APPLICATION OF FUNCTIONAL ADDITIVES IN POULTRY PRODUCTION

A Dissertation

by

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ABSTRACT

Functional additives are used in poultry production to deliver benefits beyond the nutritive value of the additives. Although benefits of functional additive administration have been previously demonstrated, the mechanisms responsible for their beneficial action is not well characterized. We have explored mechanisms responsible for probiotic persistence in the gastrointestinal tract, effects of mineral source and administration on the gastrointestinal microbiota, intermittent administration of Lactic Acid Bacteria, and co-administration of functional feed additives. The results of these studies will provide insight on the mechanisms responsible for improved performance due to functional additives, and the administration methods needed to optimize benefits.

Lactobacillus species are common inhabitants of the gastrointestinal tract with a record of safe use as probiotics in animal health. While they are widely used, the mechanisms necessary for their persistence in the gastrointestinal environment are not well characterized. We characterized bile salt hydrolase (BSH) activity in poultry *Lactobacillus* and evaluated overexpression of BSH on survivability and growth of *L. gallinarum*. Overexpression increased deconjugation of tauro- conjugated bile acids in addition to increasing survivability in bile rich environments. Our results suggest poultry *lactobacillus* isolates possess a variety of BSH enzymes to combat the bile rich environment in the duodenum, and BSH activity may be beneficial for the persistence of probiotic bacteria in the gastrointestinal tract.

The gastrointestinal microbiota is an important modulator for animal health. Dietary inclusion of micronutrients is necessary for animal health and performance. Organic copper sources, such as $Cu(HMTBa)_2$, may provide additional functionality beyond dietary copper and

modulate the gastrointestinal microbiota. In this study, we investigated the administration of different copper sources, inclusion rates, and formulation methods on the gastrointestinal microbiota. We have demonstrated that dietary copper significantly effects populations of LAB and *C. perfringens* in the GI tract, and our data suggests a potentially mutualistic relationship between *Clostridium* and LAB. The inclusion rate and application of functional additives determines how they affect the gastrointestinal microbiota and may play a role in the scope of their non-nutritive benefit.

Alterations in nutrient and ingredient levels during feed changes is a cause of gastrointestinal stress during broiler production. Intermittent administration of probiotics may mitigate the perturbations caused during feed changes. In this study, we investigated the effects of intermittent administration of probiotic LAB during diet changes on growth performance, GI microbiota, and histomorphometry in broiler chickens. We have demonstrated the intermittent administration of LAB in drinking water improved growth performance, mitigated the decrease in LAB over feed changes, and mitigated the decrease in VH:CD over the feed change period. Our findings suggest targeted administration of functional feed additives during periods of stress may maximize their ability to improve animal health and performance.

Multiple feed additives are often administered to broilers in order to defend against a variety of stresses associated with commercial production. While their individual benefits have been characterized, the effect of co-administration is not well understood. Co-administration of *Bacillus licheniformis* with additional functional feed additive blends was investigated. Administration of *B. licheniformis* improves growth performance and additional functional feed additive blends improve gut health. However, there was insufficient data to suggest a synergistic rather than an additive effect of co-administration on broiler health and production.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This research was conducted under the supervision of a dissertation committee consisting of Dr. Tri Duong and Dr. Jason Lee of the Department of Poultry Science, Dr. Tryon Wickersham of the Department of Animal Science, and Dr. Robert Alaniz of the Department of Microbial Pathogenesis and Immunology.

Bile salt deconjugation data used in chapter 2 was conducted by Dr. Cory Klemashevich of the Texas A&M Integrated Metabolomics Analysis Core. Broilers studied in chapter 3 were raised by Ariel Bergeron with the assistance of the McElroy lab. Assistance in feed manufacturing and performance data collection for broilers studied in chapters 4 and 5 was provided by the Lee lab. Microbial data in chapter 2, 3, 4 and 5 was collected with the assistance of the Duong lab.

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1. INTRODUCTION: MECHANISMS OF *LACTOBACILLUS* PERSISTANCE AND COLONIZATION IN THE GASTROINTESTINAL TRACT OF POULTRY

1.1 INTRODUCTION

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [1]. The benefits of probiotic administration in humans and animals include inhibition of pathogens [2, 3], improved digestive function [4, 5], and modulation of immune responses [6-8]. Antibiotics have been widely used in the production of poultry and other livestock for the treatment and prevention of disease, and to improve growth performance. Concern over antibiotic resistant pathogens, and pressure from both consumers and regulatory agencies, has led to a reduction in the use of antibiotic growth promoters in poultry production [9]. Because of the reported benefits of their administration, probiotics have received significant interest as potential alternatives to the use of antibiotics in poultry production [10, 11].

Lactobacillus species are common inhabitants of the gastrointestinal tract with a record of safe use as probiotic microorganisms in humans and animals [12]. Administration of probiotic *Lactobacillus* to poultry has been demonstrated to improve digestive health [13, 14], enhance growth performance [15-17], and stimulate immune responses [18-20]. In addition to improving animal health and economic performance, *Lactobacillus* administration has also been demonstrated to improve microbial food safety of poultry and poultry products through reduced gastrointestinal colonization of human food borne pathogens including *Campylobacter* [21, 22], *Clostridium* [23], and *Salmonella* [24, 25]. Although *Lactobacillus* and other probiotics are used widely in animal agriculture, the mechanisms important to their beneficial functionality are not well understood.

Colonization and persistence in the gastrointestinal tract is thought to be critical for the realization of probiotic benefits [26, 27]. The ability of *Lactobacillus* species to persist in the gastrointestinal tract of poultry has been demonstrated [28, 29]. However, microbial factors important to gastrointestinal colonization of *Lactobacillus* in poultry are not well characterized. The ability of probiotic lactobacilli to colonize the gastrointestinal tract is strain dependent and multifactorial. However, the ability to adhere to mucosal surfaces and tolerate the harsh conditions in the gastrointestinal environment including acid and bile are likely contributing significantly to persistence and colonization in the gastrointestinal tract of poultry [30]. Understanding the mechanistic basis for these important traits will contribute to improved selection of probiotic bacteria for use in direct fed microbial (DFM) products and their improved application in poultry production.

1.2 ADHESION TO MUCOSAL SURFACES

Adhesion to mucosal surfaces is prerequisite for colonization of bacteria in the gastrointestinal tract [31]. Adhesion to the mucosal substrata allows bacterial cells to resist the peristaltic movements of the gastrointestinal tract [32] permitting their persistence in the environment. Additionally, since they occupy similar binding sites as pathogenic microbes, mucosal adhesion is likely to also contribute to the ability of beneficial microbes to competitively exclude pathogens from the gastrointestinal tract [33]. Because of its importance to the functionality of probiotics, mucosal adhesion has been used widely as a criterion for the selection of probiotic cultures [34, 35]. Although their adherence to epithelial tissues of poultry has been reported widely [36, 37], the bacterial factors which contribute to the adhesion of *Lactobacillus* to poultry mucosal surfaces in poultry are not well understood. Because of the increased interest in the role of probiotic microorganisms, the growing body of research of *Lactobacillus* in the human gastrointestinal tract can provide insight into the functionality of these organisms in poultry and other livestock species.

1.2.1 Adhesion Proteins

Adhesion of *Lactobacillus* to the mucosa is mediated by the binding of bacterial surface proteins, called adhesins, to mucus, epithelial tissues, and extracellular matrix. Analysis of bacterial genome sequences has allowed for the identification and characterization of putative adhesins including mucus binding proteins, fibronectin binding proteins, and others. Although these studies have contributed important insights, the role of these proteins in mucosal adhesion and colonization of poultry has not been widely investigated. Until recently few genomes of *Lactobacillus* strains isolated from poultry have been sequenced. However, the availability of the genome sequences for *Lactobacillus crispatus* ST1 [38] and *Lactobacillus gallinarum* JCM2011,

have allowed the identification of putative adhesins to serve as targets for functional studies (**Figure** 1).

1.2.2 Mucus Binding Proteins

Mucus, composed largely of mucin secreted by goblet cells, forms a protective layer covering the epithelial cells in the intestine [39]. In *Lactobacillus*, mucus binding proteins (Mub) are surface-associated proteins containing a mucus binding protein domain (MucBP domain) responsible for mediating adhesion to mucus [40]. In addition to MucBP domains, *Lactobacillus* Mub proteins have features typical of Gram-positive surface-associated proteins: N terminal signal peptides which target the protein for secretion and a C terminal LPxTG motif for sortase-dependent cell wall anchoring [41]. Comparative genomic analyses of Lactic Acid Bacteria (LAB) identified 48 genes which contained at least one MucBP domain [42]. The distribution of these MucBP domain containing proteins varied across species with 13 MucBP domain proteins encoded in the genome of *Lactobacillus gasseri* ATCC 33323 and 1 in the *Lactococcus lactis* SK11 genome. The increased number of Mub proteins present in intestinal LAB as compared to fermentative LAB supports the importance of Mub proteins to the gastrointestinal lifestyle of these organisms.

The potential importance of *Lactobacillus* mucus binding proteins to mucosal adhesion has been characterized previously. Chimeric fusion proteins containing MucBP domains from *Lactobacillus reuteri* 1063 adhered porcine gastric mucus and chicken intestinal mucus in vitro [40]. Inactivation of Mub in *Lactobacillus acidophilus* NCFM reduced adhesion to Caco-2 cells by 65% [43]. Lactobacilli have also been demonstrated to modify expression of mucus binding

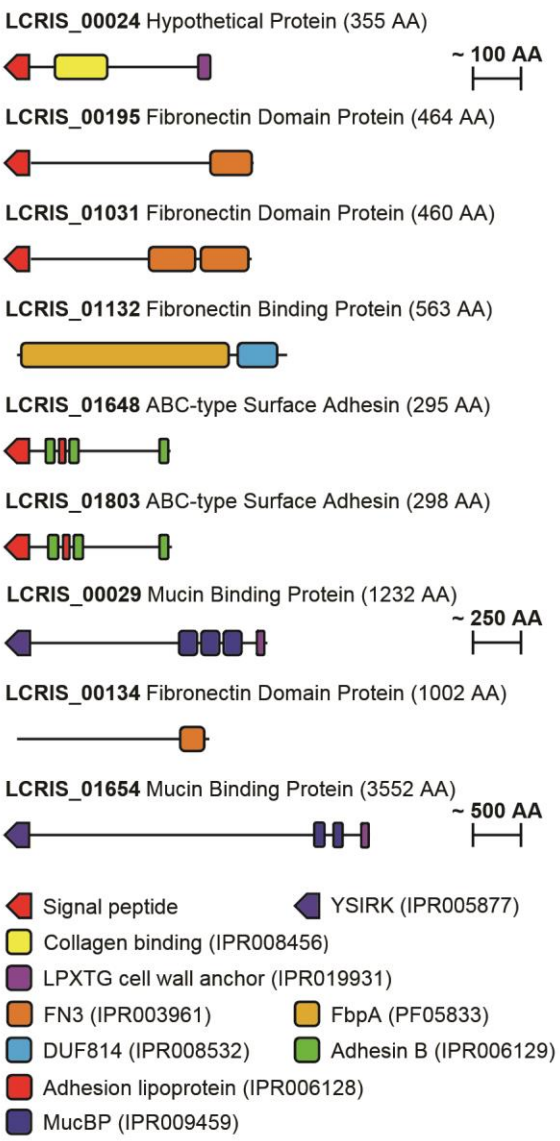

Figure 1.1 Select features of putative adhesion proteins of *L. crispatus* ST1. The Locus ID, predicted function, and predicted domain architecture of putative *L. crispatus* ST1adhesins is shown

proteins in conditions simulating the gastrointestinal environment [44]. Gene expression analysis in *Lactobacillus plantarum* 423 revealed expression of the *mub* and *mapA* (mucus adhesion-promoting protein) genes were upregulated in the presence of 0.01% and 0.05% mucin. Additionally, exposure to bile and pancreatin at pH 6.5 upregulated *mapA* expression and downregulated *mub* expression. This suggests differential gene expression under specific conditions may possibly be used to pre-adapt probiotic cultures to improve their colonization in the gastrointestinal tract.

The pilus gene cluster (*spaCBA*) of *Lactobacillus rhamnosus* GG has been demonstrated to mediate mucin binding [45]. The pilin protein SpaC was demonstrated to adhere to human intestinal mucus. Binding of radio-lableled SpaC to mucus was inhibited by non-radiolabed SpaC in a dose-dependent manner and adhesion of *L. rhamnosus* GG to mucus was significantly reduced by pre-treatment with SpaC antiserum. Finally, deletion of the *spaC* gene also reduced adhesion of the culture to mucus.

1.2.3 Fibronectin-binding Proteins

Fibronectin is an extracellular matrix glycoprotein present in the mucosa, connective tissue matrix, and basement membrane [46]. The contribution of fibronectin binding proteins in adhesion and infection of host cells by pathogens including *Listeria* and *Streptococci* has been well defined [47, 48]. Thus, fibronectin binding proteins, including those containing fibronectin binding protein A (FbpA) and fibronectin-like (FN3) domains, are expected to also be important to the adhesion proteins in *Lactobacillus* and other probiotic microorganisms [43, 48, 49].

Lactobacillus fibronectin binding protein A (FbpA) domain-containing proteins contribute to adhesion by anchoring the bacterial cell to fibronectin in the gastrointestinal tract. Similar to mucus binding proteins, FbpA domain containing proteins consist of an N-terminal

secrection signal, one or more FbpA domains, and a C-terminal LPxTG cell wall anchor motif [50]. Inactivation of FbpA in *L. acidophilus* NCFM reduced adhesion to the Caco-2 cell line by 76 % [43]. Binding of His-tagged *Lactobacillus casei* BL23 FbpA to immobilized fibronectin was demonstrated to be dose dependent and saturable [51]. Although inactivation of FbpA reduced cell-surface hydrophobicity and fibronectin binding of *L. casei* BL23, adhesion to the HT-29 cell line was not affected [51].

1.2.4 Surface Layer Proteins

The bacterial surface layer (S-layer) is a crystalline array of protein or glycoprotein which forms the outermost layer of the cell of some bacteria [52]. The S-layer performs a variety of functions including acting as a protective barrier and contributing to cellular structure [53, 54]. The S-layer is possessed by many *Lactobacillus* species [55] and has been demonstrated to contribute to their adhesion to epithelial cells. Removal of the S-layer using lithium chloride significantly reduced adhesion of *Lactobacillus crispatus* K313 to the HT-29 cell line [56].

S-layer proteins and their contributions to epithelial adhesion have been characterized for many *Lactobacillus* species [55]. Insertional inactivation of *slpA* (surface layer protein A) reduced adhesion of *L. acidophilus* NCFM to the Caco-2 cell line as compared to a *lacL*$^{-}$ (β-galactosidase) control [43]. Additionally, the SlpA deficient mutant had reduced adherence to a dendritic cell ligand suggesting S-layer proteins mediate adhesion to and interaction with immune cells as well [57]. Purified *L. crispatus* K313 SlpB [58] and *L. crispatus* JCM 5810 CbsA [59] have been demonstrated to bind immobilized sub-intestinal extracellular matrix collagens, while translational-fusion with *Lactobacillus brevis* ATCC 8287 SlpA increased adhesion of chimeric *E. coli* FliC to fibronectin.

Lactobacillus S-layer proteins have also been demonstrated to inhibit adhesion of pathogens to epithelial cells *in vitro* suggesting they may also contribute to competitive exclusion. S-layer proteins isolated from *Lactobacillus crispatus* ZJ001 reduced adhesion of *Salmonella* Typhimurium SL1344 and *Escherichia coli* 0157:H7 to HeLa cells by 44.4% and 73.0% , respectively, as compared to assays performed in the absence of the S-layer proteins [60]. Additionally, S-layer preparations from *Lactobacillus kefir* cultures were demonstrated to inhibit binding and invasion of *Salmonella* to Caco-2 cells. Inhibition of *Salmonella* and *E. coli* adhesion to epithelial cells suggests that *Lactobacillus* S-layer proteins occupy binding sites similar to those used by these two pathogens.

1.2.5 Moonlighting Proteins

Several enzymes present on the cell surface of *Lactobacillus* species have been demonstrated to adhere to extracellular matrix proteins suggesting they may function as moonlighting proteins contributing to the adhesion of *Lactobacillus* to gastrointestinal mucosal tissues. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), important in glycolysis, has been suggested to also have a non-metabolic function [61]. Quantitative surface plasmon resonance (SPR) analysis of cell surface proteins from *Lactobacillus plantarum* LA318 revealed a 40 kDa protein responsible for adhesion to human colonic mucus which was identified to be GAPDH by amino acid sequence. Agglutination assays using anti-GAPDH antibodies demonstrated the presence of this typically cytosolic protein on the cell surface.

Inactivation of *Lactobacillus plantarum* LM3 *enoA1,* encoding an α-enolase normally present on the cell surface, reduced binding to fibronectin coated microtiter plates wells [62]. His-tagged glutamate synthetase (GS) and gluco-6-phosphate isomerase (GPI) from *L.crispatus* ST1 were demonstrated to bind immobilized fibronectin, collagen I, and laminin using SPR [63].

These moonlighting adhesins were also demonstrated to mediate adhesion of *L. crispatus* ST1 to basement membrane matrix proteins in vitro. *L. crispatus* ST1 bound Matrigel matrix at pH 4 but was unbound at pH 8. Under acidic conditions, GS and GPI are bound to the cell wall. However, these moonlighting proteins are released from the bacterial surface under basic conditions rendering them unable to mediate adhesion [63]. Mucins care composed of many sugar moieties which are likely to be recognized as binding sites by these carbohydrate metabolizing enzymes. Elongation factor Tu (EF-Tu), normally important in protein synthesis, is a additional surface protein demonstrated to mediate *Lactobacillus johnsonii* La1 adhesion in a pH dependent manner [64]. At pH 5, EF-Tu is able to bind to mucin, HT29 cells and Caco-2 cells but is no longer adhesive at pH of 7.2 in a manner consistent with other mucin binding proteins [40].

1.3 POULTRY MODELS OF *LACTOBACILLUS* ADHESION AND COLONIZATION

Bacterial cell aggregation, cell wall hydrophobicity, and adherence to extracellular components including intestinal mucus, fibronectin, and basement membrane matrix have been used as *in vitro* measures of the adherence of *Lactobacillus* strains to poultry epithelia [28, 30, 37, 65-67]. Additionally, dissected tissue sections [33, 37], primary chicken intestinal epithelial cells [36, 68, 69], and cultured mammalian epithelial cell lines including the HEp-2 [65] and Caco-2 [70] human epithelial cell lines have been used to evaluate the potential of *Lactobacillus* cultures for use in poultry.

Cultured epithelial cell lines are the most widely applied adhesion models, [71]. However, only recently has a poultry-derived epithelial cell line been used to evaluate the adhesion of *Lactobacillus* cultures to poultry epithelium. Spivey, Dunn-Horrocks [29] evaluated the ability of *L. crispatus* and *L. gallinarum* cultures to the chicken LMH epithelial cell line.

Although, *L. gallinarum* JCM 8782 adhered to the LMH cells at levels similar to *L. crispatus* ST1 and *L. gallinarum* ATCC 33199 *in vitro*, the culture's poor intestinal colonization *in vivo* was similar to the weakly adherent *L. crispatus* JCM 5810 (Figure 2) suggesting factors additional adhesion are also important in gastrointestinal colonization.

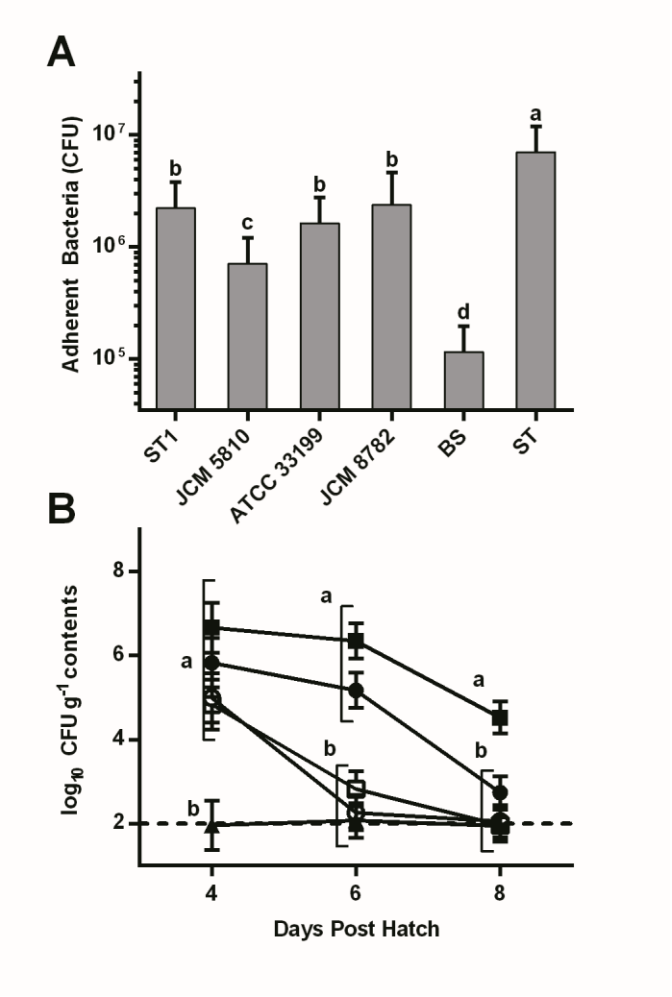

Figure 1.2 ***Lactobacillus*** **adhesion and gastrointestinal colonization**. (A) Adhesion of Rif *L. crispatus* (ST1 and JCM 5810) and *L. gallinarum* (ATCC 33199 and JCM 8782) cultures to chicken LMH cells. *B. subtilis* ATCC 23857 (BS) and *Salmonella Typhimurium* (ST) were used as low and high adherent controls, respectively. The geometric mean + 95% Cl adherent bacteria (bars) and percent adherent bacteria ± SEM (■) of inoculum. (B) Recovery of Rifr *Lactobacillus* from the ileum of broiler chicks administered Rif *L. crispatus* STl ('), *L. crispatus* JCM 5810 (O), *L. gallinarum* ATCC 33199 (■), *L. gallinarum* JCM 8782 (□), or a mock inoculation (□). Different letters indicate means are significantly different ($P < 0.05$). Adapted from Spivey et al. [29].

1.4 SURVIAL IN THE GASTROINTESTINAL ENVIRONMENT

While adhesion to the mucosal tissues of the digestive tract is critical, other factors also likely contribute to gastrointestinal colonization of probiotic *Lactobacillus* species. Once adherent to the mucosal substrata, bacterial persistence is dictated by the ability to survive the adverse environmental conditions in the gastrointestinal tract [72]. Thus, tolerance to gastrointestinal conditions is often used a criterion to the selection of probiotic cultures [73]. Residence and passage through the stomach exposes bacterial to gastric acids, while entrance to the intestine will expose a bacterium to bile.

1.4.1 Acid Resistance

The pH of the digestive tract of poultry varies from approximately 4.5 in the proventriculus, to 3 in the gizzard, then 6.4 in the intestine [74]. When extracellular pH is less than their $pK_{a,}$ protonated acids diffuse through the bacterial membrane and become dissociated in the cytoplasm [75]. This can decrease intracellular pH resulting in reduced enzyme activity, DNA damage, and altered transmembrane ΔpH [76, 77]. Additionally, acid stress can increase susceptibility to other environmental stressors including bile [78]. Because of their importance as starter cultures in lactic acid fermentations, the physiological response of *Lactobacillus* to acid stress is an active area of investigation [79]. Several mechanisms have been characterized and have been found to include both acid-specific and general stress responses (Table 1.1).

1.4.2 pH gradient

Lactobacillus and other LAB maintain a proton gradient across the cell membrane such that external pH variations do not drastically affect cytoplasmic pH [80].

Table 1.1. Acid factors of *Lactobacillus*

Organism/Locus	Function	Mutant/Phenotype	Reference
L. acidophilus NCFM			
atpBEFHAGDC	F_0F_1-ATPase	acid stress induced	[81]
LBA0057	Glutamate antiporter	acid sensitive mutant	[82]
LBA0867	Transcriptional regulator	acid sensitive mutant	
L. brevis ATCC 367			
Lvis-0199	Na^+/H^+ antiporter	acid stress induced	[83]
Lvis-0378	FabG, Membrane synthesis	acid stress induced	
L. plantarum ZDY2013			
Pyk	Pyruvate kinase	acid stress induced	[84]
L. reuteri ATCC 55730			
lr1516	Cell envelope esterase	acid sensitive mutant	[85]
lr1864	ClpL w/ chaperone activity	acid sensitive mutant	
L. rhamnosus GG			
atpBEFHSGDC	F_0F_1-ATPase	acid stress induced	[86]

The *atp* operon encoding for F_0F_1-ATP*ase*s activity in *L. acidophilus* NCFM was determined to be induced when pH of a culture was reduced from pH 5.6 to pH 3.5 [81]. The membrane bound F_0F_1-ATPase consists of a catalytic component (F_1) and an integral membrane channel (F_0) [87] and functions as a proton pump to maintain membrane ΔpH or for ATP synthesis using the proton motive force [76]. Under acid stress, its primary function is proton export for the maintenance of intracellular pH [81]. *Lactococcus lactis* subsp. *cremoris* MG1363 was unable to be cultured in the absence of nisin when the F_0F_1-ATPase of was placed under control of the nisin inducible *nis*A promoter [88]. This demonstrates that the F_0F_1-ATPase is necessary for maintenance of cytoplasmic pH and essential for growth.

The active transport of K^+ can also affect ΔpH through ion exchange. *Streptococcus lactis* ATCC7692 was demonstrated to accumulate of K^+ in exchange for H^+ ions at pH 5 allowing the organism to maintain a more basic intracellular pH [89]. Although K^+ lowered the electrical gradient of the cell as demonstrated by the fluorescence of membrane potential sensitive cyanine dye, the ΔpH was increased signaling an increased intracellular pH.

Amino acid decarboxylation

Although this response may be inducible only under extreme conditions, the activity of amino acid decarboxylases may also contribute to the maintenance of intracellular pH [90]. Many amino acid decarboxylation reactions can consume intracellular protons and result in an increase in intracellular pH [91]. These decarboxylases are often paired with antiporters in order to generate ATP through the proton motive force [92, 93].

Decarboxylation of glutamate to γ-aminobutyric acid (GABA) and glutamate-GABA antiport has been demonstrated to contribute to acid resistance of *Lactobacillus reuteri* 100-23 [94]. Addition of glutamate increased survival of *L. reuteri* 100-23 at pH 2.5 by 2 log cfu.

However, the protective effect of glutamate was not observed for an isogenic *gadB* (Glutamate decarboxylase B) mutant. Additionally, GABA production by the *gadB* mutant was also not observed. Several Amino acid-dependent acid resistance systems have been characterized in other intestinal organisms including the lysine decarboxylase/ lysine-cadervine antiporter systems in *Bacillus cereus* ATCC 14579 [95], *E. coli* EP314 [96], and *Salmonella* Typhimurium DT104 [97] and the arginine deiminase/arginine-ornithine antiporter system in *Lactobacillus plantarum* Lp60 [98]. Expression of the amino acid decarboxylase and antiporter genes of these amino acid-dependent acid resistance systems has been demonstrated to be inducible under pH stress as well.

1.4.3 Bile Tolerance

Bile is secreted into the duodenum and aids in the digestion of fats through the detergent action of its constituent bile acids [99]. Primary bile acids are synthesized in the liver and are composed of steroid moiety, cholic acid or chenodeoxycholic acid, and an amino acid conjugate, either glycine or taurine, linked by an amide bond [100]. Once secreted in the gastrointestinal tract, they may become converted to secondary bile acids through microbial activities including deconjugation of the amino acid [101]. The detergent properties of bile acids also confer antimicrobial activity, primarily through emulsification of bacterial membranes [102]. Bile acids may cause damage to DNA, RNA, and proteins [103]. Potentially important mechanisms of bile detoxification and physiological responses to bile stress have been characterized for several *Lactobacillus* strains **(Table 2)**. However, their role in bile tolerance in vivo and its contribution to gastrointestinal persistence is unclear.

Recently, resistance to bile has been implicated as a factor likely to contribute to the persistence of *Lactobacillus* in the digestive tract of broiler chicks [29]. Although, their adhesion

to chicken LMH epithelial cells was similar, *L. gallinarum* JCM 8782 was a weaker colonizer than *L. gallinarum* ATCC 33199. The maximum growth rate of *L. gallinarum* ATCC 33199 in media supplemented with 0.10% and 0.25% oxgal was greater than that of *L. gallinarum* JCM 8782, indicating the greater bile tolerance of *L. gallinarum* ATCC 33199. These results suggest that difference in gastrointestinal colonization between the two strains may have been due in part to the differences in bile tolerance.

1.4.4 Bile salt hydrolases of Lactobacillus species

Bile salt hydrolases (BSHs) are classified as members of the choloylglycine hydrolase family which catalyze the hydrolysis of the amide bond linking primary bile acids to their conjugated amino acid[100, 104]. BSHs are have been identified in many intestinal bacteria including *Bacteroides fragilis* NCTC9343[105], *Bifidobacterium bifidum* ATCC11863 [106], *Clostridium perfringens* 13 [107], and *L. johnsonii* 100-100 [108]. Their wide distribution among intestinal bacteria suggests their importance in the gastrointestinal lifestyle of these organisms. Bile salt hydrolases have been characterized in several *Lactobacillus* species. However, their contribution to survival in the gastrointestinal tract remains unclear [109]

Table 1.2. *Lactobacillus* bile tolerance factors

Species/Locus	Function/Activity	Reference
Bile Salt Hydrolases		
L. acidophilus NCFM		
bshA	Hydrolysis of chenodeoxycolic bile acids	[110]
bshB	Hydrolysis of taurine conjugated bile acids	
L. plantarum WCFS1		
bsh1	Hydrolysis of bile acids	[111]
L. salivarius NRRL B-30514		
bsh1	Hydrolysis of bile acids	[112]
Transport Proteins		
L. acidophilus NCFM		
LBA0552	Bile salt efflux/MDR transporter	[113]
LBA1429	Bile salt efflux	
LBA1446	Bile salt efflux	
LBA1679	Bile salt efflux	
L. johnsonii 100-100		
cbsT2	TCA/CA antiporter	[114]
L. plantarum WCFS1		
lp_0085	Bile efflux	[115]
L. reuteri ATCC 55730		
lr1265	Bile efflux/MDR transporter	[116]
Bile Stress Responsive Genes		
L. acidophilus NCFM		
LBA1425	Hypothetical protein	[117]
LBA1426	Unknown protein	
LBA1427	Oxidoreductase	
LBA1428	Hypothetical protein	
LBA1429	Multidrug efflux transport protein	
LBA1430	2 CRS Histidine Protein Kinase	
LBA1431	2 CRS Response Regulator	
LBA1432	Hypothetical protein	
L. reuteri ATCC 55730		
lr0004	Clp Chaperone	[116]
lr1516	Putative esterase	
L. rhamnosus GG		
dltD	Synthesis of D-alanyl esters in LTA	[118]
LGG00777	D-alanyl carrier protein ligase	[119]
LGG00779	D-alanyl carrier protein	

BSH activity of *bshA* (bile salt hydrolase A) and *bshB* knock-out mutant strains of *L. acidophilus* NCFM was significantly reduced as compared to a to wild-type control [110]. Additionally, *bshA* mutants were unable to hydrolyze conjugated chenodeoxycholic acid bile salts revealing the specificity of this enzyme is centered on the primary bile acid, while *bshB* mutants were unable to deconjugate taurine-conjugated bile salts revealing the amino acid conjugate specificity of this enzyme. Inactivation of either enzyme did not affect survival of the culture in oxgal. However, a mutant deficient for both BSH activities would be required to determine their effect on bile resistance.

Analysis of the *L. plantarum* WCFS1 genome predicted the presence of four *bsh* genes [120]. Deletion of *bsh1* suggested it to encode the majority of BSH activity of *L. plantarum* WCFS1 [121] and decreased the threshold tolerance to glycoldeoxycholic acid from 0.7% to 0.1% [111]. Heterologous expression all four putative *L. plantarum* WCFS1 BSHs revealed that while hydrolysis of conjugated bile salts was the primary enzymatic activity of BSH1, the remaining BSHs had significant penicillin acylase suggesting that they may be involved in conversion of other substrates present in the gastrointestinal environment.

A BSH gene (*bsh1*) encoded on the megaplasmid of *Lactobacillus salivarius* UCC 118 was found to be widely conserved on the megaplasmids of 26 *L. salivarius* strains [122], while a second (*bsh2*) was found in only two of the strains. Of the strains characterized, only the two strains which encoded both *bsh1* and *bsh2* were found to have strong BSH activity, suggesting their activity was due to the presence of *bsh2*. Although the BSH1 was determined to have weak activity, inactivation of this protein reduced the bile resistance of *L. salivarius* UCC 118. Additionally, the *bsh2* encoding isolates were found to have greater resistance towards glycoconjugated than to tauroconjugated bile salts, suggesting a substrate preference of *bsh2*

towards glycoconjugates. A BSH from *Lactobacillus salivarius* NRRL 30514 was also demonstrated to have a substrate preference towards glycoconjugates.

Bile detoxification by BSH

Bile salt inhibition of *Lactobacillus* is thought to be pH dependent, with the protonated bile salts entering the cell, dissociating, and causing a reduction in intracellular pH. Glycoconjugates have been demonstrated to have greater toxicity than tauroconjugates [123]. This is likely attributed to the difference in pK_a with glycodeoxycholic acid (GDCA) (pK_a 3.9) more easily entering the cell in a protonated form than taurodeoxycholic acid (TDCA) (pK_a 1.0). This has been suggested to account for the relative abundance of glycoconjugate specific BSHs over tauroconjugate specific BSHs observed in *Lactobacillus* species [124]. Deconjugation of bile salts has been proposed as detoxification mechanism with BSH enzymes potentially playing an important role in survival of *Lactobacillus* in the gastrointestinal tract. Hydrolysis of primary bile salts produces weaker unconjugated bile acids which are able to reassociate with the proton and be exported from the cell (**Figure 2**). Although bile salt hydrolysis may result in the removal of free protons in the cell, the resulting unconjugated bile acids from bile salt hydrolysis have greater in vitro antimicrobial activity than conjugated bile acids [124]. However, they may be removed by localized precipitation due to lactic acid produced by *Lactobacillus* and other LAB in the gastrointestinal tract. Additionally, dihydroxylation by 7-α-dehydroxylase producing bacteria including *Clostridium* and other species [125] may also cause precipitation.

Bile Stress Responses

In addition to deconjugation of bile acids by BSHs, additional bacterial factors include the induction of general stress proteins [126], transmembrane transporters [117], and cation efflux [127]which contribute to bile tolerance have been characterized.

Gene expression analysis in *L. acidophilus* NCFM using DNA microarrays identified a bile-induced operon containing a two component regulatory system (2CRS) [117]. Inactivation of the histidine protein kinase (HPK), response regulator (RR), and a transporter encoded in the operon resulted in decreased viability under bile stress. These genes demonstrated that bile stress resulted in decreased recovery of viable cells with the exception of HPK in TDCA. It is likely that HPK and the response regulator exert transcriptional control on proteins involved with bile tolerance. Additionally, transcription of genes associated with active cell growth were downregulated in the presence of bile while putative adhesion genes were upregulated [117], suggesting that bile may signal arrival of the bacterium in the intestine.

Gene expression analysis of *L. plantarum* WCFS1 identified 62 genes which were differentially expressed after exposure to 0.1% porcine bile [128]. In addition to BSH genes, expression of the stress proteins glutamate decarboxylase (lp 3420) and glutathione reductase (lp1253) and stress related sigma factors was induced. Teichoic acid synthesis genes (lp2019 and lp2020) were also induced, while several cell-membrane transporters were repressed, suggesting increased maintenance of the cell surface structure and reduced production of non-essential membrane proteins is used to compenstate for membrane damage caused by the detergent properties of bile salts [124].

1.5 FUTURE PERSPECTIVES

Colonization and persistence of *Lactobacillus* and other microorganisms in the gastrointestinal tract is critical to the realization of their probiotic benefits. Because of the increasing use of probiotic cultures as therapeutics and for health promotion in humans, there is a growing body of work investigating mechanisms important to the colonization of *Lactobacillus* cultures in the gastrointestinal tract. The identification of bacterial factors of *Lactobacillus* important in adhesion host tissues and survival in the host gastrointestinal environment in humans has contributed valuable insight. However, little research investigating colonization of probiotics has been performed in poultry. Although probiotics are widely administered in poultry production, their overall effectiveness is mixed. Probiotic strains have traditionally been selected from the native gastrointestinal microbiota with an understanding of the desired benefits but with little understanding of the mechanisms important for achieving that benefit *in vivo*. A mechanistic understanding of probiotic functionality will provide the foundation based on structure-function relationships for a rational approach to the selection of novel probiotic cultures. The elucidation of mechanisms important to persistence and colonization of probiotic *Lactobacillus* cultures in poultry provide improved criteria for the selection of probiotic cultures and contribute to their improved application in poultry production.

2. CHARACTERIZATION OF BILE TOLERANCE AND PUTATIVE BILE SALT HYDROLASES IN *LACTOBAILLUS*

2.1 INTRODUCTION

Bile is secreted into the duodenum and aids in the digestion of fats through the detergent action of its constituent bile salts [99]. Primary bile salts are synthesized in the liver and are composed of steroid moiety, cholic acid or chenodeoxycholic acid, and an amino acid conjugate, either glycine or taurine, linked by an amide bond [100]. Once secreted in the gastrointestinal tract, they may become converted to secondary bile acids through microbial activities including deconjugation of the amino acid [101]. The detergent properties of bile salts also confer antimicrobial activity, primarily through emulsification of bacterial membranes [102]. Bile salts may cause damage to DNA, RNA, and proteins [103]. Potentially important mechanisms of bile detoxification and physiological responses to bile stress have been characterized for several *Lactobacillus* strains**.** However, their role in bile tolerance in vivo and its contribution to gastrointestinal persistence is unclear.

Bile salt hydrolase activity has previously been reported in isolates from the small intestine of chickens [129]. Recently, resistance to bile has been implicated as a factor likely to contribute to the persistence of *Lactobacillus* in the digestive tract of broiler chicks [29]. Although, their adhesion to chicken LMH epithelial cells was similar, *L. gallinarum* JCM 8782 was a weaker colonizer than *L. gallinarum* ATCC 33199. The maximum growth rate of *L. gallinarum* ATCC 33199 in media supplemented with 0.10% and 0.25% oxgal was greater than that of *L. gallinarum* JCM 8782, indicating the greater bile tolerance of *L. gallinarum* ATCC 33199. These results suggest that difference in gastrointestinal colonization between the two strains may have been due in part to the differences in bile tolerance.

Bile salt hydrolases (BSHs) are classified as members of the choloylglycine hydrolase family which catalyze the hydrolysis of the amide bond linking primary bile acids to their conjugated amino acid [100, 104]. BSHs are have been identified in many intestinal bacteria including *Bacteroides fragilis* NCTC 9343 [105], *Bifidobacterium bifidum* ATCC 11863 [106], *Clostridium perfringens* 13 [107], and *L. johnsonii* 100-100 [108]. Their wide distribution among intestinal bacteria suggests their importance in the gastrointestinal lifestyle of these organisms. Bile salt hydrolases have been characterized in several *Lactobacillus* species. However, their contribution to survival in the gastrointestinal tract remains unclear [109]. In this study, we investigate the ability of poultry *Lactobacillus* isolates to deconjugate bile salts and its effect on bacterial growth.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial Strains, Plasmids, and Growth Conditions

Bacterial Strains and plasmids used in this study are listed in **Table 2.1**. *Lactobacillus crispatus* and *Lactobacillus gallinarum* strains were cultured using de Man, Rogosa, and Sharpe (**MRS**) (BD, Franklin Lakes, NJ) medium, supplemented with 5 µg mL^{-1} erythromycin (**Erm**) (EMD Chemicals, Inc., San Diego, CA) as appropriate, incubated at 37°C in 10% CO_2. *Escherichia coli* strains were cultured using Luria-Bertani (**LB**) broth (**BD**) and Brain Heart Infusion (**BHI**) agar (**BD**), supplemented with 150 µg mL^{-1} Erm or 100 µg mL^{-1} kanamycin (**Kan**) (ThermoFisher Scientific, Waltham, MA) as appropriate, incubated at 37°C aerobically.

2.2.2 Characterization of Bile Salt Hydrolase Activity

Hydrolysis of specific bile salts by *Lactobacillus* strains was evaluated qualitatively using the method of McAuliffe, Cano [130] with minor modification. Single isolated *Lactobacillus* colonies were transferred onto MRS agar plates and incubated for 36 h. Plates were then overlaid with molten MRS agar supplemented with 4 mM glycocholic acid (**GCA**), glycodeoxycholic acid (**GDCA**), taurocholic acid (**TCA**), or taurodeoxycholic acid (**TDCA**) and incubated for an additional 48 h. Hydrolysis of bile salts was indicated by formation of a precipitate around the colony.

2.2.3 Growth Curve Analysis

Bile tolerance of *Lactobacillus* cultures was evaluated using growth curve analysis according to the method of Spivey, Dunn-Horrocks [29]. *Lactobacillus* were cultured in 96-well microtiter plates using MRSbroth supplemented with 1 % (w/v) oxgall (BD) or 4 mM conjugated bile salts as indicated, incubated under microaerobic conditions at 37°C, and the absorbance

Table 2.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant Characteristics	Source
Lactobacillus crispatus		
ST1	Chicken crop isolate, genome sequenced strain	[38]
JCM 5810	Chicken fecal isolate	JCM[1]
Lactobacillus gallinarum		
ATCC 33199^T	Type strain, chicken crop isolate, genome sequenced strain	ATCC[2]
JCM 8782	Chicken fecal isolate	JCM
TDC 062	Em^r, ATCC 33199 with pTRK882	[17]
TDC 063	Em^r; rPhyA$^+$; ATCC 33199 with pTD003	[17]
TDC 102	Em^r; rCbh$^+$; ATCC 33199 with pTD018	This Study
Escherichia coli		
TOP10	Propagation host for pMK-RQ and derivatives	ThermoFischer
MC 1061	Str^r, propagation host for pTRK882 and derivatives	[131]
TDC 101	Erm^r, MC 1061 with pTD018	This Study
Plasmids		
pTRK882	4.5 kb; Em^r, constitutive expression under control of P_{pgm} from *Lactobacillus acidophilus* NCFM	[132]
pTD003	5.6 kb; pTRK882::*phyA*; recombinant overexpression of PhyA (phytatse) from *Bacillus subtilis* 168	This Study
pTD017	3.3 kb; Kan^r, pMK-RQ*::r-cbh*; intermediate plasmid host for cloning of *cbh* from *L. crispatus* ST1	This Study
pTD018	5.5 kb, pTRK882::*cbh;* recombinant overexpression of Cbh (chologycine bile hydrolase) from *L. crispatus* ST1	This Study

[1] Japan Collection of Microorganisms; [2] American Type Culture Collection; [T] Type strain for species

(O.D. $_{600nm}$) was recorded at 15 min intervals over 24 h using a SpectroFluor Plus (Tecan Systems, Inc. San Jose, CA) microtiter plate reader. Wells containing sterile (uninoculated) medium were used for blank correction. Maximum growth rate (μ_{max}) was determined by fitting blank-corrected growth curves to a modified Gompertz equation [133]. The relative μ_{max} was calculated as the quotient of μ_{max} in supplemented MRS and μ_{max} in neat MRS × 100 %.

2.2.4 Recombinant expression of L. crispatus ST1 *cbh*

A 978bp putative chologlycine bile hydrolase (*cbh*) (CDS: CBL50353.1) was identified in the genome of *L. crispatus* ST1 [38]. The *L cristpatus* ST1 *cbh* gene was synthesized

commercially in a pMK-RQ plasmid (Thermo Fisher Scientific, Waltham, MA) with NotI and EcoRI restriction sites added at the 5' and 3' ends, respectively, to facilitate subcloning into pTRK882. The *cbh* was subcloned into pTRK882 for constitutive expression; and the resulting plasmid, pTD 18, was propagated in *E. coli* TDC 101 and transformed into *L. gallinarum* ATCC 33199.

E. coli plasmid DNA was isolated using QIAprep Spin Miniprep kits (Qiagen, Inc., Valencia, CA), whereas *Lactobacillus* DNA was isolated using the method of Walker and Klaenhammer [134] for use in confirmation of transformation. Restriction endonucleases, T4 Ligase, Antarctic phosphatase, and *Taq* DNA polymerase were used according to manufacturer's instructions (New England Biolabs, Ipswich, MA). Primers for PCR confirmation of plasmid insertion were designed using Clone Manager (Scientific and Educational Software, Cary, NC) and synthesized commercially (Integrated DNA Technologies, Coralville, IA).

Electrocompetent *E. coli* TOP10 and EC1000 were prepared and transformed according to manufacturer's directions (ThermoFisher) and standard methods [135], respectively. Electrocompetent *Lactobacillus gallinarum* was prepared using 3.5× Sucrose MgCl Electroporation Buffer and electrotransformed as described by Luchansky, Tennant [136] using a GenePulse Xcell electroporator (Bio-Rad, Hercules, CA).

2.2.5 Characterization of Bile Acid De-conjugation

Overnight cultures of *Lactobacillus* strains were inoculated at 1 % into neat MRS broth or MRS broth supplemented with 2.0 mM GCA, GDCA, TCA, or TDCA. After 24 hr of incubation, cultures were harvested by centrifugation (5000 ×g; 10 min, 4^{o}C). The resulting supernatant was filter sterilized and analyzed using liquid chromatography mass spectrometry (LC-MS). Targeted analysis for de-conjugated bile acids was performed in conjunction with the

Texas A&M Integrated Metabalomics Analysis Core. Results were reported as µmol of de-conjugated bile acids. Bile acid de-conjugation was analyzed using liquid chromatography mass spectrometry (LC-MS).

2.2.6 Survival of L. gallinarum strains

L. gallinarum cultures were grown to mid-log phase ($O.D._{600}$ ~ 0.5) in MRS, harvested by centrifugation (5000 ×g; 10 min, 4^{o}C), washed and re-suspended using PBS, and aliquoted into separate tubes containing 1% (w/v) oxgall or 10 mM TDCA in PBS or neat PBS. *Lactobacillus* suspensions were incubated at 37 ^{o}C in 10% CO_2 for 6 h, and enumerated at 0.25, 2, 4, and 6 h using MRS agar. Relative survival was calculated as the quotient of the viable count of culture suspended in oxgall or TDCA and suspended in neat PBS at each time point × 100%.

2.2.7 Statistical Analysis

Univariate tests were used to verify normality and homoscedasticity of data so that all assumptions of ANOVA were fulfilled. Replicate measurements from independent assays were arranged as blocks when applicable. The General Linear Model was used to determine significant differences, and means with a p-value ≤ 0.05 were separated using Duncan's Multiple Range Test.

2.3 RESULTS

2.3.1 Characterization of Bile Salt Hydrolase Activity

Bile salt hydrolase (BSH) activity of chicken isolates of *Lactobacillus* was evaluated using a qualitative assay in which deconjugation of bile salts was visualized as the formation of a precipitate around a colony (**Table 2.2**). *L. crispatus* ST1 was observed to deconjugate TCA, GCA, TDCA, and GDCA. However, *L. crispatus* JCM 5810 was only observed to deconjugate TCA and GCA. *L. gallinarum* 33199 was not observed to deconjugate any of the four bile salts assayed and was unable to grow on media supplemented with GDCA, whereas JCM 8782 was able to deconjugate GCA only.

Table 2.2. Hydrolysis of bile salts by *Lactobacillus* cultures

	Bile Salts[1]			
Strain	GCA	GDCA	TCA	TDCA
L. crispatus				
ST1	+	+	+	+
JCM 5810	+	-	+	-
L. gallinarum				
ATCC 33199^T	-	*ng*[2]	-	-
JCM 8782	+	-	-	-

[1] GCA, glycocholic acid; GDCA, glycodeoxycholic acid; TCA, taurocholic acid; TDCA, taurodeoxycolic acid;
[2] *ng = no growth*

2.3.2 Growth Curve Analysis

The effect of bile salts on growth of *Lactobacillus* cultures was evaluated relative to growth in neat MRS (**Figure 2.1**). A significant treatment effect was observed on the relative maximum growth rate (μ_{max}) of *Lactobacillus* cultures across all bile salts ($P < 0.01$). Relative growth rate in GCA was greatest for *L. gallinarum* JCM 8782 and *L. crispatus* JCM 5810 (**Figure 2.1 A**), whereas *L. gallinarum* ATCC 33199 and *L. crispatus* ST1 had the lowest relative μ_{max}. In GDCA, *L. gallinarum* JCM 8782 had the greatest relative μ_{max}, whereas growth rate was most heavily depressed in *L. gallinarum* ATCC 33199 and *L. crispatus* JCM 5810 (**Figure 2.1 B**). Relative growth rate in TCA was greatest for *L. crispatus* JCM 5810, followed by *L. gallinarum* ATCC 33199, *L. gallinarum* JCM 8782, and *L. crispatus* ST1 respectively (**Figure 2.1 C**). In TDCA, *L. gallinarum* JCM 8782 had the highest relative μ_{max}, whereas *L. gallinarum* ATCC 33199 and *L. crispatus* ST1 had the lowest (**Figure 2.1 D**).

2.3.3 Characterization of recombinant cbh overexpression in L. gallinarum ATCC 33199

The effect of *cbh*-overexpression on bile-salt hydrolase activity of *L. gallinarum* ATCC 33199 was evaluated. De-conjugation of bile salts by wild-type *L. gallinarum* ATCC 33199, rPhyA over-expressing *L. gallinarum* (TDC 63), and rCbh expressing *L. gallinarum* (TDC 102) was determined using LC-MS (**Figure 2.2**). A significant treatment effect was observed on the deconjugation of TCA ($P = 0.02$). Deconjugation of TCA by *L. galliarum* TDC 102 was greater than that of wild-type *L. gallinarum* ATCC 33199. Although TCA deconjugation by *L. gallinarum* TDC 63 was not significantly different from the other strains. A significant treatment effect was observed on the deconjugation of TDCA ($P < 0.01$). Deconjugation of TDCA by TDC 102 rCBH was significantly greater than either *L. gallinarum* TDC 63 rPhyA or wild type. No significant treatment effect was observed on deconjugation of GCA or GDCA.

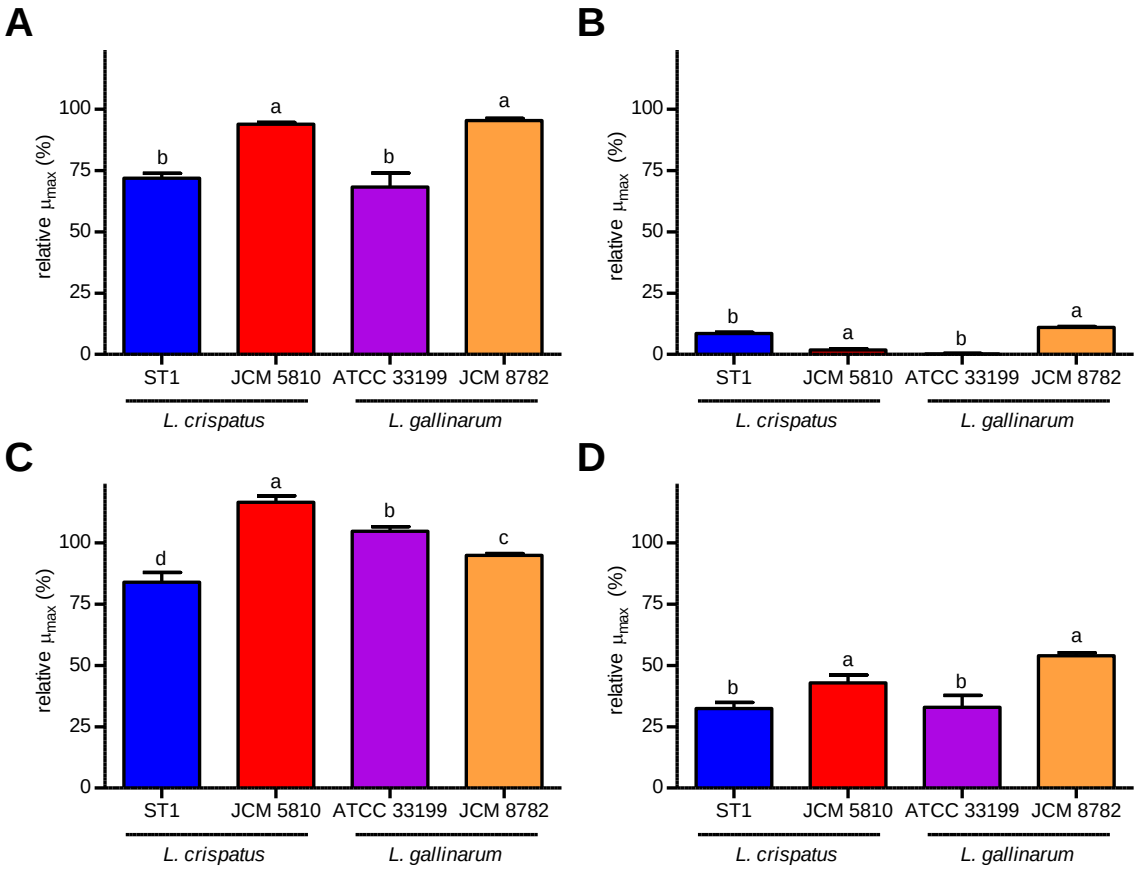

Figure 2.1 Sensitivity of *Lactobacillus* cultures to bile salts. The maximum growth rate (μ_{max}) of *Lactobacillus crispatus* (ST1 and JCM 5810) and *Lactobacillus gallinarum* (ATCC 33199 and JCM 8782) strains cultured in MRS broth supplemented with 4 mM **(A)** glycocholic acid, **(B)** glycodeoxycholic acid, **(C)** taurocholic acid, or **(D)** taurodeoxcholic acid was determined. The mean ± SEM relative μ_{max} (%) as compared with growth in neat MRS of triplicate wells from 4 independent assays is reported. Means not sharing common letters differ significantly ($P \leq 0.01$).

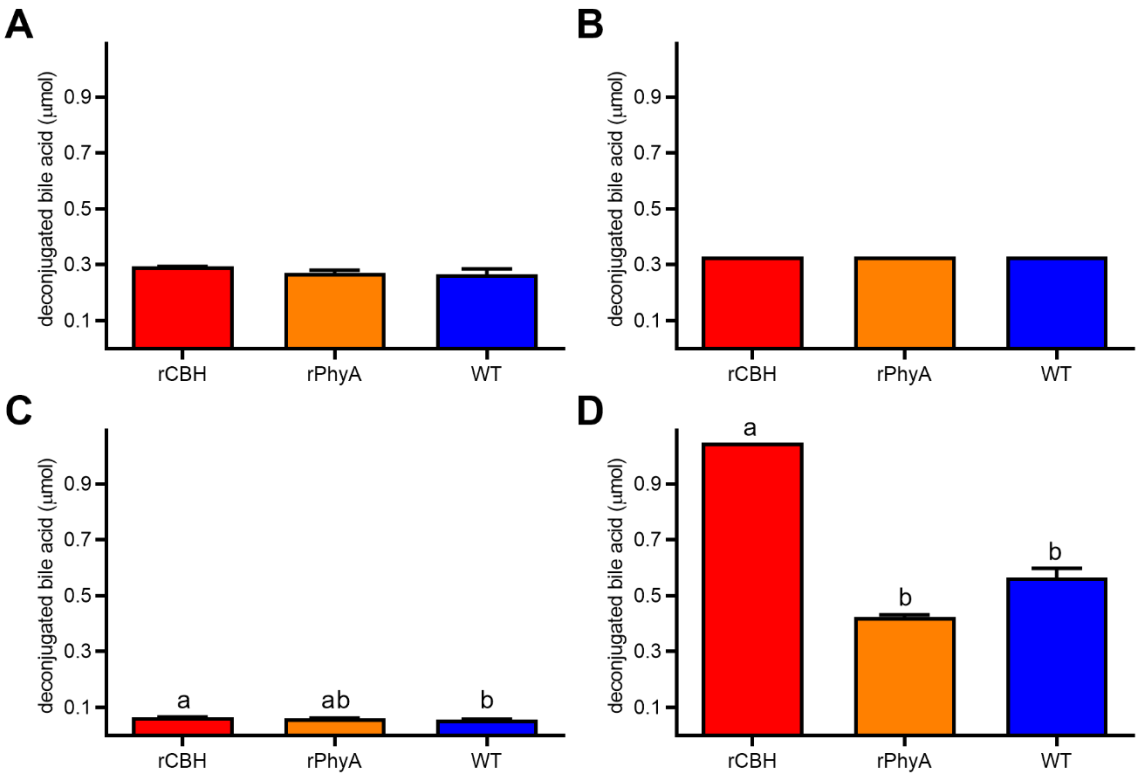

Figure 2.2 Effect of Cbh overexpression on bile deconjugation of *Lactobacillus gallinarum.* *L. gallinarum strains* were cultured in MRS broth supplemented with 2mM (**A**) glycocholic acid, (**B**) glycodeoxocholic acid, (**C**) taurocholic acid, or (**D**) taurodeoxcholic acid and deconjugation of bile salts was determined using LC-MS. The mean ± SEM µmol bile acid decojugated of triplicate measurements 3 independent cultures is reported. rCbh^{+} *L. gallinarum* (red); rPhyA^{+} *L. gallinarum* (orange); WT *L. gallinarum* ATCC 33199 (blue). Means not sharing common letters are significantly different ($P \leq 0.05$).

The effect of *cbh*-overexpression on growth of *L. gallinarum* in TDCA was evaluated (Figure 2.3). The relative μ_{max} of *Lactobacillus* strains in MRS supplemented with TDCA as compared to neat MRS was determined. A significant treatment effect was observed for the maximum growth rate of *Lactobacillus* cultures in MRS supplemented with TDCA relative to neat MRS ($P < 0.01$) (**Figure 2.3**). Greater μ_{max} was observed in *L. gallinarum* TDC 102 rCBH. No difference was reported between *L. gallinarum* 33199 strains containing rPhyA or pTRK882.

2.3.4 Survivability of Recombinant CBH

Survivability of recombinant CBH in oxgall was determined (**Figure 2.4 A**). A significant treatment effect was observed on survivability at 0.3 and 4-hours post-exposure to oxgall relative to recovery in neat PBS. Survivability of *L. gallinarum* ATCC 33199 containing rCBH was significantly greater than those containing rPHY or pTRK882 ($P < 0.05$) at both 0.25 and 4 hours. Additionally, survivability tended to be higher in rCBH at 2 and 6 hours post exposure ($P < 0.10$) compared to the rPHY and pTRK882.

Survivability of recombinant CBH in 10mM TDCA was determined relative to recovery in neat PBS (**Figure 2.4 B**). A significant treatment effect was observed on survivability at 6 hours post-exposure to TDCA. Survivability of *L. gallinarum* ATCC 33199 rCBH was significantly greater than those containing rPhyA or pTRK 882 ($P < 0.05$). No significant treatment effect was observed at 0.3, 2, or 4 hours.

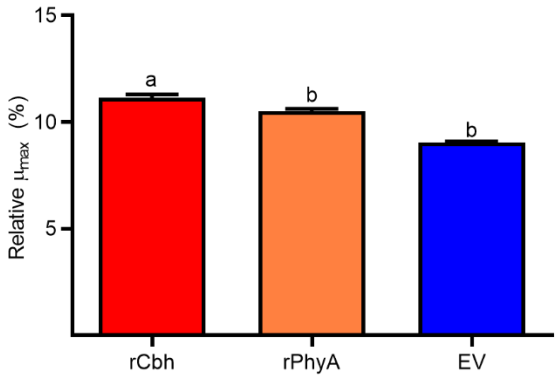

Figure 2.3 Effect of rCbh overexpression on growth of *L. gallinarum* in TDCA. The maximum growth rate (μ_{max}) of rCbh or rPhyA overexpressing *L. gallinarum* strains or the empty vector (EV) cultured in MRS broth supplemented with 4 mM taurodeoxcholic acid (TDCA) was determined. The mean ± SEM relative μ_{max} (%) of growth compared to neat MRS of triplicate wells from 3 independent assays is reported. Means not sharing common letters differ significantly ($P \leq 0.05$).

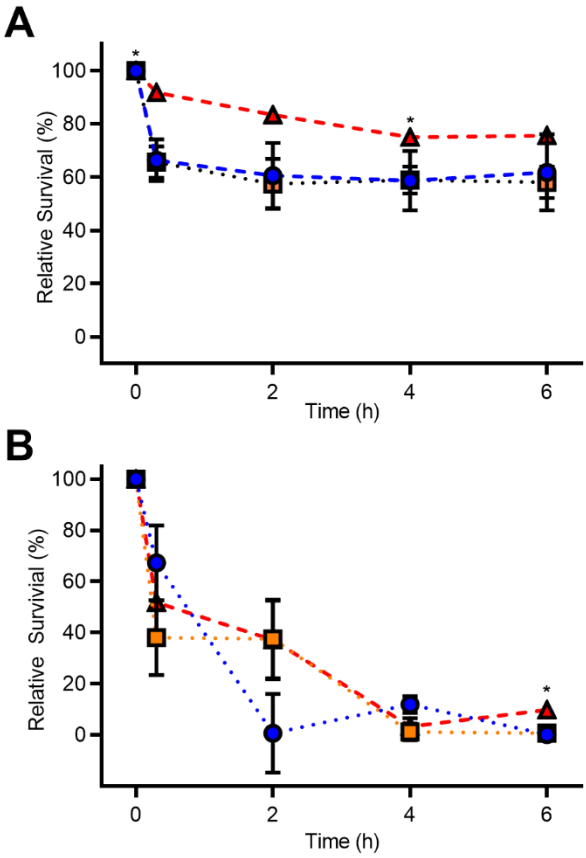

Figure 2.4 Survivability of recombinant *L. gallinarum* strains. Survival of recombinant *L. gallinarum* strains in **(A)** 1% (w/v) oxgall and **(B)** 10 mM TDCA was evaluated. Relative survival is reported as the mean ± SEM viable bacteria (%) recovered from oxgall or TDCA as compared to recovery in neat PBS of triplicate measurements from 3 independent cultures. rCbh$^+$ *L. gallinarum* (red); rPhyA$^+$ *L. gallinarum* (orange); EV *L. gallinarum* ATCC 33199 (blue). * indicates means are significantly different ($P \leq 0.05$).

2.4 DISCUSSION

Bile is secreted into the duodenum and aids in the digestion of fats through the detergent action of its constituent bile salts [99]. Primary bile salts are synthesized in the liver and are composed of a steroid moiety, cholic acid or chenodeoxycholic acid, and an amino acid conjugate, either glycine or taurine, linked by an amide bond [100]. Once secreted in the gastrointestinal tract, they may become converted to secondary bile acids through microbial activities including deconjugation of the amino acid [101]. Detergent properties of bile salts also confer antimicrobial activity, primarily through emulsification of bacterial membranes and damage to nucleic acids [102, 103]. In order to survive in the GI tract of poultry, *Lactobacillus* species must cope with bile salts in the duodenum. However, the influence of BSH activity on the survivability and growth of *Lactobacillus* is not well understood.

Because of their role in the emulsification and absorption of lipids, deconjugated bile acids may be less efficient and impair fat digestion [137]. Indeed, reductions in *L. salivarius* A6 counts in broilers were associated with greater concentrations of conjugated bile acids and increased fat digestibility [138] suggesting reduced microbial BSH activity improves the digestion and absorption of fat. Therefore, research pertaining to the importance of BSH activity to the growth and survivability of probiotic bacteria the gastrointestinal environment is significant to maximizing dietary efficiency in broilers. A cbh gene was identified in the genome of *L. crispatus* ST1 with identical protein structure to a predicted cbh in *L. gallinarum* ATCC 33199. Overexpression of cbh was used to analyze the role of bile hydrolase enzymes in poultry gastrointestinal bacteria. In this study, we investigate bile salt hydrolase activity of poultry *Lactobacillus* isolates and its effect on growth and survivability of *Lactobacillus* in media enhanced with bile salts.

Lactobacillus strains containing multiple BSH enzymes with different substrate specificities have been previously reported [130]. In this study, *Lactobacillus crispatus* strains exhibited greater ability to de-conjugate bile salts than *L. gallinarum*. *L. crispatus* ST1 was observed to deconjugate all four bile salts used in this study, whereas *L. crispatus* JCM 5810 deconjugated only the cholic acid derivatives and *L. gallinarum* JCM 8782 only deconjugated GCA. Bile salt hydrolase enzymes often exhibit a broad range of substrate specificities [139] with enzymes often favoring glyco- [111] or tauro- [140] conjugates. Previous research suggests BSH enzymes recognized substrates primarily at their respective amino acid moiety as opposed to the cholate moiety [141]. However, individual BSH enzymes may not have a broad spectrum of activity against bile salts due to differences in both amino acid groups and the cholate steroid nucleus between bile acids [142]. Indeed, *L. crispatus* JCM 5810 used in this study displayed BSH activity towards cholic acid derivatives, however this ability was not observed in *L. gallinarum*.

Phenotypic analysis of BSH activity does not fully translate to the bacteria's ability to grow in media containing individual bile salts. Bile salts are amphipathic molecules and, therefore, possess detergent properties that enable disruption of bacterial membranes [143] and misfolding of proteins [144]. Additionally, bile acids are able to cause damage to nucleic acids upon entry to the bacterial cell [145]. Bile salt hydrolase activity has been suggested to mitigate these effects [123]. In this study, relative μ_{max} of *Lactobacillus* cultures was determined by comparing maximum growth in MRS supplemented with individual bile salts relative to neat MRS. The presence of bile salts largely decreased the relative μ_{max} of *Lactobacillus* cultures. The growth rate of poultry *Lactobacillus* isolates has been previously demonstrated to be reduced in the presence of bile [146].Across *Lactobacillus* species, glyco- conjugates were more inhibitory

than tauro- conjugates. However, GDCA had the greatest reduction of max growth rate across *Lactobacillus* species. Previous research has demonstrated DCA conjugates have greater toxicity in their unconjugated form, suggesting that increased BSH activity yields a greater level of inhibition [123].

In this study, we investigated the effect of *cbh* overexpression in *L. gallinarum* ATCC 3199. Previous research has utilized site specific homologous recombination to inactivate BSH in *Lactobacillus* [147], however overexpression has not been well characterized. An uncharacterized chologlycine hydrolase enzyme was identified in the genome of *L. crispatus* ST1 and used to form a recombinant plasmid which was inserted in *L. gallinarum* 33199. rCBH expression by *L. gallinarum* ATCC 33199 significantly increased the deconjugation of TCA and TDCA. Previous research has demonstrated substrate specificity for tauro conjugates by some BSH enzymes, however lactobacilli BSH has typically shown substrate preference towards glycocholate [148].

Maximum growth rate is often used as a metric for evaluating the inhibition of microbial growth by chemical compounds [149]. Recombinant overexpression of CBH increased relative μ_{max} of *L. gallinarum* ATCC 33199 in TDCA. BSH activity has been previously corelated with increased growth rate in media containing bile [150]. Secondary bile acids derived from deconjugation of primary bile acids by BSH has an inhibitory effect on some bacterial strains [151]. Indeed, bile salt detoxification mechanisms have been observed in *Lactobacillus* to precipitate deconjugated bile acids [152]. Addition of erythromycin to the media to preserve strain integrity may have contributed to the depressed growth rates across *L. gallinarum* ATCC 33199 strains compared to the wild type in TDCA.

Survival in the gastrointestinal tract is generally considered an important feature for probiotics to deliver their health promoting effects [153]. Bile concentrations in the gastrointestinal tract of chickens are highest in the jejunum where they range from14 to 23 mM [154, 155]. Recombinant overexpression of CBH increased survivability of *L. gallinarum* ATCC 33199 in oxgall and TDCA. Significant increases in survivability of rCBH bacteria were seen as early as 0.3 hours, supporting previous research detailing antibacterial effects of bile salts [156]. In poultry, retention time in the digestive tract is 5-6 hours, of which 1 hour is in the duodenum [157], underscoring the importance of characterizing acute stress responses in poultry probiotics. However, associations between BSH activity and survivability of bacteria under bile stress are not entirely clear [109]. Bile stress responses are multifaceted, which may contribute to the discrepancy in BSH activity and bile susceptibility in some species. In this study recombinant CBH expression increased survivability, however a difference was only reported after 6 hours of exposure. Gene expression analysis in *Lactobacillus* has detected expression of general stress proteins, transmembrane transporters, and cation efflux pumps that all contribute to bile tolerance [152, 158].

In this study, we characterized BSH activity in *Lactobacillus* isolated from poultry and investigated the role of a putative BSH, *cbh* from *L. crispatus* ST1, in the deconjugation of bile salts and survivability of *Lactobacillus* using recombinant expression. We have demonstrated that within *Lactobacillus* species there are varying BSH with affinities for different bile acids. Although variable, the phenotypic characterization of BSH activity did not necessarily translate to a significant increase in relative μ_{max}. Additionally, we were able to overexpress BSH derived from *L. crispatus* ST1 in *L. gallinarum* ATCC 33199. Overexpression increased deconjugation of tauro- conjugated bile acids in addition to increasing survivability in environments containing

TDCA or oxgall. Our results suggest poultry *lactobacillus* isolates possess a variety of BSH enzymes to combat the bile rich environment in the duodenum. Although the bile salts present significantly affected relative μ_{max}, overexpression of BSH was still able to improve survivability in oxgall. Our findings suggest BSH activity in poultry *Lactobacillus* may increase survivability and growth in the gastrointestinal tract. Continued research is needed on the mechanisms of bile detoxification by gastrointestinal microorganisms and their effect on both growth rate and survivability under gastric stress. Additional in-vivo studies in broilers are needed to determine the effect overexpression of BSH has on fat digestion.

3. EVALUATION OF COPPER SOURCES ON GASTROINTESTINAL BACTERIA IN BROILER CHICKENS

3.1 INTRODUCTION

Copper is an essential micronutrient for proper growth and development of broilers. Copper is required for enzyme activity, the release of iron, and synthesis of phospholipids [159], with deficiencies in poultry causing anemia [160], reduced pigmentation [161], or cardiac hypertrophy [162]. However, dietary inclusion of copper in commercial poultry production typically far exceeds the nutritional requirements of modern broilers [163]. Excess copper supplementation has been used in poultry because of its growth promoting properties [164]. There are a wide variety of copper sources used in poultry production including organic and inorganic salts. Due to the variabilities in copper sources and inclusion levels, bioavailability of copper is also variable in the diet [165, 166].

Dietary copper is supplemented commonly in broiler diets as inorganic salts including copper(II) sulfate, tribasic copper chloride (**TBCC**), and copper(I) oxide, or organic salts including copper proteinate, copper lysine complex, and copper methionine hydroxy analogue chelate (**HMTBa**). Copper sulfate began to be used in animal premixes due to its increased bioavailability compared to TBCC, however its acidic and hygroscopic nature may result in reduced vitamin stability when included as a premix component [167]. TBCC has fewer limitations compared to other inorganic forms as its structure allows slower release and increased absorption which ultimately limits excretion, and it has been found to be less reactive in feed [168-170]. Organic copper exists as a complex, consisting as copper with an organic carrier compound, or as a chelate. One form of copper chelate, Cu-$(HMTBa)_2$, consists of two HMTBa ligands covalently bonded to a copper atom, with HMTBa being a hydroxyl analog of

methionine. Chelation of copper allows for reduced dissociation in the upper GI tract and increased absorption across the luminal wall due to the neutral charge for Cu-$(HMTBa)_2$. Therefore, organic trace minerals exhibit greater bioavailability than inorganic salts [171], however their expense compared to inorganic sources affects their inclusion in premixes.

Previous research generated mixed results regarding the effect of copper source on gastrointestinal (GI) microbiota. In vitro studies suggest the bactericidal actions of copper is based upon free ionic copper concentrations [172]. Copper montmorillonite has been shown to reduce *Clostridium* and *E. coli* counts in the cecum and small intestine of broilers, whereas copper(II) sulfate did not significantly effects microbial populations [173]. In swine, copper sulfate reduces coliform populations in the colon and total Lactic Acid Bacteria (**LAB**) counts in the stomach [174]. Due to the variety of copper sources on the market, and ranging dietary copper inclusion rates, it is important to understand the affect copper source and level may have on the GI microbiota as a potential mechanism for their growth promoting effects.

In this study, we examine the effect of copper source on the gastrointestinal microbiota of broiler chickens over a 40-day production period.

3.2 MATERIALS AND METHODS

3.2.1 *Experimental Animals and Husbandry*

Male broilers chicks (Ross 708) were obtained from a commercial hatchery on day of hatch and vaccinated for *Eimeria* (Advent, Huvepharma Inc, Peachtree City, GA), weighed, wing banded, and assigned randomly to treatment pens with statistically similar starting weights. Experimental animals were raised in floor pens on built-up litter under conditions simulating commercial poultry production and provided access to experimental rations and water *ad libitum* for the 41 d duration of the study. An industry lighting program was used in accordance with the

Texas A&M standard operating procedures [175] with temperature guidelines following the breeder's recommendations [176]. All experimental procedures were performed as approved by the Texas A&M University Institutional Animal Care and Use Committee.

3.2.2 *Experimental Design and Diets*

The effect of different copper sources on gastrointestinal bacteria was evaluated. Broiler chicks (n=2200) were allocated to 5 experimental treatment groups with 20 replicate pens of 18 birds arranged as a randomized complete block design. The 5 experimental treatment groups were as follows: tribasic copper chloride [$Cu_3(OH)_3Cl$] included at 10 ppm (**TBCC**; Micronutrients, Inc, Amersfoort, NL); TBCC incorporated at 125 ppm from 0-19 d and 20-29 d with 110ppm from 30-42 d; Copper-2-hydroxy-4-(methylthio)butanoate Cu-$(HMTBa)_2$; (Novus International, Inc., St. Charles, MO) at 60ppm without additional dietary methionine accounted for in formulation [Cu-$(HMTBa)_2$ (-)]; Cu$(HMTBa)_2$ at 60ppm with a met credit [Cu-$(HMTBa)_2$ (+)], and 40 ppm organic copper [Cu$(HMTBa)_2$ (+)] with a met credit.

Experimental diets were fed for the duration of the study using 3 dietary phases: starter (0 to 19 d post-hatch, crumble), grower (20 to 29 d post-hatch, pellet), and finisher (30 to 41 d post-hatch, pellet). All treatments received the same basal ration, with differing copper levels and sources accounted for in the premix. Methionine credit was taken from supplemental DL-methionine.

3.2.3 Tissue Sample Collection

When indicated, one representative (median weight ± 5%) bird was selected from each pen, killed humanely, dissected aseptically, and an ileal section of approximately 3 cm was taken at the midpoint between the ileocecal junction and Meckel's diverticulum.

3.2.4 Bacterial Enumeration

Ileal samples were homogenized and diluted serially using Fluid Thioglycolate Medium (FTM; BD, Franklin Lakes, NJ). *Clostridium perfringens* were enumerated using Tryptose Sulfite Cycloserine – Egg Yolk agar (TSC-EY; BD) and incubated at 37^{o}C under anaerobic conditions (Coy Laboratory Products, Inc., Grass Lake, MI) for 48 hr. *Lactobacillus* were enumerated using de Mann, Rogosa, Sharpe (MRS; BD) agar with 100µg mL^{-1}cylcoheximide (Amresco, Solon, OH) and incubated at 37^{o}C in 10% CO_2 for 24 hr. Ileal homogenate was selectively enriched for *Clostridium* using Iron Milk Media (HiMedia; Mumbai, India).

3.2.5 Statistical Analysis

Univariate tests were used to verify normality and homoscedasticity of data so that all assumptions of ANOVA were fulfilled. bacterial counts were $\log_{10}$ transformed for analysis. The General Linear Model was used to determine significant differences and means with a p-value $\leq$ 0.05 were separated using Duncan's Multiple Range Test.

3.3 RESULTS

3.3.1 Gastrointestinal Microbiota

3.3.1.1 Lactic Acid Bacteria

A significant treatment effect was observed on total LAB counts at 20 d post-hatch ($P < 0.001$) (**Figure 3.1 A**). Counts of total LAB were greater when broilers were fed the TBCC and 40ppm [$Cu(HMTBa)_2$ (+)] diets than when compared with the other treatments at 20 d post-hatch. A significant treatment effect was observed ($P < 0.001$) on LAB counts at 40 d post-hatch (**Figure 3.1 B**). Counts of LAB in the ileum were greatest in broilers fed 10ppm TBCC and 60ppm [$Cu(HMTBa)_2$ (+)] . Although not statistically similar to broilers fed 10ppm TBCC and 60ppm [$Cu(HMTBa)_2$ (+)] , counts of total LAB in broilers fed 125ppm TBCC were greater than those fed 40ppm [$Cu(HMTBa)_2$ (+)].

Incorporation rate and formulation method of $Cu(HMTBa)_2$ was further evaluated. Student's t-test was used to directly compare differences between inclusion of 40 or 60 ppm in groups fed [Cu-($HMTBa)_2$ (+)] with Met credit and directly compare differences between groups fed the 60 ppm Cu-($HMTBa)_2$ diets with (+), or without (-), Met credit. A significant difference for LAB was observed between incorporation rates of [$Cu(HMTBa)_2$ (+)] ($P < 0.001$). At day 20, LAB counts were significantly greater in broilers with inclusion of [$Cu(HMTBa)_2$ (+)] at 40 ppm whereas 40 d LAB counts were significantly greater in broilers with 60 ppm [$Cu(HMTBa)_2$ (+)]. A significant treatment effect on LAB was observed between broilers given 60 ppm $Cu(HMTBa)_2$ (+) or [Cu-($HMTBa)_2$ (-)] ($P < 0.001$) at 40 d. Counts of LAB were greater when birds were fed 60 ppm [$Cu(HMTBa)_2$ (+)] than those fed [$Cu(HMTBa)_2$ (-)].

When observing all broilers, a positive ($r = 0.204$. $P = 0.042$) correlation was observed between LAB on day 40 and cumulative FCR from 0-29 d. In broilers fed either 40 or 60 ppm

$Cu(HMTBa)_2$ (+) broilers, significant correlations of 20 d LAB were reported with 0 – 19 d ABW (r = 0.317, P = 0.047) and counts of LAB at 40 d (r = -0.432, P = 0.005). In broilers fed 60 ppm [$Cu(HMTBa)_2$ (+)] or [$Cu(HMTBa)_2$ (-)] , a significant correlation (r = 0.402, P = 0.010) was observed between LAB counts at d 40 and *Clostridium* counts at d 40. No significant differences were reported in broiler performance [177].

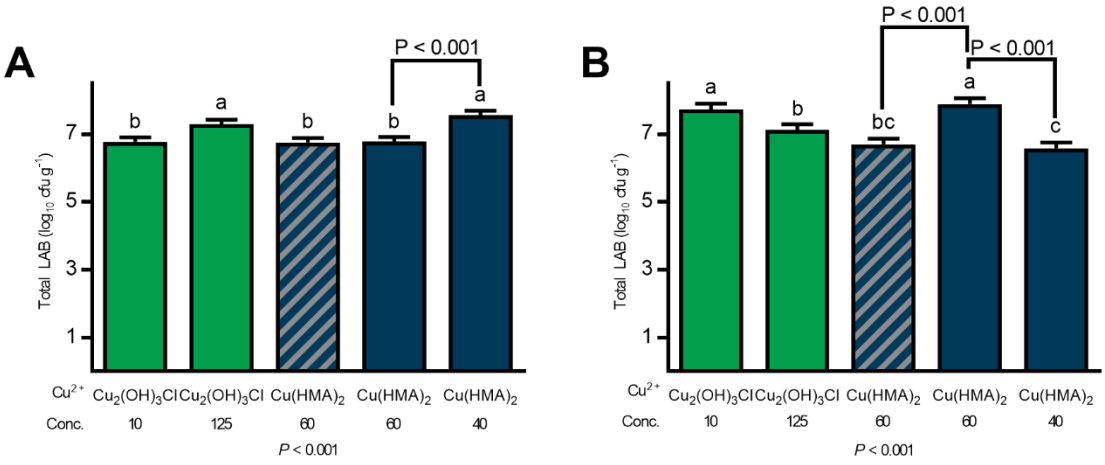

Figure 3.1 Enumeration of LAB from the ileum of broilers.

LAB were enumerated from the ileum of broilers at (**A**) 20 d and (**B**) 40 d post-hatch. Counts are reported as the $\log_{10}$ CFU g^{-1} of tissue. Means were deemed significantly different at $P \leq 0.05$ with means separated using Duncan's multiple range test. Hashed bars represent treatments not given Met credit in the formulation. Connecting brackets indicate significant differences between treatments using student's t-test.

3.3.1.2 Clostridium perfringens

No significant treatment effect on *C. perfringens* was observed at 20 d post-hatch (P = 0.843) (**Figure 3.2 A**), however a significant effect was observed at 40 d post-hatch (P = 0.007) (**Figure 3.2 B**). *C. perfringens* counts were higher in broilers given 60ppm $Cu(HMTBa)_2$ (+) compared to 10 ppm TBCC, 125ppm TBCC, and 60ppm [$Cu(HMTBa)_2$ (-)]. 40ppm [$Cu(HMTBa)_2$ (+)] was not significantly different from any other treatment group.

Incorporation rate and formulation method of $Cu(HMTBa)_2$ was further evaluated. Student's t-test was used to directly compare differences between inclusion of 40 or 60 ppm in groups fed [$Cu(HMTBa)_2$ (+)] with Met credit and directly compare differences between groups fed the 60 ppm Cu-$(HMTBa)_2$ diets with (+), or without (-), Met credit. No significant differences were observed for $Cu(HMTBa)_2$ concentration on *C. perfringens*. A significant treatment effect of Met credit at 60ppm $Cu(HMTBa)_2$ on 40 d *C. perfringens* (P = 0.002). Higher counts of *C. perfringens* were reported in broilers fed [$Cu(HMTBa)_2$ (+)] than those fed [$Cu(HMTBa)_2$ (-)].

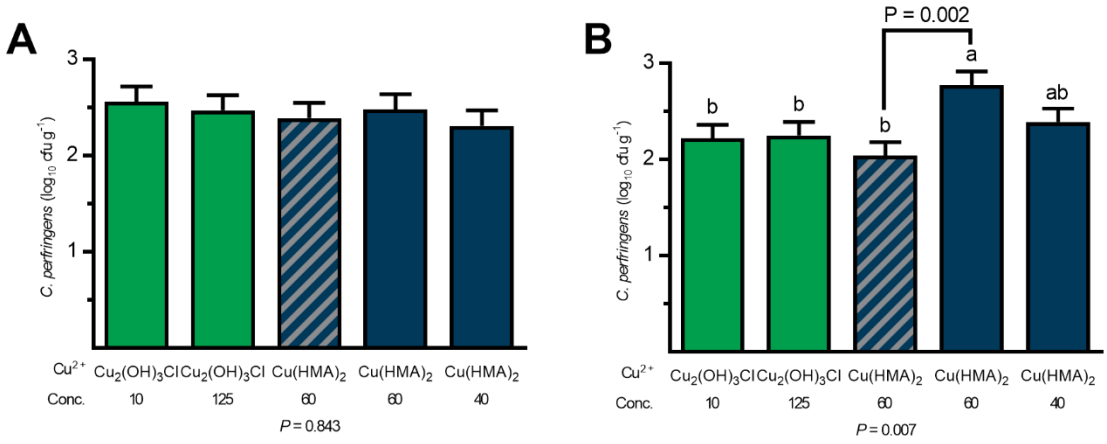

Figure 3.2 Enumeration of *C. perfringens* from the ileum of broilers

Clostridium perfringens were enumerated from the ileum at (**A**) 20 d and (**B**) 40 d post-hatch. Counts are reported as the $\log_{10}$ CFU g^{-1} of tissue. Means were deemed significantly different at $P \leq 0.05$ with means separated using Duncan's multiple range test. Hashed bars represent treatments not given Met credit in the formulation. Connecting brackets indicate significant differences between treatments using student's t-test.

3.4 DISCUSSION

The objective of this study was to evaluate the effect of copper source, dietary inclusion level, and formulation method on the gastrointestinal microbiota of broiler chickens. A variety of copper sources are used in commercial poultry premixes, with nutritionists having to balance cost and efficacy. Dietary copper source has been previously demonstrated to affect growth performance and bacterial populations in the gut. GI microbiota has been recognized as an important modulator for animal health. Although differences in bioavailability have been previously reported between organic and inorganic copper sources, the effect of copper sources and inclusions on the gastrointestinal microbiota are not well understood. In this study, we evaluated the effect of incorporating different copper sources, inclusion levels, or formulation methods on populations of gastrointestinal microorganisms in broiler chickens over a 42-d growth period.

The gastrointestinal microbiota is recognized as an important modulator of animal health. In this study, copper source, level, and formulation method were all observed to effect LAB and *C. perfringens* populations in the ileum of broilers. Antimicrobial properties of copper can be attributed to two primary mechanisms. In the presence of oxygen, copper is able to catalyze the production of hydroxyl radicals via the Fenton and Haber–Weiss reactions [178], which in turn damage microbial proteins and nucleic acids [179]. Microbial protein structure can also be disrupted through interactions of copper with polypeptide backbones or amino acids, thus excluding other metal cofactors from their intended ligands [180]. Dietary copper level, which is associated with copper content of ileal and colonic digesta, has been reported to significantly affect estimators of microbial species richness and diversity within the gastrointestinal tract [181].

Inorganic copper sources have been reported to decrease populations of *Clostridium* spp. [182] and *Lactobacillus* spp. in the hindgut of pigs. Nutrient levels in the hindgut can be modulated based on their dietary inclusion. Organic copper, by nature of being more bioavailable, reduces copper excretion when fed at the same level as inorganic copper [183]. Increased dietary supplementation of copper proteinate, an organic copper source, was reported not to significantly effect microbial counts in the ileum of broilers [184]. In our study, increased copper level was not observed to have an effect on counts of *C. perfringens* in the ileum of broilers fed $Cu(HMTBa)_2$. It is likely that the greater bioavailability of Cu-HMTBa resulted in increased absorption by the host and decreased concentration of copper in the distal portion of the small intestine. However LAB results were significant yet inconsistent, as 40 ppm $Cu(HMTBa)_2$ inclusion yielded more LAB on day 20 but less on day 40 compared to 60 ppm $Cu(HMTBa)_2$. Inclusion of a dietary Met credit in formulation yielded greater counts of both LAB and *C. perfringens* at d 40. This could potentially be attributed to interactions between LAB and *C. perfringens* [185].

A strong positive correlation was observed between *Lactobacillus* and *C. perfringens* counts in broilers fed 60ppm $Cu(HMTBa)_2$. Lactic Acid Bacteria are commonly detected as bacteria that accompany hydrogen producers, and produce lactic acid as a product of carbohydrate fermentation [186]. Although LAB contribute very little to the production of hydrogen, lactate can be utilized by other microorganisms as a substrate in hydrogen producing fermentations by other microorganisms suggesting a possibly mutualistic relationship between LAB and some *Clostridium* spp. [185, 187]. Lactate is oxidized into pyruvate by lactate dehydrogenase. In the conversion of pyruvate to acetyl-CoA, pyruvate synthase forms H_2 thus increasing the hydrogen concentration with the increased availability of lactate. Counts of *Lactobacillus* on day 40

positively correlated with 0-29 d FCR in broilers given 60ppm $Cu(HMTBa)_2$. *Lactobacillus spp.* have been previously reported to increase FCR in broilers [188, 189]. Among $Cu(HMTBa)_2$ broilers with a Met credit, 20 d LAB counts were positively correlated with 0 – 19 d ABW in. *Lactobacillus* have been previously reported to increase in abundance in broilers with increased body weight [190]. Ileal LAB populations are not always indicative of growth performance [191] as certain LAB species or strains appear neutral in their effect on broiler performance [192]. A possible difference in predominant *Lactobacillus* strain could account for these opposing correlations in $Cu(HMTBa)_2$ copper level and Met credit.

In this study, we investigated the effect of copper source on the gastrointestinal microbiota of broiler chickens over a 40-day production period. Additionally, we compared the effect of $Cu(HMTBa)_2$ inclusion rate, and formulation method was evaluated. $Cu(HMTBa)_2$ level and methionine credit has been previously reported to significantly effect FCR and ABW[193], however the effect on the microbiota is not well understood. The source and inclusion rate of copper used in a premix influences the availability of copper at various points in the digestive tract. We have demonstrated that dietary copper significantly effects populations of LAB and *C. perfringens* in the GI tract. Counts of LAB at 20 d were greater in broilers given 125ppm TBCC or 40ppm [$Cu(HMTBa)_2$ (+)], however 40 d LAB counts were greater in 10 ppm TBCC and 60ppm [$Cu(HMTBa)_2$ (+)]. *C. perfringens* counts were significantly lower in broilers fed 60ppm [$Cu(HMTBa)_2$ (-)] than those with [$Cu(HMTBa)_2$ (+)]. Some of these bacterial results may suggest a mutualistic relationship between *Clostridium* and LAB. Although there were no observed performance differences between TBCC and $Cu(HMTBa)_2$, inclusion rate and formulation method for $Cu(HMTBa)_2$ significantly affected the GI microbiota of broilers. Because of the variety in copper sources and methods of application, nutritionists must consider

effects on the GI microbiota while formulating to maximize functionality while meeting or exceeding the dietary requirements of broilers.

4. INTERMITTENT PROBIOTIC ADMINISTRATION

4.1 INTRODUCTION

Lactic Acid Bacteria (**LAB**) are important inhabitants of the gastrointestinal (**GI**) tract of poultry and have been used widely as probiotics in the production of broiler chickens [194]. Administration of probiotic LAB has been demonstrated to promote digestive health [195], improve growth performance [196], and modulate the immune responses of broilers [197]. In addition to their growth performance and health benefits, probiotic LAB administration has also been demonstrated to improve microbial food safety of poultry and poultry products through reduced gastrointestinal colonization of human food borne pathogens including *Campylobacter* [198] and *Clostridium perfringens* [199]. Despite their many benefits, their use in poultry production has fallen out of favor due to their inconsistent results and difficulty incorporating them into pelleted feeds.

In order to maximize performance under least cost formulation, multiple dietary changes are made throughout a traditional grow-out in regard to both ingredient and nutrient levels [200]. It has been previously demonstrated that feed ingredient profiles have the ability to change the microbial community in the GI tract [201]. Shifts have been observed in the GI community as succession occurred from a transient community to one of increasing complexity as the birds aged, with some periods of stability [202]. Perturbations of the normal GI microbiota which occur during feed changes can damage the intestinal mucosa and innate protective mechanisms, leading to a potential loss in growth performance characteristics.

The primary methods of LAB administration in poultry are inclusion in-feed or administration in water lines [203]. Whereas pelleting feed is advantageous in broiler production [204], it provides challenges for LAB inclusion as immobilization and microencapsulation is often required to help maintain elevated survivability of LAB in animal feeds [205, 206].

Inclusion in pelleted feed also reduces the flexibility in modulating dose or administration time. Water-based administration the increases the viability of LAB during the treatment period and also improves production statistics when compared with in-feed administration of the same probiotic cultures [207, 208]. Administration method can significantly affect performance statistics and the immune competence of broiler chickens, with in-water administration being more advantageous than supplementation in feed [209].

Intermittent administration of probiotic LAB has been demonstrated to improve performance parameters suggesting that it may serve to mitigate perturbations in the GI microbial community [207]. In addition, LAB supplementation may increase intestinal villus height and goblet cell counts [210], whereas intermittent administration may increase mucin expression [211]. Without continuous use, the reduced input cost also allows for greater economic return for producers using intermittent administration [212].

In this study, we investigated the effect of intermittent administration of probiotic LAB through drinking water during diet changes on growth performance, gastrointestinal microbiota, and ileal histomorphometry in broiler chickens over a 42 d production period.

4.2 MATERIALS AND METHODS

4.2.1 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 treatment pens with statistically similar starting weights. Experimental animals were raised in 3.85 m^2 floor pens on built-up litter; provided age-appropriate heat, ventilation and lighting according to breeder's recommendations [213]; and provided access to experimental rations and water *ad libitum* for the 42 d duration of the study. All experimental procedures were performed as approved by the Texas A&M University Institutional Animal Care and Use Committee.

4.2.2 Experimental Design and Diets

The effect of intermittent administration of a multi-strain culture of probiotic LAB through drinking water to broiler chicks during feed changes was evaluated. Experimental animals (n = 1200) were allocated to three treatment groups with 10 replicate pens of 40 birds arranged as a randomized complete block design. The three experimental treatment groups were as follows: administered untreated water (**Unt**), administered 10^6 cfu $bird^{-1}$ day^{-1} of probiotic LAB culture containing *Pediococcus acidilactici* FM 18 and *Weisella confusa* FM 46 for 1 d after placement and 1 d pre- feed changes (**Low)**, administered 10^8 cfu $bird^{-1}$ day^{-1} of the probiotic LAB culture for 2 d after placement and 2 d pre- and post-feed changes (**High**).

A commercial-type non-medicated diet was formulated to meet or exceed industry dietary requirements for three phases: starter (0 to 14 d post hatch, crumble), grower (14 to 28 d post hatch, pellet), and finisher (28 to 42 d post-hatch, pellet) [159]. Dietary ingredients were chosen to promote hindgut fermentation and perturb the GI tract microbiota during feed changes.

Experimental rations were held constant for all treatment groups (**Table 4.1)**. Full matrix values for enzyme contribution of P, Ca, Na, AA, and ME were used as recommended by the manufacturer.

Table 4.1. Ingredient composition and nutrient content of the diet fed to broiler chickens

Item (%)	Starter	Grower	Finisher
Ingredients			
Corn	60.40	58.85	51.30
Soybean Meal	27.05	22.05	51.30
Meat and Bone Meal	5.00	2.50	0.00
Bakery Meal	5.00	0.00	0.00
Wheat Bran	0.00	3.00	7.50
Wheat Midds	0.00	0.00	5.00
Corn DDGS	0.00	7.50	0.00
DL-Met	0.32	0.26	0.17
Lysine HCL	0.28	0.29	0.02
Soy Oil	0.46	3.41	7.13
Limestone	0.54	0.95	1.23
Monocalcium PO_4	0.18	0.32	0.56
Salt	0.24	0.24	0.36
Sodium Bicarbonate	0.13	0.24	0.14
Trace Minerals[1]	0.05	0.05	0.05
Vitamins[2]	0.25	0.25	0.25
Phytase[3]	0.01	0.01	0.01
Calculated Nutrients			
Ca	0.90	0.82	0.74
Available P	0.56	0.56	0.55
ME (kcal kg^1)	3047	3101	3168
dig Met	0.61	0.54	0.43
dig TSAA	0.87	0.79	0.69
dig Lys	1.18	1.04	0.89
dig Trp	0.20	0.18	0.20
dig Thr	0.77	0.69	0.61
Na	0.20	0.20	0.20
Analyzed Nutrients[4]			
Dry Matter	87.68	87.80	86.78
Crude Protein	22.10	19.20	18.30
Crude Fat	5.99	5.82	6.81
Crude Fiber	3.10	2.60	2.40
Ash	4.87	5.30	3.99

[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. 748 units kg^{-1} feed
[4]Midwest Laboratories, Inc., Omaha, NE

4.2.3 Water Treatments

Probiotic-treated water was prepared daily during the treatment periods and administered using individual bucket drinkers for each pen. A chlorine binder (Novozymes, Bagsvaerd, DK) was used for all water during treatment periods at a final concentration of 0.7 g l^{-1}. Water consumption was estimated using breeder's management guidelines [213] for calculation of the appropriate LAB concentration in the drinking water for each treatment. Water consumption was monitored gravimetrically for calculation and verification of proper dosage.

Samples of probiotic treated water were collected from all treatments prior to distribution to individual pens for enumeration of total LAB using de Mann, Rogosa, Sharp (**MRS**; Difco, Franklin Lakes, NJ) agar supplemented with 100 μg mL^{-1} cylcoheximide (Amresco, Solon, OH) and incubated at in 10 % CO_2 at 37 ˚C.

4.2.4 Growth Performance Measurements

Experimental animals and residual feed were weighed by pens at 0, 14, 28 and 42 d post hatch for determination of BW and feed consumption. Mortalities and post-mortem weight were recorded for calculation of mortality, ADG, ADFI, and mortality corrected FCR.

4.2.5 Gastrointestinal Tissue Samples

One representative (median weight ± 5%) bird was selected from each pen at 2, 12, 16, 26, and 30 d post-hatch for collection of ileal specimens, whereas three representative birds were selected at 42 d post-hatch for collection of ileal and cecal specimens. Selected experimental animals were killed humanely and dissected aseptically for the collection of GI tissues. Ileal sections were taken at the midpoint between the ileocecal junction and Meckel's diverticulum. The proximal 1/3 of the ileal specimens and the ceca were used for enumeration of bacteria when

indicated, whereas the remaining 2/3 of the ileul specimens were used for histmorphometric analysis when indicated.

4.2.6 Enumeration of Gastrointestinal Bacteria

Ileal specimens were pooled by pen, as appropriate, and total LAB and *Clostridium perfringens* were enumerated using MRS agar supplemented with 100μg mL^{-1} cylcoheximide incubated in 10% CO_2 at 37 ºC and Tryptose Sulfite Cycloserine-Egg Yolk (**TSC-EY**) agar (Difco) incubated anaerobically (Coy Laboratory Products, Inc., Grass Lake, MI) at 37 ºC, respectively. Cecal specimens were pooled by pen, and *Campylobacter* was enumerated using Campy Cefex Agar (**CCA**; Hardy Diagnostics, Santa Maria, CA) incubated in 10% CO_2 at 37 ˚C. *C. perfringens* was selectively enriched from the ileum using Fluid Thioglycolate Medium (Difco) and Iron Milk Medium (HiMedia Laboratories, Mumbai, India), and *Campylobacter* was selectively enriched from the cecum using Bolton's Enrichment Broth (Hardy) and CCA.Specimens for which no colonies appeared on enumeration plates but were positive by selective enrichment were assigned the limit of detection enumeration (2.0 $\log_{10}$ cfu g^{-1}) for statistical analysis.

4.2.7 Histomorphometric Analysis

When indicated, ileal subsections were flushed and fixed using sterile ice cold PBS (ThermoFisher Scientific, Waltham, MA) and 10% neutral buffered formalin (ThermoFisher), respectively. Fixed ileal specimens were trimmed, embedded in paraffin, sectioned, and prepared on slides for analysis using Alcian Blue and Periodic Acid Schiff staining. Measurements were taken from five intact villi and crypts for each sample over three cross-sections using an image analyzer at 100× magnification. Villus heights and crypt depths were used to calculate the villus height to crypt depth ratio (**VH:CD**).

4.2.8 Statistical Analysis

Univariate tests were used to verify normality and homoscedasticity of data so that all assumptions of ANOVA were fulfilled. Percent mortality was arcsine square root transformed for analysis [214], whereas bacterial counts were $\log_{10}$ transformed for analysis. The General Linear Model in SPSS (IBM Corporation, Armonk, NY) was used to determine significant treatment effects, with significantly different means separated *post-hoc* using Duncan's Multiple Range Test ($P \leq 0.05$). When indicated, changes within treatment before and after feed changes was analyzed using Student's-*t* test. Correlations between growth performance, histomorphometry, and bacterial counts were analyzed using Pearson's *r*.

4.3 RESULTS

4.3.1 Growth Performance

No significant treatment effects were observed on BW, ADG, ADFI, or mortality during the course of this study (**Table 4.2**). However, a significant treatment effect was observed on FCR over 0 to 28 d post-hatch ($P = 0.01$). Feed conversion ratio was lower when broilers were administered probiotic LAB through drinking water during feed changes as compared with the untreated broilers. No significant treatment effects were observed on FCR during any other period.

Table 4.2. Growth performance of broiler chickens

	Treatments[1]				Pooled
Item	Unt	Low	High	*P*	SEM
BW (kg)					
Starter[1]	0.406	0.407	0.412	0.63	0.003
Grower	1.407	1.404	1.407	0.99	0.009
Finisher	2.619	1.407	2.612	0.96	0.017
ADG **(**g bird-day^{-1})					
Starter	25.4	25.9	26.2	0.29	0.2
Grower	71.1	71.2	70.7	0.93	0.1
Finisher	85.6	83.6	83.7	0.58	0.8
ADFI **(**g bird-day^{-1})					
Starter	33.3	33.5	33.7	0.70	0.2
Grower	112.3	110.7	110.1	0.38	0.8
Finisher	155.9	151.3	152.7	0.38	1.3
Mortality corrected FCR (Feed:Gain)					
Starter	1.308	1.297	1.287	0.14	0.005
Grower	1.580	1.556	1.557	0.15	0.007
Finisher	1.823	1.812	1.826	0.86	0.009
0 to 28 d	1.503[a]	1.483[b]	1.480[b]	0.01	0.004
0 to 42 d	1.645	1.627	1.631	0.35	0.005
Mortality (%)					
Starter	1.19	0.86	2.00	0.49	0.36
Grower	3.60	5.26	5.69	0.40	0.61
Finisher	6.83	3.89	6.38	0.24	0.67

[a,b] Means within a row lacking a common superscript differ significantly ($P \leq 0.05$)
[1] Treatments: Unt, untreated; Low, low LAB dose; High, high LAB dose
[2] Starter: 0 to 14 d, Grower: 14 to 28 d. Finisher: 28 to 42 d

4.3.2 Gastrointestinal Microbiota

Total LAB were enumerated from ileum of broiler chickens at 2 d post-hatch, 2 d before and after each feed change, and at termination of the study. A significant treatment effect was observed on total LAB counts ($P < 0.01$) at 16 d post-hatch but was not observed at the remaining time points (**Table 4.3**). More LAB were recovered from broilers administered the high probiotic dose than from the other treatments at 16 d post-hatch. Additionally, although the effect of the treatments on total LAB at 42 d was not significant ($P=0.10$), more LAB tended to be recovered from broilers administered probiotic LAB over feed changes than from those that did not. At termination of the study, *C. perfringens* and *Campylobacter* were enumerated from the ileum and cecum, respectively (**Table 4.3**). A significant treatment effect was not observed on *C. perfringens* counts but was observed on *Campylobacter* counts ($P < 0.01$). Fewer *Campylobacter* were recovered from broilers administered the low dose probiotic LAB treatment than untreated broilers or administered the high dose.

Table 4.3. Enumeration of gastrointestinal microbiota (log_{10} cfu g^{-1})

	Treatments[1]				
Item	Unt	Low	High	*P*	SEM
Total LAB[2]					
0 d	8.24	8.41	8.20	0.83	0.09
12 d	8.25	7.78	8.23	0.36	0.16
16 d	7.66[b]	8.29[b]	9.36[a]	< 0.01	0.17
26 d	8.23	7.60	7.94	0.34	0.16
30 d	7.55	7.64	7.72	0.85	0.16
41 d	7.87	8.29	8.05	0.10	0.09
Campylobacter					
41 d	6.85[a]	5.97[b]	6.46[a]	0.01	0.10
Clostridium perfringens					
41 d	4.73	4.302	5.05	0.67	0.31

[a,b] Means within a row lacking a common superscript differ significantly ($P \leq 0.05$)
[1] Treatments: Unt, untreated; Low, low LAB dose; High, high LAB dose
[2] LAB, Lactic Acid Bacteria

The changes in total LAB counts pre- and post-feed changes was evaluated between treatments and within the treatments. A significant treatment effect was observed on the change in LAB counts between treatments over the Starter to Grower [ΔLAB(12 to 16 d)] feed change (P = 0.01) but not over the Grower to Finisher feed change [ΔLAB(12 to 16 d)] (**Figure 4.1**).

Within each treatment over the Starter to Grower (12 to 16 d) feed change (**Figure 4.1A**), fewer LAB were recovered after the feed change than before when broilers were not administered probiotic LAB (P < 0.05), whereas total LAB counts did not change over the feed change when boilers were administered the low probiotic dose and increased when broilers were administered the high probiotic dose (P < 0.05). However, within treatments over the Grower to Finisher (26 to 30 d) feed change (**Figure 4.1B**), total LAB counts were not significantly different for broilers administered probiotic LAB, whereas fewer LAB were recovered after the feed change than before when broilers were not administered probiotic LAB (P < 0.05).

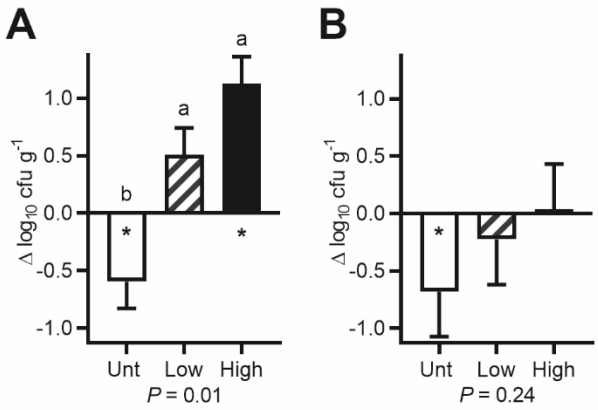

Figure 4.1. Change in total LAB counts across feed changes. The difference in total LAB recovered from the ileum of broilers over the (**A**) Starter-Grower [Δ(12 to 16 d)] and (**B**) Grower-Finisher [Δ(26 to 30 d)] feed changes was evaluated. Difference (Δ) in LAB counts are reported as the mean ± SEM $\log_{10}$ CFU g^{-1} of digestive contents. Unt, untreated; Low, low LAB dose; High, high LAB dose. * indicates difference in total LAB counts pre- and post-feed change is significant within a treatment group. [a,b] Means lacking a common letter are significantly different (P ≤ 0.05).

Associations between populations of GI microorganisms and changes in LAB counts with FCR were also evaluated (**Table 4.4**). Strong negative correlations were observed between LAB counts at 30 d post-hatch with grower phase (r = -0.431, *P*=0.02) and cumulative (r = -0.409, *P* = 0.03) FCR and between LAB counts at 42 d post-hatch with grower FCR (r = -0.443, *P* = 0.01). Strong negative correlations were also observed between LAB [Δ(12 to 16 d)] with grower phase FCR (r=-0.450, P = 0.01) and between LAB [Δ(26 to 30 d)] with finisher phase (r=-0.431, P=0.02) and cumulative (r=-0.443, P=0.01) FCR. Additionally, positive correlations were observed between *C. perfringens* and grower phase (r=0.357, P=0.05) and cumulative (r=0.433, P=0.02) FCR. However, *Campylobacter* counts at 42 d post-hatch were not observed to be associated with FCR.

Table 4.4. Correlation of bacterial counts and FCR

		FCR		
Bacterial Counts		Grower	Finisher	0 to 41 d
Total LAB[1]				
30 d	r	-0.431	-0.232	-0.409
	P	0.02	0.22	0.03
41 d	r	-0.443	-0.059	-0.312
	P	0.01	0.76	0.09
Δ(12 to 16 d)	r	-0.450	-0.094	-0.353
	P	0.01	0.62	0.06
Δ(26 to 30 d)[3]	r	-0.307	-0.431	-0.443
	P	0.10	0.02	0.01
Clostridium perfringens				
41 d	r	0.357	0.275	0.433
	P	0.05	0.14	0.02
Campylobacter				
41 d	r	0.105	-0.142	0.017
	P	0.58	0.46	0.93

[1] LAB, Lactic Acid Bacteria
[2] Δ, change in value over the period indicated

4.3.3 Ileal Histomorphometry

Intestinal villus height (**VH**), crypt depth (**CD**), and VH to CD ratio (**VH:CD**) were evaluated at 12, 16, 26, and 30 d post-hatch (**Table 4.5**). A significant treatment effect was observed on CD ($P = 0.01$) and VH:CD ($P < 0.01$) at 16 d post-hatch. Crypts of broilers administered the high dose probiotic treatment were more shallow when compared with untreated broilers, while VH:CD was greater when broilers were administered probiotics when compared to the untreated broilers. A significant treatment effect ($P < 0.01$) was observed on CD at 30 d. Broilers administered probiotic LAB had shallower crypts than untreated broilers.

Table 4.5. Ileal histomorphometry of broiler chickens (μm)

	Treatments[1]				
Item	Unt	Low	High	*P*	SEM
12 d					
VH[2]	575.7	581.9	544.4	0.61	18.1
CD	144.4	140.4	133.3	0.67	5.7
VH:CD	4.21	4.30	4.24	0.94	0.12
16 d					
VH	507.6	564.2	523.9	0.56	20.3
CD	144.0[a]	123.5[ab]	106.9[b]	0.01	4.6
VH:CD	3.62[b]	4.80[a]	5.13[a]	<0.01	0.17
26 d					
VH[2]	425.3	393.6	404.7	0.51	19.17
CD	96.7	91.9	101.7	0.26	4.05
VH:CD	4.50	4.29	3.98	0.32	0.24
30 d					
VH[2]	469.5	427.4	378.3	0.09	27.12
CD	121.8	99.2	94.4	<0.01	4.42
VH:CD	3.89	4.31	3.97	0.49	0.26
Δ(12 to 16 d)[3]					
VH	-68.1	-17.6	-20.5	0.75	28.7
CD	-0.364	-16.952	-26.5	0.33	6.9
VH:CD	-0.59[b]	0.50[a]	0.89[a]	<0.01	0.18
Δ(26 to 30 d)3					
VH	44.3	33.8	-26.4	0.27	32.33
CD	25.2	7.3	-7.3	<0.01	6.26
VH:CD	-0.61	0.02	-0.01	0.32	0.33

[a,b] Means within a row lacking a common superscript differ significantly ($P \leq 0.05$)

[1] Treatments: Unt, untreated; Low, low LAB dose; High, high LAB dose

[2] VH, villus height; CD, crypt depth;VH:CD, VH to CD ratio

[3] Δ, change in values over the period indicated

When evaluating the change in histomorphometric measures over the feed change period Δ(12 to 16 d), there was a significant treatment effect in VH:CD ($P < 0.01$) (**Figure 4.2A**). VH:CD in untreated broilers decreased from day 12 to 16 whereas broilers given LAB showed an increase in VH:CD ratio. Over the grower to finisher Δ(26 to 30 d) feed change, there was a significant treatment effect in VH:CD ($P < 0.01$) (**Figure 4.2B**). Crypt depth in untreated broilers increased whereas broilers administered high dose of probiotic LAB had shallower crypts. Low dose administration of LAB had an intermediate effect.

Within each treatment over the starter to grower (12 to 16 d) feed change (**Figure 4.2A**), VH:CD after the feed change was lower than before when broilers were not administered probiotic LAB ($P < 0.05$), whereas VH:CD increased over the feed change when boilers were administered the low or high probiotic dose ($P < 0.05$). Within treatments over the grower to finisher (26 to 30 d) feed change (**Figure 4.2B**), CD was significantly ($P < 0.01$) greater in untreated broilers at 30 d compared to 26 d. Although change in VH:CD within treatments over the grower to finisher (26 to 30 d) feed change was not significant, VH:CD was maintained in broilers administered probiotics whereas VH:CD decreased in untreated broilers from 26 to 30 d ($P < 0.01$).

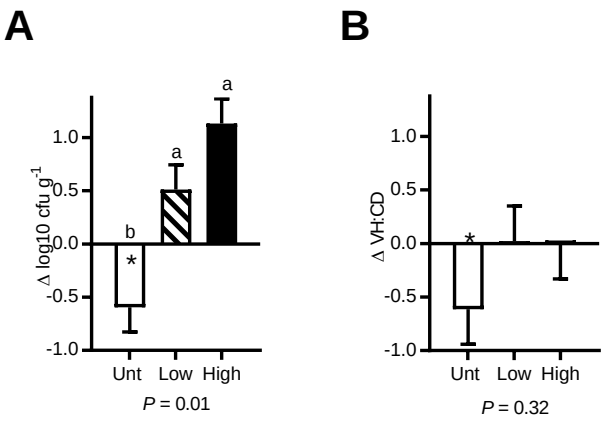

Figure 4.2. Changes in VH:CD ratio across feed changes. The difference in VH:CD were evaluated over the (**A**) Starter-Grower [Δ(12 to 16 d)] and (**B**) Grower-Finisher [Δ(26 to 30 d)] feed changes was evaluated. * Indicates difference in VH:CD pre- and post-feed change is significant within a treatment. [a,b] Means lacking a common letter are significantly different ($P \leq 0.05$).

Associations between populations of LAB and changes in histomorphometry over the Starter to Grower feed change [Δ(12 to 16 d)] with performance were also evaluated (**Table 4.6**). Strong positive correlations were observed between VH with grower phase (r = 0.447, *P* = 0.01) and 0 to 28 d FCR (r = 0.452, *P* = 0.01) and CD with grower phase (r = 0.428, *P* = 0.02) and 0 to 28 d FCR (r = 0.451, *P* = 0.01). Additionally, strong negative correlations were observed between VH with grower phase (r = -0.483, *P* = 0.01) and 0 to 28 d ADG (r = -0.530, *P* < 0.01) and CD with grower phase (r = -0.485, *P* = 0.01) and 0 to 28 d ADG (r = -0.556, *P* < 0.01). Strong positive correlations were observed between VH:CD [Δ(12 to 16 d)] with 16d (r = 0.377, *P* = 0.04) and [Δ(12 to 16 d)] (r = 0.447, *P* = 0.01) LAB. A strong negative correlation was detected between CD and [Δ(12 to 16 d)] LAB (r = -0.570, *P* < 0.01). Lastly, strong positive correlations were detected between VH and 12d (*r* = 0.715, *P* < 0.01), 16d (*r* = 0.454, *P* < 0.01), and [Δ(12 to 16 d)] (*r* = 0.728, *P* < 0.01) CD.

Table 4.6. Correlation of histomorphometry with performance and GI microbiota

		Δ(12 to 16 d)[1]		
Item		VH[2]	CD	VH:CD
Mortality adjusted FCR (Feed:Gain)				
14 to 28 d	r	0.447	0.428	-0.003
	P	0.01	0.02	0.99
0 to 28 d	r	0.452	0.451	-0.040
	P	0.01	0.01	0.84
Average Daily Gain (g bird-day^{-1})				
14 to 28 d	r	-0.483	-0.485	-0.012
	P	0.01	0.01	0.95
0 to 28 d	r	-0.530	-0.556	0.060
	P	<0.01	<0.01	0.75
Total LAB counts (log_{10} cfu g^{-1})				
16 d	r	0.055	-0.170	0.377
	P	0.77	0.37	0.04
Δ(12 to 16 d)	r	-0.306	-0.570	0.447
	P	0.10	<0.01	0.01

[1] Δ, change in value over the period indicated
[2]VH, villus height; CD, crypt depth; VH:CD, VH to CD ratio

Associations between populations of LAB and changes in histomorphometry over the Starter to Grower feed change [Δ(26 to 30 d)] with performance were also evaluated (**Table 4.7**). A strong positive correlation was observed between VH:CD with finisher phase (r = 0.373, *P* = 0.04) FCR. Additionally, moderate negative correlations were observed between VH with finisher phase (r = -0.355, *P* = 0.05) and 0 to 42 d ADG (r = -0.361, *P* = 0.05) and VH:CD with finisher phase (r = -0.485, *P* = 0.01) and 0 to 42 d ADG (r = -0.365, *P* = 0.05). Strong positive correlations were observed between CD [Δ(26 to 30 d)] with 26d (r = 0.418, *P* = 0.02) and [Δ(26 to 30 d)] (r = -0.436, *P* = 0.02) LAB.

Table 4.7. Correlation of Δ(26 to 30 d) histomorphometry with performance and GI microbiota

		Δ(26 to 30 d)[1]		
Item		VH[2]	CD	VH:CD
Mortality adjusted FCR (Feed:Gain)				
28 to 41 d	r	0.306	-0.034	0.373
	P	0.10	0.86	0.04
0 to 41 d	r	0.300	0.221	0.096
	P	0.11	0.24	0.61
Average Daily Gain (g bird-day^{-1})				
28 to 41 d	r	-0.355	0.068	-0.485
	P	0.05	0.72	0.01
0 to 42 d	r	-0.361	-0.064	-0.365
	P	0.05	0.74	0.05
Total LAB counts (log_{10} cfu g^{-1})				
26 d	r	0.258	0.418	-0.137
	P	0.17	0.02	0.47
Δ(26 to 30 d)	r	-0.303	-0.436	0.078
	P	0.10	0.02	0.68

[1] Δ, change in value over the period indicated
[2]VH, villus height; CD, crypt depth; VH:CD, VH to CD ratio

4.4 DISCUSSION

The objective of this study was to evaluate the effect of intermittent administration of probiotic LAB through drinking water during diet changes on the mitigation of perturbations during feed changes. During broiler grow-out, a variety of feedstuffs and nutrition guidelines are used to maximize production and minimize cost [200]. It has been previously demonstrated that different feed ingredient profiles have the ability to disrupt the gastrointestinal microbiome [201], which could lead to a potential loss in performance characteristics. Intermittent administration of LAB could allow for the mitigation of these perturbations; as this method has been shown to improve performance parameters [207]. In this study, we evaluated the effect of the administration of LAB during feed changes using a water-based LAB blend at two application rates on growth performance GI microbiota, and histomorphometry in broilers.

Intermittent administration of LAB through drinking water significantly decreased FCR of broiler chickens from 0 to 28 d compared to untreated broilers. Although the effects of LAB administration on BW and ADFI were not significant, the observed numeric increases in BW and decreases in ADFI result in a significant treatment effect when combined to calculate FCR. Improvements to FCR have been reported widely when broilers were administered probiotic LAB [215]. Reductions in FCR have also been observed when broilers been administered cell-free supernatants from LAB suggesting these organisms produce metabolites produced by these organisms may be beneficial to digestive efficiency [216]. Organic acids have been demonstrate to increase secretion and activity of endogenous digest enzymes Reduced GI pH has been suggested to increase digestive enzyme activity resulting in improved nutrient digestibility [217]. Lactic Acid Bacteria are able to alter the GI pH, which may increase enzymatic activity and nutri Reduction in pH from lactic acid production optimizes conditions for pepsin activity and can

contribute to improved protein digestibility [217, 218]. Additionally, improved apparent energy, fiber, and protein digestibility [218] and increased digestive enzyme activity [219, 220] have been observed when poultry have been administered probiotic LAB suggesting exogenous enzymes produced in the GI tract by these organisms in situ are likely to contribute to host digestion [221, 222]. Indeed, administration of recombinant *Lactobacillus* strains which heterologously expressed *Bacillus* phytase has been demonstrated to improve BWG of broilers a fed phosphorous-deficient diet [223], providing proof-of-principle for this beneficial functionality of LAB. .

Lactic Acid Bacteria are common inhabitants of the GI tract with long history of safe use as probiotics [152] and have been reported widely to improve growth performance [224], promote host immunity [225], and reduce colonization by human foodborne pathogens [226] when administered to poultry and other livestock animals. Because of the beneficial role of both autochthonous and allochthonous LAB in host health, they have been suggested as a potentially important indicator of GI health [227]. In this study, administration of probiotic LAB during feed changes increased counts of total LAB in the ileum at 16 d post-hatch compared to untreated broilers. Administration of LAB should increase LAB counts compared to untreated broilers due to naturally occurring LAB being present in addition to supplemented LAB which adhere to poultry epithelial tissue [228].

Campylobacter spp. are commensal organisms of poultry and an the major cause of poultry-associated foodborne illness in humans [229]. Cell-free supernatants from LAB have been demonstrated to inhibit *Campylobacter* in vitro [230, 231], suggesting their potential as a pre-harvest intervention for reducing *Campylobacter* colonization in poultry. *Campylobacter* inhibiting antimicrobial peptides have been are produced by some stains of LAB [232], whereas

lactic acid, produced universally by LAB, is widely reported to inhibit *Campylobacter* [233] and has been demonstrated to induce alteration structural phospholipids in the cytoplasmic membrane [22]. In our study, the low dose LAB treatment was observed to reduce *Campylobacter* counts in the cecum of broilers at 42 d by almost 1.0 $\log_{10}$ cfu g^{-1} contents. The administration of probiotic LAB has been demonstrated previously to reduce *Campylobacter* colonization [234] and prevalence [235] in broiler chickens. A quantitative risk modelling of *Campylobacter* contamination in broilers estimated that a decrease in carcass contaminationof 1 $\log_{10}$ cfu would reduce the burden of foodborne illness by 85%,[236], suggesting that modest decreases in *Campylobacter* contamination would result in significant improvements to the microbial food safety of poultry.

[Comment] Counts of total LAB from untreated broilers decreased significantly over the course of each feed change demonstrating the effect that the changes in ingredient profiles over diet phases can have in disrupting the established microbiota present in the GI tract. The decrease in total LAB counts observed in the untreated broilers likely resulted from changing nutrient levels, ingredient composition, and feed properties in each diet phase... Multiphase feeding programs are designed to closely meet the age-specific nutrient requirements of broilers and can result in excess available nutrients at the conclusion of feeding phases [237]. Feed ingredients are often altered between feeding phases in order to achieve least-cost formulations. However, differences in the digestibility of the substituted carbohydrate and protein sources can change the fermentable substrates available for microorganisms in the distal portion of the GI tract and induce shifts in the composition of the microbiota [238]. . LAB are predominantly saccharolytic and have variable growth depending on carbohydrate sources available [239], and the changing carbohydrate sources likely favored other organisms and contributed to their reduction over feed

change periods [240]... In our study, probiotic treatment increased counts of total LAB in the ileum \ over the starter to grower feed change and maintained LAB counts over the grower to finisher feed change and mitigated the reductions in LAB counts observed in the untreated broilers. Prior research in other animal species has indicated grain type can influence microbial changes in the GI tract, with exogenous LAB supplementation mitigating microbial changes associated with cereal grain fermentation [240, 241]. Further feed properties, such as NSP type and level, also effect the microbiota [242], which can be exacerbated during feed change periods. In swine, LAB administration during weaning significantly increased *Lactobacillus* populations throughout the GI tract [243] although process of weaning typically reduces the abundance of *Lactobacillus* in the jejunum and ileum [244].

The mitigation of these LAB reductions through intermittent administration can improve gastrointestinal health and broiler performance. In our study, significant strong correlation with decreased FCR in the grower phase and 0 to 28 d post-hatch. Positive shifts in LAB over feed changes improves measures of the gastrointestinal epithelium and ultimately leads to decreased FCR through energy sparing.

Although the treatments were not observed to have a significant effect on counts of *C. perfringens*, we observed a strong positive correlation between *C. perfringens* counts and FCR. Significant strong positive correlations between *C. perfringens* counts in the ileum with FCR have been reported previously [227], suggesting less efficient feed conversion is associated with higher *C. perfringens* counts. Reduced weight gain and increased FCR have been reported when high numbers of *C. perfringens* were recovered from broilers [245, 246], and negative effects on growth performance have been reported when broilers were experimentally infected with *C. perfringens* under conditions of both sub-clinical [247] and clinical [248] necrotic enteritis.

Overall, the results of ours and previous studies suggest FCR is lowest in broilers with a positive shift in LAB during feed changes, and fewer counts of *C. perfringens* in the ileum.

The GI epithelium is an important interface between diet and metabolism, and the development of intestinal morphology may reflect the health of the GI tract [249]. In this study, intermittent LAB administration significantly increased VH:CD via significantly decreasing crypt depth and numerically increasing villus height. Villi are crucial to the digestion and absorption of nutrients by housing digestive enzymes and providing greater surface areas for nutrient absorption. Increasing villus height suggests an increase in the capacity to absorb nutrients [250]. Intestinal crypts are found between villi and serves to produce enterocytes, goblet, enteroendocrine, and Paneth cells [251]. Deepening of crypts corresponds with an increase in crypt cell production rate due to cell turnover in the intestine [252], which can reduce digestive and absorptive efficiency [253, 254]. The villus height to crypt depth ratio is often used as a metric for gut health, as a lower ratio is indicative of small villi and deeper crypts whereas a higher ratio indicates increased villus height and shallower crypts. Lower VH:CD ratios have been associated with the presence of microbial toxins or other GI stressors [255]. The resulting inflammatory process in the gut mucosa due to these disruptions can lead to decreased villus height and crypt depth [256].

During the starter to grower feed change period, VH:CD was significantly reduced in untreated broilers. LAB administration mitigated this loss and yielded an increase in VH:CD over the feed change period. Intermittent administration mitigated the loss in villus height while the intestinal crypts became shallower. Additionally, positive changes [Δ(12 to 16 d)] in GI histomorphometry were significantly correlated with an increase in [Δ(12 to 16 d)] LAB. LAB and their metabolites have been demonstrated to protect the GI epithelium by excluding

pathogens [257], strengthening tight junctions [258], and production of volatile fatty acids [259]. LAB have been previously demonstrated to mitigate damage to epithelial tissue caused by periods of stress [260]. Feed changes and heat stress cause changes in GI bacterial populations [242, 261]. Acute stressors can increase adherence of pathogens in the ileum and effect epithelial structure [262]. During periods of heat stress, administration of LAB metabolites has been demonstrated to modulate the microbiota in favor of LAB while reducing pathogens, resulting in an increased villus height and decreased crypt depth in broilers [263]. Intermittent administration of LAB mitigates reductions in LAB over feed change periods and increases LAB [Δ(12 to 16 d)]. Although the exact mechanism in unclear, intermittent LAB administration is able to mitigate the reduction in LAB over feed change periods which positively effects epithelial histomorphometry by preserving the villi and decreasing crypt depth.

Correlation analysis between crypt depth and performance parameters revealed increasing crypt depth from 12 to 16 d post hatch was correlated with a significant increase in FCR and decrease in ADG for both the grower phase and cumulative starter and grower. Reduced tissue turnover in the intestine lowers maintenance requirements which can result in an increased growth rate or efficiency [264, 265]. Deepening of crypts may be indicative of villi atrophy and sloughing due to intestinal pathogens or perturbations as the host compensates for such losses [249, 266]. Deeper crypts were correlated with taller villi, supporting the importance of cypt depth in the regeneration of the intestinal epithelium. However, current data indicates the energetic cost of this process decreases production efficiency. Inflammation in the gut mucosa elevates energy expenditure and protein catabolism in the host [267]. Metabolic inflammation, an inflammation generated by excessive nutrient intake and metabolic surplus of nutrients, [268] could be a result of excess nutrients available at the end of feeding phases [237]. While

characterized as a chronic low grade inflammation, metabolic inflammation stimulates the innate immune system at the expense of production through decreased digestibility and absorptive capacity [269]. Perturbations of the gastrointestinal tract due to dietary changes can yield inflammatory responses in the intestinal mucosa. Intermittent LAB administration mitigated the negative effects of feed changes on gut morphology, although the mechanism responsible for this intervention is still unclear. By reducing inflammation and mitigating deleterious changes to the gut mucosa, intermittent LAB administration may improve efficiency as evidenced by reduced FCR.

In this study, we investigated the effects of intermittent administration of probiotic LAB during diet changes on growth performance, GI microbiota, and histomorphometry in broiler chickens. We have demonstrated the intermittent administration of LAB in drinking water improved growth performance through decreased FCR. Additionally, we have demonstrated that intermittent LAB administration increases LAB and decreases *Campylobacter* counts in the GI tract. Intermittent administration mitigates the decrease in LAB over feed changes between the starter to grower and grower to finisher phases. Additionally, intermittent administration increases VH:CD, mitigating the decrease in VH:CD over the feed change between the starter and grower phase seen in untreated broilers. Our results suggest intermittent LAB administration increases LAB in the ilium of broilers, encourages shallowing of intestinal crypts and maintenance of villi height over feed changes which promotes energy sparing compared to untreated broilers as evidenced by a significant improvement in FCR amongst broilers administered LAB. Perturbations of the gastrointestinal microbiota can occur during periods of stress in broiler production caused by feed changes, vaccine cycling, or elevated temperature. Our findings suggest intermittent administration of LAB via drinking water may be applied to

mitigate fluctuations in the microbiota, preserve epithelial structure and improve feed efficiency during these periods of stress.

5. FUNCTIONAL FEED ADDITIVE ADMINISTRATION

5.1 INTRODUCTION

The administration of sub-therapeutic antibiotics has been used widely to increase weight gain [270], improve feed efficiency [271, 272], and reduce poultry and human foodborne pathogens [273, 274] in poultry production. Although they have been applied in poultry for over 50 years, the use of antibiotic growth promoters (**AGP**) has declined [275] due to consumer preferences [276] and regulations [277] resulting from concerns over the development of antibiotic resistance in bacteria [278, 279]. As the demand for antibiotic-free (**ABF**) production of poultry and other livestock continues to grow, the continued development of alternatives to antibiotics will become increasingly important. Because the beneficial effects of antibiotics are attributed to their activities on the microbial community in the gastrointestinal (**GI**) tract [280, 281], the GI microbiota is an important target for the development of alternatives to AGP [282].

The United States Food and Drug Administration has defined direct-fed microbial products as those that "are purported to contain live microorganisms [283]", and the International Scientific Association for Probiotics and Prebiotics has defined a prebiotic as "a substrate that is selectively utilized by host microorganisms conferring a health benefit [284]". Phytogenic preparations consist of plant derived products used in animal diets to improve productivity or feed quality [285]. The use of Direct-Fed Microorganisms (**DFM**), prebiotics, and phytogenic preparations as functional additives, ingredients that may provide a benefit beyond satisfying traditional nutrient requirements [286], is seen as a potentially important alternative to the use of AGP in poultry production. When administered to poultry individually or in combination, DFM, prebiotics, and phytogenic preparations have been demonstrated to promote growth and performance [287, 288], reduce GI colonization by human foodborne and poultry pathogens [227, 289], and improve measures of intestinal function [290].

Because of their benefits, interest in the administration of functional additives as alternatives to the use of AGP has increased. Although the benefits of the administration of DFM, prebiotics, and phytogenic preparations have been widely reported, their application in the poultry industry is inconsistent, their overall effectiveness is mixed, and the functionalities of specific additives are not well understood. In this study, we investigated the co-administration of *Bacillus licheniformis* as a DFM with functional additive blend including dietary prebiotics and phytogenic preparations on the growth performance and GI microbiota of broiler chickens.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Animals and Husbandry

Male broilers chicks (Cobb 500) were obtained from a commercial hatchery on day of hatch and vaccinated for *Eimeria* (Advent, Huvepharma Inc, Peachtree City, GA), weighed, wing banded, and assigned randomly to treatment pens with statistically similar starting weights at an initial stocking density of 0.096 m^2 per bird. Experimental animals were raised in floor pens on built-up litter under conditions simulating commercial poultry production and provided access to water and experimental rations *ad libitum* for the 21 d duration of the study. An industry lighting program was used in accordance with standard operating procedures of the Texas A&M University Poultry Research Center [287] with temperature guidelines following the breeder's recommendations [213]. All experimental procedures were performed as approved by the Texas A&M University Institutional Animal Care and Use Committee.

5.2.2 Experimental Design and Diets

The effect of functional feed additive administration on growth performance, GI microbiota, ileal histomorphometry, and serum antioxidant capacity was evaluated in comparison to an AGP. Broiler chicks (n=1960) were allocated to 5 experimental treatment groups with total of 49 pens of 40 birds arranged, due to housing constraints, as a randomized incomplete block design and fed experimental rations supplemented with functional additives using the manufacturers' recommended incorporation rates. The 5 experimental treatment groups were as follows: bacitracin methylene disalicylate (**BMD**) treated (50 g ton^{-1}) feed (10 pens); untreated (**UNT**) feed (9 pens); administered *Bacillus licheniformis* DSM 28710 (**BL**) in-feed at 1.6 $x10^{9}$ cfu kg^{-1} feed (Huvepharma, Inc., Peachtree City, GA) as a DFM (10 pens); co-administered BL with a functional feed additive blend (**BL+A**) consisting of a multi-strain DFM culture of *Lactobacillus acidophilus* and *Enterococcus faecium* in-feed at 4.4 $x10^{7}$ cfu kg^{-1} feed, yeast cell wall extract at 113.40 g ton^{-1} feed (Phileo, Marcquen-Baroel, FR), and a phytogenic preparation of capsicum, cinnamaldehyde, and carvacrol at 45.36 g ton^{-1} (Allied Nutrition, Doringkloof, ZA) (10 pens); or administered a synbiotic (**SYN**) combination of BL and a yeast cell wall extract (Altech, Lexington, KY) at 226.79 g ton^{-1} (10 pens).

Experimental rations (**Table 5.1**) were fed for the duration of the study in two phases: starter (0 to 14 d post-hatch, crumble) and grower (14 to 21 d post-hatch, pellet). For each phase, feed was manufactured as a single commercial-type corn/soybean meal basal diet with 5 % distiller's dried grains with solubles and added phytase and xylanase and divided for inclusion of dietary treatments as appropriate. Full matrix values for enzyme contribution of aP, Ca, Na, digestible AA, and ME as recommended by the manufacturer were used.

Table 5.1. Ingredient composition and nutrient content of the basal diet

Item (%)	0 to 14 d	14 to 21d
Ingredients		
Corn	60.76	65.74
Soybean Meal	26.56	22.25
Meat and Bone Meal	5.00	4.43
Corn DDGS	5.00	5.00
Fat, A/V blend	0.66	0.77
DL-Met	0.26	0.25
Lysine HCL	0.27	0.25
Limestone	0.65	0.55
$CaH_4(PO_4)_2$	0.12	0.00
NaCl	0.37	0.27
$NaHCO_3$	0.04	0.19
Trace Minerals[1]	0.05	0.05
Vitamins[2]	0.25	0.25
Phytase[3]	0.01	0.01
Xylanase[4]	0.01	0.01
Calculated Nutrients		
Available P	0.45	0.40
dig Met	0.57	0.54
dig TSAA	0.84	0.78
dig Lys	1.18	1.04
dig Trp	0.20	0.18
dig Thr	0.69	0.62
Analyzed Nutrients[5]		
Dry Matter	88.86	89.13
Crude Protein	21.60	19.20
ME (kcal kg^1)	3014	3124
Crude Fat	4.17	4.89
Crude Fiber	4.20	3.50
Ash	4.73	4.29
Ca	0.80	0.77
Total P	0.97	0.50
Na	0.22	0.17

[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. 748 units kg^{-1} feed
[4]Hostazym X, Huvepharma. Peachtree City, GA. 1500 units kg^{-1} feed
[5]Performed by Midwest Laboratories, Inc., Omaha, NE

5.2.3 Growth Performance Measurements

Experimental animals and residual feed were weighed by pens at 0, 14, and 21 d post-hatch for determination of BW and feed consumption. Mortalities and post-mortem weight were recorded for calculation of percent mortality, ADG, ADFI, and mortality corrected FCR.

5.2.4 Tissue Sample Collection

At 21 d post-hatch, one representative (median weight ± 5%) experimental animal was selected from each pen, killed humanely, and dissected aseptically for the collection of tissues. Ileal sections of approximately 6 cm taken at the midpoint between the ileocecal junction and Meckel's diverticulum were collected from each bird and divided in half with the proximal and distal segments being used for enumeration of ileal microbiota and histomorphometry, respectively. Additionally, the ceca and whole blood were collected from each bird for enumeration of cecal microbiota and determination of serum antioxidant capacity, respectively.

5.2.5 Bacterial Enumeration

Ileal specimens were homogenized and diluted serially using Fluid Thioglycolate Medium (**FTM**; BD, Franklin Lakes, NJ). One cecal specimen from each broiler was homogenized and diluted serially using sterile phosphate buffered saline (**PBS**, ThermoFisher Scientific, Waltham, MA), whereas the other was placed in 10 mL Bolton's Enrichment Broth (**BEB**; Hardy Diagnostics, Santa Maria, CA).

Clostridium perfringens was enumerated from the ileal specimens using Tryptose Sulfite Cycloserine-Egg Yolk agar (BD) incubated at 37 ^{o}C anaerobically (Coy Laboratory Products, Inc., Grass Lake, MI) for 48 h; *Campylobacter* spp. were enumerated using Campy Cefex agar (Hardy) incubated at 42 ^{o}C in 10% CO_2 for 48 h; and *Lactobacillus* spp. were enumerated from

the ileum and cecal specimens using Rogosa Selective Lactobacilli agar (BD) supplemented with 100 μg mL^{-1} cylcoheximide (Amresco, Solon, OH). *C. perfringens* were selectively enriched from the ileum using FTM and Iron Milk Media (HiMedia; Mumbai, India), whereas *Campylobacter* spp. were selectively from the cecum using BEB and Campy Cefex Agar. 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}).

5.2.6 Histomorphometry

Ileal specimens were flushed and fixed using sterile PBS and 10% neutral buffered formalin (ThermoFisher), respectively. Fixed ileal specimens were trimmed, embedded in paraffin, sectioned, and prepared on slides for analysis using Alcian Blue and Periodic Acid Schiff staining. Measurements of five intact villi and crypts were recorded over three cross-sections for each broiler at 100× magnification. Villus heights and crypt depths were used to calculate the villus height to crypt depth ratio (**VH:CD**).

5.2.7 Serum Antioxidant Capacity

Whole blood was collected post-mortem using blood collection tubes (SST Plus, BD), incubated (room temperature, 30 min) and centrifuged (1000× g, 10 min, 4°C), and serum was collected as the resultant supernatant. Antioxidant capacity was determined as the Trolox equivalent inhibition of metmyoglobin-induced oxidation of 2,2'-azino-di-(3-ethylbenzthiazoline sulphonate) (**ABTS**) according to the manufacturer's instructions (Cayman Chemical Co, Ann Arbor, MI). Oxidation of ABTS was monitored colorimetrically using absorbance at 405 nm (Tecan Systems Inc., San Jose, CA). Serum antioxidant capacity was reported as mM Trolox equivalents.

5.2.8 Statistical Analysis

Univariate tests were used to verify normality and homoscedasticity of data so that all assumptions of ANOVA were fulfilled. Percent mortality was arcsine square root transformed for analysis [291], whereas bacterial counts were $\log_{10}$ transformed for analysis. The General Linear Model was used to determine significant treatment effects, and significantly different means were separated *post-hoc* using Duncan's Multiple Range Test ($P \leq 0.05$).

5.3 RESULTS AND DISCUSSION

Although the administration of sub-therapeutic doses of antibiotics has been used to great benefit in the production of poultry and other livestock, growing consumer [276] and regulatory pressures [276] have increased the need for the development of alternatives to the use of AGP. Antibiotics have been suggested to improve growth and performance of livestock through competition for nutrients between GI microbiota and the host animal, decreased production of toxins and other growth depressing metabolites by the microbiota, and inhibition of subclinical infections [292, 293]. Because the growth promoting effects of antibiotics come as a result from their activities on the microbial community in the GI tract [281], the GI microbiota is an important target for the development of alternatives to AGP [282]. Because their beneficial effects on growth promotion and animal health are mediated through their activities on the GI microbiota, the administration of DFM prebiotics, and phytogenic preparations as functional additives, ingredients that may provide a benefit beyond satisfying traditional nutrient requirements [286], is seen as a potentially important alternative to the use of AGP in poultry production. Although the administration of DFM is used widely in poultry production, their effects when co-administered with other functional additives is not well understood. In this

study, we investigated the co-administration of DFM with other functional additives, including prebiotics and phytogenic preparations, as potential alternatives to AGP in poultry production.

Table 5.2. Growth performance of 21 d broiler chickens

	Treatments[1]						Pooled
Item	BMD	UNT	BL	BL+A	SYN	*P*-value	SEM
BW (kg)							
0 d	0.044	0.044	0.044	0.044	0.044	0.891	0.000
14 d	0.439	0.436	0.441	0.442	0.440	0.800	0.016
21 d	0.888	0.888	0.903	0.887	0.894	0.555	0.004
ADG (kg bird-day^{-1})							
0 to 14 d	0.031	0.031	0.031	0.032	0.031	0.697	0.000
14 to 21 d	0.071	0.072	0.074	0.071	0.072	0.417	0.001
0 to 21 d	0.042	0.042	0.043	0.043	0.042	0.492	0.000
ADFI (kg bird-day^{-1})							
0 to 14 d	0.035	0.035	0.035	0.035	0.035	0.986	0.000
14 to 21 d	0.096	0.098	0.100	0.098	0.098	0.248	0.001
0 to 21 d	0.055	0.056	0.056	0.056	0.055	0.457	0.000
Mortality corrected FCR (Feed:Gain)							
0 to 14 d	1.239^{b}	1.263^{a}	1.239^{b}	1.243^{ab}	1.248^{ab}	0.049	0.004
14 to 21 d	1.350	1.361	1.357	1.369	1.353	0.717	0.004
0 to 21 d	1.301	1.317	1.305	1.314	1.305	0.272	0.003
Mortality (%)							
0 to 14 d	1.111	0.333	0.667	0.667	1.000	0.794	0.222
14 to 21 d	0.000	0.333	0.333	0.000	0.800	0.581	0.110
0 to 21 d	1.111	0.667	0.911	0.667	1.800	0.386	0.301

[a,b] Superscripts indicate significant differences between treatments ($P \leq 0.05$)

[1] Treatments: BMD, bacitracin methylene disalicylate; UNT, untreated; BL, direct-fed *B. licheniformis*; BL+A, BL with additive blend; SYN, synbiotic

5.3.1 Growth Performance

The effects of functional additive administration on the growth performance of broiler chickens was evaluated in comparison to antibiotic-treated and untreated control groups (**Table 5.2**). No significant treatment effects were observed on BW, ADG, ADFI, or mortality over the 21-d course of the study. However, a significant treatment effect on FCR was observed over 0 to 14 d post-hatch (P = 0.049). Feed conversion ratio was highest when broilers were fed the untreated diet. Administration of BMD and direct-fed *B. licheniformis* (**BL**) decreased FCR when compared to the untreated broilers. Although FCR over 0 to 14 d was not significantly reduced when compared to the untreated broilers, co-administration of direct-fed *B. licheniformis* and the feed additive blend (**BL+A**) or the synbiotic (**SYN**) did reduce FCR to a level similar to that of BMD-treated broilers. No significant treatment effect was observed on FCR over 14 to 21 or 0 to 21 d post-hatch.

Improvements in BW, body weight gain, or feed consumption are often not observed in the absence of a disease or stress challenge [294], whereas significant improvements to growth have been reported previously when broilers are raised under conditions of experimentally applied stress [295-297]. Poor *Eimeria* vaccine cycling or absence of a direct microbial challenge due to low litter moisture [298] may have contributed the lack of an observable growth response in our study. Administration of *B. licheniformis* has been reported previously to improve FCR during the starter phase [294, 299]. *B. licheniformis* and other *Bacillus* spp. are valued as industrial microorganisms because of their production of important digestive enzymes including amylases, phytases, and proteases [299-302]. Increased digestive enzyme activity has been observed when administered in poultry [299] suggesting enzyme production in situ by *Bacillus* spp. may directly improve digestibility of feed and increase feed efficiency. Indeed,

administration of heterologous phytase producing recombinant *Lactobacillus* spp. has been demonstrated to improve growth of broilers a fed phosphorous-deficient diet [303], underscoring the significance of microbial enzyme production in animal production.

5.3.2 Gastrointestinal Microbiota

A significant treatment effect was observed on *Clostridium perfringens* counts (P = 0.027) in the ileum (**Figure 5.1A**). *C. perfringens* counts were highest when broilers were fed the untreated diet and lowest when broilers were fed diets treated with BMD or SYN. Although they were not significantly different from the untreated control, administration of BL and BL+A reduced colonization of *C. perfringens* to levels similar to BMD. Bacitracin, a non-ribosomal peptide (**NRP**) antibiotic produced commercially using strains of *B. licheniformis* [304], and its derivatives, BMD and zinc-bacitracin, have been used widely as AGP and for mitigation of necrotic enteritis in poultry because of their antibacterial activity on *C. perfringens* [273, 305].

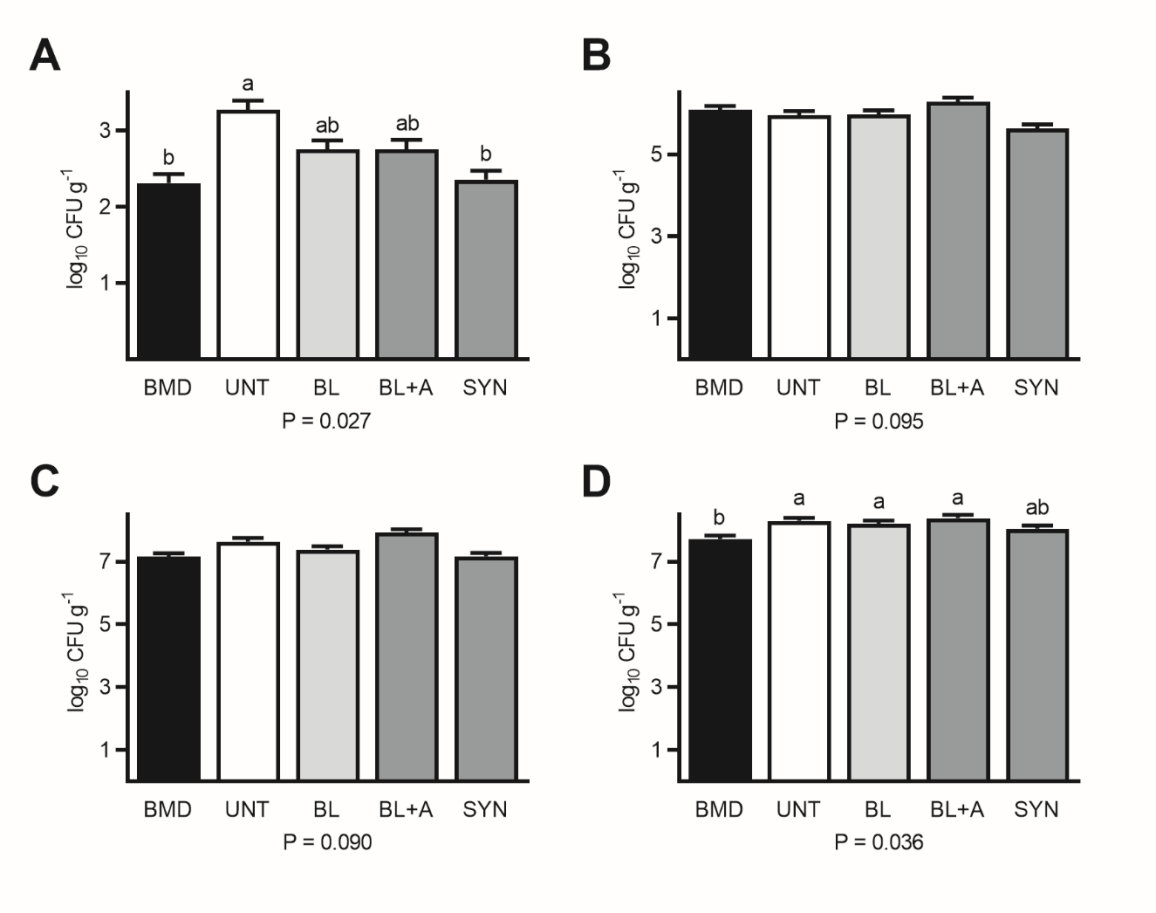

Figure 5.1. Enumeration of gastrointestinal bacteria from broiler chickens at 21 d post-hatch. (**A**) *Clostridium perfringens* and (**B**) *Campylobacter* spp were enumerated from the ileum and cecum, respectively; *Lactobacillus* spp. were enumerated from the (**C**) ileum and (**D**) cecum. . BMD, bacitracin methylene disalicylate; UNT, untreated; BL, direct-fed *B. licheniformis*; BL+A, BL with additive blend; SYN, synbiotic. Counts are reported as mean ± SEM $\log_{10}$ cfu g^{-1} digestive contents from 9 UNT broilers or 10 broilers for all other treatments. Means not sharing common letters differ significantly ($P \leq 0.05$).

Homologs of bacitracin synthetase (*bac*) genes are distributed widely among *B. licheniformis* strains, with over half of the strains screened being reported to harbor homologs of the *bac* gene cluster [306], suggesting that production of an antimicrobial NRP in situ in the GI tract may be important to the functionality of direct-fed *B. licheniformis* strains as potential alternatives to AGP. Production of bacitracin by *B. licheniformis* in situ the GI tract of gnotobiotic mice has been demonstrated previously to inhibit experimental *C. perfringens* infection [307]. The reduction of inflammation induced by *C. perfringens* during subclinical infection has been reported to promote growth by sparing energy otherwise lost to the immune system [308, 309] and likely contributed to the observed improvements to the FCR of *B. licheniformis*-treated broilers in our study. Indeed, administration of DFM to broiler chickens has been reported previously to repartition energy away from a proinflammatory response in the GI tract to other host tissues [310]. Furthermore, rapid selection of bacitracin-resistant *C. perfringens* was observed when the antibiotic was administered in-feed to experimentally infected mice [307]. However, the same study reported that bacitracin-resistant bacteria were not observed when mice were administered *B. licheniformis*, suggesting a potentially important advantage to the production of antibiotics in situ by DFM in the GI tract over the use of AGP. Further characterization will be required in order to determine whether *B. licheniformis* DSM 28710 produces an antimicrobial NRP and whether it is capable of doing so in situ in the GI tract.

Poultry are a commensal host for *Campylobacter* spp. [311] and serve as a primary reservoir for foodborne *Campylobacter* infection in humans [312]. The treatments were not observed to have a significant effect (P=0.095) on *Campylobacter* counts in the cecum of broiler chickens (**Figure 5.1B**). However, fewer *Campylobacter* tended to be recovered from SYN-treated broilers as compared with those administered BL+A. Although the 0.6 $\log_{10}$ cfu reduction

was not observed to be significant, a quantitative risk assessment model suggested that *Campylobacter* reductions of a similar degree should result in a 30-50 % reductions in the burden of *Campylobacter*-associated foodborne illness from poultry [313].

Administration of hydrolyzed yeast-cell wall extracts, composed largely of mannan-oligosaccharides (**MOS**), β-glucans, and other prebiotics, has been demonstrated previously to reduce cecal *Campylobacter* counts [289, 314]. However, the effectiveness of various yeast-derived prebiotics in reducing *Campylobacter* colonization is mixed and their interaction with other functional additives has not been well characterized [315]. The difference in MOS composition or interactions between MOS and the additional functional additives in the functional additive blend administered to the BL+A-treated broilers may have contributed to this difference. The yeast-derived prebiotic administered to the SYN-treated was a more purified MOS fraction whereas the yeast-derived prebiotic administered to the BL+A broilers contained a yeast fraction rich in both MOS and β-glucans. Previous research has shown statistically insignificant 0.6 log difference in *Campylobacter* counts between different mannan-rich fractions used at manufacturer recommended inclusion levels [315]. Although MOS has not yet been demonstrated to agglutinate *Campylobacter* [316], it has been demonstrated to inhibit *Campylobacter* adhesion to poultry epithelial cells in vitro [317] suggesting inhibition of adhesion in the GI tract may be important to the functionality of yeast cell-wall derived prebiotics in reducing pathogen colonization.

Lactobacillus spp. and other Lactic Acid Bacteria (LAB) are recognized widely as beneficial organisms because of their beneficial effects on GI health and host immunity [318, 319]. The treatments were not observed to have a significant effect ($P = 0.090$) on counts of *Lactobacillus* in the ileum (**Figure 5.2C**) but were observed to have a significant effect (P

=0.036) in the cecum (**Figure 5.1D**). Although the effect in the ileum was not significant, more lactobacilli tended to be recovered from broilers fed the BL+A treated diet as compared to those fed the SYN-treated diet. However, in the cecum, fewer *Lactobacillus* were recovered when broilers were fed the BMD-treated diet as compared to the remaining treatments. BMD administration has been demonstrated previously to reduce *Lactobacillus* and other LAB in the GI tract of poultry [320, 321] due to their sensitivity to the activity of BMD against Gram-positive bacteria [322, 323]. The *Lactobacillus acidophilus* included in the feed additive blend administered to the BL+A treatment is likely contributed to increased recovery of *Lactobacillus* as compared to other treatments in the ileum and as compared to BMD-treated broilers in the cecum [324]. Administration of *B. licheniformis* DSM 28710 has been reported previously to reduce GI pH [325], which may promote energy sparing and nutrient availability through the reduction of harmful bacteria [326]. *Lactobacillus* spp. possess an array of acid tolerance factors that increase their survivability in low pH environments and could allow for their persistence in the GI tract of broilers fed *B. licheniformis* [319].

5.3.3 Histomorphometry

The effects of the administration of functional feed additives on intestinal morphology were evaluated to serve as an indicator of GI function (**Figure 5.2**). A significant treatment effect was not observed on villus height ($P = 0.116$) but was observed on crypt depth ($P = 0.009$) and villus height:crypt depth (**VH:CD**) ratio ($P < 0.001$). Crypts of broilers administered BL, BL+A, or SYN were shallower as compared to BMD treated broilers. Crypts of broilers administered DFM alone or in combination with additive blend B were also shallower when compared with the untreated broilers. Greater crypt depth is indicative of increased cell turnover and is associated with higher energy expenditure due to the increased nutrient requirement for

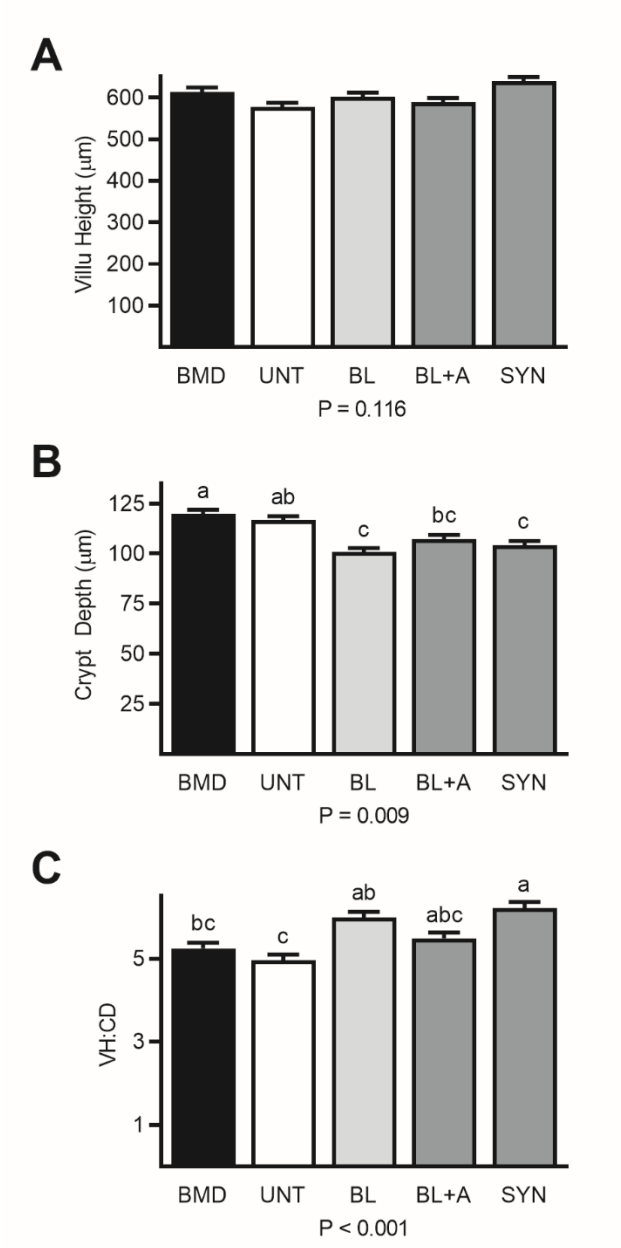

Figure 5.2. Ileal histomorphometry in broiler chickens. Ileal sections were sampled at 21d post-hatch for determination of (**A**) villus height and (**B**) crypt depth and calculations of (**C**) VH:CD. BMD, bacitracin methylene disalicylate; UNT, untreated; BL, direct-fed *B. licheniformis*; BL+A, BL with additive blend; SYN, synbiotic. Villus height and crypt depth are reported as mean ± SEM µm of 3 ileal sections from 9 UNT broilers or 10 broilers for all other treatments. Means not sharing common letters differ significantly ($P \leq 0.05$).

maintenance [327]. Additionally, VH:CD was also greater when broilers were administered BL or SYN as compared with the untreated control, with VH:CD of SYN-treated broilers also being greater than that of BMD-broilers. Increased VH:CD [328] and decreased crypt depth [329] have been reported previously when broilers were administered *Bacillus* spp. as DFM. Additionally, α-amylase produced in situ by *B. licheniformis* [330] may contribute to improved morphology as increased energy available to the host through increased carbohydrate degradation and absorption allows for positive structural development of the small intestine [331]. Improved VH:CD through inclusion of *B. licheniformis* and functional feed additives was driven by shallower crypts. Deeper crypts drive cell turnover in the small intestine which increases maintenance requirements and decreases efficiency[332]. Although a small fraction of body weight, the gastrointestinal tract accounts for upwards of 20% of energy expenditures [333, 334]. The reduction in FCR by *B. licheniformis* and functional feed additives to levels similar to BMD may be facilitated by reduced energy expenditure due to shallower crypts.

5.3.4 Serum Antioxidant Capacity

Oxygen and nitrogen free radicals, products of normal metabolic activity and immune function, can damage host DNA, proteins, and lipids [335]. Antioxidant capacity measures the ability of antioxidants in the serum to quench free radicals compared against a trolox standard [336]. A significant treatment effect was not detected ($P = 0.055$) for serum antioxidant capacity (**Figure 3**). However, antioxidant capacity of broilers administered BL+A or SYN tended to be greater when compared to those administered DFM alone. Neither the positive or negative control was distinguishable from the treated groups. Functional feed additives are capable of increasing total antioxidant capacity in broilers [337]. Administration of phytogenic preparations and synthetic antioxidants as functional feed additives has been demonstrated previously to

increase serum antioxidant capacity in addition to reducing lipid oxidation in broiler meat [338-340]. *Saccharomyces*-derived MOS have been reported scavenge reactive oxidative radicals and exhibit anti-mutagenic activity *in vitro* [341]. Thus, the yeast-derived MOS administered in BL+A and SYN treatments likely contributed to the increased antioxidant capacity compared to BL alone. When administered to poultry, yeast or MOS has been reported to increase the activity of antioxidative enzymes including catalase and glutathione peroxidase and other antioxidants in blood [342, 343]. An increase in antioxidant capacity may be beneficial to the immune response by mitigating inflammation in the gastrointestinal tract. Reactive oxygen species in the mucosa cause inflammation which hinders digestion and absorption of nutrients [344]. Additionally, oxidative stress increases lipid peroxidation which can induce metabolic disturbances [345]. By increasing the capacity to mitigate influxes in reactive species, broilers are better able to tolerate disease and environmental stressors that would otherwise cause oxidative damage to lipids, proteins, or tissues [346].

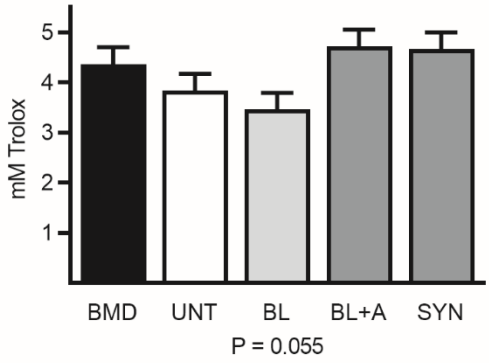

Figure 5.3. Serum antioxidant capacity of broiler chickens. Serum was separated from whole blood and collected at 21 d post-hatch. Antioxidant capacity is reported as the treatment mean ± SEM mM trolox equivalents from 9 UNT broilers or 10 broilers for all other treatments. Means not sharing common letters differ significantly ($P \leq 0.05$).

In this study, we investigated the effects *Bacillus licheniformis* and its co-administration with functional additive blends consisting of dietary prebiotics and phytogenic preparations in broiler chickens. Administration of direct-fed *B. licheniformis* alone and in conjunction with functional additives improves performance parameters, gastrointestinal health, and intestinal morphology. Feed conversion ratio was lower for broilers administered direct-fed *B. licheniformis* as compared to untreated broilers through 14 d post-hatch. Co-administration of DFM with functional additives decreased counts of *Clostridium perfringens* compared to the untreated control and increased counts of *Lactobacillus* spp. compared to antibiotic treated broilers. Administration of Direct-Fed *B. licheniformis* was observed to increase the villus height crypt to depth ratio (VH:CD) compared to the untreated control, whereas co-administration of DFM with functional additives increased VH:CD ratio compared to both the untreated and antibiotic treated control. Direct-Fed *B. licheniformis* and functional feed additives are able to improve feed efficiency, promote positive shifts in populations of gastrointestinal microbiota, and improve measures of gastrointestinal function. Although their independent contributions improve performance and health metrics, there is not sufficient data to indicate a synergistic relationship between *Bacillus licheniformis* and functional additives in broiler production.

5.4 CONCLUSIONS AND APPLICATION

1. Administration of *Bacillus licheniformis* decreased FCR to levels statistically similar to an antibiotic control compared to untreated feed.
2. Functional feed additives promote a healthier GI microbiota by decreasing *Clostridium perfringens* and increasing total Lactic Acid Bacteria.
3. *Bacillus licheniformis* and functional additives improve measures of gut function (VH:CD)

6. CONCLUSIONS

6.1 CONCLUDING REMARKS

Previous research has supported the use of functional feed additives to improve animal performance, however their application and mode of action remains poorly understood. By characterizing mechanisms important to the functionality of these additives, they can be better applied to solving the health and nutritional challenges in commercial poultry production. In this dissertation, we characterized BSH activity in poultry *Lactobacillus*. Our findings suggested BSH activity may increase survivability and growth in the gastrointestinal tract, which may contribute to improved probiotic functionality when administered to poultry. We also investigated the inclusion rate and formulation method for $Cu(HMTBa)_2$ on the GI microbiota. Our results suggest both inclusion rate and formulation method for $Cu(HMTBa)_2$ affect *Clostridium* and LAB populations, potentially contributing to the additional benefits associated with copper supplementation. Targeted intermittent administration of LAB was investigated. We have demonstrated that intermittent administration mitigated the decrease in LAB counts over feed changes and improves measures of gut health. Targeted administration of functional additives during periods of gastrointestinal stress may mitigate fluctuations in the microbiota, preserve epithelial structure and improve feed efficiency. Lastly, we demonstrated *Bacillus licheniformis* alone and in conjunction with other functional feed additives promotes gut health and improves performance. However, there was not sufficient data to suggest a synergistic effect among functional additives. Functional additives can be used in poultry production to improve gut health, animal performance, and promote positive shifts in the microbiota. Formulation method, rate of dietary inclusion, administration method, and co-administration all contribute to the role of these additives in poultry and must be considered to maximize their performance when applied in poultry production.

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