybean meal (Table 2) and supplemented with the synbiotic (PoultryStar® Me, Biomin, San Antonio, TX). The synbiotic supplement was added to the feed at a rate of 1 g/kg for the first 3 weeks, then reduced to 0.5 g/kg until the end of wk 23 of age, and increased back to 1 g/kg inclusion until the end of the project at wk 28 of age.

At 14 wk of age the birds were weighed. At 14 wk of age, 32 birds in both the synbiotic and control groups (64 birds in total) were vaccinated with a Salmonella vaccine resulting in a 2 (control and synbiotic supplementation) X 2 (vaccinated and non-vaccinated group) factorial arrangement of treatments. For pre-Salmonella infection, the number of replication was 16 for the vaccinated group (n = 16) and 8 for the unvaccinated groups (n = 8). Each replication had 8 pullets. Birds were vaccinated at 14 wk of age, with a booster dose on 17 wk of age, with 0.3 cc of *Salmonella* vaccine (Poulvac® SE, Zoetis, Florham Park, NJ) subcutaneously in the breast muscle. Body weight was measured at 17,
18, and 20 wk of age. Eggs were collected and recorded daily from day of first egg until the end of the project.

At 24 wk of age, half of the birds in the vaccinated groups and all the birds that were not vaccinated were challenged with *Salmonella* resulting in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. The number of replication was 8 for all the treatment groups post-*Salmonella* infection (n = 8). Each replication had eight birds.

A primary isolate of wild-type *Salmonella enterica* Enteritidis was used. *Salmonella* was grown in tryptic soy broth for 12 h. The cells were washed three times with 1X PBS by centrifugation (5,000 RPM), and the concentration of the bacteria in the media was determined spectrophotometrically. The concentration of the bacteria was further confirmed by serial dilution plating of the inoculum on Xylose Lysine Tergitol-4 (XLT) agar plates. Birds were gavaged with 250 µl of 1 X 10⁹ CFU/bird on wk 24.

On days -5, 3, 8, 10, 17, 22, 24, and 30 post-challenge, one bird was randomly chosen from six out of the eight replication for sample collection.

*RNA Isolation, DNA Isolation, and Real-Time PCR*

Single-cell suspensions of cecal tonsils were collected from six replication pens (n = 6) 5 days pre-challenge and 3, 8, 10, 17, 22, 24, 30d birds (n = 6) post-challenge. One cecal tonsil from each bird was used and a quarter of the total cells were suspended in 750µl of Tri Reagent (Molecular Research Center, Cincinnati, OH). 200 µl of chloroform was added to the solution, incubated for 5 minutes, and centrifuged at 11,000 X g for 15
minutes. After centrifugation, the top aqueous layer was removed and added to 500 μl isopropyl alcohol to precipitate the RNA, incubated for 10 minutes, and centrifuged at 13,000 X g for 10 minutes. The supernatant was removed and the pellet was washed in 75% ethanol. After centrifugation and drying, based on the size of the pellet, it was dissolved in 20 – 200 μl of TE buffer. RNA concentration and quality was determined using the NanoDrop (Thermo Scientific). The mRNA was reverse transcribed into complementary DNA [145]. The mRNA was analyzed for IL-10 and TNFα, mRNA by real-time PCR (iCycler, BioRad, Hercules, CA) using SyBr green (Qiagen, Valencia, CA) using the primers noted in Table 3. Cytokine samples were normalized to the housekeeping gene β-actin mRNA. Fold change from the reference was calculated by the $2^{-\Delta\Delta CT}$ equation. The CT was determined by the iQ5 software (Biorad) when the fluorescence rose exponentially 2-fold times above background. The reference group was the group that had the lowest expression of that particular cytokine or gene.

The total DNA was isolated from the cecal scrapings and cecal content as described earlier (Amit-Romach et al., 2004) with the modification of adding 600 μl lysis buffer (1 M Tris-HCl, 500 mM EDTA, 5 M NaCl, 20% SDS) and 32 μl Proteinase K, incubating for 5 minutes at 80°C, and extracting via isopropanol and 70% ethanol. Cecal microflora (L. reutri, P. acidilacti, B. animalis, E. faecium, and S. enterica) was analyzed by real-time PCR described earlier [2] using the primers noted in Table 3. The threshold cycle (Ct) values were determined by iQ5 software (Bio-Rad, Hercules, CA) when the fluorescence rises exponentially 2-fold above background. The Ct values were normalized to the Ct values of the universal primer 16S. To evaluate the relative proportion of each examined
bacteria, all Ct values were expressed relative to the Ct value of the universal primers, and proportions of each bacterial group are presented where the total of the examined bacteria was set at 100% as described earlier (Amit-Romach et al., 2004).

**CD4⁺ Cell, CD8⁺ Cell, and CD4⁺CD25⁺ Cell Percentages**

At -5, 3, 8, 10, 17, 22, 24, and 30 d post-challenge, the cecal tonsils were collected from six replication groups (n = 6). Single cell suspensions of the cecal tonsils were concentrated for mononuclear cells by density centrifugation at 400 X g for 20 minutes over Histopaque (1.077 g/mL; Sigma Aldrich, St. Louis, MO). Production and phycoerythrin-linking of mouse anti-chicken CD25⁺ were conducted as described earlier. Approximately 1 X 10⁶ cells were incubated with 10 μg/mL of primary phycoerythrin-linked mouse anti-chicken CD25, 1:200 dilution of fluorescein isothiocyanate-conjugated mouse anti-chicken CD4 (Southern Biotech, Birmingham, AL) or 1:200 fluorescein isothiocyanate-conjugated mouse anti-chicken CD8 (Southern Biotech), and 1:200 unlabelled mouse IgG for 45 min. Unbound primary antibodies were removed by centrifugation at 400 X g for 20 min. The percentages of CD4⁺, CD8⁺, and CD4⁺CD25⁺ cells in cecal tonsils were analyzed by flow cytometry (Guava EasyCyte, Millipore, Billerica, MA). The CD4⁺ and CD8⁺ cells were expressed as a percentage of mononuclear cells. The percentage of CD4⁺CD25⁺ cells were expressed as a percentage of CD4⁺ cells to facilitate comparison between samples [146]. Unbound antibodies were removed by washing 3 times and the samples were analyzed in a flow cytometry [146].
Determination of Salmonella-specific IgA Concentrations

An enzyme-linked immunosorbent assay (ELISA) was performed to determine Salmonella-specific IgA concentrations in the chicken plasma and bile. The concentrations of different reagents were established using checkerboard titrations with dilutions of plasma, bile, antigens, and conjugates. Salmonella was grown as previously stated and lysed by glass beads size 425-600 µm (Sigma, St. Louis, MO) in a TissueLyser LT (Qiagen Hilder, Germany) for 5 minutes at 50 1/s, two times to be used as an antigen to coat the wells of the microtiter plates. Flat-bottomed 96-well microtitration plates were coated with 67.2 µl of the antigen (1.435 ug/ml) diluted in 0.1M carbonate buffer and incubated overnight at 4°C. The plates were washed three times with PBS-Tween 20 (50mM Tris, pH 7.4, containing 150mM sodium chloride and 0.05% Tween 20) and non-specific immune sites were blocked by PBS-Tween 20 – 2.5% nonfat dry milk incubated for one h at 37°C. The plasma was diluted to 1:10 and the bile was diluted to 1:200 in PBS-Tween 20-2.5% nonfat dry milk and added to the plates in duplicates (100 µl/well) and incubated for 1 h at room temperature. After washing, HRP-labeled anti-chicken IgA diluted 1:100,000 in PBS-Tween 20 – 2.5% nonfat dry milk was added to each well (100 µl/well) and incubated for 1 h at room temperature. After washing, peroxidase activity was revealed by adding 100 µl of TMB solution (3 M sodium acetate, TMB, hydrogen peroxide). The reaction was blocked by adding 100 µl of 2M sulfuric acid and the optical density (OD) value was measured at 490 nm in a microplate reader.
Statistical Analysis

A two way ANOVA was used to examine the interaction effects of vaccination X synbiotic on dependent variables collected between 14 wk to 24 wk of age. A two way ANOVA was used to examine the interaction effects of vaccination/challenge X synbiotic on dependent variables collected between 24 wk to 28. When the interaction effects were not significant (P > 0.05), the main effects of symbiotic was analyzed if there were no main effects of vaccine/challenge. When interaction or main effects were significant (P < 0.05), differences between means were analyzed by Tukey’s least square means comparison.

Results

Effect of synbiotic supplementation on bird body weights between 14-28 wk of age

There were significant interaction effects of vaccine and synbiotic supplementation on bird body weight at 17 (P = 0.02), 18 (P = 0.03), and 20 (P = 0.01) wk of age (Figure 2). At 17 wk of age, birds supplemented with the synbiotic product and not vaccinated were significantly heavier than birds not supplemented and unvaccinated. At 18 and 20 wk of age, birds fed synbiotics in both vaccinated and unvaccinated group had higher body weights than that in the no synbiotic fed group. After 20 wk of age, body weight did not differ significantly among the different treatment groups (data not shown).
Effect of synbiotic supplementation on weekly hen day egg production (HDEP) pre-
Salmonella challenge.

There were no significant interaction effects between vaccine and synbiotic supplementation on egg production at 19 (P = 0.68), 20 (P = 0.41), 21 (P = 0.29), 22 (P = 0.69), and 23 (P = 0.59) wk of age (Figure 3). There were no significant main effects of vaccine on egg production at wk 19 (P = 0.68), 20 (P = 0.09), 21 (P = 0.05), and 23 (P = 0.24) wk of age. At 22 wk of age, there was a significant main effect (P = 0.03) of vaccine on egg production. Because there were no significant interaction effects and the main effects of vaccine was not significant on egg production, the main effects of synbiotic supplementation was analyzed. Birds supplemented with synbiotic had higher number of eggs, as calculated as weekly HDEP, compared to the group with no synbiotic supplementation between 19-23 wk of age. Birds fed synbiotics had 0.7%, 17.8%, 21.7%, 3%, and 4.2% greater HDEP at 19, 20, 21, 22, 23 wk of age, compared to the birds fed no synbiotics, respectively.

Effect of synbiotic supplementation on weekly hen day egg production (HDEP) post-
Salmonella challenge

There were no significant interaction effects between vaccine/challenge and synbiotic supplementation on egg production at 24 (P = 0.34), 25 (P = 0.34), 26 (P = 0.13), 27 (P = 0.63), and 28 (P = 0.86) wk of age (Figure 4). There were no significant main effects of vaccine on egg production at 24 (P = 0.48), 25 (P = 0.95), 26 (P = 0.13), 27 (P = 0.46) and 28 (P = 0.86) wk of age. Because there were no significant interaction effects
and the main effects of vaccine on egg production were not significant, the main effect of synbiotic supplementation was analyzed. Birds supplemented with synbiotic had higher number of eggs, as calculated as weekly HDEP, compared to the group with no synbiotic supplementation between 24-28 wk of age. Birds fed synbiotic had 3%, 6.7%, 4.3%, 12.5%, and 14.4% greater HDEP at 24, 25, 26, 27, and 28 wk of age, compared to the birds fed no synbiotic, respectively.

**Effect of synbiotic supplementation on supplemented probiotic strains content in the ceca post-Salmonella challenge**

There were significant interaction effects between vaccine/challenge and synbiotic supplementation on *P. acidilacti* content at 8 (P = 0.01) and 17 (P < 0.01) d post-Salmonella challenge (Figure 5). At 8 d post-Salmonella challenge, birds fed synbiotic and challenged with *Salmonella* had higher percentage of *P. acidilacti* in the ceca than all other treatment groups. At 17 d post-Salmonella challenge, birds fed synbiotic, vaccinated and challenged with *Salmonella* had higher percentage of *P. acidilacti* in the ceca than all other treatment groups.

There were no significant interaction effects between vaccine/challenge and synbiotic supplementation on cecal *P. acidilacti* content at 10 (P = 0.42) and 22 (P = 0.15) d post-Salmonella challenge (Figure 6). There were no significant main effects of vaccine/challenge on cecal *P. acidilacti* content at 10 (P = 0.42) and 22 (P = 0.15) d post-Salmonella challenge. Because there were no significant interaction effects and the main effect of vaccine/Salmonella was not significant, the main effect of synbiotic
supplementation was analyzed. Birds supplemented with synbiotic had higher number of cecal *P. acidilacti* content, compared to the group with no synbiotic supplementation. Birds fed synbiotic had 0.003% and 0.0003% greater *P. acidilacti* content at 10 and 22 d post-*Salmonella* challenge, compared to the birds fed no synbiotics, respectively.

There was significant interaction effects at 8 (P = 0.03) and 24 (P = 0.03) d post-*Salmonella* challenge (Figure 7a, 7b) on *L. reuteri* in the cecal content. There were no significant main effects of vaccine/*Salmonella* or synbiotic on *L. reuteri* load in the cecal content. At both 8 and 24 d post-challenge, the group that was supplemented, vaccinated, and challenged had the highest amount of cecal content *L. reuteri* than all other treatment groups.

*Enterococcus faecium* was undetectable in the ceca in any of the treatment groups.

**Effect of synbiotic supplementation on *S. Enteritidis* cecal content load post-*Salmonella* challenge**

*Salmonella* was undetectable in the cecal content at day -5 post challenge. At 3 d post-*Salmonella* challenge, *Salmonella* was detected in the cecal content of all treatment groups, except the groups with no *Salmonella* challenge (data not shown). At 10 d post-*Salmonella* challenge, *Salmonella* was undetectable in the cecal content of all treatment groups (data not shown). At 8d post-*Salmonella* challenge, there were significant interaction effects between vaccine/challenge and synbiotic supplementation on *S. Enteritidis* cecal content (P = 0.03) (Figure 8). Irrespective of the vaccination status, birds fed synbiotics and challenged with *Salmonella* had lower *S. Enteritidis* content compared
to that in the no synbiotic supplemented and unvaccinated treatment group. Among the *Salmonella* challenged birds, birds in the synbiotic supplemented and unvaccinated treatment group had comparable S. Enteritidis content to the vaccinated group with no synbiotics.

*Effect of synbiotic supplementation on bile and plasma anti-Salmonella IgA content post-Salmonella challenge*

There were significant interaction effects between vaccine/challenge and synbiotic supplementation on bile anti-*Salmonella* IgA content at 8 (P = 0.01) and 22 (P = 0.02) d post-*Salmonella* challenge (Figure 9). At 8 d, birds in the unvaccinated and synbiotic un-supplemented group and challenged with *Salmonella* had the highest IgA content. Among birds with no *Salmonella* challenge, birds in synbiotic supplemented and unvaccinated treatment group had comparable bile IgA content to that in the vaccinated and synbiotic un-supplemented group. At 22 d post-*Salmonella* infection, birds in synbiotic supplemented, vaccinated and challenged with *Salmonella* had the highest bile IgA content.

There were no significant interaction effects between vaccine/challenge and synbiotic supplementation on plasma anti-*Salmonella* IgA content at 8 (P = 0.11) and 22 (P = 0.38) d post-*Salmonella* challenge (Figure 10).
Effect of synbiotic supplementation on cecal tonsil IL-10 mRNA content post-Salmonella challenge

There were significant interaction effects between vaccine/challenge and synbiotic supplementation on cecal tonsil IL-10 mRNA content at 3 (P = 0.04) and 8 (P = 0.02) and 24 (P = 0.01) d post-Salmonella challenge (Figure 11). At 3 d post-Salmonella challenge, among birds unvaccinated and challenged with Salmonella, synbiotic supplementation increased IL-10 mRNA content in the cecal tonsils. At 8 d post-salmonella challenge, birds fed synbiotic, vaccinated and challenged with Salmonella had the highest relative amounts of IL-10 mRNA in the cecal tonsils compared to all other treatment groups.

Effect of synbiotic supplementation on cecal tonsil TNFα mRNA content post-Salmonella challenge

There were no significant interaction effects between vaccine/challenge and synbiotic supplementation on TNFα mRNA content at 8 (P = 0.19) and 10 d (P = 0.35) d post-Salmonella challenge. There were no significant main effects of vaccine/challenge on TNFα mRNA content at 10 (P = 76) and 22 (P = 56) d post-Salmonella challenge. Because there were no significant interaction effects and the main effects of vaccine/Salmonella was not significant, the main effect of synbiotic supplementation was analyzed. At 8 (P = 0.01) and 10 (P = 0.02) d post-Salmonella challenge, synbiotic supplementation increased TNFα mRNA content in the cecal tonsils (Figure 12).
Effect of synbiotic supplementation on cecal tonsil $CD4^+$ cell percentage post-Salmonella challenge.

There were significant interaction effects between vaccine/challenge and synbiotic supplementation on cecal tonsil $CD4^+$ cells at 3 (P = 0.04), 10 (P = 0.01), 17 (P = 0.01), and 22 (P = 0.04) d post-Salmonella challenge (Figure 13). At all-time points, the group un-supplemented with synbiotic and unchallenged with Salmonella had the highest $CD4^+$ cell percentage. The $CD4^+$ cell percentages in the cecal tonsil was comparable between the group fed synbiotic but unvaccinated and the group un-supplemented with synbiotic but vaccinated.

Effect of synbiotic supplementation on cecal tonsil $CD8^+$ cell percentage post-Salmonella challenge

There were significant interaction effects between vaccine/challenge and synbiotic supplementation on cecal tonsil $CD8^+$ cells at 3d (P = 0.04) post-Salmonella challenge (Figure 14). At 3 d post-Salmonella challenge, the group un-supplemented with synbiotic and unchallenged with Salmonella had the highest $CD8^+$ cell percentage. The $CD8^+$ cell percentages in the cecal tonsil was comparable between the group fed synbiotic but unvaccinated and the group un-supplemented with synbiotic but vaccinated.

Effect of synbiotic supplementation on cecal tonsil $CD4^+CD25^+$ cell percentage post-Salmonella challenge
There were significant interaction effects between vaccine/challenge and synbiotic supplementation on cecal tonsil \( \text{CD}^{4+}\text{CD}^{25^+} \) cell percentages at 10 (\( P = 0.01 \)) and 24 (\( P = 0.01 \)) d post-\textit{Salmonella} challenge (Figure 15). At 10 d post \textit{Salmonella} challenge, the unvaccinated group fed synbiotic and challenged with \textit{Salmonella} had the lowest percentage of \( \text{CD}^{4+}\text{CD}^{25^+} \) cells. Among the birds challenged with \textit{Salmonella}, the \( \text{CD}^{4+}\text{CD}^{25^+} \) cell percentages in the cecal tonsil was comparable between the group fed and not fed synbiotic.

**Discussion**

In agreement with previous studies [121, 147, 148], our study showed an increase in performance of birds supplemented with probiotics and prebiotics. Synbiotic supplemented birds showed increased body weight gain, and egg production as well as decreased age of the bird at first lay. This is in contrast to other studies which have failed to find increased performance from the supplementation of pro and prebiotics [122, 149]. It is likely the increased egg performance is due to the increase in body weights observed in the supplemented groups.

A specific function of probiotics and prebiotics is to influence the gut microflora so that there is an increase in beneficial native and supplemented bacteria and a decrease in pathogenic bacteria [84]. Early in life, the typical microflora in the ceca of chickens includes \textit{Peptostreptococcus}, \textit{Propionibacterium Eubacterium}, \textit{Bacteriodes}, and \textit{Clostridium} [150]. In our study, supplementation with the synbiotic increased numbers of \textit{P. acidilacti} and \textit{L. reuteri} in the ceca. However, even with supplementation, this study we
failed to identify *E. faecium* in the ceca suggesting that this species did not colonize the ceca.

Previous studies [138, 151, 152] have concluded that the supplementation of pre and probiotics in the diets of chickens can minimize bacterial infections, including *Salmonella*. This study identified that although all the birds cleared the *Salmonella* infection by 10 d post-*Salmonella* infection, synbiotic supplemented groups were able to clear the infection faster than the un-supplemented groups. The synbiotic group was as efficient as the vaccinated group in clearing *Salmonella* from the ceca. It can be concluded that supplementation of the synbiotic product can be beneficial in potentially clearing a *Salmonella* infection of chickens.

This study identified that unvaccinated birds and synbiotic un-supplemented birds challenged with *Salmonella* had the highest amount of IgA in the bile at 8 d post-*Salmonella* infection. This is likely due to the continued presence of *Salmonella* infection in this particular group at 8 d post-*Salmonella* infection while the other groups had cleared the infection by that time point. Interestingly, birds fed synbiotics and challenged birds, though had high amounts of IgA, had cleared the *Salmonella* infection by 8 d post-*Salmonella* infection suggesting that the higher IgA in synbiotic supplemented group could have contributed to the *Salmonella* clearance. The relatively lower amounts of *Salmonella*-specific IgA in the plasma compared to in the bile can be expected because IgA is seen primarily in the bile and in mucosal secretions.

Studies by Uchiya *et al* and Li *et al* have shown that IL-10 is up-regulated in response to *Salmonella* infection [153, 154] – IL-10 mRNA expression was significantly
higher in the synbiotic fed unvaccinated birds and challenged with *Salmonella* as well as the group vaccinated but had no synbiotic supplementation or challenge. Although there is limited data regarding TNFα responses to *Salmonella*, many *Salmonella* serovars do not typically mount strong inflammatory responses in the gut [39]. This study identified that following infection, TNFα mRNA expression was higher in synbiotic fed groups. The higher IL-10 in the synbiotic supplemented and challenged group might be a host mechanism to counter the higher TNFα in synbiotic fed groups.

The cecal tonsils of chickens are gut-associated lymphoid tissues that play a role as an immunological organ [99]. T-cells are cells of the immune system which play a part in the adaptive immunity of the bird. CD4 cells (T-helper cells) secrete cytokines and activate B-cells. CD8+ (cytotoxic T-cells) kill cells infected with the pathogen. After the pathogen infects a bird, T-cells migrate to the site of infection and the time it take for T cells to migrate to the site of infection is dependent upon previous exposure [43]. In this study, CD4+ and CD8+ cell percentages increased with infection and decreased after the birds cleared the infection. Additionally, we see that vaccinated bird have a much faster response than the other groups, as expected due to previous exposure. In this study, synbiotic supplemented groups tend to have lower percentages of CD4+ and CD8+ cells than the non-supplemented groups. We theorize this is because of the decreased *Salmonella* infection in this group and the increased levels of IL-10.

CD4+CD25+ cells are considered to be T-regulatory cells and are immune suppressors [45]. *Salmonella* has been shown to upregulate Tregs to suppress the immune system and thereby can survive the immune system. This study identified that at day 10
post-\textit{Salmonella} infection, the group fed synbiotic and challenged with \textit{Salmonella} had the lowest amount of T regulatory cells suggesting that supplementing synbiotics can counter the \textit{Salmonella} induced increase in T regulatory cells.

We conclude from this study that supplementation of synbiotic product to layer diets can improve growth, improve production performance, and protect against \textit{S. Enteritidis} infection.

\textbf{Acknowledgements}

Animal husbandry help from K. Patterson, J. Sidle, J. Snell, and J. Welsh, technical help from R. Shanmugasundaram, and product help from G. Raj Murugesan are acknowledge
Chapter 5: Effect of organic acid and phytochemical supplement on production parameters with and without Salmonella vaccine

Abstract

This experiment studied the effects of a blend of organic acid and phytochemical supplement (Biotronic® Top3) consisting of formic, propionic, and acetic acids combined with cinnamaldehyde on pullet growth and hen egg production parameters with and without Salmonella vaccination. The organic acid and phytochemical supplement was added to the feed at a rate of 1 g/kg from day-of-hatch until the end of the project at 23 wk of age. At 14 wk of age, birds were vaccinated with 0.3 cc Poulvac SE (Salmonella Enteritidis bacterin; phage types 4, 8 & 13a) subcutaneously and were given a booster at wk 17 of age. At 18 wk of age, birds were brought into production by increasing light hours to 16 hours a day. Body weights were collected every two weeks and egg production was measured every day following first egg. Supplementation of organic acid and phytochemical supplement increased body weight from day-of-hatch through 23 wk of age (P < 0.05). Birds which received diets supplemented with organic acid and phytochemical supplement had increased body weight (P < 0.05) and organic acid and phytochemical supplement increased weekly hen day egg production (P < 0.05). It could be concluded that
supplementing pullet and layer diets with the organic acid and phytochemical supplement can increase production performance.

**Introduction**

Due to the increase in restrictions for using antibiotic growth promoters in poultry feed, other substances have been looked at as replacement to enhance production performance and growth in poultry. These products include organic acids and essential oils. Organic acids and essential oils are unlikely to be acceptable as a substitute separately, but in combination they could provide a broader spectrum of activity [155].

Organic acids are partly dissociated, weak acids. Organic acids are typically used as supplements in drinking water or as acidifiers in feed. Many of these acids are also available as salts [156]. In poultry, organic acids have been shown to have competitive exclusion properties as well as increase nutrient utilization and growth and feed conversion efficiency [155, 157]. These organic treatments have also been shown to have similar effects as with antibiotics [158]. Acetic, formic, and propionic acids specifically have been shown to inhibit Gram-negative bacteria by decreasing DNA replication [159].

Essential oils are phytochemicals derived from plants and have received increased attention as growth performance enhancers [160]. Essential oils are a cocktail of secondary plant metabolites and consist of phenylpropenes and terpenes [161]. Essential oils have been linked with several proposed health benefits, among them is increased lipid metabolism, digestive stimulation, antimicrobial effects, and anti-inflammatory effects [162]. A major component of bark extract of cinnamon, cinnamaldehyde, has been shown
to have antimicrobial effects towards many foodborne pathogens [163]. Cinnamaldehyde has also been shown to be effective in killing S. Enteritidis in chickens, when presented in drinking water [164].

The present study was carried out to determine the effects of a commercially available organic acid and phytochemical supplement (Biotronic® Top3, Biomin, San Antonio, TX) supplementation on production performances of layer birds. The organic acid and phytochemical supplement contained formic, propionic, and acetic acids combined with cinnamaldehyde.

**Materials and Methods**

**Birds**

384 1-d-old specific-pathogen-free White Leghorn chicks (Hy-Line North America; Johnstown, OH) were provided ad libitum water and feed, housed in battery cages (pullet and layer), and raised using standard animal husbandry practices. The feed formulations are provided in Table 2. Experiment was approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. The birds were housed in pullet cages for the first 12 wk of age after which they were transferred to layer cages. At 18 wk of age, the light hours were increased to 16 hours to photostimulate them.

**Treatments**

The birds were fed a layer starter diet between 0-8 wk of age and a layer grower diet between 8-18 wk of age and a layer finisher diet between 19 to end of the experiment.
A total of 384 one-day-old layer chicks were randomly distributed to one of the two treatment groups, control and growth enhancer supplemented groups. Each treatment was replicated in 24 feeders of 8 chicks per replication (n=24). The basal diet was based on corn and soybean meal (Table 2) and supplemented with organic acid plus phytochemical supplement (Biotronic® Top3, Biomin, San Antonio, TX). The organic acid plus phytochemical supplement was added to the feed at a rate of 1g/kg from day-of-hatch until the end of the project at wk 23.

At 14 wk of age the birds were weighed. At 14 wk of age, 32 birds in both growth enhancer and control group (64 birds in total) were vaccinated with a Salmonella vaccine resulting in a 2 (control and organic acid plus phytochemical supplementation) X 2 (vaccinated and non-vaccinated group) factorial arrangement of treatments. The number of replication was 16 for the vaccinated group (n = 16) and 8 for the unvaccinated groups (n=8). Each replication had 8 pullets. Birds were vaccinated on 14 wk of age, with a booster dose on 17 wk of age, with 0.3 cc of Salmonella vaccine (Poulvac® SE, Zoetis, Florham Park, NJ) subcutaneously in the breast muscle. Body weights were measured at 17, 18, and 20 wk of age. Eggs were collected and recorded daily from day of first egg until the end of the project.

Statistical Analysis

A two way ANOVA was used to examine the interaction effects of vaccination X organic acid plus phytochemical supplement on dependent variables collected between 14 to 23 wk of age. When the interaction effects were not significant (P > 0.05), the main
effects of organic acid plus phytochemical supplement was analyzed if there were no main effects of vaccine. When interaction or main effects were significant (P < 0.05), differences between means were analyzed by Tukey’s least square means comparison.

Results

Effect of organic acid plus phytochemical supplementation on bird body weights between 14-23 wk of age

There were no significant interaction effects of vaccine and organic acid plus phytochemical supplementation on bird body weight at 17 (P = 0.76), 18 (P = 0.28), 20 (P = 0.49), 22 (P = 0.29), and 23 (P = 0.72) wk of age, however there was an interaction effect at 14 (P < 0.01) wk of age. There was also no significant main effect of vaccine on body weight for any time point but there was a significant main effect of organic acid plus phytochemical supplementation on body weight for wk 14 (P = 0.01), 17 (P = 0.01), 18 (P = 0.01), 20 (P = 0.01), 22 (P = 0.01), and 23 (P = 0.02) (Figure 16). Because there were no significant interaction effects and the main effect of vaccine was not significant on body weight, the main effect of organic acid plus phytochemical supplement was analyzed. Birds supplemented with organic acid plus phytochemical had higher body weight compared to the group with no organic acid plus phytochemical supplement between 17-23 wk of age. Birds fed organic acid plus phytochemical supplement had 8.4%, 7.5%, 8.8%, 4.6%, and 9% greater body weight at 17, 18, 20, 22, 23 wk of age, compared to the birds fed no organic acid plus phytochemical supplement, respectively.
Effect of organic acid plus phytochemical supplementation on weekly hen day egg production (HDEP)

There were no significant interaction effects between vaccine and organic acid plus phytochemical supplementation on egg production at wk 19 (P = 0.05), 20 (P = 0.75), 21 (P = 0.06), 22 (P = 0.06), and 23 (P = 0.81) wk of age (Figure 17). There were no significant main effects of vaccine on egg production at wk 20 (P = 0.52), 21 (P = 0.32), 22 (P = 0.41) and 23 (P = 0.79) wk of age. At 19 wk of age, there was a significant main effect (P =0.04) of vaccine on egg production. Because there were no significant interaction effects and the main effects of vaccine was not significant on egg production, the main effects of organic acid plus phytochemical supplementation was analyzed. Birds supplemented with organic acid plus phytochemical had higher number of eggs, as calculated as weekly HDEP, compared to the group with no organic acid plus phytochemical supplementation between 19-23 wk of age. Birds fed growth enhancer had 0.62%, 12.5%, 19.3%, 4.2%, and 3.6% greater HDEP at 19, 20, 21, 22, 23 wk of age, compared to the birds fed no growth enhancer, respectively.

Discussion

Supplementation with the organic acids, produced heavier birds from pullet to layer stages and as a result increased egg production in those same birds. It has been shown previously that supplementation with organic acids improve feed efficiency by reducing feed pH, which allows for increased protein digestibility due to increased enzymatic activity [165].
Increased body weight and performance due to organic acid supplementation, specifically with short chain fatty acids has been previously observed in broilers [166, 167]. Our study showed significant increases in body weight in pullets and layers.

The results of this study is consistent with other studies [168, 169] in that organic acid supplementation not only induced laying earlier in the supplemented hens, but those hens had a consistent increase in production. This increased production performance can be attributed to the increased body weight.

In conclusion, this study has shown that dietary organic acid plus phytochemical supplementation to layer hens improved production performances and induced laying at an earlier age. It can be concluded that organic acid plus phytochemical supplementation to layer hens improves production performance in layer hens, however, further investigation is needed to evaluate feed efficiency and other metabolic effects.

Acknowledgements

Animal husbandry help from K. Patterson, J. Sidle, J. Snell, and J. Welsh, technical help from R. Shanmugasundaram, and product help from G. Raj Murugesan are acknowledged.
Chapter 6: Conclusion

*Salmonella enterica* Enteritidis, the causative agent of Salmonellosis in humans, is a Gram-negative pathogen that is transferred from poultry to humans through contaminated meat and egg products. With the emergence of antibiotic-resistant salmonellae in addition to the strict regulations regarding the use of antibiotics in poultry feed, alternatives are needed to control *Salmonella* in poultry flocks. Studies have found that non-nutritive feed additives, particularly in combination, are effective in enhancing production performance as well as protecting against pathogenic bacteria, such as *Salmonella*, in both broilers and layers.

One such feed additive combination is synbiotics. Synbiotic supplementation in chicken feed has been shown to increase both broiler and layer body weight and production performance as well as reduce *Salmonella* incidence in the gastrointestinal tract. Synbiotic products take advantage of the mechanisms of both prebiotics and probiotics, including competitive exclusion, the production of anti-microbial substances, and pH manipulation.

A second feed additive combination is organic acids and phytochemicals (OP). OP supplementation in chicken feed has also been shown to increase both broiler and
layer body weight and production performance and is effective in reducing *Salmonella* infections. OP products are able to decrease feed pH as well as inhibit DNA and ATP production in pathogenic bacteria.

The objectives of this thesis were to

1. Determine the inhibitory potential of four probiotic strains (*L. reuteri, P. acidilacti, B. animalis*, and *E. facecium*) against *Salmonella*.
2. Determine the effect of synbiotic product on layer growth and performance and *Salmonella* colonization.
3. Determine the effect of organic acid plus phytochemical product on pullet growth and layer performance.

The study presented in chapter 3 confirmed the inhibitory potential of four live probiotic strains (*Lactobacillus reuteri, Bifidobacteria animals, Pediococcus acidilaci*, and *Enterococcus faecium*) against *Salmonella* in vitro. Among these strains, *L. reuteri* and *B. animalis* had the highest inhibitory potential against *S. Enteritidis*. These results suggested that the probiotic strains could be fed to poultry to reduce *Salmonella* shedding.

The study presented in chapter 4 evaluated the effects of synbiotic supplementation on layer growth, egg production, and immune responses following an experimental *Salmonella* challenge. Synbiotic supplementation increased layer body weights and egg production pre- and post-*Salmonella* infection and allowed the birds to clear the infection earlier than the non-supplemented birds. The ability of the supplemented birds to clear the infection faster can be contributed to the synbiotic’s effect on body weight and the effect
on the immune response. Synbiotic supplementation increased cecal tonsil IL-10 mRNA content, increased cecal tonsil TNF-a mRNA content, and decreased cecal tonsil CD4+CD25+ cells, all suggesting that the synbiotics counter *Salmonella* mediated immune suppression. Additionally, synbiotic supplementation produced comparable results to the vaccination in several immune parameter studied. It can be concluded from this study that supplementation of synbiotic product to layer diets can improve growth, improve production performance, and protect against *S. Enteritidis* infection.

The study presented in chapter 5 evaluated the effects of an organic acid plus phytochemical (OP) product on pullet growth and layer performance. OP supplementation improved pullet and layer body weights and contributed to the increase egg production seen in those birds. We conclude from this study that supplementation of OP product to layer diets can improve growth and production.

In conclusion,

1. *L. reuteri, B. animals, P. acidilaci,* and *E. faecium* have inhibitory potential against *S. Enteritidis in vitro.*
2. Synbiotic supplementation increases pullet body weight and egg production.
3. Synbiotic supplementation decreases *S. Enteritidis* detection in the ceca.
4. Synbiotic supplementation has an effect on layer immune responses to *S. Enteritidis.*
5. OP supplementation increases pullet and later body weight and egg production.
References


## Appendix A-Tables

<table>
<thead>
<tr>
<th>Probiotic</th>
<th>Dilution of probiotic supernatant</th>
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<tbody>
<tr>
<td></td>
<td>1:1</td>
<td>1:2</td>
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<tr>
<td><strong>CFUs of S. Enteritidis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>26</td>
<td>81</td>
</tr>
<tr>
<td><em>L. reuteri</em></td>
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<td>45</td>
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<tr>
<td><em>B. animalis</em></td>
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<td>134</td>
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<tr>
<td><em>P. acidilactici</em></td>
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<td>151</td>
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</table>

**Table 1.** Number of CFUs of *Salmonella* Enteritidis after being plated with each probiotic strain at each dilution and incubated at 37°C for 24h.
<table>
<thead>
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<th>Ingredient</th>
<th>Pullet</th>
<th>Grower</th>
<th>Layer</th>
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<tr>
<td>Blended fat</td>
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Table 2. Basal feed formulation.

The birds were fed a layer starter diet between 0-8 wk of age and a layer grower diet between 8-18 wk of age and a layer finisher diet between 19 to end of the experiment. Vitamins and minerals were fed at a level above the minimum NRC requirements.
<table>
<thead>
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<th>Target</th>
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<td>-r</td>
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Table 3. PCR primers.
Appendix B-Figures

Figure 1. Effect of probiotic culture supernatant on inhibition of *Salmonella* proliferation *in vitro*.

Probiotic strains *E. faecium* (*E.f.*), *L. reuteri* (*L.r.*), *B. animalis* (*B.a.*) and *P. acidilacti* (*P.a.*) and *Salmonella* were each grown in MRS medium separately. Probiotic supernatant was added to *Salmonella* culture at 1:1 and 1:2 dilutions (continued)
Figure 1 continued. and allowed to grown an additional 24 h at 37°C. The bacterial concentration was measured in a microplate reader and reported as optical density (OD) values. Bars (+ SE) with no common superscript differ (P < 0.05). P Values: E.f. P=0.01, L.r. P=0.01, B.a. P= 0.01, P.a. P=0.01.
Figure 2. Effect of synbiotic supplementation on bird body weights

Birds were fed either basal diet (Control) or supplemented with synbiotic product and vaccinated or not vaccinated with *Salmonella* vaccine age in a 2 (control and vaccinated) X 2 (control and synbiotic) factorial design. Synbiotic product was fed from day-of-hatch and birds were vaccinated with *Salmonella* vaccine at 14 wk of age. At 17, 18, and 20 wk of age, average body weights were measured. Bars (+ SE) with no common superscript differ (P < 0.05). P Values: Wk 17 Synbiotic X Vaccine P=0.02, Synbiotic P<0.01, Vaccine, P=0.15. Wk 18 Synbiotic X Vaccine P=0.03, Synbiotic P<0.01, Vaccine, P<0.01. Wk 20 Synbiotic X Vaccine P=0.01, Synbiotic P<0.01, Vaccine, P=0.01.
Figure 3. Effect of synbiotic supplementation on weekly hen day egg production (HDEP) pre-Salmonella challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from day-of-hatch through 23 wk of age and were vaccinated with Salmonella vaccine at 14 wk of age in a 2 (control and vaccinated) X 2 (control and synbiotic) factorial design.

Egg production was measured daily and at 19, 20, 21, 22, and 23 wk of age, weekly HDEP was analyzed. Because there were no significant interaction or main effects of vaccine on egg production, the main effects of synbiotic supplementation was analyzed.

Bars (+ SE) with no common superscript differ (P < 0.05). P Values: Wk 19 Synbiotic X Vaccine P=0.68, Synbiotic P=0.01, Vaccine, P=0.68. Wk 20 Synbiotic X Vaccine P=0.42, Synbiotic P<0.01, Vaccine, P=0.09. Wk 21 Synbiotic X Vaccine P=0.29, Synbiotic P<0.01, Vaccine, P=0.05 (continued)
Figure 3 continued. Wk 22 Synbiotic X Vaccine P=0.69, Synbiotic P=0.11, Vaccine, P=0.03. Wk 23 Synbiotic X Vaccine P=0.59, Synbiotic P=0.01, Vaccine, P=0.24
Figure 4. Effect of synbiotic supplementation on weekly hen day egg production (HDEP) post-Salmonella challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from day-of-hatch through 28 wk, were either vaccinated or not vaccinated with Salmonella vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of S. Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. Egg production was measured daily and at 24, 25, 26, 27, and 28 wk of age, and weekly HDEP was analyzed. Because there were no significant interaction or main effects of vaccine, the main effects of synbiotic supplementation was analyzed. Bars (+ SE) with no common superscript differ (P < 0.05). P Values: Wk 24 Synbiotic X Vaccine P=0.34, Synbiotic P=0.03, Vaccine, P=0.48. Wk 25 Synbiotic X Vaccine P=0.34, Synbiotic P=0.01, Vaccine, P=0.95. Wk 26 Synbiotic X Vaccine P=0.13, Synbiotic P=0.10, Vaccine, P=35 (continued)
Figure 4 continued. Wk 27 Synbiotic X Vaccine P=0.63, Synbiotic P=0.06, Vaccine, 
P=0.46. Wk 28 Synbiotic X Vaccine P=0.86, Synbiotic P=0.01, Vaccine, P=0.86.
Figure 5. Effect of synbiotic supplementation on cecal *P. acidilacti* content load post-*Salmonella* challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from day-of-hatch through 28 wk, were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10⁹ CFU of *S. Enteritidis* at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. Cecal content samples were collect on 8 and 17 d post-challenge and the relative proportion of *P acidilacti* in the cecal content were measured by real time PCR after normalizing to the total DNA content of the cecal content. The relative proportion of *P acidilacti* in the cecal content were measured by real time PCR after normalizing to the total DNA content of the cecal content (continued).
Figure 5 continued. The proportions of \textit{P. acidilicati} is presented where the total of the examined bacteria was set at 100%. Bars (+ SE) with no common superscript differ (P < 0.05). P values: Day 8 Synbiotic X Treatment P = 0.01, Synbiotic P = 0.01, Treatment P = 0.01. Day 17 Synbiotic X Treatment P < 0.01 Synbiotic P = 0.01, Treatment P < 0.01.
Figure 6. Effect of synbiotic supplementation on *P. acidilacti* cecal content load post-challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from day-of-hatch through 28 wk, were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10⁹ CFU of *S. Enteritidis* at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. Cecal content samples were collect on 10 and 22 d post-challenge and the relative proportion of *P acidilacti* in the cecal content were measured by real time PCR after normalizing to the total DNA content of the cecal content. The proportions of *P. acidificati* is presented where the total of the examined bacteria was set at 100%. Bars (+ SE) with no common superscript differ (P < 0.05) (continued).
**Figure 6 continued.** P values: Day 10 Synbiotic X Treatment P =0.42, Synbiotic P<0.01, Treatment P=0.42. Day 22 Synbiotic X Treatment P=0.15, Synbiotic P<0.01, Treatment P=0.15.
Figure 7. Effect of synbiotic supplementation on *L. reuteri* cecal content load post-challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from day-of-hatch through 28 wk of age, were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either (continued)
**Figure 7 continued.** 0 or $1 \times 10^9$ CFU of *S. Enteritidis* at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. Cecal content samples were collected on days 8 and 10 (a) and 24 (b) post-challenge and the relative proportion of *L. reuteri* in the cecal content were measured by real time PCR after normalizing to the total DNA content of the cecal content. The proportions of *L. reuteri* is presented where the total of the examined bacteria was set at 100%. Bars (+ SE) with no common superscript differ (P<0.05). P values: Day 8: Synbiotic X Treatment P =0.02, Synbiotic P=0.41, Treatment P=0.02. Day 10: Synbiotic X Treatment P=0.67, Synbiotic P=0.37, Treatment P=0.28. Day 24: Synbiotic X Treatment P=0.02, Synbiotic P=0.02, Treatment P=0.01.
Figure 8. Effect of synbiotic supplementation on S. Enteritidis cecal content load post-
Salmonella challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from
day-of-hatch through 28 wk, were either vaccinated or not vaccinated with Salmonella
vaccine at 14 wk of age and challenged with either 0 or 1 \times 10^9\text{ CFU} of S. Enteritidis at
24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and
synbiotic) factorial design. Cecal content samples were collect on 8 d post-challenge and
the relative proportion of S. Enteritidis in the cecal content were measured by real time
PCR after normalizing to the total DNA content of the cecal content. The proportion of S.
Enteritidis is presented where the total of the examined bacteria (continued)
Figure 8 continued. was set at 100%. Bars (+ SE) with no common superscript differ (P < 0.05). P values: Day 8 Synbiotic X Treatment P=0.03, Synbiotic P=0.03, Treatment P=0.04.
Figure 9. Effect of synbiotic supplementation on bile anti-\textit{Salmonella} IgA content post-\textit{Salmonella} challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from day-of-hatch through 28 wk, were either vaccinated or not vaccinated with \textit{Salmonella} vaccine at 14 wk of age and challenged with either 0 or \(1 \times 10^9\) CFU of \textit{S}. Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. Bile samples were collected on 8 and 22 days post-challenge and analyzed for \textit{Salmonella}-specific IgA content through ELISA and results are reported as average optical density (OD) values (continued).
**Figure 9 continued.** Bars (+ SE) with no common superscript differ (P < 0.05). P values:

Day 8 Synbiotic X Treatment P =0.01, Synbiotic P=0.84, Treatment P<0.01. Day 22

Synbiotic X Treatment P =0.02, Synbiotic P=0.90, Treatment P=0.07.
Figure 10. Effect of synbiotic supplementation on plasma anti-Salmonella IgA content post-Salmonella challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from day-of-hatch through 28 wk, were either vaccinated or not vaccinated with Salmonella vaccine at 14 wk of age and challenged with either 0 or 1 X 10⁹ CFU of S. Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. Plasma samples samples were collected on 8 and 22 d post-challenge and analyzed for Salmonella-specific IgA content through ELISA and results are reported as average optical density (OD) values. Bars (+ SE) with no common superscript differ (P < 0.05) (continued)
**Figure 10 continued.** P values: Day 8 Synbiotic X Treatment P = 0.11, Synbiotic P = 0.51, Treatment P = 0.26. Day 22 Synbiotic X Treatment P = 0.38, Synbiotic P = 0.05, Treatment P = 0.01.
Figure 11. Effect of synbiotic supplementation on cecal tonsil IL 10 mRNA content post-\textit{Salmonella} challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from day-of-hatch through 28 wk, were either vaccinated or not vaccinated with \textit{Salmonella} vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of S. Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. At 3, 8, and 24 d post-\textit{Salmonella} challenge, cecal tonsils were collected and analyzed for IL-10 mRNA content after correcting for β-actin mRNA content and normalizing to the mRNA content of the vaccine group (continued),
**Figure 11 continued.** so all bars represent fold change compared to the vaccine group.

Bars (+ SE) with no common superscript differ (P < 0.05). P values: Day 3 Synbiotic X Treatment P =0.01, Synbiotic P=0.53, Treatment P=0.04. Day 8 Synbiotic X Treatment P =0.02, Synbiotic P=0.05, Treatment P=0.23. Day 24 Synbiotic X Treatment P =0.001, Synbiotic P=0.001, Treatment P=0.01.
Figure 12. Effect of synbiotic supplementation on cecal tonsil TNFα mRNA content post-*Salmonella* challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from day-of-hatch through 28 wk, were either vaccinated or not vaccinated with *Salmonella* vaccine at 14 wk of age and challenged with either 0 or 1 X 10⁹ CFU of *S. Enteritidis* at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. At 8 and 10 d post-*Salmonella* challenge, cecal tonsils were collected and analyzed for TNFα mRNA content after correcting for β-actin mRNA content and normalizing to the mRNA content of the vaccine group, so all bars represent fold change compared to the vaccine group. Because there were no significant interaction or main effects of vaccine, the main effects of synbiotic (continued)
Figure 12 continued. supplementation was analyzed. Bars (+ SE) with no common superscript differ (P < 0.05). P values: Day 8 Synbiotic X Treatment P =0.20, Synbiotic P=0.001, Treatment P=0.32. Day 10 Synbiotic X Treatment P =0.35, Synbiotic P=0.02, Treatment P=0.76
Figure 13. Effect of synbiotic supplementation on cecal tonsil CD4⁺ cell percentage post-
Salmonella challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from
day-of-hatch through 28 wk, were either vaccinated or not vaccinated with Salmonella
vaccine at 14 wk of age and challenged with either 0 or 1 X 10⁹ CFU of S. Enteritidis at
24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and
synbiotic) factorial design. At 3, 10, 17, and 22 d post-Salmonella challenge, cecal tonsils
were collected and stained with fluorescent-conjugated anti-chicken CD4 and analyzed in
a flow cytometer. CD4⁺ cell percentage was expressed as a percentage of total cecal
tonsil mononuclear cells. Bars (+ SE) with no common superscript differ (P < 0.05). P
values: Day 3 Synbiotic X Treatment P =0.04, Synbiotic P=0.31, Treatment P=0.17. Day
10 Synbiotic X Treatment P =0.01, Synbiotic P=0.60, Treatment P=0.01 (continued).
Figure 13 continued. Day 17 Synbiotic X Treatment P =0.01, Synbiotic P=0.01, Treatment P=0.01. Day 22 Synbiotic X Treatment P =0.04, Synbiotic P=0.05, Treatment P=0.93.
**Figure 14.** Effect of synbiotic supplementation on cecal tonsil CD8\(^+\) cell percentage post-
_Salmonella_ challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from
day-of-hatch through 28 wk, were either vaccinated or not vaccinated with _Salmonella_
 vaccine at 14 wk of age and challenged with either 0 or 1 X \(10^9\) CFU of S. Enteritidis at
24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and
synbiotic) factorial design. At 3 d post-_Salmonella_ challenge, cecal tonsils were collected
and stained with fluorescent-conjugated anti-chicken CD8 and analyzed in a flow
cytometer. CD8\(^+\) cell percentage was expressed as a percentage of total (continued)
Figure 14 continued. cecal tonsil mononuclear cells. Bars (+ SE) with no common superscript differ (P < 0.05). P values: Day 3 Synbiotic X Treatment P =0.04, Synbiotic P=0.82, Treatment P=0.01.
Figure 15. Effect of synbiotic supplementation on cecal tonsil CD4+CD25+ cell percentage post-Salmonella challenge.

Birds were fed either basal diet (Control) or supplemented with synbiotic product from day-of-hatch through 28 wk, were either vaccinated or not vaccinated with Salmonella vaccine at 14 wk of age and challenged with either 0 or 1 X 10^9 CFU of S. Enteritidis at 24 wk of age in a 3 (vaccinated, challenged, vaccinated+challenged) X 2 (control and synbiotic) factorial design. 10 and 24 d post-Salmonella challenge, cecal tonsils were collected and stained with fluorescent-conjugated anti-chicken CD4 and CD25 and analyzed in a flow cytometer. CD4+CD25+ cell percentage was expressed as a percentage of CD4+ cells. Bars (+ SE) with no common superscript differ (P < 0.05) (continued).
Figure 15 continued. P values: Day 10 Synbiotic X Treatment P = 0.01, Synbiotic P = 0.84, Treatment P = 0.20. Day 24 Synbiotic X Treatment P = 0.01, Synbiotic P = 0.02, Treatment P = 0.26.
Figure 16. Effect of organic acid plus phytochemical supplementation on bird body weights.

Birds were fed either basal diet (Control) or supplemented with organic acid plus phytochemical supplement (OP) from day-of-hatch through 14 wk of age. At 14 wk of age, body weights were measured. Bars (+ SE) with no common superscript differs (P < 0.05).

P Values: Wk 14 P<0.01. Birds were fed either basal diet (Control) or supplemented with organic acid plus phytochemical supplement (OP) and vaccinated or not vaccinated with Salmonella vaccine age in a 2 (control and vaccinated) X 2 (control and synbiotic) factorial design. OP product was fed from day-of-hatch and birds were vaccinated with Salmonella vaccine at 14 wk of age. At wk 17, 18, 20, 22, and 23 wk of age, average body weights were measured. Bars (+ SE) with no common superscript differ (P < 0.05) (continued).
Figure 16 continued. P Values: Wk 17 OP x Vaccine P=76, OP P<0.01, Vaccine, P=0.13.
Wk 18 OP x Vaccine P=0.28, OP P<0.01, Vaccine, P=0.36. Wk 20 OP x Vaccine P=0.49, OP P<0.01, Vaccine, P=0.84. Wk 22 OP x Vaccine P=0.29, OP P<0.01, Vaccine, P=0.43.
Wk 23 OP x Vaccine P=0.72, OP P=0.02, Vaccine, P=0.26.
Figure 17. Effect of organic acid plus phytochemical supplement (OP) on weekly hen day egg production (HDEP).

Birds were fed either basal diet (Control) or supplemented with organic acid plus phytochemical supplement (OP) product from day-of-hatch through 23 wk of age and were vaccinated with *Salmonella* vaccine at 14 wk of age in a 2 (control and vaccinated) X 2 (control and synbiotic) factorial design. Egg production was measured daily and at 19, 20, 21, 22, and 23 wk of age, weekly HDEP was analyzed. Because there were no significant interaction or main effects of vaccine on egg production, the main effects of OP supplementation was analyzed. Bars (+ SE) with no common superscript differ (P<0.05).

P Values: Wk 19: OP X Vaccine P=0.05, OP P=0.04, Vaccine, P=0.05. Wk 20: OP X Vaccine P=0.75, OP P<0.01, Vaccine, P=0.53 (continued)
Figure 17 continued. Wk 21: OP X Vaccine P=0.06, OP P<0.01, Vaccine, P=0.03. Wk 22 OP X Vaccine P=0.06, OP P=0.02, Vaccine, P=0.41. Wk 23 OP X Vaccine P=0.81, OP P=0.02, Vaccine, P=0.79.