EVALUATING THE IMMUNE RESPONSE AND PERFORMANCE PARAMETERS OF BROILERS TO *BACILLUS SUBTILIS* AND MANNAN OLIGOSACCHARIDES AND ASSESSING THEIR EFFICACY IN REDUCING NECROTIC ENTERITIS IN BROILERS

By

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(Under the Direction of RAMESH SELVARAJ)

ABSTRACT

Necrotic enteritis (NE) is one of the major enterotoxemic diseases in poultry. *In vitro* and *in vivo* studies have been performed to determine the effectiveness of *Bacillus subtilis* (*B. subtilis*) and mannan oligosaccharides (MOS) in challenged and unchallenged birds with necrotic enteritis. In vitro assays were done to determine the survival ability of *B. subtilis* in different gastric conditions. *B. subtilis* was able to survive in acidic conditions ($P < 0.05$) and was able to proliferate in 1.5% bile salt concentrations ($P > 0.05$). In the *in vivo* experiment, supplementation of feed with 0.1% *B. subtilis* and 0.1% MOS was able to increase enteric *Bifidobacterium animalis* and *Lactobacillus reuteri* ($P < 0.05$). Reduction in feed conversion ratio and increased body weight gain were observed in birds supplemented with *B. subtilis* ($P < 0.05$). In experiment 2, there was a decrease in intestinal permeability in birds fed *B. subtilis* challenged with NE ($P < 0.05$). Increased mRNA levels of zonula occluden ($P > 0.05$) and claudin 1 ($P < 0.05$) was observed in the ileum of the birds supplemented with *B. subtilis*. The upregulation of anti-inflammatory and downregulation of pro-inflammatory cytokines was observed in birds fed with *B. subtilis* and MOS. An increase in

IgA antibody levels was observed in the challenged birds with MOS ($P < 0.05$). Our studies found that *B. subtilis* and MOS supplementation increased the proliferation of *Lactobacillus reuteri* and *Bifidobacterium animalis. B. subtilis* and MOS can be used to reduce the lesions caused by NE and stimulate the immune responses in birds induced with NE.

INDEX WORDS: *Clostridium perfringens*, Mannan oligosaccharide, Necrotic enteritis, Immune responses

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DEDICATION

I dedicate this dissertation to my dear father, Janardhana Reddy Reddyvari, mother, Nageswaramma Guddeti, and my brother, Joseph Prem Swaroop Reddy Reddyvari. Thank you for your inspiration, support, and unconditional love.

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CHAPTER 1

INTRODUCTION

The occurrence and severity of outbreaks for necrotic enteritis (NE) are influenced by many unknown factors, making it a complex disease. Many predisposing factors lead to NE development, such as nutrition, coccidiosis, poor management, stress, and pathogenic *Clostridium perfringens* (*C. perfringens*) strains (Waeyenberghe et al., 2016). Recent years have seen NE infections worsened by the withdrawal of antimicrobial growth promoters and ionophore coccidiostats. NE occurs in both acute clinical and subclinical forms. Both acute clinical and subclinical conditions lead to huge economic losses. In the acute clinical form, an increase in mortality is observed. There is less mortality in subclinical form, and signs like reduced weight gain, bloody diarrhea, and depression are noticed. NE typically occurs around the age of 2 to 6 weeks as maternal antibodies begin to decrease over this time. There are no efficient strategies to control NE yet. Some of the alternatives to antibiotics in controlling NE are bacteriophages, probiotics, prebiotics, organic acids, enzymes, and vaccines.

In current years, the use of probiotics and prebiotics in feed has attracted a lot of attention. *Lactobacillus reuteri* and *Bifidobacterium animalis* are beneficial microflora present in the gut. *Lactobacillus reuteri* (Tavella et al. 2018) and *Bifidobacterium animalis* (Elbaz 2020) can inhibit the pathogenic bacteria's growth and modulate the immune system. Probiotics and prebiotics are able to increase the growth of the beneficial bacteria in the gut. Different research studies have been conducted to evaluate the effect of probiotics and prebiotics in reducing NE. Many studies conducted with probiotics and prebiotics have reported decreasing lesions and mortality (Hofacre

et al. 2003, Guo et al., 2017). *Bacillus subtilis* is a spore-forming bacteria and is able to secrete bacteriocins and short-chain fatty acids. Mannan oligosaccharide is a yeast cell wall extract consisting of β-glucans and mannans that help stimulate the immune system (Sadeq et al., 2015). The broad objective was to assess the efficacy of *Bacillus subtilis* and mannan oligosaccharide in reducing NE in broilers. Our specific objectives were

Aim 1: *Bacillus subtilis in-vitro* characterization and effects of *B. subtilis* and Mannan oligosaccharide on performance parameters, immune responses, *Lactobacillus reuteri* and *Bifidobacterium animalis* load in broilers

Aim 2: Effects of *Bacillus Subtilis* and mannan oligosaccharide supplementation on decreasing necrotic enteritis severity in broilers induced with necrotic enteritis

CHAPTER 2

LITERATURE REVIEW

Review of *Clostridium perfringens* in commercial poultry

Introduction

Clostridium perfringens (C. perfringens) is a common bacterial pathogen found in many enteric diseases of animals (Chalmers et al. 2008). *C. perfringens* is classified based on its ability to produce toxins labeled from A to E (Mwangi et al. 2018). *C. perfringens* is one of the primary sources of foodborne illnesses. *C. perfringens* have the shortest generation time, which is around 7 to 8 minutes, which allows the bacteria to spread more rapidly (Labbé and Juneja 2013).

Clostridium perfringens as a foodborne pathogen

C. perfringens is one of the major causes of foodborne illness in developed countries. In the US, it ranks second next to *Salmonella* in the estimated annual number of cases due to bacterial pathogens (Labbé and Juneja 2000). *C. perfringens* is expected to cause 26% of food-related bacterial illness (Scharff 2012). In 1968, 28% of the food poisoning cases were due to *C. perfringens,* 49% of the cases were due to *Salmonella, Staphylococcus, Shigella, and Clostridium botulinum* combined (Duncan 1969). From 1998 to 2010, there were many foodborne outbreaks and illnesses between 1998 to 2010, out of which 92% of these outbreaks were from beef and poultry meat. People around 20 to 49 age group were affected (Grass et al., 2013). Food poisoning due to *C. perfringens* is due to the consumption of contaminated meat and poultry products. The *C. perfringens* spores in raw or cooked meat germinate under anaerobic conditions, which upon ingestion, sporulate and cause the release of enterotoxin, which causes disease in humans (Grass

et al. 2013). The infective dose for *C. perfringens* is 100,000 to 1,000,000 cells/spores per gram of food (FDA 2012).

Clostridium perfringens characteristics

Clostridium perfringens is an anaerobic, Gram-positive, endospore-forming bacterial pathogen (Brynestad and Granum 2002). Spores are tough dormant structures, which can survive at high temperatures (Schneider et al. 2014) while another competing flora is killed (Brynestad and Granum 2002). It was found that *C. perfringens* is closest relative to *Clostridium pasteurianum* based on analyses of 16s rRNA (Labbé and Juneja 2000). Soil and intestinal tracts of humans and other warm-blooded animals are the natural habitats of *C. perfringens. C. perfringens* vegetative cells can grow at 15 to 50 ^{o} C (Grass et al. 2013). *C. perfringens* appears slightly opaque and shiny colonies, which usually show dual-zone hemolysis on agar plates (Brynestad and Granum 2002). Spores, if exposed to low cooking temperatures and then allowed to cool at temperatures between 54^{o} F (12^{o} C) and 140^{o} F (60^{o} C) for several hours, can change spores into potentially harmful vegetative cells (Schneider et al. 2014). Usually, *C. perfringens* spores are able to grow at a higher pH (pH 9 compared to 7.5). Higher salt concentrations, lower temperatures, and pH cause an inhibitory effect on *C. perfringens* (Doyle and Ph 2002). *C. perfringens* is able to resist a temperature of 100 ^{o}C for 30 mins (Robert and Sanitary 1962). Cultural characteristics of *C. perfringens* include reduction of nitrate, liquefaction of gelatin, and fermentation of lactose. The generation time for *C. perfringens* is reported to be 20 min between 33 and 49 ^{o}C (Labbé and Juneja 2000).

Classification of *Clostridium perfringens* based on toxins

The virulence of *C. perfringens* is majorly due to its toxins. *C. perfringens* is classified into five toxin types from A to E with four significant toxins, namely alpha (CPA), beta

(CPB), epsilon (ETX), and iota (ITX) with 16 toxins in various combinations (Songer 1997). Apart from these toxins, some *C. perfringens* strains produce additional toxins likes *C. perfringens* enterotoxin (CPE) or necrotic enteritis B-like toxin (NetB), which were identified later (Li et al. 2013). It was stated that the beta toxin (pore-forming toxin) produced by *C. perfringens* is related to alpha-toxin of *Staphylococcus aureus.* Earlier it was believed that the main virulent toxin in chickens was alpha-toxin, but now studies have proved that NetB toxin is a critical virulent toxin (Keyburn et al. 2008). *C. perfringens* type A, which is positive for CPE toxin, causes major foodborne poisoning outbreaks (Li et al. 2013). *C. perfringens* strains A, C, and D have a primary amino acid sequence identical to CPE protein. *C. perfringens* types B and D produces a neurotoxin called epsilon, which causes edema of lungs and brain damage and enterotoxaemia in ruminants (Tironi-farinatti et al. 2009)

Necrotic enteritis in poultry

Necrotic enteritis (NE) is predominantly caused by *C. perfringens* type A in poultry. Necrotic enteritis is an enteric disease which causes hemorrhages and perforations in the intestinal tract of chickens. Parish first described necrotic enteritis in 1961. The worldwide losses of NE are over $US 2 billion (Keyburn et al. 2008). Toxins such as alpha-toxin (Van Immerseel et al. 2004) and newly identified NetB toxins constitute the main factors for NE occurrence (Keyburn et al. 2008). There are several predisposing factors for the emergence of NE in poultry, such as feed and water contamination with *C. perfringens*, a diet high in protein, non-starch polysaccharides, high-quality fish meal, increased viscosity in the gut (Van Immerseel et al. 2004). Concurrent infection with coccidia like *Eimeria necatrix, Eimeria maxima, or Eimeria acervulina* also plays an essential role in the induction of NE in chickens. The caecum is the leading site of *C. perfringens* colonization, and it carries diverse microbiota. Thus, if GIT microbiota is impaired by NE

predisposing factors (fish meal and Eimeria), these effects are likely to be seen in the cecum (Wu et al. 2014). It was noted that there was a low percentage of mortality in birds infected with *C. perfringens* alone compared to birds with coccidia infection (Al-Sheikhly and Al-Saieg 1980). Normal *C. perfringens* count in healthy broilers ranges between 10^0 to 10^5 colony forming units /gram (CFU/g) of intestinal contents. In contrast, in birds affected with necrotic enteritis, the load of C. perfringens ranges from 10^6 to 10^8 CFU/g of intestinal contents (Long et al. 1974).

NE typically occurs in birds 14 to 60 days old, with more cases occurring in 3 weeks old birds (S. Bryant and Helmboldt 1971, Long 1973). *C. perfringens* induces NE in poultry, both clinical and subclinical forms (Songer 1997). The mortality rate is typically high in clinical per acute way and death occurs within 1 to 2 hours, unlike in subclinical form where there are no evident clinical symptoms and no peak mortality (Timbermont et al. 2011). In subclinical form, damage to the intestinal mucosa, diminished weight gain, decreased digestion, and absorption is observed. Common lesions are usually seen in the lower part of the small intestine and occasionally in organs like the kidney and liver (Kaldhusdal et al. 2001). The intestine might be filled with gas, and the mucosa would be typically covered with a diphtheritic membrane devoid of the villi tips (S. Bryant and Helmboldt 1971). Due to liver colonization with a high number of *C. perfringens*, cholangiohepatitis could be seen during subclinical infection (Kaldhusdal et al. 2001).

MECHANISM OF INFECTION

Introduction

C. perfringens is a serious pathogen that produces histotoxic, enteric, or enterotoxemic diseases (Navarro and Mcclane 2018). It is essential to know the mechanism of action to find the best way to intervene with its invasion.

Mechanism of CPE toxin

Initially, *C. perfringens* involves binding to the receptor on the plasma membrane of target cells, accompanied by activation of intracellular pathways and various cytopathic effects that finally lead to cell death (Navarro and Mcclane 2018). After completing sporulation and lysis of cells in the intestine, CPE is released into the lumen and binds to the claudin receptors present at the apical tips of intestinal villi (Chen and Ma 2014). After CPE is bound to the receptor, it oligomerizes into hexameric pores and then extends to a β-hairpin loop. It forms a β-barrel, which extends to the membrane and forms an active pore (Robertson et al. 2007), allowing Ca^{2+} influx into cells and a K^{+} efflux from cells (Freedman et al., 2016). The influx of Ca^{2+} stimulates calpain and calmodulin activation, leading to the activation of two specific cell death pathways, i.e., apoptosis and necrosis (Freedman et al., 2018).

Mechanism of Alpha toxin

Alpha toxin belongs to the phospholipase C (PLC) family that can degrade the eukaryotic membrane components phosphatidylcholine (PC) and sphingomyelin (SM) (Titball 1993). The alpha-toxin has two biologically active domains: an N terminal α helical domain and a C terminal β sandwich domain (Li et al. 2013). The N-terminal domain contains phospholipase C's active site, and the C-terminal domain is involved in membrane binding, cytolytic, and toxic activity (Oda et al., 2015). The toxin causes leakage of carboxyfluorescein (CF) and phosphorylcholine's secretion from PC or SM composed liposomes, which leads to membrane damage. The toxin's membrane damaging potential depends on the fluidity, choline-containing phospholipids, cholesterol (Nagahama et al. 1996), and penetration of the C domain into the lipid bilayer membrane (Jun Sakurai et al. 2004). Studies in rabbits have shown that this toxin induces erythrocyte hemolysis by activating PIP2-specific phospholipase C and/or phospholipase D in the erythrocyte membranes

(Sakurai et al. 1993). *C. perfringens* is capable of escaping macrophages in host tissues via membrane disruption effects of perfringolysin O (PFO) and alpha-toxin (PLC) (Brien and Melville 2004). The alpha-toxin has been shown to cause an increase in phosphatidic acid in rats erythrocytes leading to thrombus formation, which eventually leads to ischemia and tissue necrosis (J. Sakurai et al. 1993). The alpha-toxin causes leukostasis and impairs blood supply to the infected tissue, causing anoxia and tissue necrosis (A. E. Bryant et al.).

Mechanism of NetB toxin

The first step in colonization involves the adhesion of bacteria to an epithelial cell-mediated by fimbriae and non-fimbriae appendices (Parker and Sperandio 2009). It was shown that there was an increase in neuraminidase and toxin production when *C. perfringens* strains adhere to the cells (Mcclane and Mcclane 2010). Many studies have shown that NetB is a β pore-forming toxin closely related to the alpha-hemolysin toxin family. NetB consists of 16 β strands and an α helix. The rim region comprises four-stranded antiparallel β sheets and a loop that is involved in the membrane recognition and binding to cells. R230 and W287 residues on the NetB rim domain and S254 residues are postulated to play a role in forming oligomers on the phospholipid bilayer (Yan et al., 2013). The levels of cholesterol affected the performance of the NetB toxin. NetB had shown that the addition of cholesterol by 50 mole% increased the oligomerization by 10-fold. The presence of cholesterol increases the efficiency of NetB toxin (Savva et al., 2013). *In vitro* studies showed that NetB was cytotoxic to Leghorn male hepatoma (LMH) cells, and the strains which do not produce NetB are not toxic to LMH (Keyburn et al. 2008). The exact mechanism of NetB in inducing NE in chickens is yet to be known.

Role of Coccidia in NE

Certain common factors like high wheat in feed, high moisture, etc., can worsen both NE and coccidiosis. The causative agents for both diseases share common sites in the poultry gut, so interactions between the two diseases are predicted (Branton et al. 1987). *Eimeria*-aided induction of NE can be associated with damage done to epithelium releasing serum and other nutrients which help in the colonization and proliferation of *C. perfringens* in the broiler gut (Wu et al. 2014). As the increase in mucus secretion creates an anaerobic environment, it promotes the growth of *C. perfringens*. Studies have shown that coccidia-infected birds have increased the number of goblet cells, mucin secretion, which in turn has favored the growth of *C. perfringens* and increased alpha-toxin levels (Collier et al. 2008). NE lesions are generally seen in regions that are affected by various coccidia species. In *E. necatrix* inoculated birds, the lesion scores and *C. perfringens* load were substantially higher in the jejunum and duodenum than in uninoculated birds (Ikemoto et al. 1997).

Table 1: Eimeria and their location in different parts of the intestine (Pandey 2018)

Eimeria	Location
E. acervuline	Duodenum, Jejunum
E. brunetti	Ileum, Rectum
E. maxima	Duodenum, Jejunum, Ileum
E. necatrix	Jejunum, Ceca
E. tenella	Ceca

PREBIOTICS IN COMMERCIAL POULTRY

Introduction

Prebiotics are defined as "a non-digestible feed ingredient that favors the growth of beneficial bacteria in the colon (Gibson and Roberfroid 1995). Prebiotics are one of the several approaches used as alternatives to antibiotics in animal feed.

Prebiotics

Prebiotics contribute to the development of beneficial microbiota like *Bifidobacteria* and/or *lactobacilli* and exert potential health-promoting effects at the cost of harmful pathogens (Hajati and Rezaei 2010). Prebiotics usually consist of oligosaccharides, which have proven effective in stimulating the beneficial growth of bacteria within the gastrointestinal (GI) tract (Gibson et al. 2003). To be considered prebiotic, a food ingredient must i) not be hydrolyzed or absorbed in the proximal part of the alimentary tube, ii) be a suitable substrate for one or a small number of beneficial colon bacteria, iii) be able to alter colonic flora in favor of healthier composition iv) induce beneficial luminal or systemic effects on host health (Gibson and Manning 2004).

Prebiotic oligosaccharides are capable of supplying the requisite energy to specific species of bacteria that are capable of producing lactic, acetate, and other organic short-chain acids. They are primarily mannan oligosaccharides (MOS), FOS (including short-chain fructooligosaccharides, scFOS), and trans-galactooligosaccharides (TOS, including galactooligosaccharides, GOS) (Hélène et al. 2011). Prebiotics help reduces the growth of certain pathogenic bacteria like clostridia, Bacteroides, and enterococci by preventing their attachment to the intestinal mucosa (Biggs et al. 2007). Fructooligosaccharides (FOS) help reduce the load of *C. perfringens* in broilers (Bortoluzzi et al., 2019).

One of the functional benefits of prebiotics includes reducing gut pH, which aids in mineral absorption and enhances nutritional absorption (Hélène et al., 2011). Animal studies have shown that prebiotics such as inulin and oligofructose have improved the absorption of calcium and magnesium from the colon and reduced calcium loss from bone tissue (Greger 1999). There are various ways to introduce prebiotics into the avian gastrointestinal tract; however, prebiotics must be administered to an animal as early as possible in life to achieve the desired efficacy. In-fed or in-water supplementation was typically used in the first hours/days after hatching (Biggs et al. 2007). Research studies suggest that prolonged and costly in-water supplementation of broiler chickens with these bioactive compounds can be replaced with *in ovo* prebiotic delivery route. *In ovo,* prebiotic delivery on embryonic day 12 has shown an increase in the number of *Bifidobacteria* in newly hatched chicks (Bednarczyk et al., 2016).

Mechanism of action

Laboratory animal and human studies indicate that prebiotics increases bacterial species' growth like *Lactobacillus* and *Bifidobacteria* in the intestinal tract (Damaskos and Kolios 2008). It is difficult to know if the prebiotic effects on immunity are direct or indirect as some of these effects are consequences of the shift in gut microbiota (Flaherty et al. 2010). The gut epithelial cell releases some antimicrobial protein that serves as natural antibiotics, which destroys the harmful microbes directly by acting on the cell wall. Prebiotics can activate these epithelial cells and produce an increased amount of antimicrobial compounds (Brink et al. 2006). Immunological maturity of gut-associated lymphoid tissue (GALT) depends on the gut delivered antigens, gut mucosal integrity, and the gut microbiome. Prebiotics help maintain the above functions by modifying mucus production, reducing the bacterial linkage to the epithelial barrier, and enriching the tight junctions (Nawaz et al., 2018). The positive effects of prebiotics are mainly

due to short-chain fatty acids (SCFA) generation, which contributes to blood lipid regulation, energy sources leading to the proliferation of intestinal cells, and enhanced gut barrier function (Tungland and Meyer 2002). Acetate, propionate, and butyrate are the main SCFA produced by prebiotics. Butyrate has been found to increase apoptosis, an innate cellular defense mechanism in human colonic tumor cell lines. It helps induce glutathione S transferases, which in turn help in detoxifying dietary carcinogens (Macfarlane 2010).

SCFA has anti-inflammatory properties and contributes to intestinal mucosal immunity (Nawaz et al., 2018). A study in calves showed that prebiotics in feed influenced both the cellular and mucosal immunity. In tylosin treated calves, there was seen downregulation of interleukin 1(IL-1), interleukin 2 (IL 2), and tumor necrosis factor α (TNF α) (Szymańska-Czerwińska et al. 2009). It has been observed that yeast-derived carbohydrates extended the serum immunoglobulin G (IgG) levels in 3 weeks old broilers that had already lost their protection from maternal antibodies. Extended defense by maternal antibodies helps in protecting the birds from early infections (Alizadeh et al., 2017). Toll-like receptors (TLRs) play a role in identifying structural motifs known as pathogen-associated molecular patterns expressed by microbes. Upregulation of toll-like receptors 2b (TLR2b) and interleukin 10 (IL 10) in cecal tonsils were observed in pullets supplemented with yeast-derived carbohydrates (Yitbarek et al. 2005).

Overview of Mannan oligosaccharide as prebiotic

Mannan oligosaccharides are used as potential substitutes for antibiotic growth promoters. MOS is found in the yeast cell wall and has been demonstrated to modify livestock's microbial communities (Biggs et al., 2007). The broilers fed with MOS at 1g/kg of feed alternative metabolizable energy (AME) were improved, and the feed conversion ratio (FCR) was increased (Yang et al. 2009). MOS favors the elimination of gram-negative bacteria like *Salmonella* and *E.*

coli. Mannan residues are specific to the type 1 fimbriae of the pathogenic bacteria, and thus MOS plays a role in preventing the adhesion of the pathogenic bacteria to the GI tract (Hélène L et al. 2011). An increase in the villi's length and the number of goblet cells were observed in broilers fed with MOS. Goblet cells produce mucin that serves as the first line of host defense (Baurhoo et al. 2004). MOS favors an increase in the count of *Lactobacilli* and *Bifidobacteria,* which contributes to the increase in the length of the villi (Baurhoo and Phillip 2006). Various studies have reported that MOS enhances secretory immunoglobulin A (IgA) levels in the gut mucosa. Mannan supplementation in weaned piglets showed a decline in neutrophils in the blood and a rise in lymphocytes suggesting an alteration in the inflammatory response (Davis et al. 2001). Mannose-binding proteins in the blood can act as opsonins and bind to mannose-containing bacteria and viruses and activate the host immune system's complement cascade (Halas and Nochta 2012). Studies in cows showed an improvement in serum immunoglobulin G (IgG) and enhanced immune response to rotavirus (Franklin et al. 2005). MOS induces anti-inflammatory cytokine IL10 and decreases in levels of pro-inflammatory cytokine TNF α (Che et al. 2011).

PROBIOTICS IN COMMERCIAL POULTRY

Introduction

In 1974 Parker coined the term "probiotic" to describe living microorganisms whose activity was the opposite to antibiotics ("pro" versus "anti"). Fuller (1992) defined probiotics as live microorganisms that improve animals' and humans' health and well-being and affect all host mucosal surfaces. The usage of antibiotics in the poultry diet leads to many problems like antibiotic resistance. In Europe, since the year 2000, antibiotic growth promoters usage in animals was banned, which lead to the use of other alternatives like probiotics, prebiotics, organic acids. (Bedford, 2000).

Probiotics

The common probiotics used in the poultry industry are *Lactobacillus, Bacillus, Bifidobacterium, Enterococcus, Streptococcus,* and *Lactococcus* spp. and, in some cases, yeast-like *Saccharomyces* spp. (Kabir, 2009). Different research studies have shown that the use of probiotics can reduce the pathogenic bacteria such as *Salmonella, Clostridium, Campylobacter jejuni, E. coli*; besides, there was also an increase in the production parameters (Torres-Rodriguez et al. 2007). Probiotics improve the overall health of humans or animals by maintaining a balance and improving the gut microbiome. Probiotics also help in weight gain and an increase in feed efficiency in animals (Yirga 2015). The minimum effective dose of probiotics that need to be added is approximately 10^6 to 10^7/gram of feed but need to be adjusted based on the effective dose curve. Probiotics should be administered regularly for at least 1 to 2 months to see beneficial effects in animals (Tournut 1989).

Probiotics have many health benefits in humans and animals, like reducing irritable bowel syndrome, antimicrobial properties, and suppressing diarrhea. (Bermudez-Brito et al., 2012). Different studies have shown that feeding piglets with probiotics have helped protect them from serious gastrointestinal diseases, restore intestinal mucosal thinning, and improve local resistance to infections (Durand 2010). Probiotics must be able to withstand acidic pH and bile salts to survive their passage through the stomach and small intestine. The other factor considered when selecting a probiotic is its ability to adhere to the intestinal epithelial cells. Studies have shown that probiotics do not need to colonize the intestines to demonstrate their beneficial effects (Bezkorovainy 2001).

Probiotics can be administered through the routes like feed, water, litter, and oral gavage in animals. A study was done to learn the efficacy of *Lactobacillus johnsonii* administered in various

ways, and it was found that *Lactobacillus johnsonii* was found in the ileum in all four routes (Olnood et al. 2015). Early microbe colonization helps prevent pathogenic bacteria from colonizing and stimulates the immune system's development and maturation. Research has been conducted to test the effect of *in ovo* inoculation of the probiotics, but still, further research is required to standardize this method for commercial application (Oliveira et al., 2014).

Mechanism of action of probiotics

Probiotics in the gut stimulate the immune system either by migrating through the gut wall and multiplying to a limited extent or absorbing the antigens released by the dead microorganisms (Ganguly 2013). The probiotics mechanism of action usually includes increased adhesion to the intestinal mucosa, enhancing the epithelial barrier, competitive exclusion of pathogens, production of antimicrobial substances such as bacteriocins, and regulation of the immune system (Bermudez-Brito et al. 2012). The intestinal barrier is one of the crucial defensive mechanisms. Once the barrier is disrupted, pathogens and antigens can cause inflammatory responses, leading to intestinal disorders. Recent data indicate that some of the probiotic bacteria can enhance the gene expression of tight junction proteins such as zonula occluden and protein kinase C (PKC), contributing to the reconstruction of the tight junction complex (Hooper 2012). Mucins are glycoproteins which prevent the pathogenic bacteria from adhering and invading the intestinal epithelial cells. Probiotics like *Lactobacillus* increased the expression of MUC2 and MUC3 mucin genes, which increase mucus secretion and improve the barrier function (Mack et al. 2003).

Probiotics produce antibacterial compounds known as bacteriocins. *Bifidobacterium* strains produce bacteriocins like bifidocin B and bifidin. Bifidin mostly inhibits gram-negative bacteria's growth, unlike bifidocin B, which inhibits the growth of gram-positive bacteria like *Listeria, Enterococcus,* and *Pediococcus* (Yildlrim and Johnson 1998). *Lactobacillus* strains produce anti-

fungal substances like benzoic acid, mevalolactone, lactic acid (Lindgren and Dobrogosz 1990), and other inhibitory compounds like lactic acid, hydrogen peroxide, diacetyl, and bacteriocins. Lactic acid contributes to the lowering of pH and disruption of cell metabolism, which delays pathogens' growth. Diacetyl interferes with the arginine utilization of gram-negative bacteria and inhibits their growth (Mishra and Lambert 1996). Spore-forming probiotics such as *B. subtilis* can survive extremes environmental and gut conditions, which helps them sustain and increase germination (Elshaghabee et al. 2017).

It is hypothesized that probiotics or their soluble factors might activate antigen-presenting cells in the gastrointestinal tract, which leads to immunomodulatory activities (Fiona Long et al. 2015). In the intestinal epithelium, probiotics might modulate the immune system by influencing the antimicrobial peptides and cytokines like IL-12, IFN-γ, IL-10, and TNF-α or by enhancing phagocytosis or proliferation of immune cells (Ajuwon 2016). Studies have shown that probiotic *Lactobacillus* stimulates antigen-presenting cells through the NF-kB and STAT signaling pathway, which leads to the production of IFN-gamma and IL-12, which alter the equilibrium towards Th1. The immunological effects are also species and strain-specific (Ohashi and Ushida 2009).

Overview of *Bacillus subtilis* as probiotic

Bacillus subtilis (*B. subtilis*) is a spore-forming, gram-positive, and facultative aerobic bacteria found in the soil and gastrointestinal tract of animals and humans. Spores of *Bacillus* can survive in very acidic conditions. They can tolerate bile salts, and apart from this, due to their stable nature, they are used in several pharmaceutical preparations and food storage (Adewumi et al., 2014). Many researchers have studied the action of *Bacillus* against various pathogens. For example, Vero and CaCo- 2 cells were protected from the cytotoxic effects of

Clostridium perfringens by the probiotic *B. clausii* O/C strain (Elshaghabee et al., 2017). *B. subtilis* reduced the heat stress in birds by maintaining the microbiota modulated immunity (Walsh 2018). Based on the recent findings, some of the *B. subtilis* strains can produce bacteriocins. Such strains are used in food industries as bio preservatives and as antimicrobials in infections (Elshaghabee et al., 2017). Some of the bacteriocins produced against gram-positive bacteria by *B. subtilis* subgroups are subtilin, ericin S, and ericin A (Teixeira et al., 2013). Probable mechanisms of action *Bacillus spp*. against pathogenic bacteria include stimulation of the immune system, producing antimicrobials such as bacteriocins, enzymes, and gut microbiome regulation (Elshaghabee et al. 2017). Studies have shown that the interaction of *B. subtilis* spores with macrophages plays a crucial part in activating innate and adaptive immunity (Suva et al. 2016). Increased lymphocyte expression was seen when *B. subtilis* spores were given orally as treatment (Caruso et al. 1993). *B. subtilis* can enhance the growth of lactobacilli by the production of subtilisin and catalase. The enzymes produced by *B. subtilis* were seen to be active in live and dead cells (Sorokulova 2013). Experiments done in humans showed that *B. subtilis* CU1 had aided in increasing IgA's secretory activity, which is essential for maintaining gut immunity and microbiome (Suva et al. 2016). Various studies have shown that the host's immunomodulatory effects and antimicrobial activities are different for different *Bacillus* strains.

AVIAN IMMUNE SYSTEM

Birds, like all other vertebrate animals, have a strong immune system. In some respects, the bird's immune system varies from that of mammals, most notably in possession of a bursa of fabricius by the bird and the lack of organized lymph nodes. The immune system's key feature is the identification of organisms and substances perceived to be alien to the body or "non-self." The

immune system contains two branches, namely Innate immunity and adaptive immunity, which function together to protect the birds from disease-causing agents.

Innate Immunity

Innate immunity is 'non-specific' and the first line of defense against invading pathogens. It consists of physical and chemical barriers like skin mucosal epithelium and gastric secretions, blood proteins called complement, which work in conjunction with antibodies and cellular components, including macrophages, heterophils, and natural killer (NK) cells.

Host cells initiate classical innate responses after microbial molecular motifs are recognized by pattern recognition receptors (PRRs). Avian β-defensins (AvBDs) and cathelicidins (CTHLs) are important classes of antimicrobial peptides during early embryonic development that have a broad spectrum of activity against bacteria and fungi. Toll-like receptors (TLR) are the best characterized PRRs usually present on the surface of macrophages, heterophils, and dendritic cells. In chickens, 10 TLRs have been recognized, out of which TLR-2, TLR-4, and TLR-5 play an essential role in identifying bacterial pathogen-associated molecular patterns (PAMPS). Avian TLRs vary significantly in the absence of the TLR9 compared to vertebrates (Chen et al., 2013). PAMPs are unique molecular patterns present on various pathogens. Some of the examples for PAMPs include lipopolysaccharide of gram-negative bacteria, lipoteichoic acids of gram-positive bacteria, peptidoglycans, lipoproteins, etc. The interaction of PRRs with PAMPS activates intracellular signaling pathways leading to the activation of effector molecules, including chemokines, cytokines.

Innate immune system activation contributes to the recruitment of phagocytic heterophils and macrophages. Pathogens are recognized by heterophils through TLRs. Unlike neutrophils in mammals, heterophils in birds lack myeloperoxidase, so they are less reliant on killing bacteria

through the oxidative burst. Heterophils produce heterophil extracellular traps (HETs) made up of chromatin and serine proteases, which help capture bacteria for phagocytosis (Paul Wigley 2013). Macrophages play a crucial role as antigen-presenting cells and possess a range of TLRs that allow them to recognize various pathogens. Leucocytes express effector peptides like β defensins to attract the effector cells like macrophages and mast cells and kill the pathogenic microbes. Dendritic cells and macrophages act as a bridge between innate and adaptive immune responses (Kaiser and Kaiser 2010). They present the antigens to the naïve CD4+ cells activating the adaptive immune responses and leading to the maturation of naïve CD8+ T cells to cytotoxic T cells.

Adaptive immune response

The adaptive immune system is a more targeted approach towards pathogens as it recognizes specific features and can distinguish closely related antigens. One of the unique properties of the adaptive immune system is immunological memory, which makes it highly efficient and rapid. Adaptive immunity is divided into two categories humoral and cell-mediated immunity. Humoral immunity plays a role in eliminating extracellular pathogens such as extracellular bacteria, protozoa, and helminths. Cell-mediated immunity helps in inducing immunity against intracellular pathogens such as viruses, intracellular bacteria, and protozoa (Erf 2004).

The humoral or noncellular immunity consists of immunoglobulins (antibodies) and cells produced by B lymphocytes. The major immunoglobulins produced in chickens on exposure to antigens are IgM, IgG, and IgA (Butcher and Miles 2018).

Table 2: Antibodies major location and their function (Carlander et al.,2010)

Antibody	Major location	Function
IgY	Yolk	Produce early immunity in chicks
IgM	Blood	Complement activation
IgG	Blood	Opsonization, antibody-dependent cell-mediated cytotoxicity
IgA	Mucus secretion	Mucosal immunity

Cell-mediated immunity consists of T lymphocytes and various cytokines released in response to antigen. T lymphocytes consist of helper T cell, which expresses CD4+ and cytotoxic T cells, which express CD8+ molecules on their surface. Both CD4 + and CD25+ cells are called T regulatory cells, and they are specialized in immune suppression. They produce high amounts of IL-10, TGF-β, and CTLA-4 (Shanmugasundaram and Selvaraj, 2013). The T cells which play a primary role in adaptive immunity are T helper (Th) cells. Major histocompatibility complex (MHC) class II presents antigens to the T cell receptors (TCRs) on Th cells, whereas MHC class presents antigens to cytotoxic T cells. Recognition of antigen with TCR initiates the release of cytokines and activation signals responsible for activating innate and adaptive immunity. Type 1 Th (Th1) cells play an essential role in cell-mediated immunity, whereas type 2 Th (Th2) favor humoral immune responses. Th1 cells typically produce cytokines such as interferon-γ (IFN-γ), tumor-necrosis factor-α, and interleukin (IL)-2, whereas Th2 cells generate cytokines such as IL-4, IL-5, transforming growth factor-β, and IL-10 (Wigley and Kaiser 2003, Pete and Staheli 2014).

Table 3: Cytokines and their functions (Wigley and Kaiser 2003)

Cytokines	Functions
IL-1	Supports activation of T and B lymphocytes, activates NF-kB, pro-inflammatory
IL-10	Anti-inflammatory, inhibits IL-1, TNF-α, IL-12 and MHC class II expression, downregulates IFN- γ
INF- γ	The primary activator of macrophages stimulates NK cells, induced MHC class II expression
IL-6	Both anti and pro-inflammatory modulates Th1 and Th2 responses, plays a role in hematopoiesis
TNF-α	Major cytokine during parasitic, viral, and bacterial infection
IL-2	Activates T cell proliferation, activates macrophages
TGF-β	Anti-inflammatory, induces apoptosis
IL-4	induces B cell proliferation, upregulates MHC class II

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CHAPTER 3

BACILLUS SUBTILIS IN-VITRO CHARACTERIZATION AND EFFECTS OF *B. SUBTILIS AND* MANNAN OLIGOSACCHARIDE ON PERFORMANCE PARAMETERS, IMMUNE RESPONSES, *LACTOBACILLUS REUTERI AND BIFIDOBACTERIUM ANIMALIS* LOAD IN BROILERS.

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ABSTRACT

After the ban of antibiotics usage in animal production, probiotics and prebiotics are used to alter gastrointestinal flora favorably, thus improving animal health and productivity. This study aimed to assess the efficacy of *Bacillus subtilis in vitro* and *in vivo*. For the *in vitro* experiments, *B. subtilis* was cultured in different pH ranges and bile salts concentrations. For the *in vivo* experiment, 144 broilers were randomly assigned to four treatments with six birds per pen in 6 replicates (n=6). Experimental diets consisting of the basal diet (control), a basal diet supplemented with 0.1% MOS or 0.1% *B. subtilis*, or 0.1% MOS + *B. subtilis* were fed to the birds for 17 days. At 17 d of age, all birds were fed a control diet for 72h so that *B. subtilis* in the feed does not confound the recovery of *B. subtilis* from the intestine. *B. subtilis* showed maximal growth at pH 1.5 and 2.5 ($P < 0.05$). At d20, the BWG of birds was higher in MOS, *B. subtilis*, and MOS + *B. subtilis* compared to control ($P < 0.05$). At d17 and d20, *Lactobacillus reuteri* (*L. reuteri*) load in cecal and jejunal contents and *Bifidobacterium animalis* (*B. animalis*) load in ceca on d17 were higher in the 0.1% MOS, 0.1% *B. subtilis* and MOS + *B. subtilis* groups compared to control ($P<0.05$). At d17, IL-1β mRNA amounts were higher in 0.1% MOS, 0.1% *B. subtilis* and MOS + *B. subtilis* groups compared to control ($P>0.05$). At d17, IL-10 mRNA amounts were higher in the 0.1% MOS and MOS + *B. subtilis* groups and lower in 0.1% *B. subtilis* compared to control ($P>0.05$). Findings demonstrate that *B. subtilis* can grow in acidic pH. 0.1%*B. subtilis* and 0.1%MOS and 0.1% MOS + *B. subtilis* feeding have significant effects on body weight gain and enteric *L. reuteri* and *B. animalis* load in ceca.

INTRODUCTION

The term “probiotic” means “for life,” and Mechnikov first suggested the concept in 1907, and the term prebiotics was coined in 1995 by Glenn Gibson. Several scientific studies have shown that probiotics and prebiotics play a role in improving animal health, stimulating immune responses, enhancing beneficial bacteria growth, and improving performance parameters (Markowiak and Ślizewska 2018). As antibiotics have been prohibited, the use of pro-and prebiotics has increased as alternatives to antibiotic growth promoters in animal feed. For probiotics to produce their beneficial effects, it's necessary that they survive in the gut (Patterson and Burkholder 2003). Bile salts and pH play an essential role in the survival of probiotics in the animal gut (Terpou et al., 2019).

Probiotics play a role in increasing enteric lactic acid bacteria growth and inhibiting the proliferation of pathogenic bacteria such as *Salmonella enterica, Campylobacter jejuni,* and *E. coli* (Patterson and Burkholder 2003, Park et al. 2020). A research study showed that various *lactobacillus* strains produced inhibitory organic acids against *C. jejuni*, and *L. crispatus decreased the colonization of C. jejuni in birds (Neal-McKinney et al. 2012)*. Studies have shown that *B. subtilis* prevents the reactive oxygen species from causing oxidative stress and thus helps birds escape from protein and tissue damage (Bai et al., 2017). *B. subtilis* exhibited immunomodulatory effects when introduced to the animal diet, such as increased production of IgA antibodies, increased IL 10 gene expression in thermoneutral and heat-stressed birds (Wang et al. 2018).

The use of prebiotics or fermentable sugars has increased as they modify the immune system and serve as an energy source for the gut microflora. Fructo-oligosaccharides (FOS) have been shown to increase the proliferation of certain strains of *Bifidobacteria*, which in turn inhibit the survival of pathogenic bacteria and help to increase the absorption of amino acids in the small

intestine (Hajati and Rezaei 2010). Studies have shown that the addition of mannan oligosaccharides (MOS) as an alternative to antibiotics has improved feed efficiency and antimicrobial activity in the duodenum and ileum (Samarasinghe et al., 2003).

There are only fewer studies which evaluated the immunological effects in the birds fed with probiotics and prebiotics. The present study aimed at assessing the pH and bile tolerance of *B. subtilis in vitro,* and for the *in vivo* study, the performance parameters, cecal tonsil immune genes expression, and the enteric *L. reuteri* and *B. animalis* bacterial load were studied in the birds fed with 0.1% MOS or 0.1% *B. subtilis* or combination of 0.1% MOS and *B. subtilis*.

MATERIALS AND METHODS

In vitro study

pH tolerance test

B. subtilis was cultured in De Man, Rogosa and Sharpe (MRS) broth (Sigma-Aldrich, MO, USA) and incubated at 37°C for 24hrs. 200µl of *B. subtilis* broth and was resuspended in 10 ml of MRS broth with pH of 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.0. The pH of MRS broth was adjusted using 1M HCl. To calculate the optical density, a Spectrophotometer was used at an absorbance of 600 nm at 4 hours after incubation at 37°C. This assay was repeated at three different times.

Bile tolerance test

Bacterial cells were resuspended in 10 ml of MRS broth supplemented with 0, 1, 1.5, or 2% bovine bile salts. We used a spectrophotometer to measure the optical density at 600 nm at 4 hours after incubation at 37°C. This assay was repeated at three different times.

***In vivo* study**

A total of 144 Cobb 500 male broilers were randomly assigned to four treatments: basal-fed treatment (control) or 0.1% MOS or 0.1% *B. subtilis* or 0.1% MOS and 0.1% *B. subtilis*. The basal feed was a corn and soybean meal diet (Table 3). Birds were fed the supplementation of basal diet until 17d of age. On day 17, all birds were fed the same basal diet with no supplementation. The probiotic species in the feed are expected not to confound the probiotic species' recovery from the intestine. One bird per pen was sacrificed, and cecal, jejunum, and ileal contents were collected to quantify *L. reuteri* and *B. animalis* by using PCR on day 17 and 20. Cecal tonsils were collected on day 17 and 20 for mRNA expression analysis of IL-1 and IL-10. Treatments were replicated in six-floor pens (n = 6) with six birds/pen.

Effect of *B. subtilis*, MOS and *B. subtilis* + MOS supplementation on performance parameters

Birds and feed were weighed regularly every week. BWG and feed conversion ratio were calculated.

Effect of *B. subtilis*, MOS and *B. subtilis* + MOS supplementation on enteric *L. reuteri* and *B. animalis* load in cecal, jejunal and ileal contents

On days 17 and 20, the cecal, jejunal, and ileal contents were collected in the 2ml Eppendorf tubes. About 0.1 gram of the cecal, jejunal or ileal contents diluted with 1ml of 1x PBS and washed twice. In EDTA, the cell pellet was resuspended, and then 20 mg/mL lysozyme was added. The samples were incubated for 30 min at 37◦C. Following incubation, the samples were centrifuged, and the supernatant was discarded. The samples were treated with lysis buffer containing 20% SDS and 0.1 mg / mL proteinase K (Sigma Aldrich, St Louis MO) for 5 min at 80^{o} C. The samples were

incubated for 30min with 5 µL of RNase at 37^{o} C. Cell lysate was incubated on ice for 10 minutes with 6 M sodium chloride. After incubation, the supernatant was collected by centrifuging the cell lysate at 400× g for 10 min. DNA was precipitated by adding isopropanol to the supernatant and washed once in ice-cold ethanol. The samples were centrifuged, and the DNA pellet was collected and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at −20◦C until further use. The DNA extracted from the various parts of the intestine was examined by real-time PCR for *L. reuteri* using primers (5′- CAGGATCGGTAATTGATG -3′and, 3′- TGGATATGGAAGTTCGTC-5′), and *B. animalis* using primers (5′- GTGGAGACACGGTTTCCC -3′ and 3′- CACACCACACAATCCAATAC -5′) (Revathi Shanmugasundaram et al. 2015). The annealing temperature for *L. reuteri* was 54^{0} C, and *B. animalis* was 57.4^{0} C. Quantified DNA was reported as Log10CFU/g.

Effect of *B. subtilis*, MOS and *B. subtilis* + MOS supplementation on IL-1 and IL-10

Cecal tonsils were collected on days 17 and 20 into 2 ml Eppendorf tubes filled with 1 ml RNA later (Qiagen). The samples were kept at 4 ° C for 7 days to allow the RNA later to permeate into the tissue and stabilize the RNA. After 7 days, excess RNA later was removed, and the samples were stored in -80^{0} C until further analysis. RNA extraction was carried using the phenol-chloroform method with some modifications (Selvaraj and Klasing 2006). Briefly, about 0.1g of tissue was homogenized in 1ml of Tri-reagent (Sigma Aldrich, St.Louis, MO) and incubated for 10 mins at room temperature. Chloroform was then added. After 10 mins, the tubes were centrifuged at 8000 xg for 15 mins, and the supernatant was collected. The collected supernatant was treated with isopropanol to precipitate RNA, followed by a 70% ethanol wash. Nuclease free water was used to resuspend the RNA pellet. A nanodrop was used to test the purity and concentration of extracted RNA. The total extracted RNA was reverse transcribed into cDNA. The

mRNA analyzed was IL-1β (5′-CTACACCCGCTCACAGTCCT-3′ and 5′-TCACTTTCTGGCTGGAGGAG-3′) (R Shanmugasundaram et al. 2011) and IL-10 (5'-CATGCTGCTGGGCCTGAA-3' and 5'-CGTCTCCTTGATCTGCTTGATG-3') (Rothwell et al. 2004) by using real-time PCR (CFX96 Touch Real-Time System, BioRad). Both the genes were normalized using the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5'-TCCTGTGACTTCAATGGTGA-3' and 5'-CACAACACGGTTGCTGTATC-3') (Dube et al. 2014). The annealing temperatures of IL-1, IL-10, and GAPDH are 57.5^0 C, 55^0 C, and 55^0 C, respectively. The $2^{(Ct\ Sample-\ Housekeeping)}/2^{(Ct\ Comparison-Housekeeping)}$ comparative Ct approach was used to measure the fold shift from the reference, where Ct is the threshold cycle. The Ct was calculated when the fluorescence increases exponentially 2 folds above the baseline by iQ5 software (Biorad).

Statistical analysis

A one-way ANOVA (JMP Software, Cary, NC) was used for in vitro assays to analyze the influence of various parameters studied on dependent variables. For the in vivo study, a one-way ANOVA was used to examine the effect of *B. subtilis*, MOS, and MOS + *B. subtilis* effect and their interaction on the dependent variables. With Tukey's HSD post hoc test, variations between means were analyzed. Significant effects at $P < 0.05$ were reported.

RESULTS

***In vitro* assays**

pH tolerance test

B. subtilis was highly tolerant to acidic pH of 1.5 and 2.5, moderately tolerant to pH of 3.5, 6.5, and 7.0, and less tolerant to pH of 4.5 and 5.5 ($P < 0.005$, Figure 1.1)

Bile tolerance test

There were no statistically significant differences of *B. subtilis* proliferation in different bile concentrations (P > 0.05, Figure 1.2).

***In Vivo* Experiment**

Effect of *B. subtilis*, MOS and *B. subtilis* + MOS supplementation on performance parameters

There were significant effects on body weight gain and feed conversion ratio from 0 to 20 days of age (P < 0.05). The FCR was lower for the *B. subtilis* treatment group with FCR 1.3, followed by the *B. subtilis* + MOS group with FCR 1.35 (P < 0.05, Figure 1.4). Body weight gain was highest for the MOS group with an average of 92.7g/day and *B. subtilis* + MOS group with an average of 88.6g/day, followed by *B. subtilis* group with an average of 55.4g/day when compared with the control group (P < 0.05, Figure 1.3)

Effect of *B. subtilis*, MOS and *B. subtilis* + MOS supplementation on enteric *L. reuteri* and *B. animalis* load in cecal, jejunal and ileal contents

On day 17, *L. reuteri* cecal load was higher in the MOS, *B. subtilis*, and *B. subtilis* + MOS groups by 0.8, 0.9, and 1.1 Log10CFU/g, respectively, when compared with the control group (P < 0.05, Figure 1.5). On day 20, *L. reuteri* cecal load was higher in the MOS, *B. subtilis*, and *B. subtilis* + MOS groups by 0.7, 0.8, and 0.7 Log10CFU/g, respectively, when compared with the control group (P < 0.05, Figure 1.5). On day 17, *L. reuteri* jejunal load was higher in the *B. subtilis* + MOS groups by 1.0 Log10CFU/g compared with the control group (P < 0.05, Figure 1.6). On day 20, *L. reuteri* jejunal load was higher in the MOS groups by 1.0 Log10CFU/g, when compared with the control group (P < 0.05, Figure 1.6). On day 17, *L. reuteri* load in the ileum was higher in the MOS

by 1.1 Log10/CFU/g and *B. subtilis* + MOS group by 1.0 Log10CFU/g when compared with the control group ($P < 0.05$, Figure 1.7). On day 20, *L. reuteri* ileal load was higher in the *B. subtilis* + MOS groups by 1.1 Log10CFU/g compared with the control group ($P < 0.05$, Figure 1.7).

On day 17, *B. animalis* cecal load was higher in the MOS by 0.7Log10CFU/g compared with the control group ($P < 0.05$, Figure 1.8). On day 20, *B. animalis* cecal load was higher in the *B. subtilis* groups by 0.5Log10CFU/g compared with the control group ($P < 0.05$, Figure 1.8). On day 17, *B. animalis* jejunal load was higher in the MOS, *B. subtilis*, and *B. subtilis* + MOS groups by 0.7, 0.6, and 0.7 Log10CFU/g, respectively, when compared with the control group ($P < 0.05$, Figure 1.9). On day 20, *B. animalis* jejunal load was not statistically significant between the treatments ($P > 0.05$, Figure 1.9).

Effect of *B. subtilis*, MOS and *B. subtilis* + MOS supplementation on IL-1 and IL-10

On d17, IL-1β mRNA amounts in the supplemented treatment groups were not significantly different from that in the control group ($P > 0.05$, Figure 1.10). On d20, IL-1β mRNA amounts in the supplemented treatment groups were not significantly different from that in the control group ($P > 0.05$). On d17, IL-10 mRNA amounts in the supplemented treatment groups were not significantly different from that in the control group ($P > 0.05$, Figure 1.11). On d20, IL-10 mRNA amounts in the supplemented treatment groups were not significantly different from that in the control group ($P > 0.05$, Figure 1.11).

Discussion

In this study, *B. subtilis* was able to tolerate and proliferate at various pH levels. The addition of *B. subtilis* and MOS to the feed enhanced o proliferation of *L. reuteri* and *B. animalis* in various parts of the chicken gut. In chickens, the pH of the digestive tract varies between 1.5 to 7.0 (Svihus

2014). To colonize the intestine, probiotics should be able to survive in the acidic environment to transmit their beneficial effects and inhibit the growth of the pathogenic bacteria (Tannock 2004). *B .subtilis* can survive the acidic conditions that make it resistant to the severe gastric conditions and helps in the survival and proliferation in the gut. Bile salts have antimicrobial activity and disorganize the cell membrane. For the probiotics to survive and transit in the gut, they should tolerate the bile salts. Strains of *Lactobacilli* and *Bifidobacteria* have shown to be resistant to bile salts (Ruiz et al., 2013). There were no statistically significant differences for the tolerance of *B. subtilis* to different bile salts concentrations. Findings demonstrate that *B. subtilis* can withstand gastrointestinal conditions and can survive in the chickens' gut.

There were statistically significant differences in the performance parameters like body weight gain and feed conversion ratio in this study. FCR was lower for the group supplemented with B. subtilis compared to the control group. Feeding poultry with probiotics like *Lactobacillus* spp. (Apata 2008; Mountzouris et al. 2005), Bifidobacterium spp. (Jung et al. 2001; Elbaz 2020), and *Bacillus* spp. (Zhang et al. 2013; Lillehoj et al. 2018) and prebiotics like FOS (Ragheb and Mohammed 2019) and MOS (Yang et al. 2009) enhanced growth performance. Probiotics and prebiotics are capable of increasing the surface area, enhancing digestive enzymes activity, and augmenting nutrient availability and absorption, contribute to animal growth performance (Ragheb and Mohammed 2019; Elbaz 2020)

Treatment groups supplemented with *B. subtilis*, MOS, and *B. subtilis* + MOS showed that they significantly affected the enteric *L. reuteri* and *B. animalis* in various parts of the intestine. Treatment groups were fed with a basal diet without additional supplements for 72h to ensure that the infeed *B. subtilis* is not confounded in the gut, and only the *B. subtilis* colonized in the gut will help in the proliferation of the enteric bacteria. *L. reuteri* concentrations expressed as Log10

CFU/g was highest in the treatment groups in the cecum, jejunum, and ileum. The growth of *L. reuteri* and *L. murinus* increased when *B. subtilis* was co-cultivated with lactobacilli. Bacteriocins like catalase and subtilisin produced by *B. subtilis* help in enhancing the growth and viability of the lactic acid bacteria (Hosoi et al.) In the intestine, bacillus spores germinate, allowing to create an anaerobic or microaerobic environment that, in turn, promotes lactobacilli growth (Zhang et al. 2014).

Prebiotic fermentation by gut bacteria leads to the production of lactic acid, SCFA, and butyric acid production. The fermentative metabolites produced by one bacteria can act as substrates of growth for other bacterial community members (Holscher 2017). Many studies have shown that the addition of prebiotics like FOS, GOS, MOS, and inulin has increased the growth of saccharolytic bacteria like *Bifidobacterium* and *Lactobacillus*. SCFA produced by the prebiotics decreases the pH, which inhibits some of the bacteria like *Bacteroides* and *Clostridium* (Gibson and Roberfroid 1995, Gibson et al. 2017, S. Wang et al. 2020).

In this study, the enteric load of *L. reuteri* and *B. animalis* was decreased when the birds were withdrawn from the feed supplemented with *B. subtilis* and MOS ($P < 0.05$). Findings demonstrate that *B. subtilis* and MOS enhanced *L. reuteri* and *B. animalis* in the gut and *Bacillus spp*. spores do not colonize the gut (Otutumi et al., 2012).

In our current study, we did not find any statistically significant differences in the mRNA amount of IL-1β and IL-10 in the MOS, *B. subtilis*, and *B. subtilis* + MOS group compared to the control ($P > 0.05$). Various studies have reported that *Lactobacilli* strains usually induce the production of both anti-inflammatory cytokines and pro-inflammatory cytokines (Morita et al. 2014). Few strains of *Lactobacilli* have shown to induce pro-inflammatory cytokines like IL-12 and TNFα than the anti-inflammatory cytokines. Probiotics like *E. coli* have been shown to

activate both pro and anti-inflammatory cytokines. The induction of cytokines depends on the activated target cell and the type of probiotics (Cross et al. 2004). *B. subtilis* B10 induced the proinflammatory cytokines like IL-1β, INF-α, and IFN-γ and downregulated the anti-inflammatory cytokine IL-10 in the RAW 264.7 cells. *B. subtilis* B10 spores were speculated to facilitate inflammatory reactions (Huang et al. 2013). The activation of pro-inflammatory cytokines by *B. subtilis* could be triggered due to the secretion of lipoteichoic acids which activates the nuclear factor-kB. Nuclear factor-kB activation results in the subsequent activation and release of pro-inflammatory cytokines (Xu et al., 2012). It was hypothesized that activation of IL-10 in parallel with pro-inflammatory cytokines occurs as an autoregulatory effect and part of a negative feedback mechanism that modulates acute inflammatory responses (Xu et al. 2012; Shimauchi et al. 1999). Our results suggest that there was a balance between the Th1 and Th2 cytokine responses.

It can be concluded that *B. subtilis* can tolerate acidic conditions and survive in the gut. *B. subtilis* and MOS enhance the growth performance in broilers. *B. subtilis* and MOS play a role in improving the growth of enteric load of *L. reuteri* and *B. animalis*.

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Basal diet ingredients and calculated nutrient composition (Table 4)

	Starter (0 – 35 d)
Ingredients	%
Corn	58.47
Soybean Meal, 48% CP	35.15
Soybean Oil	2.27
Monocalcium phosphate 21%	1.38
Limestone	1.59
DL-Methionine	0.21
L-Lysine-HCL, 78%	0.14
Salt (NaCl)	0.35
Vitamin premix[1]	0.08
Mineral premix[2]	0.35
Total:	100
Calculated Nutrient Composition	
ME, kcal/kg	3,050
Crude Protein, %	21.44
Crude Fat, %	4.55
Crude Fiber, %	2.17
Calcium, %	0.95
Total Phosphorus, %	0.71
Avail. Phosphorus< %	0.45
Sodium, %	0.16

Potassium, %	0.92
Chloride, %	0.27
Lysine, %	1.31
Methionine, %	0.56
TSAA, %	0.91
Threonine, %	0.87
Tryptophan, %	0.29
Arginine, %	1.50

[1]Vitamin mix provided the following (per kilogram of diet): thiamin-mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B12 (cobalamin), 12.0g; pyridoxine-HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-reinyl acetate, 2,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.
[2]Trace mineral mix provides the following (per kilogram of diet): manganese (MnSO4.H2O), 101 mg; iron (FeSO4.7H2O), 20 mg; zinc (Zn)), 80 mg; copper (CuSO4.5H2O), 3 mg; iodine (ethylene diamine dihydroiodide), 0.75 mg; magnesium (MgO), 20 mg; selenium (sodium selenite), 0.3 mg.

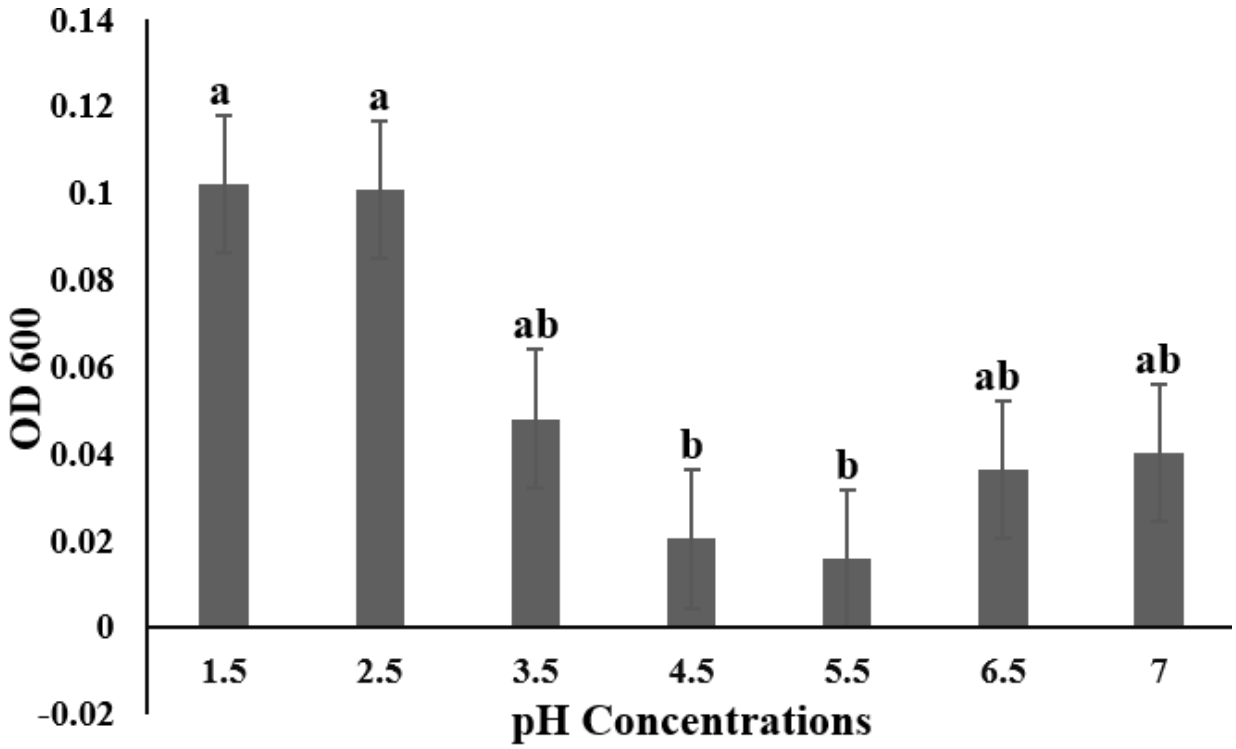

Figure 1.1. Effect of pH on *Bacillus subtilis* proliferation: *B. subtilis* was incubated at 37 ^{o}C in MRS broth of 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, and 7.0 pH. Optical density (OD) was measured using a spectrophotometer after 4 hours of incubation. Bars (mean ± SEM) with no common superscripts within a bacterial species differ significantly ($P < 0.05$). n = 3.

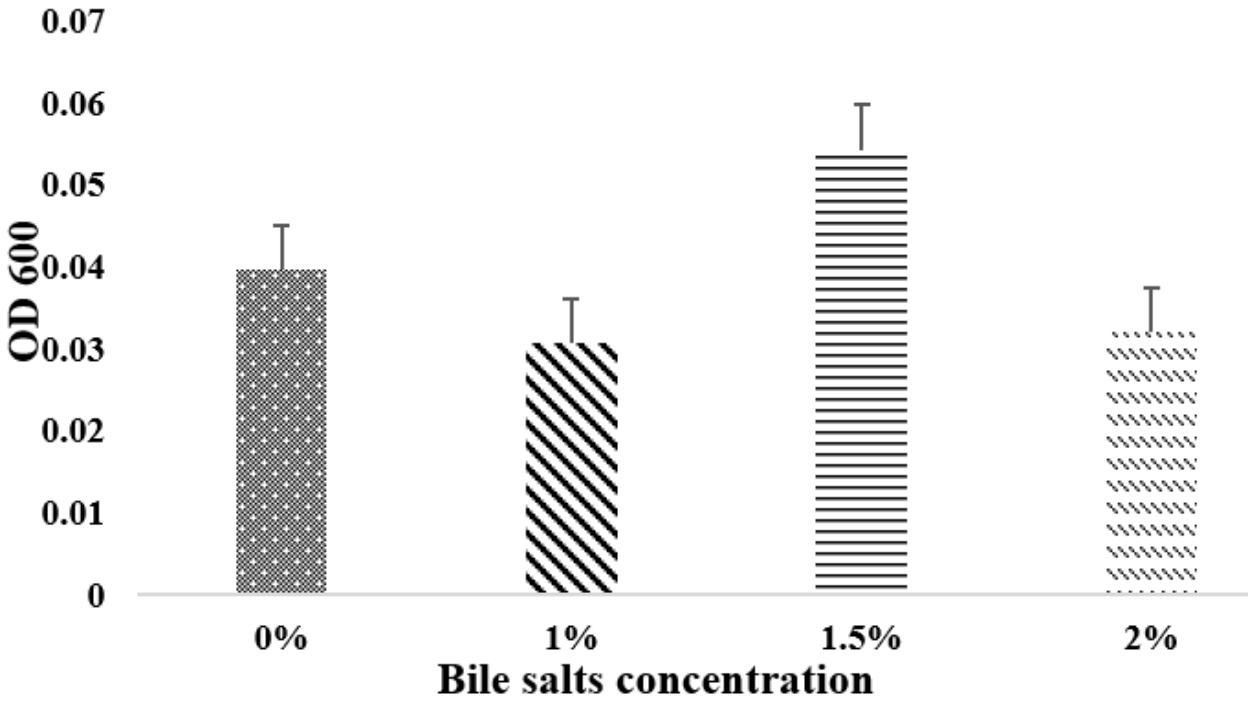

Figure 1.2. Effect of bile salts on *Bacillus subtilis* proliferation: *B. subtilis* was incubated at 37 ^{o} C in MRS broth with 0, 1, 1.5, and 2.0% bovine bile salts. Optical density (OD) was measured by a spectrophotometer after 4 hours of incubation. Bars (mean ± SEM) with no common superscripts within a bacterial species differ significantly (P >0.05). n = 3.

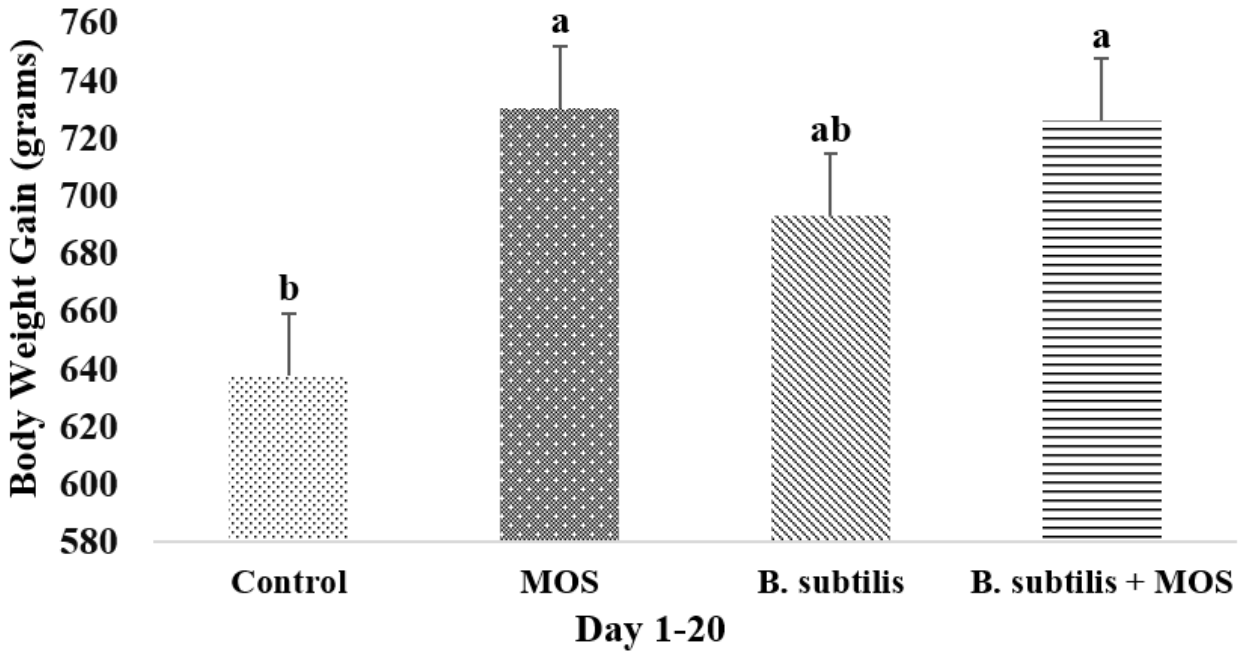

Figure 1.3. Effect of *B. subtilis*, MOS, and *B. subtilis* + MOS supplementation on body weight gain from day 1 to 20: Birds were randomly assigned to one control and three treatment groups: MOS, *B. subtilis*, and *B. subtilis* + MOS group. At 17 d of age, all birds were fed control diets for 72hrs. Means with no common superscript within a column differ significantly ($P < 0.05$). ($n = 6$).

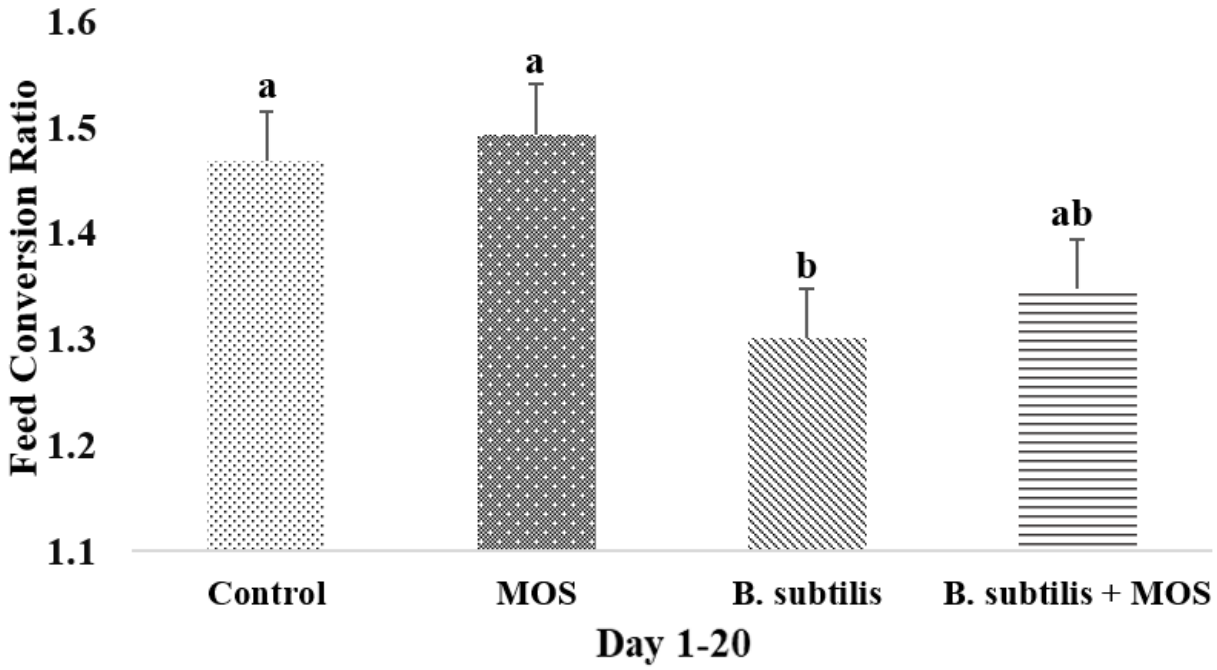

Figure 1.4. Effect of *B. subtilis*, MOS, and *B. subtilis* + MOS supplementation on feed conversion ratio from day 1 to 20: Birds were randomly assigned to one control and three treatment groups: MOS, B. subtilis, and *B. subtilis* + MOS group. At 17 d of age, all birds were fed control diets for 72hrs. Means with no common superscript within a column differ significantly ($P < 0.05$). ($n = 6$).

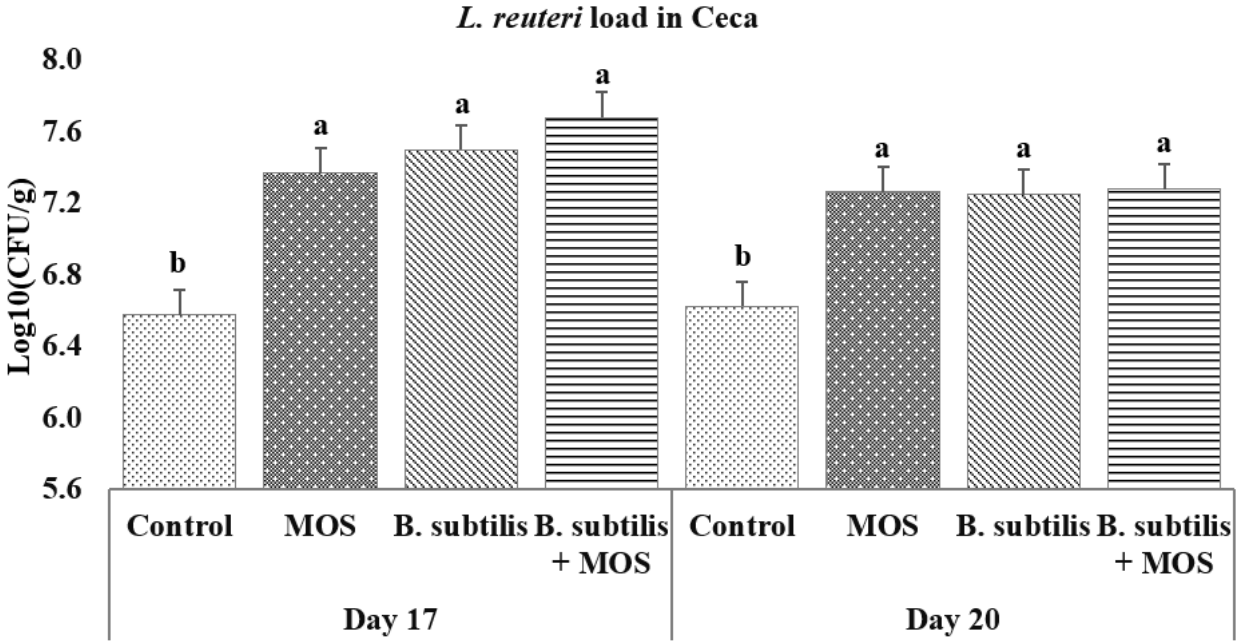

Figure 1.5. Effect of *B. subtilis*, MOS, and *B. subtilis* + MOS supplementation on enteric *L. reuteri* load in cecal contents. Birds were randomly distributed to one control and three treatment groups: 0.1% MOS, 0.1% *B. subtilis*, and 0.1% *B. subtilis* + MOS group. At 17 d of age, all birds were fed control diets for 72hrs. At d17 and d20, samples from ceca were collected and analyzed for *L. reuteri* using real-time PCR and reported as Log10 CFU/g. Means with no common superscript within a column differ significantly ($P < 0.05$). ($n = 6$).

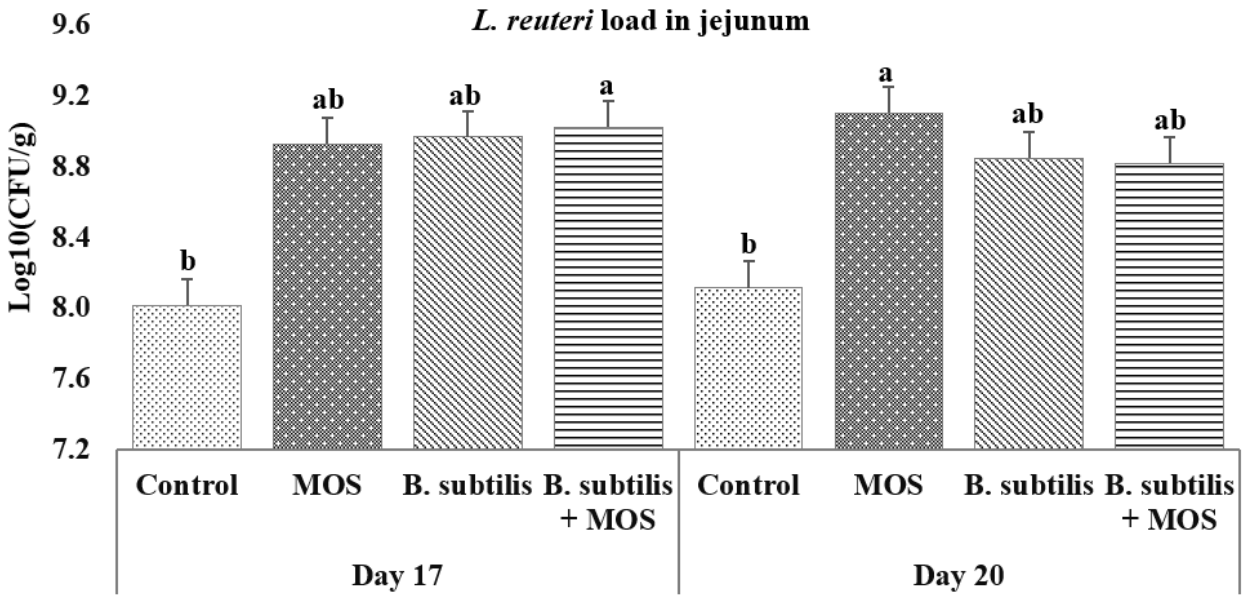

Figure 1.6. Effect of *B. subtilis*, MOS, and *B. subtilis* + MOS supplementation on enteric *L. reuteri* load in jejunal contents: Birds were randomly distributed to one control and three treatment groups: 0.1% MOS, 0.1% *B. subtilis*, and 0.1% *B. subtilis* + MOS group. At 17 d of age, all birds were fed control diets for 72hrs. At d17 and d20, samples from jejunum were collected and analyzed for *L. reuteri* using real-time PCR and reported as Log10 CFU/g. Means with no common superscript within a column differ significantly ($P < 0.05$). ($n = 6$).

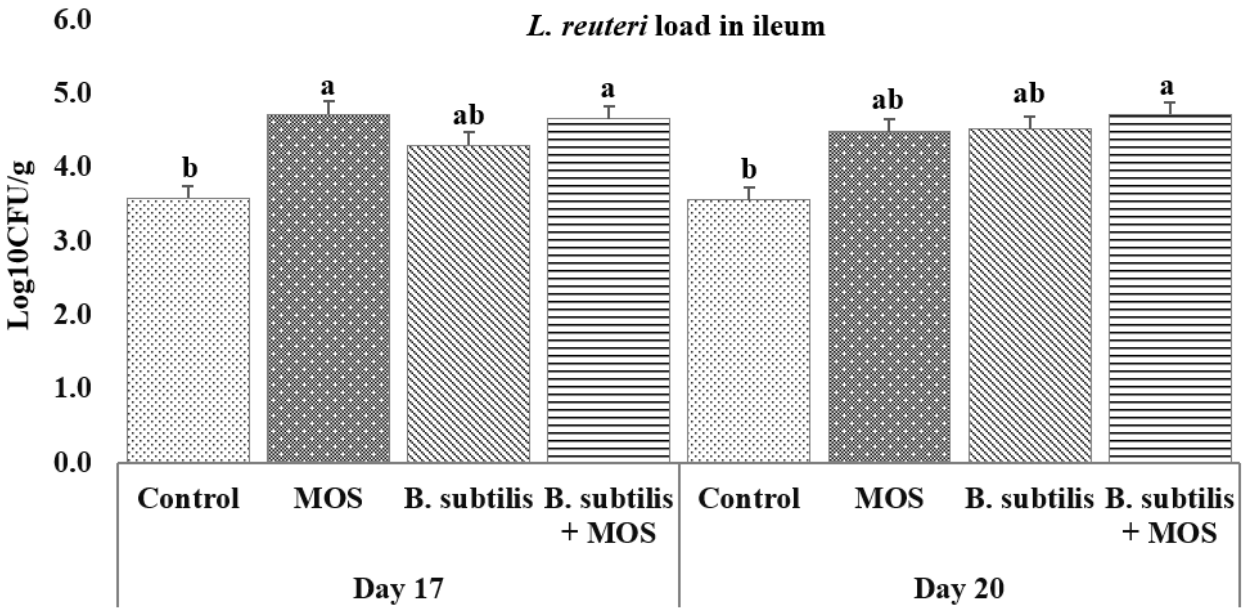

Figure 1.7. Effect of *B. subtilis*, MOS, and *B. subtilis* + MOS supplementation on enteric *L. reuteri* load in ileal contents. Birds were randomly distributed to one control and three treatment groups: 0.1% MOS, 0.1% *B. subtilis*, and 0.1% *B. subtilis* + MOS group. At 17 d of age, all birds were fed control diets for 72hrs. At d17 and d20, samples from ileum were collected and analyzed for *L. reuteri* using real-time PCR and reported as Log10 CFU/g. Means with no common superscript within a column differ significantly ($P < 0.05$). ($n = 6$).

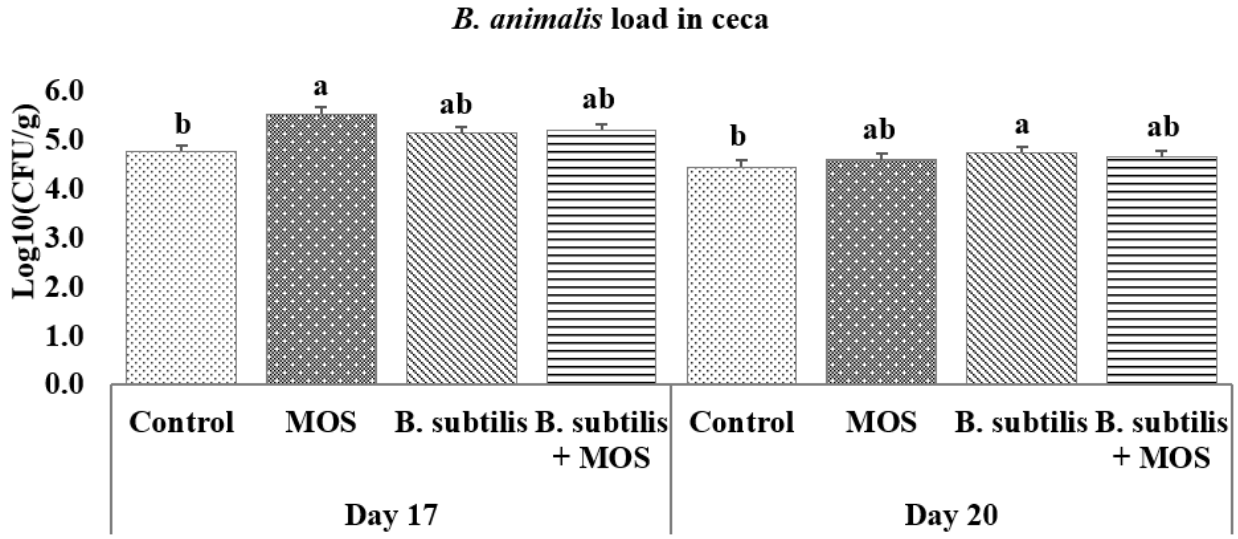

Figure 1.8. Effect of *B. subtilis*, MOS, and *B. subtilis* + MOS supplementation on enteric *B. animalis* load in cecal contents. Birds were randomly distributed to one control and three treatment groups: 0.1% MOS, 0.1% *B. subtilis*, and 0.1% *B. subtilis* + MOS group. At 17 d of age, all birds were fed control diets for 72hrs. At d17 and d20, samples from ceca were collected and analyzed for *B. animalis* using real-time PCR and reported as Log10 CFU/g. Means with no common superscript within a column differ significantly ($P < 0.05$). ($n = 6$).

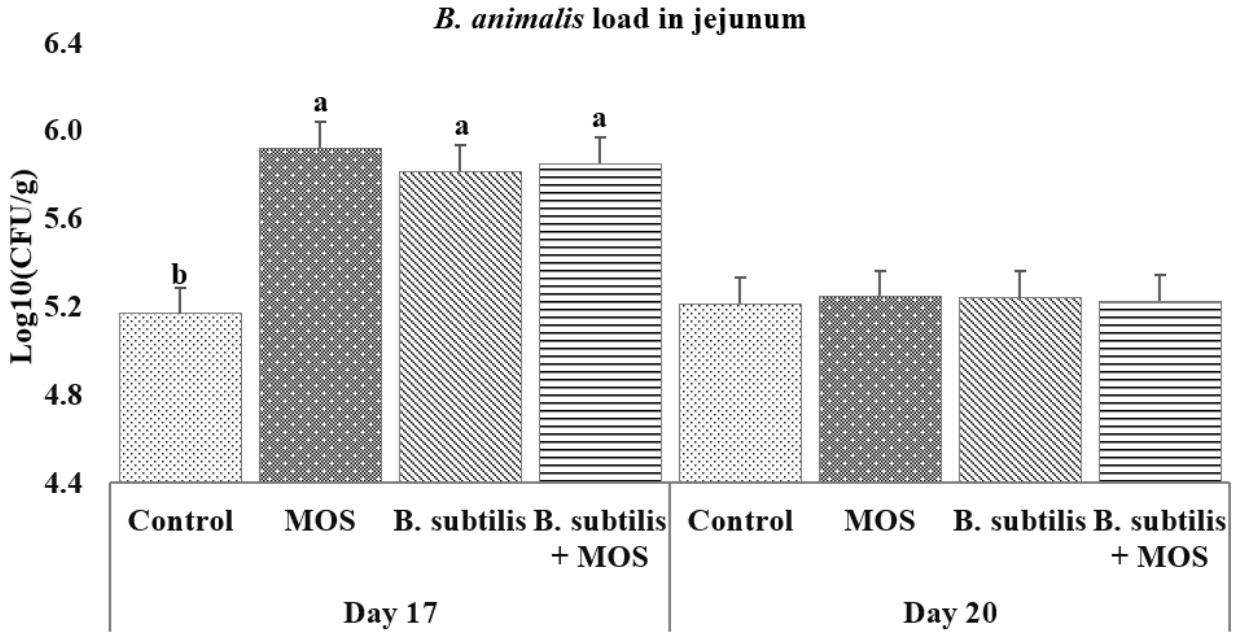

Figure 1.9. Effect of *B. subtilis*, MOS, and *B. subtilis* + MOS supplementation on enteric *B. animalis* load in jejunal contents. Birds were randomly distributed to one control and three treatment groups: 0.1% MOS, 0.1% *B. subtilis*, and 0.1% *B. subtilis* + MOS group. At 17 d of age, all birds were fed control diets for 72hrs. At d17 and d20, samples from jejunum were collected and analyzed for *B. animalis* using real-time PCR and reported as Log10 CFU/g. Means with no common superscript within a column differ significantly ($P < 0.05$). ($n = 6$).

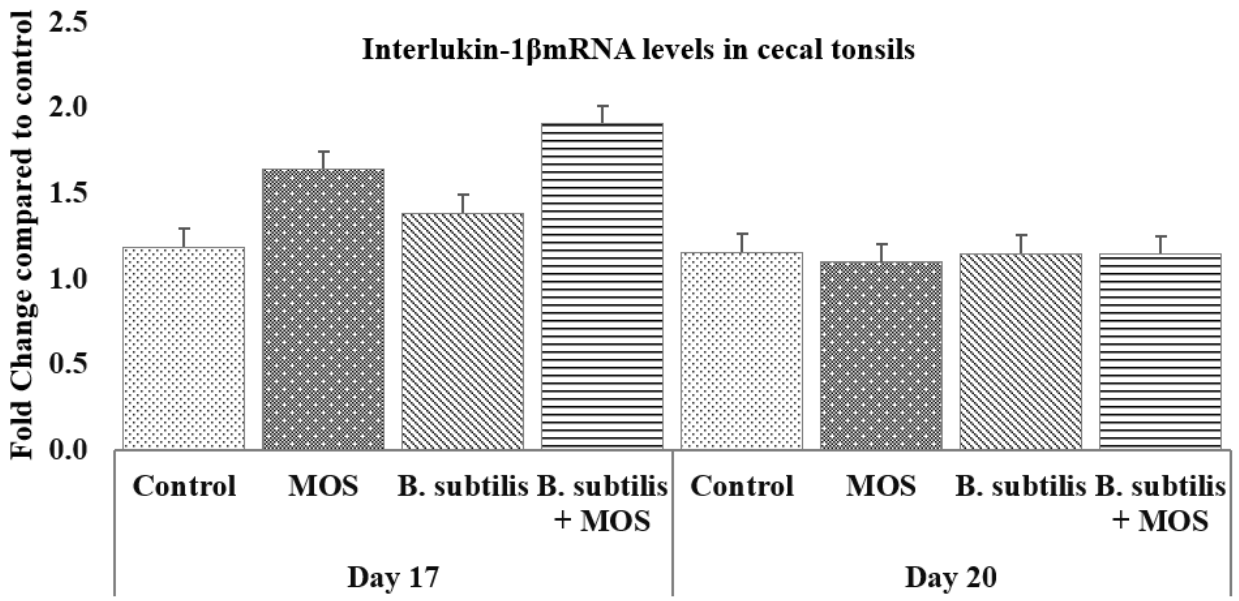

Figure 1.10. Effect of *B. subtilis*, MOS, and *B. subtilis* + MOS supplementation on Interleukin-1β. Birds were randomly distributed to one control and three treatment groups: 0.1% MOS, 0.1% *B. subtilis*, and 0.1% *B. subtilis* + MOS group. At d17 and d20, cecal tonsil samples were collected. Relative IL-1β mRNA content was analyzed after correcting for GAPDH mRNA and normalizing to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

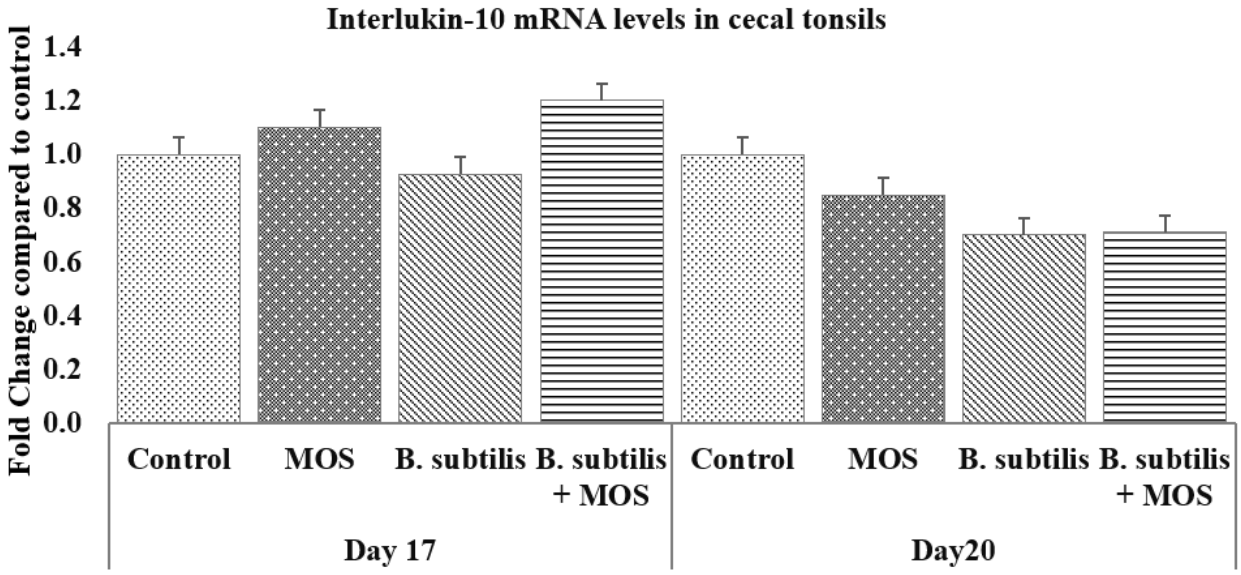

Figure 1.11. Effect of *B. subtilis*, MOS, and *B. subtilis* + MOS supplementation on Interleukin-10. Birds were randomly distributed to one control and three treatment groups: 0.1% MOS, 0.1% *B. subtilis*, and 0.1% *B. subtilis* + MOS group. At d17 and d20, cecal tonsil samples were collected. Relative IL-10 mRNA content was analyzed after correcting for GAPDH mRNA and normalizing to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

CHAPTER 4

EFFECTS OF *BACILLUS SUBTILIS* AND MANNAN OLIGOSACCHARIDE SUPPLEMENTATION ON DECREASING NECROTIC ENTERITIS SEVERITY IN BROILERS INDUCED WITH NECROTIC ENTERITIS

2

[2] R. Reddyvari, R. Shanmugasundaram, R.K Selvaraj. To be submitted to PLOS ONE

ABSTRACT

Due to the ban of antibiotics usage for growth promotion, probiotics and prebiotics have become potential replacements to control pathogenic diseases in animals. The motive of this study was to analysis the effectiveness of probiotic *Bacillus subtilis* and prebiotic mannan oligosaccharide (MOS) on birds induced with necrotic enteritis (NE). *In vitro* study was conducted to test *B. subtilis* cell-free supernatants (CFS) to decrease *Clostridium perfringens* proliferation. *B. subtilis* CFS reduced the proliferation of *C. perfringens* at 5:1 and 10:1 supernatant-to-pathogen dilution *in vitro* ($P < 0.05$). For the *in vivo* study, 192 broiler chicks were randomly assigned to four treatments: control unchallenged (basal diet), control challenged, 0.05%*B.subtilis* + challenged, or 0.05%MOS + challenged with six replicates per treatment and eight birds per pen. On day 14, the challenge groups were orally gavaged with 0.5 mL/bird of ~5000 *Eimeria maxima* oocysts. On days 19, 20, and 21, birds were challenged with 1 x 10^8 CFU/ml *C. perfringens*. Birds in the control unchallenged treatment were orally mock challenged with 0.5 mL/bird of 1X Phosphate Buffer Saline (PBS). On day 21, there was a decrease in the intestinal permeability and increased levels of claudin-1 mRNA in the ileum in the *B. subtilis* + challenged ($P < 0.05$) group. On day 35, there were increased levels of IL-1 mRNA in control challenged group ($P < 0.05$). On days 21 and 35, there were increased TGF-β mRNA levels in the *B. subtilis* + challenged and MOS + challenged groups. On day 28, birds in the *B. subtilis* treatment group had significantly greater anti-ECP IgA relative to those in the control unchallenged group ($P < 0.05$). The results suggest that *B. subtilis* and MOS can modulate the immune system and improve gut health during an NE challenge.

INTRODUCTION

Necrotic enteritis (NE) usually causes mortality in chickens, with mortality rates up to 50% within 24hrs of infection, which makes it challenging to treat the disease (Kaldhusdal et al. 2001). NE usually occurs shortly after the switch from starter to grower diet due to disturbances in the intestinal microbiome caused by diet change (Timbermont et al. 2011). The development of necrotic enteritis could be promoted by toxin production or the predisposing factors like infection with coccidia, high protein diet, stress that facilitates excessive bacterial growth and slows feed passage rate in the small intestine. Bacitracin, lincomycin, and penicillin are antibiotics used to treat NE (Hagris, 2020). Since the ban on using antibiotics in poultry, alternatives to antibiotics like probiotics, prebiotics, vaccines, enzymes, and organic acids can play an essential role in reducing NE incidence in chickens (Caly et al. 2015). *Eimeria* and *C. perfringens* are one of the main predisposing factors of NE. It was found that intestinal damage and the *C. perfringens* load were higher in NE disease when Eimeria and *C. perfringens* were co-infected than Eimeria alone (Park et al. 2008). The upregulation of INF-γ in the jejunum was seen in *E. maxima*-infected chickens, which aided in the clearance of infection (Laurent et al. 2001).

The toxins released by *C. perfringens* increase the paracellular permeability across the enterocytes either by altering cellular permeability or destroying the actin cytoskeleton or by obliterating the tight junction proteins. Due to this, increased translocation of bacteria from the gut epithelium to internal organs, increased concentrations of plasma LPS, and leakage of plasma proteins into the intestinal lumen are observed, which creates ideal conditions for *C. perfringens* growth (Latorre et al. 2018). Tight junction proteins like claudin either increase or decrease intestinal permeability. Claudin-2 and claudin-16 are the pore-forming proteins, whereas claudin-1, claudin-3, and claudin-5 are the barrier formation proteins. Zonula occluden (ZO) like ZO-1 and

ZO-2 are tight junction proteins which help in improving the gut integrity (Günzel and Yu 2013; Awad, and Hess 2017).

By altering the roles of dendritic cells, macrophages, T and B lymphocytes, probiotics regulate the host's innate and adaptive immune responses. The probiotic blend of *Lactobacillus* and *Bifidobacterium spp*. upregulated the suppressor activity of Tregs and decreased cytokine Th1 and Th2 responses (Yan 2014). The probiotics have been found to exhibit *in vitro* and *in vivo* antimicrobial activity against *C. perfringens* and *Eimeria* spp. in various studies. Studies with *B. subtilis* showed that they might promote the competitive exclusion of the pathogenic bacteria like *Salmonella*, increase the villus height, and secretion of defensive mucins (Abudabos et al., 2019). Short-chain fatty acids (SCFA) are released when prebiotics are broken down by probiotics or commensal bacteria. SCFA enhances the production of host defensin proteins and plays a role in improving intestinal immunity (Vanessa, n.d.; Gibson et al., 2017). Prebiotics boost gut immune responses, and oligosaccharides such as beta-glucans improve phagocytosis and prevent pathogenic bacteria from adhering to the intestine. Feeding MOS in poultry has increased the villi height, increased the IgA antibody titers, and increased body weight gain (Adhikari and Kim 2017).

This study evaluates the effect of feeding *B. subtilis* and MOS to birds challenged with NE and assessing their impact on *C. perfringens* load, immune responses, and intestinal permeability.

MATERIALS AND METHODS

In vitro experiment

The anti-microbial activity of *B. subtilis* cell-free supernatant

This anti-microbial assay was carried out based on Santini et al. with several modifications (Santini et al. 2010). Single isolated colonies from a *B. subtilis* strain were inoculated in MRS broth and incubated overnight. The *B. subtilis* broth was centrifuged at 4,500 X g. The supernatant was filter-sterilized using a 0.22µm Millipore filter (Sigma Aldrich, St Louis, MO) to get cell-free supernatant (CFS). Four separate experiments were conducted. For the first experiment, a volume of 10 µl of an overnight culture of *C. perfringens* was added in each well of the 96-well plate and incubated with four different supernatants: microbial dilutions 0:1, 10:1, 5:1, or 1:1 in triplicates. The total incubated volume was 110 µl adjusted using TGB broth and was incubated for 8h under microaerobic conditions at 37°C. The absorbance was measured after 8h at 600nm. The effect of *B. subtilis* culture supernatants on the inhibition of *C. perfringens* proliferation *in vitro* was reported as optical density (O.D) values.

To identify the nature of the anti-microbial compound released by *B. subtilis,* the assay was repeated by adding proteinase K (1mg/ml) and neutralizing the CFS by adding 1M NaOH. For the second experiment, CFS was treated with 1mg/ml of proteinase K for 5mins, and later, the activity of proteinase K was inhibited by heating the CFS at 100 ^{o}C for 30 mins. For the third experiment, CFS was neutralized to pH 7.2 by adding 1M NaOH. For the fourth experiment, CFS was neutralized and treated with proteinase K. For all these experiments, the supernatants: microbial dilutions were 0:1, 10:1, 5:1, or 1:1, and the absorbance was measured after 8h at 600nm. The recorded data reflect the mean value for four separate experiments; each experiment was repeated three times.

In vivo experiment

The Institutional Animal Care and Use Committee at the University of Georgia approved all the animal protocols.

Birds and Necrotic enteritis challenge

A total of 192 Cobb 500 male broilers were randomly assigned to four treatments: basal-fed unchallenged (control) or basal-fed challenged or 0.05% (0.5kg/ton) of *B. subtilis* + challenged or 0.05% (0.5kg/ton) of MOS + challenged treatments. The basal feed was a corn and soybean meal diet (Table 4). At the Poultry Research Center at the University of Georgia, birds from the two treatments were housed in two separate BSL-2 rooms. In the unchallenged control treatment, birds were orally mock challenged with a 0.5 mL/bird of 1X PBS. Birds in all the challenge treatments were orally gavaged with 0.5 mL/bird of ~5000 *E. maxima* oocysts on day 14, and on day 19, 20, and 21, birds were challenged with 1 x 10^8 CFU/ml *C. perfringens*.

E. maxima oocysts were received from Dr. Lorraine Fuller from the Department of Poultry Science, University of Georgia. *C. perfringens* was incubated in thioglycolate broth (TGB) for 24h in an anaerobic atmosphere at 37°C. The challenge stock, 1 x 10^8 CFU/mL of *C. perfringens*, was confirmed by serial dilutions and direct plating on blood agar.

Production parameters

Birds and feed were weighed weekly. BWG and FCR were calculated.

Effect of *B. subtilis* and MOS lesion scores in birds induced with NE

Two birds were picked randomly from each pen on days 21, 28, and 35; they were sacrificed, weighed, and examined for NE lesions on jejunum and ileum. The lesion scoring was done by a

person blinded to the treatments based on a zero to three score (0 = no lesions, 1 = mild, 2 = moderate, and 3 = severe) (Hofacre et al. 2011)

Effect of *B. subtilis* and MOS on intestinal permeability in birds induced with NE

One bird per pen was orally gavaged with 1ml of FITC-d solution (2.2mg FITC-d/ml). Blood was drawn from the heart of the chicken after 2 hrs. The serum was collected by centrifuging the samples at 1000 xg. Serum samples were diluted and read in the spectrophotometer at the excitation wavelength of 485 nm and an emission wavelength of 528nm. Fluorescence levels in the samples were transformed based on a standard curve to the respective FITC-d ng per mL of serum.

Effect of *B. subtilis* and MOS on *C. perfringens* load in cecal and ileal colonization in birds induced with NE

On days 21, 28, and 35, Ceca and ileum samples were collected aseptically in stomacher bags, were placed on ice, and transferred to the laboratory. A rubber mallet was used to macerate the Ceca and ileum samples, and 3X (wt/vol) 1X PBS was added and homogenized for 60 seconds, and the samples were transferred into 50ml tubes and stored in -80^0 C. 1ml of the samples was taken and used for DNA extraction. The DNA extracted was analyzed for *C. perfringens* by real-time PCR using primers (5′- AAAGGAAGATTAATACCGCATAA- 3′) and (5′- ATCTTGCGACCGTACTCCCC- 3′) (Shanmugasundaram et al. 2011). The annealing temperature was 56^0 C.

Effect of *B. subtilis* and MOS on IL-1β, IL-10, TGF-β4, TNF-α, and iNOS in birds induced with NE

Cecal tonsils were collected on days 21, 28, and 35 in 2 ml Eppendorf tubes filled with 1 ml of RNA later (Qiagen). Excess RNA later from the tubes was drained, and the samples were kept at -80 ° C. The total extracted RNA was reverse transcribed into cDNA. The mRNA analyzed was IL-1β(5′-CTACACCCGCTCACAGTCCT-3′ and 5′-TCACTTTCTGGCTGGAGGAG-3′) (Shanmugasundaram et al. 2011), IL-10 (5'- CATGCTGCTGGGCCTGAA-3' and 5'-CGTCTCCTTGATCTGCTTGATG-3') (Rothwell et al. 2004), TGF-β4 (5'-AGGATCTGCAGTGGAAGTGGAT-3' and 5'-CCCCGGGGTTGTGTGTTGGT-3') , TNF-α (5'-ATCCTCACCCCTACCCTGTC-3' and 5'-GGCGGTCATAGAACAGCA-3') and iNOS (5'-AGTGGTATGCTCTGCCTGCT-3' and 5'- CCAGTCCCATTCTTCTTCC-3') (Selvaraj and Klasing 2006) by using real-time PCR (CFX96 Touch Real-Time System, BioRad). All the genes were normalized using the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5'-TCCTGTGACTTCAATGGTGA-3' and 5'-CACAACACGGTTGCTGTATC-3') (Dube et al. 2014). The annealing temperatures of IL-1, IL-10, TGF-β4, TNF-α, iNOS, and GAPDH are 58^0 C, 55^0 C, 58^0 C, 58^0 C, 60^0 C, and 55^0 C, respectively. The $2^{(Ct\ Sample-\ Housekeeping)}/2^{(Ct\ Comparison-Housekeeping)}$ comparative Ct approach was used to measure the fold shift from the reference, where Ct is the threshold cycle. The Ct was calculated when the fluorescence increases exponentially 2 folds above the baseline by iQ5 software (Biorad).

Effect of *B. subtilis* and MOS on Zonula occluden and Claudin-1 in birds induced with NE

On days 21, 28, and 35, approximately 2 cm of the proximal jejunum and ileum tissue was excised and collected into 2 ml Eppendorf tubes filled with 1 ml of RNA later (Qiagen). Excess RNA later from the tubes was drained, and the samples were kept at -80^{o}C. The total extracted RNA was

reverse transcribed into cDNA. The mRNA analyzed was zonula occluden (ZO) using the primers (5'-TGTAGCCACAGCAAGAGGTG-3', and 5'-CTGGAATGGCTCCTTGTGGT-3') and Claudin-1 (5'-CATACTCCTGGGTCTGGTTGGT-3', and 5'-GACAGCCATCCGCATCTTCT-3') with annealing temperatures of 57.4^0C and 57.5^0C respectively (Oxford and Selvaraj 2019).

Effect of *B. subtilis* and MOS on IgG and IgA antibodies titers in birds induced with NE.

Samples of serum and bile were obtained at age d21, 28, and 35 from one bird per cage (total 6 birds/treatment). ELISA plates were coated with Native extracellular proteins (ECP) of *C. perfringens* with 20 μg / ml for analysis of IgA or 5 μg / ml for analysis of IgG (Nunc Maxisorp™, ThermoFisher Scientific, Waltham, MA). Bile has been diluted to 1:50, and serums have been diluted to 1:10 using 2.5% PBS, non-fat dried milk, and 0.1% Tween 20 (VWR, Radnor, PA). A 1:50,000 dilution of Horseradish peroxidase (HRP) conjugated polyclonal goat anti-chicken IgG (Bethyl, Montgomery, TX) or 1:100,000 dilution of HRP-conjugated polyclonal goat anti-chicken IgA (Southern Biotech, Birmingham, AL) was used as a secondary antibody. The spectrophotometer (Biochek, Scarborough, ME) was used to calculate the absorbance at 450 nm, and the values are reported at OD_{450}.

STATISTICAL ANALYSIS

For lesion scoring, statistical analysis was done using the nonparametric Wilcoxon rank test (JMP Software, Cary, NC). A one-way ANOVA was used for *in vitro* assays to analyze the influence of various parameters studied on dependent variables. For the *in vivo* study, a one-way ANOVA was used to examine the effect of *B. subtilis*, MOS, and challenge control group and their interaction on the dependent variables. With Tukey's HSD post hoc test, variations between means were analyzed. Significant effects at $P < 0.05$ were reported.

RESULTS

In vitro experiment

Anti-microbial activity of *B. subtilis* supernatant

The CFS of *B. subtilis* showed anti-microbial activity at 5:1 and 10:1 dilutions ($P < 0.01$, Figure 2.1). The nature of the anti-microbial compound was evaluated by assessing the effects of proteolytic molecule proteinase K and neutralizing the pH on CFS inhibitory activity against *C. perfringens*. When the CFS was treated with proteinase K, anti-microbial activity was observed at 5:1 and 10:1 dilutions compared with 0:1 dilution. When the CFS was neutralized to a pH of 7, there was no anti-microbial activity in any of the dilutions ($P < 0.05$, Figure 2.1). When the CFS is treated with both proteinase K and neutralized pH, there was no anti-microbial activity in any dilutions ($P < 0.05$, Figure 2.1).

In vivo experiment

Effect of *B. subtilis* and MOS on performance parameters in birds induced with NE.

No significant differences were found in FCR and BWG between the control, control challenged, *B. subtilis* + challenged, and MOS + challenged treatments ($P > 0.05$, Figure 2.16, 2.17).

Effect of *B. subtilis* and MOS on lesion scores in birds induced with NE.

NE lesions were higher in the challenged control group compared to the unchallenged control and challenged groups supplemented with *B. subtilis* and MOS. NE lesions scores were lower in the *B. subtilis* + challenged group compared to the control challenged and MOS + challenged groups ($P < 0.05$, Table 6).

Effect of *B. subtilis* and MOS on intestinal permeability in birds induced with NE.

On day 21, there was a 92.8% increase in FITC-d in the control challenged group than the unchallenged control. Birds in the *B. subtilis* + challenge and MOS + challenge had a 26% and 18.5% decrease in serum FITC-d than the control challenged group ($P < 0.05$, Figure 2.2). On day 28, there were no statistically significant differences in all the NE challenged treatments compared with the unchallenged control group.

Effect of *B. subtilis* and MOS on *C. perfringens* load in cecal and ileal colonization in birds induced with NE.

On d21, d28, and d35, the *C. perfringens* control challenge treatment had a significantly higher *C. perfringens* load in the ceca compared with the control unchallenged group. On day 28, there was a 1.1 Log10 CFU/g increase in *C. perfringens* cecal load in control challenged group compared to the unchallenged control and 0.5 and 0.6 Log10 CFU/g higher compared with the *B. subtilis* + challenged and MOS + challenged groups ($P < 0.01$, Figure 2.3). On day 28, there was 1.1 Log10 CFU/g increase in *C. perfringens* ileal load in the control challenged group compared to the unchallenged control and 0.5 and 0.6 Log10 CFU/g higher when compared with the *B. subtilis* + challenged and MOS + challenged groups ($P < 0.05$, Figure 2.4).

Effect of *B. subtilis* and MOS on IL-1β, IL-10, TGF-β4, TNF-α, and iNOS in birds induced with NE.

On d21, there was a 1.1-fold increase in cecal tonsil TGF- β mRNA amounts in the *B. subtilis* + challenged group compared to the challenged control group ($P < 0.05$, Figure 2.5). On d28, there were no statistically significant differences in the TGF- β mRNA amounts between all the challenged groups compared to the unchallenged control group ($P > 0.05$, Figure 2.5). On d35,

there was a 3.4- fold increase in cecal tonsil TGF- β mRNA amounts in the *B. subtilis* + challenged group compared to the control unchallenged and a 0.8-fold increase when compared to the control challenged group ($P < 0.05$, Figure 2.5). On d21, d28, and d35, there were no statistically significant differences in the IL-10 mRNA amounts between all the challenged groups compared to the unchallenged control group ($P > 0.05$, Figure 2.6). On d21, d28, and d35, there were no statistically significant differences in the TNF-α mRNA amounts between all the challenged groups when compared to the unchallenged control group ($P > 0.05$, Figure 2.7). On both d21 and d28, there were no statistically significant differences in the IL-1β mRNA amounts between all the challenged groups when compared to the unchallenged control group ($P > 0.05$, Figure 2.8). On day 35, there was a 3.2-fold increase in cecal tonsil IL-1 β mRNA amounts in the control challenged group than the unchallenged control group ($P < 0.05$, Figure 2.8). On d21, d28, and d35, there were no statistically significant differences in the iNOS mRNA amounts between all the challenged groups when compared to the unchallenged control group ($P > 0.05$, Figure 2.9).

Effect of *B. subtilis* and MOS on Zonula occluden and Claudin-1 in birds induced with NE.

On d21, d28, and d35, there were no statistically significant differences in the zonula occluden mRNA amounts in jejunum between all the challenged groups when compared to the unchallenged control group ($P > 0.05$, Figure 2.10). On d21, d28, and d35, there were no statistically significant differences in the zonula occluden mRNA amounts in ileum between all the challenged groups when compared to the unchallenged control group ($P > 0.05$, Figure 2.11). On d21, d28, and d35, there were no statistically significant differences in the claudin-1 mRNA amounts in jejunum between all the challenged groups when compared to the unchallenged control group ($P > 0.05$, Figure 2.12). On d21, there was a 2.9- fold increase in claudin-1 mRNA amounts in the ileum in the *B. subtilis* + challenged group when compared to the control unchallenged and

a 2.1-fold increase when compared to the control challenged group ($P < 0.05$, Figure 2.13). On d28 and d35, there were no statistically significant differences in the claudin-1 mRNA amounts in ileum between all the challenged groups when compared to the unchallenged control group ($P > 0.05$, Figure 2.13).

Effect of *B. subtilis* and MOS on IgG and IgA antibodies titers in birds induced with NE.

On day 21, there was a 46.8% decrease in bile IgA levels in the control challenged group compared to the unchallenged control group ($P < 0.05$, Figure 2.14). On day 21, there was a 39% decrease in bile IgA levels in MOS + challenged birds compared to the unchallenged control group ($P < 0.05$, Figure 2.15). On day 28, there was a 75.8% increase in bile IgA levels in MOS + challenged birds compared to the unchallenged control group ($P < 0.05$, Figure 2.15). On d35, there were no statistically significant differences in bile IgA levels between all the challenged groups when compared to the unchallenged control group ($P > 0.05$, Figure 2.15). On d21, d28, and d35, there were no statistically significant differences in the serum IgG levels between all the challenged groups when compared to the unchallenged control group ($P > 0.05$, Figure 2.16).

DISCUSSION

Anti-microbial activity is considered as one of the potential benefits of probiotics. *Bacillus* is recognized as the producer of bacteriocins. It was reported that *B. clausii* exhibited antimicrobial activity against gram-positive bacteria, and the antimicrobial compound was characterized as bacteriocin (Urdaci et al. 2004). Findings from this study demonstrated that *B. subtilis* CFS has anti-microbial activity at 5:1 and 10:1 dilutions of *B. subtilis* CFS: *C. perfringens* ($P < 0.05$). When CFS was treated with proteinase K, there was no effect on the anti-microbial activity, but when the CFS was neutralized, there was a loss in anti-microbial activity. This makes us assume that the

anti-microbial activity might be due to SCFA. In an *in vitro* experiment, *L. acidophilus* and *L. fermentum* were able to inhibit the growth of *C. perfringens* in acidic pH, but no inhibition was observed in the neutral pH. *C. perfringens* is sensitive to acidic pH (Guo et al. 2017). *B. subtilis PB6* produced SCFA in the ceca and helped in the recovery of NE challenged birds (Aljumaah et al. 2020). SCFA are known to lower the pH, and the acidic conditions inhibit the growth of pathogenic bacteria (Fooks and Gibson 2002).

This study *in vivo* experiment assesses the effects of *B. subtilis* and MOS supplementation on performance parameters, intestinal lesion scores, *C. perfringens* load, intestinal membrane permeability, and immune parameters in broilers experimentally infected for necrotic enteritis (NE). There were no statistically significant differences in FCR and BWG between the supplemented groups and the control group.

On day 28, the challenged treatments supplemented with *B. subtilis* and MOS had decreased by 0.5 and 0.4 log10CFU/g of *C. perfringens* in ceca compared with the control challenged group. On day 28, 0.5 and 0.7 log10CFU/g of *C. perfringens* load in the ileum was decreased in the challenged treatments supplemented with *B. subtilis* and MOS compared to the control challenged group. By producing SCFA like butyrate, MOS reduces the intestinal pH, which helps suppress pathogenic bacterial growth (Mohamed and Hafez 2011). MOS supplementation decreases the *C. perfringens* load in turkeys of 6wks of age (Sims et al. 1996). In the *in vitro* experiment conducted in our lab, it was seen that *B. subtilis* CFS has an anti-microbial activity, which might be attributed to the production of SCFA. The NE challenged control group had higher intestinal lesion scores compared to the other challenged and unchallenged treatments. The challenged group supplemented with *B. subtilis* and MOS had lower intestinal lesions than the control diet-fed

challenged group. This shows that *B. subtilis* and MOS (Hofacre et al. 2011)can reduce the effects caused due to NE.

NE is associated with the disruption of the gut epithelial barrier and necrotizing the tissue. Maintaining gut integrity is crucial since it enables the free permeation of essential elements, nutrients, water, and serves as the first line of defense against pathogenic microbes. FITC-d does not leak into the circulation unless there is an impairment in the intestine's tight junctions, which typically happens during enteric infections (Latorre et al., 2018). FITC-d levels in serum from challenged control birds were significantly higher than unchallenged birds. On day 21, the *B. subtilis* + challenge group had lower levels of serum FITC-d followed by the MOS + challenge group when compared with the control challenge group. This indicates that *B. subtilis* and MOS were able to reduce significant damage to the intestinal epithelium. A study had shown that *B. licheniformis* could enhance the gut barrier function and prevent endotoxins and pathogens passing from the intestinal mucosa into blood (Xu et al. 2018). It was reported that *Bacillus* direct-fed microbial also improved the gut integrity in birds challenged with *S. typhimurium* and NE (Hernandez-patlan et al. 2019).

In this study, we noticed there were no statistically significant differences for the zonula occluden mRNA amounts between treatments. On day 21, we observed that there were significant differences in Claudin-1 gene expression in the ileum. *B. subtilis* supplemented group had the highest claudin-1 mRNA levels between all the groups. ZO-1, ZO-2, and ZO-3 bind to the actomyosin belt, and disruption of the actin cytoskeleton disrupts the tight junction proteins and results in a leaky gut. Metabolites and bioactive molecules produced by probiotics have been known to maintain tight junction proteins and enhance the intestinal epithelial barrier function (Rao and Samak 2013). *Lactobacillus* probiotics were able to strengthen the barrier function and

upregulated the ZO-1 in rats infected with necrotizing enterocolitis (Blackwood et al., 2017). Claudin-1, 3, -4, -5 are pore sealing proteins and claudin-2, -7, -12 are pore-forming proteins. Increased expression of claudin-1 enhances the barrier function and prevents the passage of pathogens and toxins from the mucosa (Awad et al., 2017).

IL-1 and TNF-α are pro-inflammatory cytokines, whereas IL-10 and TGF-β are anti-inflammatory cytokines. In NE, pro-inflammatory cytokines are upregulated, whereas anti-inflammatory cytokines are downregulated. This study noticed that *B. subtilis* upregulated the gene expression of TGF- β. Increased mRNA levels of TGF- β also contributes to increased gut barrier function. Studies conducted with different probiotics like *L. fermentum* (Cao et al. 2009), *E. faecium* (Wu et al. 2019), and *L. delbruekii (Hegazy and El-bedewy 2010)* showed that these probiotics ameliorated the inflammatory conditions and increased the expression of anti-inflammatory cytokines. IL-1 mRNA levels were higher in control challenged group compared with the unchallenged group. Significant differences were found on the d35 *B. subtilis*, and MOS supplemented challenged group had low IL-1 mRNA levels. A study with *L. reuteri* showed that it downregulates the pro-inflammatory cytokines like TNF-α during inflammation (Lin, Thibodeaux, and Pen 2008). In rats, it was seen that butyrate produced by the prebiotics inhibited the production of pro-inflammatory cytokines INF-γ and IL-2. The impact of prebiotics on IL-1 was found to be inconsistent (Shokryazdan et al., 2017).

IgA has a vital role in mucosal immunity as it counteracts the inflammatory effects of other antibodies, strengthens nonspecific defensive mechanisms, and suppresses pathogenic functions. In the current study, MOS supplementation induced the IgA in bile on day 28 compared with control. On day 21, the unchallenged control group had higher bile IgA levels. A study conducted in dogs saw an increase in the IgA levels and enhanced protection against pathogenic bacteria

(Swanson et al. 2002). *L. johnsonii* reduced the IgA levels in the ileum in subclinical necrotic enteritis infected birds (Wang et al. 2018). *B. subtilis* B10 showed an increase in IgA concentrations and improved mucosal immunity in broilers (Rajput et al. 2011). There were no statistically significant differences in the serum IgG levels.

In conclusion, supplementation with *B. subtilis* and MOS decreased the *C. perfringens* load in ceca and ileum, increased the intestinal barrier function, enhanced tight junction mRNA expression, and increased the IgA levels. Supplementation of feed with *B. subtilis* and MOS might be helpful in reducing the *C. perfringens* load and intestinal disruption caused by necrotic enteritis.

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Basal diet ingredients and calculated nutrient composition (Table 5)

	Starter (0 – 35 d)
Ingredients	%
Corn	58.47
Soybean Meal, 48% CP	35.15
Soybean Oil	2.27
Monocalcium phosphate, 21%	1.38
Limestone	1.59
DL-Methionine	0.21
L-Lysine-HCL, 78%	0.14
Salt (NaCl)	0.35
Vitamin premix[1]	0.08
Mineral premix[2]	0.35
Total:	100
Calculated Nutrient Composition	
ME, kcal/kg	3,050
Crude Protein, %	21.44
Crude Fat, %	4.55
Crude Fiber, %	2.17
Calcium, %	0.95
Total Phosphorus, %	0.71
Avail. Phosphorus< %	0.45
Sodium, %	0.16

Potassium, %	0.92
Chloride, %	0.27
Lysine, %	1.31
Methionine, %	0.56
TSAA, %	0.91
Threonine, %	0.87
Tryptophan, %	0.29
Arginine, %	1.50

[1]Vitamin mix provided the following (per kilogram of diet): thiamin-mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B12 (cobalamin), 12.0g; pyridoxine-HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-reinyl acetate, 2,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.
[2]Trace mineral mix provides the following (per kilogram of diet): manganese (MnSO4.H2O), 101 mg; iron (FeSO4.7H2O), 20 mg; zinc (Zn)), 80 mg; copper (CuSO4.5H2O), 3 mg; iodine (ethylene diamine dihydroiodide), 0.75 mg; magnesium (MgO), 20 mg; selenium (sodium selenite), 0.3 mg.

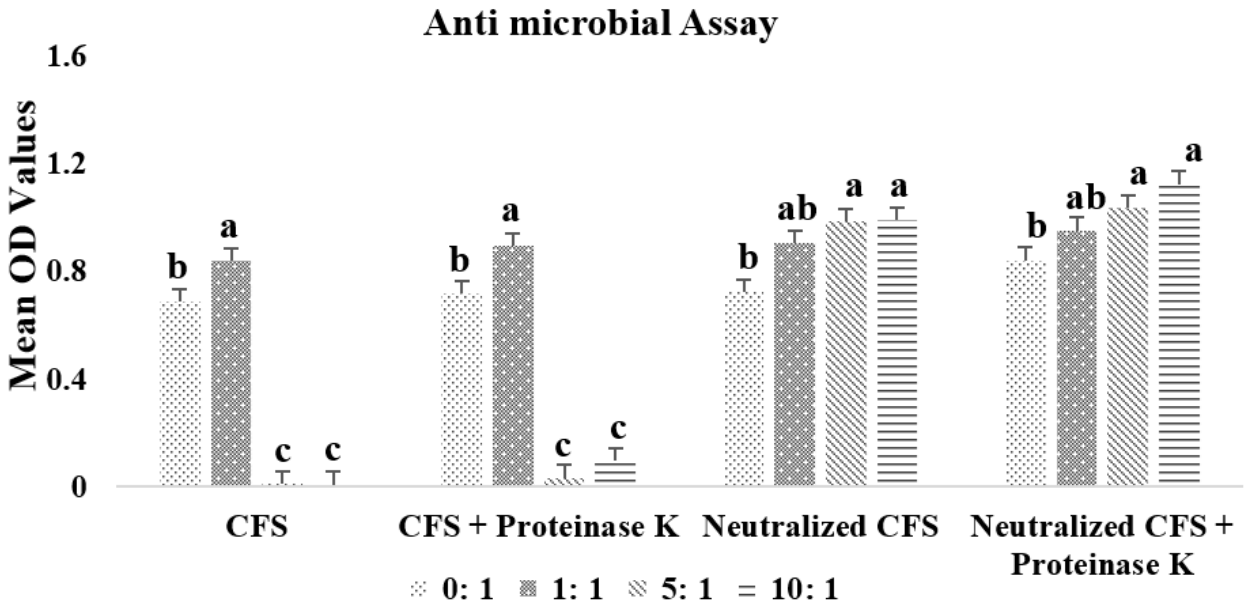

Figure 2.1. The anti-microbial activity of *B. subtilis* cell-free supernatant: Effect of cell-free *B. subtilis* supernatants (CFS) on *C. perfringens in vitro* proliferation. Overnight culture of single isolated colonies of *B. subtilis* strain was centrifuged at 4,500 × *g* for 10 min to collect supernatants. The supernatant was filtered sterilized using a 0.22-µm filter to collect cell-free supernatant. *C. perfringens* overnight culture was incubated with 0:1, 10:1, 5:1, or 1:1 cell-free supernatant-to-clostridial dilutions. To identify the nature of the anti-clostridial compounds, proteinase K was added to the CFS (CFS + Proteinase K), or CFS was neutralized by 1M NaOH (Neutralized CFS) or CFS was treated by both Proteinase K and 1M NaOH (Neutralized CFS + Proteinase K). A spectrophotometer was used to measure absorbance was at 600 nm at 8 hrs. n = 3. Bars (+SEM) with no common superscript differ significantly ($P < 0.05$). *P* values: CFS P < 0.05; CFS + Proteinase K P < 0.05; Neutralized CFS P < 0.05; Neutralized CFS + Proteinase K P < 0.05.

Table 6:- Effect of *B. subtilis* and MOS on lesion scores in birds induced with NE.

Level	Rank Sum (Day 21)
Challenged	121.000[a]
MOS+ Challenged	85.000[ab]
B. subtilis + Challenged	73.000[b]
Unchallenged	21.000[c]

Level	Rank Sum(Day 28)
Challenged	97.000[a]
MOS + Challenged	71.500[ab]
B. subtilis + Challenged	71.500[ab]
Unchallenged	60.000[b]

Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). On day 21 ($P < 0.05$), and day 28 ($P > 0.05$) lesions from 2 birds/cage were scored from 0 to 3.

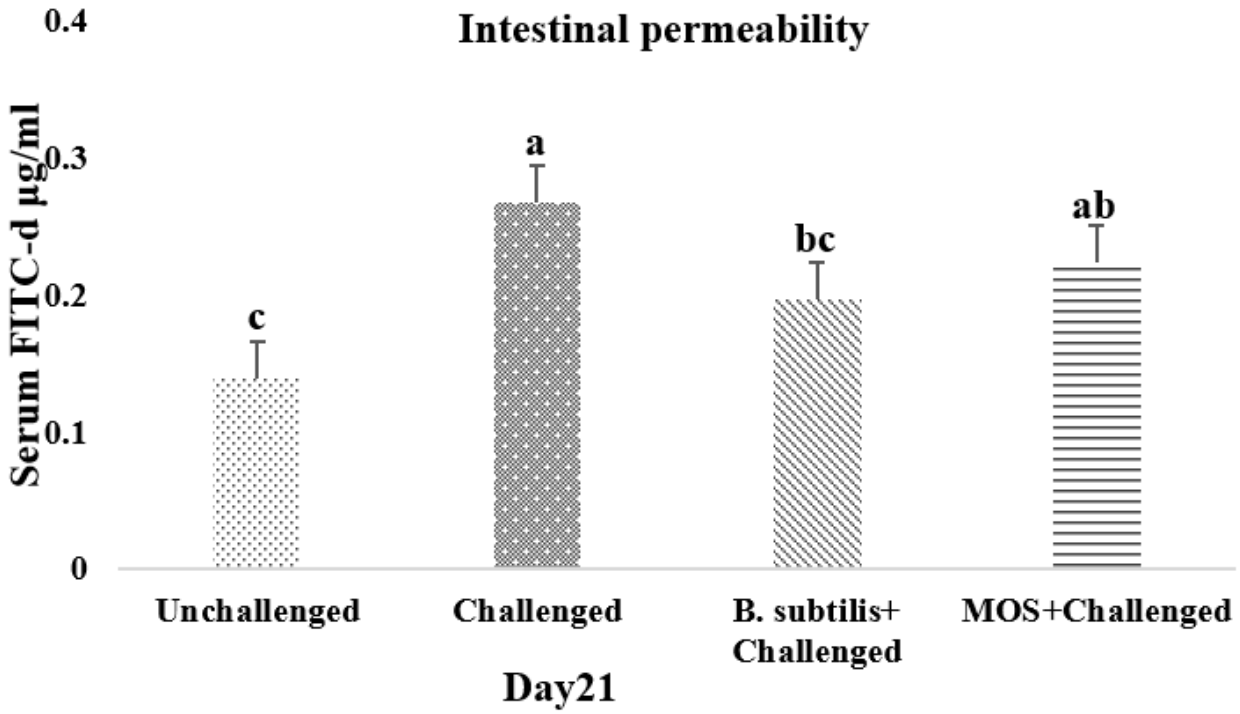

Figure 2.2. Effect of *B. subtilis* and MOS on intestinal permeability in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On day 14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on days 19, 20, and 21, birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). On d21, birds were orally gavaged with FITC-d, and blood samples were collected after 2hrs. Serum samples were diluted and read in the spectrophotometer at an excitation wavelength of 485 nm and an emission wavelength of 528nm. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

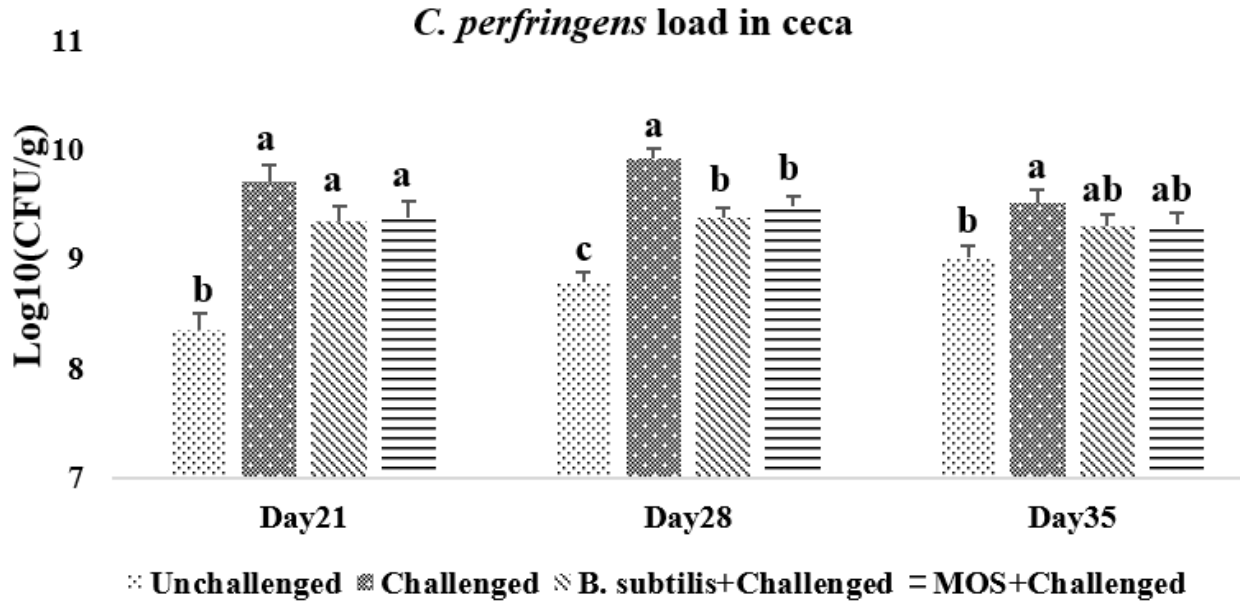

Figure 2.3. Effect of *B. subtilis* and MOS on cecal *C. perfringens* load in birds induced with NE: Birds were randomly assigned to one control unchallenged, and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On day 14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20, and 21, birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28 and 35 cecal contents were collected analyzed for *C. perfringens* using real-time PCR and reported as Log10 CFU/g Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

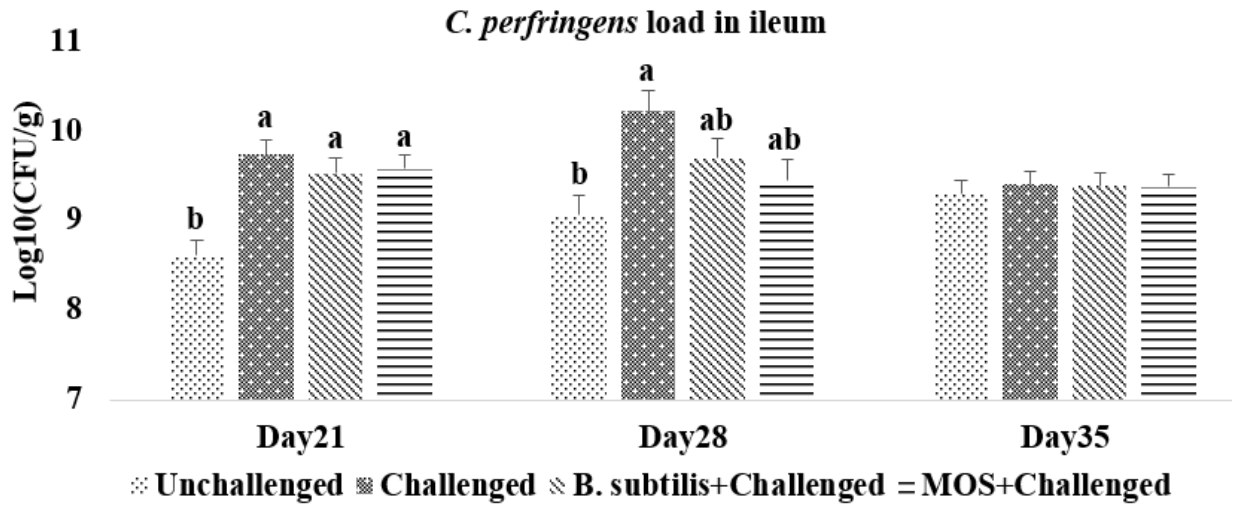

Figure 2.4. Effect of *B. subtilis* and MOS on ileal *C. perfringens* load in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28 and 35 ileal contents were collected analyzed for *C. perfringens* using real-time PCR and reported as Log10 CFU/g Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

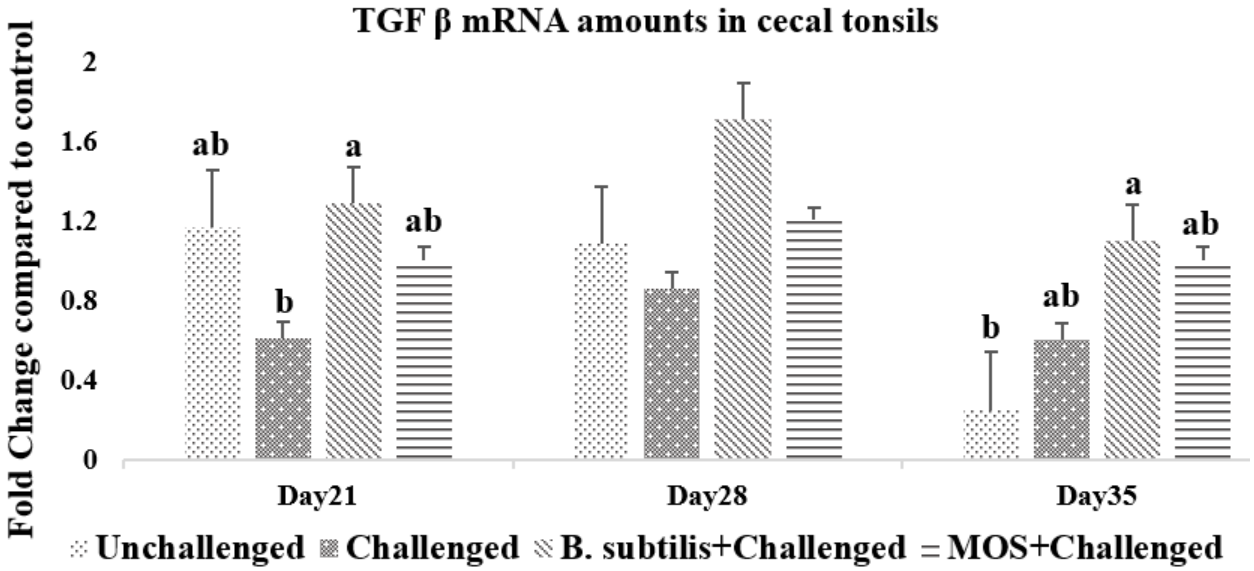

Figure 2.5. Effect of *B. subtilis* and MOS on TGF-β in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28, and 35, cecal tonsil samples were collected. Relative TGF-β mRNA content was measured for GAPDH mRNA and normalized to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

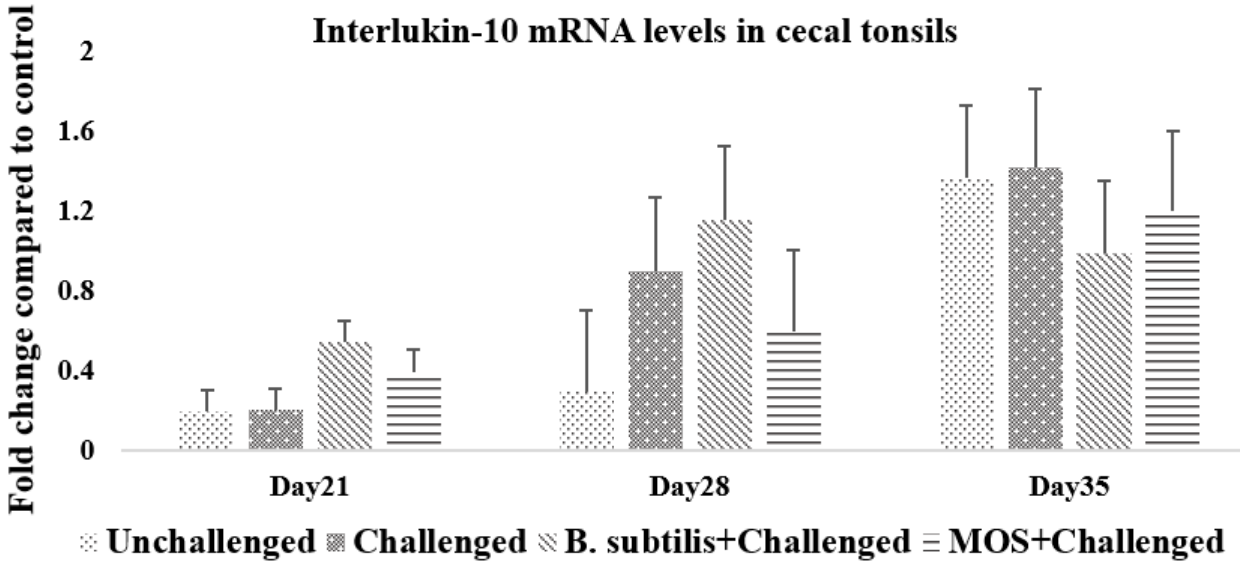

Figure 2.6. Effect of *B. subtilis* and MOS on IL-10 in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28, and 35, cecal tonsil samples were collected. Relative IL-10 mRNA content was measured for GAPDH mRNA and normalized to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

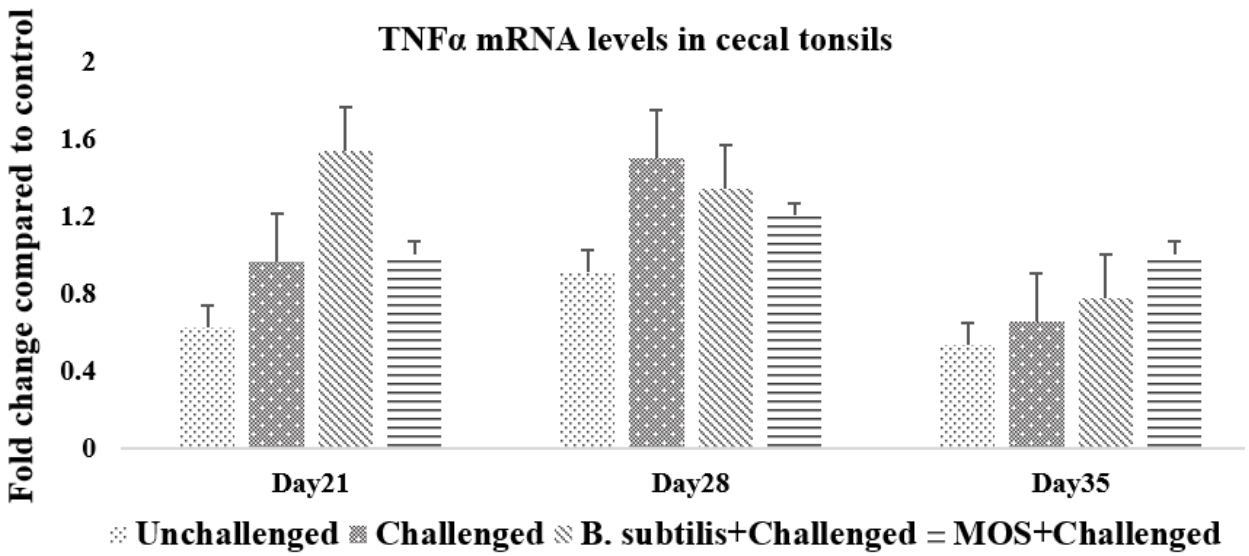

Figure 2.7. Effect of *B. subtilis* and MOS on TNF-α in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28, and 35, cecal tonsil samples were collected. Relative *TNF-α* mRNA content was measured for GAPDH mRNA and normalized to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

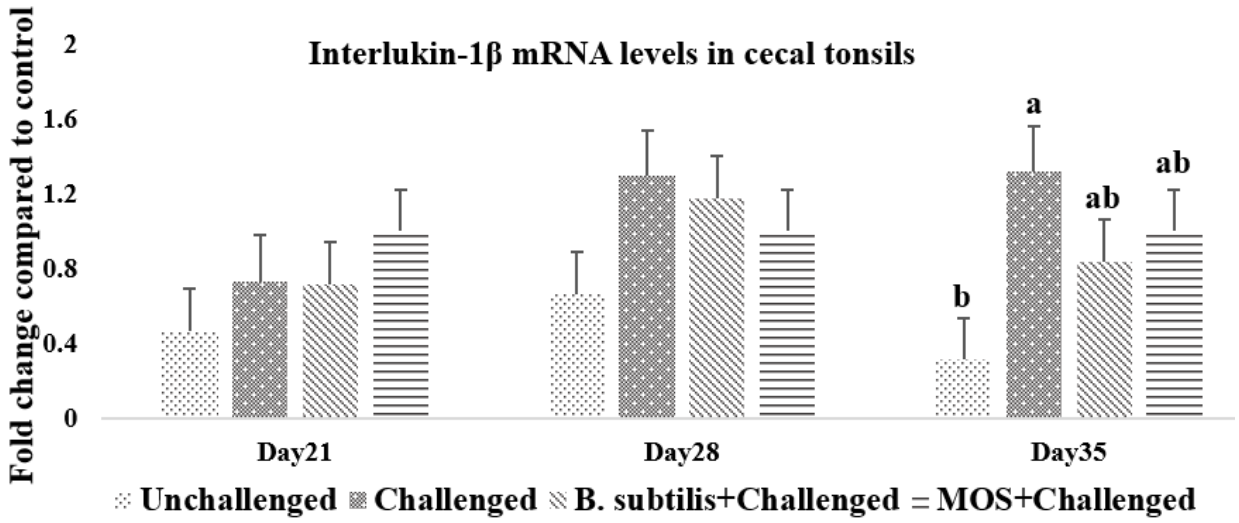

Figure 2.8. Effect of *B. subtilis* and MOS on IL-β in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28, and 35, cecal tonsil samples were collected. Relative IL-β mRNA content was measured for GAPDH mRNA and normalized to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

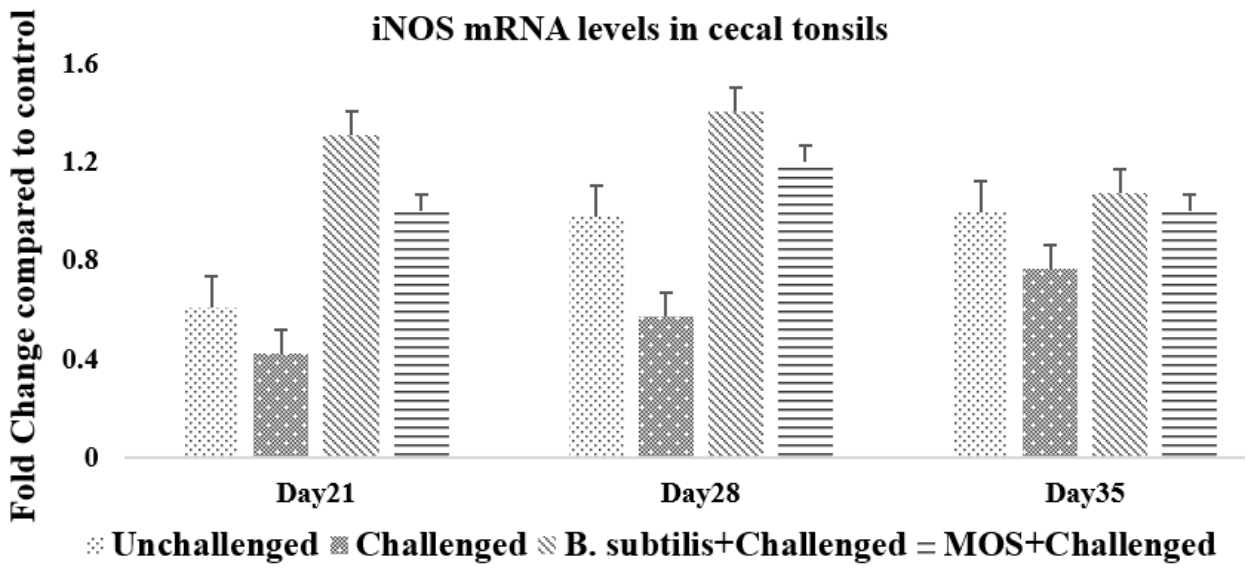

Figure 2.9. Effect of *B. subtilis* and MOS on iNOS in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28, and 35, cecal tonsil samples were collected. Relative iNOS mRNA content was measured for GAPDH mRNA and normalized to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

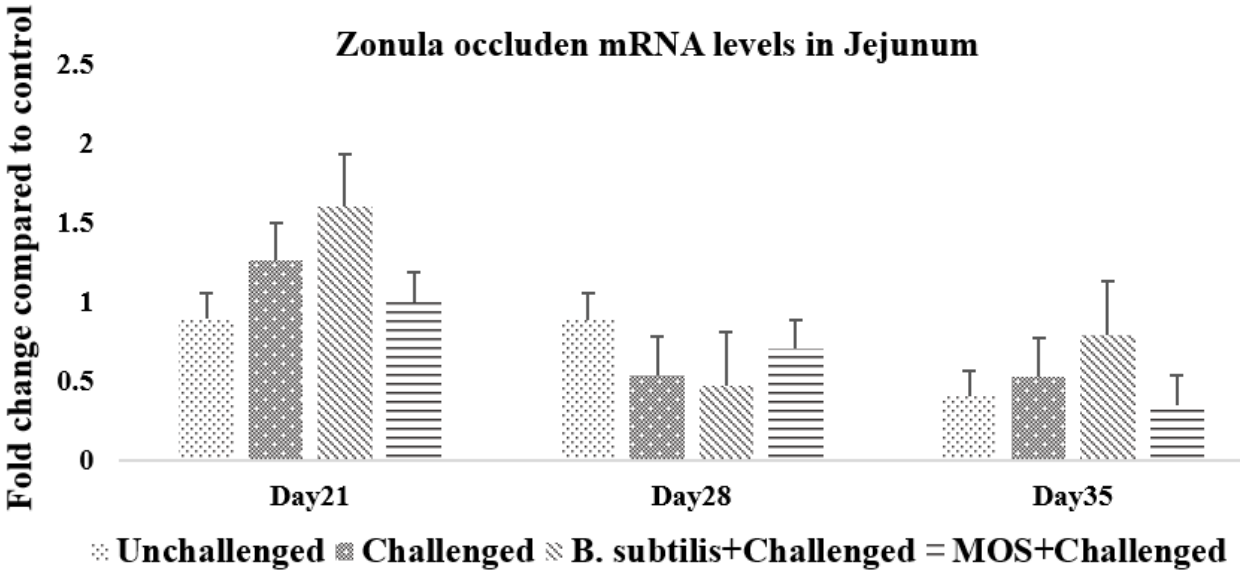

Figure 2.10. Effect of *B. subtilis* and MOS on zonula occluden in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28, and 35, jejunum samples were collected. Relative zonula occluden mRNA content was measured for GAPDH mRNA and normalized to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

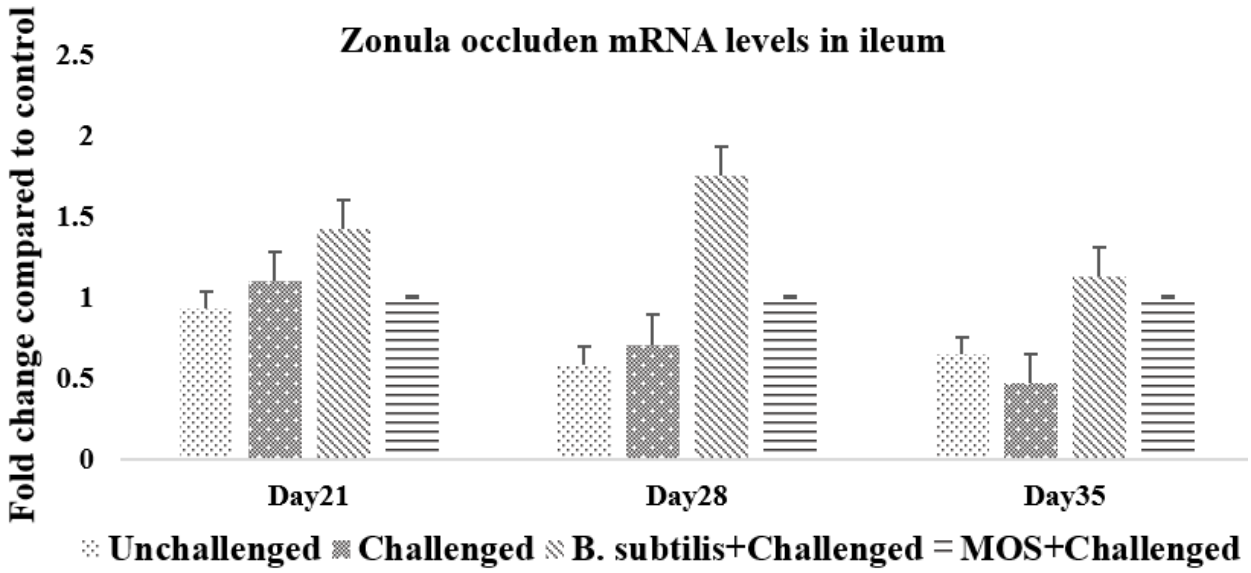

Figure 2.11. Effect of *B. subtilis* and MOS on zonula occluden in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups:- control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28 and 35 ileum samples were collected. Relative zonula occluden mRNA content was measured for GAPDH mRNA and normalized to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

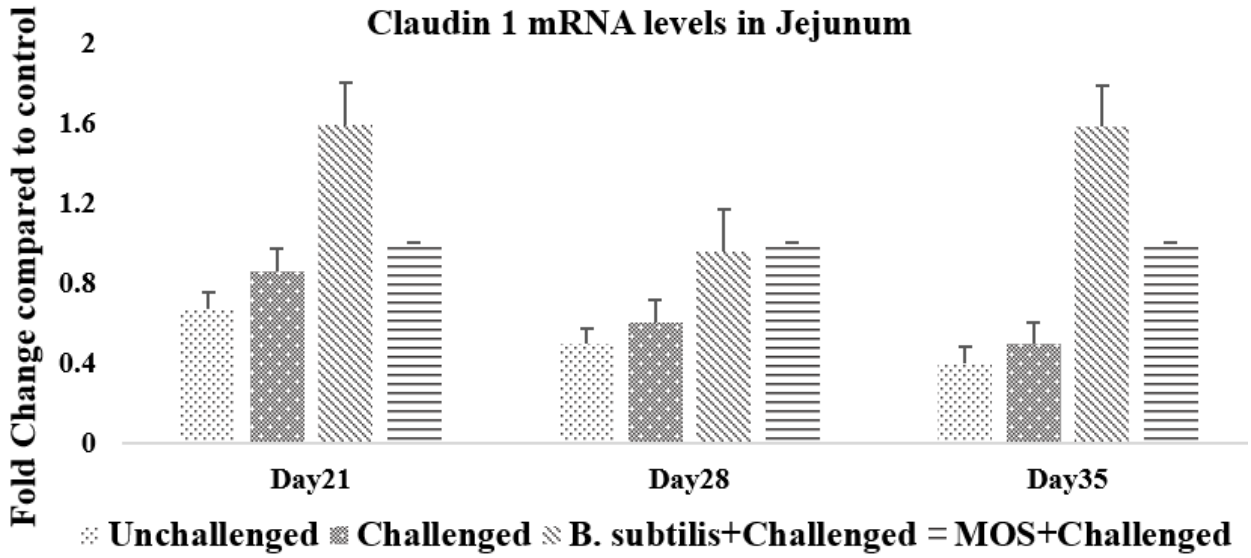

Figure 2.12. Effect of *B. subtilis* and MOS on jejunal claudin-1 in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28, and 35, jejunum samples were collected. Relative claudin-1 mRNA content was measured for GAPDH mRNA and normalized to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

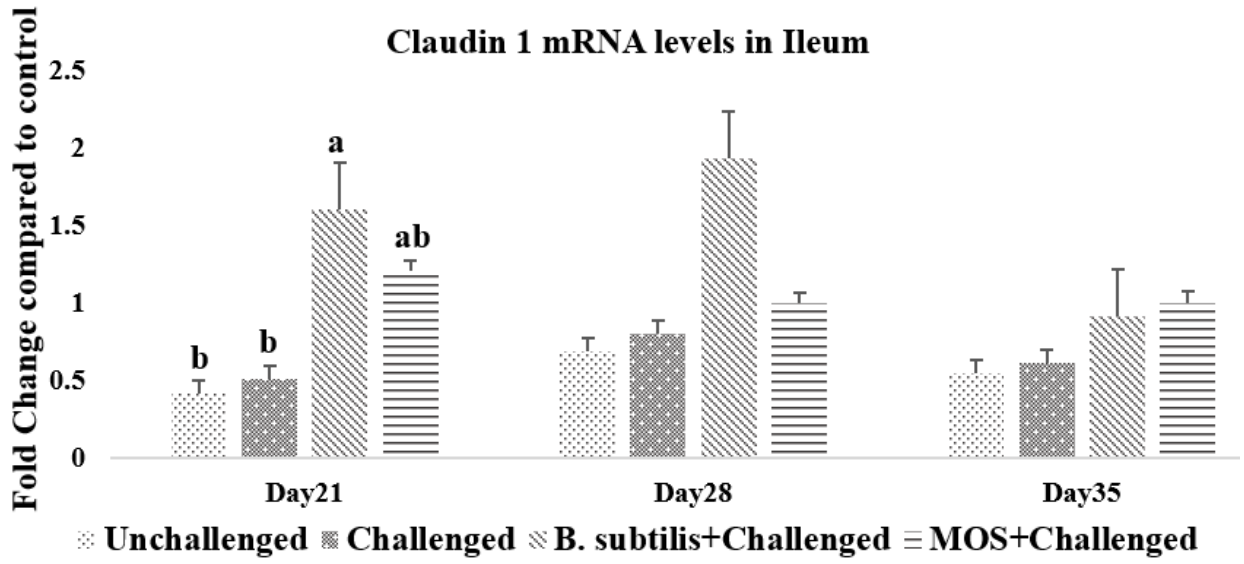

Figure 2.13. Effect of *B. subtilis* and MOS on ileal claudin-1 in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28 and 35 ileum samples were collected. Relative claudin-1 mRNA content was measured for GAPDH mRNA and normalized to the mRNA content of the control group. Bars (+ SEM) with no common superscript are significantly different ($P < 0.05$), ($n = 6$).

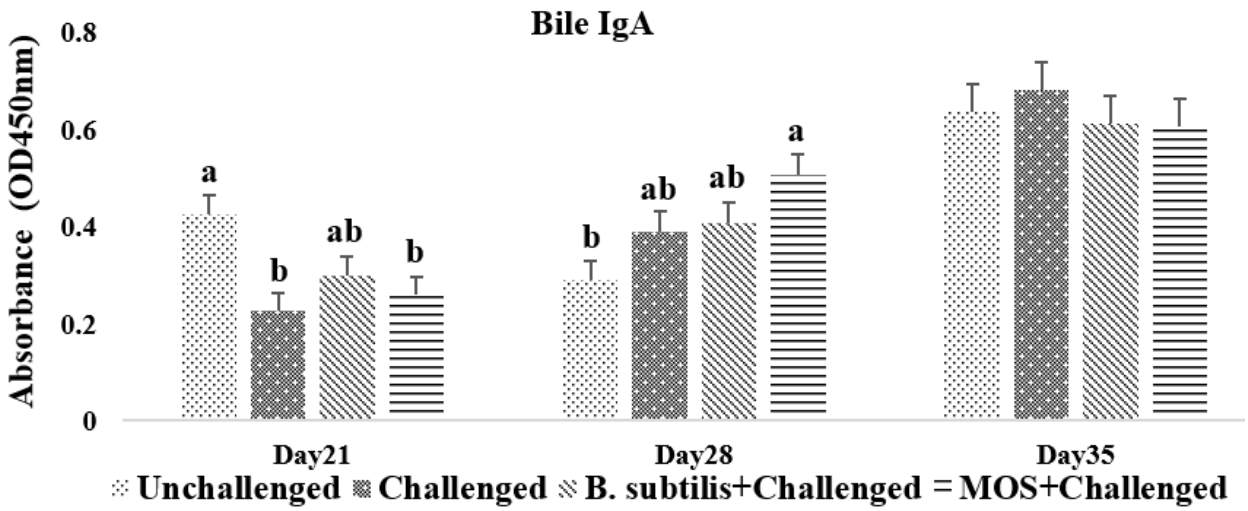

Figure 2.14. Effect of *B. subtilis* and MOS on IgA antibodies titers in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28 and 35 bile samples were collected for IgA amounts were analyzed by ELISA, and values reported as optical density (OD) values. Mean + SEM. n=6. ($P < 0.05$)

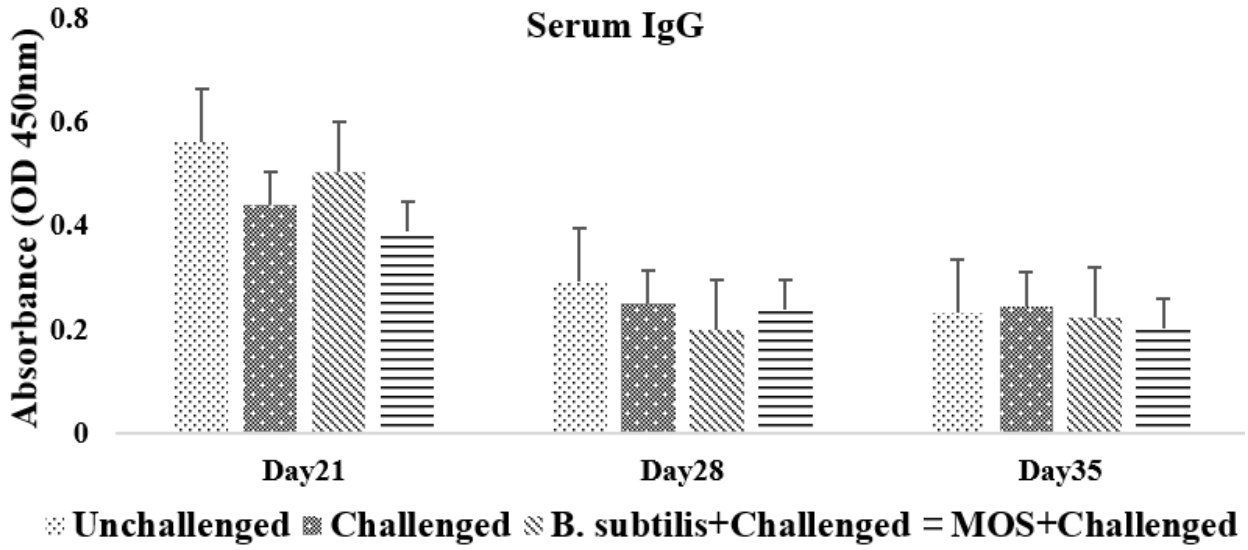

Figure 2.15. Effect of *B. subtilis* and MOS on IgG antibodies titers in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). At d21, 28 and 35 blood samples were collected for anti-ECP IgG amounts were analyzed by ELISA, and values reported as optical density (OD) values. Mean + SEM. n=6. ($P < 0.05$)

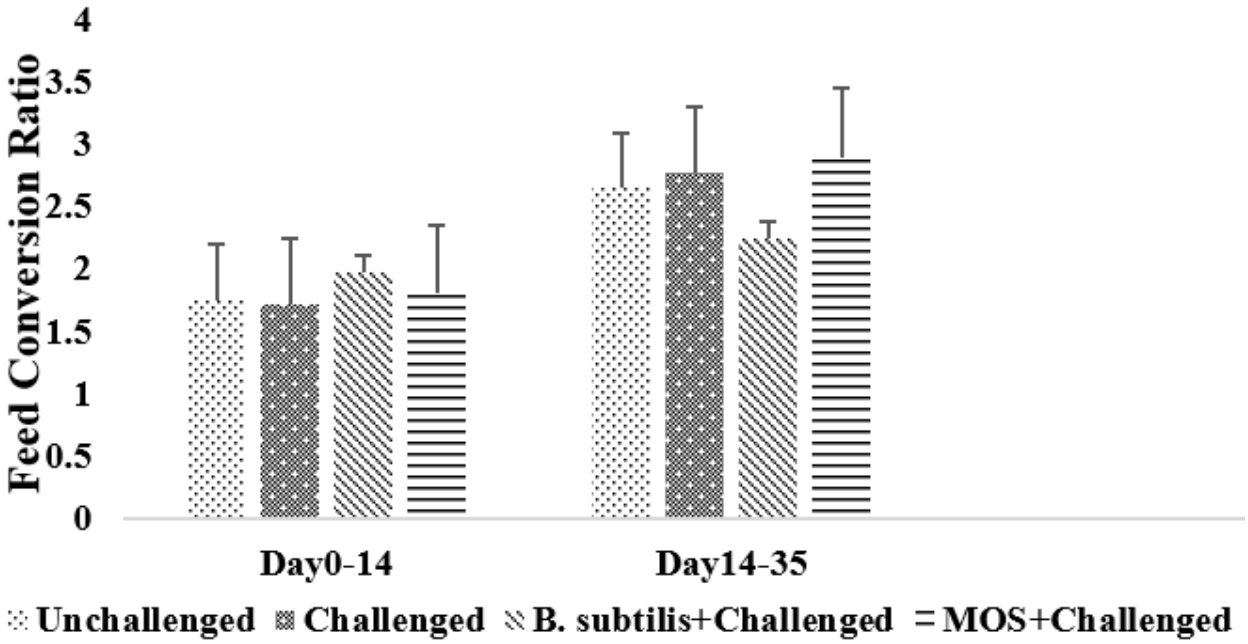

Figure 2.16. Effect of *B. subtilis* and MOS on feed conversion ratio in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). Birds and feed were weighed once every week. Means with no common superscript within a column differ significantly Mean + SEM ($P < 0.05$). ($n = 6$).

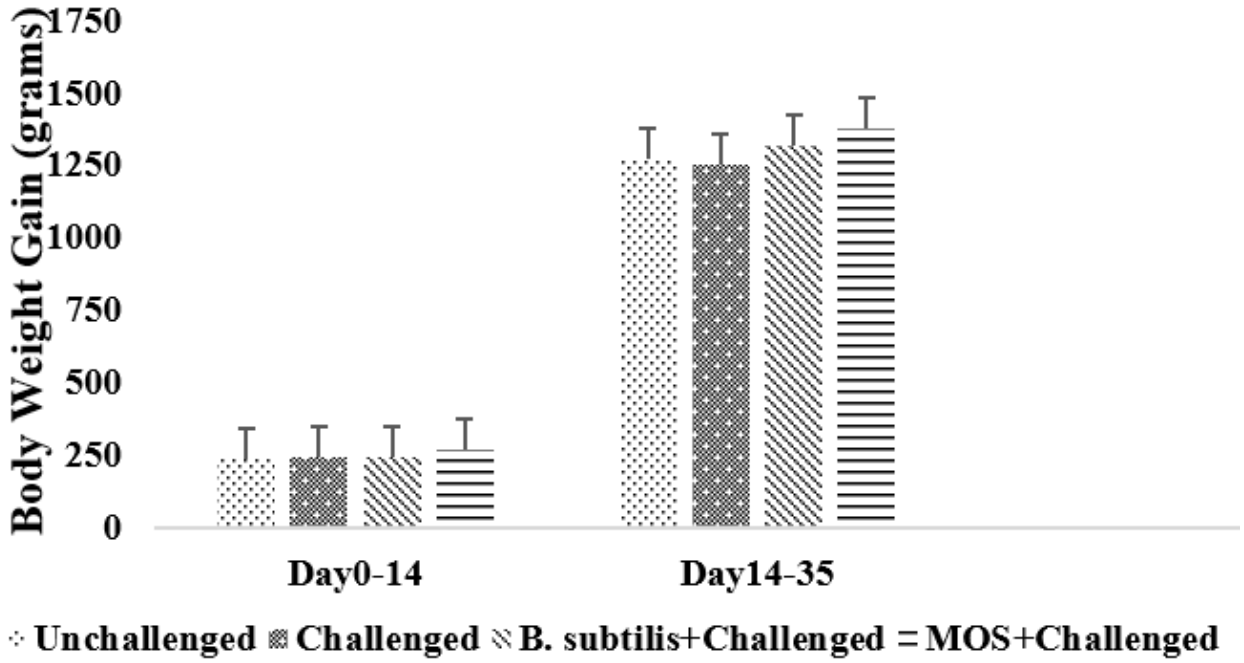

Figure 2.17. Effect of *B. subtilis* and MOS on body weight gain in birds induced with NE: Birds were randomly assigned to one control unchallenged and three treatment challenged groups: control challenged, 0.05% *B. subtilis* + challenged, and 0.05% MOS + challenged group. On d14, challenged treatment groups were orally gavaged with ~5000 *Eimeria* oocysts (0.5m1/bird), and on d19, 20 and 21 birds were orally gavaged with 1 X 10^8 CFU/bird of *C. perfringens* (0.5m1/bird). Birds and feed were weighed once every week. Means with no common superscript within a column differ significantly Mean + SEM ($P < 0.05$). ($n = 6$).