EFFECTS OF SYNBIOTIC SUPPLEMENTATION AS AN ANTIBIOTIC GROWTH PROMOTER REPLACEMENT ON CECAL *CAMPYLOBACTER JEJUNI* LOAD IN BROILERS CHALLENGED WITH *C. JEJUNI*

by

EMILY CASON

(Under the Direction of Ramesh Selvaraj)

ABSTRACT

Campylobacter jejuni is a pathogen of great concern to the poultry industry as the consumption of undercooked poultry and poultry meat products is the leading source of human infection. Recent legislation and shifting public opinion on antibiotic growth promoter usage in food animal agriculture has removed the historical means of controlling *C. jejuni* and the poultry industry is exploring new means of foodborne pathogen control. In this thesis, we established a *C. jejuni* animal challenge model to effectively colonize broiler ceca, alter tight junction protein expression, and elicit strong immune responses. Successful, enduring cecal colonization and immunomodulation with challenge of 1 X 10^8 CFU/bird *C. jejuni* at 21 days was established. This challenge model to evaluate the efficacy of a synbiotic supplement as an antibiotic growth promoter alternative and identified it decreases *C. jejuni* loads in broilers and beneficially modulate the immune response to challenge, compared to antibiotic supplementation.

INDEX WORDS: *Campylobacter jejuni*, Nutrition, Immunology, Broilers, Poultry, Synbiotics, Antibiotic Growth Promoters, Animal Challenge Models

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EMILY CASON

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by

EMILY CASON

Major Professor:	Ramesh Selvaraj
Committee:	Todd Applegate
	Woo Kim

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
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CHAPTER 1

INTRODUCTION

In this thesis, a thorough review of current literature pertaining to *Campylobacter jejuni* in poultry and synbiotic supplementation as an antibiotic growth promoter (AGP) alternative, a research study evaluating the effect of *C. jejuni* challenge on microbiological and immunological parameters with the aim of establishing a challenge model, and a research study examining the effects of a synbiotic supplement as an AGP replacement on production, microbiological, and immune parameter performance in *C. jejuni* challenged broilers will be presented.

In the first study, Immune Responses, Gut Permeability, and Cecal *Campylobacter jejuni* Loads in an Experimental *C. jejuni* Challenge Model in Broilers, we had two specific aims:

- *Specific Aim 1:* To determine the effects of 1 X 10^4 and 10 X 10^8 CFU/bird *C. jejuni* challenge at 21 days of age on load in the ceca at 0, 3, 7, and 14 dpi.
- *Specific Aim 2:* To determine the effects of 1 X 10^4 and 1 X 10^8 CFU/bird *C. jejuni* challenge at 21 days of age on immune parameters at 0, 3, 7, 14 dpi.

The purpose of these specific aims was to establish an effective *C. jejuni* challenge model in broilers. This data in important because there is not a consistent poultry *C. jejuni* challenge model in use and the dose dependent effects of *C. jejuni* challenge on the immune and microbiological parameters is not well characterized. We evaluated performance, cecal *C. jejuni* load, anti-*C. jejuni* IgA concentration in the bile, pro- and anti-inflammatory cytokine and tight junction protein expression, gut permeability, immune gene expression, and $CD4^+$:$CD8^+$ ratio in birds challenged with 1 X 10^4 and 1 X 10^8 CFU/bird *C. jejuni*. We expected the *C. jejuni* to colonize the ceca of birds at 3, 7, and 14 dpi and for *C. jejuni* to induce immune responses

following challenge. Broilers challenged with 1 X 10^8 CFU/mL *C. jejuni* were expected to demonstrate heightened anti-*C. jejuni* IgA production, increased pro- and anti-inflammatory cytokine expression, decreased tight junction protein amounts, increased gastrointestinal permeability, upregulated immune gene expression, and an elevated $CD4^+$:$CD8^+$ ratio.

In the second study, Effects of Synbiotic Supplementation as an Antibiotic Growth Promoter Replacement on Cecal *Campylobacter jejuni* Load in Broilers Challenged with *C. jejuni*, we had three specific aims:

- *Specific Aim 1:* To determine the effects of synbiotic (PoultryStar®) supplementation as an AGP replacement on decreasing *Campylobacter jejuni* in market age broilers challenged with *C. jejuni.*
- *Specific Aim 2:* To determine the effects of synbiotic and AGP supplementation on immune parameters in broilers challenged with *C. jejuni*.
- *Specific Aim 3:* To determine the effects of synbiotic supplementation as an AGP replacement on broilers challenged with *C. jejuni* on production performance.

The purpose of these specific aims was to evaluate the viability of a specific synbiotic product as an AGP alternative in broilers. Our findings are important as the industry has moved away from AGP usage in commercial production and an alternative is needed. We evaluated cecal, hepatic, and ileal *C. jejuni* loads, performance, anti-*C. jejuni* IgA concentration in bile, pro- and anti-inflammatory cytokine and tight junction protein expression, gut permeability, immune gene expression, $CD4^+$:$CD8^+$ ratio, regulatory T cell activation, and nitric oxide production by splenocyte mononuclear cells (MNCs) in challenged birds supplemented with an AGP or synbiotic. Compared to AGP supplemented broilers, we expected synbiotic supplementation to improve performance, decrease *C. jejuni* load in the ceca, ileum, and liver, modulate anti-*C. jejuni* IgA production, decrease pro- and anti-inflammatory cytokine expression, elevate tight junction protein expression, decrease gut permeability, reduce immune gene expression, lower $CD4^+$:$CD8^+$ ratio and Treg activation, and reduce nitric oxide production by splenocyte MNCs.

CHAPTER 2

CAMPYLOBACTER JEJUNI AND SYNBIOTIC SUPPLEMENTATION AS AN ANTIBIOTIC GROWTH PROMOTER ALTERNATIVE

Introduction

Campylobacter jejuni is the most reported bacterial cause of foodborne illness in the United States each year (CDC, 2021). The discovery of the bacterium occurred in 1886 by Theodor Esherich, after visualization in human fecal material, but it was not until 1972 when *C. jejuni* was found to be a pathogen of human concern, responsible for enteric symptoms, gastroenteritis, and gastrointestinal disease (Skirrow & Butzler, 2000). *Campylobacter* comes from the family *Campylobacter*aceae and typically occurs as a commensal organism within human and animal hosts (Vandamme, 2000). There are 31 distinct species and 10 sub-species within the genus *Campylobacter*, with two clinically relevant species, *C. jejuni* and *Campylobacter coli*, being responsible for greater than 95% of human clinical cases annually (Facciola et al., 2017; Garcia-Sanchez et al., 2018). In humans, both animal and environmental vectors can be responsible for transmission, with the consumption or improper handling of poultry and poultry meat products being the primary sources of infection (Hermans et al., 2012). It takes fewer than 100 colony-forming units of *C, jejuni* to induce human infection, with the primary human symptom being acute diarrhea, of which *Campylobacter* is the leading global cause (Tribble et al., 2010; Allos et al., 2013; Skirrow, 1977). Campylobacteriosis human clinical manifestations, which develop after a 1–7-day incubation period and are typically self-

limiting, frequently include fever, abdominal pain, cramping, and malaise but systemic infections have also occurred causing septic thrombophlebitis, endocarditis, neonatal sepsis, pneumonia, bloodstream infections, acute *coli*tis of inflammatory bowel disease, and acute appendicitis (Igwaran and Okoh, 2019). Post-infection, *C. jejuni* can continue to cause enduring morbidity in the form of arthritis, Reiter syndrome, Guillain-Barré syndrome, and Miller-Fisher syndrome can occur in humans (Bryan & Doylez', 1995; Snelling et al., 2005; Skarp et al., 2016).

Microbiology

Growth Characteristics and Requirements

Within *C. jejuni*, there exist two subspecies, ssp. *doylei* and spp. *jejuni*. As little research has been done focused on spp. *doylei*, this literature review will exclusively refer to spp. *jejuni* in all mentions. *C. jejuni* is a gram-negative S-shaped bacterium, capable of motility due to its polar flagella, which enables the cork-screw motility characteristic of this bacterium (Frirdich et al., 2017; Lertsethtakarn et al., 2011). As an adaptive response to environmental stress, *C. jejuni* changes its shape to become filamentous or coccoid (Gaynor et al., 2005). The dual polar, unsheathed flagella of *C. jejuni* are critical to not only motility, but also to viability, gastrointestinal mucosa colonization, dissemination out of the gut, and pathogenesis of the disease (Fields & Swerdlow, 1999; Man, 2011). *C. jejuni* is an obligate microaerobic bacteria, that grows best in a microaerobic environment with an atmosphere of 85% N2, 10% CO2, and 5% O2, and at temperatures of 37-43°C in the lab, which mimics the avian gut (Koenraad et al., 1997; Mortada et al., 2021; Rautelin et al., 1999). *C. jejuni* is notoriously difficult to grow *in vitro*, as it requires a rich, complex growth medium, has narrow growth conditions, easily loses viability and culturability, and has a 90–180-minute doubling time (Konkel et al., 2007).

Considered a commensal organism, *C. jejuni* is a non-saccharolytic and non-spore-forming bacteria with a size of 0.2-0.8 μm by 0.5-5.0 μm (Griffiths & Park, 1990; Ottosson & Stenstrom, 2003). The bacterium is catalase, oxidase, and hippurate hydrolysis positive, which are beneficial in the identification of and distinction between *C. jejuni* and other *Campylobacter* (Day et al., 2000; Morris et al., 1985; Steinbrueckner et al., 1999). *C. jejuni* is also unique compared to other foodborne pathogens in that is highly sensitive to processing-related stressors including drying, freezing, and heating (Doyle & Roman, 1982; Rusin et al., 1997; Stern & Kotula, 1982).

C. jejuni is an opportunistic pathogen with a high proficiency for multiplication in the hostile environment that is the gastrointestinal tract. It is capable of not only readily colonizing the gut but also of capitalizing on the available nutrients and outcompeting the native microbiota (Hofreuter, 2014). *C. jejuni* is fastidious in its growth and can be isolated from many environments, despite its previously mentioned high sensitivity to processing stressors, within poultry processing and production. The apathogenic pathogen is temperature resistant and can remain viable for moderate amounts of time in the presence of oxygen, endure sudden pH changes, and persist in osmotic or nutrient-deficient environments (Murphy et al., 2006; Park, 2002). This implies that *C. jejuni* is highly adaptable and variable in nature.

Genome

The genome of *C. jejuni* is just over 1.6 million base pairs in length, with few repeating sequences, no insertion sequences or phage-associated sequences, and many hypervariable sequences (Parkhill et al., 2000; Pearson et al., 2007). The small size of this foodborne pathogen's genome, in combination with its highly variable nature, has been proposed as responsible for the complicated growth requirements and unique pathogenesis and

microbiological characteristics of the microorganism (Hofreuter et al., 2007). Dasti et al. propose that variation in the clinical manifestations of *Campylobacter*iosis is due to the significant genetic diversity present between *C. jejuni* isolates (2009).

Pathogenesis and Virulence Factors

Despite the importance of *C. jejuni* as the most prevalent global cause of foodborne disease, little is known about the bacteria's mechanisms of virulence. *C. jejuni* is a unique bacterial enteric pathogen in that it does not exhibit typical virulence factors associated with bacteria like *Salmonella* spp. and *Escherichia coli*, but it does possess a multitude of fitness and virulence factors to allow it to persist within a host (Gaytan et al., 2016; Park et al., 2018). These virulence factors include flagellum, other motility and chemotaxis proteins, outer-membrane binding and adhesion proteins, internalization mechanisms and proteins, cytolethal-distending toxins, and heat shock and stress response proteins (Dasti et al., 2010). There are nine critical phases of *C. jejuni* invasion of the intestinal epithelium in which pathogenicity and virulence factors are expressed; these are: motility, chemotaxis, oxidative stress defense, adhesion, invasion, toxin production, iron acquisition, temperature stress response, and coccoid dormant stage (van Vliet and Ketley, 2001).

Motility

C. jejuni is a facultative intracellular pathogen, incapable of intracellular replication, that relies on its discreet part-time intracellular nature to evade host immune responses by simply reducing its metabolism and persisting (Watson & Galan, 2008). The O-linked glycosylated flagella of *C. jejuni* enable its initial interaction with the environment, and the motility conveyed

by the polar flagella is essential to colonization and attachment to host intestinal epithelial cells (Dasti et al., 2010). The cork-screw motility characteristic of *C. jejuni* is used to penetrate the mucosal membrane covering the epithelial cells of the gastrointestinal tract (Szymanski et al., 1995). *C. jejuni* with mutant *flaA*, the primary structural gene of the flagella, experience reduced motility and adhesion and an inability to invade intestinal epithelial cells (Yao et al., 1994). Flagella facilitated motility is an important virulence factor for motility through the environment as well as in the lumen and mucosa of the intestine (Van Vliet and Ketley, 2001). The chemotaxis pathway of *C. jejuni*, critical motility-associated virulence factor when the bacteria are in the intestinal lumen, is facilitated by adaptation proteins including *tlp1*, *tlp4*, *tlp10*, methylesterase *CheB*, *CheR*, *CheY*, and various flagellum genes (Dasti et al., 2010; Stephens et al., 2006; Vegge et al., 2009).

Pathogenesis and Factors of Virulence

Once *C. jejuni* has successfully traversed the intestinal lumen and reached the mucosa, adhesion, invasion, iron acquisition, and oxidative stress defense must occur for colonization to be successful. The binding and adhesion proteins active in the lumen include flagellum, LOS, *CadF*, *PEB1*, *JlpA*, and *O*- and *N*-linked glycans (Hermans et al., 2011). *Peb1a* has been demonstrated to be critical for colonization and the protein likely works as an adhesin essential for the utilization of aspartate and glutamate amino acids as carbon sources (Flanagan et al., 2009). *CadF* is important to *C. jejuni* as it binds to the fibronectin of the gastrointestinal tract extracellular matrix, enabling adhesion (Konkel et al., 1997). Adherence and invasion cannot occur if the *PEB1* or *CadF* adhesion proteins are mutated (Snelling et al., 2005). *LOS* is another surface-accessible carbohydrate structure of *C. jejuni* that is important to host immune response

evasion, adhesion, and invasion of the intestinal epithelium (Guerry et al., 2000). The addition of sialic acid to *LOS* reduces the host immune reactivity to the pathogen and increases cellular invasion potential (Guerry et al., 2000; Habib et al., 2009).

The superoxide dismutase protein, *SodB,* is the major player in *C. jejuni's* defense against oxidative stress and is critical for intracellular survival (Pesci et al., 1994). Iron is crucial for *C. jejuni* colonization and cellular function as it is required for electron transfer, as an enzyme cofactor, and for the generation of hydroxyl radicals (Palyada et al., 2004). Hermans et al. suggest a link between *PerR* regulated oxidative stress mechanisms and *Fur* regulated iron metabolism and that both are necessary for successful and effective intestinal colonization (2011).

Internalization and cellular invasion of *C. jejuni* is accomplished by utilizing microfilaments and microtubules of host cells and biochemical cross-talk between host and pathogenic cells (Biswas et al., 2000, 2003). Invading *C. jejuni* interacts with host intestinal epithelial cells by generating signaling cascades that stimulate the remodeling of the cytoskeleton and ultimately the invasion, and internalization, of the pathogen (Kopecho et al., 2001). *C. jejuni* is internalized by host cells into a vacuole from which it must then escape through exocytosis to gain the freedom to the intracellular matrix (Forsythe, 2000).

Cytolethal distending toxin (CDT), the only toxin produced by *C. jejuni,* has been found to cause cell cycle arrest, cellular apoptosis, and IL-8 secretion by the host immune response (Hickey et al., 1999; Kopecho et al., 2001; Pickett and Whitehouse, 1999). CDT is a *C. jejuni* virulence factor whose role in pathogenicity is not well understood, though its requirement for binding to host cells, importance to cytotoxic activity, and immune-modulatory roles have been proven (Newell, 2001; Purdy et al., 2000). It is interesting to note that CDT only induces

neutralizing antibody production in humans, not in poultry hosts, which indicates possible host-specific recognition of *C. jejuni* antibodies (Elmi et al., 2021; Young et al., 2007).

Campylobacter jejuni in Poultry

C. jejuni is capable of surviving at all levels of poultry production and processing, indicating many environmental niches within the poultry industry. Gastrointestinal tract microbial populations play a crucial role in digestion, productivity, pathogenicity, disease susceptibility, and the overall health of poultry. Poultry are considered a natural biological reservoir of *C. jejuni* and is among the most frequently cited sources of human infection as the skin, organs, and meat all are capable of supporting *C. jejuni* colonization (Altekruse et al., 1999). It is probable that up to 80% of broiler flocks are positive for *Campylobacter* worldwide, though there is great variation regionally and between countries (Hermans et al., 2012). In the United States, the Food Safety and Inspection Service estimates that 46.7% of young chickens have *C. jejuni* infections (USDA FSIS, 2009). The transmission of *Campylobacter* is entirely horizontal, with vectors including humans, wild animals, litter, equipment, water, feed, rodents, and insects (Shanker & Sorrell, 1986). Young chicks are protected from colonization by *C. jejuni* due to anti-*C. jejuni* maternally transferred antibodies from hatch until 21 days of age under field conditions, though colonization can be induced through 1 X 10^8 CFU challenge doses experimentally at an earlier age (Sahin et al., 2003). *C. jejuni* contamination often originates on the farm and the pathogen is shed and spread during transport and processing (Bryan & Doylez', 1995) (*Figure 2.1*).

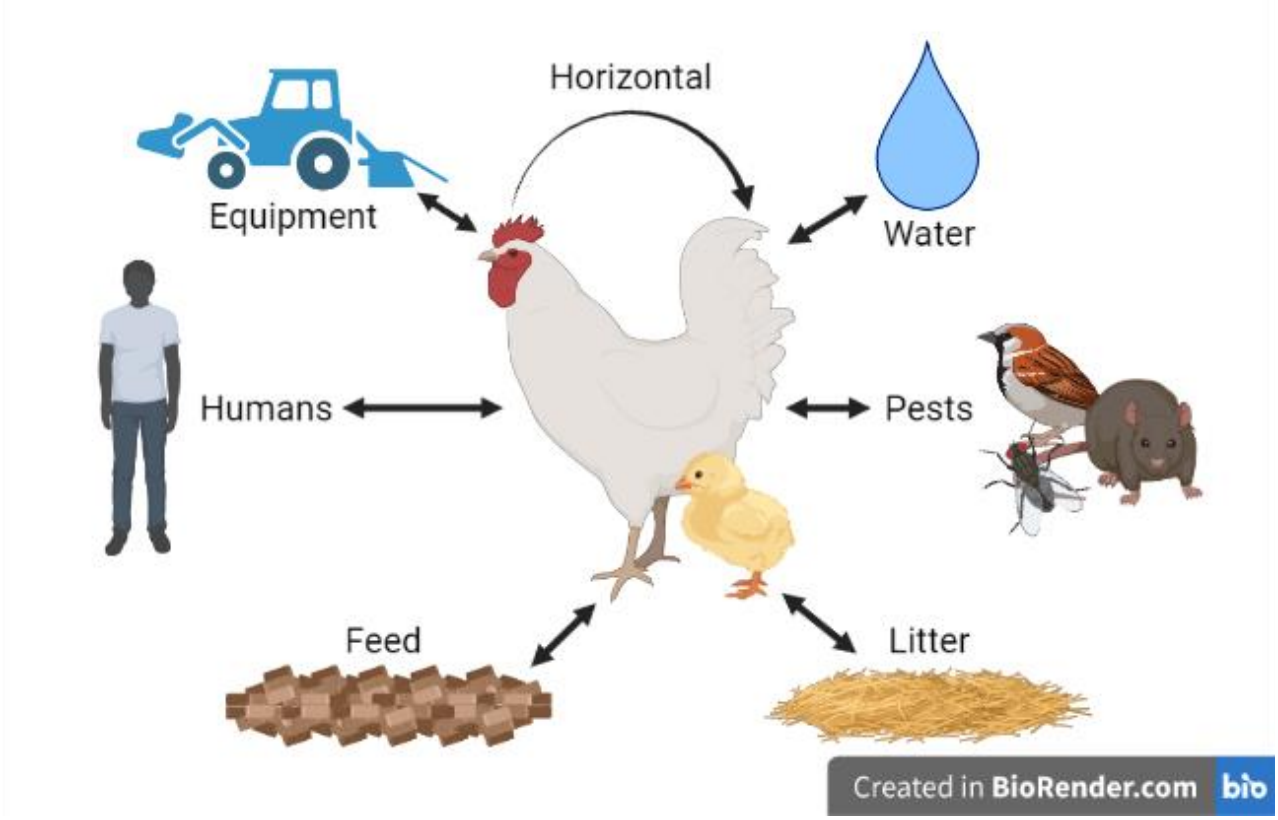

Figure 2.1. Vehicles and vectors of *Campylobacter jejuni* transmission to broilers at the farm level. Created with BioRender.com.

Colonization

Subsequent to ingestion, *C. jejuni* enters the host intestine via the stomach acid barrier and then colonizes the intestinal mucosa and ceca, releasing chemotactic virulence factors including heat shock proteins (Ketley, 1997). Though the exact mechanism through which *C. jejuni* induces disease is currently unknown, it is thought that symptoms are a result of cytolethal-distending toxin-induced host cell death and the subsequent inflammatory response (Snelling et al., 2005). Colonization by *C. jejuni* in poultry initially occurs in the gastrointestinal tract of the birds, with the greatest pathogenic load concentrated in the ceca (Mortada et al., 2021). Experimentally and commercially, cecal *C. jejuni* load in the ceca has been positively

correlated with whole carcass load of *C. jejuni* in broilers post-harvest (Allen et al., 2007; Reich et al., 2008). In the gastrointestinal tract, *C. jejuni* increases intestinal paracellular permeability by destroying the integrity of and the tight junctions between intestinal epithelial cells, to facilitate its escape and dissemination to other organs of the body, such as the liver and spleen (Awad et al., 2020; Knudsen et al., 2006). Challenging chicks with *Campylobacter* leads to ineffective activation of the developing immune system and reduced antimicrobial peptide gene expression (Hermans et al., 2012). Once colonized with *C. jejuni*, poultry never clear the infection and will continue to shed the pathogen for the rest of their lives, especially during critical stress periods (Achen et al., 1998). These stressors include the period of feed withdrawal, transportation, temperature changes, population density changes, handling, and instances of illness (Whyte et al., 2001).

Transmission

While virtually all flocks are contaminated with *C. jejuni* across the globe, the bacteria act apathogenically in poultry and are described as commensal and part of the natural avian microbiome (Hendrixson & DiRita, 2004). Chickens colonized by *C. jejuni* typically display no clinical signs and experience no marked productivity losses associated with the disease (Awad et al., 2016; Cean et al., 2015). Colonization is optimized by the poorly developed gut microbiota of young chicks and the ideal temperature, 42 ^{o}C, of the gut for growth and proliferation (Snelling et al., 2005). The pathogen rapidly colonizes and spreads rapidly from bird to bird and from flock to flock without notice. In one study, a 5% initial introduction rate of *C. jejuni* challenge to a flock resulted in 95% of the flock testing positive for the pathogen within 6 days, without any clinical manifestations of disease, elevated mortality, or notable decreases in

productivity (Van Gerwe et al., 2005). As a pathogen responsible for illness in 14.3 per every 100,000 Americans annually and estimated annual costs of $1.7 billion in the United States each year, *C. jejuni* represents critical biosecurity and human health concerns (Gilliss et al., 2012; Hoffmann et al., 2012).

Controlling *Campylobacter jejuni*

Control of *C. jejuni* contamination in poultry meat production is accomplished at every step within the vertically integrated industry. These control measures are undertaken at the feed mill, broiler breeder, hatchery, broiler grow out, transportation, processing, further processing, and storage levels of production. *C. jejuni* control in all of these combined areas together minimizes the likelihood of carcass and parts contamination, ultimately resulting in an increased likelihood of human disease incidence. This effective approach to reducing *C. jejuni* in final poultry meat and poultry meat products is an example of what is known as the hurdle concept: the combined factors, steps, and treatments used to limit microbial growth that acts more effectively together than any single agent alone (Leistner, 2000; Leistner & Gould, 2012).

Feed Mill

Past work has shown that poultry feed can be a vector responsible for *C. jejuni's* introduction to broiler and broiler breeder farms (Julien et al., 2013; Oliveira et al., 2008; Sommer et al., 2013). In stored starter and finisher feed, *C. jejuni* is capable of surviving for at least 5 days at both high and low storage temperatures and is viable and culturable (Alves et al., 2016). To control pathogenic microbial contamination of feed, feed mills have established Hazard Analysis and Critical Control Point programs in place. The greatest hurdles at the feed

mill level include pest control and ingredient quality control, the sourcing of pathogenic microorganism-free feedstuffs (Butcher & Miles, 2017). Feed ingredients readily transmit *C. jejuni* and other foodborne pathogens to final mixed feed and then on to the live poultry to which it is fed. The American Feed Industry Association states that the most impactful means of minimizing pathogenic microorganism contamination of finished feed products include pest management, screening of feed ingredients, implementation of functional biosecurity programs, overall mill cleanliness, moisture and temperature control of raw feed ingredients, regular vehicle and equipment sanitation, segregated airflow, employee traffic patterns, dust minimization, segregation of feed types, and sourcing of high-quality feed ingredients (1990). During feed formulation and mixing, thermal and chemical processing are used as hurdles to reduce foodborne pathogenic contamination. Pelleting, extruding, rendering, baking, and chemical control compound addition are all effective processing procedures used by poultry feed mills (American Feed Industry Association, 2010; Jones, 2011). The AFIA goes on to explain that thermal processing intensity and duration, moisture level minimization in the final feed product and physical processing in the form of pressure application may be sufficient to kill low levels of pathogenic organisms present in the raw feed inputs (2010). The production and delivery of pathogen-free feed are of vital importance to the maintenance of a *C. jejuni*-free flock (Alves et al., 2016).

Broiler Breeder

At the broiler breeder level, maintenance of a *C. jejuni*-free environment and flock is essential to the production of *C. jejuni*-free chicks. A study of broiler breeders colonized with *C. jejuni* by Callicott et al. found that vertical transmission is not a factor in *C. jejuni* passage from

parent to progeny (2006). *C. jejuni* is a pathogen traditionally thought of as being gastrointestinal tract specific, with some dissemination to the other internal organs as pathogenesis advances. Limited data exists supporting the association of *C. jejuni* with the reproductive tract and reproductive organs of breeding hens and roosters, suggesting that it is at most a rare occurrence without any significance to commercial fertile broiler egg production (Callicot et al., 2006; Newell et al., 2011; Shanker et al., 1986; Van de Giessen et al., 1992). Despite the practically nonexistent role of vertical and transovarial transmission, *C. jejuni* can still be transmitted to chicks from broiler breeders. Egg passage has recently been proven to be a significant source of *C. jejuni* transmission, responsible for not only chick contamination but also hatchery contamination with the pathogen (Cox et al., 2012). Egg passage of *C. jejuni* offers in the form of fecal contamination of the eggshell, shell membrane, and albumin of fertile, freshly lain eggs (Cox et al., 2012). Poor sanitary conditions of arriving hatching eggs represent a major source of hatchery contamination. When chicks emerge from their shells, ingestion of *C. jejuni* can occur, leading to intestinal colonization, shedding, and transmission of the pathogen to other birds. Preventing dirty eggs from reaching the hatchery is an important control measure for reducing the transmission of *C. jejuni* from broiler breeders to their offspring. Anti-*C. jejuni* maternal antibody transfer also has been shown to contribute to low levels of disease occurrence in chicks under 21 days of age and could represent a strong immunization-based approach to controlling *C. jejuni* colonization and infection in broiler flocks (Sahin et al., 2003; Shoaf-Sweeney et al., 2008).

Hatchery

C. jejuni is commonly found within commercial hatcheries and is regarded as a biosecurity and sanitation concern. The pathogen can be found on eggshells, feces, chick fluff, embryos, and equipment surfaces, as well as in water samples (Chaudhary et al., 1989; Cox et al., 2002; Doyle, 1984; Hiett et al., 2002). While *C. jejuni* may be commonplace in the hatchery, the pathogen is infrequently isolated from commercially hatched, day-old chicks (Rearson et al., 1996; Stern et al., 2001; Herman et al., 2003). The controls in place at the hatchery level for the reduction of *C. jejuni* include water sanitation, proper ventilation, and air filtration, coil, grill, and fan sanitation, full hatcher cleanouts, equipment, and surface sanitization within the hatchery and transport trucks, hatch basket, tray, and egg flat sanitization, fogging and fumigation of hatchers, thermal and humidity controls, and routine monitoring (French, 1987; Lively, 2020; Taylor, 1997). Reducing the level of *C. jejuni* and other pathogens within the hatchery level relies chiefly on three factors: the procurement of clean eggs, hatchery and truck sanitation, and good biosecurity.

Broiler Farm

On the farm, not even the strictest of control measures are always successful at eliminating intestinal carriage by poultry, but great efforts are made by sanitizing at cleanout, insect and rodent control, chlorine water treatment, and minimizing human interaction to control horizontal transmission (Altekruse et al., 1999; Hermans et al., 2012). Lake and Cressy describe three general approaches to *C. jejuni* control in poultry at the broiler farm level: biosecurity to reduce environmental exposure, increasing bird resistance to *C. jejuni* colonization and transmission, and the use of antimicrobials or antimicrobial alternative supplements (2013). As

previously mentioned, once *C. jejuni* enters a flock, it rapidly colonizes and spreads to virtually all birds within a house (Lindblom et al., 1986). *C. jejuni* is a microorganism naturally found in the farm environment within the soil, water, dust, contact surfaces, and the air (Ellis-Iversen et al., 2012). The pathogen has been isolated from feed, drinking water, farmers and farm visitors, wild birds, insect and rodent pests, equipment, and litter (Hakeem & Lu, 2021). In the United States, broiler house litter is frequently used for a year or more without replacement between flocks and plays a role in the perpetuation and transmission of *C. jejuni* from one flock to another (Montrose et al., 1985).

As *C. jejuni* colonization of a flock poses no financial repercussions, in terms of mortality or production losses to the farmer, prevention of infection at the farm level has proven incredibly difficult. Typical prevention measures a farmer can undertake are limited to good management practices and biosecurity while in grow out, and sanitation between flocks. Biosecurity is described as the combined hygiene practices put in place to minimize the risks posed by disease (Defra, 2021). Management practices essential to *C. jejuni* control include eliminating standing water, proper temperature maintenance, pest control, and stress minimization (Genigeorgis et al., 1986; Newell et al., 2011). Farm-level biosecurity measures include eliminating pest entry to broiler houses, minimizing human visits and guest entry to houses, feeder and drinker line sanitation between flocks, the addition of fresh litter or complete litter replacement between flocks, equipment sanitation, and PPE usage (Newell et al., 2011; Royden et al., 2021). Additionally, *C. jejuni* can be partially mitigated at the farm level using antibiotics, prebiotics, probiotics, synbiotics, enzymes, organic acids, mycotoxins, vaccines, essential oils, and aromatic plants (Abd El-Hack, 2022). These approaches have varying levels of efficacy and application in experimental and commercial applications, but many show promise as the commercial broiler

industry moves away from antimicrobial growth promoter usage due to increased legislation and changing consumer perception of antibiotic use in food animal production.

Pre-Harvest Feed Withdrawal and Transport

Feed withdrawal is a practice in broiler production during which birds are removed from feed for a period of time lasting 3-18 hours, immediately before processing, including catching and transportation time, done to decrease carcass contamination during commercial processing (Willis et al., 1996). The effects of feed withdrawal on *C. jejuni* in broilers are significantly increased crop and overall carcass contamination with the pathogen following feed withdrawal, compared to before feed withdrawal (Byrd et al., 1998). Transportation from the broiler farm to the processing plant represents a critical stress point in the lives of the birds and *C. jejuni* transmission. Stress-induced pathogen shedding coupled with the crowding of birds and usage of inadequately cleaned transport crates creates the perfect environment for *C. jejuni* spread and fecal contamination (Newell & Fearnley, 2003; Slader et al., 2002). A study by Clark and Bueschkens found that up to 70% of broilers that were *C. jejuni*- negative before transport became colonized following exposure to contaminated transport crates (1988). To reduce the incidence of *C. jejuni* shedding and spread during transportation, poultry overcrowding must be minimized, transport crates must be sanitized between uses, and stress must be minimized.

Processing

A bird entering the processing plant may harbor as many as 1 X 10^9 cells/g of *C. jejuni* in its cecal content (Stern et al., 2001). Control at the processing plant level provides an opportunity for *C. jejuni* reduction in poultry carcasses, particularly at stunning, scalding, de-feathering, evisceration, giblet harvesting, and chilling where the greatest shedding occurs (Altekruse et al., 1999). At stunning, fecal material is produced and can contaminate local skin and feathers in addition to other birds (Bryan & Doylez', 1995; Shane, 1992). The process of scalding causes the shedding of pathogens into the scald water where they have the potential to redistribute themselves onto other carcasses but reduces the viability of the pathogen (Berrang and Dickens, 2000). Scalding also opens up feather follicles, generating a new niche for *C. jejuni* to accumulate in and become trapped once chilling closes the feather follicles (Thomas & McMeekin, 1980). Upon de-feathering, the transfer of *C. jejuni* between birds is common with the transfer of organic and fecal material between carcasses passing through the pickers, leading to both direct and indirect contamination by pathogens (Bryan & Doylez', 1995; Shane, 1992). Evisceration is the most critical step in the cross-contamination of poultry carcasses. Here, the colonized gastrointestinal tract of a carcass is removed and any rupture or leakage facilitates contamination of the original carcass and others (Northcutt et al., 2003). Rapid immersion chilling in water offers another opportunity for pathogens to be dislodged from one carcass and redistributed to others or trapped within follicles (Demirok et al., 2013). Dipping typically results in cross-contamination of carcasses but this cross-contamination is reduced and managed via chlorination of the wash water (Bilgili et al., 2002; Demirok et al., 2013). Spray washing effectively removes visible fecal and organic material contamination from carcasses but aerosolizes any pathogens present, facilitating their spread (Keener et al., 2004). Antimicrobials

are used in poultry processing to minimize and eliminate the spread of pathogens between carcasses. Up to 5 log reduction of *C. jejuni* can be achieved experimentally using common antimicrobials including acidified sodium, chlorite, cetylpyridinium, chlorine, chlorine dioxide, peroxyacetic acid, and trisodium phosphate (Hakeem and Lu, 2021). These antimicrobials have markedly less efficacy in commercial use due to the presence of high quantities of lipids and proteins causing inactivation, pathogen evasion of antimicrobials, thermal inactivation or efficacy reduction of antimicrobials, low water quality, insufficient sanitation, and high organic material presence (Tresse et al., 2017).

Controlling *C. jejuni* contamination before and at the processing level is essential due to legislation in place by the USDA-FSIS dictating performance standards for *Campylobacter* contaminated poultry meat carcasses, parts, and comminuted meat. The performance standards state that 8 of 51, 1 of 52, and 4 of 52 broiler carcass, comminuted meat, and parts, respectively, may, at maximum, test positive for *Campylobacter* (USDA-FSIS, 2019).

Post-Harvest

Proper meat storage, hand washing following direct or indirect meat contact, effective temperature control during storage, and reduction of opportunities for cross-contamination all additionally play roles in the control of *C. jejuni* following processing. While heat processing and proper food preparation are by far the best means of *C. jejuni* elimination, controlling and reducing the colonization of pathogenic *C. jejuni* in the poultry intestinal tract is the most effective means of reducing the global incidence of *Campylobacter*iosis in humans who consume commercial poultry meat and poultry meat products.

Antibiotic Resistance of *Campylobacter jejuni*

Antibiotics

The World Health Organization has identified the zoonotic *C. jejuni* as a high priority pathogen on its list of antibiotic-resistant bacteria due to its resistance to fluoroquinolone, macrolides, and other antibiotic classes, while the CDC has labeled *C. jejuni* as a serious threat to public health due to its multi-drug resistant status and high level of annual human cases (CDC, 2019; WHO, 2018). An antibiotic compound is a therapeutic intervention targeting bacteria that are used to treat or prevent infections by either preventing bacterial reproduction or disrupting vital bacterial functioning or processes (Calhoun et al., 2022). The effects of antibiotics are, thus, either bacteriostatic or bactericidal. Since 1951, antibiotics, also known as antimicrobial growth promoters, have been used in the United States to treat as well as prophylactically prevent illness in poultry flocks and, additionally, as growth-promoting feed additive agents (Castanon, 2007; WHO, 2012). The rise of antibiotic resistance in pathogenic bacteria is a concern of global proportion and impacts humans, animals, and plants. The use of antibiotic growth promoters in food animal production has been a controversial topic in recent years. Antibiotics have been used in food animals for almost as long as they have been in people, but public perception on the matter began to change in the early 2000s, and mounting consumer pressure has since been applied to poultry producers and foodservice establishments alike, to produce food grown with fewer antibiotics of human health importance. In 2015, the FDA published Final Rule of the Veterinary Feed Directive went into effect and disallowed the use of antibiotics in humans and animals as growth-promoting supplements (FDA, 2015). The Final Rule additionally required that all antibiotics of human medical importance intended to be used in food animal agriculture must only be prescribed and overseen by a licensed veterinarian (FDA, 2015).

Human Disease

In human *Campylobacter*iosis infection, most cases do not require antibiotics, and the typically mild, self-limiting disease is most commonly managed with increased fluid intake (CDC, 2019). However, for humans with compromised immune systems, the young, and the elderly, clinical treatment with antibiotics might be required to control and eliminate the disease. Some *C. jejuni* have developed resistance to the antibiotics historically used to treat infection, making infections persist longer, increasing disease severity, and elevating the bacterium's public health threat. The increasing antibiotic resistance of *Campylobacter* has led to higher rates of treatment failures for the disease and an elevated threat level to the immunocompromised and others already at a greater risk of severe complications (Hussein et al., 2016). The estimated cost of treatment in the United States for multi-drug resistant bacteria was calculated to be approximately $20 billion in 2014, with drug-resistant *Campylobacter* infections directly incurring an estimated $270 million in medical costs each year (CDC, 2019; WHO, 2014).

Poultry Disease

Campylobacter is ever-present in poultry as a commensal element of the poultry gastroenteric microbiome (Ohishi et al., 2017; Zhao et al., 2010). One proposed route of antibiotic-resistant *Campylobacter* strain introduction to humans is via the consumption of poultry meat and poultry meat products that contain antibiotic-resistant *Campylobacter* strains (Sahin et al., 2015; Thakur et al., 2010; US Food and Drug Administration, 2014). This possibility of antibiotic-resistant bacteria transmission from poultry is significant due to the association of poultry as the most important source of human infection with *Campylobacter* (Altekruse & Tollefson, 2003; Paravisi et al., 2020). In human clinical cases of

*Campylobacter*iosis, macrolides such as erythromycin, and fluoroquinolone antimicrobials including ciprofloxacin are used as a broad-spectrum treatment of enteric pathogens, while systemic *Campylobacter* infection is more frequently treated with tetracyclines and gentamicin (Allos, 2001; Blaser & Engberg, 2008; Engberg et al., 2001). A study of chicken carcasses post-slaughter in Poland found that over 93% of *Campylobacter* isolates from poultry were resistant to at least one antibiotic class (Wieczorek et al., 2020). According to the CDC, the amount of *Campylobacter* with decreased susceptibility to ciprofloxacin has risen from 18% to 28% in just 20 years, while 4% of *Campylobacter* have decreased susceptibility to azithromycin, 29% have decreased susceptibility to ciprofloxacin or azithromycin, and 2% have decreased susceptibility to both ciprofloxacin and azithromycin, in the United States (CDC, 2019). The increase in the antibiotic resistance of *C. jejuni* human infections in recent years has been attributed to overuse and excessive application of antibiotics in animal husbandry practices and human medicine (Guévremont et al., 2006; Lévesque et al., 2007).

Antibiotic Resistance Mechanisms

C. jejuni uses a variety of antibiotic resistance mechanisms independently or in synergy to promote colonization and pathogenesis within a host, which may be chromosomal or plasmid-borne, including the four mechanisms described by Iovine (2013): antibiotic target alteration, antibiotic inactivation, decreased membrane permeability, and antimicrobial efflux pump expression. Antibiotics may be evaded by *Campylobacter* as point mutations naturally occur and accumulate during DNA replication and may effectively reduce antibiotic target site binding affinity and avidity, thus rendering an antibiotic ineffective (Lin et al., 2007; Ling et al., 2003; Vetting et al., 2011). When antibiotics are not present in a system, point mutations that alter

antibiotic target sites are not favorable to the bacteria; when antibiotics are present, point mutations can confer benefits to *Campylobacter* in the form of resistance, which can be passed on as the bacteria reproduce, eventually becoming a dominant mutation (Ling et al., 2003).

Antibiotic inactivation is often the result of the biotransformation of the antimicrobial compound within the bacteria itself (Norris & Serpersu, 2013). Biotransformation is described as a process through which a drug compound is converted from its original form to a new form, which may be more or less effective than the original (Zumstein & Helbling, 2019). Aminoglycosides are resisted by *Campylobacter* via modifications to the antibiotic by aminoglycoside-modifying enzymes AphA, AadE, and Sat, resulting in inactivation (Iovine, 2013; Lambert et al., 1985). Alteration of membrane permeability is one of the most common mechanisms of antimicrobial resistance which functions to prevent the diffusion of antimicrobial substances to the intracellular matrix of a bacteria (Pumbwe et al., 2004; Yang et al., 2019).

In *Campylobacter*, specifically, the expression of transmembrane proteins known as porins is modified to prevent the entry of antibiotics through pores of reduced size (Bolla et al., 1995; Labesse et al., 2001; Page et al., 1989). In *C. jejuni*, CmeABC multidrug-resistant efflux pump is critical for colonization of the human and animal intestine due to its mediation of bile salts and, additionally, is important, along with other efflux pumps, resistance to many antibiotic classes (Sharifi et al., 2021; Vieira et al., 2017). Efflux pumps are used by *Campylobacter* to purge aminoglycosides from the intracellular environment and prevent an antibiotic from reaching the genetic material of the bacteria (Mamelli et al., 2003). The efflux effect of these pumps is nonspecific to one antibiotic class, and can, therefore, act on multiple antibiotic classes effectively (Yao et al., 2016). In the case of beta-lactam antibiotics, efflux through CmeABC decreased membrane permeability, and antibiotic inactivation all play roles in resistance

to *Campylobacter* (Griggs et al., 2009; Lachance et al., 1993). Fluoroquinolone is resisted by *Campylobacter* via DNA gyrase target mutations to Thr-86-Ile; also Asp-90-Asn, Ala-70-Thr, and also via efflux through CmeABC (Endtz et al., 1991; Hooper, 2001; Iovine, 2013Unicomb et al., 2006). Mutations to 23s rRNA and ribosomal proteins L4 and L22, efflux through CmeABC, and decreased membrane permeability by *Campylobacter* are responsible for resistance to macrolides, the drug of choice in human *Campylobacter*iosis treatment (Cagliero et al., 2006; Iovine, 2013; Lin et al., 2007). Tetracycline resistance is the result of modifications to the target binding site by TetO, efflux through CmeABC, and decreased membrane permeability due to MOMP (Cagliero et al., 2006; Iovine et al., 2013; Lehtopolku et al., 2011; Page et al., 1989; Salyers et al., 1990). All of these factors come together to contribute to the rise of antibiotic resistance, multimodally, and highlight the importance of the evolving and threatening development of multi-drug resistant *Campylobacter*.

The prevention of emergent and control of present antibiotic-resistant *Campylobacter* strain infections must be addressed using a multifaceted approach. Prevention of drug resistance can only be achieved if the antibiotic application in both humans and animals is appropriate and specific, if on farm biosecurity practices are strenuously applied to manage *Campylobacter* transmission, and if effective disease prevention and minimization strategies at the farm level are implemented to reduce antibiotic need within poultry flocks.

Synbiotic Supplementation and *Campylobacter jejuni*

As antibiotic-resistance increases and antimicrobial usage decreases, alternatives to antibiotic growth promoters needed to be found that could act in a similar way to benefit poultry. Antibiotic growth promoters exert growth-promoting effects in challenged animals, reduce the

incidence of subclinical infection, decrease competition for nutrients within the gut microbiome, beneficially shift the intestinal microflora, strengthen the gut walls, improve nutrient absorption, and induce immune reactions (Brennan et al., 2003; Pedroso et al., 2006; Snyder and Wostmann, 1987; Teirlynck et al., 2009; Wise and Siragusa, 2007). One antibiotic replacement that has been used in poultry production extensively is synbiotics, due to their similar mode of action to antibiotic growth promoters. Synbiotics can be described as nutritional additives containing both probiotic(s) and prebiotic(s) which specifically favor the probiotic bacteria in a supplement, that act synergistically within a host (Schrezenmeir & de Verse, 2001). Probiotics are defined as live bacterial supplements which confer beneficial effects on a host and are designed to compete with pathogenic microbes in the gut (Fuller, 1989; Nava et al., 2005). Prebiotics are complex, non-viable, indigestible macromolecule additives that provide nutrients to and stimulate probiotic bacteria selectively (FAO, 2007; Gibson & Roberfroid, 1995; Hamasalim, 2016; Patterson & Burkholder, 2003). Without a prebiotic in the synbiotic supplement, the probiotic component does not survive passage through the intestinal system to successfully colonize the gut (Bhupinder & Saloni, 2010).

Antimicrobial Growth Promoter Alternatives

An antimicrobial growth promoter alterative should possess several essential attributes: it should effectively improve production performance, not be of importance to human or veterinary medicine, not confer adverse effects on the normal microbiome, not be associated with transmissible drug resistance or cause cross-resistance to drugs, not be capable of escaping the gut, not promote pathogenic shedding, not be mutagenic or carcinogenic, not pollute the environment, be biodegradable, and be non-toxic to both the host animal and human

administrators of the alternative supplement (Yadav et al., 2016). In broilers, an alternative must have the same beneficial conferences as an antibiotic growth promoter, including increased nutrient availability, improved overall health, and production performance parameter enhancement in the form of decreased feed conversion ratio and increased body weight gains (Huyghebaert *et al*., 2011; Seal *et al*., 2013). Perhaps the most critical requirement of an antibiotic growth promoter in broiler production, is that the cost of the supplement must not be superfluous to the benefits conveyed by the supplement, in the form of production performance enhancement: the supplement must be cost-effective to an integrator.

Prebiotics

According to Piniero et al. (2008), a prebiotic must meet several criteria: resistance to upper intestinal absorption, hydrolysis resistance, and specificity as substances only capable of supporting their intended bacterial targets. Prebiotics can be either derived from plants or synthesized by microorganisms (Gadde et al., 2017). The Compendium of Methods for the Microbiological Examination of Foods states that there are more than 50 prebiotic products currently in use in production agriculture in the United States (Salfinger & Tortorello, 2015). Prebiotics has been shown to convey to their host increased intestinal integrity of the tight junctions, improved production performance growth values, and improved gut microbiome composition (Gao et al., 2008; Roto et al., 2015; Zhang et al., 2005). The gut microbial population modification effects of prebiotics come in the form of interference with the pathogenic attachment to the gut wall and via the production of lactic acid to decrease gut pH (Mc Donald et al., 2002; Okumara et al., 1994). Prebiotics selectively enhance the proliferation and rate of growth of target microbes within the intestines to, additionally, alter gastrointestinal

microflora, stimulate the immune system, reduce pathogenic invasion, and ultimately reduce production costs with appropriate application (Cummings & Macfarlane, 2002; Ghiyasie al., 2007; Khksar et al., 2008; Peric et al., 2009). The immunological benefits of prebiotic supplementation include promotion of IgA secretion, mimicking of pathogenic attachment sites, destruction of pathogens, promotion of endocytosis, and modulation of cytokine and chemokine activity (Di Bartolomeo et al., 2013; Iji & Tivey, 1998; Nakamura et al., 2004). Common prebiotic supplements used in commercial poultry feed production include inulin, yeast, yeast cell walls, yeast metabolites, ß-glucans, mannan-oligosaccharides, and xylooligosaccharides (Patterson & Burkholder, 2003; Steiner, 2006). According to Swennen et al., an ideal prebiotic is one that is active at a low dose, lacks associated side effects, survives passage through the gut, is easily stored and is stable through processing, controls microflora, and is cost-effective (2006).

Probiotics

Probiotics offer an effective means of improving production parameters and reducing bacterial load, spread, and contamination in poultry by improving the animal's natural ability to ward off pathogenic bacteria via competitive inhibition (Cean et al., 2015; Nava et al., 2005). In poultry, probiotics are administered as live microorganisms included in the feed, as water additives, or as injections *in ovo*. Probiotics act by improving the natural gut microbial balance within poultry and therefore the animals' abilities to defend against pathogenic bacteria (Santini et al., 2010). Probiotic microorganisms are live, beneficial bacteria with a high affinity for attachment to the gastrointestinal mucosal wall that, additionally, produce inhibitory compounds and adjust to immune responses while living as non-pathogenic organisms within a chicken host (Willis & Reid, 2008). There exist around 130 approved commercial probiotic products

containing one or more probiotic strains in a culture (Salfinger & Tortorello, 2015). The probiotic mechanisms of action are: improved immune response, improved gastroenteric function, bacteriocin production, and improved gut homeostasis (Gaggia et al., 2010; Yang et al., 2009). An ideal probiotic must be non-pathogenic, tolerant of the acid and bile rich enteric environments through which it must pass to colonize in the gut, effective at adhering to the gut wall, have a short generation time, must survive processing and be shelf-stable, must be genetically stable, must produce lactic acid, and possess anti-genotoxic properties (Pandey et al., 2015). Probiotic mechanisms of action include competitive exclusion of pathogen colonization, acid production to lower intestinal pH, antagonistic activity towards pathogens, bile salt de-conjugation, digestive enzymatic activity, anti-mutagenic and anti-carcinogenic activity, suppression of ammonia production and urease activity, anti-cholesteremic activity, and mucosal immuno-modulation (De Rodas et al., 1996; Fox, 1988; Gilliland, 1990; Goldin & Gorbach, 1977; Juven et al., 1991; Martini et al., 1991; Mc Donald et al., 2002; Nahason et al., 1994; Sissons, 1989; Yeo & Kim, 1997). Benefits of probiotic supplementation include maintenance of gut homeostasis, pathogenic colonization inhibition, improved production performance, improved nutrient absorption, reduced circulating cholesterol levels, enhanced vaccine efficacy, mycotoxin detoxification, stress reduction, vitamin B synthesis, improved litter quality, elevated short-chain fatty acid production, no residues in poultry products, and decreased environmental pollution (Yadav et al., 2016). Commonly used probiotic microorganisms include *Bacillus, Lactobacillus, Enterococcus, Bifidobacterium, Escherichia, Streptococcus, Saccharomyces cerevisiae, Aspergillus oryzae, A. niger*, (EFSA, 2013; Joerger and Ganguly, 2017; Roberts et al., 2015; Ricke, 2015; Simmering & Blaut, 2001).

Synbiotics

The use of synbiotics depends on the concept of synergism, in which the interactions of the components generate a combined effect greater than the sum of the effects of the individual components. Synbiotics not only introduce beneficial bacteria to the gut, but they also selectively promote the proliferation of healthy gut microflora and modulate the avian gut microbiome (Yang et al., 2009). The development of synbiotics occurred as a means of ensuring probiotic survival in the upper intestinal tract and to ensure effective colonization on the intestinal walls to promote intestinal homeostasis and overall health (Pandey et al., 2015; Peña, 2007). In a synbiotic mixture, which does not come with a withdrawal period or produce residues like an antibiotic, the most common prebiotics included are oligosaccharides (fructo-oligosaccharide, galactooligosaccharides, and xylose-oligosaccharide) and inulin, while the most common probiotics used are *Lacbobacilli, Bifidobacteria*, *Saccharomyces*, and *Bacillus* (Pandey et al., 2015; Sharma et al., 2018). The synbiotic mechanism of action combines the modes of action of prebiotics and probiotics. To ultimately improve production performance, synbiotics are immunomodulatory, support the gastrointestinal tract, and stabilize the gastrointestinal tract (Sharma et al., 2018; Zhang et al., 2010). Gastrointestinal stabilization occurs as synbiotics decrease pathogenic presence, decrease inflammation, and free up more nutrients and energy sources for beneficial elements of the microflora (Kulkarni, 2013). Synbiotic support of the gastrointestinal tract occurs as bacterial translocation is decreased, thus decreasing the incidence of localized bacterial infections and septicemia, and as gut integrity increases in the form of increased villi height and crypt depth within the mucosa of the intestinal lining (Awad et al., 2009; Jung et al., 2008; Kulkarni, 2013; Sohail et al., 2012). Stimulation of the immune system occurs due to the modulatory effects of synbiotics on vaccine response, decreased incidence of

pathogens in the gut and of related disease, supported immune organ development, modulated interleukin secretion, environmental antigenic tolerance, enhanced production of protective cytokines, and promoted epithelial cell regeneration (Isolauri et al, 2001; Kulkarni, 2013; Szczypka et al., 2021; Żbikowski et al., 2020).

Advantages and Disadvantages of Synbiotics

Synbiotic supplementation has been shown to reduce *C. jejuni* colonization in broilers and to positively impact their gut microbiome composition (Baffoni et al., 2017; Śliżewska et al., 2020). During critical stress points of a broiler's life, including periods of heat stress, disease, and transportation, a synbiotic application has also been proven an effective antibiotic alternative that improves stress tolerance, immune function, body weight, feed conversion ratio, and physiological responses to stressors (Ghareeb & Bohm, 2009; Hu et al., 2022). Synbiotics have also been proven to improve mortality, body weight, average daily gain, feed efficiency, and carcass yield percentage, even more so than probiotic supplements in broilers (Ashayerizadeh *et al*., 2009; Awad et al., 2009). The potential benefits of synbiotic supplements as an antimicrobial growth promoter alternative are significant, but there do exist several critical drawbacks to synbiotic application that should be carefully considered by integrators before inclusion in commercial poultry production. Drawbacks include inconsistent performance impacts in broiler production, product inconsistency, cannot be used in combination with antibiotics, shelf life and stability concerns, and the threat of gut escape by the synbiotic components (Jung et al., 2008; Piewngam et al., 2018; Szlufman et al., 2021; Willis et al., 2007). These potential disadvantages can largely be mitigated and sometimes eliminated through good management and production

practices, biosecurity, and the selection of appropriate prebiotic and probiotic components to a synbiotic mixture.

As the poultry industry continuously moves away from the use of antibiotics and has eliminated the use of antibiotic growth promoters, synbiotic supplementation proposes an effective means of controlling pathogenic bacteria, improving production performance, and reducing the need for antibiotic usage in broilers.

PoultryStar® Me

Synbiotic Supplementation in Broilers

PoultryStar® is a synbiotic product produced by BIOMIN that is specific to poultry and composed of a combination of one prebiotic and multiple probiotic microorganism strains (*PoultryStar®*, n.d.). This synbiotic is comprised of inulin containing a high proportion of fructooligosaccharides (FOS), *Bifidobacterium animalis, Pediococcus acidilactici, Lactobacillus reuteri, Lactobacillus salivarius,* and *Enterococcus faecium.* The probiotic components of PoultryStar® were first isolated from the gastrointestinal tract of healthy hosts then intensively evaluated for safety, adaptability, and efficacy as an antimicrobial growth promoter alternative (*Poultrystar-probiotic*, n.d.). The primary prebiotic constituent of PoultryStar®, FOS, was chosen to selectively stimulate the growth, colonization, and proliferation of the probiotic strains within the synbiotic supplement (*Poultrystar-probiotic*, n.d.).

There exist three forms of PoultryStar® currently on the market, each with distinct applications. PoultryStar® is made in feed, water, and gel forms of application. The gel form of PoultryStar® is applied in the hatchery setting to promote bird growth and to establish a healthy gut microbiota in the early days of life and has been shown to improve foot pad dermatitis, blood

metabolites, cecal microbiome, and performance in poultry (Brugaletta et al., 2020). The water-soluble form of PoultryStar® is applied in drinking water to broilers in an effort to modulate the immune response to challenge, improve the gut microbiota composition, and reduce pathogenic colonization under challenge conditions (Ghareed et al., 2012; Markazi et al., 2018). The feed additive form of PoultryStar® is microencapsulated to survive processing at the feed mill and can be administered in pelleted or crumbled form to promote a healthy gut microbiome, to inhibit pathogenic colonization of the ceca and carcass, and to promote growth (*PoultryStar*, n.d.; Shanmugasundaram et al., 2019). PoultryStar® Me is created through the careful microencapsulation of the prebiotic and probiotic components of the feed additive. Microencapsulation protects the live bacteria from oxygen, heat, and environmental stresses during storage, feed formulation, and feed processing (Crittenden et al., 2006; Kailasapathy, 2002). Without microencapsulation, the probiotic bacteria of PoultryStar® ME would be unable to survive the high thermal processing temperatures required to pellet poultry feed (Wang et al., 2018). PoultryStar® can be effectively applied to broilers, broiler breeders, layers, and pullets of both purposes to promote immune, physiological development, microbiological, and production performance (Abdulhasan, 2018; Anwar et al., 2016; Wideman et al., 2012; Yan et al., 2019). The overall benefits of PoultryStar® as a nutritional supplement are summarized in *Figure 2.2.* and include the effective competitive exclusion of pathogens, microbiome modulation, performance enhancement, improved immune function, a reduction in mortality and morbidity, improved litter quality, and the lack of a withdrawal time prior to processing (Brugaletta et al., 2020; Shanmugasundaram et al., 2019; Valenzuela, 2019).

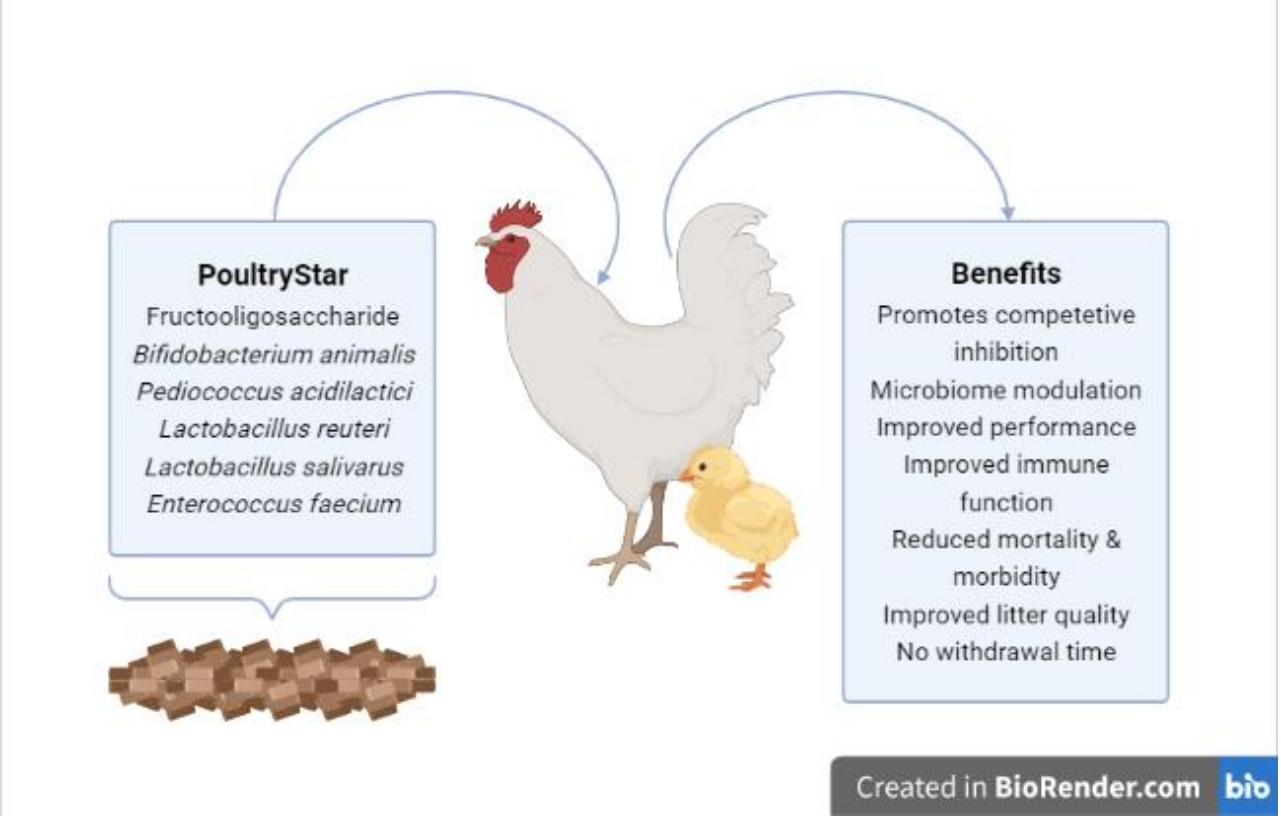

Figure 2.2. PoultryStar® Me composition and benefits to poultry. Created with BioRender.com.

Fructooligosaccharides

FOS is a nondigestible carbohydrate that constitutes the prebiotic component of PoultryStar® (Murphy, 2001). FOS is a naturally occurring oligosaccharide found in plants, made up of beta (2-1) linked fructose units in linear chain formation (Sabater-Molina et al., 2009). This thermally stable, slightly acidic (pH 4.0-7.0), water-soluble prebiotic is hygroscopic and possesses a high water-holding capacity (Mussatto & Manchilha, 2007; Yun, 1996). There are three critical aspects to the FOS as a prebiotic: FOS resists digestion, it is fermented selectively by the intestinal microbiota, and it selectively stimulates microbiota that promote a healthy gastrointestinal tract (Gibson et al., 2004). The mechanism of action for FOS relies upon a lowering of the luminal pH as short chain fatty acids (SCFA) are produced as a product of FOS

fermentation to inhibit pathogens, in addition to selective stimulation of beneficial bacteria within the intestines (Roberfroid, 1993; Wang & Gibson, 1993).

Within the intestinal microbiome, FOS produce a positive effect by reducing the incidence of gastrointestinal disease and infection through the probiotic effect (Flores-Maltos et al., 2014). The beneficial effect of FOS in PoultryStar® relies upon its use as a substrate for the beneficial microflora of the large intestine (Mao et al., 2018). When FOS is broken down by bacteria into SCFAs, the SCFAs work to positively influence host metabolism as they are absorbed by host tissues (Miller & Wolin, 1996). Additionally, FOS has been associated with the immunoregulation of IgA and IFN-γ, expression of small intestinal immunoglobulin receptors, and the development of gut-associated lymphoid tissue (Boehm et al., 2004; Roberfroid & Delzenne, 1998; Seifert & Watzl, 2007). LOS is specifically fermented by the probiotic lactobacilli strains and *B. animalis* within PoultryStar® which go on to competitively inhibit the colonization of pathogenic bacteria within the intestinal tract (Malaguarnera et al., 2007; Mao et al., 2018; Rousseau et al., 2005).

Bifidobacterium animalis

B. animalis is a gram-positive, rod-shaped, lactic acid producing, anaerobic, non-motile, and non-spore forming bacteria of the large intestine that is frequently isolated as a component of the healthy gastrointestinal tract microbiota (Jungersen et al., 2014; Juntunen et al, 2001; Reuter, 2001). *B. animalis* is an autochthonous species that is capable of adhering to and colonizing the gastrointestinal mucosal surfaces of poultry (Jungersen et al., 2014). *B. animalis* has been found to tolerate bile, gastric acid, and bile salts in the gastrointestinal system, important to its use as a probiotic species (Garrigues et al., 2005; Kim et al., 2009; Matsumoto et al., 2004). This

probiotic bacterium improves gut passage and digestion, has high immunomodulatory activity, enhances intestinal barrier function, and can inhibit pathogenic colonization. Araújo et al. found that *B. animalis* increases gut passage, reduces gastroenteric symptoms associated with enteric disease, and promotes healthy digestion (2022). Immunomodulatory activity occurs as *B. animalis* communicates with and effects the intestinal mucosal immune system (Ezendam et al., 2008; Kim et al., 2008). *B. animalis* induces dendritic cell maturation as well as chemokine and cytokine expression (Latvala et al., 2008; López et al., 2010; Oliveira et al, 2017). Enhancement of enteric barrier function by *B. animalis* occurs in the form of increased tight junction strength and colonization induced protection of the enteric epithelium (Collado et al., 2007a). Experimentally, *B. animalis*, in combination with a prebiotic, has been shown to have a protective effect inhibiting the growth of *Bacteroides vulgatus*, *Clostridium histolyticum*, *Enterobacter aerogenes C. jejuni, Bacillus cereus*, *Clostridium difficile*, *C. perfringens*, *E. coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Shigella flexneri*, *Shigella sonnei* and *Staphylococcus aureus* (Collado et al., 2007b; Matrins et al., 2009; Schoster et al., 2013). Microbiome modulation within the intestine by *B. animalis* occurs as this bacterium competitively inhibits the colonization of pathogenic or undesirable bacteria (Jungersen et al., 2014).

Pediococcus acidilactici

P. acidilactici is a gram-positive, facultatively anaerobic, pediocin antimicrobial peptide producing cocci, lactic acid bacteria which is commonly found in the gastrointestinal tract of healthy poultry (Bhunia et al., 1991; Carr et al., 2002; Daeschel & Klaenhammer, 1985). This probiotic bacterium is capable of colonizing the gastrointestinal tract due to strong affinity for

adhesion to the mucosal epithelium of the intestines and tolerates gastrointestinal passage well, in addition to a great tolerance to gastric acid, lysozyme, bile salt, low pH, high temperature, and osmotic pressure changes (Attri et al., 2015; Daeschel & Klaenhammer, 1985; Noohi et al., 2016; Sarkar et al., 2020). *P. acidilactici* supplementation has been found to improve egg weight, eggshell thickness, eggshell relative weight, egg specific gravity, egg yolk fatty acid composition, and FCR while decreasing the quantity of broken eggs, shell-less eggs, downgraded eggs, and egg yolk cholesterol content in laying hens (Mikulski et al., 2012; Mikulski et al., 2020). In broilers, *P. acidilactici* supplementation promotes body weight gain and visceral organ development (Alkhalf et al., 2010; Yu et al., 2020). *P. acidilactici* also works within the intestinal tract as an immune modulator (Jha et al., 2020). The bacterium induces IL-10 producing T regulatory type 1 cells, TNFa, IELs, goblet cells, circulatory neutrophil counts, and monocyte levels, and antioxidant activity including phenols, ascorbic acid, catechol, and dipeptidyl peptidase-III (Attri et al., 2015; Castex et al., 2010; Standen et al., 2013; Takata et al., 2011). *S*upplementation of *P. acidilactici* has been concluded to decrease *Eimeria tenella* and *Eimeria acervulina* fecal oocyte shedding, and to competitively inhibit or express antibacterial activity against *S. enteritidis*, *Salmonella enterica*, *E. coli*, *L. monocytogenes*, *Shigella sonnei*, *Klebsiella oxytoca*, *Enterobacter cloaca*, *Streptococcus pyrogenes*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus bifermentans*, *Leuconostoc mesenteroides*, and *C. difficile* (Abbasiliasi et al., 2017; Gonzalez & Kunka, 1987; Lee et al., 2007; Nielsen et al., 1990; Noohi et al., 2016).

Lactobacillus reuteri

Lactobacillus spp. are widely used probiotic strains naturally found in the small intestinal tract that produces antimicrobial molecules, inhibits pathogen colonization, remodels the commensal microbiome composition, modulates the host immune system, and strengthens the host intestinal barrier (Mu et al., 2018; Sinkiewicz, 2010). *L. reuteri* is an anaerobic, heterofermentative, gram-positive, rod-shaped lactic acid bacteria that produces reuterin, an antimicrobial substance potent to a wide range of bacteria (Axelsson et al., 1989; Gänzle et al., 2000; Hou et al., 2015; Spinler et al., 2008). This probiotic can tolerate acid and bile salts, inhibits pathogenic bacteria, can adhere to the intestinal wall, secretes β-glucanase, is immunomodulatory, decreases digesta viscosity, and improves production performance in broilers (Hou et al., 2015; Nakphaichit et al., 2011; Yu et al., 2007; Yu et al., 2008). *L. reuteri* produces lactic acid, acetic acid, ethanol, and reutericyclin as antimicrobial molecules to inhibit pathogenic colonization of the intestinal epithelium (Burge et al., 2015; Ganzle & Vogel, 2003; Gopi et al., 2015; Jacobsen et al., 1999). Additionally, *L. reuteri* regulates the gastrointestinal microbiome through competitive exclusion, pH modulation, lactic acid production, and the secretion of antimicrobial and antiviral metabolites (Ang et al., 2016; Jones & Versalovic, 2009; Yu et al., 2008). Probiotic *L. reuteri* influences the host immune system by decreasing pro-inflammatory cytokine production, promotes T cell development, increases antioxidant activity, and modulates LPS-induced inflammation and TNF production (Jones & Versalovic, 2009; Liu et al., 2008; Lin et al., 2010; Tzang et al., 2017; Yu et al., 2008). Within the intensinal epithelium, *L. reuteri* was additionally found to upregulate expression of tight junction proteins (Mu et al., 2017). *S. enteritidis*, *C. jejuni*, *E. coli*, and *Cryptosporidium parvum* colonization of

the intestine is resisted by *L. reuteri* (Casas & Dobrogosz, 2000; Edens et al., 1997; Nakphaichit et al., 2019; Śmiałek et al., 2021).

Lactobacillus salivarius

Like *L. reuteri*, *L. salivarius* is a rod-shaped, gram-positive, facultatively anaerobic, lactic acid bacterium demonstrating bacteriocin and probiotic activity (Chapot-Chartier et al., 2014). This probiotic strain thrives in the gastrointestinal tract and is capable of pathogenic bacterial suppression via bacteriocin production, much like *L. reuteri* (Messaoudi et al., 2013; Neville & O'Toole, 2010). *L. salivarius* has proven abilities to modulate the microbiome of the gastrointestinal tract, produces antimicrobial bacteriocins, stimulates the immune system, and acidifies the gut beneficially via SCFA production (Messaoudi et al., 2012; Messaoudi et al., 2013). This lactic acid bacterium resists acid and bile, adheres to intestinal cells (Corr et al., 2007; Messaoudi et al., 2012). *L. salivarius* modulates the intestinal barrier by increasing mucin secretion to block pathogenic adherence, upregulates tight junction expression, trigger heat shock protein synthesis, and stabilize the cytoskeleton of immune cells (Klingberg et al., 2005; Lavoie et al., 1993; Mack et al., 2003; Malago et al., 2010). *L. salivarius* modulates the immune system by reducing β-glucosidase and β-glucuronidase, improving intestinal histmorphology, decreasing TLR2 and TLR4, improving nitric oxide production, and by inducing IL-10, IL-6, IL-12, natural killer cells, β-defensin 2, monocytes, and anti-inflammatory cytokines (Messaoudi et al., 2012; O'Flaherty and Klaenhammer, 2009; Peran et al., 2005; Pérez-Cano et al., 2010; Sierra et al., 2010; Shokryazdan et al., 2017; Sun et al., 2020; Zhang et al., 2011). Probiotic application of *L. salivarius* in poultry has been shown to improve egg production and reduce egg yolk cholesterol content (Kalsum et al., 2012). Reduction of *P. aeruginosa*, *C. jejuni*, *Helicobacter pylori*, *E. coli*,

L. monocytogenes, *C. perfringens*, and *Salmonella* Enteritidis colonization of the poultry gastrointestinal tract is resultant of *L. salivarius* supplementation (Aiba et al., 1998; Ayala et al. 2017; Corr et al., 2007; Kizerwetter-Świda & Binek, 2009; Messaoudi et al., 2012; Pascual et al., 1999; Saint-Cyr et al., 2017).

Enterococcus faecium

E. faecium is a gram-positive, facultative anaerobic, coccoid shaped, non-hemolytic, lactic acid bacterium considered to be both a commensal component of the human digestive tract and a multi-drug resistant pathogens capable of generating opportunistic infections in humans (Bhardwaj et al., 2010; Ryan & Ray, 2003; Top et al., 2008). While it possesses the potential to be opportunistically pathogenic, *E. faecium* is commonly used in animal production agriculture and in food preparations (Bhardwaj et al., 2010; Top et al., 2008). This probiotic survives gastrointestinal transit and adheres to the intestinal wall of its host where it has been proven to stimulate the adhesion and colonization of other lactic acid bacteria, increase villus height and crypt depth, and modulates the gut microbiome (He et al., 2021; Lund et al., 2002; Scharek et al., 2005; Vahjen et al., 2002). *E. faecium* is thermal stress resistant, acid resistant, and non-cytotoxic to epithelial cells (He et al., 2021). Immunologically, *E. faecium* reduces IgG levels and cytotoxic T-cell expression, increases immune cell proliferation, activates macrophages, stimulates IL-6, IL-4 and TNF-α production, improves IgA antibody production following vaccination, stimulates immune organ development, downregulates mucin-2, decreases inflammation and oxygen stress, (Beirão et al., 2018; Johansson et al., 2011; Royan, 2018; Scharek et al., 2005; Sim et al., 2018). Broiler performance is enhanced by *E. faecium* supplementation in the form of improved FCR and weight gains (Awad et al., 2009; Samli et al.,

2007). Additionally, meat quality is enhanced supplementation with this probiotic in the form of increased meat color, water holding capacity, and pH of the breast muscle and decreased abdominal fat (Zheng et al., 2014). *E. faecium* supplementation has been found to be an effective control against *C. jejuni*, *Salmonella* Enteritidis, *Salmonella* Pullorum, *Salmonella* Typhimurium, and *E. coli* infection of the gastrointestinal tract (Audisio et al., 2000; Carter et al., 2017; Letnická et al., 2017; Scharek et al., 2005; Sim et al., 2018).

PoultryStar® is a synbiotic product with a wide range of uses including against pathogens, to promote growth, and to mitigate the negative effects of stress in poultry. The PoultryStar® mode of action is multi-faceted and relies on the inherent synergistic nature of synbiotics. The probiotic bacteria work to effectively colonize the intestinal tract of poultry via competitive exclusion while the prebiotic stimulates the probiotics by providing a selective nutrient source (*PoultryStar*, n.d.). The synbiotic has been proven effective against many common pathogens of poultry, including *Salmonella*, *Campylobacter*, *E. coli*, and *Clostridium perfringens*, within the ceca, intestinal tracts, and whole carcass (Ghareeb et al., 2012; Huff et al., 2015; Markazi et al., 2018; Mora et al., 2020; Shanmugasundaram et al., 2020). PoultryStar® application has also been shown to mitigate the effects of temporary and prolonged rearing under heat, cold, and transportation stress conditions (Huff et al., 2015; Mohammed et al., 2018; Mohammed et al., 2021). Early use of antibiotics in chicks has been shown to negatively impact immune system development but PoultryStar® application during and following antibiotic withdrawal has been shown to improve immune development and growth (Valenzuela, 2019). In pullets and broilers, PoultryStar® improves performance, uniformity, and intestinal microbiota composition while in layers and breeders, it improves egg productivity and quality (*PoultryStar*, n.d.).

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CHAPTER 3

IMMUNE RESPONSES, GUT PERMEABILITY, AND CECAL *CAMPYLOBACTER JEJUNI* LOADS IN AN EXPERIMENTAL *C. JEJUNI* CHALLENGE MODEL IN BROILERS.[1]

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[1]Cason E.E., Al Hakeem W.G., Adams D., Shanmugasundaram R., Selvaraj R. To be submitted to The Journal of Poultry Science.

Abstract

Campylobacter jejuni is a foodborne pathogen that colonizes the ceca and disseminates from the gut to the internal organs by compromising gut integrity. This study aimed to characterize the host immune response of broilers and broiler cecal *C. jejuni* loads to establish an experimental infection model by determining an effective dose of *C. jejuni* that will colonize the poultry ceca at 35 days of age. A total of 90 broiler chicks were randomly divided into three experimental groups in six replications. The three experimental groups were 0, 1 X 10^4, and 1 X 10^8 CFU/bird *C. jejuni*. At 21 d of age, birds were orally inoculated with PBS (control) or 1 X 10^4 or 1 X 10^8 CFU/bird of *C. jejuni*. Feed intake and body weight were measured weekly. On 3 and 7 dpi, gut permeability was measured by FITC-dextran assay. On 3, 7, and 14 dpi, *C. jejuni* loads in cecal content were quantified by plating, bile anti-*C. jejuni* IgA concentration was measured, and cecal tonsil $CD4^+$:$CD8^+$ cell ratio, immune gene, and tight junction protein expression were quantified by flow cytometry and qPCR. Data were statistically analyzed using JMP Pro 15, by One Way ANOVA, followed by Tukey's test. There were no significant differences ($P > 0.05$) in BWG and FCR between the treatment groups at any of the time points studied. Birds inoculated with 1 X 10^4 and 1 X 10^8 CFU of *C. jejuni* had significantly increased ($P < 0.05$) *C. jejuni* loads in the ceca compared to the control group, respectively, which had no detectable *C. jejuni* load. At 7 dpi, the $CD4^+$:$CD8^+$ cell ratio was ($P < 0.05$) higher in the 1 X 10^8 CFU of *C. jejuni* group than that in the 0 and 1 X 10^4 CFU/bird *C. jejuni* groups. There were no significant differences ($P > 0.05$) in serum FITC-d concentration or bile anti-*C. jejuni* IgA concentration between the treatment groups at any of the time points studied. Tight junction proteins were downregulated, and T cell differentiation genes were upregulated in the cecal tonsils at 3 dpi in the challenged groups ($P < 0.05$) while both pro- and anti-inflammatory

cytokines were upregulated at 3, 7, and 14 dpi in the challenged groups ($P < 0.05$). This study found that 1 X 10^8 CFU/bird *C. jejuni* challenge produced greater cecal colonization and increased the $CD4^+$:$CD8^+$ cell ratio, compared to the Control and 1 X 10^4 CFU/bird *C. jejuni* groups, at 35 days of age. It can be concluded that 1 X 10^8 CFU/bird *C. jejuni* inoculation at 21 days of age colonized the ceca at market age and induced host immune responses.

Introduction

Campylobacter jejuni is the most reported bacterial cause of foodborne illness in the United States each year (CDC, 2021). Poultry are considered a natural biological reservoir of *C. jejuni* and is among the most frequently cited sources of human infection (Altekruse et al., 1999). *C. jejuni* is capable of surviving at all levels of poultry production and processing, indicating many environmental niches within the poultry industry. Gastrointestinal tract microbial populations play a crucial role in digestion, productivity, pathogenicity, disease susceptibility, and the overall health of poultry. It is probable that up to 80% of broiler flocks are positive for *Campylobacter* worldwide, though there is great variation regionally and between countries (Hermans et al., 2012). In the United States, the Food Safety and Inspection Service estimates that 46.7% of young chickens have *C. jejuni* infections (USDA FSIS, 2009).

Colonization by *C. jejuni* in poultry initially occurs in the gastrointestinal tract of the birds, with the greatest pathogenic load concentrated in the ceca (Mortada et al., 2021). In the gastrointestinal tract, *C. jejuni* increases intestinal paracellular permeability by destroying the integrity of and the tight junctions between intestinal epithelial cells, to facilitate its escape and dissemination to other organs of the body, such as the liver and spleen (Awad et al., 2020; Knudsen et al., 2006). Challenging chicks with *Campylobacter* leads to ineffective activation of

the developing immune system and reduced antimicrobial peptide gene expression (Hermans et al., 2012). Once colonized with *C. jejuni*, poultry never clear the infection and will continue to shed the pathogen for the rest of their lives, especially during critical stress periods including during feed withdrawal, transportation, temperature changes, population density changes, handling, and instances of illness (Achen et al., 1998; Whyte et al., 2001). Chickens colonized by *C. jejuni* typically display no clinical signs and experience no marked productivity losses associated with the disease (Cean et al., 2015).

Because of the difficult nature of working with *C. jejuni* in animal models due to its complicated, narrow growth condition requirements, and difficulty to isolate from internal organs following passage through poultry, we lack an effective challenge model for *C. jejuni* infection (Koenraad et al., 1997; Konkel et al., 2007; Mortada et al., 2021; Rautelin et al., 1999). Past broiler challenge models have noted conflicting cecal colonization, inconsistent effects on performance and immunological parameters, age and dose dependent interactions on colonization, and inconsistent shedding and transmission patterns with varying challenge dosages at various challenge application time points (Achen et al., 1998; Awad et al., 2016; Awad et al., 2020; Beery et al., 1988; Connerton et al., 2018; Dhillon et al., 2006). This experiment seeks to evaluate the effects of two common challenge dosages administered at 21 days of age, following the dissipation of maternally derived protective antibodies against *C. jejuni*, on the immunological and microbiological implications of *C. jejuni* challenge in broilers (Sahin et al., 2003).

The best way to prevent drug resistant cases of human campylobacteriosis is to control infection at the broiler live production level. Due to increased antimicrobial resistance and multi-drug resistance of *Campylobacter*, understanding the pathogenesis of *C. jejuni* within poultry and

the immune response of chickens challenged with *C. jejuni* is critical to the establishment of a challenge model in broilers. An effective challenge model in broilers is useful in the generation of hurdles and for experimentation with antimicrobial growth promoter alternatives important to poultry and human health against foodborne pathogens.

Materials and Methods

Birds, diets, and *C. jejuni* challenge: 90 day-old male Cobb by-product breeder chicks were randomly assigned to three treatment groups, 0, 1 X 10^4, and 1 X 10^8 CFU/bird *C. jejuni*, with 5 birds per pen and six floor pens per treatment (n = 6). Birds in challenged groups received 1 X 10^4 or 1 X 10^8 CFU/bird of *C. jejuni* or mock challenge with 1 mL of PBS via oral gavage at 21 days of age. Throughout the trial, days 0-35, all birds were fed a corn and soybean-meal based starter diet (*Table 3.1*). One bird per cage was euthanized by cervical dislocation and ceca, cecal tonsil, spleen, liver, blood, and bile were collected on 3, 7, and 14 dpi for microbiological and immunological parameters. Body weight and feed intake were recorded weekly, and body weight gain and feed conversion ratio were analyzed. The FCR was corrected to account for bird mortality over time.

Basal Diet	
Ingredient	**%**
Corn	58.47
Soybean meal	35.15
Soybean oil	2.269

Limestone	1.593
Biofos	1.387
NaCl	0.35
Vitamins premix[1]	0.35
Dl-methionine	0.21
Lysine HCL	0.137
Trace mineral premix[2]	0.08
Total	100
Calculated Nutrient Composition	
ME, kcal/kg	3050
Crude protein, %	21.44
Crude fat, %	4.55
Lysine, %	1.31
Calcium, %	0.95
TSAA, %	0.91
Threonine, %	0.87

Methionine, %	0.56
Available phosphorus, %	0.45

[1] Vitamin mix provided the following per kg of diet: 2.4 mg thiamin-mononitrate, 44 mg nicotinic acid, 4.4 mg riboflavin, 12 mg D-Ca pantothenate, 12 g vitamin B12, 2.7 mg pyridoxine-HCl, 0.11 mg D-biotin, 0.55 mg folic acid, 3.34 mg menadione sodium bisulfate complex, 220 mg choline chloride, 1,100 IU cholecalciferol, 2,500 IU trans-reinyl acetate, 11 IU all-rac-tocopherol acetate, and 150 mg ethoxyquin

[2] Trace mineral mix provided the following per kg of diet: 101 mg $MnSO_4.H_2O$, 20 mg $FeSO_4.7H_2O$, 80 mg Zn, 3 mg $CuSO_4.5H_2O$, 0.75 mg ethylene diamine dihydroiodide, 20 mg MgO, and 0.3 mg sodium selenite.

Table 3.1. Basal diet with ingredients and nutrient composition calculations.

***Campylobacter jejuni* challenge:** *C. jejuni* (strain 33560) was cultivated on Campy-CEFEX agar supplemented with Park and Sanders (Sigma-Aldrich, St. Louis, MO) and Remel laked horse blood (Thermo Fisher Scientific, Lenexa, KS) and incubated for 48 hours in a microaerobic atmosphere (85% N_2, 10% CO_2, and 5% O_2) at 42°C. Following incubation, bacterial cells were collected and resuspended in PBS to create a suspension with an optical density of 0.27 at 540 nm with a Spec-20 (Milton-Roy Spectrophotometer, Thermo Spectronics, Madison, WI). An optical density value of 0.27 is equivalent to 1 X 10^8 CFU/mL of *C. jejuni* (Cosby et al., 2017). The challenge stock was verified utilizing serial dilutions and direct plating on Campy-CEFEX. Prior to challenge, the *C. jejuni* was evaluated via dark field microscopy to evaluate cork-screw motility, indicative of viability and essential for colonization to occur.

Effect of *Campylobacter jejuni* challenge on *Campylobacter* load in the ceca: On 3, 7, and 14 dpi, whole ceca tissue and digesta samples were excised and collected in stomacher bags, then

stored on ice. The samples were macerated and 3 X (weight/volume) BPW was added to each sample. Samples were stomached for 60 seconds until homogenized. Samples were diluted and a plated at a volume of 10 µl/plate, on campy-CEFEX agar supplemented with Park and Sanders (Sigma-Aldrich, St. Louis, MO) and Remel laked horse blood (Thermo Fisher Scientific, Lenexa, KS). Plates were incubated under microaerobic conditions (85% N_2, 10% CO_2, and 5% O_2) at 42°C for 48 hours then colonies were enumerated. *C. jejuni* CFUs were confirmed by microscopic observation of characteristic cork-screw motility and further confirmed using SyBr green qPCR using primers directed at the *C. jejuni mapA* gene sequence. Enumeration data was recorded as CFU/g then converted to $\log_{10}$ CFU/g for statistical evaluation.

Effect of *Campylobacter jejuni* challenge on *Campylobacter jejuni mapA* gene in the ceca: On 3, 7, and 14 dpi, ceca were excised and collected in stomacher bags, then stored on ice. Whole ceca samples were macerated and 3 X (weight/volume) BPW was added to each sample. Samples were stomached for 60 seconds until homogenized. Bacterial DNA was extracted from the whole ceca samples and quantified using qPCR with SyBr green, and primers directed at the *C. jejuni mapA* gene sequence. Quantified data was reported as 40-Ct and statistically evaluated. *mapA* gene is specific to *C. jejuni* and differentiates it from *Campylobacter coli*.

Effect of *Campylobacter jejuni* challenge on FITC-d permeability through the gastrointestinal tract: On 3 and 7 dpi, one bird per pen was administered 2.2 mg/mL mw 4,000 fluorescein isothiocyanate-dextran (FITC-d) (Sigma-Aldrich, St. Louis, MO) via oral gavage of 1 mL/bird. After 2 hours, blood was collected directly from the heart and stored on ice in the dark prior to transport to the laboratory. Tubes were centrifuged at 1,500 X g for 12 minutes to allow serum collection. A standard curve was prepared and plated then 100 µl from each sample was plated on black 96 well plates (Krystal Biotech, Pittsburgh, PA) in triplicate. FITC-d was

measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using an Epoch microplate spectrophotometer (BioTek, VT, USA), and 485/528 values recorded. The FITC-d concentration in the serum samples was determined using the equation derived from the standard curve of serially diluted FITC-d Vs. 485/528 values.

Effect of *Campylobacter jejuni* challenge on bile IgA anti-*Campylobacter jejuni* antibodies: On 3, 7, and 14 dpi, bile was collected and stored at -20°C until analysis. Specific anti-*C. jejuni* IgA antibodies were measured via enzyme-linked immunosorbent assay (ELISA). Whole cell *C. jejuni* antigens were generated by lysing 1 X 10^9 CFU/mL of *C. jejuni* through seven cycles of bead beating (TissueLyser LT, Qiagen, Germantown, MD), freezing, and thawing. ELISA plates (Nunc Maxisorp™, ThermoFisher Scientific, Waltham, MA) were coated in 10 µg/ml of *C. jejuni* antigen, diluted in coating buffer (carbonate/bicarbonate, pH 9.6). Bile samples were diluted to 1:800 in PBS containing 8 % non-fat dry milk and 0.1 % Tween-20 (VWR, Radnor, PA). HRP-conjugated polyclonal goat anti-chicken IgA (SouthernBiotech, Birmingham, AL) was added to each well at 1:100,000 as a secondary antibody. 3, 3, 5, 5-tetramethylbenzidine (Ken-En-Tec, Taastrup, DK) was added at 100 µL/well and incubated for 10 minutes, prior to halting the reaction using HCl. Absorbance was measured at 450 nm using Epoch microplate spectrophotometer (BioTek, VT, USA) and antibody levels were reported as OD 450 values.

Effect of *Campylobacter jejuni* challenge on cecal tonsil CD4+ and CD8+ T lymphocytes and CD4+:CD8+ cell ratio: On 3, 7, and 14 dpi, cecal tonsil tissues were excised and collected in 2 mL tubes filled with 1.5 mL of incomplete RPMI media then stored on ice. A flow cytometer was used to analyze the samples for $CD4^+$ and $CD8^+$ cells. Single-cell suspensions of the cecal tonsils (1 X 10^6 cells) were generated via straining and incubated with PE-conjugated mouse anti-chicken CD4 and FITC-conjugated mouse anti-chicken CD8 (Southern Biotech,

Birmingham, AL) at 1:250 and 1:313 dilutions, respectively, and unlabeled mouse IgG at 1:100 dilution in a 96-well plate for 20 minutes at 4°C. Following incubation, cells were washed three times by centrifugation at 400 X g for 5 minutes to remove unbound antibodies using a wash buffer (1 X PBS, 2 mM EDTA, 1.5% FBS). Following washing, CytoSoft software (Guava Easycyte, Millipore, Billerica, MA) was used to analyze the cells, and $CD4^+$ and $CD8^+$ cells were reported as percentage of gated cells and $CD4^+$:$CD8^+$ cell ratios calculated.

Effect of *Campylobacter jejuni* challenge on immune gene expression: On 3, 7, and 14 dpi, cecal tonsil tissues were excised and collected in 2 mL tubes filled with 1.5 mL of RNAlater (Qiagen, Germantown, MD). Samples were stored on a desktop at room temperature for 7 days until RNAlater thoroughly permeated the tissue samples and stabilized the RNA. After seven days, excess RNAlater was discarded and samples were stored at -80°C until total RNA was extracted. Following total RNA extraction from the samples, the RNA was converted via reverse transcription to cDNA then stored at -20°C until analysis. The cDNA was analyzed for IL-10, TGF-β, TLR-4, IL-1β, LITAF, iNOS, K60, IL-6, IL-4, CLAU-2, and ZO-1 by qPCR (CFX96 Touch Real Time System, BioRad) using SyBr green after normalization using GAPDH (*Table 3.2*). Fold change from the housekeeping gene was calculated using $2^{(\text{Ct Sample—Housekeeping})}/2^{(\text{Ct Reference—Housekeeping})}$. Ct, representing the threshold cycle, was determined using the iQ5 software (Biorad) when fluorescence rose exponentially 2-fold above the background.

Primers			
Target Gene	**Sequence (5'-3')**	**Annealing Temperature**	**Reference**
IL-10 F	CATGCTGCTGGGCCTGAA	58.0 °C	Rothwell et al., 2004

IL-10 R	CGTCTCCTTGATCTGCTTGATG		
TGF-β F TGF-β R	CATACTCCTGGGTCTGGTTGGT GACAGCCATCCGCATCTTCT	58.0 °C	Kogut and Arsenault, 2016
TLR-4 F TLR-4 R	ACCTACCCATCGGACACTTG TGCCTGAGAGGTCAGGTT	60.0 °C	Shanmugasundaram and Selvaraj, 2011
IL-1β F IL-1β R	GCATCAAGGGCTACAAGCTC CAGGCGGTAGAAGATGAAGC	58.0 °C	Selvaraj et al., 2010
LITAF F LITAF R	ATCCTCACCCCTACCCTGTC GGCGGTCATAGAACAGCACT	58.0 °C	Markazi et al., 2018
iNOS F iNOS R	AGTGGTATGCTCTGCCTGCT CCAGTCCCATTCTTCTTCC	60.0 °C	Selvaraj and Klasing, 2006
K60 F K60 R	ATTTCCTCCTGCCTCCTACA GTGACTGGCAAAAATGACTCC	55.0 °C	Hong et al., 2006
IL-6 F IL-6 R	CAAGGTGACGGAGGAGGAC TGGCGAGGAGGGATTTCT	57.5 °C	Hong et al., 2012
IL-4 F IL-4 R	AACATGCGTCAGCTCCTGAAT TCTGCTAGGAACTTCTCCATTGAA	57.5 °C	Renu et al., 2020

CLAU-2 F CLAU-2 R	CCTGCTCACCCTCATTGGAG GCTGAACTCACTCTTGGGCT	55.0 °C	Chen et al., 2017
ZO-1 F ZO-1 R	TGTAGCCACAGCAAGAGGTG CTGGAATGGCTCCTTGTGGT	55.0 °C	Oxford and Selvaraj, 2017
GAPDH F GAPDH R	TCCTGTGACTTCAATGGTGA CACAACACGGTTGCTGTATC	55.0 °C	Dube et al., 2014
mapA F *mapA* R	CTGGTGGTTTTGAAGCAAAGATT CAATACCAGTGTCTAAAGTGCGTTTAT	55.0 °C	Best et al., 2003

Table 3.2. RTqPCR primers and conditions.

Statistical Analysis: Data were statistically analyzed using JMP Pro 15, by One Way ANOVA, followed by Tukey's test. The significance level was set at ($P < 0.05$). Floor pen was used as the experimental unit ($n = 6$).

Results

Effect of *Campylobacter jejuni* challenge dosage on production performance

There were no significant differences in FCR, BWG, or FI between the treatment groups ($P > 0.05$) (*Table 3.3*).

Parameter	**Control**	**1 X 10^4 CFU *C. jejuni***	**1 X 10^8 CFU *C. jejuni***	**SEM**	***P* value**

Body Weight Gain (kg)					
0-7	0.10	0.09	0.10	0.01	0.95
7-14	0.21	0.21	0.18	0.01	0.15
14-21	0.36	0.35	0.31	0.02	0.11
21-28	0.56	0.59	0.55	0.03	0.54
28-35	0.58	0.62	0.54	0.04	0.43
0-35	1.80	1.87	1.68	0.08	0.23
Feed Intake (kg)					
0-7	0.22	0.20	0.22	0.01	0.24
7-14	0.44	0.44	0.42	0.03	0.85
14-21	0.67	0.64	0.59	0.05	0.46
21-28	0.91	0.97	0.84	0.06	0.38
28-35	1.01	1.12	0.98	0.04	0.10
0-35	2.60	2.73	2.41	0.11	0.17
Feed Conversion Ratio					
0-7	2.34	2.17	2.44	0.19	0.60
7-14	2.13	2.11	2.36	0.17	0.50
14-21	1.88	1.82	1.89	0.15	0.93
21-28	1.64	1.67	1.54	0.12	0.76
28-35	1.78	1.97	1.85	0.23	0.85
0-35	1.44	1.50	1.44	0.10	0.91

Table 3.3. Effect of *Campylobacter jejuni* challenge dosage on production performance: Day old chicks were distributed into three treatment groups: Control, 1 X 10^4 CFU *Campylobacter jejuni*,

and 1 X 10^8 CFU *C. jejuni* (n = 6). Birds in challenge groups received 1 X 10^4 and 1 X 10^8 CFU/bird of *C. jejuni* or mock challenge with 1 mL of PBS via oral gavage. Body weight and feed intake were measured on days 0, 7, 14, 21, 28, and 35 to calculate body weight gain (BWG) and feed conversion ratio (FCR). Means with no common superscript differ significantly ($P < 0.05$).

Effect of *Campylobacter jejuni* challenge on *Campylobacter* load in the ceca.

At 3 dpi, the 1 X 10^4 and 1 X 10^8 CFU groups had significantly higher *Campylobacter* load in the ceca by 5.08 and 5.17 $\log_{10}$ CFU/mL, respectively, when compared to the Control group (*Figure 3.1*). There was a significant increase at 7 dpi in cecal *Campylobacter* load of 5.69 and 6.02 $\log_{10}$ CFU/mL in the 1 X 10^4 and 1 X 10^8 groups, respectively, when compared to the Control group. At 14 dpi, the 1 X 10^4 and 1 X 10^8 CFU groups had significantly higher *Campylobacter* load in the ceca by 4.84 and 5.16 $\log_{10}$ CFU/mL, respectively, when compared to the Control group. Log_{10} CFU/mL *C. jejuni* in the ceca peaked at 7 dpi and decreased at 14 dpi ($P < 0.05$).

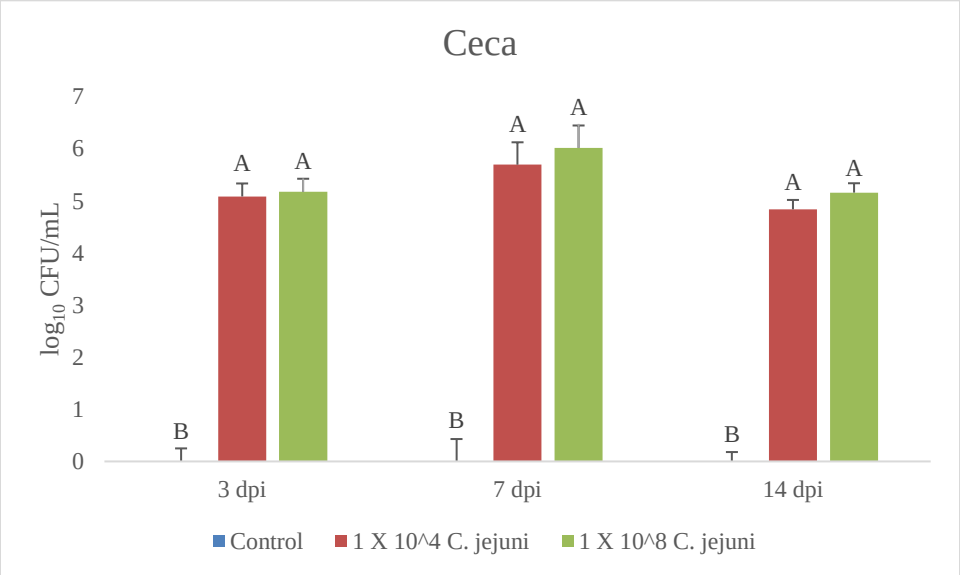


Figure 3.1. Effect of *Campylobacter jejuni* challenge on *Campylobacter* load in the ceca: Day old chicks were distributed into three treatment groups: Control, 1 X 10^4, and 1 X 10^8 (n = 6). Birds in challenge groups received 1 X 10^4 and 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. *C. jejuni* load of the whole ceca was estimated using micro-dilutions (CFU/mL) and then log transformed to $\log_{10}$ CFU/mL for statistical analysis. Results were expressed as mean + SEM. Means with no common superscript differ significantly (P < 0.05).

Effect of *Campylobacter jejuni* challenge on *mapA* gene in the ceca.

Cecal *mapA* expression was significantly higher in the 1 X 10^4 and 1 X 10^8 CFU groups by 4.83 and 4.38 Ct-40 at 3 dpi, 4.91 and 4.73 Ct-40 at 7 dpi, and 3.74 and 5.43 Ct-40 at 14 dpi, respectively, compared to the Control group (P < 0.05) (*Figure 3.2*).

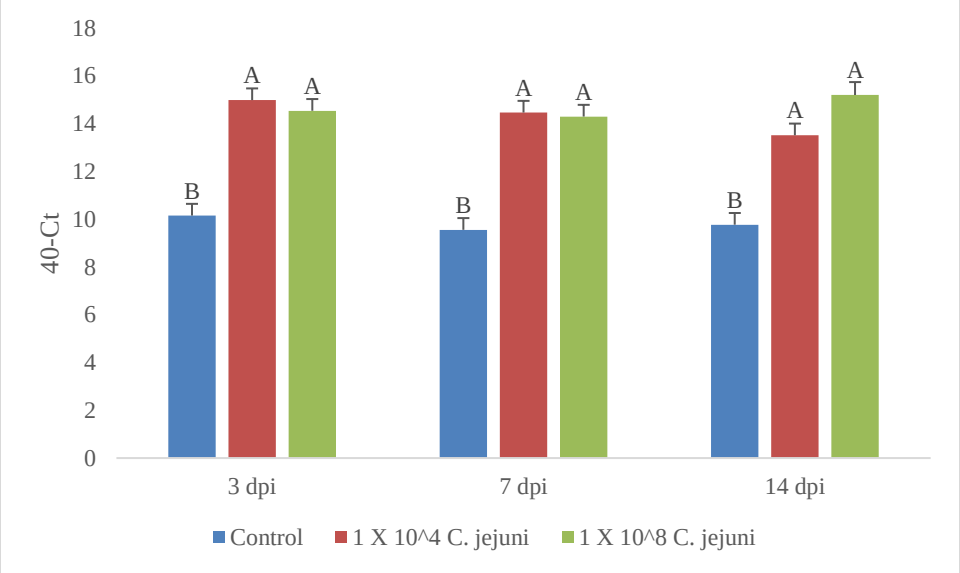


Figure 3.2. Effect of *Campylobacter jejuni* challenge on *mapA* gene in the ceca: Day old chicks were distributed into three treatment groups: Control, 1 X 10^4, and 1 X 10^8 (n = 6). Birds in challenge groups received 1 X 10^4 and 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Whole cecal *C. jejuni* load was quantified via SyBr

green qPCR with primers designed to target the *mapA* gene of *C. jejuni*. Results were expressed as mean + SEM. Means with no common superscript differ significantly ($P < 0.05$).

Effect of *Campylobacter jejuni* challenge on gut permeability of FITC-d.

From 3 to 7 dpi, intestinal permeability increased across all treatment groups (*Figure 3.3*). The concentration of serum FITC-d was greatest in the Control group, followed by the 1 X 10^4 CFU *C. jejuni*, and 1 X 10^8 CFU *C. jejuni* groups, respectively, at 7 dpi though no significance was observed ($P > 0.05$).

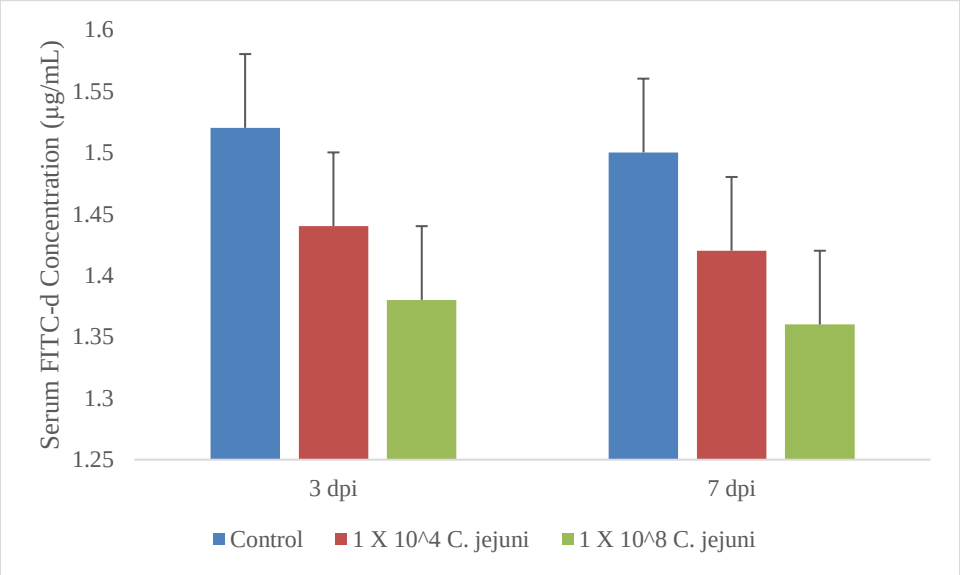


Figure 3.3. Effect of *Campylobacter jejuni* challenge on gut permeability of FITC-d: Day old chicks were distributed into three treatment groups: Control, 1 X 10^4, and 1 X 10^8 (n = 6). Birds in challenge groups received 1 X 10^4 and 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. 1 mL of FITC-d was administered to each bird via oral gavage at 3 and 7 dpi to measure intestinal permeability. Blood was collected and serum was analyzed for FITC-d concentration. Results were expressed as mean + SEM. Means with no common superscript differ significantly ($P < 0.05$).

Effect of *Campylobacter jejuni* challenge on bile anti-*C. jejuni* IgA antibody levels.

No significance was observed in bile anti-*C. jejuni* IgA antibody levels (P > 0.05) (*Figure 3.4*).

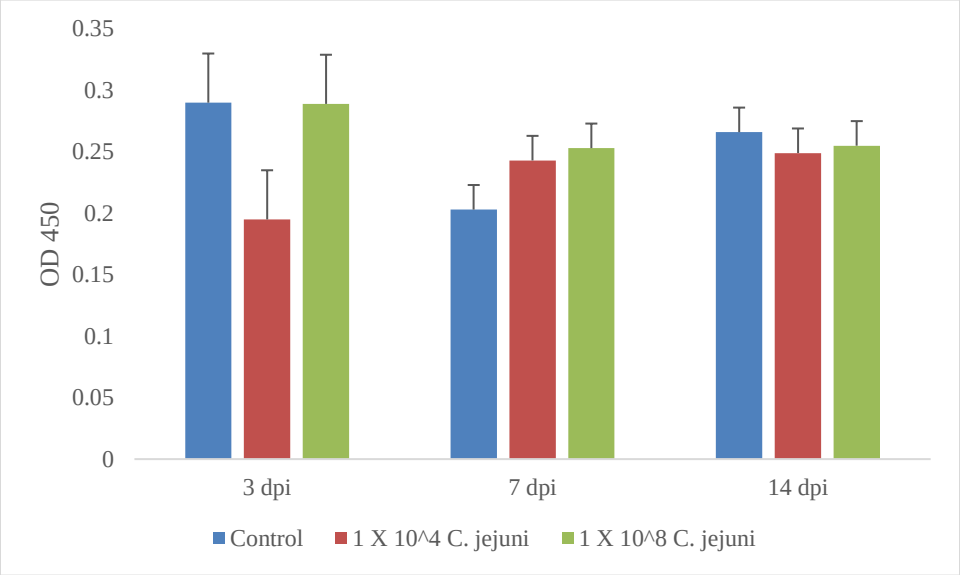
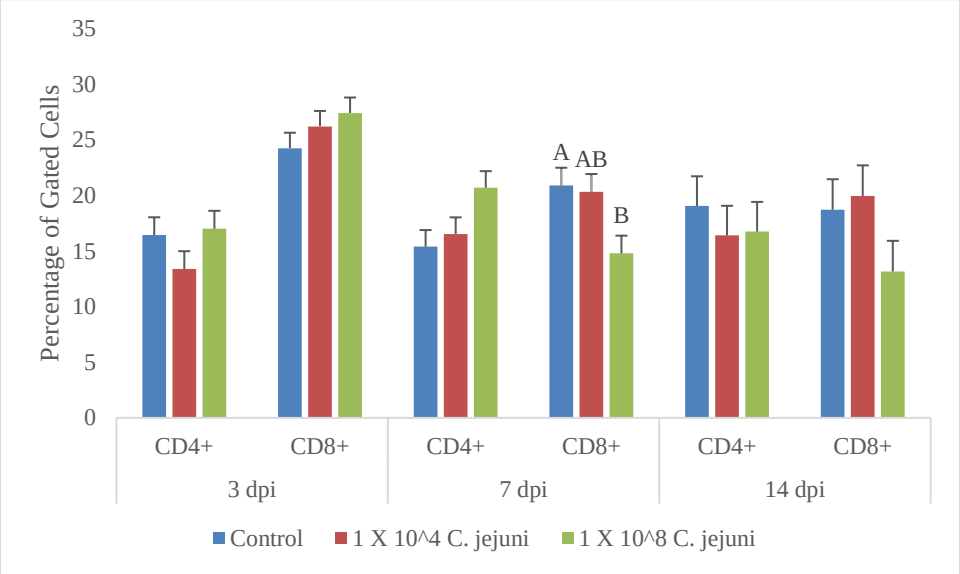


Figure 3.4. Effect of *Campylobacter jejuni* challenge on bile anti-*C. jejuni* IgA antibody levels: Day old chicks were distributed into three treatment groups: Control, 1 X 10^4, and 1 X 10^8 (n = 6). Birds in challenge groups received 1 X 10^4 and 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. At 3, 7, and 14 dpi, bile was collected, and anti-*C. jejuni* IgA antibodies were detected using direct enzyme-linked-immunosorbent assay (ELISA). Results were expressed as mean + SEM OD 450 values. Means with no common superscript differ significantly (P < 0.05).

Effect of *Campylobacter jejuni* challenge on cecal tonsil $CD4^+$ and $CD8^+$ T lymphocytes, and $CD4^+$:$CD8^+$ cell ratio.

At 3 dpi, the Control, Control, 1 X 10^4, and 1 X 10^8 groups had similar percentages of $CD4^+$ (16.42, 13.37, and 17.00, respectively), $CD8^+$ (24.24, 26.20, and 27.41), and $CD4^+$:$CD8^+$ cell ratio (0.72, 0.53, and 0.63) (*Figure 3.5*). 7 dpi, there was an increase in $CD4^+$ percentage and a significant decrease in $CD8^+$ percentage of the 1 X 10^8 group, as well as a significantly lower $CD4^+$:$CD8^+$ ratio in the Control compared to the challenged groups ($P < 0.05$). The $CD4^+$:$CD8^+$

ratio at 14 dpi was 2.39 and 2.62 greater in the 1 X 10^8 group than the Control and 1 X 10^4 groups, respectively.



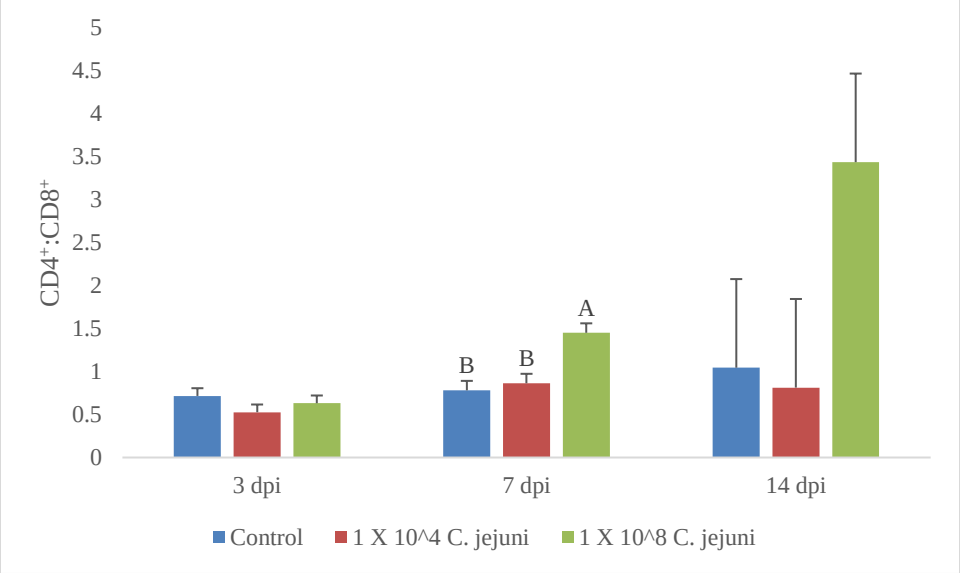
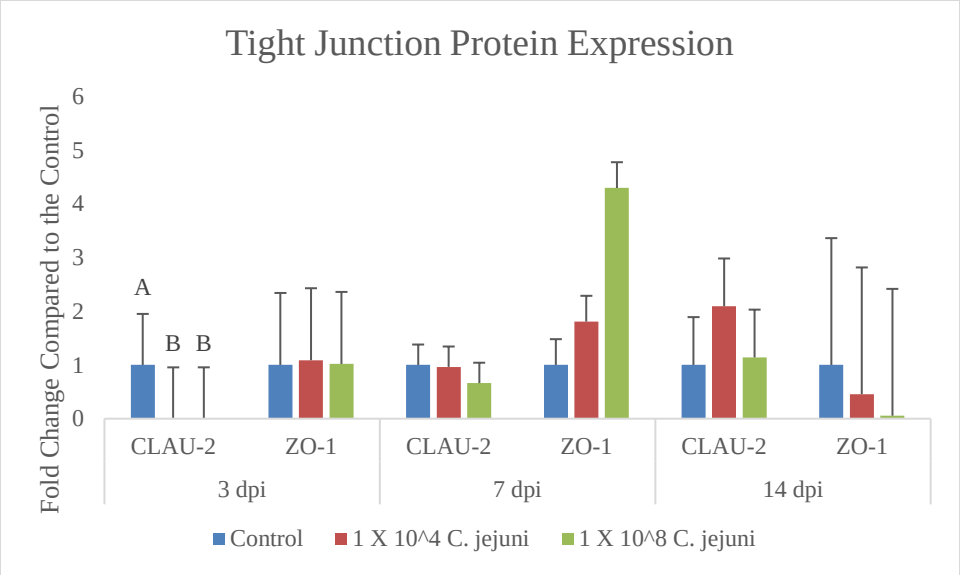


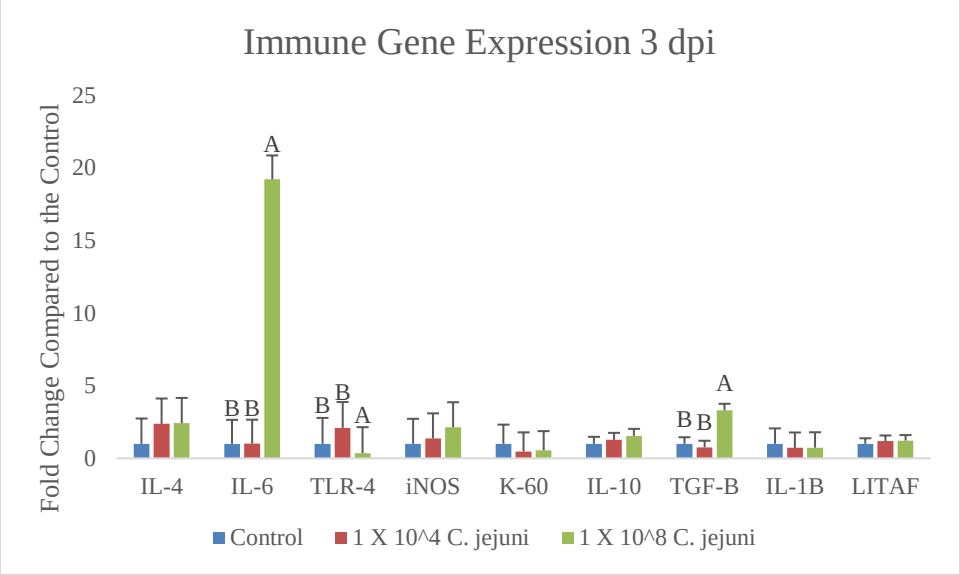
Figure 3.5. Effect of *Campylobacter jejuni* challenge on cecal tonsil $CD4^+$ and $CD8^+$ T lymphocytes, and $CD4^+$:$CD8^+$ cell ratio: Day old chicks were distributed into three treatment groups: Control, 1 X 10^4, and 1 X 10^8 (n = 6). Birds in challenge groups received 1 X 10^4 and 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Cecal tonsils were strained to single-cell suspensions (1 × 10^6 cells) and incubated with PE-

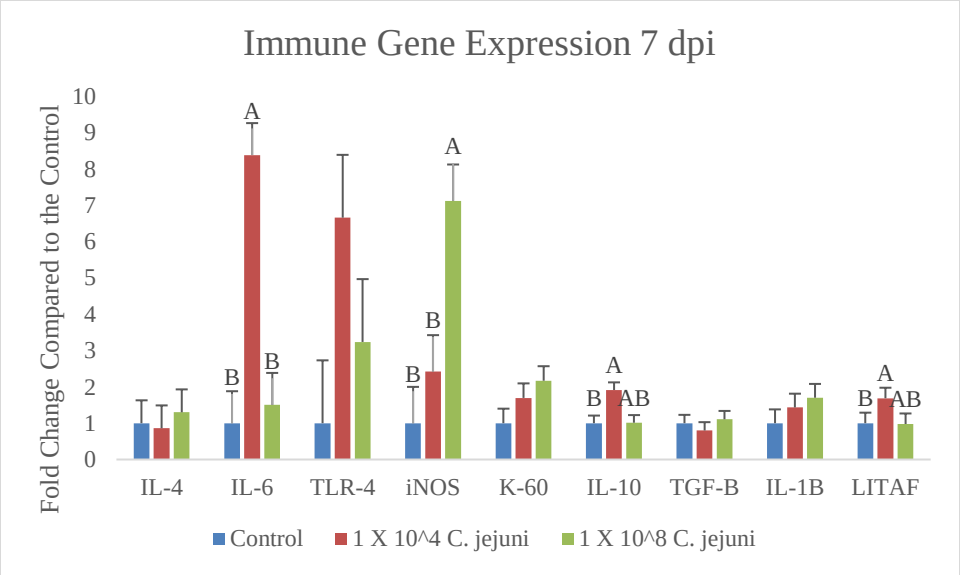
conjugated mouse anti-chicken CD4 and FITC-conjugated mouse anti-chicken CD8 at 1:200 dilution, and unlabeled mouse IgG at 1:500 dilution in a 96-well plate for 20 minutes. $CD4^+$ and $CD8^+$ cells were reported as percentage of gated cells and $CD4^+$:$CD8^+$ ratio was calculated. Results were expressed as mean + SEM. Means with no common superscript differ significantly ($P < 0.05$).

Effect of *Campylobacter jejuni* challenge on immune gene and tight junction protein expression.

At 3 dpi, CLAU-2 expression was significantly higher in the Control group than in the challenged groups, with the 1 X 10^4 group being significantly lowest ($P < 0.05$) (*Figure 3.6*). Pro-inflammatory IL-6 and iNOS were significantly higher in the 1 X 10^8 groups at 3 dpi ($P < 0.05$). 7 dpi, IL-6 and iNOS were significantly high in the challenged groups while anti-inflammatory IL-10 and pro-inflammatory IL-1β were significantly higher in the challenged groups than in the Control group ($P < 0.05$). Expression of TLR-4 and K-60 at 14 dpi was significantly downregulated in the challenged groups compared to the Control group ($P < 0.05$). At 14 dpi, IL-10 and IL-1β were significantly lower in the challenged groups compared to the Control ($P < 0.05$).


Tight Junction Protein Expression
Fold Change Compared to the Control
6
5
4
3
2
1
0
A
B B
CLAU-2
ZO-1
CLAU-2
ZO-1
CLAU-2
ZO-1
3 dpi
7 dpi
14 dpi
Control
1 X 10^4 C. jejuni
1 X 10^8 C. jejuni


Immune Gene Expression 3 dpi
Fold Change Compared to the Control
25
20
15
10
5
0
A
B B
B B
A
B B
A
IL-4
IL-6
TLR-4
iNOS
K-60
IL-10
TGF-B
IL-1B
LITAF
Control
1 X 10^4 C. jejuni
1 X 10^8 C. jejuni



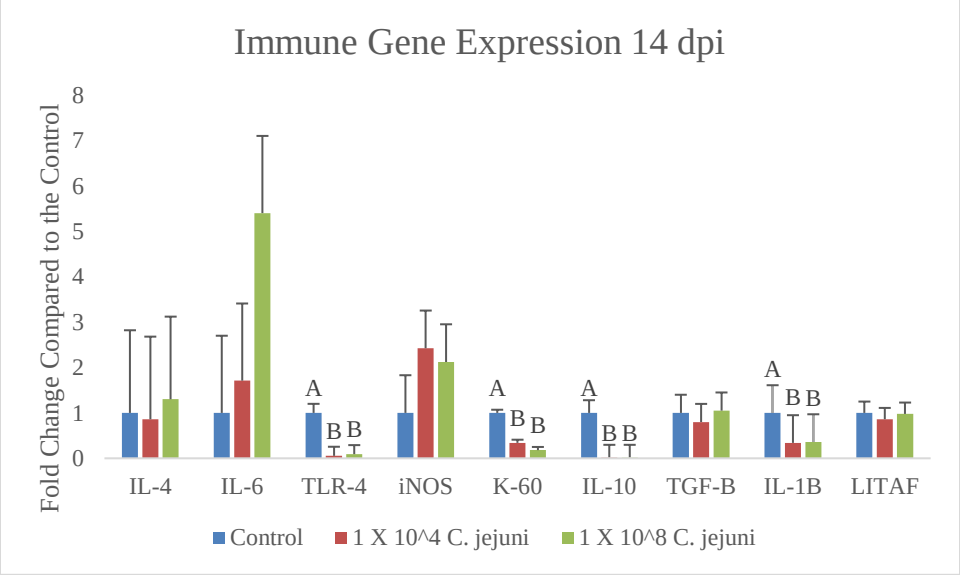


Figure 3.6. Effect of *Campylobacter jejuni* challenge on immune gene and tight junction protein expression: Day old chicks were distributed into three treatment groups: Control, 1 X 10^4, and 1 X 10^8 (n = 6). Birds in challenge groups received 1 X 10^4 and 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Gene expression was determined by qPCR of cecal tonsil mRNA, converted to cDNA. Results were expressed as the mean + SEM fold change in tissue mRNA levels in the challenge treatment as compared to

the mock-challenged control treatment. Means with no common superscript differ significantly from the Control group ($P < 0.05$).

Discussion

This study aimed to characterize the immune and microbiological responses, gut permeability, and cecal *C. jejuni* load of broilers challenged with 1 X 10^4 or 1 X 10^8 CFU/bird of *C. jejuni* strain 33560, in order to establish an effective challenge model. Colonization by *C. jejuni* in poultry initially occurs in the gastrointestinal tract of the birds, with the greatest pathogenic load concentrated in the ceca, and has been found to disseminate to other organs pre-harvest (Mortada et al., 2021). Virtually all flocks are contaminated with the ubiquitous *C. jejuni* across the globe, the bacteria typically act apathogenically within their poultry hosts and have been described as a commensal part of the natural avian microbiome (Hendrixson & DiRita, 2004). Chickens colonized by *C. jejuni* typically display no clinical signs and experience no marked productivity losses associated with the disease (Awad et al., 2016). In our study, we saw no significance between treatment groups' BWG, FI, or FCR at any time point measured. These results agree with past research showing that *C. jejuni* infection in broilers is clinically asymptomatic and does not impact production performance parameters critical to the commercial broiler industry. Some research has found *C. jejuni* experimental challenge to decrease BWG and FI while negatively impacting FCR in broilers (Awad et al., 2020; Nooreh et al., 2021; Visscher et al., 2018). These opposing performance results could be due to challenge dosage, broiler breed, bird age at challenge, challenge strain, or overall bird health.

In our study, *C. jejuni* challenge was introduced to the broilers at 21 days of age, as young chicks are protected from colonization prior to this at levels up to 1 X 10^8 CFU due to

anti-*C. jejuni* maternally transferred antibodies (Sahin et al., 2003). The ceca of challenged birds was colonized from 3 dpi through 14 dpi ($P < 0.05$), at which time the trial concluded at 35 days of age. Cecal colonization was evaluated via direct plating of the ceca to evaluate load, which has previously been established as directly linked and positively correlated with whole carcass load of *C. jejuni* (Allen et al., 2007; Reich et al., 2008). *mapA* is a gene used in PCR analysis for differentiation between *C. jejuni* and *C. coli* isolates (Begum et al., 2015). In this experiment, we evaluated *mapA* levels in the ceca and found that the expression level of the gene corresponded with our direct plating data, in that the *mapA* expression was significantly higher in the challenged groups than in the Control group ($P < 0.05$), which is consistent with past research (Johnson et al., 2014). This confirms that our challenge models effectively colonized the broiler ceca.

FITC-d is a non-digestible indicator of intestinal paracellular permeability commonly used in broilers to evaluate gut permeability following challenge (Liu et al., 2021). Serum FITC-d concentration differences between treatment groups was insignificant in this experiment ($P > 0.05$), which contradicts past research demonstrating increased intestinal paracellular permeability with *C. jejuni* challenge (Awad et al., 2016; Awad et al., 2020; Boehm et al., 2012). CLAU-2 and ZO-1 are tight junction proteins expressed in the chicken intestinal paracellular epithelium where they regulate paracellular permeability and serve as scaffolding proteins (Van Itallie et al., 2009; Venugopal et al., 2019). Tight junction protein expression of CLAU-2 and ZO-1 within broiler cecal tonsils in this experiment supported the results of our FITC-d assay. The lack of significantly increased intestinal permeability in the FITC-d and tight junction protein assays between treatment groups in this study could be due to our challenge strain, broiler breed, or feed formulation.

In poultry, IgA is produced as a secretory product of B-cells produced in the bursa of Fabricius and stored in the gallbladder (Lacharme-Lora et al., 2017). The purpose of IgA is to protect the intestinal epithelium from colonization by invading pathogens by acting as the immunoglobulin of mucosal immunity (Macpherson et al., 2008; Ohland & MacNaughton, 2010). Concurrent with past studies, we did not see significance in our anti-*C. jejuni* IgA assay results (Hermans et al., 2014; Widders et al., 1996). This is in contrast with other research which found heightened IgA response following challenge (Mortada et al., 2021; Myszewski & Stern, 1990). The lack of significance could be attributed to the feed formulation or immune status of the birds.

$CD4^+$:$CD8^+$ cell ratio is used in poultry immunology to indicate immunocompetence (Char et al., 1990). The numeric value associated with the $CD4^+$:$CD8^+$ cell ratio gives an indication of the overall health of poultry, with values in the range of 0.95 – 1.9 being associated with clinically healthy broiler flocks (Arthanari et al., 2012; Brindle et al., 2006). In this study, we observed significantly ($P < 0.05$) elevated $CD4^+$:$CD8^+$ cell ratios within the challenged treatments, compared to the Control group and a significantly lower percentage of gated $CD8^+$ cells in the challenged groups than the Control group at 7 dpi. In typical disease instances, the $CD4^+$:$CD8^+$ cell ratio is expected to be significantly lower in challenged compared to control treatments as $CD8^+$ cells are important to the clearance of bacterial intracellular infections in poultry (Fimlaid et al., 2014). In our experiment, the statistically low $CD8^+$ found in the challenge groups and statistically high $CD4^+$:$CD8^+$ cell ratio at 7 dpi ($P < 0.05$) could provide evidence for the commensal nature of *C. jejuni* in poultry and is supported by past research (Huang et al., 2010). $CD8^+$ T lymphocyte depression and an inverted $CD4^+$:$CD8^+$ cell ratio in *C. jejuni* animal challenge models has been linked to *C. jejuni* dissemination to the liver and

enduring *C. jejuni* colonization within the liver and gastrointestinal tract, which supports our results (Vučković et al., 2006).

At 3 dpi, TGF-β, iNOS, and IL-6 expression were statistically higher in the 1 X 10^8 group ($P < 0.05$). The elevated expression of these genes together in the cecal tonsils during early infection drives Th-17 cellular differentiation, thus promoting the inflammatory response to challenge (Sanjabi et al., 2009). 7 dpi, IL-10, IL-6, iNOS, and IL-1β were elevated in challenge groups ($P < 0.05$). Elevated IL-10 is associated with an absence of clinical signs of infection, acts as an anti-inflammatory cytokine, and has been suggested as a mediator of tolerance to enduring high load *C. jejuni* infections in poultry (Humphrey et al., 2014; John et al, 2017). IL-6, a pro-inflammatory pleiotropic cytokine, critically supports the host immune response in the acute phase response to infection (Gabay, 2006). Enhanced production of iNOS by $CD4^+$ cells within the cecal tonsil T cells negatively regulates Th17 differentiation and could serve to counteract the effects of the elevated TGF-β and IL-6 expression at 3 dpi (Xue et al., 2018). At 14 dpi, IL-10, TLR-4, and IL-1β expression by the challenged groups were significantly lower than the Control group. Low expression of TLR-4 helps combat systemic infection by limiting inflammation and regulating response to LPS (Ciesielska et al., 2020). Suppressed IL-10 and IL-1 expression in birds indicates tolerance and persistence of the inflammation and infection within the gastrointestinal tract (Humphrey et al., 2014). K-60 is a chemokine that instigates immune cell migration and activates T cells and macrophages (Sun et al., 2021). Low K-60 expression in the challenged groups during late infection at 14 dpi indicates tolerance of *C. jejuni* colonization within the broiler ($P < 0.05$).

Conclusions

In this study, we found that challenge dosage had no effect on performance parameters, FITC-d permeability of the gastrointestinal tract, or anti-*C. jejuni* IgA concentration within the bile. Cecal colonization with *C. jejuni* increased in a dose dependent manner with the greatest colonization and $CD4^+$:$CD8^+$ cell ratios occurring in the 1 X 10^8 CFU/bird *C. jejuni* group. Tight junction protein expression was downregulated in the cecal tonsils following challenge while both pro- and anti-inflammatory cytokine expression was upregulated. Our study found that 1 X 10^8 CFU/bird *C. jejuni* challenge dosage at 21 days of age yielded the greatest cecal colonization and immunomodulatory effects in broilers which persisted to at least 35 days of age.

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CHAPTER 4

EFFECTS OF SYNBIOTIC SUPPLEMENTATION AS AN ANTIBIOTIC GROWTH PROMOTER REPLACEMENT ON CECAL *CAMPYLOBACTER JEJUNI* LOAD IN BROILERS CHALLENGED WITH *C. JEJUNI*.

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[1]Cason E.E., Al Hakeem W.G., Adams D., Shanmugasundaram R., Selvaraj R. To be submitted to The Journal of Poultry Science.

Abstract

Synbiotic supplementation alters the gut microbiota to increase beneficial bacteria and decrease pathogenic bacteria. Synbiotics can improve production performance, decrease cecal *Campylobacter jejuni* load and colonization, and improve immunological responses and can be used as an alternative to antibiotic growth promoters in poultry production. This study aimed to determine the effects of synbiotic supplementation as an antibiotic replacement in broilers challenged with *C. jejuni*. A total of 336 day-old broiler chicks were distributed into four treatment groups: Control, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge. There were six replicate pens per treatment, with 14 birds per pen. At 21 days of age, birds were orally inoculated with PBS or 1 X 10^8 CFU/bird of *C. jejuni*. Antibiotic (Virginiamycin) and synbiotic (PoultryStar® Me, DSM) were supplemented in feed at 20 mg/kg and 20 g/1000 birds/day, respectively. Feed intake and body weight were measured weekly. On 3, 7, and 14 dpi, gut permeability was measured by FITC-dextran assay, anti-*C. jejuni* IgA concentration in the bile was measured via ELISA, *C. jejuni* loads in cecal content, ileal tissue, and livers were quantified by plating, splenocyte adherent mononuclear cell nitric oxide production was quantified using Ricca reagent, and cecal tonsil $CD4^+$:$CD8^+$ and $CD4^+$:$CD25^+$, immune gene, and tight junction protein expression were measured by flow cytometry and qPCR. Data were statistically analyzed using JMP Pro 15, by One Way ANOVA, followed by Tukey's test. There were no significant differences ($P > 0.05$) in BWG, FCR, FITC-d permeability, or bile anti-*C. jejuni* IgA concentration between the treatment groups at the time points studied. At 7 and 14 dpi, birds in the Synbiotic + Challenge group had a significantly lower ($P < 0.05$) cecal *C. jejuni* load compared to the Antibiotic + Challenge group. At 7 dpi, cecal tonsil $CD4^+$:$CD8^+$ was significantly higher ($P < 0.05$) in the Synbiotic + Challenge and Antibiotic + Challenge groups

than in the Synbiotic and Control groups, respectively. At 3 dpi, cecal tonsil $CD4^+$:$CD25^+$ was significantly higher ($P < 0.05$) in the Synbiotic + Challenge than in the Synbiotic group. On the day of challenge, tight junction proteins were upregulated significantly ($P < 0.05$) in the Synbiotic and Synbiotic + Challenge groups. At 0, 3, and 7 dpi, T cell differentiation genes, pro-, and anti-inflammatory cytokine expression were all impacted significantly by synbiotic supplementation and challenge. It can be concluded that synbiotic supplementation decreases *C. jejuni* loads in broilers and beneficially modulates the immune response to challenge, compared to antibiotic supplementation, and that synbiotics can be applied to replace antibiotic growth promoter usage during *C. jejuni* infections of poultry.

Introduction

Campylobacteriosis is the most reported bacterial foodborne illness in the United States each year (Altekruse et al., 1999). Poultry are a natural biological reservoir of *Campylobacter jejuni* and is the most frequently cited source of human infection (Bryan & Doylez', 1995). *C. jejuni* is a commensal organism of poultry with great persistence that evokes no clinical signs/lesions, no marked productivity losses, rapid colonization, and high disease transmission/shedding (Cean et al., 2015, Shane, 1992; Snelling et al., 2005). *C. jejuni* is apathogenic to poultry and quickly spreads between birds and flocks (Hermans et al., 2012). Immunologically, the response by the immune system to *C. jejuni* infection is mild and perpetuates disease persistence (Mortada et al., 2021). Colonization and shedding endure for the life of the birds and present a significant problem at processing where the bacteria readily spread and represent a critical food safety concern (Garcia-Sánchez et al., 2007). As the poultry industry has moved away from antibiotic usage, synbiotics propose an effective means of improving

production parameters and reducing bacterial load, spread, and contamination in poultry through improving the animal's natural ability to resist colonization by pathogens of human concern.

Controlling *C. jejuni* contamination before and at processing is essential due to legislation in place by the USDA-FSIS dictating performance standards for *Campylobacter* contaminated poultry meat carcasses, parts, and comminuted meat. As antibiotic-resistance increases and antimicrobial usage decreases, alternatives to antibiotic growth promoters needed to be found that could act in a similar way to benefit poultry. Antibiotic growth promoters exert growth-promoting effects in challenged animals, reduce the incidence of subclinical infection, decrease competition for nutrients within the gut microbiome, beneficially shift the intestinal microflora, strengthen the gut walls, improve nutrient absorption, and induce immune reactions (Brennan et al., 2003; Pedroso et al., 2006; Snyder and Wostmann, 1987; Teirlynck et al., 2009; Wise and Siragusa, 2007). One antibiotic replacement that has been used in poultry production is synbiotics, due to their similar mode of action to antibiotic growth promoters. Synbiotics can be described as nutritional additives containing both probiotic(s) and prebiotic(s) which specifically favor the probiotic bacteria in a supplement, that act synergistically within a host (Schrezenmeir & de Verse, 2001). To ultimately improve production performance, synbiotics are immunomodulatory, support the gastrointestinal tract, and promote anergy within the gastrointestinal tract (Sharma et al., 2018; Zhang et al., 2010).

Synbiotic supplementation has been shown to reduce *C. jejuni* colonization in broilers and to positively impact their gut microbiome composition (Baffoni et al., 2017; Śliżewska et al., 2020). During critical stress points of a broiler's life, including periods of heat stress, disease, and transportation, a synbiotic application has also been proven an effective antibiotic alternative that improves stress tolerance, immune function, body weight, feed conversion ratio, and

physiological responses to stressors (Ghareeb & Bohm, 2009; Hu et al., 2022). Synbiotics have also been proven to improve mortality, body weight, average daily gain, feed efficiency, and carcass yield percentage, even more so than probiotic supplements in broilers (Ashayerizadeh *et al*., 2009; Awad et al., 2009). Past studies have demonstrated performance promotion, increased tight junction expression, and the immunomodulatory effect of synbiotics on *C. jejuni* challenged broilers (Abdel-Wareth et al., 2019; Arthanari et al, 2012; Awad et al., 2020; Humphrey et al., 2014; Markazi et al., 2018; Quinn et al., 2020).

To our knowledge, no comprehensive study has been conducted to evaluate and compare synbiotic versus antibiotic growth promoter supplementation in broilers challenged with *C. jejuni* on performance, immunological, and microbiological parameters. Recent regulatory changes put forth by the Veterinary Feed Directive, changes in public perception regarding antibiotic usage in animals, and a rise in antimicrobial resistant *C. jejuni* have effectively ended antibiotic usage within the poultry industry but the pathogens they were once used to control persist and an effective new control mechanism must be found and its impacts on immune, microbiological, and performance aspects characterized.

Materials and Methods

Birds, diets, and *C. jejuni* challenge: 336 day-old male Cobb by-product breeder chicks were randomly assigned to four treatment groups, Control, Synbiotic, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge, with 14 birds per cage and six floor pens per treatment (n = 6). Birds in challenge groups received 1 X 10^8 CFU/bird of *C. jejuni* while Control and Synbiotic treatment birds were mock challenged with 1 mL of PBS via oral gavage at 21 days of age. Antibiotics (Virginiamycin) and synbiotics (PoultryStar® Me) were supplemented in feed

for the duration of the experiment at 20 mg/kg and 20 g/1000 birds/day respectively. Throughout the trial, days 0-35, all birds were fed a corn and soybean-meal based starter diet (*Table 4.1*). One bird per cage was euthanized by cervical dislocation and ceca, cecal tonsil, spleen, liver, blood, and bile were collected on the day of challenge (0 dpi), 3, 7, and 14 dpi for microbiological and immunological parameters. Body weight and feed intake were recorded weekly, and body weight gain and feed conversion ratio were analyzed. The FCR was corrected to account for bird mortality over time.

Basal Diet	
Ingredient	**%**
Corn	58.47
Soybean meal	35.15
Soybean oil	2.269
Limestone	1.593
Biofos	1.387
NaCl	0.35
Vitamins premix	0.35
Dl-methionine	0.21
Lysine HCL	0.137

Trace mineral premix	0.08
Total	100
Calculated Nutrient Composition	
ME, kcal/kg	3050
Crude protein, %	21.44
Crude fat, %	4.55
Lysine, %	1.31
Calcium, %	0.95
TSAA, %	0.91
Threonine, %	0.87
Methionine, %	0.56
Available phosphorus, %	0.45

[1] Vitamin mix provided the following per kg of diet: 2.4 mg thiamin-mononitrate, 44 mg nicotinic acid, 4.4 mg riboflavin, 12 mg D-Ca pantothenate, 12 g vitamin B12, 2.7 mg pyridoxine-HCl, 0.11 mg D-biotin, 0.55 mg folic acid, 3.34 mg menadione sodium bisulfate complex, 220 mg choline chloride, 1,100 IU cholecalciferol, 2,500 IU trans-reinyl acetate, 11 IU all-rac-tocopherol acetate, and 150 mg ethoxyquin

[2] Trace mineral mix provided the following per kg of diet: 101 mg $MnSO_4.H_2O$, 20 mg $FeSO_4.7H_2O$, 80 mg Zn, 3 mg $CuSO_4.5H_2O$, 0.75 mg ethylene diamine dihydroiodide, 20 mg MgO, and 0.3 mg sodium selenite.

Table 4.1. Basal diet with ingredients and nutrient composition calculations. UGA trace mineral and vitamin premixes were used in this study.

***Campylobacter jejuni* challenge:** *C. jejuni* (strain 33560) was cultivated on Campy-CEFEX agar supplemented with Park and Sanders (Sigma-Aldrich, St. Louis, MO) and Remel laked horse blood (Thermo Fisher Scientific, Lenexa, KS) and incubated for 48 hours in a microaerobic atmosphere (85% N_2, 10% CO_2, and 5% O_2) at 42°C. Following incubation, bacterial cells were collected and resuspended in PBS to create a suspension with an optical density of 0.27 at 540 nm with a Spec-20 (Milton-Roy Spectrophotometer, Thermo Spectronics, Madison, WI). An optical density value of 0.27 is equivalent to 1 X 10^8 CFU/mL of *C. jejuni* (Cosby et al.,, 2017). The challenge stock was verified utilizing serial dilutions and direct plating on Campy-CEFEX. Prior to challenge, the *C. jejuni* was evaluated via dark field microscopy to evaluate cork-screw motility, indicative of viability and essential for colonization to occur.

Effect of synbiotic supplementation on *Campylobacter* load in the ceca, ileum, and liver: On 3, 7, and 14 dpi, whole ceca, ileum, and liver samples were excised and collected in stomacher bags, then stored on ice. The samples were macerated and 3 X (weight/volume) BPW was added to each sample. Samples were stomached for 60 seconds until homogenized. Samples were diluted and a plated at a volume of 10 µl/plate, on campy-CEFEX agar supplemented with Park and Sanders (Sigma-Aldrich, St. Louis, MO) and Remel laked horse blood (Thermo Fisher Scientific, Lenexa, KS). Plates were incubated under microaerobic conditions (85% N_2, 10% CO_2, and 5% O_2) at 42°C for 48 hours then colonies were enumerated. *C. jejuni* CFUs were

confirmed by microscopic observation of characteristic cork-screw motility and further confirmed using SyBr green qPCR using primers directed at the *C. jejuni mapA* gene sequence. Enumeration data was recorded as CFU/g then converted to $\log_{10}$ CFU/g for statistical evaluation.

Effect of synbiotic supplementation on *Campylobacter jejuni mapA* gene in the ceca and ileum: On 3, 7, and 14 dpi, whole ceca and ileal tissue were excised and collected in stomacher bags, then stored on ice. Whole ceca and ileal samples were macerated and 3 X (weight/volume) BPW was added to each sample. Samples were stomached for 60 seconds until homogenized then stored at 0°C until analyzed. Bacterial DNA was extracted from the whole ceca samples and quantified using qPCR with SyBr green, and primers directed at the *C. jejuni mapA* gene sequence. Quantified data was reported as 40-Ct and statistically evaluated. *mapA* gene is specific to *C. jejuni* and differentiates it from *Campylobacter coli*.

Effect of synbiotic supplementation on FITC-d permeability through the gastrointestinal tract: On 3, 7, and 14 dpi, 1 bird per pen was administered 2.2 mg/mL mw 4,000 fluorescein isothiocyanate-dextran (FITC-d) (Sigma-Aldrich, St. Louis, MO) via oral gavage of 1 mL/bird. After 2 hours, blood was collected directly from the heart and stored on ice in the dark prior to transport to the laboratory. Tubes were centrifuged at 1,500 X g for 12 minutes to allow serum collection. A standard curve was prepared and plated then 100 µl from each sample was plated on black 96 well plates (Krystal Biotech, Pittsburgh, PA) in triplicate. FITC-d was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using an Epoch microplate spectrophotometer (BioTek, VT, USA), and 485/528 values recorded. The FITC-d concentration in the serum samples was determined using the equation derived from the standard curve of serially diluted FITC-d Vs. 485/528 values.

Effect of synbiotic supplementation on bile IgA anti-*Campylobacter jejuni* antibodies: On 0, 3, 7, and 14 dpi, bile was collected and stored at -20°C until analysis. Specific anti-*C. jejuni* IgA antibodies were measured via enzyme-linked immunosorbent assay (ELISA). Whole cell *C. jejuni* antigens were generated by lysing 1 X 10^9 CFU/mL of *C. jejuni* through seven cycles of bead beating (TissueLyser LT, Qiagen, Germantown, MD), freezing, and thawing. ELISA plates (Nunc Maxisorp™, ThermoFisher Scientific, Waltham, MA) were coated in 10 μg/ml of *C. jejuni* antigen, diluted in coating buffer (carbonate/bicarbonate, pH 9.6). Bile samples were diluted to 1:800 in PBS containing 8 % non-fat dry milk and 0.1 % Tween-20 (VWR, Radnor, PA). HRP-conjugated polyclonal goat anti-chicken IgA (SouthernBiotech, Birmingham, AL) was added to each well at 1:100000 as a secondary antibody. 3, 3, 5, 5-tetramethylbenzidine (Ken-En-Tec, Taastrup, DK) was added at 100 μL/well and incubated for 10 minutes, prior to halting the reaction using HCl. Absorbance was measured at 450 nm using Epoch microplate spectrophotometer (BioTek, VT, USA) and antibody levels were reported as OD 450 values.

Effect of synbiotic supplementation on cecal tonsil $CD4^+$ and $CD8^+$ T lymphocytes and $CD4^+$:$CD8^+$ cell ratio: On 0, 3, 7, and 14 dpi, cecal tonsil tissues were excised and collected in 2 mL tubes filled with 1.5 mL of incomplete RPMI media then stored on ice. A flow cytometer was used to analyze the samples for $CD4^+$ and $CD8^+$ cells. Single-cell suspensions of the cecal tonsils (1 X 10^6 cells) were strained and incubated with PE-conjugated mouse anti-chicken CD4 and FITC-conjugated mouse anti-chicken CD8 (Southern Biotech, Birmingham, AL) at 1:250 and 1:313 dilutions, respectively, and unlabeled mouse IgG at 1:100 dilution in a 96-well plate for 20 minutes at 4°C. Following incubation, cells were washed three times by centrifugation at 400 X g for 5 minutes to remove unbound antibodies using a wash buffer (1 X PBS, 2 mM EDTA, 1.5% FBS). Following washing, CytoSoft software (Guava Easycyte, Millipore,

Billerica, MA) was used to analyze the cells, and $CD4^+$ and $CD8^+$ cells were reported as percentage of gated cells and $CD4^+$:$CD8^+$ cell ratios calculated.

Effect of synbiotic supplementation on cecal tonsil $CD4^+$ and $CD25^+$ T lymphocytes and $CD4^+$:T-reg cell ratio: On 0, 3, 7, and 14 dpi, cecal tonsils were excised and collected in 2 mL tubes filled with 1.5 mL of incomplete RPMI media then stored on ice. A flow cytometer was used to analyze the samples for $CD4^+$ and $CD25^+$ cells. Single-cell suspensions of the cecal tonsils (1 X 10^6 cells) were generated and incubated with mouse anti-chicken $CD25^+$ antibody (Shanmugasundaram and Selvaraj, 2011) at 1:54 dilution and unlabeled mouse IgG at 1:99 dilution in a 96-well plate for 50 minutes at 4°C. Following incubation, PE-conjugated mouse anti-chicken CD4 (Southern Biotech, Birmingham, AL) was added at 1:13.5 per well then incubated for 20 minutes at 4°C. Following incubation, cells were washed three times by centrifugation at 400 X g for 5 minutes to remove unbound antibodies using a wash buffer (1 X PBS, 2 mM EDTA, 1.5% FBS). Following washing, CytoSoft software (Guava Easycyte, Millipore, Billerica, MA) was used to analyze the cells, and $CD4^+$ and T-reg cells were reported as percentage of gated cells and $CD4^+$:T-reg cell ratios calculated.

Effect of synbiotic supplementation on nitric oxide production from adherent splenocyte MNCs: On 0, 3, 7, and 14 dpi, spleens were excised and collected in 5 mL tubes with 3 mL of incomplete RPMI media then stored on ice. Spleens were strained through a 45μm cell strainer (Fisher scientific) to achieve a single-cell suspension. 3 mL of the single-cell suspension was layered over 3 mL of Histopaque (1.077 g/mL, Sigma-Aldrich, St. Louis, MO) and spun in a centrifuge at 1200 X g for 10 minutes at 10ºC with centrifuge brakes off. The mononuclear cells were then washed with complete RPMI and resuspended in 8 mL of complete RPMI-1640 media, supplemented with 4 % FBS, 2 % chicken serum, and 1 % penicillin plus streptomycin.

MNCs were allowed to incubate 24 hours in T75 cell culture flasks in an incubator with a 5 % CO_2 atmosphere at 42°C. Following incubation, non-adherent splenocytes were removed via trypsinization (5 ml of 0.4% trypsin supplemented with 0.025% EDTA) and adherent cells were stimulated with complete RPMI and counted using a hemocytometer. Next the MNCs were diluted to get 100 µL of 10^6 cells/mL/well and seeded in triplicate in 96-well plates. Cells were stimulated with 100 µL of complete RPMI media supplemented with 20 µg/mL of *Salmonella* Enteritidis LPS (Sigma Chemicals, MO, USA) or 20 µg/mL of lysed *C. jejuni*. The plates were incubated for 24 and 48 hours at 42°C and 5 % CO_2. Following incubation, plates were centrifuged at 2000 RPM for 5 minutes and the supernatant removed. Nitrite level in 100 µL of the supernatant was determined, as compared to the plated standard, by adding 100 µL of RICCA color reagent, for nitrite determination (RICCA Chemical Solutions, Arlington, TX), then allowing color to develop for 5 minutes in darkness. Following incubation, absorbance was measured at 540 nm using an Epoch microplate spectrophotometer (BioTek, VT, USA), and OD 540 values were recorded. Nitrite concentration values were determined using the standard curve generated by the serially diluted sodium nitrite.

Effect of synbiotic supplementation on immune gene expression: On 0, 3, 7, and 14 dpi, cecal tonsils were excised and collected in 2 mL tubes filled with 1.5 mL of RNAlater (Qiagen, Germantown, MD). Samples were stored on a desktop at room temperature for 7 days until RNAlater thoroughly permeated the tissue samples and stabilized the RNA. After seven days, excess RNAlater was discarded, and samples were stored at -80°C until total RNA was extracted. Following total RNA extraction from the samples, the RNA was converted via reverse transcription to cDNA then stored at -20°C until analysis. The cDNA was analyzed for IL-10, TGF-β, TLR-4, LITAF, iNOS, K60, IL-6, IL-4, CLAU-2, and ZO-1 by qPCR (CFX96 Touch

Real Time System, BioRad) using SyBr green after normalization using GAPDH (*Table 4.2*). Fold change from the housekeeping gene was calculated using $2^{(\text{Ct Sample—Housekeeping})}/2^{(\text{Ct Reference—Housekeeping})}$. Ct, representing the threshold cycle, was determined using the iQ5 software (Biorad) when fluorescence rose exponentially 2-fold above the background.

Primers			
Target Gene	**Sequence (5'-3')**	**Annealing Temperature**	**Reference**
IL-10 F IL-10 R	CATGCTGCTGGGCCTGAA CGTCTCCTTGATCTGCTTGATG	58.0 °C	Rothwell et al., 2004
TGF-β F TGF-β R	CATACTCCTGGGTCTGGTTGGT GACAGCCATCCGCATCTTCT	58.0 °C	Kogut and Arsenault, 2016
TLR-4 F TLR-4 R	ACCTACCCATCGGACACTTG TGCCTGAGAGGTCAGGTT	60.0 °C	Shanmugasundaram and Selvaraj, 2011
LITAF F LITAF R	ATCCTCACCCCTACCCTGTC GGCGGTCATAGAACAGCACT	58.0 °C	Markazi et al., 2018
iNOS F iNOS R	AGTGGTATGCTCTGCCTGCT CCAGTCCCATTCTTCTTCC	60.0 °C	Selvaraj and Klasing, 2006
K60 F	ATTTCCTCCTGCCTCCTACA	55.0 °C	Hong et al., 2006

K60 R	GTGACTGGCAAAAATGACTCC		
IL-6 F IL-6 R	CAAGGTGACGGAGGAGGAC TGGCGAGGAGGGATTTCT	57.5 ℃	Hong et al., 2012
IL-4 F IL-4 R	AACATGCGTCAGCTCCTGAAT TCTGCTAGGAACTTCTCCATTGAA	57.5 ℃	Renu et al., 2020
CLAU-2 F CLAU-2 R	CCTGCTCACCCTCATTGGAG GCTGAACTCACTCTTGGGCT	55.0 ℃	Chen et al., 2017
ZO-1 F ZO-1 R	TGTAGCCACAGCAAGAGGTG CTGGAATGGCTCCTTGTGGT	55.0 ℃	Oxford and Selvaraj, 2017
GAPDH F GAPDH R	TCCTGTGACTTCAATGGTGA CACAACACGGTTGCTGTATC	55.0 ℃	Dube et al., 2014
mapA F *mapA* R	CTGGTGGTTTTGAAGCAAAGATT CAATACCAGTGTCTAAAGTGCGTT TAT	55.0 ℃	Best et al., 2003

Table 4.2. RTqPCR primers and conditions.

Statistical Analysis: Data were statistically analyzed using JMP Pro 15, by One Way ANOVA, followed by Tukey's test. The significance level was set at ($P < 0.05$). Floor pen was used as the experimental unit ($n = 6$).

Results

Effect of synbiotic supplementation on production performance

There were no significant differences in FCR, BWG, or FI between the treatment groups (*Table 4.3*). Synbiotic supplementation numerically decreased the 0-35 d FCR by 11 points, compared to the Control group. Birds in the Antibiotic + Challenge group had a 23 point increase in FCR ($P > 0.05$) while birds in the Synbiotic group had a 16 point lower FCR than the Synbiotic + Challenge group.

Parameter	**Control**	**Synbiotic**	**Synbiotic + Challenge**	**Antibiotic + Challenge**	**SEM**	***P* value**
Body Weight Gain (kg)						
0-7	0.10	0.10	0.10	0.10	0.01	0.69
0-14	0.34	0.36	0.35	0.34	0.01	0.68
0-21	0.76	0.82	0.76	0.77	0.03	0.41
0-28	1.37	1.45	1.37	1.38	0.05	0.60
0-35	2.24	2.28	2.07	2.16	0.08	0.32
Feed Intake (kg)						
0-7	0.19	0.18	0.18	0.18	0.01	0.71
0-14	0.56	0.55	0.53	0.55	0.02	0.79
0-21	1.37	1.29	1.26	1.32	0.04	0.20
0-28	2.61	2.46	2.53	2.63	0.07	0.35
0-35	3.87	3.7	3.68	4.00	0.09	0.06
FCR						

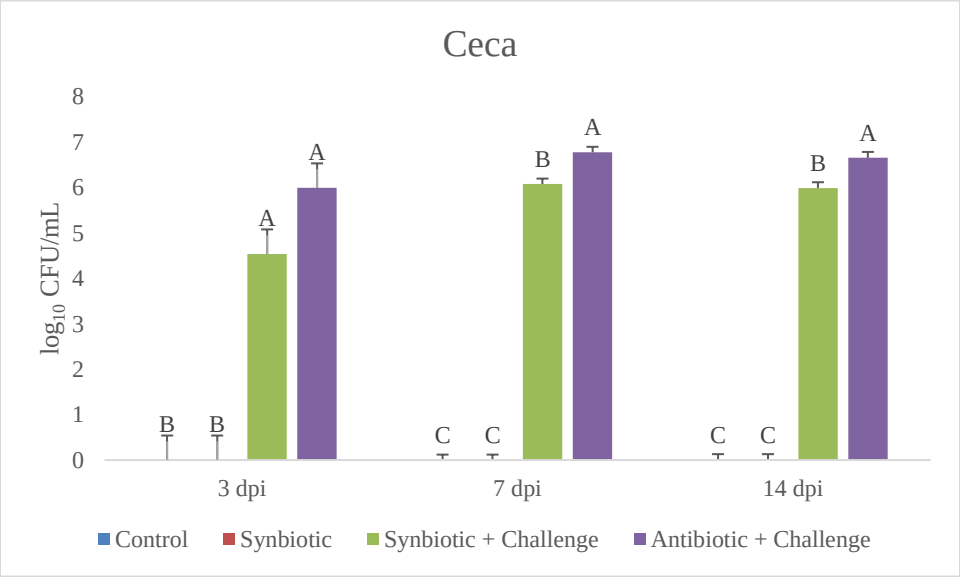
0-7	1.94	1.78	1.80	1.91	0.09	0.49
0-14	1.63	1.53	1.52	1.60	0.04	0.20
0-21	1.82	1.58	1.67	1.74	0.07	0.13
0-28	1.91	1.71	1.86	1.93	0.10	0.41
0-35	1.74	1.63	1.79	1.87	0.08	0.21

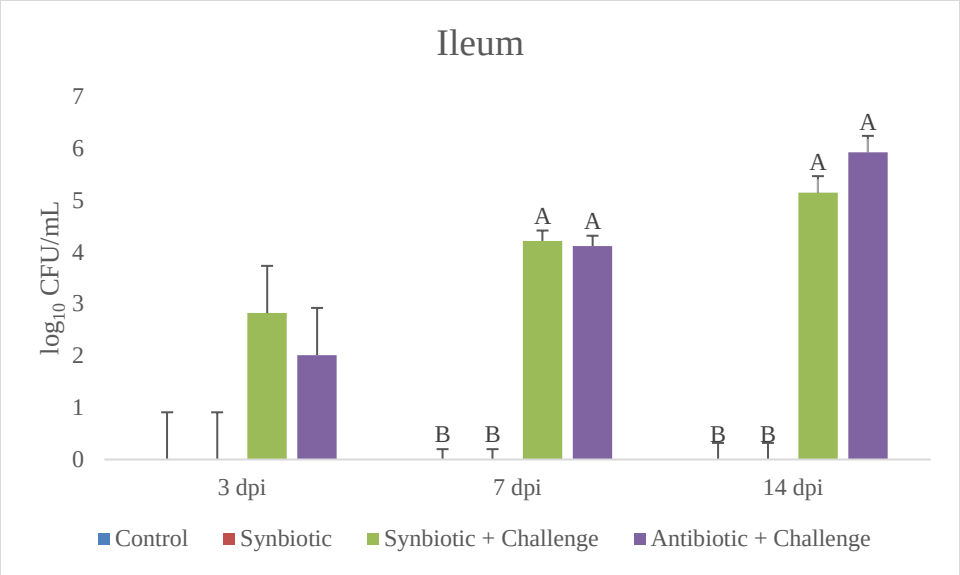
Table 4.3. Effect of synbiotic supplementation on production performance: Day old chicks were distributed into four treatment groups: Control, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge (n = 6). Birds in challenge groups received 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Antibiotics (Virginiamycin) and synbiotics (PoultryStar®) were supplemented in feed for the duration of the experiment at 20 mg/kg and 20 g/1000 birds/day respectively. Body weight and feed intake were measured on days 0, 7, 14, 21, 28, and 35 to calculate body weight gain and feed conversion ratio.

Effect of synbiotic supplementation on *Campylobacter* colonization in the ceca, ileum, and liver.

At 3 dpi, the cecal load of *Campylobacter* was significantly higher in the Antibiotic + Challenge and Synbiotic + Challenge groups than the Control and Synbiotic groups by 5.54 and 5.99 $\log_{10}$ CFU/mL, respectively ($P < 0.05$) (*Figure 4.1*). The ileal load of *Campylobacter* at 3 dpi was 2.82 $\log_{10}$ CFU/mL higher in the Synbiotic + Challenge and 2.01 $\log_{10}$ CFU/mL higher in the Antibiotic + Challenge groups ($P < 0.05$). At 7 dpi, *Campylobacter* load in the ceca was significantly greater in the Antibiotic + Challenge group than the Synbiotic + Challenge group by 0.70 $\log_{10}$ CFU/mL, in the Synbiotic + Challenge group compared to the Control and Synbiotic groups by 6.08 $\log_{10}$ CFU/mL, and in the Antibiotic + Control group compared to the Control group by 6.78 $\log_{10}$ CFU/mL ($P < 0.05$). In the ileum at 7 dpi, *Campylobacter* load was

significantly higher in the Synbiotic + Challenge and Antibiotic + Challenge groups compared to the Control and Synbiotic groups by 4.21 and 4.11 $\log_{10}$ CFU/mL, respectively ($P < 0.05$). In the ceca at 14 dpi, *Campylobacter* load was significantly greater in the Antibiotic + Challenge group than the Synbiotic + Challenge group by 0.67 $\log_{10}$ CFU/mL, in the Synbiotic + Challenge group compared to the Control and Synbiotic groups by 5.99 $\log_{10}$ CFU/mL, and in the Antibiotic + Control group compared to the Control group by 6.66 log10 CFU/mL ($P < 0.05$). At 14 dpi, the load of *Campylobacter* in the ileum was significantly greater in the Synbiotic + Challenge and Antibiotic + Challenge groups than the Control and Synbiotic groups by 5.14 and 5.92 $\log_{10}$ CFU/mL, respectively ($P < 0.05$). In the liver at 3, 7, and 14 dpi, there was no significant difference or increase in *Campylobacter* load, though levels in the Synbiotic + Challenge and Antibiotic + Challenge groups did rise, peaking at 7 dpi ($P < 0.05$).





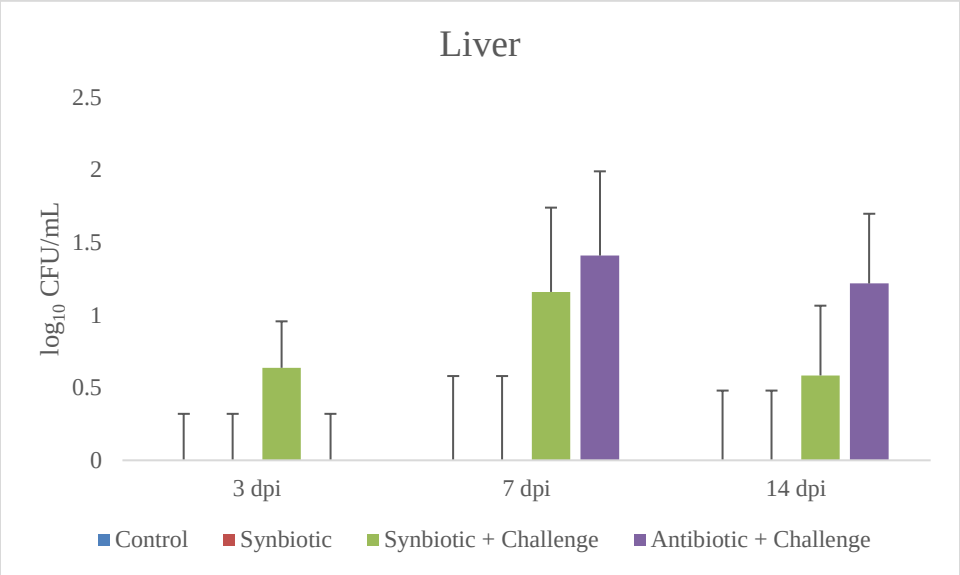


Figure 4.1. Effect of synbiotic supplementation on *Campylobacter* colonization in the ceca, ileum, and liver: Day old chicks were distributed into four treatment groups: Control, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge (n = 6). Birds in challenge groups received 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Antibiotics (Virginiamycin) and synbiotics (PoultryStar®) were supplemented in feed for the duration of the experiment at 20 mg/kg and 20 g/1000 birds/day respectively. Colonization of the ceca (a), ileum (b), and liver (c) were estimated using micro-dilutions (CFU/mL) and then log

transformed to log_{10} CFU/mL for statistical analysis. Results were expressed as mean + SEM. Means with no common superscript differ significantly ($P < 0.05$).

Effect of synbiotic supplementation on *mapA* gene expression.

Expression of *mapA* in the ileum and ceca was significantly higher in all challenged groups at 3, 7, and 14 dpi than in the Control and Synbiotic groups ($P < 0.05$) (*Figure 4.2*).

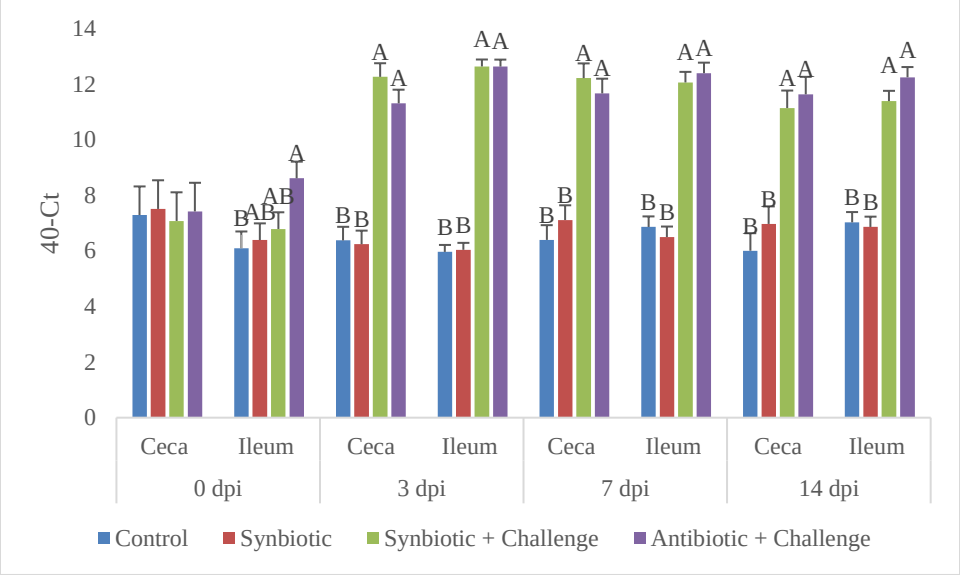


Figure 4.2. Effect of synbiotic supplementation on *mapA* gene expression: Day old chicks were distributed into four treatment groups: Control, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge (n = 6). Birds in challenge groups received 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Antibiotics (Virginiamycin) and synbiotics (PoultryStar®) were supplemented in feed for the duration of the experiment at 20 mg/kg and 20 g/1000 birds/day respectively. Cecal and ileal *C. jejuni* loads were quantified via SyBr green qPCR with primers designed to target the *mapA* gene of *C. jejuni*. Results were expressed as mean + SEM. Means with no common superscript differ significantly ($P < 0.05$).

Effect of synbiotic supplementation on gut permeability of FITC-d.

At 3, 7, and 14 dpi, intestinal permeability was insignificant between treatment groups (*Figure 4.3*) ($P > 0.05$).

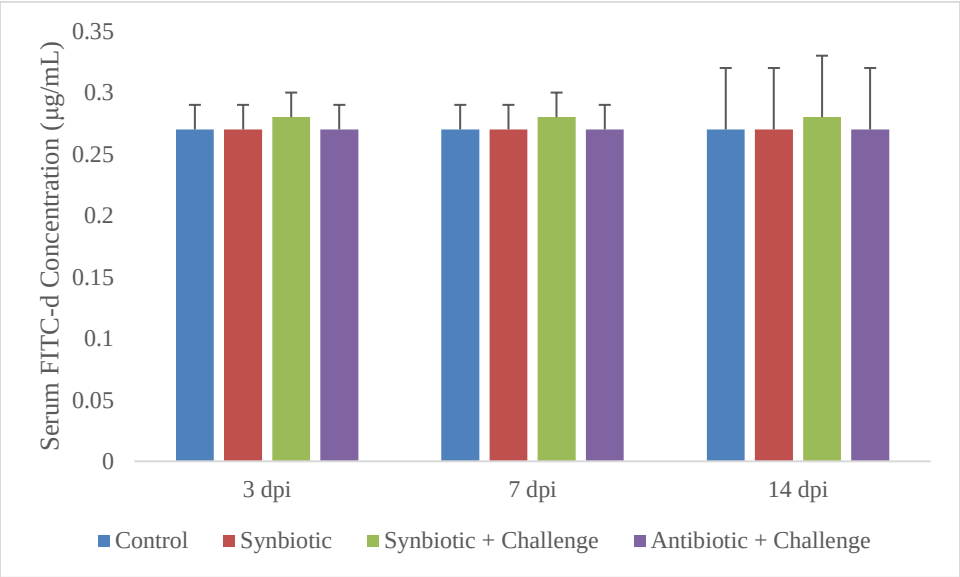


Figure 4.3. Effect of synbiotic supplementation on gut permeability of FITC-d: Day old chicks were distributed into four treatment groups: Control, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge (n = 6). Birds in challenge groups received 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Antibiotics (Virginiamycin) and synbiotics (PoultryStar®) were supplemented in feed for the duration of the experiment at 20 mg/kg and 20 g/1000 birds/day respectively. 1 mL of FITC-d was administered to each bird via oral gavage at 3, 7, and 14 dpi to measure intestinal permeability. Blood was collected and serum was analyzed for FITC-d concentration. Results were expressed as mean + SEM. Means with no common superscript differ significantly ($P < 0.05$).

Effect of synbiotic supplementation on bile anti-*C. jejuni* IgA antibody levels.

No significance was observed in bile anti-*C. jejuni* IgA antibody levels (P > 0.05) (*Figure 4.4*).

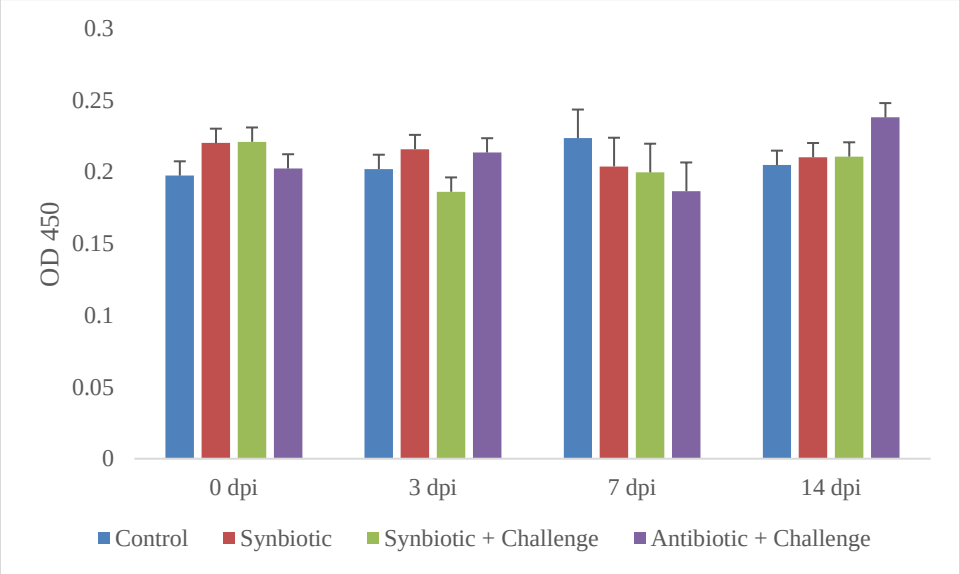
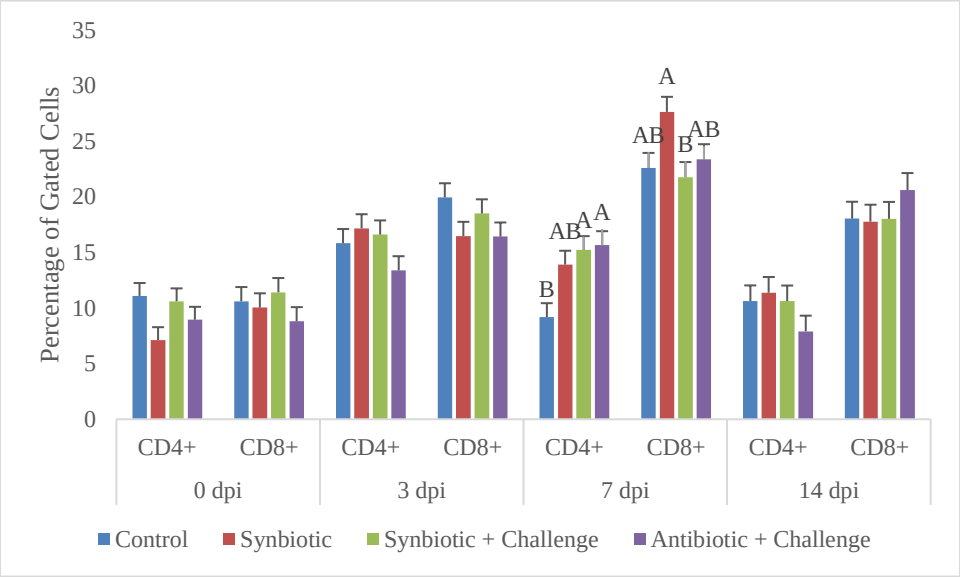


Figure 4.4. Effect of synbiotic supplementation on bile anti-*C. jejuni* IgA antibody levels: Day old chicks were distributed into four treatment groups: Control, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge (n = 6). Birds in challenge groups received 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Antibiotics (Virginiamycin) and synbiotics (PoultryStar®) were supplemented in feed for the duration of the experiment at 20 mg/kg and 20 g/1000 birds/day respectively. At 0, 3, 7, and 14 dpi, bile was collected, and anti-*C. jejuni* IgA antibodies were detected using direct enzyme-linked-immunosorbent assay (ELISA). Results were expressed as mean + SEM OD 450 values. Means with no common superscript differ significantly (P < 0.05).

Effect of synbiotic supplementation on cecal tonsil $CD4^+$:$CD8^+$ cell ratio.

Between 0 and 3 dpi, there was an increase in the percentage of gated $CD4^+$ and $CD8^+$ cells in all treatment groups (*Figure 4.5*). At 3 dpi, the percentage of gated cells was generally higher in $CD8^+$ than $CD4^+$. At 7 and 14 dpi, the percentages of $CD4^+$ cells in every treatment group were

lower than the percentages of gated $CD8^+$ cells. At 7 dpi, the Control group had the lowest percentage of $CD4^+$ gated cells and was significantly less than groups ($P < 0.05$). The $CD4^+$:$CD8^+$ ratio of the Synbiotic and Control groups at 7 dpi were statistically less than the ratios of the challenged groups. At 14 dpi, the Antibiotic + Challenge group had the lowest percent of gated $CD4^+$, highest percentage of gated $CD8^+$, and lowest $CD4^+$:$CD8^+$ ratio of all the treatment groups.



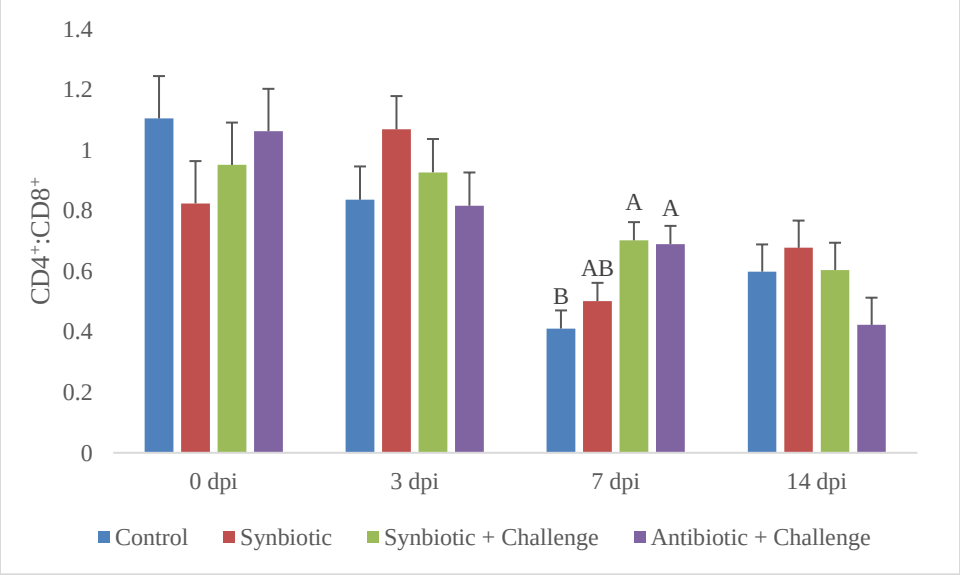
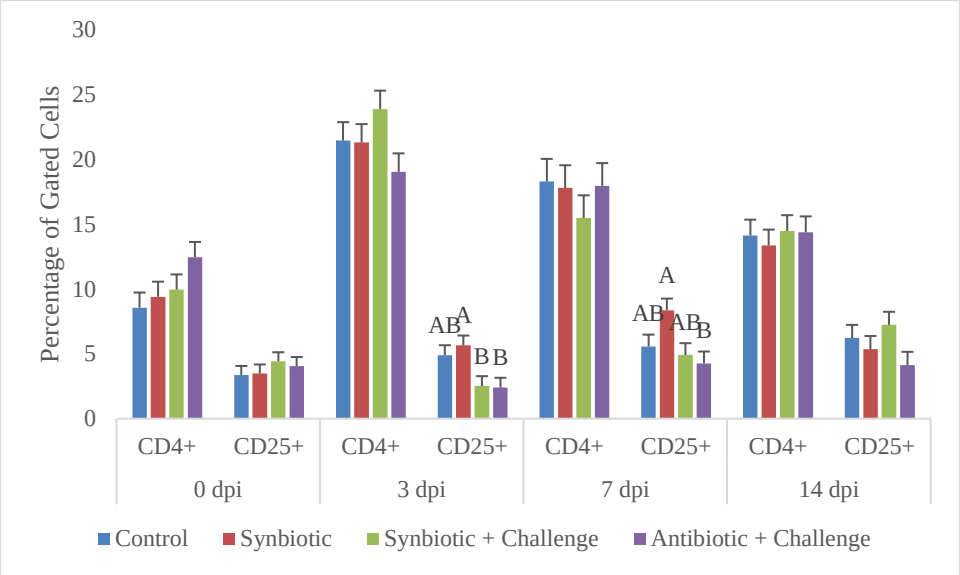


Figure 4.5. Effect of synbiotic supplementation on cecal tonsil $CD4^+$:$CD8^+$ cell ratio: Day old chicks were distributed into four treatment groups: Control, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge (n = 6). Birds in challenge groups received 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Antibiotics (Virginiamycin) and synbiotics (PoultryStar®) were supplemented in feed for the duration of the experiment at 20 mg/kg and 20 g/1000 birds/day respectively. Cecal tonsils were strained to single-cell suspensions (1×10^6 cells) and incubated with PE-conjugated mouse anti-chicken CD4 and FITC-conjugated mouse anti-chicken CD8 at 1:200 dilution, and unlabeled mouse IgG at 1:500 dilution in a 96-well plate for 20 minutes. (a) $CD4^+$ and $CD8^+$ cells were reported as percentage of gated cells and (b) $CD4^+$:$CD8^+$ ratio was calculated. Results were expressed as mean + SEM. Means with no common superscript differ significantly ($P < 0.05$).

Effect of synbiotic supplementation on cecal tonsil regulatory T cells, and $CD4^+$:T-reg cell ratio.

From 0 to 3 dpi, the percentage of gated $CD4^+$ cells increased then decreased on 7 and 14 dpi (*Figure 4.6*). At 3 and 7 dpi, the Synbiotic groups had statistically high percentages of gated T-regs, and the challenged groups were lower had lower percentages of gated T-regs than the Synbiotic and Control groups ($P < 0.05$). At 3 dpi, the $CD4^+$:$CD8^+$ ratios of the Synbiotic + Challenge and Antibiotic + Challenge groups were greater than the Synbiotic and Control groups by 7.16 and 1.83, respectively ($P > 0.05$).



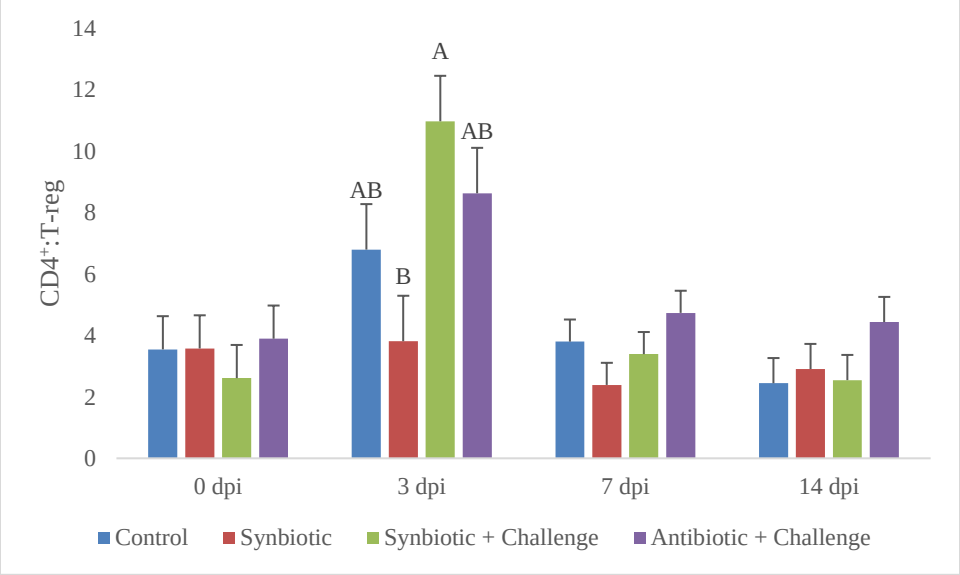
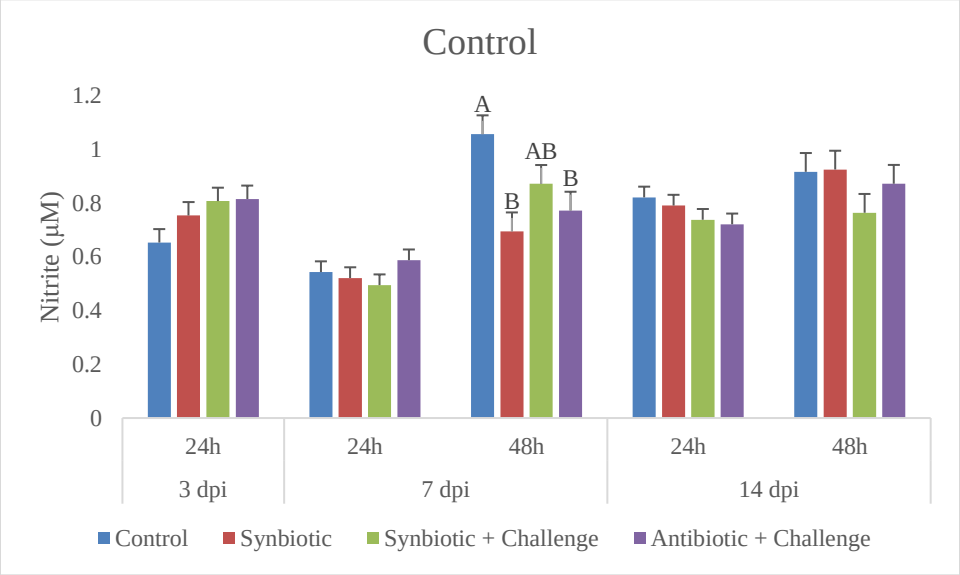


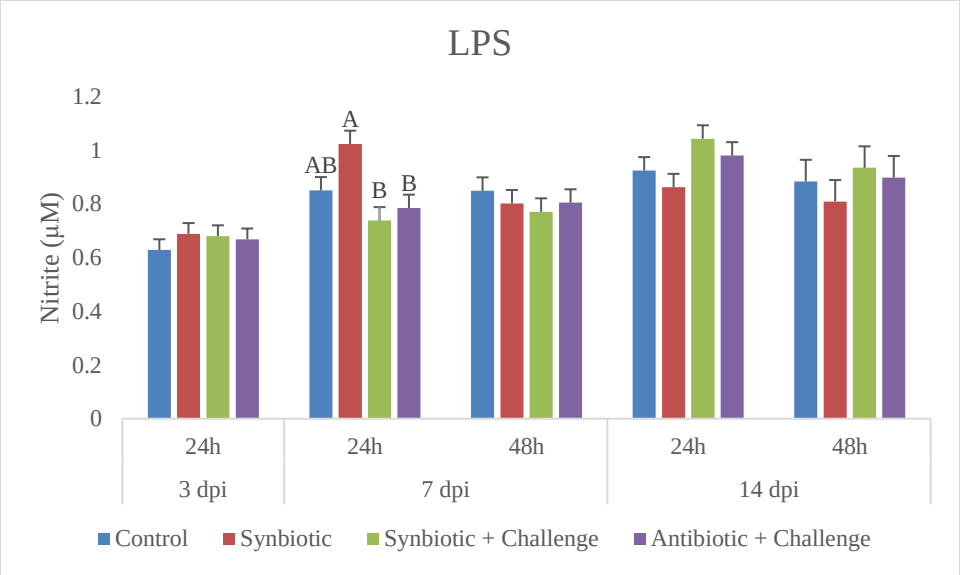
Figure 4.6. Effect of synbiotic supplementation on cecal tonsil regulatory T cells, and $CD4^+$:T-reg cell ratio: Day old chicks were distributed into four treatment groups: Control, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge (n = 6). Birds in challenge groups received 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Antibiotics (Virginiamycin) and synbiotics (PoultryStar®) were supplemented in feed for the duration of the experiment at 20 mg/kg and 20 g/1000 birds/day respectively. Cecal tonsils were strained to single-cell suspensions (1×10^6 cells) and incubated with $CD25^+$ and FITC-

conjugated mouse anti-chicken $CD4^+$ at 1:200 dilution, and unlabeled mouse IgG at 1:500 dilution in a 96-well plate for 20 minutes. (a) $CD4^+$ and T-reg cells were reported as percentage of gated cells and (b) $CD4^+$:T-reg ratio was calculated. Results were expressed as mean + SEM. Means with no common superscript differ significantly ($P < 0.05$).

Effect of synbiotic supplementation on nitric oxide production by adherent splenocyte mononuclear cells stimulated *ex vivo*.

At 7 dpi, nitrite production at 48 hours post-stimulation with *C. jejuni* in the Synbiotic + Challenge and Antibiotic + Challenge groups were significantly lower by 0.26 and 0.18, respectively, compared to the Synbiotic and Control groups ($P < 0.05$) (*Figure 4.7*).





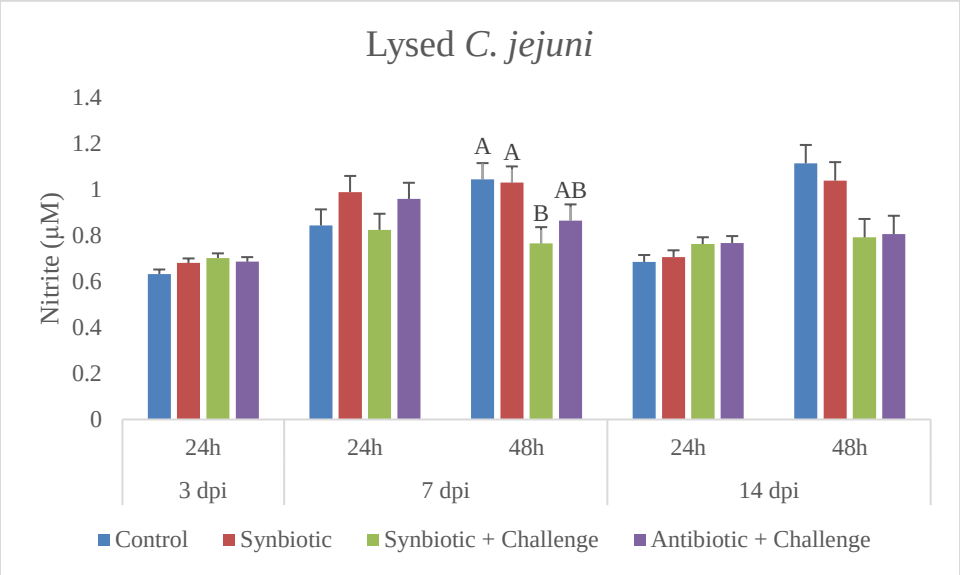
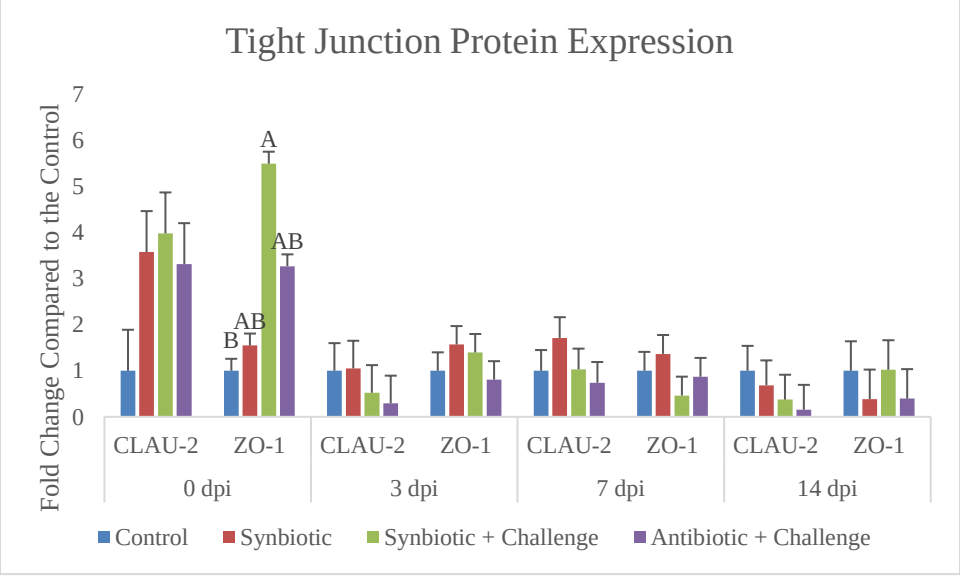


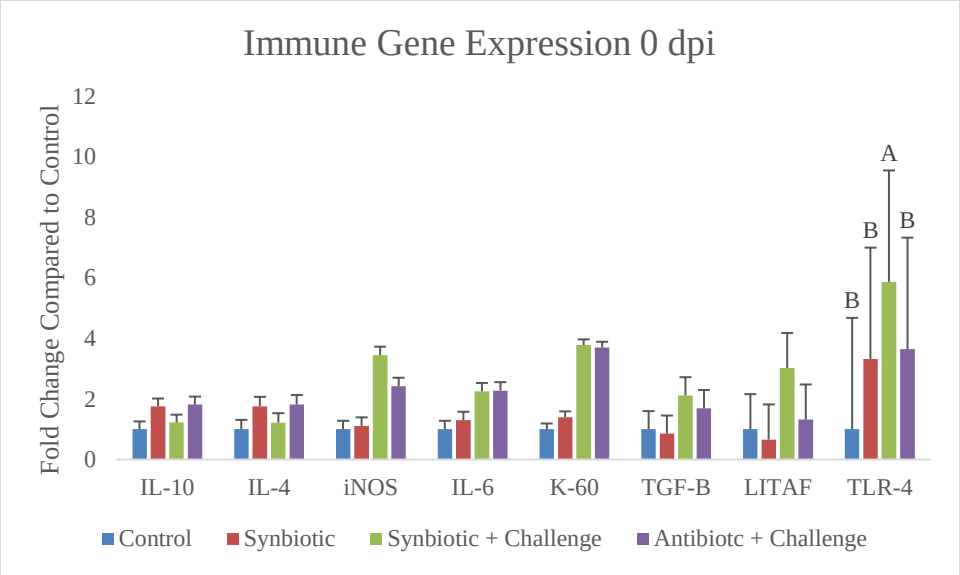
Figure 4.7. Effect of synbiotic supplementation on nitric oxide production by adherent splenocyte mononuclear cells stimulated *ex vivo*: Day old chicks were distributed into four treatment groups: Control, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge (n = 6). Birds in challenge groups received 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Antibiotics (Virginiamycin) and synbiotics (PoultryStar®) were supplemented in feed for the duration of the experiment at 20 mg/kg and 20 g/1000 birds/day respectively. Adherent splenocyte MNCs (5 x 10^4 cells) were stimulated with 10

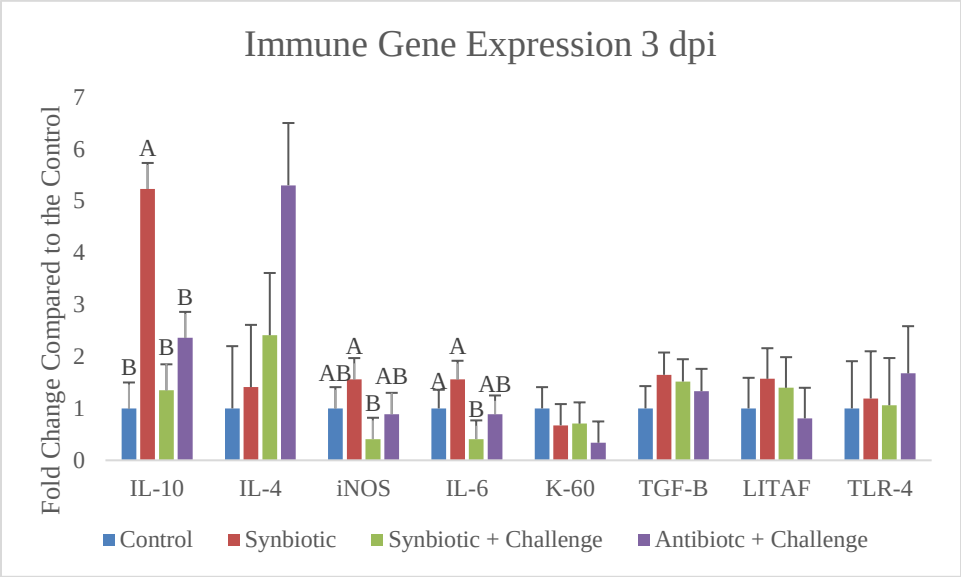
μg/mL of LPS or 20 μg/mL of lysed *C. jejuni*. After 48 h of stimulation, NO production was measuring using the Griess assay. Results were expressed as mean + SEM. Means with no common superscript differ significantly ($P < 0.05$).

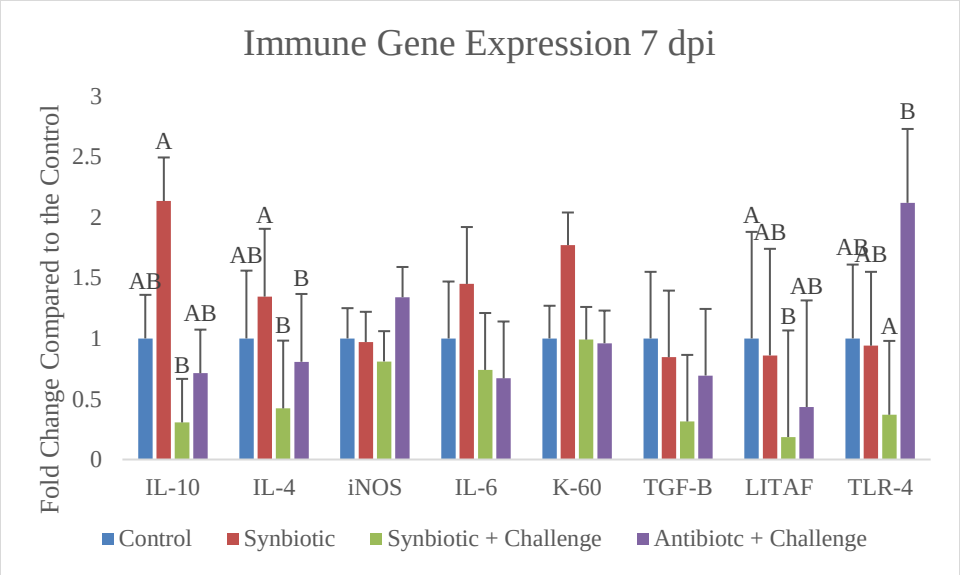
Effect of synbiotic supplementation on immune gene expression.

At 0 dpi, expression of TLR-4 was significantly high in the Synbiotic + Challenge group ($P < 0.05$) (*Figure 4.8*). iNOS and anti-inflammatory IL-10 expression at 3 dpi was significantly higher in the Synbiotic than in the Synbiotic + Challenge group ($P < 0.05$). Pro-inflammatory Il-6 expression at 3 dpi was significantly higher in the Control and Synbiotic groups than in the Antibiotic + Challenge and Synbiotic + Challenge groups, respectively ($P < 0.05$). 7 dpi, IL-10 expression was significantly higher in the Synbiotic than Synbiotic + Challenge group, while IL-4, LITAF, and TLR-4 were significantly higher in the Control and Synbiotic groups than in the Antibiotic + Challenge and Synbiotic + Challenge groups, respectively ($P < 0.05$).




Immune Gene Expression 0 dpi
Fold Change Compared to Control
12
10
8
6
4
2
0
A
B
B
B
IL-10
IL-4
iNOS
IL-6
K-60
TGF-B
LITAF
TLR-4
Control
Synbiotic
Synbiotic + Challenge
Antibiotc + Challenge


Immune Gene Expression 3 dpi
Fold Change Compared to the Control
7
6
5
4
3
2
1
0
A
B
B
B
AB
A
AB
A
A
B
AB
IL-10
IL-4
iNOS
IL-6
K-60
TGF-B
LITAF
TLR-4
Control
Synbiotic
Synbiotic + Challenge
Antibiotc + Challenge



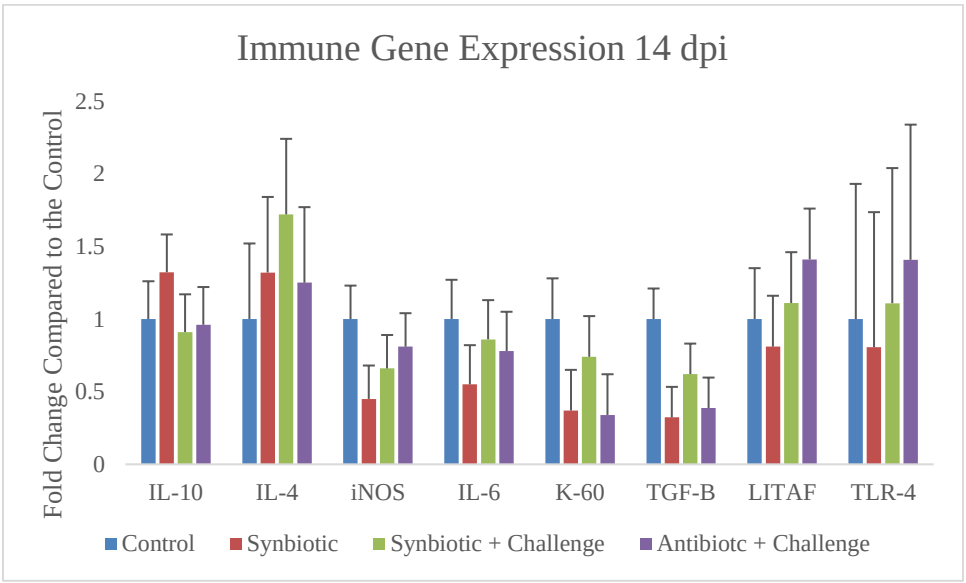


Figure 4.8. Effect of synbiotic supplementation on immune gene expression: Day old chicks were distributed into four treatment groups: Control, Synbiotic, Antibiotic + Challenge, and Synbiotic + Challenge (n = 6). Birds in challenge groups received 1 X 10^8 CFU/bird of *Campylobacter jejuni* or mock challenge with 1 mL of PBS via oral gavage. Antibiotics (Virginiamycin) and synbiotics (PoultryStar®) were supplemented in feed for the duration of the experiment at 20 mg/kg and 20 g/1000 birds/day respectively. Gene expression was determined by qPCR of cecal tonsil mRNA, converted to cDNA. Results were expressed as the mean + SEM

fold change in tissue mRNA levels in the challenge treatment as compared to the mock-challenged control treatment. Means with no common superscript differ significantly from the Control group ($P < 0.05$).

Discussion

This study aimed to characterize the effects of synbiotic supplementation in broilers challenged with *C. jejuni* strain 33560, with regards to cecal load and immune, microbiological, and performance parameters. The purpose of synbiotic supplementation in this trial was to evaluate their viability as an antibiotic growth promoter replacement. Synbiotics not only introduce beneficial bacteria to the gut, but they also selectively promote the proliferation of healthy gut microflora and modulate avian gut immune responses to challenge (Yang et al., 2009). Synbiotics have been shown in past works to decrease cecal *C. jejuni* load and total carcass load post-harvest, improve feed efficiency and body weight gains, positively modulate microbiome composition, decrease mortality, and improve carcass yields at processing (Abdel-Wareth et al., 2019; Ashayerizadeh *et al*., 2009; Awad et al., 2009; Baffoni et al., 2012). In our study, we saw no significance between treatment groups' BWG, FI, or FCR at any time point measured. These results are in contrast with past work that found synbiotic supplementation to improve production performance compared to antibiotic growth promoters in *C. jejuni* challenged broilers (Wahyuni et al., 2021). In the Wahyuni et al. study, the challenge dose was administered to the same breed of broiler on the same day of age, in birds grown out to an identical day of age. The differing performance parameter results between our two studies could be related to the growth environment, challenge dosage and strain, the type of synbiotic and antibiotic growth promoters used, the sexes of the broilers, or feed composition differences.

Colonization load of *C. jejuni* in the avian ceca has previously been directly linked to and positively correlated with the whole carcass *C. jejuni* load in poultry (Allen et al., 2007; Reich et al., 2008). In our study, *C. jejuni* colonization of the challenged groups was observed beginning at 3 dpi and persisting through the duration of the experiment in the ceca and ileum of the broilers ($P < 0.05$). Liver colonization was observed in the Synbiotic + Challenge group at 3 dpi but by 7 dpi was found in both challenge groups ($P < 0.05$). Progressive colonization of the liver by *C. jejuni* following challenge indicates the dissemination of the challenge from the gut to other internal organs and the establishment of a systemic infection. At 7 and 14 dpi in the ceca, *C. jejuni* load was significantly higher in the Antibiotic + Challenge group than in the Synbiotic + Challenge group ($P < 0.05$). Pre-challenge, PCR analysis of cecal *mapA* expression, which differentiates *C. jejuni* from *C. coli* infection, was consistent between all treatment groups (Begum et al., 2015). By 3, 7, and 14 dpi, *mapA* expression in the ceca and ileum was significantly higher in the challenged groups than in the Control and Synbiotic groups, supporting the direct plating results. The *mapA* results combined with our direct plating data confirm that our broilers were colonized with *C. jejuni* following challenge.

The non-digestible intestinal paracellular permeability marker FITC-d has long been used in broilers as a mechanism to monitor gut permeability over time following disease challenge (Liu et al., 2021). In this experiment, we did not find significance between treatment groups in FITC-d permeability from the intestinal tract to blood serum. In past studies, *C. jejuni* challenge has been found to degrade the tight junction proteins of the avian gut wall and to allow *C. jejuni* to escape the gastrointestinal tract and disseminate to other organs (Awad et al., 2016; Awad et al., 2020; Boehm et al., 2012). ZO-1 is a tight junction protein of the avian intestinal paracellular epithelium that critically regulates paracellular permeability and acts as a structural protein (Van

Itallie et al., 2009). Cecal ZO-1 expression was significantly elevated at 3 dpi in the Synbiotic + Challenge group, suggesting that synbiotic supplementation in the face of early infection with *C. jejuni* promotes tight junction functionality and expression ($P < 0.05$). Despite a lack of increased serum FITC-d concentration and decreased tight junction protein expression in this study, we found that dissemination of *C. jejuni* to the liver in challenged birds occurred as early as 3 dpi and endured until the conclusion of the trial at 14 dpi (day 35), providing definitive proof of gut permeability facilitating *C. jejuni* translocation.

IgA is the immunoglobulin of mucosal immunity produced by B-cells and responsible for protecting the mucus lined intestinal epithelium from colonization by pathogenic bacteria (Lacharme-Lora et al., 2017; Macpherson et al., 2008; Ohland & MacNaughton, 2010). In our study, anti-*C. jejuni* IgA concentration in the bile was consistent between all treatment groups at each timepoint measured. IgA is heavily involved in pathogen clearance and the lack of increased IgA in response to challenge could be responsible for facilitating the enduring colonization of *C. jejuni* in poultry and the lack of clinical manifestations of disease (Quinn et al., 2020). Past work done in other studies supports our findings that anti-*C. jejuni* IgA concentration in the bile was not affected by *C. jejuni* challenge (Hermans et al., 2014; Widders et al., 1996). In past studies, prebiotics and probiotics were found to upregulate IgA responses and to facilitate the colonization of the paracellular epithelium by beneficial bacteria (Donaldson et al., 2018; Xiao et al., 2019). Our lack of significance could be attributed to the synbiotic components we used, feed formulation, or the immune status of the birds.

Evaluating the $CD4^+$:$CD8^+$ cell ratio of broilers enables us to judge and quantify the immunocompetence level of the birds and to assign a value to the overall health of the birds (Char et al., 1990). In poultry, clinically healthy flocks are known to express a $CD4^+$:$CD8^+$ cell

ratio of around 0.95 at market age (Arthanari et al, 2012). In our birds, the $CD4^+$:$CD8^+$ cell ratio at 0 dpi ranged from 0.8 – 1.1 while at 14 dpi it had decreased to between 0.4 – 0.7. At 7 dpi, the percentage of gated $CD4^+$ was significantly higher in the Synbiotic + Challenge compared to the Synbiotic group, in the Antibiotic + Challenge compared to the Control group, and in the Synbiotic compared to the Control group ($P < 0.05$). The Synbiotic and Antibiotic + Challenge groups had significantly higher percentages of gated $CD8^+$ cells at 7 dpi than the Control and Synbiotic + Challenge groups, respectively ($P < 0.05$). The $CD4^+$:$CD8^+$ cell ratio at 7 dpi was significantly higher in the Synbiotic, Synbiotic + Challenge, and Antibiotic + Challenge groups compared to the Control, Synbiotic, and Control groups, respectively ($P < 0.05$). An elevated $CD4^+$:$CD8^+$ cell ratio under *C. jejuni* challenge has previously been shown to be related to *C. jejuni* dissemination to the liver and colonization persistence within both the liver and gastrointestinal tract (Vučković et al., 2006). Regulatory T cells are a subset of mature T lymphocytes with important immunosuppressive roles during disease and in immunotolerance (He et al., 2021; Piccioni et al., 2014). During infection, $CD4^+$:T-reg cell ratio is expected to be significantly higher in instances of disease, which supports our results at 3 dpi that the ratio was higher in the Synbiotic + Challenge group than the Synbiotic group ($P < 0.05$). Depressed T-reg expression in disease challenged treatments at 3 and 7 dpi is supported by past research ($P < 0.05$) (Ferreira et al., 2017).

Nitric oxide is a messaging molecule with critical functional roles in host cell-defense, pro-inflammatory response to pathogens, neurotransmission, and immune cell recruitment (Snyder & Bredt, 1992). iNOS is cytokine induced and expressed by T cells, macrophages, and dendritic cells to regulate immune cell function and differentiation induced by NO production (Xue et al, 2018). In this study, we found that NO production by adherent solenocyte

mononuclear cellular response to challenge *ex vivo* with LPS at 7 dpi was significantly lower in challenged groups than in non-challenged groups ($P < 0.05$). Also at 7 dpi, NO production in response to *C. jejuni* challenge was significantly lower in the Synbiotic + Challenge compared to the Synbiotic and Antibiotic + Challenge groups and in the Antibiotic + Challenge group than in the Control group ($P < 0.05$). At 3 dpi, iNOS expression in the cecal tonsils was significantly lower in the Synbiotic + Challenge group than in the Synbiotic group. These results together indicate that *C. jejuni* infection in broilers is poorly controlled by NO and iNOS expression, as supported by the findings of a past study (Elvers et al., 2004). This indicates a poor Th1 immune response to *C. jejuni* challenge by the broilers in this study.

TLR-4 is a sensing receptor for the lipopolysaccharide of gram-negative cells (Molteni et al., 2016). At 0 dpi, TLR-4 expression in the Synbiotic + Challenge group was significantly higher than in the other treatment groups ($P < 0.05$). Increased TLR-4 expression within the cecal tonsils could indicate a background, subclinical infection or stress in our broilers (Aster et al., 2021; Molteni et al., 2016). IL-10 is an anti-inflammatory cytokine that has been proposed as a mediator of tolerance to *C. jejuni* infection in poultry (Humphrey et al., 2014; John et al, 2017). IL-6 is a pro-inflammatory cytokine that is critical to the acute phase response by host immune cells to infection (Gabay, 2006). At 3 dpi, IL-10 and IL-6 expression within the cecal tonsils was significantly higher in the Synbiotic group than in the Synbiotic + Challenge group while IL-6 expression in the Antibiotic + Challenge group was significantly lower than the Control and significantly higher than the Synbiotic + Challenge group ($P < 0.05$). This indicates that the immune system of the broilers in our study is actively responding to the challenge while balancing pro- and anti-inflammatory cytokine and Th1 and Th2 responses. IL-4 is a cytokine responsible for inducing helper T and B cell differentiation and activation, B cell class switching

to IgE, and regulation of MHC class II (Hershey et al., 1997). At 7 dpi, IL-4 expression was significantly higher in the Synbiotic group and significantly low in the challenged treatments ($P < 0.05$). Anti-inflammatory IL-10 and IL-4 cytokine expression at 7 dpi was significantly high in the Synbiotic group and significantly low in the Synbiotic + Challenge group ($P < 0.05$). TLR-4 expression 7 dpi was significantly high in the Antibiotic + Challenge group and significantly low in the Synbiotic + Challenge group ($P < 0.05$). LITAF is a gene responsible for regulating autophagy in B cells and lysosomal degradation of proteins (Myokai et al., 1999). At 7 dpi, LITAF expression was significantly lower in the Synbiotic + Challenge and Antibiotic + Challenge groups ($P < 0.05$). A combined decrease in LITAF and pro-inflammatory cytokine expression within the synbiotic supplemented groups has previously been observed (Lin et al., 2008; Markazi et al., 2018).

Conclusions

In this study, we found that synbiotic supplementation to *C. jejuni* challenged broilers had no effect on performance parameters, FITC-d permeability of the gastrointestinal tract, tight junction protein expression, or anti-*C. jejuni* IgA concentration within the bile. Cecal colonization with *C. jejuni* was significantly lower in Synbiotic + Challenge treated birds than in the Antibiotic + Challenge group with the $CD4^+$:T-reg cell ratio at 3 dpi being significantly elevated in the Synbiotic + Challenge group compared to the Antibiotic + Challenge group. Immunomodulation occurred in the form of altered cellular differentiation genes, pro-, and anti-inflammatory cytokine expression within the cecal tonsils following challenge in the synbiotic treated group. Our study concludes that synbiotic supplementation decreases *C. jejuni* loads in broilers and beneficially modulates the immune response to challenge, compared to antibiotic

supplementation. We propose that synbiotics can be applied as a nutritional supplement to replace antibiotic growth promoter usage during *C. jejuni* infections of poultry and during specific pathogen free broiler production.

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CHAPTER 5

CONCLUSIONS

In Immune Responses, Gut Permeability, and Cecal *Campylobacter jejuni* Loads in an Experimental *C. jejuni* Challenge Model in Broilers, we found that production performance parameters, FITC-dextran permeability through the gastrointestinal tract, and anti-*C. jejuni* IgA bile concentration were not effected by challenge dosage and did not statistically differ between the 1 X 10^4 and 1 X 10^8 CFU/bird *C. jejuni* treatment groups. It was found that the cecal load of *C. jejuni* increased significantly in a dose dependent manner, along with the $CD4^+$:$CD8^+$ cell ratio, in the 1 X 10^8 CFU/bird *C. jejuni* group, compared to the 1 X 10^4 CFU/bird *C. jejuni* group. The expression of tight junction proteins within the cecal tonsils was downregulated with challenge while the expression of both pro- and anti-inflammatory cytokines was upregulated. We conclude that birds could successfully be challenged with *C. jejuni* at 21 days of age and that 1 X 10^8 CFU/bird *C. jejuni* challenge dosage was an effective challenge dose to colonize the ceca of broilers and additionally to elicit microbiological and immunological responses to challenge.

In Effects of Synbiotic Supplementation as an Antibiotic Growth Promoter Replacement on Cecal *Campylobacter jejuni* Load in Broilers Challenged with *C. jejuni*, we found that the supplementation of a synbiotic or antimicrobial growth promoter (AGP) to broilers challenged with 1 X 10^8 CFU/bird of *C. jejuni* did not impact production performance parameters, FITC-d permeability of the gastrointestinal tract, or anti-*C. jejuni* IgA concentration in the bile.

Colonization within the ceca, which is reflective of whole bird *C. jejuni* load, was significantly lower in the synbiotic supplemented birds, compared to the AGP supplemented birds at 7 and 14 dpi. At 3 dpi, the $CD4^+$:T-reg cell ratio was significantly elevated in the Synbiotic + Challenge group compared to the Antibiotic + Challenge group. The expression of immune cell differentiation genes, as well as pro- and anti-inflammatory cytokine expression, was additionally modulated by synbiotic supplementation. Our study found that synbiotic supplementation contributes to decreasing *C. jejuni* loads in broiler ceca and beneficially modulates the immune response to challenge, compared to antibiotic supplementation. We conclude that synbiotics can be applied as a nutritional supplement to replace AGP usage during *C. jejuni* infections of poultry and during broiler production without *C. jejuni* challenge.