PREBIOTIC AND PROBIOTIC MICROBIOLOGY IN POULTRY

A Dissertation

by

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ABSTRACT

The *overall goal* of this research was to investigate antibiotic alternatives for use in poultry production.

Antibiotic administration in poultry production has declined due to changes in consumer preferences and governmental regulations that limit or ban their use. Their growth promoting properties have been attributed to impacts on the gastrointestinal microbiota, therefore, the gastrointestinal microbiota is thought to be an ideal target for the development of antibiotic alternatives. The *objective* of this research was to investigate the effects of probiotics and prebiotics in broilers and the mechanisms important to the functionality of prebiotics administered to poultry.

Specific Aim 1: Evaluate effects of a poultry prebiotic on growth performance and the colonization of human foodborne pathogens in broiler chickens

We administered two doses of a dietary prebiotic composed of refined functional carbohydrates derived from *Saccharomyces cerevisiae* with yeast culture to broiler chickens. Increased body weights, body weight gain, and feed intake were observed with administration of the high prebiotic dose, and administration of either dose reduced cecal *Campylobacter* counts.

Specific Aim 2: Evaluate effects of prebiotics and Direct-Fed Microorganisms on growth performance and microbial populations in broiler chickens

We administered a dietary prebiotic, two direct-fed *Bacillus*, and a synbiotic to broilers. We observed improved feed efficiency and body weights and a reduction of

Campylobacter with administration of each functional feed ingredient, and Lactic Acid Bacteria increased with prebiotic administration.

Specific Aim 3: Evaluate the effects of prebiotic compounds on adhesion of *Salmonella* Typhimurium and *Campylobacter jejuni* to epithelial tissue *in vitro*

We performed adhesion assays using the LMH cell line to conduct a dose response of a poultry prebiotic product on the adherence of *Salmonella* Typhimurium and *Campylobacter jejuni*. We then evaluated the adhesion reduction of both human foodborne pathogens with purified components of the prebiotic and four commercial prebiotic products and observed significantly different reductions for both comparisons.

DEDICATION

For my sweet Mae.

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NOMENCLATURE

ABF	Antibiotic-free
ADFI	Average Daily Feed Intake
ADG	Average Daily Gain
AGP	Antibiotic Growth Promoters
BMD	Bacitracin Methylene Disalicylate
BW	Body Weight
d	Day
DFM	Direct-Fed Microorganisms
FCR	Feed Conversion Ratio
FOS	Fructooligosaccharides
GI	Gastrointestinal
GOS	Galactooligosaccharides
h	Hour
LAB	Lactic Acid Bacteria
MOI	Multiplicity of Infection
MOS	Mannanoligosaccharides
RFC	Refined Functional Carbohydrates
SCFA	Short-Chain Fatty Acid
VFA	Volatile Fatty Acid

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW OF PROBIOTICS AND PREBIOTICS IN POULTRY PRODUCTION

Introduction

Sub-therapeutic doses of antibiotics have been administered widely in livestock production because of their ability to improve growth performance (Moore et al., 1946; Jukes et al., 1950) and prevent and mitigate disease (Glisson et al., 1989; Hu and McDougald, 2002). However, the use of antibiotic growth promoters (AGP) has declined due to concerns regarding the consequences to human and animal health resulting from the development of antibiotic-resistant bacteria (Silbergeld et al., 2008). In addition, consumer demand for antibiotic-free (ABF) production has increased (Hume, 2011), resulting in regulations that ban AGP use by the European Union (Cogliani et al., 2011) and limit AGP use in the United States (Department of Health and Human Services, 2015). The reduction in AGP use has led to decreased animal growth and feed efficiency, and increased burden of disease (Wierup, 2001; Dibner and Richards, 2005). Thus, development of effective alternatives to antibiotics will help ensure that poultry remains an efficient, inexpensive, and safe source of animal protein for the consumer (Singer and Hofacre, 2006; Gaucher et al., 2015).

Modification of the host microbiota by antibiotics has been suggested to improve growth performance of livestock through inhibition of subclinical infections (Barnes et al., 1978), reduced competition for nutrients between the microbiota and host-animal

(Monson et al., 1954; Eyssen, 1962), decreased production of growth depressing metabolites by the resident microbiota (Dang et al., 1960), and enhanced absorption of nutrients through the thinner intestinal wall of antibiotic-fed animals (Eyssen and Desomer, 1963; Boyd and Edwards, 1967). The growth-promoting activity of antibiotics is attributed to their effect on the gastrointestinal (GI) microbiota (Dibner and Richards, 2005), and such increased growth has been observed with antibiotic administration to animals with normal microbiota (Moore et al., 1946; Stokstad and Jukes, 1950; Miles et al., 2006) but not observed in germ-free animals (Coates et al., 1963). This suggests the metabolic activities of intestinal microorganisms are competitive with growth performance of the host animal (Gaskins et al., 2002). Because the growth promoting activities of AGP are a result of their effects on the gastrointestinal microbiota, the microbiota is an important target for the development of alternatives to antibiotics.

Probiotics and dietary prebiotics are important functional feed additives, those used to provide a health benefit beyond satisfying basic nutritional requirements (Marriot, 2000), seen widely as important potential alternatives to AGP. Their administration has been demonstrated to improve growth performance parameters, including body weights (Awad et al., 2009; Shivaramaiah et al., 2011) and feed efficiency (Cavazzoni et al., 1998; Mookiah et al., 2014). In addition, the administration of probiotics and prebiotics has been shown to modify the GI microbiota resulting in the promotion of populations of beneficial bacteria such as the Lactic Acid Bacteria (LAB) (Knarreborg et al., 2008) and the reduction of important poultry pathogens, such as *Clostridium perfringens* (Sims et al., 2004; Knap et al., 2010), and food-borne human

pathogens, such as *Salmonella* (Huff et al., 2013; Jeong and Kim, 2014) and *Campylobacter* (Arsi et al., 2015; Froebel et al., 2019).

Although the benefits of probiotic and prebiotic use have been widely reported, their overall effectiveness is mixed and the mechanisms responsible are not well understood. In this review, we will explore the mechanisms of probiotic and prebiotic functionality important to their application in poultry production as alternatives to AGP for growth promotion and pathogen reduction.

Probiotics and Prebiotics

Probiotics and Direct-Fed Microorganisms

Probiotics are defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014)."When applied in animal agriculture, the term *probiotic* is often and incorrectly used interchangeably with *direct-fed microbial*. Although the former is an expert consensus definition rather than a legal definition, there is a regulatory basis for the latter. The United States Food and Drug Administration defines *direct-fed microbial products* as "products that are purported to contain live (viable) microorganisms (FDA, 1995)." This definition does not state or imply claims for any benefit of their use, and, depending on the manner in which any claims are made, they may be cause for regulatory action as an adulterant. Because the suffix *-ial* is used to form adjectives from nouns, the grammatically correct term would, in fact, be Direct-Fed Microorganism (DFM) rather than the more commonly used *direct-fed microbial*. Indeed, the Official Publication of the Association of American

Feed Control Officials contains a list of species approved for use as D*irect-Fed Microorganisms* (AAFCO, 2019).

Studies investigating the effects of administering beneficial microorganisms to livestock typically only measure and report growth performance results. Although it is presumed that any improvements to growth performance are the result of some beneficial health effect, the term *probiotic*, should be reserved to reference to microorganisms for which there are published reports of benefits to *bone fide* markers of health including pathology, immune status, or histomorphology. Further, although DFM can be used in reference to any live microorganism administered to animals as a broader term, probiotics may be considered to be a sub-set of DFM.

Prebiotics

Although the term has continued to be revised since its introduction (Gibson and Roberfroid, 1995), a prebiotic is defined currently, also by expert consensus from ISAPP, as "a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017)", and when administered orally are referred to specifically as dietary prebiotics (Bindels et al., 2015). Prebiotic products often include indigestible carbohydrates that are able to pass minimally digested through the upper portion of the GI tract and reach the lower portion intact with the ability to be utilized selectively by intestinal microbiota (Grizard and Barthomeuf, 1999; Vandeplas et al., 2010). Examples of dietary prebiotics used in poultry production include fructooligosaccharides, galactooligosaccharides, transgalacto-oligosaccharides, xylo-oligosaccharides, and yeast cell wall mannan-oligosaccharide**.**

Probiotics and Prebiotics as Alternatives to Antibiotics

The many benefits of probiotic and prebiotic administration have been widely reported and reviewed previously (Patterson and Burkholder, 2003; Chichlowski et al., 2007; Dhama et al., 2011). However, their overall effectiveness is still questioned. Probiotics and prebiotics are often applied with an understanding of the desired benefit but with little understanding of the mechanisms important to their functionality. Much of the development of their application has been empirical, and their specific benefits have been attributed *post-hoc*. It is likely that specific benefits of AGP alternatives are often attributed to the broad classes of products, when, in fact, many benefits are likely to be very specific for individual strains of bacteria (Rhayat et al., 2017) or individual prebiotic molecules (Ajuwon, 2015).

A mechanistic understanding of probiotic and prebiotic functionality will contribute to more effective discovery and application of these potentially important alternatives in poultry production. Based on an understanding of the mode of action of AGP, analogous activities of any potential interventions can be identified that will potentially be important to their development and application as alternatives to AGP. Therefore, mechanisms of probiotic and prebiotic functionality important to their application in poultry production described later in this paper are related to the mode of actions of AGP.

Improved Nutrient Digestion and Utilization by the Host

Probiotics and prebiotics improve growth performance in poultry through increased gain (Alkhalf et al., 2010) and decreased feed conversion ratio (Eeckhaut et al.

2016), however, there is often variation in these results (Otutumi et al. 2012). The GI microbiota play an important role in the augmentation of host metabolism by improving the capacity to digest and absorb nutrients. As nutrients enter the GI tract, microbial populations utilize them for their own energetic benefit. End products, such as those produced from exogenous enzymes or microbial fermentation, are then able to be used by the host. Probiotic may harness these mechanisms to better host performance while also improving gut health and absorptive capacity. The totality of energy spared due to probiotic administration has been approximated to be 63 kcal kg^{-1} feed (Harrington et al., 2015), representing a substantial energy saving that can be utilized for growth. While probiotic and probiotic administration is often associated with improved broiler performance, the exact mechanisms require further analysis.

Exogenous Enzyme Production by Probiotics

Bacillus and *Lactobacillus* species have been previously characterized as divers of microbial fermentation and are commonly used in the industrial production of enzymes (Schallmey et al., 2004). Improved nutrient availability is believed to be one mechanism contributing to improved growth parameters in poultry. Probiotic bacteria modulate enzyme activity in the host through increased microbial enzymes and stimulation of host enzyme synthesis (Wang et al., 2017).

Amylase activity in the duodenum of broilers is increased after administration of *Bacillus* coagulans NJ0516 (Wang and Gu, 2010), a result which has been corroborated through studies using *Lactobacillus acidophilus* I26 and a mixed culture of *Lactobacillus* spp. where probiotic inclusion significantly increased amylolytic enzyme activity (Jin et

al., 2000). *Lactobacillus* administration is associated with increased BW and decreased FCR in broilers, likely due to a significant increase α-Amylase activity in the small intestine as *Lactobacillus* are capable of producing extracellular amylase *in vitro* (Jin et al. 2000). Amylolytic *Lactobacillus* species have been isolated from the crop of chickens with the ability to hydrolyze amylopectin into maltose, maltotriose and glucose (Champ, Szylit et al. 1983, Jin et al., 2000). This gives credence to the notion that probiotic type bacteria directly introduce digestive enzymes into the GI tract, however the full mechanism for increased enzymatic activity is likely multifaceted.

Previous research indicated that the natural microbiota is capable of proteolytic activity as conventional birds demonstrated improved protease activity in the cecum compared to germ free birds (Philips and Fuller 1983). Dietary administration of *Lactobacillus bulgaricus* in feed increased apparent nitrogen digestibility when fed to broiler chickens (Apata 2008). *L. bulgaricus* constitutively expresses a cell-wall protease, PrtB, which provides peptides to maximize microbial growth (Courtin et al., 2002). Additionally, specific proteolytic activity of *L. bulgaricus* derived proteases *in vitro* was increased at temperatures above 37°C, with the average body temperature of a chicken at 41°C (Abraham et al., 1993). Analysis of differential abundance of proteins in broilers fed *Enterococcus facium* CGMCC 2516 showed up-regulation of host genes involved in peptidase expression and amino acid metabolism, which could improve the metabolic capacity of the GI tract (Luo, Zheng et al. 2013). While proteolytic activity of intestinal microbes is mechanistically favorable to their own growth, the further digestion of proteins into peptide chains is thought to be advantageous to the host.

Although *in vitro* studies suggest exogenous enzyme production as a mechanism for improved nutrient utilization, it remains unclear whether the increased activity *in vivo* is due to stimulation of endogenous enzyme production, microbial enzymes, or a combination of the two. To further examine the role of individual enzymes on performance, phytase has been recombinantly expressed in *Lactobacillus gasseri*. Recombinant expression of phytase, an enzyme not produced endogenously by monogastrics, gives credence to the notion that exogenous enzyme production by bacteria *in situ* as recombinant cultures produced 10 to 50-fold greater activity. When fed to broilers on a phosphorus deficient diet over 21 days, *Lactobacillus gasseri* recombinantly expressing *phyA* from *B. subtilis* increased body weight to a level significantly similar to broilers given a diet optimized for phosphorus (Askelson et al. 2014). This reinforces the notion that bacteria exhibit phytate-degrading activity and that specific *Lactobacillus* degrade phytate after being exposed to simulated gastric conditions (González-Córdova et al., 2016).

Interspecies Hydrogen Transfer

Indigestible carbohydrates, host cell debris, and unabsorbed nutrients from the proximal gastrointestinal tract contribute to the nutritive environment of the cecum. The bacterial community in the cecum can reach densities of 10^{11} CFU per gram of contents, taking advantage of these undigested polysaccharides as substrates in the synthesis of short-chain fatty acid (SCFA) (Barnes et al., 1978). Volatile fatty acid (VFA) production in game birds can account for energy production up to 7% of free living energy requirements, with other monogastrics, including rats, swine, and laying hens, extracting

5-11% of energy requirements through VFA production (Annison et al., 1968; Gasaway 1976; Bergman, 1990; Józefiak, Rutkowski, and Martin 2004). During the fermentative process, the buildup of certain products serves to limit the reaction. Excess hydrogen accumulation inhibits the oxidation of NADH to NAD^+ and H_2, resulting in the reduction of acetyl Co-A to ethanol instead of acetate (Wolin, Miller, and Stewart 1997). Microbes that are able to utilize H_2 may serve a vital role in the cecal environment as they alleviate this rate limiting step.

Glucose fermentation results in the formation of between 2.67 and 4 H^+ depending on the VFA end product, which can result in the rapid accumulation of rate limiting hydrogen (Van Lingen et al., 2016). The presence of a hydrogen sink allows for more productive fermentation to acetate and increases short chain-fatty acid production which could lead to an increase in recovered energy from feed (Sergeant et al. 2014). Metagenome analysis has identified potential pathways that could act as hydrogen sinks within the cecal environment. Of those, uptake hydrogenases and reductive acetogenesis pathways were the predominant mechanisms, with *Campylobacter* associated uptake hydrogenases representing the largest quantity of genes (Sergeant et al. 2014). The presence of genes encoding these enzymes is significant as hydrogen is the most energy efficient substrate which has the ability to increase respiration 50-100 fold higher when used as an electron donor (Hoffman and Goodman, 1982). Genome sequencing has resulted in the identification of *hynS* and *hynL* NiFe-H_2ases in *Campylobacter* (Vignais et al., 2001).This is an energy conserving membrane bound uptake hydrogenase dependent upon exogenous nickel (Howlett et al. 2012). Because of *Campylobacter's*

ability to act as a hydrogen sink, it can create an environment where acetate is continually favored which benefits the microbial community of the cecum and supports the fermentation process that feeds back secondary metabolites to *Campylobacter* (Park et al. 2017). This interplay is exemplified by the relationship of *Campylobacter* and *Clostridium perfringens* where high *Clostridium* levels were associated with high *Campylobacter* counts as *Clostridium* produces hydrogen which *Campylobacter* can utilize to reduce nitrate (Skanseng et al., 2006; Laanbroek et al., 1978). This interplay demonstrates the importance of substrate utilization within the cecum and the impact some bacterial species have in creating a more favorable fermentation environment.

While microbes such as *Campylobacter* and *Clostridium* may fill needs in the energetic environment of the cecum, their presence remains undesirable for the safe and efficient production of poultry (van Immerseel et al., 2004). Ecological niches filled by these bacteria may be replaced through a consortium of organisms that can rebuild the specific pathways.

Enhanced Absorption of Nutrients

Probiotic supplementation has been associated with improved nutrient absorption through increased uptake via alterations of intestinal morphology and nutrient transport. Improvements of total dry matter digestibility without significant differences in ileal digestibility indicate improved nutrient uptake as a mechanism for improved performance (Reis et al. 2017). Nutrient uptake is greatly influenced by villi height, transport mechanism, and the secretory role of crypt cells (Kiela and Ghishan 2016). Slow renewal of cells in the mucosa is associated with increased villus height and

shallower crypts, resulting in greater villus area and higher enzymatic activity (Nousiainen, 1991).

There is a strong association between increased villi height and improved performance parameters. *Bacillus subtilis* increased villus height in laying hens (Samanya and Yamauchi 2002) and *Enterococcus faecium* increased villi height in broilers (Samli et al. 2007) which is believed to increase the absorptive capacity of the enterocyte due to an increase in surface area allowing for greater nutrient transport and greater digestive enzyme action (Laudadio et al. 2012). Organic acid and bacteriocin production by probiotics may reduce intestinal colonization and therefore inflammation in the mucosa (Beski and Al-Sardary, 2015). Decreased inflammation in the mucosa allows for the increase in villus height and villus function (Adil et al. 2010). Enzymatic activity also impacts villus height as alpha amylase activity by *Bacillus licheniformis* is also attributed to improved morphology (Divakaran et al., 2011). Administration of dietary amylase improves gastrointestinal morphology (Ritz et al. 1995), which could be attributed to increased energy available to the host through increased carbohydrate degradation and absorption.

Bacillus have been previously shown to increase villus height:crypt depth ratio (Lei et al., 2015) due to decreased crypt depth (Latorre et al. 2017). An increase in villus depth is indicative of higher cell turnover and therefore energy expenditure (Yason, Summers, and Schat 1987) which increases nutrient requirement for maintenance. By slowing the turnover rate through shallower crypts or a higher villus height:crypt depth ratio, a greater growth rate or growth efficiency can be achieved (van Nevel et al., 2005).

Higher cell turnover can be in response to inflammation from toxins, pathogens or other deleterious conditions of the gut. The role probiotics and prebiotics play in shallowing intestinal crypts in multifaceted. Improved intestinal protective factors, protective mucosal immunity (Deng et al. 2012), strengthening tight junctions (Mennigen et al. 2009) and excluding pathogens (Santin et al. 2001) all play a role in protecting the intestinal epithelium thus reducing cell turnover. Individual nutrients can also be impacted as *Lactobacillus* increases active glucose transport in vitro (Awad et al. 2008). Under heat stress conditions, *Lactobacillus* supplementation increased expression of GLUT2, GLUT5, and SGLT4, all of which are trans*por*ters of glucose (Jahromi et al. 2016). Further metabolites, potentially SCFA or polyamines, produced by *L. acidophilus* may be responsible for the non-genomic upregulation of glucose transporters such as SGLT1 as demonstrated using Caco-2 cells (Rooj et al., 2010). Stimulated short circuit current values in gut mucosal tissues of broilers fed *Lactobacillus* had a greater increase from basal values after exposure to glucose indicating a greater capacity for sodium-glucose co-transport (Awad, Ghareeb, and Böhm 2010). Increased expression of glucose transporters and electrophysiological parameters to improve glucose transport can lead to the improvement in performance parameters associated with probiotic administration.

PepT1 is a transport protein for oligopeptides important to protein utilization and weight gain in broilers. It has been suggested that one mechanism for increased body weights and lowered FCR associated with probiotic administration is due to either increased absorption by or expression of PepT1 (Etmektedir 2017). Incubation of Caco-2 cells with *L. casei* increased absorption of labeled glycine without differences in PepT1

mRNA expression, indicating increased PepT1 activity is responsible for increased amino acid uptake (Neudeck et al., 2004). Increased absorption by PepT1 can potentially be attributed to increased protein kinase C activity, leading to elevated plasma amino acid concentrations due to probiotic administration (Chen et al. 2010). mRNA expression of *PepT1* is increased with *Lactobacillus* administration under challenge conditions, which could be an effect of improved villi height and function due to probiotic administration and mitigate the effects of toxin and pathogen exposure which has a detrimental effect on the intestinal morphology.

Pathogen Reduction/ Inhibition

Although AGP were originally approved for use as growth promoters, withdrawal of these products has highlighted animal health promotional effects of AGP consistent with the prophylaxis of important food-animal infections. Consequently, sudden AGP removal has led to reduced of disease resistance in animals and decreased animal welfare (Friis et al., 2003). For example, necrotic enteritis (NE), a multifactorial disease typically characterized by an over-proliferation of *Clostridium perfringens,* a Gram-positive, spore-forming, opportunistic pathogen (Williams, 2005; Collier et al., 2008), was conventionally lessened by antibiotics (Peek and Landman, 2011). NE was relatively rare when sub therapeutic antibiotics were included in poultry feed (Hofacre et al., 2003), as producers were able to prevent disease and manage losses using antibiotics. The limiting of tools available for the management of diseases initiated an increased need to develop of alternatives to AGP for poultry production (van der Fels-Klerx et al., 2011).

Control of pathogenic bacteria in poultry production is important for the well-being of both humans and poultry alike. High loads of *Salmonella* can cause lesions in poultry associated with diseases, such as pullorum disease and fowl typhoid (Porter, 1998) and is a leading cause of foodborne illness with over 1 million people infected a year (CDC, 2019). In addition to previously noted animal health consequences, *Clostridium perfringens* has also been associated with human foodborne illnesses, infecting an estimated 970,000 individuals a year (Scallan et al., 2011). *Campylobacter* spp., are considered commensal organisms that colonize the in gastrointestinal tract of poultry (Corry and Atabay, 2001; Hermans et al., 2012). However, consumption of undercooked poultry can cause human foodborne illness (Domingues et al., 2012), with approximately 1.3 million people infected each year (Scallan et al., 2011).

It is thought that the inhibition of pathogenic bacteria improves the gastrointestinal environment for the retention of nutrients in the animal, and that beneficial bacteria, such as lactic acid bacteria (LAB), are useful in reducing the incidence of pathogens which cause disease in poultry in addition to human foodborne pathogens. The direct mode of action for individual probiotics and prebiotics are still not fully understood, however, they have been shown to inhibit pathogenic bacteria using mechanisms including modulation of GI bacteria populations (Patterson and Burkholder, 2003), competitive exclusion (Rantala and Nurmi, 1973), and antimicrobial substance secretion (Lin and Zhang, 2017).

Modulation of Microbial Populations by Prebiotics

Prebiotics reach the lower portion minimally digested and intact with the ability to interact with intestinal microbiota. These indigestible carbohydrates have been shown to increase populations of bacteria thought to be beneficial to the gastrointestinal health of poultry, including *Lactobacillus* and *Bifidobacterium* (Orban et al., 1997; Patterson et al., 1997; Collins and Gibson, 1999; Patterson and Burkholder, 2003; Yang et al., 2009) and decrease populations of pathogenic bacteria (Sims et al., 2004; Baurhoo et al., 2007; Allart et al., 2013). It is suggested that prebiotics modulate the microbial population of the GI tract by a variety of mechanisms, including selective utilization and changes in GI composition. One potentially important functionality of *Bacillus* spores is their ability to create an anaerobic environment in the GI tract of poultry through rapid oxygen intake while germinating; this environment is thought to favor proliferation of LAB (Jeong and Kim, 2014).

The selective utilization of dietary prebiotics has been suggested to promote populations of beneficial bacteria. Bacteria can metabolize polysaccharides and monosaccharides. For example, it has been demonstrated that *L. acidophilus* uses the API50 sugar fermentation pattern to use complex dietary carbohydrates that are not digested in the upper GI tract, such as fructooligosaccharides (Gibson and Roberfroid, 1995; Altermann et al., 2005). In addition, genome sequencing of bacteria has shown probiotics and beneficial resident microbes have genes that code for specific sugar transferase systems to utilize prebiotic carbohydrates correlated with persistence of probiotics in the GI (Altermann et al., 2005; Denou et al., 2008) or ATP-binding cassette

carbohydrate specific transporters (Fukuda et al., 2011) that allow for the internal usage of prebiotic compounds by probiotic bacteria. However, not all strains of probiotics can utilize prebiotic carbohydrates internally. Such probiotic species often have been shown to secrete extracellular hydrolases which depolymerize oligosaccharides for uptake by other bacteria (Pokusaeva et al., 2008; Porcheron et al., 2011). Import and intracellular hydrolysis may provide a selective advantage through the non-altruistic utilization of FOS and other prebiotic oligosaccharides. Whether any poultry GI tract-associated microorganisms are capable of similar non-altruistic utilization of MOS or other prebiotic oligosaccharides has not been determined.

Mannan oligosacarhides (MOS) have been administered similarly to other indigestible prebiotic carbohydrates and has been demonstrated to increase potentially beneficial bacteria (Kocher et al., 2005; Yang et al., 2008), however, it is thought that their mode of action is not primarily through the enhanced growth of beneficial bacteria. It is suggested that MOS compounds are able to agglutinate to the mannose-specific lection of gram-negative bacteria that express Type-1 fimbrae, such as *Salmonella* and *E. coli*, and once bound, the pathogens are thought to be no longer infectious and passed through the animal (Spring et al., 2000; Thomas et al., 2004; Baurhoo et al., 2009). Further, administration of MOS has been shown to increase the synthesis goblet cells that secrete glycoproteins, including mucin, which contain mannosyl receptors shown to bind to the type-1 fimbriae and can assist with the removal of the pathogens from the GI tract (Baurhoo et al., 2009).

Competition for Shared Binding Sites

Probiotic bacteria are thought to compete with pathogenic bacteria for mucosal binding sites and available nutrients within the gastrointestinal tract (Freter et al., 1983; Patterson and Burkholder, 2003; Schneitz, 2005; Askelson and Duong, 2015). However, very little is known about the mechanism of competitive exclusion (Schneitz, 2005), but studies suggest it is an initial protection in predominantly a physical occurrence (Mead et al., 1989), and the physical blocking of opportunistic pathogens via the binding of niche sites in the intestinal tract results in this exclusionary nature (Chichlowski et al., 2007). Although originally studied in broilers for the control of *Salmonella*, competitive exclusion has been shown experimentally exclude *E. coli*, *Campylobacter* spp., and *Clostridium perfringens* (Schneitz, 2005). Probiotic LAB have been shown to reduce *Campylobacter jejuni* (Willis and Reid, 2008), *Salmonella* (Vilà et al., 2009), and *E. coli* (La Ragione et al., 2004) in poultry through competitive exclusion.

Colonization and persistence are thought to be an important quality for probiotic bacteria (Bernet et al., 1994; Mack et al., 1999). Although the ability of probiotic lactobacilli to colonize the GI tract of poultry is multifactorial, adhesion to the mucosal surfaces of the GI tract is a thought to be a significant mechanism related to colonization (Rosenberg et al., 1983 because it allows bacterial cells to persist against peristaltic movements (Granato et al., 1999). Therefore, the mucosal adhesion is likely to play a role in the ability of beneficial bacteria to competitively exclude pathogens from the GI tract of poultry (Fuller and Brooker, 1974; MacKenzie et al., 2010). However, the

mechanisms responsible are not well characterized, but will be important for the development of future probiotic cultures used in the poultry industry.

The adhesion of *Lactobacillus* cultures to a poultry-derived epithelial cell line has been investigated, and similarities between *in vitro* adherence and *in vivo* colonization were observed (Spivey et al., 2014). In addition to adherence, bile resistance of probiotics has been identified as a potentially important mechanism that promotes colonization in the GI tract of poultry. Bile salt hydrolases catalyze the hydrolysis of the amide bond that links bile acids to their conjugated amino acids (Price et al., 2006) and are thought to be important to bile resistance of probiotics (Brashears et al., 2003; Taheri et al., 2009); Spivey et al., 2014). However, the correlation between bile salt hydrolase activity and probiotic colonization of the GI tract is not well characterized (Moser and Savage, 2001). Although strains with different abilities to adhere to cells *in vitro* have been demonstrated to have different abilities to reduce pathogen colonization under challenge. Only the use of isogenic mutants of a single strain will be able to definitively determine the role of adhesion in inhibiting pathogen binding.

Antimicrobial Molecules

Probiotic bacteria, including *Lactobacillus* spp., can produce organic acids, such as lactic acid, as end products of metabolism. Such acids have been shown to decrease populations of *Salmonella* spp., *Escherichia coli* spp., and *Pseudomonas* spp. (Alakomi et al., 2000; Neal-McKinney et al., 2012) by reductions in pH, cell membrane permeabilizing, and acting as a potentiator for the effects of other antibacterial

substances. The pathogen reduction is often attributed to the decrease in intracellular pH that disrupts transmembrane proton motive forces when the undissociated form of organic acids breaches the cell membrane (Ray and Sandine, 1992). In addition, organic acids have been demonstrated to play a part in in inhibiting the growth of *Campylobacter* spp. by destablizing the cell membrane structure not solely as a result of decreased pH (Neal-McKinney et al., 2012). In addition, organic acids can disintegrate the outer membrane of pathogens by causing lipopolysaccharide to release from the outer membrane, making bacteria more suspectable to detergents, enzymes, such as lysozyme, and bacteriocins (Cutter and Siragusa, 1995; Alakomi et al., 2000).

In addition to producing organic acids that reduce pathogen populations, probiotics produce antimicrobial compounds including, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, reuterin, and bacteriocins which have been demonstrated to reduce pathogens (Gibson and Wang, 1994; Joerger, 2003. Lin and Zhang, 2017). Bacteriocins are small, heat stable peptides produced by many bacterial species, including many probiotic strains with potential antimicrobial activity primarily for Gram-positive bacteria (Klaenhammer, 1993; Cotter et al., 2005). It has been demonstrated that the pathogen reducing effect of some probiotic species is directly related to production of bacteriocins (Corr et al., 2007). Specifically, it was shown that a non-bacteriocin producing mutant of *L. salivarius* did not reduce *Listeria monocytogenes*, suggesting bacteriocin production can be a primary mediator of protection against microorganisms. In addition, strains with and without an immunity gene were equally infectious, thus suggesting that bacteriocins act directly against the

microorganism, not through an intermediate mechanism. The mode of action for bacteriocins is quite varied. For example, bacteriocins have been shown to prevent proper cell wall synthesis by binding to the main transporter of peptidoglycan (Brötz et al., 1998), disrupt important enzymatic reactions necessary for cell wall synthesis Pag and Sahl, 2002), or to bind cell membranes and activate pore formation and influence cell membrane permeability that leads to rapid cell death (Wiedemann et al., 2001; Martinez et al., 2008).

Immune Modulation

Eimeria, the causative agent of coccidiosis, are intestinal parasites ubiquitous to commercial poultry production. These parasites are controlled through anticoccidial drugs or administration of live vaccines (Peek and Landman, 2011). Vaccination with live coccidia hasn't historically been used as much as anticoccidials, but with drug resistance concerns and removal of ionophores from many production facilities, this alternative has begun to grow in popularity (Williams, 2002; Chapman and Jeffers, 2014; Witcombe and Smith, 2014). It has known for many years that *Eimeria* and host bacteria have an interaction, whether through the increase severity of coccidial infections in conventional chickens when compared with gnotobiotic chickens, or as a predisposing factor of necrotic enteritis (Ruff et al., 1975; Fukata et al., 1987; Collier et al., 2008). Dietary probiotic supplementation has been shown to mitigate losses from *Eimeria* infections through reduced oocyst shedding and lesion severity, increased *Eimeria* specific antibodies and body weights (Lee et al., 2007; Abderlrahman et al., 2014; Giannenas et al., 2014). Probiotics may reduce severity of lesions, while allowing oocyst

cycling, promoting immunity development (Dalloul et al., 2003; Dalloul et al., 2005; Stringfellow et al., 2011). This would allow them to be used in combination with live coccidia vaccines (Bozkurt et al., 2014; Ritzi et al., 2016). Exact mechanisms on *Eimeria* infection immune responses have not been elucidated but probiotics have been demonstrated to impact the systemic and mucosa-associated immune responses in rodents orally receiving lactic acid bacteria (Hwang et al., 2015).

Concluding Remarks

The development of effective antibiotic alternatives is important for the poultry industry to prevent and mitigate diseases and ensure poultry continues to be an inexpensive and safe source of animal protein for consumers. Functional feed additives, including probiotics and dietary prebiotics, are viewed as potentially important alternatives, however their overall effectiveness is varied, and their mechanisms are not well defined. Understanding the mechanisms of probiotics and prebiotics analogous to the mode of actions of AGP provides opportunity for the development and application of probiotics and prebiotics in poultry production.

Probiotics and prebiotics have been shown to improve growth performance parameters, such as body weights and feed conversion, through a variety suggested mechanisms. It is believed that enzyme production by bacteria increases the availability of nutrients to the host and that indigestible carbohydrates, endogenous proteins, and residual nutrients from the proximal GI tract contribute to a nutritive environment in the cecum, resulting in improved bird growth. In addition, probiotics have been shown to

increase nutrient uptake with changes to the intestinal morphology and nutrient transport.

Although much of the focus on developing AGP alternatives is centered on mechanisms that improve growth, alternatives with properties analogous to antibiotics' mode of action of reduced subclinical infections are viewed as highly important to the poultry industry to improve animal welfare and reduce disease. Modulation of the microbial population of the GI tract is a suggested mechanism by which probiotics and prebiotics do both. Prebiotics are thought to alter microbial composition of the GI by a variety of mechanisms, such as selective utilization, that results in increased levels of beneficial bacteria such as LAB. In addition, probiotic administration has been shown to modulate the microbial population by reducing pathogens through competitive exclusion, antimicrobial compound production, and immune modulation. This mechanistic understanding of probiotic and prebiotic functionality will contribute to more effective discovery and application of these potentially important alternatives in poultry production.

CHAPTER II

ADMINISTRATION OF DIETARY PREBIOTICS IMPROVES GROWTH PERFORMANCE AND REDUCES PATHOGEN COLONIZATION IN BROILER CHICKENS[*]

Introduction

Antibiotic have been used widely in poultry production because of their ability to increase weight gain (Moore et al., 1946), reduce the gastrointestinal (GI) colonization of pathogens (Lev et al., 1957; Stutzet al., 1983), and improve feed efficiency (Emborg et al., 2002). However, the use of antibiotic growth promoters (AGP) has declined due to increased concerns regarding the development of antibiotic-resistant bacteria with consequences to human and animal health (Silbergeld et al., 2008) and growing consumer demand for antibiotic-free food production (Hume, 2011). In response, AGP use has been banned by the European Union (Cogliani et al., 2011) and limited in the United States by the Veterinary Feed Directive (Department of Health and Human Services, 2015). Therefore, the development of alternatives to AGP is of significant interest to the poultry industry. Because growth promotion by antibiotics is attributed to their effects on GI microorganisms (Visek, 1978; Gaskins et al., 2002), the GI

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microbiota is thought to be an important target for the development of alternatives to AGP.

Defined by expert consensus from the International Scientific Association for Probiotics and Prebiotics, a prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit (Gibson et al., 2017)", and, when administered orally, prebiotics are referred to specifically as dietary prebiotics (Bindels et al., 2015). The administration of dietary prebiotics has been shown to enhance digestive functionality of the poultry GI tract (Nahashon et al., 1994) and positively affect animal performance by increasing BW (Torres-Rodriguez et al., 2007) and improving feed efficiency (Salianeh et al., 2011). Additionally, the administration of prebiotics has been shown to promote populations of Lactic Acid Bacteria (LAB) and other beneficial microorganisms in the GI tract that are thought to compete with pathogenic bacteria for mucosal binding sites (Patterson and Burkholder, 2003; Askelson and Duong, 2015; Broderick and Duong, 2016). The administration of prebiotics has been shown to reduce pathogens of poultry, such as *Clostridium perfringens* (Yang et al., 2008; Allaart et al., 2013). Further, the administration of prebiotics has been shown to reduce human foodborne pathogenic bacteria, including *Salmonella* (Xu et al., 2003; Chung and Day, 2004) and *Campylobacter* (Fernandez et al., 2000; Baurhoo et al., 2009), thus improving the microbial food safety of poultry products.

Indigestible carbohydrates are often administered as dietary prebiotics because they pass through the proximal portion of the GI tract with minimal digestion and reach the distal portion intact with the ability to interact with intestinal microbiota (Grizard and

Barthomeuf, 1999; Vandeplas et al., 2010). Refined functional carbohydrates (RFC), including mannanoligosaccharides, β-glucan, and D-mannose which account for 20 to 30% of the cell dry mass, derived from the cell wall of *Saccharomyces cerevisiae*, are a readily available source of prebiotics for human and animal use (Dallies et al., 1998). In previous studies, the administration of RFC as dietary prebiotics has been demonstrated to increased BW of broilers (Walker et al., 2018) and decrease the colonization of foodborne human bacterial pathogens in broiler chickens (Walker et al., 2018), broiler breeder hens and their progeny (Walker et al., 2017), and turkeys during transport stress (Huff et al., 2013).

Although the ability of prebiotics to increase performance and reduce foodborne pathogens has been widely reported, their overall effectiveness when administered to poultry is mixed. The beneficial effects of their administration are often inappropriately attributed broadly across all prebiotic products as a general class of functional feed ingredients. However, the ability to confer specific benefits is dependent on upon the individual constituent components of a prebiotic product (Askelson and Duong, 2015). Thus, research investigating the functionality of specific prebiotic products is required. In this study, we evaluated the effects of a dietary prebiotic product composed of RFC with yeast culture on growth performance and GI and environmental microbiota when administered in-feed and through water to broiler chickens as a potential alternative to AGP.

Materials and Methods

Experimental Animals and Husbandry

Male broiler chicks (Cobb) were obtained from a commercial hatchery on day of hatch, vaccinated for Eimeria (Advent, Huvepharma Inc, Peachtree City, GA), weighed, wing banded, and assigned randomly to pens to ensure statistically similar starting pen weights. Experimental animals were raised in 3.35 m^2 floor pens on built-up litter; provided age appropriate heat, ventilation; and given access to potable water and experimental rations ad libitum. Broilers were placed at an initial stocking density of 0.075 m^2 per broiler; temperature was monitored, recorded daily, and adjusted in response to bird comfort; and the lighting program followed the standard operating procedure for broilers raised at the Texas A&M University Poultry Science Research Center (Flores et al., 2019) according to the breeder's recommendations (Cobb-Vantress, 2018). All experimental procedures were performed as approved by the Texas A&M University Institutional Animal Care and Use Committee.

Experimental Design and Diets

The effects of dietary prebiotic administration on growth performance and GI colonization of *Campylobacter* spp., *Clostridium perfringens*, and total LAB were evaluated in comparison to an AGP. Broiler chicks (n = 1,720) were allocated to 6 experimental treatment groups with a total of 40 pens of 43 birds arranged, due to housing constraints, as a randomized incomplete block design and fed experimental rations with dietary prebiotic administered in-feed (Celmanax SCP, Arm and Hammer Animal and Food Production, Princeton, NJ) or through drinking water (Celmanax

Liquid NC, Arm and Hammer Animal and Food Production, Princeton, NJ) using the manufacturer's recommended dosages. The 6 experimental treatment groups were as follows: bacitracin methylene disalicylate (BMD)-treated (50 g t−1) feed (7 pens); untreated feed (7 pens); low-dose (50 g t−1) prebiotic RFC in-feed (7 pens); high-dose (100 g t−1) of prebiotic RFC in-feed 125 (7 pens); low-dose prebiotic RFC in-feed and prebiotic RFC administered via drinking water (500 ppm) beginning at 39 D post-hatch (6 pens); and high-dose prebiotic RFC in-feed and prebiotic RFC administered via drinking water (500 ppm) beginning at 39 D post-hatch 130 (6 pens).

Broilers were fed experimental rations beginning at 0 D through 41 D post-hatch. After collecting final BW at 42 D post-hatch, feed was withdrawn for 8 h, and the study was terminated. Prebiotic-treated water was administered to the appropriate groups beginning at 39 D post-hatch (72 h prior to feed withdrawal) through study termination, while the remaining groups received untreated water over the same period. Water was provided to all treatment pens using individual hanging bucket drinkers during the water treatment period.

Experimental treatment diets (**Table 2.1**) were fed for the duration of the trial using a 3-phase feed plan: starter phase (days 0 to 14, crumble), grower phase (days 14 to 28, pellet), and finisher phase (days 28 to 42, pellet). For each phase, feed was manufactured as a single commercial-type corn/soybean meal basal diet with added phytase and 5% distiller's dried grains with solubles and divided for inclusion of dietary treatments

as appropriate. Full matrix values for phytase contribution of aP, Ca, Na, digestible amino acids, and metabolizable energy as recommended by the manufacturer were used.

Growth Performance Measures

Experimental animals and feed were weighed by pen at 0, 14, 28, and 42 D post-hatch for determination of body weight and feed consumption. Mortalities and post-mortem weight were recorded daily for the calculation of percent mortality, body weight gain, and mortality adjusted FCR.

Recovery of Gastrointestinal Microbes

Two representative (median weight ± 5%) birds were selected from each pen, euthanized, and dissected aseptically for the collection of GI tissues at 42 D post-hatch and 8 h post-feed withdrawal. An approximately 3 cm section of the ileum proximal to the midpoint between the ileocecal junction and Meckel's diverticulum and the ceca were collected from each bird on day 42 post-hatch, while, at 8 h post-feed withdrawal, only the ceca 170 were collected from each bird. Total LAB and *Clostridium perfringens* were enumerated from the ileum using cylcoheximide (100 μg mL^{-1}, Amresco, Solon, OH) Franklin Lakes, NJ) agar incubated in 10% CO_2 at 37 °C for 24 h and Tryptose Sulfite Cycloserine-Egg Yolk (BD) agar incubated at 37 °C for 48 h anaerobically (Coy Laboratory Products Inc., Grass Lake, MI), respectively. *Campylobacter* spp. were enumerated from the cecum using Campy Cefex agar (CCA; Hardy Diagnostics, Santa Maria, CA) incubated in 10% CO_2 at 37°C for 24 h. *C. perfringens* was selectively enriched from the ileum using Fluid Thioglycollate Medium (BD) and Iron Milk Medium (Hi-Media Laboratories; Mumbai, India), while *Campylobacter* spp. was

selectively enriched from the cecum using Bolton's Enrichment Broth (BEB; Hardy) and CCA. Specimens for which no colonies appeared on enumeration plates but were positive by selective enrichment were assigned the limit of detection for enumeration (100 cfu g^{-1}).

Recovery of Litter Campylobacter

Immediately prior to placement and at 42 d post-hatch, litter was collected from 5 locations in each treatment pen, pooled by pen, and homogenized using Buffered Peptone Water (HiMedia) for selective enrichment of *Campylobacter* spp. using BEB and CCA.

Statistical Analysis

Bacterial count and mortality data were $\log_{10}$ and arcsine square root transformed, respectively, for analysis. Growth performance results and bacterial counts were analyzed using ANOVA. Significantly different means were separated using Duncan's Multiple Range Test post hoc. Bacterial incidence was analyzed using Pearson's χ^2 Test. Because prebiotic treatment via drinking water did not occur until the finisher phase, the relevant treatment groups, e.g., low dose prebiotic-treated feed with and without water treatment, were combined for analysis during the starter and grower phases. Additionally, growth performance results and bacterial counts for treatment groups receiving the prebiotic feed supplement with or without prebiotic water treatment were analyzed using a 2 (dose) × 2 (water treatment) factorial ANOVA with main effects for infeed dose, water treatment, and in-feed dose × water treatment, while the

effects of dose and water treatment on bacterial incidence were analyzed using binomial logistic regression. Statistical significance was considered at $P \leq 0.05$.

Results

Growth Performance

The effects of prebiotic administration in-feed and treated water were evaluated in comparison to antibiotic-treated and untreated controls. A significant treatment effect was observed for d 42 BW ($P = 0.002$) and average daily gain (ADG) over days 0 to 42 ($P = 0.033$) (**Table 2.2**). Body weight and ADG was greatest when broilers were fed the high-prebiotic diets as compared to the low-prebiotic and control diets. Although they were not significantly greater than the controls or non-water treated low prebiotic treatments, administration of the low-prebiotic dose by feed with prebiotic-treated water improved BW and ADG to a level similar to the treatments administered the high prebiotic dose with or without treated water. No significant treatment effects were observed for BW on days 14 or 28 or ADG over days 0 to 14, days 15 to 28, or days 29 to 42.

No significant treatment effect on FCR was observed for any period of the study (**Table 2.3**). However, a significant treatment effect was observed for ADFI for days 28 to 42, ($P = 0.010$) and days 0 to 42 ($P = 0.022$) (**Table 2.3**). Over both periods, ADFI was greatest when broilers were fed the high prebiotic dose and administered treated water when compared to the other treatments. Additionally, ADFI of broilers administered high prebiotic dose alone was similar to those administered the high prebiotic dose and treated water over the finisher phase and d 0 to 42.

A significant treatment effect was observed for mortality for the grower period, days 15 to 28, (P = 0.026) and days 0 to 42 (P = 0.016) (**Table 2.4**). Although mortality was lowest when broilers were administered BMD, BMD administration did not significantly reduce mortality when compared to untreated broilers over either period. Over the grower period, mortality of broilers fed either prebiotic dose was also not different than that of the untreated group. Similarly, for days 0 to 42, mortality of broilers administered the high prebiotic dose alone or the low prebiotic dose with or without treated water was not significantly different than the BMD-treated or untreated controls. However, mortality of broilers receiving the high prebiotic dose and prebiotic-treated water was greater than the antibiotic-treated and untreated broilers. No significant treatment effects on mortality were observed over the remaining periods.

Gastrointestinal Microbiota

Cecal Bacteria. A significant treatment effect was observed on counts of *Campylobacter* spp. in the cecum at day 42 (P = 0.012) **(Figure 2.1A**). Administration of prebiotic reduced *Campylobacter* spp. up to 1.0 $\log_{10}$ cfu g^{-1} cecal contents when compared to broilers fed BMD-treated or untreated feed, with the fewest *Campylobacter* spp. being recovered from broilers administered the high prebiotic dose and treated water. Although a significant treatment effect was not observed on incidence in the cecum prior to (P = 0.253) or after (P = 0.080) feed withdrawal (**Table 2.6**), *Campylobacter* spp. tended to be detected in fewer ceca from broilers administered prebiotic-treated water during the feed withdrawal period as compared to the other treatments.

Ileal Bacteria. Although a significant treatment effect was not observed on counts of *C. perfringens* ($P = 0.057$) or total LAB ($P = 0.331$) in the ileum of broilers at d 42 (**Figure 2.1B-C**), fewer *C. perfringens* tended to be recovered from broilers fed the BMD-treated diet and the low prebiotic-treated ration with prebiotic water administration compared to broilers fed the untreated control or other prebiotic diets.

Litter Campylobacter

A significant treatment effect was not observed on *Campylobacter* spp. prevalence in the litter at days 0 or 42 (**Table 2.5**). *Campylobacter* spp. was detected in all pens prior to placement of the study. Although a significant effect was not observed on day 42 ($P = 0.283$), *Campylobacter* spp. was detected in litter from fewer pens in which broilers were administered prebiotic or BMD-treated feed than for the untreated control.

Main Effects Analyses

The main effects of prebiotic-dose in-feed and administration of prebiotic treated water were on growth performance (**Table 2.6**) and GI microbiota (not shown) were also evaluated. No significant dose × water interactions were observed for any growth performance measure. A significant main effect of prebiotic dose was observed on day 42 BW ($P = 0.002$), days 29 to 42 ADG ($P = 0.004$), and days 29 to 42 feed intake ($P = 0.012$), with the high dose increasing each performance measure. Although the effect was not significant ($P = 0.059$), FCR of broilers administered the high dose tended to be lower when compared to the low dose. However, a significant main effect of the

administration of prebiotic- treated water over the final 72 h of production was not observed for any of the growth performance measures.

No significant main effects or interactions on counts of *Campylobacter* spp., total LAB, or *C. perfringens* were observed (not shown). Additionally, no significant association of dose or water treatment was observed on the incidence of *Campylobacter* spp. in the cecum or litter.

Discussion

Sub-therapeutic antibiotics have been administered widely in livestock production because of their ability to increase growth and manage infections by bacterial pathogens. However, limitations on their use in animal production have increased need for the development of potential alternatives to AGP. Growth promotion by antibiotics is attributed to their effect on the GI microbiota (Dibner and Richards, 2005). Administration of dietary prebiotics has been demonstrated to promote populations of beneficial bacteria and decrease populations of pathogens in the GI tract in poultry (Patterson and Burkholder, 2003), and prebiotics have been suggested as potential alternatives to AGP because of their ability to improve growth performance similarly to antibiotics (Huyghebaert et al., 2011). Although their benefits are often inappropriately attributed broadly across all prebiotics as a class of functional ingredients, the ability to confer specific benefits is dependent upon the individual constituents of a prebiotic product (Askelson and Duong, 2015). Refined functional carbohydrates derived from the cell wall of *Saccharomyces cerevisiae*, including mannan oligosaccharides, β-glucan, and D-mannose, are widely used as prebiotics, and although some improvement to

animal growth has been reported (Walker et al., 2018), most research related to their effects in poultry have focused on pathogen reduction (Huff et al., 2013; Walker et al., 2017). In this study, we evaluated the effect of a prebiotic, composed of RFC with yeast culture, on growth performance and GI and environmental microbiota when administered in feed and water to broiler chickens as a potential alternative to AGP.

Overall, we observed results similar to those that have reported prebiotic administration can improve broiler growth performance parameters (Torres-Rodriguez et al., 2007; Awad et al., 2009; Mookiah et al., 2014). In our study, administration of the high prebiotic RFC dose, with or without prebiotic-treated water, increased final BW and cumulative ADG (**Table 2.2**). In a previous study, RFC administration was reported to increase BW at 28 d and 42 d of female broilers (Walker et al., 2018), while a separate study reported BWG of male broilers tended to be greater when RFC were applied as a synbiotic in combination with a direct-fed *Bacillus subtilis* culture (Gómez et al., 2012). In our study, finisher phase and cumulative ADFI was greater when broilers were administered the high dose of prebiotic with prebiotic-treated water, whereas no significant treatment effect was observed for FCR during any phase of the study (**Table 2.3**). These data suggest that the improvements in BW and ADG observed in this study were the result of increased feed intake. However, improved FCR has been reported previously when broilers were administered RFC (Gómez et al., 2012) and other dietary prebiotics (Hooge, 2004; Li et al., 2008; Salianeh et al., 2011). Evaluation of the effect of the dose of prebiotic RFC administered in-feed determined that final BW and ADG and ADFI over the finisher period was greater and FCR tended to be lower when broilers

were administered the high dose when compared to the low dose (**Table 2.6**). However, administration of prebiotic RFC via drinking water over the final 3 days of production was not observed to have a significant effect on growth performance. Further research will be required to determine the most effective dosage and timing of RFC administration in-feed or by drinking water.

The improved growth performance observed in prebiotic-treated poultry has been attributed to the effects on digestion, digestive function, and the GI microbiota reported when prebiotics are administered (Askelson and Duong, 2015). Indeed, increased ileal nutrient digestibility, nitrogen retention, villus height (Gómez et al., 2012), and colonization by *Bifidobacterium* spp. and *Lactobacillus* spp. (Yang et al., 2009) and reduced *Salmonella* prevalence (Walker et al., 2017; Walker et al., 2018) have been observed when poultry were administered RFC and other dietary prebiotics. Although the ability of prebiotics to improve GI health and reduce pathogen colonization through their modification of the GI microbiota has been reported widely, the mechanisms responsible are not well understood.

The selective utilization of dietary prebiotics has been suggested to promote populations of beneficial bacteria. Many LAB and other GI tract-associated bacteria secrete extracellular hydrolases which degrade prebiotic oligosaccharides including fructooligosaccharides (FOS) and MOS (Goh and Klaenhammer, 2015). The mono- and disaccharides products of this hydrolysis are available to be utilized by all microorganisms in the GI tract which possess the appropriate phosphotransferase system transporters (Altermann et al., 2005; Azcarate-Peril et al., 2008). However, some

bacteria including *Lactobacillus acidophilus* NCFM (Altermann et al., 2005; Barrangou et al., 2006) produce FOS-specific ATP-binding cassette (ABC) transporters which enable them to import the prebiotic oligosaccharide for hydrolysis by intracellular β-fructosidases (Barrangou et al., 2003). Import and intracellular hydrolysis may provide a selective advantage through the non-altruistic utilization of FOS and other prebiotic oligosaccharides. Whether any poultry GI tract associated microorganisms are capable of similar non-altruistic utilization of MOS or other prebiotic oligosaccharides has not been determined.

In our study, we observed reduced cecal colonization by *Campylobacter* spp. in RFC-treated broilers prior to feed withdrawal, with a reduction of greater than 1 $\log_{10}$ cfu g^{-1} of cecal contents in broilers receiving the high dose in-feed and treated water as compared to the untreated control. However, administration of the prebiotic treatment via drinking water was not observed to further reduce counts of *Campylobacter* spp. in the cecum prior to feed being withdrawn. A quantitative risk assessment estimated that a 1 $\log_{10}$ decrease in the number of *Campylobacter* spp. on a contaminated carcass would result in an 80% reduction in the cases of human foodborne illness (Rosenquist et al., 2003).

Although not a prebiotic functionality per se because it does not involve selective utilization, agglutination of bacteria by RFC has been suggested to inhibit adhesion of pathogens to the GI mucosa resulting in their passage through the GI tract without the opportunity to colonize (Oyofo et al., 1989; Spring et al., 2000; Walker et al., 2017). Mannose binding of FimH-like adhesins on type 1 fimbriae of *E. coli* and *Salmonella*

has been demonstrated to block their adhesion to the GI mucosa (Oyofo et al., 1989; Spring et al., 2000). Although *Campylobacter* spp. are not known to possess similar adhesions, mannose-binding lectins have been observed in *Campylobacter jejuni* (Day et al., 2009).

Clostridium perfringens and LAB have been suggested to be potentially important markers of GI health and mediators of performance in poultry. Indeed, Askelson et al. (2018) reported greater counts of total LAB to be correlated with reduced FCR and increased counts of *C. perfringens* to be associated with increased FCR. Prebiotics have been demonstrated previously to reduce C. perfringens counts in broilers (Moore et al., 1946; Biggs et al., 2007) and promote populations of beneficial bacteria including the LAB (Gibson and Roberfroid, 1995; Teitelbaum and Walker, 2002; Patterson and Burkholder, 2003). No significant differences in counts of *C. perfringens* or total LAB were observed in this study. However, fewer *C. perfringens* tended to be recovered from broilers that were given low dose prebiotic-treated feed and prebiotic-treated water.

Campylobacter spp. has been widely considered to be a commensal inhabitant of the GI tract of poultry and is able to contaminate poultry products during processing (Achen et al., 1998; Herman et al., 2003). Built-up litter consumed by broilers has been suggested to be a primary vector for the transfer of *Campylobacter* spp. between birds within the same flock and from one flock to the next (Montrose et al., 1985; Sahin et al., 2015). Additionally, consumption of litter by broiler chickens has been demonstrated to increase during feed withdrawal prior to processing (Corrier et al., 1999), suggesting

feed withdrawal may be a potentially important critical control point at which an intervention may be applied to reduce the incidence of human foodborne pathogens in poultry. Thus, the effects of RFC administration in-feed and through drinking water on *Campylobacter* spp. prevalence in the ceca before and after an 8 h feed withdrawal and in the litter were investigated in the current study (**Table 2.6**). In our study, a significant treatment effect was not observed on *Campylobacter* spp. prevalence pre- or post-feed withdrawal. However, it is interesting to note that the prevalence of *Campylobacter* spp. after the feed withdrawal period did tend to be lower when broilers were administered prebiotic-treated water. These data suggest administration of prebiotic RFC in drinking water may potentially be useful for reducing the risks to foodborne illness associated with increased consumption of litter during feed withdrawal. Likewise, although the effect was not statistically significant, *Campylobacter* spp. was detected in the litter from fewer pens housing RFC-treated or BMD-treated broilers than when compared to untreated control. Administration of RFC with yeast culture has been demonstrated to reduce prevalence of *Salmonella* in the cecum (Walker et al., 2017) and litter (Walker et al., 2018). However, the effects of RFC and yeast culture on *Campylobacter* spp. prevalence have not been evaluated previously, and experiments conducted using experimentally infected animals will be required to understand the effectiveness and application of RFC for reducing *Campylobacter* spp. in poultry and as a potential intervention to mitigate the increased risk of GI contamination by foodborne pathogens during feed withdrawal.

In addition to promoting growth, BMD has been administered to poultry in order to reduce mortality (Brennan et al., 2003). In this study, mortality of BMD-treated broilers was not significantly lower than the untreated broilers, and, overall, mortality of RFC-treated broilers was not observed to be significantly different from the BMD-treated or untreated control. RFC administration has not been previously reported to affect mortality of broiler chickens (Gómez et al., 2012; Walker et al., 2017; Walker et al., 2018).

RFC administration to broiler chickens on growth performance and GI and litter microbiota. We have demonstrated the administration of RFC as a dietary prebiotic improved growth performance through increased BW, ADG, and ADFI. Although the differences were not observed to be statistically significant, FCR tended to be lower with administration of the high RFC dose. Additionally, we have demonstrated that prebiotic RFC administration also reduced cecal colonization by *Campylobacter* spp. and may potentially reduce *Campylobacter* spp. prevalence in litter, possibly improving pre-harvest microbial food safety of poultry and poultry products. Our results suggest that administration of RFC with yeast culture as a dietary prebiotic may potentially be an important component of an antibiotic free production program and an intervention to improve pre-harvest food safety. Because of the effectiveness and reliability of antibiotics, it is unlikely that a single alternative product will match their efficacy. Thus, the continued development of entire ABF management programs, including feed additives and improved husbandry, will likely be required to truly replace AGP in poultry production.

Table 2.1. Ingredient composition and nutrient content of the basal control diets

Item (%)	Starter	Grower	Finisher
Ingredients			
Corn	57.95	63.65	68.45
SBM (45.6 % CP)	29.10	23.70	18.95
DL-Met	0.29	0.25	0.20
Lys HCL	0.25	0.23	0.20
L-Thr	0.09	0.08	0.07
Soy Oil	2.47	2.38	2.83
Limestone	0.87	0.69	0.66
CaH_4PO_4	0.30	0.00	0.00
NaCl	0.32	0.33	0.22
$NaHCO_3$	0.14	0.12	0.27
Trace Minerals[1]	0.05	0.05	0.05
Vitamins[2]	0.25	0.25	0.25
LO- DGGS	5.00	5.00	5.00
Pork MBM	3.00	3.35	2.99
Phytase[3]	0.01	0.01	0.01
Calculated nutrient			
Protein	22.00	19.95	17.82
Crude Fat	5.30	5.41	5.95
Crude Fiber	2.50	2.53	2.55
Ca	0.92	0.82	0.75
aP	0.46	0.41	0.38
ME (kcal/kg)	3047	3102	3168
dig Met	0.59	0.53	0.46
dig TSAA	0.87	0.79	0.69
dig Lys	1.18	1.04	0.89
dig Trp	0.21	0.18	0.16
dig Thr	0.77	0.69	0.60
Na	0.046	0.043	0.039
Analyzed nutrients[4]			
Moisture	12.60	10.84	15.38
Dry Matter	87.40	89.16	84.62
Crude Protein	20.40	20.20	19.50
Crude Fat	5.27	5.07	2.57
Fiber	3.30	3.70	3.40
Ash	4.53	4.04	3.75

[1]Trace mineral premix added at this rate yields 60.0 mg manganese, 60 mg zinc, 60 mg iron, 7 mg copper, 0.4 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.

[2]Vitamin premix added at this rate yields 22,045 IU vitamin A, 7,716 IU vitamin D3, 91 IU vitamin E, 0.04 mg B12, 11.9 mg riboflavin, 91.8 mg niacin, 40.4 mg d-pantothenic acid, 261.1 mg choline, 2.9 mg menadione, 3.50 mg folic acid, 14.3 mg pyroxidine, 5.87 mg thiamine, 1.10 mg biotin per kg diet. The carrier is ground rice hulls.

[3]OptiPhosPF, Huvepharma. Peachtree City, GA.

[4]Performed by Midwest Laboratories, Inc., Omaha, NE

Table 2.2. Body weight and average daily gain of broiler chickens

Treatments		BW (kg)				ADG (g bird-day^{-1})			
Feed[1]	Water[2]	d 0	d 14	d 28	d 42	Starter	Grower	Finisher	d 0-42
BMD	-	0.038	0.527	1.489	2.664^{b}	34.2	69.3	89.6	61.8^{b}
UNT	-	0.039	0.527	1.436	2.705^{b}	34.6	65.7	94.7	61.8^{b}
RFC-Lo	-	0.039	0.525	1.552	2.722^{b}	33.7	74.1	86.5	61.2^{b}
RFC-Hi	-	0.039	0.538	1.562	2.962^{a}	34.7	73.8	105.6	67.0^{a}
RFC-Lo	+				2.848^{ab}			98.9	64.0^{ab}
RFC-Hi	+				3.040^{a}			110.2	66.6^{a}
	P-value	0.143	0.443	0.132	0.002	0.510	0.116	0.062	0.033
	Pooled SEM	0.000	0.003	0.020	0.034	0.263	1.339	2.598	0.687

^{a,b} Means within a column not sharing a common superscript are significantly different ($P \leq 0.05$)

[1] In-feed treatments: BMD, 50 g t^{-1} bacitracin methylene disalicylate; UNT, untreated; RFC-Lo, 50 g t^{-1} RFC; RFC-Hi, 100 g t^{-1} RFC

[2] Drinking water treatment: RFC at 500 ppm beginning at 39 d post-hatch

Table 2.3. Mortality corrected feed conversion ratio and average daily feed intake of broiler chickens

Treatments		FCR (Feed:Gain)				ADFI (g bird-day^{-1})			
Feed[1]	Water[2]	Starter	Grower	Finisher	d 0-42	Starter	Grower	Finisher	d 0-42
BMD	-	1.040	1.845	2.167	1.753	38.4	126.8	181.0^{c}	112.0^{b}
UNT	-	1.057	1.887	2.111	1.683	39.6	115.3	180.2^{c}	108.0^{b}
RFC-Lo	-	1.032	1.586	2.442	1.698	37.8	115.3	189.2^{bc}	109.2^{b}
RFC-Hi	-	1.220	1.741	1.970	1.636	45.8	124.2	196.6^{ab}	114.3^{ab}
RFC-Lo	+			2.055	1.614			187.6^{bc}	108.4^{b}
RFC-Hi	+			2.011	1.700			204.4^{a}	121.0^{a}
	P-value	0.374	0.158	0.315	0.359	0.270	0.158	0.010	0.022
	Pooled SEM	0.046	0.052	0.064	0.019	0.000	1.743	2.189	2.267

^{a,b,c} Means within a column not sharing a common superscript are significantly different ($P \leq 0.05$)

[1] In-feed treatments: BMD, 50 g t^{-1} bacitracin methylene disalicylate; UNT, untreated; RFC-Lo, 50 g t^{-1} RFC; RFC-Hi, 100 g t^{-1} RFC

[2] Drinking water treatment: RFC at 500 ppm beginning at 39 d post-hatch

Table 2.4. Mortality of broiler chickens

Treatments		Mortality (%)			
Feed[1]	Water[2]	Starter	Grower	Finisher	d 0 - 42
BMD	-	3.99	0.35[b]	1.10	5.32[b]
UNT	-	4.65	1.74[ab]	0.00	6.31[b]
RFC-Lo	-	6.31	3.25[a]	0.00	8.97[ab]
RFC-Hi	-	7.75	3.15[a]	0.45	8.53[ab]
RFC-Lo	+			0.75	10.30[ab]
RFC-Hi	+			0.46	13.18[a]
	P-value	0.264	0.026	0.495	0.016
	Pooled SEM	0.53	0.41	0.19	0.68

[a,b] Means within a column not sharing a common superscript are significantly different ($P \leq 0.05$)

[1] In-feed treatments: BMD, 50 g t^{-1} bacitracin methylene disalicylate; UNT, untreated; RFC-Lo, 50 g t^{-1} RFC; RFC-Hi, 100 g t^{-1} RFC

[2] Drinking water treatment: RFC at 500 ppm beginning at 39 d post-hatch

Table 2.5. Recovery of *Campylobacter* from cecum and litter

Treatments		Cecum (%)[1]		Litter (%)[2]	
Feed[3]	Water[4]	Pre	Post	D 0	D 42
BMD	-	92.9	100.0	100.0	71.4
UNT	-	100.0	100.0	100.0	100.0
RFC-Lo	-	85.7	100.0	100.0	71.4
RFC-Hi	-	75.0	83.3	100.0	50.0
RFC-Lo	+	85.7	100.0	100.0	42.9
RFC-Hi	+	100.0	83.3	100.0	66.7
	P-value	0.253	0.080		0.283

[1] *Campylobacter* positive ceca pre- and post-feed withdrawal

[2] *Campylobacter* positive pens on d 0 and 42 post-hatch

[3] In-feed treatment: BMD, 50 g t^{-1} bacitracin methylene disalicylate UNT, untreated; RFC-Lo, 50 g t^{-1}; RFC-Hi, 100 g t^{-1}

[4] Drinking water treatment: RFC at 500 ppm

Table 2.6. Main effects of feed and water additives on growth performance of broiler chickens

Main Effects	BW	Finisher (28 – 42 d)		
	42 d (kg)	ADG (g bird-day^{-1})	FI (g bird-day^{-1})	FCR (feed:gain)
Dose[1]				
RFC-Lo	2.785	92.7	188.4	2.248
RFC-Hi	3.001	107.9	200.5	1.991
Water[2]				
Untreated	2.842	96.0	192.9	2.206
Treated	2.944	104.6	196.0	2.033
P-value				
Feed	0.022	0.004	0.012	0.059
Water	0.123	0.082	0.486	0.193
Feed×*Water*	0.707	0.408	0.292	0.113
Pooled SEM	0.038	2.823	2.483	0.072

[1] In-feed RFC dose: RFC-Lo, 50 g t^{-1}; RFC-Hi, 100 g t^{-1}
[2] Drinking water treatment: Treated, RFC at 500 ppm

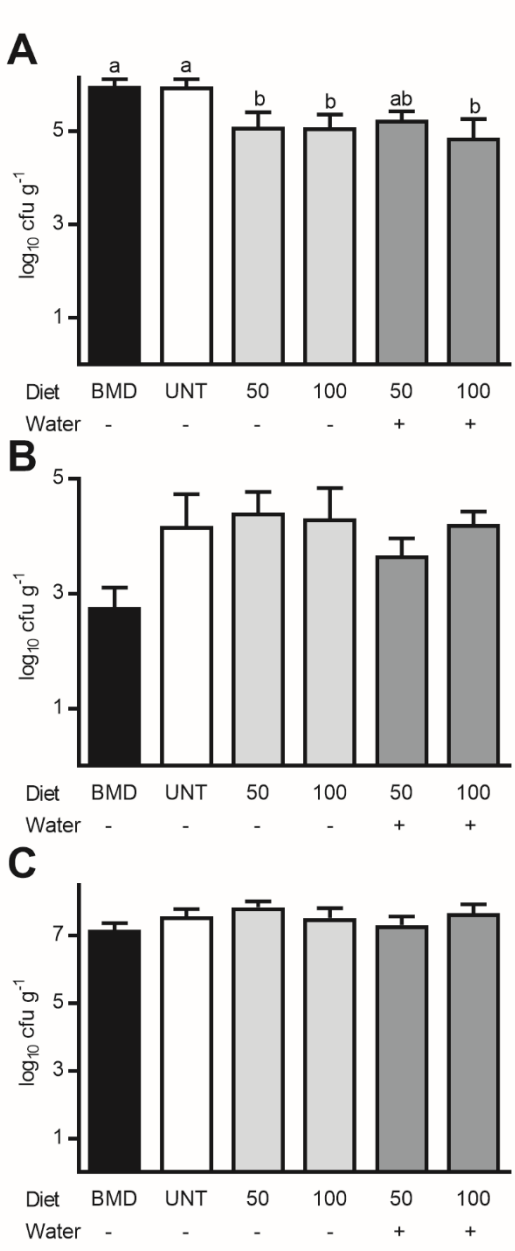

Figure 2.1. **Enumeration of bacteria from broiler chickens.** At 42 days post hatch (**A**) *Campylobacter* was enumerated from the cecum of broiler chickens, and (**B**) *C. perfringens* and (**C**) total LAB were enumerated from the ileum. Bacterial counts are reported as the mean ± SEM $\log_{10}$ cfu g^{-1} digestive contents. Means not sharing common letters are significantly different ($P \leq 0.05$).

CHAPTER III

ADMINISTRATION OF DIRECT-FED BACILLUS AND REFINED FUNCTIONAL CARBOHYDRATES TO BROILER CHICKENS IMPROVES GROWTH PERFORMANCE AND PROMOTES POSITIVE SHIFTS IN GASTROINTESTINAL MICROBIOTA

Introduction

Antibiotics have been used widely to improve growth (Bunyan et al., 1977), mitigate disease (Lanckriet et al., 2010), and reduce the colonization of human foodborne pathogens in poultry (Hofacre et al., 2007). However, consumer preferences and regulatory pressures due to increased concerns regarding antibiotic resistant bacteria with consequences to human and animal health has led to a decrease in the administration of antibiotic growth promotors (AGP) (Silbergeld et al., 2008; Hume, 2011). Therefore, the development of alternatives to AGP is of significant interest to the poultry industry. The beneficial effects of antibiotics are attributed to their effects on the microbial community in the gastrointestinal (GI) tract (Visek, 1978; Gaskins et al., 2002), thus the GI microbiota is an important target for the development of alternatives to AGP.

Probiotics, defined by expert consensus from the International Scientific Association for Probiotics and Prebiotics (ISAPP), are “live microorganisms, that when administered in adequate amounts confer a health benefit on the host” (Hill et al., 2014). Because of their beneficial health effects, they are often administered to poultry and

other livestock animals in direct-fed microbial products, “products that are purported to contain live (viable) microorganisms” (FDA, 1995) in order to promote growth. Organisms administered as Direct-Fed Microorganisms (DFM) have traditionally included non-spore forming bacteria including *Bifidobacterium* spp. and Lactic Acid Bacteria (LAB) (Patterson and Burkholder, 2003; Yang et al., 2009). However, the use of spore forming *Bacillus* spp., including *Bacillus subtilis* and *Bacillus licheniformis*, has increased because of their resistance to processing and environmental factors including high temperature and low pH (Priest, 1993; Henriques and Moran, 2000; Harrington et al., 2015).

Prebiotics, also defined by expert from ISAPP, are “substrates that are selectively utilized by host microorganisms conferring a health benefit (Gibson et al, 2017).” Indigestible carbohydrates are often administered as dietary prebiotics because they reach the lower GI tract minimally digested and intact to interact with or be metabolized by microorganisms (Bindels et al., 2015).

DFM and dietary prebiotics are potentially important alternatives to AGP. Their administration has been demonstrated to enhance digestive functionality, increase body weight (Awad et al., 2009; Shivaramaiah et al., 2011), and reduce FCR (Cavazzoni et al., 1998; Mookiah et al., 2014). The administration of direct-fed *Bacillus* spp. and prebiotics to poultry has been shown to have positive effects on the GI microbiota by increasing microbial diversity, promoting populations of beneficial bacteria including *Bifidobacterium* and LAB (Knarreborg et al., 2008; Lei et al., 2014), and reducing poultry pathogens including *Clostridium perfringens* (Sims et al., 2004; Knap et al.,

2010), and avian pathogenic *Escherichia coli* (La Ragione et al., 2001). Additionally, their administration has also been shown to reduce important human food-borne pathogenic bacteria including *Salmonella* (Huff et al., 2013; Jeong and Kim, 2014) and *Campylobacter* (Fritts et al., 2000; Arsi et al., 2015).

Because of their benefits, interest in the administration of DFM and dietary prebiotics as alternatives to the use of AGP has increased. Although performance and GI health benefits of DFM and prebiotic administration have been widely reported, their overall effectiveness is mixed, and the functionalities of specific microorganisms and prebiotic compounds are not well understood. In this study, we investigated the effects of the administration of *Bacillus* spp. as DFM and *Saccharomyces*-derived refined functional carbohydrates (RFC) as a dietary prebiotic on the growth performance and gastrointestinal microbiota of broiler chickens.

Materials and Methods

Experimental Animals and Husbandry

Male broiler chicks (Cobb) were obtained from a commercial hatchery on day of hatch and administered a live *Eimeria* vaccine (Advent, Huvepharma Inc, Peachtree City, GA) before being assigned randomly to treatment pens with similar starting weights. Chicks were raised in floor pens on built-up litter; provided age-appropriate heat, ventilation, and lighting; and given access to potable water and experimental feed *ad libitum*. All experimental procedures were performed as approved by the Texas A&M University Institutional Animal Care and Use Committee.

Experimental Design and Diets

Experimental animals (n=2,280) were allocated to six experimental treatment groups with 10 replicate pens of 38 birds arranged as a randomized complete block design and fed rations supplemented with spores of one of two *Bacillus* cultures (Arm and Hammer Animal and Food Production, Princeton, NJ), Bacillus A (1.0 × 108 cfu kg-1) or *Bacillus* B (1.5 × 108 cfu kg-1) administered as DFM; *Saccharomyces*-derived RFC administered (Arm and Hammer) as a dietary prebiotic (100 g t-1); or a combination of a *Bacillus* culture (2.5 × 108 cfu kg-1) and RFC (91 g t-1) administered as a synbiotic (Arm and Hammer). Broilers administered untreated or bacitracin methylene disalicylate (BMD)-treated (50 g t-1) feed served as control groups.

Experimental treatment diets (**Table 3.1**) were fed for the duration of the study using a 3-phase feed plan: starter phase (d 0 to 14, crumble), grower phase (d 14 to 27, pellet), and finisher phase (d 27 to 42, pellet). For each phase, feed was manufactured as a single standard corn/soybean meal basal diet with phytase and 5% DDGS and divided for inclusion of dietary treatments as appropriate.

Growth Performance Measures

Experimental animals and feed were weighed at 0, 14, 27, and 42 d post-hatch for determination of body weight and feed consumption. Mortalities and post-mortem weight were recorded daily for the calculation of percent mortality, average daily gain, and mortality adjusted FCR.

Recovery of Gastrointestinal Microbes

On d 42, two representative birds (median weight ± 5%) were selected from each pen, euthanized, and dissected for the aseptic collection of GI tissues. The proximal 1/3 from an approximately 9 cm section of the ileum taken at the midpoint between the ileocecal junction and Meckel's diverticulum and both ceca from each broiler were collected. Ileal specimens were pooled by pen and diluted serially using fluid thioglycolate medium (FTM, BD, Franklin Lakes, NJ) for enumeration of total LAB and *Clostridium perfringens* using cylcoheximide (100 μg mL^{-1}, Amresco, Solon, OH) supplemented de Mann, Rogosa, and Sharpe agar (BD) incubated in 10 % CO_2 at 37 ^oC for 24 h and Tryptose Sulfite Cycloserine-Egg Yolk agar (BD) incubated anaerobically (Coy Laboratory Products, Inc., Grass Lake, MI) at 37 ^oC for 48 h, respectively. Both ceca from each bird were pooled by pen and diluted serially using sterile PBS (Fisher Bioreagents, Pittsburgh, PA) for enumeration of *Campylobacter* spp. using Campy Cefex agar (CCA, Hardy Diagnostics, Santa Maria, CA) incubated in 10 % CO_2 at 37 ^oC for 24 h. *C. perfringens* was selectively enriched from ileal homogenates using FTM and Iron Milk Medium (HiMedia Laboratories, Mumbai, India), and *Campylobacter* was selectively enriched from cecal homogenates using Bolton's Enrichment Broth (Hardy) and CCA. Samples for which no colonies appeared on the enumeration plates but were positive by selective enrichment were assigned the limit of detection for enumeration (2.0 $\log_{10}$ cfu g^{-1}).

Statistical Analysis

Growth performance measures and $\log_{10}$ transformed bacterial counts were analyzed using ANOVA. Significantly different means were separated using Duncan's Multiple Range Test *post hoc*. Statistical significance was considered at $P \leq 0.05$.

Results and Discussion

The administration of Direct-Fed Microorganisms (DFM) and dietary prebiotics has been demonstrated to improve growth performance at levels similar to antibiotic growth promoters (AGP) (Jin et al., 1997; Awad et al., 2009) and reduce the colonization of human foodborne and poultry pathogens in the gastrointestinal (GI) tract of poultry (Griggs and Jacob, 2005; Ganan et al., 2012). Additionally, the potentially synergistic effects of the co-administration of DFM and prebiotics as synbiotics (Gibson and Roberfroid, 1995) on growth performance (Awad et al., 2009) and pathogen reduction in broilers has been demonstrated. Although DFM and prebiotics are thought to be potentially important alternatives to AGP, their effectiveness is mixed and the benefits of specific microorganisms and prebiotic compounds and their modes of action are not well characterized (Fritts et al., 2000; Willis and Reid, 2008; Flint and Garner, 2009) necessitating more thorough investigation. The use of *Bacillus* spp. as DFM has increased due to the greater resistance to heat and desiccation of spores as compared to the *Bifidobacterium* spp. and Lactic Acid Bacteria (LAB) that have been used traditionally (Barbosa et al., 2005). Refined functional carbohydrates (RFC), including mannan-oligosaccharides, β-glucan, and D-mannose, derived from the cell wall of *Saccharomyces cerevisiae* account for 20-30 % of the cell dry mass and are a readily

available source of prebiotics and for human and animal use (Dallies et al., 1998). The objective of this study was to investigate the administration of direct-fed *Bacillus* spp. and *Saccharomyces*-derived prebiotic RFC and their co-administration as a synbiotic in broiler chickens.

Growth Performance

Because of their growth enhancing effects, DFM and prebiotics are used widely as alternatives to AGP in poultry production. *Bacillus* spp. are highly valued producers of important industrial enzymes (Schallmey et al., 2004) and have been suggested to increase digestibility through the *in situ* production of enzymes in the GI tract (Askelson et al., 2014), while increased villus height observed in direct-fed *Bacillus*-treated broilers has been suggested to increase nutrient absorption by enterocytes (Samanya and Kamauchi, 2002). Prebiotic RFC have been shown to increase populations of beneficial bacteria in the GI tract (Askelson and Duong, 2015), ileal nutrient digestibility, nitrogen retention, and villus height (Gómez et al., 2012).

In this study, we observed improvements to growth performance when direct-fed *Bacillus* cultures and prebiotic RFC were administered alone or co-administered as a synbiotic (**Table 3.2**). Significant treatment effects were observed for d 14 BW (P = 0.026) and ADG during the starter phase (P = 0.022) and d 0 – 42 (P = 0.030). Broilers administered direct-fed *Bacillus* A, prebiotic RFC, synbiotic, and BMD were heavier on d 14 than the untreated control, while BW of those administered direct-fed *Bacillus* B was similar to all other treatments. Starter phase and cumulative (d 0 - 42) ADG was greatest when broilers were fed the BMD-treated diet and lowest when broilers were fed

the untreated diet. Administration of direct-fed *Bacillus*, prebiotic RFC, and synbiotic improved starter phase ADG to a level similar to that of BMD-treated broilers and when compared to untreated broilers. However, cumulative ADG was only observed to be improved in *Bacillus* A treated broilers when compared to the untreated broilers.

A significant treatment effect was observed for FCR during the starter phase (P = 0.002) (**Table 3.3**). Starter FCR was greatest when broilers were fed untreated diets. BMD and synbiotic administration improved FCR when compared to the untreated broilers. Although starter FCR was not significantly different from the untreated broilers, direct-fed *Bacillus* and prebiotic administration did improve starter FCR to a level similar to that of BMD-treated broilers. No significant treatment effects were observed for ADFI during any period of the study, suggesting the improved growth observed in this study was primarily the result of more efficient feed conversion.

The improvements in BW, ADG, and FCR observed in DFM, prebiotic, and synbiotic treated broilers in this study occurred primarily during the starter phase. Indeed, performance gains from the administration of these functional ingredients are often observed during the early phases of production. Similarly to the results of our study, improvements in BW, early phase (Spring et al., 2000; Flores et al., 2016) and cumulative ADG (Awad et al., 2009), and FCR (Gómez et al., 2002; Askelson et al., 2017) have been reported previously. However, conflicting results, including improvements in FCR (Teo and Tan, 2007; Awad et al., 2009; Lee et al., 2010) or ADG (Zhang et al., 2012) in the absence of other performance benefits, have also been reported in previous studies evaluating similar products. Although often attributed to the

entire class of products, the benefits of DFM and prebiotics are dependent upon the microbial strain and prebiotic compound being administered. Therefore, characterization of the specific mechanisms responsible for their beneficial functionalities will contribute to the improved development and application of these and other similar products.

A significant treatment effect was observed on mortality during the starter phase ($P = 0.028$), the grower phase ($P = 0.036$), and d 0-42 ($P = 0.010$) (Table 3.4). Overall, mortality was lowest when broilers were administered BMD. None of the products evaluated in this study significantly reduced mortality when compared to the untreated broilers. However, administration of direct-fed *Bacillus* A and synbiotic improved cumulative mortality to a level similar to the BMD-treated broilers, while administration of prebiotic RFC reduced mortality to a level similar to the BMD-treated during the starter phase. The overall mortality of broilers in this study was greater than is typically observed in similar studies. Although the use of built-up litter is routine in the production of poultry, we speculate that some unique condition of the recycled litter used in this study and the live *Eimeria* vaccine may have contributed to the unexpectedly high mortality observed in this study.

Gastrointestinal Microbiota

The GI microbiota is increasingly recognized as an important modulator of human and animal health (Askelson and Duong, 2015). Additionally, because growth promotion by antibiotics is attributed to their effects on the GI microbiota (Visek, 1978; Gaskins et al., 2002), the microbiota is an important target for the development of alternatives to AGP. We collected GI samples at termination of this

study (42 d) in order to evaluate the effects of DFM and prebiotic RFC administration on the GI microbiota of poultry.

Cecal Bacteria

Reduced colonization of pathogenic bacteria, including *Campylobacter* spp., has also been observed when DFM and prebiotics are administered to poultry. Probiotic bacteria are thought to reduce pathogenic bacteria likely through competition for shared attachment sites in the mucosa (Lu and Walker, 2001) or production of anti-microbial metabolites (Oelschlaeger, 2010; Neal-McKinney et al., 2012) RFC, including mannan-oligosaccharides, mannose, and β-glucans, important to the pathogen inhibition functionality of prebiotics (Oyofo et al., 1989; Spring et al., 2000) are thought to bind bacterial surface adhesins such the organisms pass through the GI tract unable to infect the host (Fernandez et al., 2002; Walker et al., 2018). In this study, a significant treatment effect was observed on counts of *Campylobacter* in the cecum ($P = 0.05$) (**Figure 3.1A**). Up to 1 $\log_{10}$ cfu g^{-1} fewer *Campylobacter* were recovered from broilers administered either direct-fed *Bacillus* culture, prebiotic RFC, or synbiotic when compared to the untreated control.

Ileal Bacteria

Lactobacillus spp. and other Lactic Acid Bacteria (LAB) are important inhabitants of the GI tract of humans and livestock animals (Klaenhammer et al., 2008) and are recognized as beneficial microorganisms (Gilliland, 1990; Priest, 1993; Mountzouris et al., 2007). Administration of probiotic LAB to poultry has been shown to improve growth performance at levels similar to AGP (Awad et al., 2009; Askelson et

al., 2014) improve digestive health (Kim et al., 2012), and stimulate immune responses (Dalloul et al., 2003; Brisbin et al., 2011) suggesting LAB as a potential indicator of GI health and possible therapeutic target for the development of alternatives to AGP. In addition, Askelson, et al. (2017) reported a negative correlation between LAB counts and FCR further suggesting these bacteria may play a role in the growth performance of broilers. Additionally, administration of direct-fed *Bacillus* has been proposed to promote the proliferation of LAB through the creation of an anaerobic environment by the rapid consumption of oxygen during the germination of spores (Jeong and Kim, 2014). In this study, a significant treatment effect on total LAB counts ($P = 0.001$) was observed (**Figure 3.1B**). Significantly more LAB were recovered from broilers that were administered either direct-fed *Bacillus* culture and prebiotic RFC with LAB counts being over 1 $\log_{10}$ cfu g^{-1} greater than when compared to the untreated control. Further, although not significantly different than the untreated broilers, LAB counts from the synbiotic-treated broilers were similar to the DFM and prebiotic treated broilers

In addition to its use as a growth promotor, BMD is administered to manage necrotic enteritis through the reduction of *Clostrdium perfringens* (Peek and Landman, 2011). The disease causes substantial increases in mortality and is associated with reduced nutrient acquisition and intestinal tissue damage (Prescott et al., 2016). Therefore, developing alternatives which reduce subclinical infections and improve growth and feed efficiency is of great importance. Decreased *C. perfringens* counts have been reported previously when broilers were administered DFM and prebiotics (Thanissery et al., 2010; Latorre et al., 2015). However, a significant treatment effect

was not observed in this study ($P = 0.249$). Although the effect was not significant, numerically fewer *C. perfringens* were recovered from broilers fed diets treated with BMD, either DFM, prebiotic, or synbiotic when compared to broilers fed the untreated diet (**Figure 3.1C**).

In this study, we investigated the administration of *Bacillus* spores as Direct-Fed Microorganisms and *Saccharomyces*-derived refined functional carbohydrates as a dietary prebiotic on the growth performance and gastrointestinal microbiota of broiler chickens. We have demonstrated the administration of direct-fed *Bacillus,* prebiotic RFC, and their co-administration as a synbiotic improved BW, ADG, and FCR of broiler chickens, overall. Additionally, we have demonstrated that DFM, prebiotic RFC, and synbiotic administration reduced *Campylobacter* spp. in the cecum and increased total LAB in the ileum, potentially improving pre-harvest microbial food safety and animal health, respectively. Our results suggest that administration of direct-fed *Bacillus* cultures and prebiotic RFC may be potentially important alternatives to be included as part of an antibiotic free poultry production program.

Table 3.1. Ingredient composition and nutrient content of the basal control diets

Item (%)	Starter	Grower	Finisher
Ingredients			
Corn	57.90	63.55	64.90
Soybean Meal	29.10	23.70	19.25
DL-Methionine	0.29	0.25	0.19
Lysine HCL	0.25	0.23	0.20
L-Threonine	0.09	0.08	0.06
Fat A&V blend	2.50	2.40	3.45
Limestone	0.87	0.69	0.74
Monocalcium PO_4	0.30	0.00	0.06
Salt	0.32	0.33	0.26
Sodium Bicarbonate	0.14	0.13	0.21
Trace Minerals[1]	0.05	0.05	0.05
Vitamins[2]	0.25	0.25	0.25
LO- DDGS	5.00	5.00	8.00
Meat and Bone Meal	3.00	3.35	2.50
Phytase[3]	0.01	0.01	0.01
Calculated nutrient content			
Protein	22.00	19.95	17.82
Crude Fat	5.30	5.41	5.95
Crude Fiber	2.50	2.53	2.55
Calcium	0.92	0.82	0.75
AV Phosphorous	0.46	0.41	0.38
ME (kcal kg^{-1})	3047	3102	3168
Digestible Methionine	0.59	0.53	0.46
Digestible TSAA	0.87	0.79	0.69
Digestible Lysine	1.18	1.04	0.89
Digestible Tryptophan	0.21	0.18	0.16
Digestible Threonine	0.77	0.69	0.60
Sodium	0.046	0.043	0.039
Analyzed nutrients[4]			
Moisture	11.73	11.93	11.75
Dry Matter	88.27	88.07	88.25
Crude Protein	21.80	19.9	17.80
Crude Fat	5.22	5.34	6.32
Fiber	3.30	3.10	3.20
Ash	4.41	4.18	4.04

[1]Trace mineral premix added at this rate yields 60.0 mg manganese, 60 mg zinc, 60 mg iron, 7 mg copper, 0.4 mg iodine, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.
[2]Vitamin premix added at this rate yields 22,045 IU vitamin A, 7,716 IU vitamin D3, 91 IU vitamin E, 0.04 mg B12, 11.9 mg riboflavin, 91.8 mg niacin, 40.4 mg d-pantothenic acid, 261.1 mg choline, 2.9 mg menadione, 3.50 mg folic acid, 14.3 mg pyroxidine, 5.87 mg thiamine, 1.10 mg biotin per kg diet. The carrier is ground rice hulls.
[3]OptiPhosPF, Huvepharma. Peachtree City, GA.
[4]Performed by Midwest Laboratories, Inc., Omaha, NE

Table 3.2. Body weight and average daily gain of broilers

	BW (kg)				ADG (g bird-day^{-1})			
Treatments	d 0	d 14	d 27	d 42	Starter	Grower	Finisher	d 0-42
BMD	0.042	0.484^{a}	1.601	3.015	32.39^{a}	85.29	95.04	72.36^{a}
UNT	0.044	0.467^{b}	1.490	2.912	29.16^{c}	78.16	95.09	65.69^{c}
Bacillus A	0.045	0.483^{a}	1.591	3.033	30.67^{ab}	84.91	95.14	70.44^{ab}
Bacillus B	0.045	0.475^{ab}	1.553	3.000	30.03^{bc}	81.88	96.63	67.70^{bc}
RFC	0.044	0.481^{a}	1.570	3.070	31.17^{ab}	82.59	100.13	69.72^{abc}
Synbiotic	0.045	0.485^{a}	1.613	2.979	31.45^{ab}	85.82	92.51	69.49^{abc}
SEM	0.000	0.002	0.012	0.019	0.28	0.862	1.37	0.61
P-value	0.386	0.026	0.061	0.242	0.022	0.122	0.745	0.030

^{a,b,c} Different superscripts within columns indicates means differ significantly ($P \leq 0.05$)

Table 3.3. Mortality corrected feed conversion ratio and average daily feed intake of broiler chickens

	FCR (Feed:Gain)				ADFI (g bird-day^{-1})			
Treatments	Starter	Grower	Finisher	d 0-42	Starter	Grower	Finisher	d 0-42
BMD	1.159^{bc}	1.463	2.088	1.479	37.55	124.22	186.27	119.06
UNT	1.214^{a}	1.629	2.142	1.735	35.34	123.97	190.96	113.87
Bacillus A	1.190^{ab}	1.505	2.056	1.645	36.50	126.88	183.27	115.52
Bacillus B	1.186^{ab}	1.534	2.084	1.683	35.58	124.09	190.35	113.92
RFC	1.170^{bc}	1.546	1.995	1.650	36.39	126.16	188.18	114.92
Synbiotic	1.144^{c}	1.474	2.109	1.662	35.96	125.76	185.30	115.39
SEM	0.005	0.190	0.032	0.010	0.27	0.57	1.19	0.79
P-value	0.002	0.168	0.852	0.053	0.285	0.534	0.323	0.504

^{a,b,c}Different superscripts within columns indicates means differ significantly ($P \leq 0.05$)

Table 3.4. Mortality of broiler chickens

	Mortality (%)			
Treatments	Starter	Grower	Finisher	d 0-42
BMD	0.53[b]	1.36[ab]	0.00	1.84[c]
UNT	6.05[a]	0.00[b]	1.26	7.11[ab]
Bacillus A	4.73[a]	0.27[ab]	0.27	5.26[abc]
Bacillus B	6.84[a]	1.88[a]	0.59	9.21[a]
RFC	3.95[ab]	1.92[a]	0.29	6.05[ab]
Synbiotic	4.74[a]	0.00[b]	0.60	5.00[bc]
SEM	0.58	0.26	0.16	0.60
P-value	0.028	0.036	0.350	0.010

[a,b,c] Different superscripts within columns indicates means differ significantly ($P \leq 0.05$)

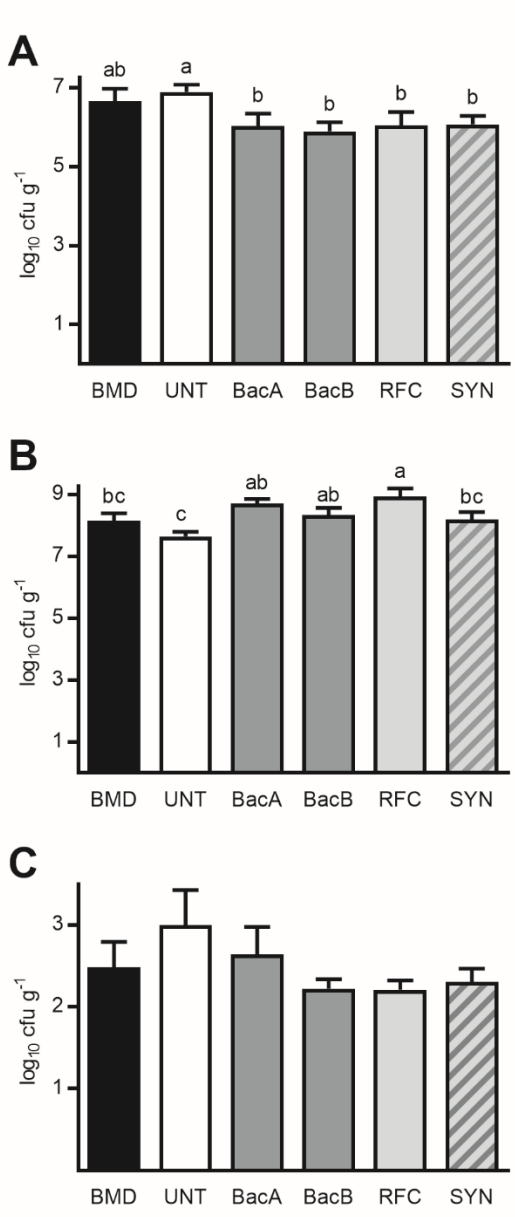

Figure 3.1. Enumeration of bacteria from broiler chickens. At 42 d post hatch, **(A)** *Campylobacter* spp. were enumerated from the cecum, and **(B)** total LAB and **(C)** *C. perfringens* were enumerated from the ileum. Counts are reported as the mean ± SEM $\log_{10}$ cfu g^{-1} digestive contents. Different letters above bars indicate means are significantly different ($P \leq 0.05$).

CHAPTER IV

REFINED FUNCTIONAL CARBOHYDRATES REDUCE ADHESION OF *CAMPYLOBACTER* AND *SALMONELLA* TO POULTRY EPITHELIAL CELLS *IN VITRO*

Introduction

The Centers for Disease Control and Prevention has estimated there are approximately 48 million cases of foodborne illness in the United States each year (CDC, 2019). *Salmonella* and *Campylobacter* are the most frequently reported bacterial causes of foodborne illness with poultry because of their association with the gastrointestinal (GI) tract (Heyndrickx et al., 2002; Mead, 2002). The pathogen load in the GI tract at the beginning of processing is the main factor for the pathogen load at the end of processing (Lahellec and Colin,1985). Therefore, the development of interventions which reduce *Salmonella* and *Campylobacter* load pre-harvest will be important to the microbial food safety of poultry.

Defined by expert consensus from the International Scientific Association for Probiotics and Prebiotics (ISAPP) (Gibson et al., 2017), a prebiotic is "a substrate that is selectively utilized by host microorganisms conferring a health benefit," and when administered orally are referred to specifically as dietary prebiotics (Bindels et al., 2015). Prebiotic products commonly include indigestible carbohydrates that remain intact until reaching the lower portion of the GI tract where they interact with intestinal microbiota (Slavin, 2013). The cell wall of *Saccharomyces cerevisiae* accounts for

between 20 and 30% of the cell dry mass and is a readily available source of prebiotics for human an animal use (Dallies et al., 1998). Refined functional carbohydrates (RFC), including mannanoligosaccharides (MOS), D-mannose, and β-glucan, are derived from the cell wall of *Saccharomyces cerevisiae* (Moran, 2004; Walker et al., 2017). The administration of prebiotics has been shown to reduce important animal pathogens, such as *Clostridium perfringens* (Yang et al., 2008; Allaart et al., 2013), and human food-borne pathogens, including *Salmonella* spp. (Spring et al., 2000; Fernandez et al., 2002) and *Campylobacter* spp. (Baurhoo et al., 2009; Huff et al., 2013).

Adhesion to mucosal surfaces is important to the colonization and persistence of pathogens in the GI tract (Rosenberg et al., 1983) because it allows bacteria to resist peristaltic movements (Granato et al., 1999). Pathogen reduction by prebiotics is thought to be the result of their ability to inhibit adhesion in the GI tract of poultry. However, the inhibition of pathogen adhesion by prebiotics and the mechanisms responsible are not well characterized. Improved understanding of this important functionality will be important for the development of prebiotics and their application in the poultry industry.

The chicken LMH epithelial cell line, derived from a hepatocellular carcinoma, (Kawaguchi et al., 1987) has been used widely to investigate host-microbe interactions in the gastrointestinal tract of poultry. For example, the use of this chicken epithelial cell line has enabled the characterization of *Clostridium perfringens* NetB toxin's role in necrotic enteritis (Keyburn et al., 2008) and identification of protein adhesins important to *Campylobacter jejuni* colonization (Flanagan et al., 2009; Quiñones et al., 2009) and virulence genes important to cellular invasion by *Salmonella* Enteritidis (Shah et al., 2012)

in the GI tract of chickens. Additionally, adhesion of *Lactobacillus* spp. to poultry epithelia was characterized *in vitro* LMH cells and verified *in vivo* using broiler chicks by our own research group (Spivey et al., 2014).

In this study, we characterized the ability of prebiotic RFC to inhibit adhesion of *Salmonella* Typhimurium and *Campylobacter jejuni* to poultry epithelial cells *in vitro* using the LMH chicken epithelial cell line and compared the inhibition of pathogens by individual RFC. In addition, we compared pathogen inhibition by RFC with fructooligosaccharides (FOS), galactooligosaccharides (GOS), and raffinose.

Materials and Methods

Culture of LMH cells

Chicken LMH hepatocellular carcinoma epithelial cells (ATCC CRL-2117) were cultured in 0.1% gelatin (MilliporeSigma, Burlington, MA) coated flasks using Waymouth's MB 752/1 medium (ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; ThermoFisher). Cells were maintained at 37 °C in a humidified 5% CO_2 incubator.

Bacterial Strains

Bacterial Strains Primary poultry isolates of *Salmonella* Typhimurium (TDC 100) and *Campylobacter jeuni* (TDC 130) were obtained from the USDA-ARS Southern Plains Agricultural Research Center (College Station, TX). *Salmonella* was cultured using Tryptic Soy Broth (Difco, Franklin Lakes, NJ) or Xylose Lactose Tergitol 4 agar (XTL-4; Difco) incubated aerobically at 37 ^{o}C. *C. jejuni* was cultured using Mueller Hinton broth (BD, Franklin Lakes, NJ) or Campy Cefex agar (CCA; Hardy Diagnostics,

Santa Maria, CA) incubated in 10% CO_2 at 42 °C. For LMH cell adhesion assays, 18 h broth cultures of bacteria were harvested by centrifugation, washed 3× using assay medium (Waymouths + 1 % FBS), and re-suspended by absorbance (O.D. $_{600\ nm}$) to the appropriate multiplicity of infection (MOI) with LMH cells using assay medium. Counts of re-suspended *Salmonella* and *C. jejuni* were confirmed by enumeration using XLT-4 and CCA, respectively.

Prebiotic Oligosaccharides

Stock solutions of prebiotic RFC and oligosaccharides (**Table 4.1**) were prepared by suspending the products in Assay Medium (1 % w/v). Final concentrations were achieved when the product was inoculated into the cell culture well at the appropriate volume.

LMH Cell-Binding Assays

Inhibition of *Salmonella* and *Campylobacter* adhesion to LMH cells by prebiotic RFC and oligosaccharides was investigated using methods adapted from Spivey et al. (2014). Gelatin coated 24-well plates were seeded with LMH cells (3.0×10^5 cells $well^{-1}$) and incubated for 18 h. Wells were rinsed 3× with assay medium to remove non-adherent cells. Wells were inoculated simultaneously with approximately 1.5×10^7 cfu bacteria (100:1 bacteria per LMH cell) and appropriate treatment of product, both of which were suspended in Assay Medium.

A range of specific concentrations of prebiotic product were used for the dose response evaluation, and 0.1% inoculations of prebiotic products or RFC were used for all comparative evaluations. Plates were centrifuged at 600 ×*g* for 5 min at 20°C to

promote bacterium-host cell contact and then incubated for 30 min at 37°C in a humidified 5% CO_2 incubator. Following incubation, wells were rinsed 5×with PBS to remove non-adherent bacteria. LMH cells were lysed by the addition of 200µL of 0.1% Triton X-100 (MilliporeSigma) to each well and incubated for 10 minutes at 37°C. Bacterial suspensions were diluted in PBS and *Salmonella* and *Campylobacter* were enumerated using XLT-4 or CCA.

Comparison of Prebiotic Products and RFC

The effects of fructooligosaccharide (FOS; OPS, Orafti Active Food Ingredients, Tienen, Belgium)-, galactooligosaccharide (GOS; Oligomate 55, Yakult, Tokyo, Japan)-, or raffinose (KEB Biotechnology, Beijing, China)-based prebiotic, and the mannan oligosaccharide- based poultry prebiotic (Celmanax, Arm and Hammer Animal and Food Production, Princeton, NJ) on *Salmonella* and *Campylobacter* adhesion were evaluated. In addition, the effects of purified β-glucan (VWR, Randor, PA), mannanoligosaccharide (MOS; Sigma Chemical Co., St. Louis, MO), and D-mannose (Sigma Chemical Co.) on the adhesion of both bacteria was assessed.

Calculations and Statistical Analysis

Counts of adherent bacteria were $\log_{10}$ transformed for determination of $\log_{10}$ reduction of adherent bacteria as compared to untreated control wells. The percent reduction was calculated using:

$$\%\ \text{reduction} = (1 - 10^{-l}) \times 100\ \%$$

$$\text{where } l = \log_{10} \text{reduction}$$

The dose response data was fit to a sigmoid four-parameter logistic model. The minimum and maximum asymptotic response values were constrained at 0 % and 100 %, respectively; the inflection point was used to determine the 50 % inhibitory concentration (IC_{50}); Hill's slope factor (SF) is the slope of the curve at the IC_{50}; and r^2 was used to establish goodness-of-fit for the regression. For non-dose response assays, percent reductions were arcsine square root transformed and analyzed using ANOVA with $\alpha = 0.05$. Results from independent assays were pooled for analysis and the independent assays used as a blocking factor. Significantly different means were separated using Tukey's Honestly Significant Differences test *post-hoc.*

Results and Discussion

While gastrointestinal health benefits from prebiotic administration have been widely reported (Baurhoo et al., 2009; Alloui et al., 2013), the exact mechanisms of pathogen inhibition are not well characterized. It is thought that prebiotics act through competitive binding to prevent pathogen adherence to epithelial tissues (Ganan et al., 2012; Xu et al., 2017). This pathogen reduction may be completed by direct prebiotic-pathogen interaction that results in pathogenic bacteria attachment to the prebiotic that results in the pathogen passing through the GI tract without attachment to the epithelial tissue (Firon et al., 1987).

In addition, it has been demonstrated that microorganisms present in the GI tract can ferment prebiotics into short chain fatty acids (SCFA), specifically acetate, propionate, and butyrate (Pourabedin and Zhao, 2015). This increase in SCFA and higher rate of fermentation has been correlated to a lower pH, which has been associated

with an increased solubility of nutrients and a reduction of pathogens in the avian gastrointestinal tract (Józefiak et al., 2004). Additionally, the fermentation of prebiotics in the GI tract can lead to higher levels of potentially beneficial bacteria, including Lactic Acid Bacteria and bifidobacteria, which are thought to be important to competitively excluding pathogens (Patterson and Burkholder, 2003).

Refined functional carbohydrates (RFC), including mannan oligosaccharides, β-glucan, and D-mannose account for 20-30% of the cell dry mass of *Saccharomyces cerevisiae,* can be enzymatically or chemically extracted are a readily available source of prebiotics for human and animal use (Dallies et al., 1998). We previously evaluated the effects of administering RFC to broilers in feed at levels of 0.05% (50 g t^{-1}) and 0.1% (100 g t^{-1}) and observed significant reductions of *Campylobacter* spp. colonization in the ceca of broilers administered either dose, with over a 1 $\log_{10}$ cfu g^{-1} reduction in *Campylobacter* observed when broilers were administered the 0.1% dose (Froebel et al., 2019). Although positive effects have been reported when RFC are administered in poultry production, the mechanisms by which they reduce pathogen colonization is not well characterized.

Although the LMH cell line is derived from the liver, its suitability for the investigation of host-microbe interactions in the GI tract of poultry has been well established. The LMH cell line has been used to evaluate host-microbe interactions of human foodborne pathogens in poultry, including *Campylobacter* and *Salmonella* (Larson et al., 2008; Quiñones et al., 2009) and *Lactobacillus*-mediated competitive exclusion and virulence inhibition of pathogenic microorganisms in poultry (Spivey et

al., 2014). Thus, in the absence of a poultry-specific intestinal cell line, we used the LMH chicken epithelial cell line as a model to investigate the inhibition of pathogen adhesion by RFC and other prebiotic oligosaccharides.

Dose-Response of Prebiotic RFC

In this study, we evaluated the effect of increasing concentrations of prebiotic RFC to inhibit adhesion of *Salmonella* Typhimurium and *Campylobacter jejuni* to the LMH chicken epithelial cell line. The bacteria were incubated separately with epithelial cells treated with 0, 0.025, 0.05, 0.1, 0.25, 0.375, 0.5, 0.625, 0.75, 1, and 2 % (w/v) RFC, and the reduction of adherent bacteria as compared to untreated (0 %) cells was determined (**Figure 4.1**). The ability of prebiotic RFC to inhibit adhesion of both pathogens to the epithelial cells was dose-dependent and saturable with the reduction of *Salmonella* (IC_{50} = 0.048%, r^2=0.989) and *Campylobacter* (IC_{50} = 0.020%, r^2=0.994) increasing with the concentration of RFC. The slope for *Campylobacter* (SF=2.143) reduction was steeper than for *Salmonella* (SF=0.935), suggesting that the adhesion of *Campylobacter* is more sensitive to inhibition by RFC than *Salmonella*.

These results suggest that inhibition of adhesion to epithelial tissues may be an important mode of action through which prebiotic RFC reduce *Salmonella* and *Campylobacter* colonization in the GI tract of poultry GI. The suggested dose for in-feed administration of these prebiotic RFC in broiler chickens is 500 – 1000 ppm (0.05% - 0.1%). These were likely determined from results of performance studies and on economic analysis. The IC_{50} for *Salmonella* and *Campylobacter* we observed in this

study fall reasonably within the likely expected concentration range of prebiotic RFC in the GI tract when administered in-feed and with water consumption taken into account.

Based these results, a concentration of 0.1 % (w/v) was used to evaluate the ability of carbohydrates/oligosaccharides to reduce adhesion of *Salmonella* and *Campylobacter* to the LMH cell line in subsequent assays. Although the carbohydrates or oligosaccharides being evaluated would not be present in equimolar concentration, they would be equivalent on a mono-saccharide basis because the molecular weight of the monosaccharides from which they are composed (glucose, fructose, mannose) is identical (180.16 g mol^{-1}).

Evaluation of Individual Prebiotic Component Effects on Adhesion to LMH Cells

Mannoproteins and glucans comprise approximately 85-90% of the dry mass of the cell well of *Saccharomyces cerevisiae* (Fleet, 1991; Klis, 1994) and serve as a readily available source of prebiotics for human and animal use. Whereas the composition varies by strain and culture conditions (McMurrough and Rose, 1967; Catley et al., 1988), glucans are estimated to make up 55-60% of the cell wall with the remaining content being mannan-protein complex and cell wall-linked and periplasmic glycoproteins (Phaff, 1971). Thermal and enzymatic processing of the cell wall produces β-glucan, mannan-oligosaccharide (MOS), and D-mannose (Hunter and Asenjo, 1988). The RFC of β-glucan, MOS, and D-mannose are not present in equal concentrations with in the yeast cell wall, and therefore, we presume they are not extracted at equivalent volumes. Understanding which component is most effective at reducing pathogens will be helpful in the development of prebiotics for the poultry industry. We evaluated the

ability of these prebiotic RFC to inhibit adhesion of *Salmonella* and *Campylobacter* individually, in order to gain insight into the relative contribution of each component of the cruder extract. All three of the major constituent carbohydrates were observed to significantly inhibit ($P < 0.001$) adhesion of *Salmonella* to the epithelial cells as compared with untreated cells (**Figure 4.2A**). Reduction of adherent *Salmonella* by β-glucan (95.80 %) and MOS (90.90%) was greater than by D-mannose (32.14 %). Similarly, each of the major component carbohydrates of prebiotic RFC also significantly inhibited adhesion of *Campylobacter jejuni* ($P < 0.001$) (**Figure 4.2B**). Reduction of adherent *Campylobacter* by β-glucan (98.57%) and MOS (97.02%) than D-mannose (94.67%). Reductions in pathogen colonization has been observed with the administration of mannooligosaccharide-based products (Hooge, 2004, Baruahou 2009). Our results suggest β-glucan and MOS, are playing an important role in reducing adherence of pathogens to the epithelial lining of the poultry GI tract and possibly successively reducing colonization of pathogens. However, the mechanisms responsible for this adherence reduction is not well understood and require further investigation for a more thorough understanding. A proposed mechanism by which of prebiotics reduce pathogens in poultry relies solely on prebiotic-pathogen interactions. The dose-dependent and saturable nature of the response curves from this study suggests specific receptor-ligand binding reactions may contribute to reduced adhesion of bacteria to epithelial cells. Specifically, MOS has been shown to bind to the FimH-like adhesions on type 1 fimbriae of Gram-negative bacteria, including *Salmonella* and *E. coli* (Oyofo et al., 1989; Spring et al., 2000), causing the pathogens to not adhere gastrointestinal

epithelial cells and be removed from the lumen. Although *Campylobacter* has not been found to have such adhesins, expression of a mannose-binding lectin has been observed in a strain of *C. jejuni* (Day et al., 2009), suggesting a binding interaction may also be involved in *Campylobacter* reduction by prebiotics. Both MOS and D-mannose are hydrolyzed from mannose, however, reduction of adherent *Salmonella* was significantly greater with MOS than D-mannose, suggesting the inhibition of adherence may be related to the chains and branching in the oligosaccharide form than simply the saturation of the sites which mannose binds to. β-glucan has also been shown to have binding capabilities to bacteria, including *Streptococcus*, *Salmonella*, and *E. coli* (Mattos-Graner et al., 2001; Ganner et al., 2013). Further, some microorganisms, including *Lactobacillus* spp. and *Pediococcus* spp., secrete β-glucans for increased adhesive capabilities (Garai-Ibabe et al., 2010). Therefore, β-glucan may also bind to the epithelial lining of the GI tract to competitively exclude pathogenic microorganisms.

Many studies have been conducted using poultry prebiotics derived from the yeast cell wall (Yang et al., 2008; Morales-Lopez and Brufau, 2013; Santos et al., 2013), however, MOS is often the component most thoroughly discussed in analysis. It is likely other carbohydrates derived from the yeast cell wall, such as β-glucan, are contributing to the effectiveness of the product as well. Therefore, it is important that MOS is not the only RFC evaluated to better characterize the mechanisms by which prebiotics impact poultry.

Evaluation of Prebiotic Effects on Adhesion to LMH Cells

Dietary prebiotics have been widely studied in poultry production, however, their effectiveness and the mechanisms by which they reduce pathogen colonization is not well understood. Prebiotic products obtained by extracting RFC through the degradation of the cell wall of yeasts are a readily available source of beneficial carbohydrates that can be obtained inexpensively by using biproducts such as spent yeast. Whereas, other prebiotics must be synthesized. Therefore, in addition to the effectiveness of reducing pathogen adhesion and colonization RFC availability and costs will likely factor in to choosing an appropriate prebiotic.

In this study, compared the ability of fructooligosaccharides (FOS), galactooligosaccharides (GOS), raffinose, or the MOS-based poultry RFC to inhibit pathogen adhesion. A significant treatment effect was observed for the percent adherence reduction of *Salmonella* Typhimurium to the LMH cells ($P = 0.012$) (**Figure 4.3A**). Reduction of adherent *Salmonella* by FOS (50.79%) and raffinose (47.70%) was greater than GOS (18.44%). Adhesion of *Salmonella* with MOS-based RFC (39.09%) was reduced to a similar level as raffinose and FOS, as well as the GOS. In addition, a significant treatment effect was also observed for the percent adherence reduction of *Campylobacter jejuni* ($P < 0.001$) (**Figure 4.3B**). Reduction of adherent *Campylobacter* was greater by MOS-based RFC (95.43%) and raffinose (93.66%) than to FOS (78.79%) and GOS (78.41%).

Similar work was previously conducted to evaluate the effects of prebiotic compounds, including FOS, GOS, and raffinose, on Enteropathogenic *E. coli* adherence

to different lines of tissue culture cells (Shoaf et al., 2006). Their work demonstrated a significantly higher reduction in adhesion with GOS than other prebiotics, differing from our results. To our knowledge, our work is the first of its kind evaluating prebiotic reduced adhesion of *Salmonella* and *Campylobacter* in a poultry-specific cell line.

In this study, we evaluated the adhesion of *Salmonella* Typhimurium and *Campylobacter jejuni* to the LMH cell line in the presence of four prebiotic products. A prebiotic composed of fructooligosaccharide reduced *Salmonella* adhesion to a greater extent than other prebiotics evaluated, and a raffinose family oligosaccharides product and a poultry product of RFC, including mannanoligosaccharide, reduced *Campylobacter* adhesion greater than the others examined. In addition, we observed a dose response of adhesion with a poultry prebiotic of RFC derived from the yeast cell wall of *Saccharomyces cerevisiae*, and we evaluated the adhesion of *Salmonella* Typhimurium and *Campylobacter jejuni* in the presence of three RFC, mannanoligosaccharides, β-glucan, and D-mannose. A significantly greater reduction in *Salmonella* adherence to LMH cells was observed with β-glucan and MOS in comparison to D-mannose, and *Campylobacter* adherence was reduced to the greatest extent by β-glucan, followed by MOS and D-mannose.

Our study suggests the value of the chicken LMH cell line for *in vitro* assessment of prebiotics effects on pathogen adhesion to the epithelial lining of poultry and that a mechanism by which dietary prebiotics reduce colonization of *Salmonella* and *Campylobacter* is through reduced adhesion to the epithelial lining. Although adhesion is a considerable factor in colonization (Rosenberg, Gottlieb et al. 1983), adhesion to the

epithelial limning of the GI tract is not the only determinant. Therefore, our results are limited due to the complexity of the poultry GI tract that is not included in this model, such as mucus and extracellular matrix components, which will need to be evaluated to better understand the effect of prebiotics on *Salmonella* and *Campylobacter* colonization in poultry. This study is expected to contribute to a mechanistic understanding of prebiotic functionality in poultry and the development and selection of future prebiotics.

Table4. 1. Structure and composition of carbohydrates used in this study

Carbohydrate	Composition (%)	Source
β-glucan	80	VWR
D-mannose	99	Sigma Chemical Co.
FOS	95	OPS, Orafti Active Food Ingredients
GOS	55	Oligomate 55, Yakult
MOS	99.9	Sigma Chemical Co.
Raffinose	99	Keb Biotechnology

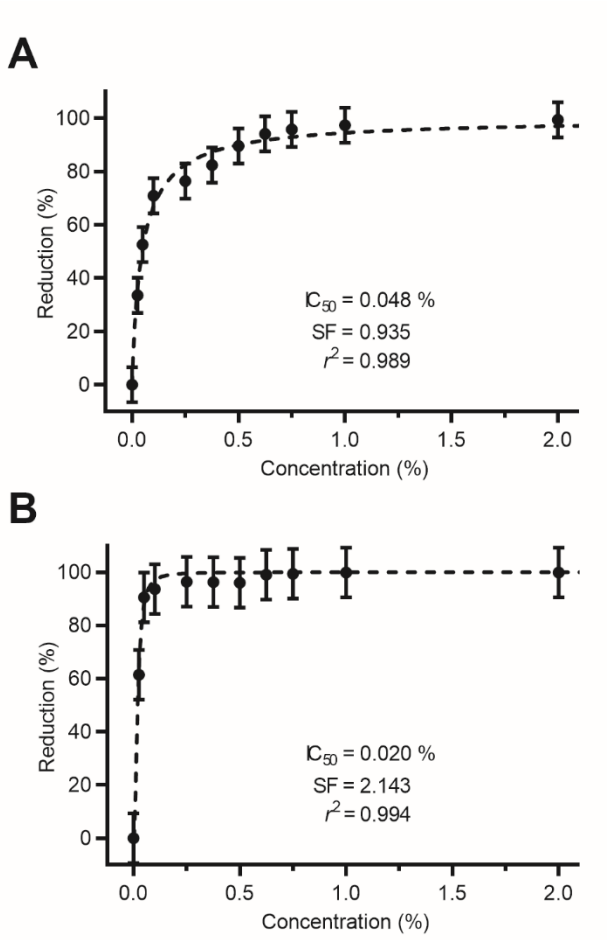

Figure 4.1. Dose response of adhesion inhibition by RFC. (A) *Salmonella* Typhimurium and **(B)** *Campylobacter jejuni* bacteria were co-incubated with LMH cells (MOI 100:1) treated with increasing concentrations of RFC, and the number of adherent bacteria was enumerated. The mean ± SEM % reduction of adherent bacteria from three independent wells is reported. IC_{50}, 50 % inhibitory concentration; SF, slope factor.

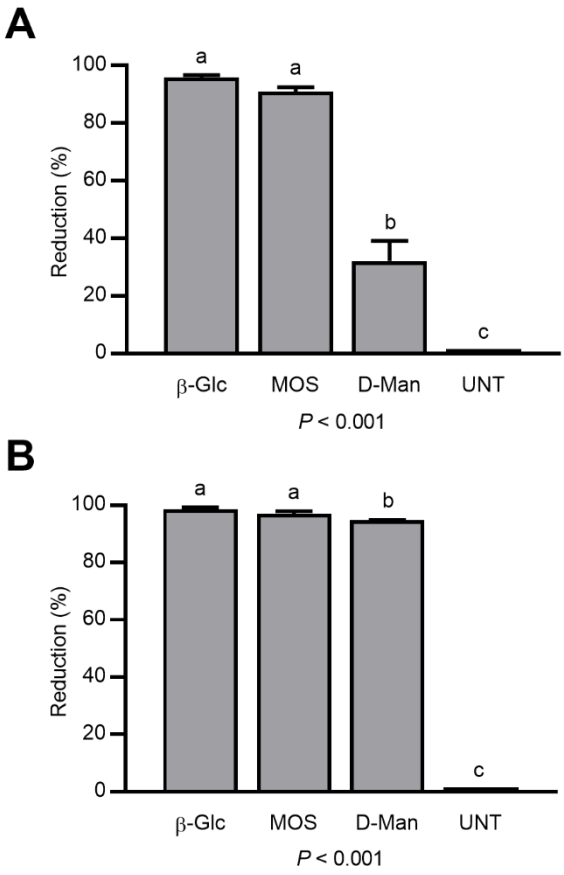

Figure 4.2. Inhibition of pathogen adhesion to chicken epithelial cells by carbohydrate components of prebiotic RFC. **(A)** *Salmonella* Typhimurium and **(B)** *Campylobacter jejuni* bacteria were co-incubated with LMH cells (MOI 100:1) treated with β-glucan (β-Glc), mannan-oligosaccharide (MOS), D-mannose (D-man), or untreated (UNT) cells, and the number of adherent bacteria was enumerated. The mean ± SEM % reduction of adherent bacteria as compared to UNT cells from 3 independent wells from 3 independent assays is reported. Means not sharing common letters are significantly different ($P \leq 0.05$).

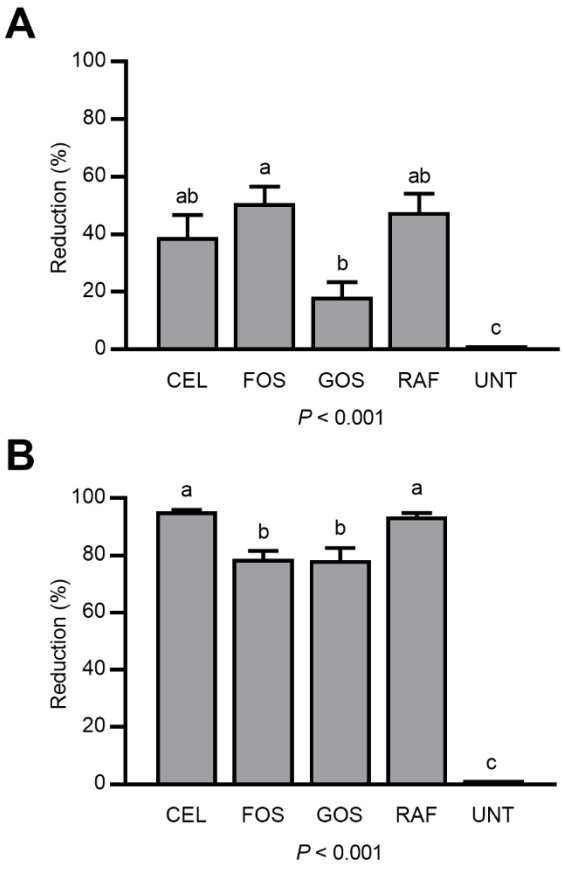

Figure 4.3. Inhibition of pathogen adhesion to chicken epithelial cells by prebiotic oligosaccharides. (A) *Salmonella* Typhimurium and **(B)** *Campylobacter jejuni* bacteria were co-incubated with LMH cells (MOI 100:1) treated refined functional carbohydrates (RFC), fructo-oligosaccharides (FOS), galacto-oligosacchardies (GOS), raffinose (RAF), or untreated (UNT) cells, and the number of adherent bacteria was enumerated. The mean ± SEM % reduction of adherent bacteria as compared to UNT cells from 3 independent wells from 3 independent assays is reported. Means which not sharing common letters are significantly different ($P \leq 0.05$).

CHAPTER V

CONCLUSION

The use of antibiotics in poultry production has greatly declined due to consumer demand and regulations that ban or limit their use. Administration of antibiotics has been acknowledged to enhance growth performance, mitigate important animal diseases, and reduce human foodborne pathogens. Thus, it is essential that effects and mechanisms of potential alternatives to antibiotics be investigated, including functional feed ingredients, such as probiotics and dietary prebiotics.

The research presented investigated the effects of a dietary prebiotic composed of refined functional carbohydrates and yeast culture hydrolyzed from the cell wall of *Saccharomyces cerevisiae* administered to broiler chickens at two doses. Body weight on d 42 and ADG for days 0 to 42 was significantly greater for broilers administered the high prebiotic diet. Further, a significant main effect of prebiotic dose was observed on day 42 BW, days 29 to 42 ADG, and days 29 to 42 feed intake, with the high dose increasing each performance measure. At d 42, significantly less *Campylobacter* spp. was recovered from the ceca of birds administered either dose of prebiotic.

The effects of administering two Direct-Fed *Bacillus* products, and one symbiotic of *Bacillus* spp. and refined functional carbohydrates, and the refined functional carbohydrates and yeast culture prebiotic was evaluated in broilers. Improvements to growth performance parameters, including body weight and feed conversion ratio, were observed with the administration of the DFM products and the prebiotic RFC,

administered individually or as a symbiotic. Also, cecal *Campylobacter* spp. counts were reduced with DFM, synbioitc, and prebiotic administration, and ileal Total Lactic Acid Bacteria were increased with prebiotic administration.

In addition to understanding the effects of probiotics and prebiotics, the poultry industry will need to continue enhancing the mechanistic understanding of prebiotics applied in poultry production to guide the selection and development of more effective products. The research presented investigated the effects of a poultry dietary prebiotic on the adhesion of *Salmonella* and *Campylobacter*, two common foodborne illness pathogens associated with poultry products, to the LMH cell line. Adhesion reduction was determined to be dose-dependent and saturable.

The functionality of individual prebiotic components, β-glucan, MOS, and D-mannose, effects on the adhesion of *Salmonella* and *Campylobacter* were also researched. All three of the major constituent carbohydrates were observed to significantly inhibit adhesion of *Salmonella* to the epithelial cells as compared with untreated cells, with reduction by β-glucan and MOS being significantly greater than by D-mannose. Each component of prebiotic RFC also significantly inhibited adhesion of *Campylobacter*, and reduction by β-glucan was significantly greater than reduction by MOS.

In this research, the ability of common prebiotics, fructooligosaccharides (FOS), galactooligosaccharides (GOS), raffinose, or the MOS-based poultry RFC, to reduce the adherence of *Salmonella* and *Campylobacter* was compared. Each prebiotic significantly inhibited the adhesion of both bacteria to the epithelial cells as compared with untreated

cells. Reduction of adherent *Salmonella* was significantly greeter by FOS and raffinose in comparison to GOS and RFC. *Campylobacter* adherence was reduced significantly greater by RFC and raffinose than by FOS and GOS.

With the limited of tools available for poultry producers to manage diseases and human foodborne pathogens, there is a true need for the development of antibiotic alternatives to ensure the poultry industry remains profitable and a source of safe animal protein. This research demonstrated the effects of probiotics and prebiotics on broiler growth performance and gastrointestinal microbiota populations. Promising results were shown to assist in the advancement of understanding probiotic and prebiotic microbiology in poultry.

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