# PROBIOTIC AND GASTROINTESTINAL MICROBIOLOGY IN THE BROILER CHICKEN

A Dissertation

by

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## ABSTRACT

Probiotics and prebiotics are used widely because of their benefits to digestive and immune health. While there is significant evidence to support their effectiveness in humans and livestock animals, interpretation of the results of this research is complicated by the wide differences in research. We have explored host-specific digestive physiology, experimental constraints, and probiotic and prebiotic functionality. The insight provided by an understanding of these important differences will provide a context in which results of host-specific studies and their broader implications to the science can be evaluated.

*Lactobacillus* species are common inhabitants of the gastrointestinal tract and are widely used as probiotics because of their health promoting benefits. When used as Direct Fed Microbials (DFM) in poultry, they have been demonstrated to promote growth, stimulate immune responses, and reduce intestinal colonization of pathogens. While they are used widely, the mechanisms responsible for their functionality are not well understood. While genetic tools available for use in lactobacilli are advanced, they have not been applied to investigate the probiotic functionality of *Lactobacillus* cultures in poultry. The objective of this study is to demonstrate the functionality of the pORI28 system in *L. gallinarum* ATCC 33199 by insertional inactivation of *lacL*, encoding β-galactosidase. The establishment of an effective chromosomal integration system for *L. gallinarum* will provide a platform for functional genomic analyses to investigate the functionality of this model probiotic culture in poultry.

DFM and exogenous enzymes have been demonstrated to improve growth performance in poultry and are potentially important alternatives to antibiotic growth promoters (AGP). We investigated the administration of a feed additive composed of a DFM products and enzymes in broiler chickens over a 42-day growth period. Evaluation of growth performance determined feed efficiency of broiler chickens which were administered the feed additive was comparable to those fed a diet containing AGPs. Characterization of the gastrointestinal microbiota using culture-dependent methods determined administration of the feed additive increased or decreased counts of bacteria enumerated from the gastrointestinal tract of the broiler chicken. Our results suggest the administration of DFMs and exogenous enzymes may potentially be an important component of antibiotic free poultry production.

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## CONTRIBUTORS AND FUNDING SOURCES

### Contributors

This work was supervised by a dissertation committee consisting of Professors Dr. Tri Duong, Dr. Jason Lee, Dr. Giridhar Athrey and Dr. David Caldwell of the Department of Poultry Science and Professor Dr. Allen Byrd of the United States Department of Agriculture.

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## 1.1 INTRODUCTION

Research in the application of probiotics and prebiotics in human and animal health has grown due to the increased interest in the gastrointestinal microbiota. The microbial community present in the gastrointestinal tract is thought to play an important role in host animal health and is a potentially important therapeutic target that can be manipulated in order to achieve positive health outcomes. Thus, the use of probiotics and prebiotics represents a powerful strategy for the manipulation of the microbial community in the gastrointestinal tract (1-4).

Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host (5), and prebiotics are selectively fermented ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota conferring benefit(s) upon host health (6). The benefits of probiotic and prebiotic application in humans and animals include inhibition of pathogens (7, 8), improved digestive function (9, 10), and modulation of immune responses (11-13). In animal agriculture, probiotics and prebiotics are thought to be an important potential al-

[1] Reprinted with permission, Tyler Edward Askelson and Tri Duong. "Chapter 30. Perspectives on Differences Between Human and Livestock Animal Research in Probiotics and Prebiotics." *Probiotics and Prebiotics: Current Research and Future Trends.* Ed. Koen Venema, Ed. Ana Paula do Carmo. Norfolk: Caister Academic Press, 2015. 447-458. Print.

ternative to the use of antibiotic growth promoters (AGP) (14, 15). When used in livestock, they have been demonstrated to promote growth performance at levels similar to AGPs (16, 17) and reduce gastrointestinal colonization by pathogens (18).

The benefits of probiotic and prebiotic use in humans and livestock animals have been well demonstrated (**Table 1.1**). However, the overall effectiveness of their application is thought to be mixed (19), and results of their use in livestock animals are perceived by some to be superior when compared to humans. A direct comparison of the overall effectiveness of probiotics and prebiotics between studies in humans and livestock animals, and between livestock animal species, is complicated by differences in the digestive physiology of the host animal, experimental constraints, and the desired experimental outcomes. Indeed, such a direct comparison may be inappropriate and irrelevant. An appreciation of host-specific differences and interpretation of research in this context will allow host-specific studies to contribute to an improved understanding of probiotic and prebiotic functionality for application in both human and animal health

**Table 1.1.** Examples of probiotic and prebiotic benefits

| **Type** | **References** |
|---|---|
| Digestion | |
| Human | (20)<br>(21)<br>(22) |
| Chicken | (23)<br>(24) |
| Cattle | (25)<br>(26)<br>(27) |
| Pathogen Inhibition | |
| Human | (28)<br>(29) |
| Pig | (18) |
| Chicken | (30) |
| Immunomodulation | |
| Human | (31)<br>(13)<br>(32) |
| Pig | (12) |
| Chicken | (33) |
| Cattle | (34)<br>(27) |

## 1.2 DIGESTIVE PHYSIOLOGY AND MICROBIOLOGY

The autochthonous gastrointestinal microbiota of humans and animals is complex and dynamic with hundreds of microbial species coexisting in a web of interdependency and antagonism, both with each other and the host animal (35-37). Host factors including age (38, 39) and diet (38, 40) have been demonstrated to affect the composition of the gastrointestinal microbiota. Thus, introduction of allochthonous microbial species or specific dietary components to these already complex environments can cause shifts in the composition of the microbial community (41, 42) and potentially produce beneficial or negative effects on the host animal.

### *1.2.1 Ruminant and Monogastric Animals.*

The anatomic features and physiology of the digestive tract vary widely between humans, swine, cattle, and chickens (**Figure 1.1**). As a result of these host-animal adaptations to dietary niches, not only is the gastrointestinal microbial community significantly different between species (37, 43), but the digestive organs of primary research interest is species-specific as well (**Table 1.2**). While the predominant bacterial genera may be similar between host animal species, the microorganisms vary at the species and subspecies levels (44, 45). Variation in the composition of the gastrointestinal microbial community between individuals of the same species is also significant (38). Thus, it is important to consider the interactions of probiotic cultures and prebiotics with the host-animal, the autochthonous microbiota, and the nutrition of the host animal.

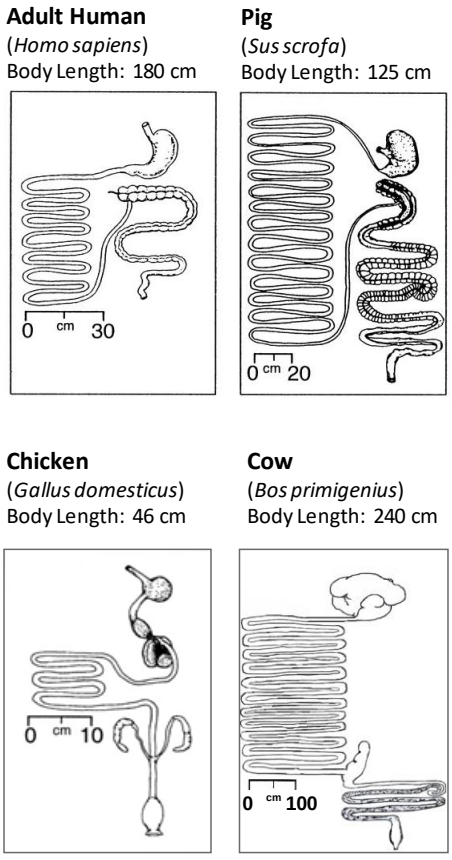


**Figure 1.1. Digestive anatomy.** Anatomic features of the human, pig, chicken, and cattle. [Human and chicken adapted from *Comparative Physiology of the Vertebrate Digestive System* with the permission of Cambridge University Press. Copyright (1995) Cambridge University Press. Pig adapted from the *American Journal of Physiology* (46). Copyright 1976 American Physiological Society].

**Table 1.2.** Features of relevant digestive organs*

| Host | Organ | Cells $g^{-1}$ or Cells $mL^{-1}$ | Organ Contents | References |
|---|---|---|---|---|
| Human | Colon | $3.2 \times 10^{11}$ | 220 g | (47) (48) |
| Pig | Colon | $5.4 \times 10^{10}$ | 9 L | (49) |
| | Cecum | $2.8 \times 10^{10}$ | 1 L | (50) |
| Chicken | Cecum | $9.5 \times 10^{10}$ | 2 g | (51) |
| Cattle | Rumen | $2.1 \times 10^{10}$ | 106 L | (52) (53) |

*Adapted from *Proceedings of the National Academy of Sciences of the United States of America* (54) with permission of the publisher. Copyright (1998) National Academy of Sciences, U.S.A.

The microbiota of cattle and other ruminant animals is considerably complex (55) when compared with monogastric animals. The bovine rumen is populated by microorganisms which ferment indigestible plant material producing volatile fatty acids (VFA) and microbial crude protein for absorption by the host animal (55). The predominant microorganisms found in the rumen include the Firmicutes *Butyrvibrio* and *Ruminococcus* and the Bacteroidete *Prevotella* (37), while the predominant microorganisms of the lower intestines include strict anaerobic species from the genera *Bifidobacterium*, *Clostridium,* and *Bacteroides* (56). Additionally, differences in microbial composition of the rumen have been demonstrated between beef and dairy cattle. Beef cattle are populated by greater numbers of cellulytic bacteria including *Fibrobacter, Ruminococcus,* and *Succiniclasticum,* likely reflecting the need for greater fiber digestion of hay-fed beef cattle (37, 38). While *Prevotella* are the most abundant genus in the rumen of cattle, they are present in lower abundance in beef cattle than in dairy cows (38).

Differences between chickens, humans, and swine demonstrate interspecific variation in the gastrointestinal microbiota of monogastric animals. The chicken ileum is populated largely with *Lactobacillus*, followed distantly by *Clostridium* and *Enterococcus,* while *Clostridium* is more prevalent than *Lactobacillus* in the cecum (44). In monogastric mammals, the human intestinal tract is dominated by *Bacteroides*, *Clostridium*, and *Bifidobacterium* (43, 45, 57), while *Lactobacillus*, *Streptococcus*, and *Selenomonas* (58) are the predominant microbes in swine.

The administration of prebiotics has been demonstrated to stimulate growth of beneficial microorganism in the gastrointestinal tract (59-61). However, the selectivity

of specific prebiotic compounds and the host animal species should be considered. A study of prebiotic administration in poultry found *Lactobacillus salivarius* and *Lactobacillus acidophilus* to be the predominant lactobacilli in chickens administered fructooligosaccharide (FOS), while *Lactobacillus reuteri* was most prevalent in chickens administered mannanoligosaccharide (MOS) (59). However, a human study found consumption of fructooligosaccharide resulted in an increase of *Bifidobacterium angulatum* (62). Additionally, host-specific differences in gastrointestinal transit time may limit the effectiveness of prebiotics. The effectiveness of prebiotics may be reduced in animals with shorter transit times due to the incomplete utilization of prebiotic substrates by gastrointestinal microorganisms (63).

## 1.3 HOST SPECIFIC EXPERIMENTAL CONSTRAINTS

The limitations placed on research performed in livestock in animals are different from those placed on research in humans. The effects of differences in these important experimental constraints likely contribute to perceptions of the effectiveness of probiotic and prebiotic application. Experimental conditions can be controlled more stringently in livestock animals than with human subjects. Thus, the confounding effects of uncontrolled or uncontrollable variables can be limited, reducing overall experimental variation and increasing experimental power.

### *1.3.1 Experimental Subjects*

The nature of research subjects is an obvious factor that affects experimental design and results from probiotic and prebiotic research. Selective breeding of production livestock animals such as broiler chickens and Holstein cows has produced homogenous

in-bred genetic lines with increased uniformity and production potential (64). As a result, homogeneity between livestock animal subjects is greater than in human subjects. An early study evaluating oligofructose administration in humans used 8 subjects, 7 men and 1 woman, ranging in age from 21 to 48 (65). A meta-analysis of sixteen studies investigating the effects of probiotic and prebiotic administration on lipid levels in humans (66) further demonstrates the wide variations in the gender composition, age range, and number of subjects that can be seen in human trials. A study of probiotic reduction of diarrhea in weaning piglets used 256 piglets only days apart in age with an even gender distribution. Probiotic and prebiotic studies in chickens are able to use up to thousands of animals of the same age reared under identical experimental conditions (67-69). Thus, experimental animals are typically nearly identical in age and have far greater size, weight, and genetic uniformity when compared to their human counterparts.

*1.3.2 Protocol Compliance*

Strict adherence to experimental protocols is necessary for the control of variables and their influence on results. These variables are more easily maintained in livestock animals than with human subjects. Research animals are typically housed in facilities where environmental conditions including lighting, temperature, and access to food and water can be controlled. However, human volunteers are not typically subjected to similarly rigorous constraints. The host diet is a particularly important factor that must be considered in studies of probiotic and prebiotic effectiveness because of its effect on the gastrointestinal microbiota. Replicate groups of research animals are pen fed experimental rations specifically formulated for the study (23, 69), while the diet of human

subjects is not nearly as easily controlled. Additionally, compliance with prescribed probiotic and prebiotic dose is more easily maintained for experimental animals than with human subjects. The removal of subjects found violating experimental protocols further reduces experimental power (70, 71).

*1.3.3 Ethical Considerations*

Significant interest in the potential of probiotics and prebiotics to prevent and treat disease has developed. The differences in ethical constraints and their effects on experimental design and study results must be considered when evaluating probiotic and prebiotic effectiveness (4). When evaluating the potential of probiotics and prebiotics to prevent infection in livestock animals, direct challenge studies in which experimental subjects are administered infectious to lethal doses of pathogenic microorganisms can be performed (72, 73). However, research in human subjects must rely on natural infection (32, 74) or direct challenge using attenuated pathogens (75). Alternatively, challenge experiments may be performed using rodents (72, 76) as intermediate models prior to natural infection experiments in humans.

The health of human subjects and severity of the potential infection are additional factors for consideration in natural infection studies and an additional complication over research in livestock animals. Two particularly interesting studies serve as examples highlighting the varying effectiveness of studies performed in healthy as compared to ill adults. A study using healthy human volunteers performed during the common cold season found consumption of a probiotic cocktail containing *Lactobacillus gasseri* PA 16/8, *Bifidobacterium longum* SP 07/3, and *Bifidobacterium bifidum* MF

20/5 reduced the duration and severity of cold symptoms (77). A study evaluating the potential of probiotics to prevent infectious complications in patients with severe pancreatitis found no difference in the incidence of infectious complications (78). However, the number of deaths was almost 3 times greater in the probiotic treated group than in the placebo group. It has been reported that known complications of the experimental probiotic treatment were not disclosed to the study subjects (79).

## 1.4 PROBIOTIC AND PREBIOTIC FUNCTIONALITY IN HUMANS AND LIVESTOCK

Probiotics and prebiotics are used in order to derive a wide array of health benefits for the human or livestock animal host including increased resistance to intestinal pathogens, improved immune health, and improved digestive function. The specific functionalities which provide these benefits are similar regardless of host species. However, the outcomes desired from probiotic and prebiotic use and the corresponding experimental endpoints used to measure their effectiveness are not universal across host species. Additionally, while improved health is the primary motivation for the use of probiotics and prebiotics in humans, their use in livestock animals is motivated primarily by the economic need for improved livestock production and performance parameters.

### *1.4.1 Digestion and Metabolism*

Consumption of probiotics and prebiotics has long been known to exert positive effects on digestion and digestive function in humans and animals. These general benefits to digestion and their contribution to nutrition and general quality of life are perhaps

the most widely understood. However, their application in digestion is continuing to expand, and novel applications of this functionality are being explored. Additionally, the effects of probiotics and prebiotics on host animal metabolism have only recently begun to be understood and represent an additional novel application area in human and animal health.

Probiotics have been demonstrated to improve digestibility of food, reducing negative effects of maldigestion while increasing the available nutrient content. Lactose intolerance is a common maldigestive disorder in humans. After the consumption of milk and dairy products, fermentation of undigested lactose by microorganisms in the large intestine causes discomfort to the host. The production of β-galactosidase by probiotic cultures in yogurt has been demonstrated to improve lactose digestion and tolerance (21, 80). Consumption of unfermented milk containing *B. longum* B6, *B. longum* ATCC 15708 (81), and *L. acidophilus* N1 (22) have also been demonstrated to improve lactose digestion. Phytic acid is a phosphorus source in livestock animal feeds (82, 83) that is underutilized in non-ruminant livestock due to its poor digestibility (84-86). Additionally, phytic acid exerts anti-nutritive effects through strong chelation of divalent cations. Administration of phytate-degrading *Lactobacillus* was demonstrated to improve weight gain of broiler chicks fed a phosphorous-deficient diet to a level comparable to those fed a phosphorus-adequate diet (**Figure 1.2**) (23). In addition to increasing bioavailability of phytate-phosphorus in monogastric livestock animals, phytate-degrading probiotic cultures may be useful in correcting malabsorption syndrome in human vegetarians (87).

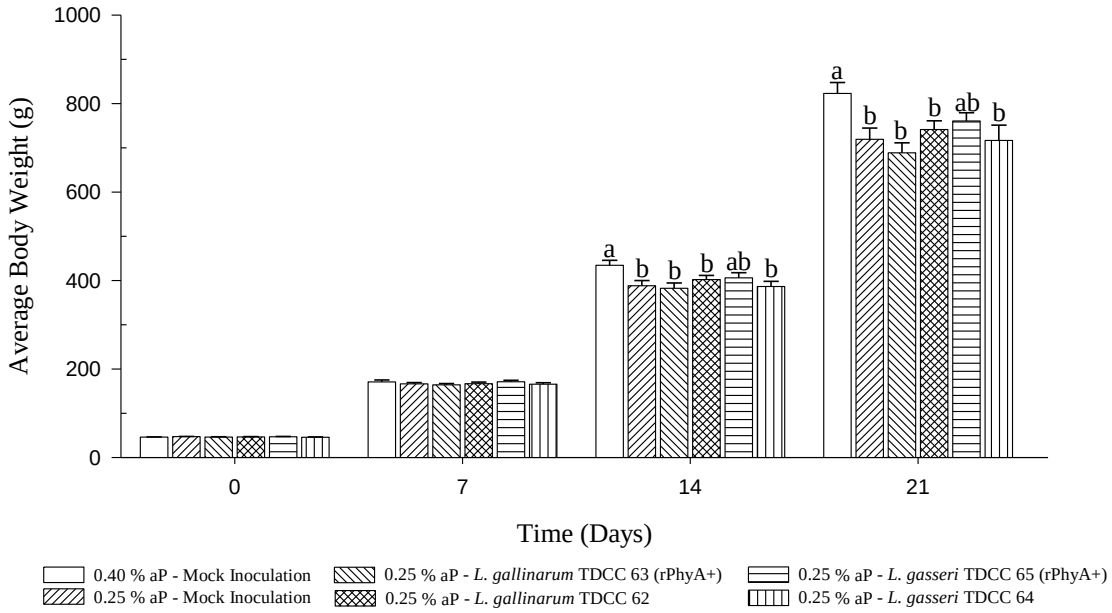


**Figure 1.2. Phytate-degrading *Lactobacillus* improves growth of broiler chickens.** Broiler chickens were fed a phosphorus adequate control diet (0.40% aP) and administered a mock inoculation, or fed a phosphorus deficient diet (0.25% aP) and administered either a mock inoculation (MRD) or cultures of *L. gallinarum* TDCC 63 (rPhyA$^+$), *L. gallinarum* TDCC 62 (Empty Vector), *L. gasseri* TDCC 65 (rPhyA$^+$), or *L. gasseri* TDCC 64 (Empty Vector). Data shown are the mean body weight ± SEM of treatment groups. Different letters indicate means are significantly different ($P < 0.05$). [Adapted from *Applied and Environmental Microbiology* (Askelson et al. 2014)] with permission of the publisher. Copyright (2014) American Society for Microbiology)].

Improved digestive function is an area that has seen significant increases in product advertising and attention in popular press. Administration of *Bifidobacterium animalis* DN173010 shortened intestinal transit time in women (88). Consumption of a probiotic beverage containing *Lactobacillus casei* Shirota (89) improved gastrointestinal symptoms in patients with chronic constipation. Additionally, a study in children found treatment with *Lactobacillus casei rhamnosus* Lcr35 (90, 91) to have similar efficacy to and causing less abdominal pain than a magnesium oxide laxative.

Results of several studies suggest probiotics and prebiotics can affect lipid metabolism and potentially reduce risk factors of coronary disease in humans. Probiotics have been reported to reduce serum cholesterol (92). While the mechanism is not clearly understood, it has been hypothesized that the probiotic microorganisms may metabolize cholesterol and bile salts (66). In two separate studies, consumption of fermented milk containing *L. acidophilus* L1 reduced serum cholesterol (20). Similarly, serum low density lipoprotein (LDL) cholesterol was reduced by 5.4% in male volunteers who consumed yogurt containing two *L. acidophilus* strains (DN 112.053 and DN 112.096) and FOS (93).

While interest in using probiotics and prebiotics to alter lipid metabolism is not limited to humans, interest in livestock production is driven by production concerns. Excess fat deposition in broiler chickens is undesirable to producers because of reduced carcass yield and to consumers that prefer a leaner product. A probiotic mixture contain-

ing 12 *Lactobacillus* strains improved body weight gain and feed conversion and reduced abdominal fat deposition in broiler chickens (94). Similar benefits of probiotic administration have also been demonstrated in egg production. *Pediococcus acidilactici* MA18/5M supplementation in egg-laying hens reduced cholesterol content and increased concentrations of polyunsaturated fatty acids, including linoleic acid and linolenic acid, in egg yolks (95). A probiotic product containing *L. acidophilus* NP51 and *Propionibacterium freudenreichii* NP24 increased milk fat percentage when given to dairy cattle (96). While similar lipid increasing effects were seen from *Bacillus subtilis* natto in a separate study (97). Increasing, rather than decreasing milk fat percentage is desirable to dairy producers because of downstream processing needs.

*1.4.2 Pathogen Inhibition*

The ability of probiotics and prebiotics to reduce colonization of bacterial pathogens in the gastrointestinal tract is an important functionality useful for both human and animal health. Probiotic microbes are able to inhibit pathogens in the gastrointestinal tract through several mechanisms. Probiotic microorganisms competitively exclude pathogens from attachment to mucosal surfaces through competition for shared binding sites (98) and steric interference of protein adhesins located on the surface of pathogenic bacteria (99, 100). In addition to preventing adhesion, an *in vitro* study demonstrated the varying ability of *L. acidophilus* TMC 0356 and *Lactobacillus rhamnosus* TMC 0503 to displace *Salmonella* Typhimurium, *Cronobacter sakazakii*, *Clostridium difficile*, and *Escherichia coli* which were already adherent to human epithelial cells (29). Inhibition of pathogen adherence is also seen in a pig intestinal mucosa model (101). These studies

demonstrate probiotic strain and host specific inhibition of pathogens, highlighting need for case-by-case selection of probiotic cultures to reduce adherence of specific pathogens.

The production of pathogen inhibiting compounds is a well understood probiotic mechanism (102). Neal-McKinney et al. (103) demonstrated that the production of lactic acid by *Lactobacillus* cultures to be an important mechanism for the reduction of *Campylobacter jejuni* in livestock animals (**Figure 1.3**). Hydrogen peroxide production by *Lactobacillus* has also been shown to inhibit *Salmonella* (104). *Campylobacter* (105) and *Salmonella* (106) are common inhabitants of the gastrointestinal tract of poultry and important human foodborne pathogens. The use of probiotics and prebiotics to reduce incidence of these organisms is motivated primarily by public health and food safety concerns rather than for the benefit of the animal. The production of bacteriocins by probiotics has the potential to prevent gastrointestinal infection in humans. A direct challenge study in mice demonstrated that bacteriocin production by *Lactobacillus salivarius* UCC118 reduced counts of *Listeria monocytogenes* by 80 % in the liver and spleen of infected mice relative to a negative control (**Figure 1.4**) (28). Additionally, while *L. salivarius* UCC118 also protected mice from infection by *Salmonella* Typhimurium, the protection was not bacteriocin mediated. While bacteriocin production by probiotic cultures is hoped to be an important alternative to antibiotics in the treatment of bacterial infections, the effectiveness of this mechanism has not yet been evaluated in humans.

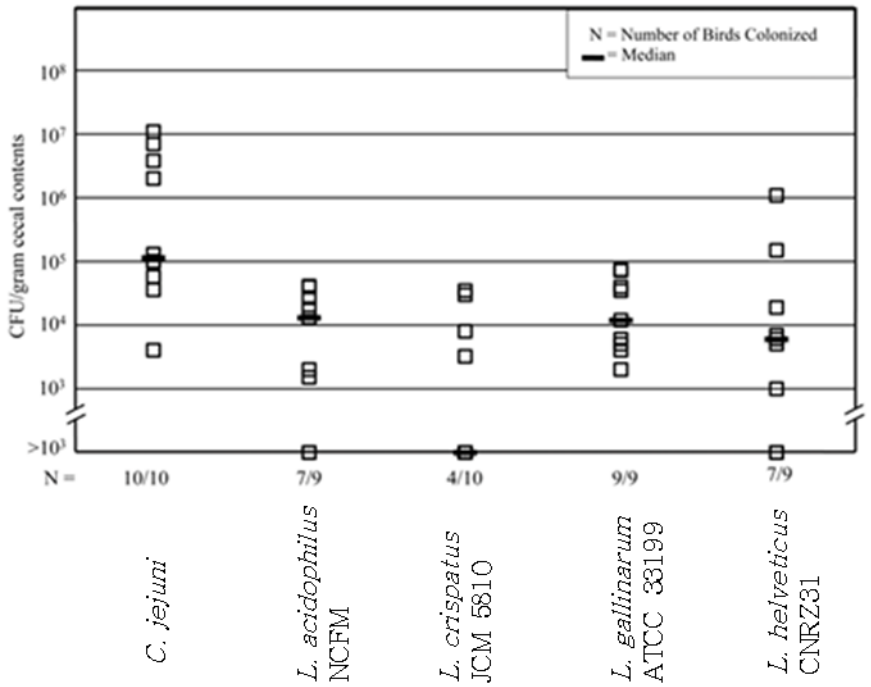


**Figure 1.3. Probiotic *Lactobacillus* cultures reduce *Campylobacter jejuni* colonization of broiler chicks.** *Campylobacter* was enumerated from the cecum of broiler chicks inoculated with probiotic *Lactobacillus* cultures or a mock inoculation and experimentally challenged with *C. jejuni*. [Adapted from PLOS ONE (103) under the terms of the Creative Commons Attribution License. Copyright (2012) Neal-McKinney *et al.*].

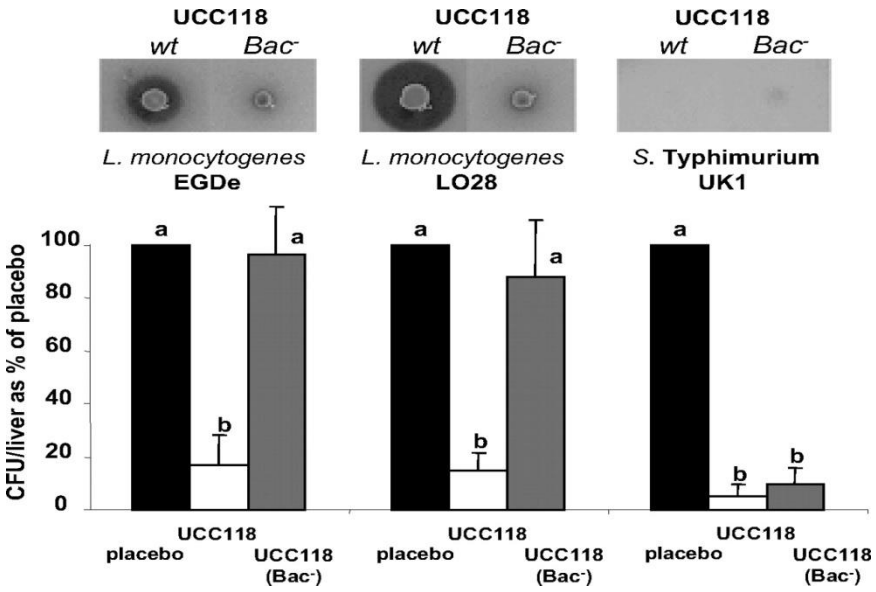


**Figure 1.4. *Lactobacillus salivarius* UCC118 bacteriocin Abp118 mediates protection against *Listeria monocytogenes* infection.** Upper, well diffusion assay. Wild type (*wt*) and Abp118 deficient (*Bac*$^-$) *L. salivarius* UCC118 cultured in media seeded with *L. monocytogenes* and *Salmonella* Typhimurium. Lower, pathogens were enumerated from the liver of mice administered a placebo (filled bars), wild-type *L. salivarius* UCC118 (open bars), or Bacteriocin deficient (*Bac*$^-$) *L. salivarius* UCC118 (gray bars) and infected with *L. monocytogenes* EGDe, *L. monocytogenes* LO28, or *Salmonella* Typhimurium. Different letters indicate means differ significantly ($P < 0.001$; n = 5). [Reprinted from the *Proceedings of the National Academy of Sciences of the United States of America* (28) with permission of the publisher. Copyright (2007) National Academy of Sciences, U.S.A.].

*1.4.3 Immune Modulation*

Significant interest has grown in the immune modulating effects of probiotics and prebiotics. Probiotics are able to directly stimulate immune responses and regulate inflammation in a strain specific manner. While prebiotic immunomodulation is thought to occur primarily in collaboration with commensal microorganisms (33), there is evidence suggesting that carbohydrate polymers can directly interact with cells of the immune system (107).

In addition to the direct action against pathogens described previously in this work, probiotics have been demonstrated to help protect the host from pathogens by enhancing host defenses. The probiotic cocktail VSL #3 improved epithelial barrier function and prevented *Salmonella* invasion of epithelial cells (108), while *L. casei* CHCC3139 stimulated the production of cytokines responsible for cell-mediated immunity (109, 110). A synbiotc combination of a commercial direct fed microbial (Milkibeef Top, Trouw Nutrition) containing *Enterococcus faecium* and prebiotic lactulose increased mRNA expression of IgA Fc receptor when administered to calves (34). Human infants fed formula containing FOS or galactooligosaccharide (GOS) had greater fecal secretory IgA than infants fed control formula (111).

Probiotic cultures including *Lactobacillus* species (112, 113) and prebiotics including mannanoligosaccharide (114) have been demonstrated to reduce expression of inflammatory cytokines. Management of inflammatory bowel disorders (115) and atopic allergy are areas where the anti-inflammatory effects of probiotics and prebiotics show promise. Several studies demonstrate the potential of probiotic bacteria (116, 117) and

yeast (118) to induce remission of active ulcerative colitis. Atopic diseases including atopic eczema, allergic asthma, and allergic rhinitis are allergic hypersensitivities. Immunomodulatory probiotics may be useful in preventing or reducing the severity of atopic disease (31). One double blind placebo controlled study observed diminished clinical signs and symptoms of atopy in infants receiving *L. rhamnosus* GG and *Bifidobacterium lactis* BB-12 (119). Oral bacteriotherapy with *L. rhamnosus* 19070-2 and *L. reuteri* DSM 122460 reduced proinflammatory eosinophil cationic protein in older children with atopic dermatitis (13). While probiotics and prebiotics may exhibit anti-inflammatory properties in livestock animals, management of chronic intestinal inflammation and atopic disease is likely to be more applicable in humans than in livestock.

## 1.5 FUTURE PERSPECTIVES

### *1.5.1 Diarrheal Disease*

The morbidity and mortality in humans (120, 121) and losses to livestock production (122, 123) due to bacterial and viral enteric disease is significant, making the reduction of diarrheal disease in humans and livestock an important global health objective. The combined pathogen inhibiting and immune stimulating functionality of probiotics and prebiotics suggests they may be able to contribute to achieving this important goal. Nosocomial infectious diarrhea is an important problem in children, prolonging hospital stays and increasing medical costs (124). Several studies have demonstrated the ability of probiotic *L. rhamnosus* GG to reduce the incidence of diarrhea in children (32, 125). Additionally, the efficacy of several probiotics in preventing antibiotic-associated diarrhea (126) and Traveler's Diarrhea (127) has also been shown. Similar reduction of

diarrhea in swine and cattle have also been demonstrated (128). Administration of probiotic *Bacillus* cultures reduced post-weaning diarrhea syndrome related mortality (12) in piglets. Additional studies have shown probiotic-mediated reduction of diarrhea and complications from pathogenic *E. coli* in piglets (129, 130) similar to *L. rhamnosus* GG in infants.

*1.5.2 Agricultural Sustainability*

Rapid growth in the use of renewable biofuels has led to a reallocation of arable land from food to fuel ethanol production (131) and forced livestock producers to becoming increasingly reliant upon secondary feedstocks that are poorly digested with lower available nutrient content. The ability of probiotics to increase digestibility and nutrient utilization from poorly digested feed constituents through biocatalysis (23) in the gastrointestinal tract will become important as feed costs continue to increase. Additionally, the immune stimulating functionality of probiotics and prebiotics will continue to be important in disease management and potentially reduce losses in livestock production (132-134). Thus, by increasing efficiency and overall productivity, probiotics and prebiotics have the potential to make important contributions to agricultural sustainability and global nutritional security.

*1.5.3 AGP Alternatives*

AGPs have been widely used to increase weight gain (135), improve feed efficiency (136, 137), and reduce mortality in livestock animal production (138, 139). Regulatory and consumer concerns over the development of antibiotic resistant microbes have led to a decline in the non-therapeutic use of antibiotics (140). However, the reduction of

their use has led to reduced livestock productivity and increased disease in production animals. Supplementation of livestock feed with probiotics and prebiotics has been demonstrated to improve growth performance to levels similar to antibiotics, thus they represent an important alternative to the use of AGPs in livestock production (15). However, while the use of probiotic cultures with growth promoting properties are desirable in livestock animal production, their use in humans is undesirable due to the growing worldwide obesity epidemic.

*1.5.4 Vaccine and Anti-infective Delivery*

Because of their long history of safe use, immune stimulating functionality, and importance in human and animal health, probiotic microorganisms have received considerable interest as potential vectors for the delivery of vaccines antigens to mucosal surfaces (141). An increasing number of studies have investigated the potential of live probiotic microorganisms as vaccines with several of examples in which they have elicited antigen specific immune responses (**Table 1.3**). Additionally, "bioshield" strategies in which probiotic microorganisms confer passive immunity through expression of receptor proteins or antibody fragments are also being explored (141). The use of recombinant probiotic cultures for this novel functionality is expected to be widely applicable in both human and animal health and for improving food safety as well.

**Table 1.3.** Examples of vaccines delivered by probiotic microorganisms

| Organism | Antigen | Host | References |
|---|---|---|---|
| *Lactobacillus acidophilus* NCFM | *B. anthracis* PA | Mouse | (142) |
| | HIV-1 Gag | Mouse | (143) |
| *Lactobacillus casei* ATCC 393 | CSFV epitope 290 Parvovirus VP2 | Pig | (144) |
| *Lactobacillus plantarum NCIMB8826* | Dust mite allergy Derp p 1 | Mouse | (145) |
| | *C. tetani* TTFC | Mouse | (146) |
| *Lactococcus lactis* E7 *Lactococcus lactis* IL-12 | HPV E7 | Mouse | (147). |
| *Lactococcus lactis* NZ9000 | Avian influenza HA | Chicken | (148) |
| *Pichia pastoris* SMD1168 | CSFV glycoprotein E2 | Pig | (149) (150) |
| *Pichia pastoris* KM71H | *C. perfringens* α toxin | Chicken | (46) |

# 2. TARGETED GENE INACTIVATION IN *LACTOBACILLUS GALLINARUM* ATCC 33199 USING CHROMOSOMAL INTEGRATION

## 2.1 INTRODUCTION

*Lactobacillus* species are important inhabitants of the gastrointestinal tracts of humans and livestock animals and are often used as probiotics because of their health promoting properties (151). Probiotics are live microorganisms, which when administered in adequate amounts confer a health benefit on the host (152). Often administered as Direct-Fed Microorganisms to poultry and other livestock animals, probiotic *Lactobacillus* have been demonstrated to stimulate immune responses (153, 154), reduce colonization of human food-borne pathogens including *Campylobacter (155)* and *Salmonella* (73), and improve growth performance at levels similar to antibiotics (156, 157). Additionally, interest in the use of probiotics in livestock animal production has grown because of increased consumer and regulatory pressure to reduce sub-therapeutic use of antibiotics (158). Although they are used widely in livestock animal production, the mechanisms responsible for the benefits of probiotic administration are not well understood.

*Lactobacillus gallinarum* ATCC 33199, originally isolated from the crop of a chicken, is a potentially important model organism for investigating mechanisms of probiotic functionality in poultry. *L. gallinarum* ATCC 33199 has been demonstrated to adhere effectively to the chicken LMH epithelial cell line *in vitro* and to be a strong colonizer of the gastrointestinal tract of broiler chickens *in vivo* (159). Administration of *L.*

*gallinarum* ATCC 33199 has been also demonstrated to reduce colonization by *Campylobacter jejuni* in experimentally challenged broiler chickens (160). Recently, the genome sequence for *L. gallinarum* ATCC 33199 (161) has been made available and is expected to provide genomic insights into the beneficial functionalities of *Lactobacillus* in the gastrointestinal tract of poultry. Additionally, the ability to be genetically transformed readily using electroporation and recombinant expression of heterologous proteins has been demonstrated in *L. gallinarum* ATCC 33199 (23). Although the genetic tractability of this organism has been demonstrated, the ability to construct targeted isogenic mutants will also be required to investigate the role of specific genes and gene products in the probiotic functionality of this organism.

The pORI28-plasmid system, based on the broad-host-range lactococcal pWV01 replicon (162), has been used widely for the targeted insertional inactivation of genes in Lactic Acid Bacteria (LAB). Targeted gene inactivation using pORI28-based plasmids is dependent upon the integration of a non-replicating plasmid containing an antibiotic resistance cassette into the host chromosome by homologous recombination. This has been demonstrated to be an efficient method for the construction of isogenic gene knockout mutants in several *Lactobacillus* species and has provided important insight into carbohydrate metabolism (163, 164), epithelial cell adhesion (165), and bile stress response of *Lactobacillus* species (166, 167). In this study, we investigated insertional inactivation of *lacL*, encoding β-galactosidase, in order to demonstrate proof-of-principle of the ef-

fectiveness of the pORI28 system in *L. gallinarum* ATCC 33199 and to provide a platform to enable functional genomic analyses investigating mechanisms of probiotic functionality in the gastrointestinal tract of poultry using this organism.

## 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 gallinarum* strains were cultured in 10% $CO_2$ at 37°C using de Man, Rogosa, and Sharpe (MRS) (Becton Dickinson, BD, Franklin Lakes, New Jersey) medium supplemented with 5 μg $mL^{-1}$ erythromycin (Erm) (EMD Chemicals, Inc., San Diego, CA) or 5 μg $mL^{-1}$ chloramphenicol (Cam) (EMD) when appropriate. *Escherichia coli* strains were cultured at 37°C using Luria-Bertani (LB) broth (Becton Dickinson) and Brain Heart Infusion (BHI) agar (Becton Dickinson) supplemented with 100 μg $mL^{-1}$ ampicillin (Amp) (Fisher BioReagents, Waltham, MA), 150 μg $mL^{-1}$ Erm, or 150 μg $mL^{-1}$ kanamycin (Kan) (Fisher) when appropriate.

**Table 2.1.** Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| *Lactobacillus gallinarum* | | |
| ATCC 33199$^T$ | Type strain, chicken crop isolate | ATCC[1] |
| TDCC 97 | ATCC 33199 w/ pTRK669 | This study |
| TDCC 98 | ATCC 33199 w/ *lacL*::pTD017 insertion, *LacL*$^-$ | This study |
| *Escherichia coli* | | |
| EC1000 | RepA$^+$, Km$^r$, replication host for pORI28- based plasmids, chromosomal insertion of *repA* in *glgB* | (168) |
| TDCC 96 | EC1000 w/ pTD017 | This study |
| Plasmids | | |
| pCR2.1 | 3.9 kb, Ori (pUC19), Am$^r$ intermediate TOPO-TA cloning vector | Invitrogen |
| pORI28 | 1.7 kb, Ori (pWV01), Em$^r$, Rep$^-$, replicates only with *repA* provided *in trans*, integration vector | (169) |
| pTRK669 | 2.9 kb, Ori (pWV01), Cm$^r$ ,Rep$^{ts}$, provides *repA in trans* | (170) |
| pTD016 | 4.6 kb pCR2.1 w/ 651-bp internal *L. gallinarum lacL* fragment | This study |
| pTD017 | 2.4 kb pORI28 w/ 651-bp internal *L. gallinarum lacL* fragment | This study |

[1] American Type Culture Collection

*2.2.2 DNA Isolation, Manipulations, and Transformations*

*E. coli* plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen Inc., Valencia, CA), whereas genomic DNA was isolated from *Lactobacillus* using the method of Walker and Klaenhammer (171). All DNA manipulations were performed using standard molecular cloning techniques (172). Restriction endonucleases, T4 Ligase, Antarctic phosphatase, and *Taq* DNA polymerase were used according to the manufacturer's instructions (New England Biolabs, Ipswich, MA). PCR Primers were designed using Clone Manager (Scientific and Educational Software, Cary, NC) and commercially synthesized (Integrated DNA Technologies, Coralville, IA). PCR products and restriction fragments were purified using the QIAquick PCR purification and gel extraction kits (Qiagen), respectively.

Electrocompetent *E. coli* TOP10 and EC1000 were prepared and transformed according to manufacturer's directions and standard methods (173), respectively. Electrocompetent *Lactobacillus gallinarum* was prepared using 3.5 × Sucrose MgCl Electroporation Buffer (SMEB) and electrotransformed as described by J. B. Luchansky et al. (174) using a GenePulse Xcell electorporator (Bio-Rad, Hercules, CA).

*2.2.3 Gene Inactivation*

The inactivation of *lacL* was performed using targeted insertion of an erythromycin resistance cassette by homologous recombination using methods adapted from W. M. Russell and T. R. Klaenhammer (170). A 651-bp internal fragment of *L. gallinarum* ATCC 33199 *lacL* (Accession PRJDB621) was PCR amplified using chromosomal template DNA and primers LGlacL_F (5' - CGGGCCATGTATGTCTATCTC - 3') and

LGlacL_R (5’ – TTGCTTCATGTCGGCTAGG - 3’). Purified PCR product was ligated into pCR2.1-TOPO-TA and subsequently subcloned via NotI and HindIII restriction sites into pORI28. The resulting plasmid, pTD016 (pORI28::*lacL*) was transformed into *L. gallinarum* TDCC 96 harboring pTRK669. Selection for chromosomal integration of pTD016 in *lacL* was performed at 42°C, a temperature non-permissive to pTRK669, in MRS broth with Erm selection. Presumptive *lacL* integration mutants were identified using blue/white selection on MRS agar supplemented with Erm, 20μg $mL^{-1}$ IPTG (RPI, Corp., Mount Prospect, IL) and 50μg $mL^{-1}$ X-gal (AMRESCO LLC, Solon, OH)

*2.2.4 Southern Hybridization*

HindIII-digested chromosomal DNA was separated using agarose gel electrophoresis, transferred onto a positively charged nylon membrane (Roche Diagnostics, Basel, Switzerland) and UV cross-linked (Stratalinker, Stratagene, La Jolla, CA). DIG-labled DNA molecular weight marker was included as a control (Roche). The membrane was blocked, probed using DIG-labled 651 bp *lacL* PCR product, and visualized using anti-DIG conjugated alkaline phosphatase and p-nitrophenyl phosphate using the Dig Easy Hyb system (Roche) according to the manufacturer’s instructions.

*2.2.5 Growth Analysis*

Wild Type and *lacL*$^{-}$ *Lactobacillus gallinarum* strains were cultured overnight using MRS medium and harvested by centrifugation (5,000 × g, 10 min, 4°C). Cells were washed and resuspended in carbohydrate-free Menon-Sturino (MS) medium (175) and inoculated at 1% (v/v) into MS broth supplemented with either 100 mM fructose (Sigma-Aldrich, St. Louis, MO), 100mM glucose (Sigma-Aldrich), 50 mM sucrose (J.

T.Baker, Center Valley, PA), and 50 mM lactose (Sigma-Aldrich). Growth of cultures at 37°C was monitored by absorbance ($O.D._{600\ nm}$) using a microplate reader (Tecan, Morrisville, NC).

*2.2.6 Stability of Integrated Plasmid*

Stability of integrated pTD016 in the chromosome of *L. gallinarum* was determined by propagating *L. gallinarum* TDCC 98 in MRS broth in the absence of antibiotic selection for 50 generations as described in W. M. Russell and T. R. Klaenhammer (170). After every 10 generations of propagation *L. gallinarum* was plated on MRS agar with X-Gal and IPTG and incubated for 48 hours. Revertant colonies were indicated by the ability to hydrolyze X-gal.

**2.3 RESULTS**

*2.3.1 Insertional Inactivation of lacL*

The *lacL* gene, putatively encoding β-galactosidase, was identified using the draft genome sequence of *L. gallinarum* ATCC 33199 (161). Presumptive integrants were selected from Erm-resistant *L. gallinarum* pTD016 transformants which formed white colonies on plates supplemented with X-gal and IPTG indicating disruption of β-galactosidase activity.

*2.3.2 Southern Hybridization Confirms Plasmid Insertion*

Disruption of the *lacL* gene by chromosomal integration of pTD016 was confirmed using Southern hybridization (**Figure 2.1**) and PCR (not shown). For the WT strain (Lane 1), the *lacL* probe hybridized to a band corresponding to the 5.7 kb HindIII

restriction fragment predicted from the *L. gallinarum* genome sequence. For the *lacL* integrant (Lane 2), the probe hybridized to two bands of approximately 6.6 kb and 1.5 kb, indicating insertion of an additional HindIII restriction site into the *lacL* locus by the integration of the plasmid. An additional restriction fragment similar in size to the pTD016 control (Lane 3) indicated amplification of the inserted plasmid within the *lacL* locus.

*2.3.3 Growth Curves*

The ability of the *lacL* mutant (*L. gallinarum* TDCC 98) to grow on various carbohydrates was evaluated (**Figure 2.2**). Cultures of wild type *L. gallinarum* were able to grow effectively in all four carbohydrates. Cultures of the *L. gallinarum lacL* integrant were able to grow in fructose, glucose, and sucrose, reaching a similar final $O.D._{600}$ as the wild type cultures. However, *lacL* integrant cultures were not able to grow in lactose indicating disruption of β -galactosidase activity due to integration of pTD016 in the *lacL* locus of *L. gallinarum*.

*2.3.4 Stability of lacL Integrants*

Loss of the integrated plasmid was assessed by the restoration of β-galactosidase activity, indicated by the appearance of blue colonies (**Figure 2.3**). The number of revertant colonies was evaluated every 10 generations over a total 50 generations. The percentage of revertants gradually increased over the first 40 generations but did not continue to increase from 40 to 50 generations. The maximum percentage of revertants reached a maximum of 1.67% after 50 generations, at a rate of 0.03% per generation

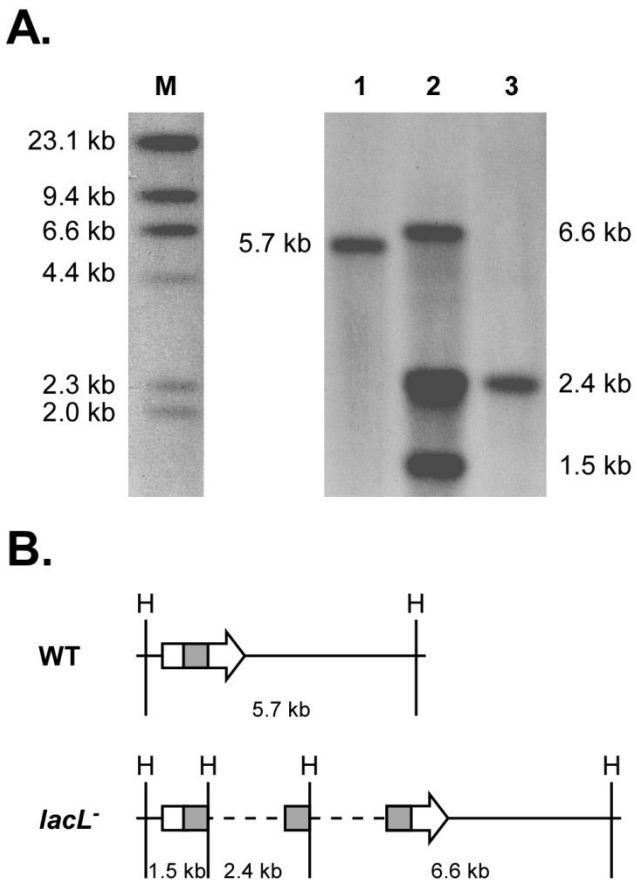


**Figure 2.1. Southern hybridization analysis confirms chromosomal integration of pTD016 in *lacL*. (A)** *L. gallinarum* ATCC 33199 (Lane 1), *L. gallinarum* TDCC 98 (Lane 2), and pTD016 (Lane 3). DNA was digested using HindIII and probed with DIG-labeled 651-bp *lacL* internal fragment. M, DIG-labeled DNA Molecular Weight Marker II. **(B)** Schematic of *lacL* locus of *L. gallinarum* ATCC 33199 (WT) and *L. gallinarum* TDCC 98 (*lacL*$^-$). Chromosomal DNA is represented by solid line, plasmid DNA is represented by dotted line, the *lacL* gene is represented by an arrow, and internal *lacL* fragment is represented by shaded box. HindIII (H) restriction sites and predicted length of restriction fragments is indicated.

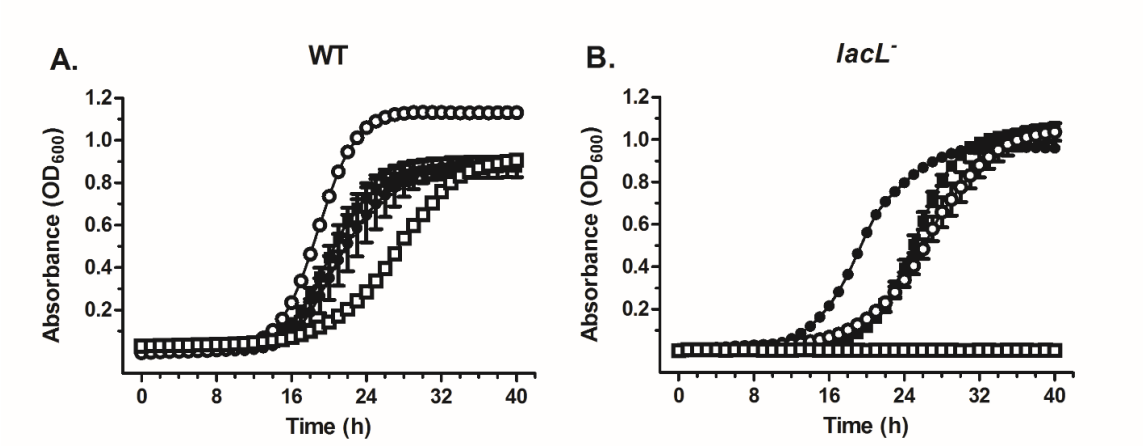


**Figure 2.2. Chromosomal integration in *lacL* abolishes growth on lactose.** Cultures of **(A)** WT and **(B)** *lacL*$^{-}$ *L. gallinarum* strains were cultured in MS broth supplemented with 100 mM Fructose (●), 100 mM glucose (■), 50 mM sucrose (○), and 50 mM lactose (□). Cell density is indicated as the mean ± SEM absorbance ($O.D._{600\ nm}$) of four independent cultures.

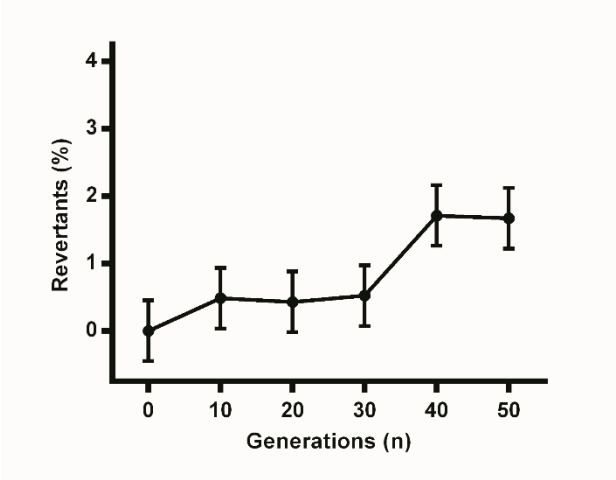


**Figure 2.3. Stability of pTD016 insertion in *L. gallinarum lacL*.** Reversion rate calculated as the percentage of revertant colonies appearing on MRS agar supplemented with 50µg $mL^{-1}$ X-Gal and 20µg $mL^{-1}$ IPTG.

## 2.4 DISCUSSION

The objective of this study was to adapt the pORI28 chromosomal integration system for use in *Lactobacillus gallinarum* ATCC 33199 and demonstrate proof-of-principle of its functionality in this strain by constructing a targeted gene insertion mutant of *lacL*. Administration of probiotic *Lactobacillus* has been demonstrated to promote growth at levels similar to antibiotics (157, 176) and reduce colonization of pathogens in the gastrointestinal tract in poultry (73, 155, 177). *Lactobacillus* strains isolated from chickens have often been reported to be recalcitrant to transformation (178). We have previously demonstrated the ability of *L. gallinarum* ATCC 33199 to be transformed readily with the pWV01 replicon by electroporation (23) and to effectively colonize the gastrointestinal tract of poultry (159). Additionally, the genome sequence of this poultry isolate has been made available (161), suggesting this strain as a potentially important model organism for investigating probiotic functionality in poultry.

The pORI28-plasmid system (169), based on the broad-host-range lactococcal pWV01 replicon (162), has been used widely for the targeted insertional inactivation of genes in Lactic Acid Bacteria (LAB)(170, 179). Derivatives of pWV01, including pGK12 (180), have been demonstrated to be efficiently replicated while maintaining a high copy number in both Gram-negative (180) and Gram-positive (181) microorganisms, including *Borrelia burgdorferi* (182) and *Listeria monocytogenes* (183). Targeted chromosomal insertion has been used widely in the construction of isogenic loss-of function mutants necessary to investigate physiology and metabolism of *Lactobacillus* (170,

184, 185) and other microorganisms. Although the CRISPR/Cas system has recently become a preferred technology for gene editing (186), the native CRISPR/Cas systems encoded in the genome of the bacterium of interest is often used. However, CRISPR/Cas elements have not been not detected in the genome sequence of *L. gallinarum* ATCC 33199 (161). Additionally, targeted chromosomal insertion of pORI28 derivatives will facilitate detection of *L. gallinarum* knock-out mutants during *in vivo* studies in poultry, with erythromycin resistance used as a selective marker and non-native sequences of the plasmid used as targets for molecular detection methods.

β-galactosidase has been used widely as a reporter in genetic studies to because of the easily observed phenotype (187-190). Targeted insertion of pTD016 into the *lacL* locus of *L. gallinarum* disrupted β-galactosidase activity and abolished growth on lactose. Growth of the *lacL* integrant cultures on the other carbohydrates was similar to the wild-type cultures, confirming their fermentation was not affected by inactivation of *lacL*. Similar results have been observed for β-galactosidase knock-out mutants constructed in other LAB (170, 191). Additionally, a lag period was observed for cultures of the *lacL* mutant compared to the wild-type when cultured in fructose, glucose, and sucrose, and has been observed previously when pORI28-based mutants were cultured under antibiotic selection (163). The inability of the mutant to grow in lactose suggests *lacL* encodes the only β-galactosidase in the genome of *L. gallinarum* ATCC 33199 and is consistent with predictions from the genome sequence for this organism (161).

Southern hybridization analysis confirmed disruption of β-galactosidase was due to integration of the pTD016 in the *lacL* locus of *L. gallinarum*. Presence of an additional band of approximately similar size to the inserted plasmid indicated amplification of the insert within the *lacL* locus. Similar amplification has been reported previously for plasmid insertions maintained under antibiotic selection in other microorganisms (162, 163, 170).

The observed total reversion of less than 2% in the absence of antibiotic selection is similar to the stability of chromosomal integrations after 50 generations reported previously for other *Lactobacillus* (170, 192) and is significantly greater than has been reported for gene knockout systems in other bacteria (193-195). *L. gallinarum* ATCC 33199 has been demonstrated to colonize chickens only transiently with its recovery decreasing significantly 4 days post-inoculation (159), suggesting that pORI28-based knock-out mutants will be sufficiently stable for application during *in vivo* studies in broiler chickens.

In this study, we constructed a knock-out mutant of *lacL* using insertional inactivation in order to demonstrate proof-of-principle of the effectiveness of the pORI28 system in *L. gallinarum* ATCC 33199. We have successfully demonstrated that integration of pTD016 (pORI28::*lacL*) into the *lacL* locus of disrupted β-galactosidase activity and abolished the ability of integrant to utilize lactose without affecting growth on other carbohydrates. To the best of our knowledge, this is the first report of targeted gene inactivation in a *Lactobacillus* culture isolated from poultry. The application of the pORI28 system will allow the construction of additional isogenic mutants to be used to support

investigation of mechanisms important to the beneficial functionality of probiotic micro-organisms in poultry.

# 3. EFFECTS OF DIRECT-FED MICROORGANISMS AND ENZYME BLEND CO-ADMINISTRATION ON INTESTINAL BACTERIA IN BROILERS FED DIETS WITH OR WITHOUT ANTIBIOTIC[2]

## 3.1 INTRODUCTION

Sub-therapeutic doses of antibiotics have been used to promote the growth of broiler chickens in the United States for more than 50 years (135, 196, 197).Antibiotic growth promoters (**AGP**) have been demonstrated to increase weight gain (135), improve feed efficiency (136, 137), and reduce mortality in livestock animals (139, 198). However, the use of AGPs has declined (140) because of increased concerns regarding the development of antibiotic resistant bacteria (199), and their use has been banned in the European Union (200) and limited in the United States by the Veterinary Feed Directive (201). Because of growing interest in low-input and antibiotic free (**ABF**) production practices, the development of effective alternatives to the sub-therapeutic use of antibiotics is of significant interest to animal agriculture.

The growth-promoting activity of antibiotics is attributed to their effect on the gastrointestinal microbiota (199) and are not observed when administered to germ-free animals (202). However, increased growth is observed when antibiotics are administered to animals with normal microbiota (135, 203, 204). Additionally, growth is depressed

[2] Reprinted with permission from "Effects of direct-fed microorganisms and enzyme blend co-administration on intestinal bacteria in broilers fed diets with or without antibiotics." Askelson, T. E., C. A. Flores, S. L. Dunn-Horrocks, Y. Dersjant-Li, K. Gibbs, A. Awati, J. T. Lee, and T. Duong. 2017. Poultry Science. pex270. doi 10.3382/ps/pex270

when germ-free animals are inoculated with normal microbiota (205), suggesting intestinal microorganisms are competitive with growth performance of the host animal (136). Modification of the host microbiota by antibiotics has been suggested to improve growth performance of livestock through inhibition of subclinical infections (206), reduced competition for nutrients between the microbiota and host-animal (207, 208), decreased production of growth depressing metabolites by the resident microbiota (209), and enhanced absorption of nutrients through the thinner intestinal wall of antibiotic-fed animals (210, 211).

Administration of probiotics, sometimes called Direct-Fed Microorganisms (**DFM**) when used in livestock animals (212), has been demonstrated to improve growth performance at levels similar to AGPs (16, 156). Additionally, they have been demonstrated to improve pre-harvest food safety of poultry by reducing colonization of human food-borne pathogens including *Salmonella* (73, 213) and *Campylobacter* (30, 214) in the gastrointestinal tract; improve poultry health by reducing colonization by poultry pathogens including *Clostridium perfringens* (215, 216) and avian pathogenic *Escherichia coli* (217); and reduce inflammation induced during *C. perfringens* associated necrotic enteritis (218).

Cereal grains commonly used in livestock animal feed contain anti-nutrients including non-starch polysaccharides (**NSP**), resistant starches, and indigestible proteins which are poorly digested by monogastric animals (219, 220). Additionally, NSPs exert anti-nutritive effects through chelation of important metal cations including calcium, iron, and magnesium (221), reduce nutrient absorption by increasing the ileal viscosity

(222), and alter the gastrointestinal microbiota (223). Digestive enzymes including xylanases, amylases, and proteases are used routinely in animal feeds to improve digestibility (224, 225) and reduce anti-nutritive effects of poorly digested feed constituents (226, 227); and their effect on growth performance has been well demonstrated (228-230). Additionally, the products of the hydrolysis of indigestible feed constituents by exogenous feed-additive enzymes may produce substrates that promote the growth or activities of beneficial bacteria (231), which suggests the administration of particular enzyme blends may confer an additive benefit when combined with appropriate Direct-Fed Microorganisms. This potential prebiotic-like effect on growth performance suggests the co-administration of enzyme blends with DFM may be an important component of ABF management programs.

The co-administration of DFMs with feed-additive enzymes has been investigated previously. In addition to improving growth performance, co-administration of *Lactobacillus plantarum* and xylanase was demonstrated to reduce fecal shedding of *Salmonella* Typhimurium in experimentally challenged broilers (232). Administration of a multi-strain DFM product containing *Bifidobacterium animalis* and several Lactic Acid Bacteria (LAB) in combination with xylanase improved growth performance when compared to either product individually (233). Dersjant-Li et al. (234) demonstrated previously that administration of a multi-strain *Bacillus amyloliquefaciens* DFM product in combination with an enzyme complex composed of xylanase, amylase, and protease (**XAP**) improved growth performance in broilers fed a diet with reduced energy and digestible amino acids. Although the use of antibiotics in poultry production is continuing

to decline, the use of antibiotics non-medically relevant, including BMD and Virginiamycin has not been prohibited, and the effect of antibiotics on the efficacy of DFM and DFM containing products is not well understood. In this study, we evaluated the effect of a feed additive containing three strains of *B. amyloliquefaciens* and XAP described previously, administered with or without AGP on the gastrointestinal microbiota and growth performance of broiler chickens.

## 3.2 MATERIALS AND METHODS

### *3.2.1 Experimental Design*

Male broilers (Cobb 500, n = 2160) were obtained from a commercial hatchery on day of hatch, randomly assigned to treatment pens with similar starting weights, and provided experimental feed and water *ad libitum* for the duration of the study. Experimental animals were allocated to 6 experimental treatment groups with 9 replicate pens of 40 broiler chicks arranged as a randomized complete block design. Experimental treatment groups were fed experimental rations which contained combinations of an AGP [control (**-AGP**), bacitracin methylene disalicylate (**BMD**), or virginiamycin (**VM**)] and a feed additive (**ADD**[3]) composed of a DFM culture containing spores of three *Bacillus amyloliquefaciens* strains ($7.5 \times 10^7$ cfu $kg^{-1}$ feed) and an enzyme blend *Trichoderma reesei* endo-xylanase (2000 U $kg^{-1}$ feed), *Bacillus licheniformis* α-amylase (200 U $kg^{-1}$ feed), and *Bacillus subtilis* serine protease (4000 U $kg^{-1}$ feed) (**XAP**) (**Table 3.1**). All animal care and experimental procedures were performed in accordance with protocols approved by the Texas A&M University Institutional Animal Care and Use

[3] (DFM + XAP, Syncra AVI, Danisco Animal Nutrition/DuPont, Marlborough, Wiltshire, UK)

Committee. Additional details including experimental design, experimental diets, animal husbandry, and growth performance measures are presented in a separate publication (235)

*3.2.2 Bacterial Enumeration*

At 21 and 42 days post-hatch, a single chicken of approximately mean pen weight (± 5%) was selected from each replicate pen, euthanized, and necropsied for the collection of tissues for the enumeration of gastrointestinal microorganisms. The ceca and a section (~ 6 cm) of the ileum centered on the midpoint between Meckel's diverticulum and the ileocecal junction were dissected aseptically from each selected chicken. Ileal specimens were homogenized and diluted using Fluid Thioglycolate Medium (**FTM**; BD, Franklin Lakes, NJ), whereas cecal specimens were homogenized and diluted using sterile phosphate buffered saline (**PBS**; Fisher Scientific, Pittsburgh, PA). *Campylobacter jejuni*, *Escherichia coli*, *Salmonella*, and total Lactic Acid Bacteria (**LAB**) were enumerated from the ceca using Campy Cefex agar (Hardy Diagnostics, Santa Maria, California), Compact Dry EC plates (**EC**; Hardy Diagnostics), Xylose-Lysine-Tergitol-4 agar (**XLT-4**; BD), and deMan, Rogosa, and Sharpe agar (**MRS**; BD) supplemented with 100 $\mu g \cdot mL^{-1}$ cycloheximide (Amresco, Solon, OH), respectively. *Clostridium perfringens* was enumerated from the ileum using Tryptose Sulphite Cycloserine Egg Yolk overlay agar (**TSC-EY**; BD). EC and XLT-4 were incubated aerobically at 37 $^{o}$C for 36 h. Campy Cefex and MRS were incubated in 10% $CO_2$ at 42 $^{o}$C and

**Table 3.1.** Feed Conversion of Broiler Chickens

| Treatment | | FCR (Feed:Gain) | | |
|---|---|---|---|---|
| AGP | ADD[1] | D 0-21 | D 22-42 | D 0-42 |
| - | - | 1.380[a] | 1.875[a] | 1.663[a] |
| - | + | 1.358[bc] | 1.830[b] | 1.625[b] |
| BMD[2] | - | 1.357[bc] | 1.824[b] | 1.625[b] |
| BMD | + | 1.356[bc] | 1.807[b] | 1.612[b] |
| VM[3] | - | 1.371[ab] | 1.831[b] | 1.636[ab] |
| VM | + | 1.352[c] | 1.806[b] | 1.612[b] |
| **One-way *P* -values** | | 0.007 | 0.018 | 0.003 |
| **Main Effects** | | | | |
| *AGP* | | | | |
| Control | | 1.369 | 1.849[a] | 1.644[a] |
| BMD | | 1.357 | 1.818[b] | 1.619[b] |
| Virginiamycin | | 1.362 | 1.816[b] | 1.624[b] |
| *Feed Additive* | | | | |
| Control | | 1.369[a] | 1.842[a] | 1.641[a] |
| ADD | | 1.356[b] | 1.813[b] | 1.616[b] |
| ***P* -values** | | | | |
| *AGP* | | 0.079 | 0.016 | 0.015 |
| *Feed Additive* | | 0.002 | 0.005 | <0.001 |
| *AGP*×*Feed Additive* | | 0.092 | 0.492 | 0.332 |
| ***Pooled SEM*** | | 0.002 | 0.007 | 0.004 |

[a - c] different superscripts within columns indicate means are significantly different ($P \leq 0.05$)

[1]DFM + XAP; [2]Bacitracin Methylene Dialicylate (50 g $t^{-1}$)
[3]Virginiamycin (20 g $t^{-1}$)

37ºC, respectively, for 36 h. TSC-EY was incubated at 37 ºC anaerobically (Coy Laboratory Products, Grass Lake, MI) for 36 h. *C. jejuni* was selectively enriched from cecal specimens using Bolton's Enrichment Broth (**BEB**, Hardy Diagnostic) incubated at 42 ºC for 24 h followed by Campy Cefex agar. *Salmonella* was from cecal specimens using Rappaport Vassiliadis R10 broth (**RV**; BD) incubated at 42ºC for 24 h and XLT-4 agar.

*C. perfringens* was selectively enriched from the ileum using FTM incubated anaerobically at 37°C for 24 h followed by Iron Milk Medium incubated at 46ºC for 3 h. Specimens for which there were no colonies appearing on enumeration plates but were positive by selective enrichment were assigned the lower limit of detection, 100 cfu $g^{-1}$ for statistical analysis.

Presumptive *C. perfringens* were confirmed using Iron Milk Medium, whereas presumptive *C. jejuni*, *E. coli,* and *Salmonella* colonies were confirmed by PCR using species-specific primers (**Table 3.2**). *C. jejuni* ATCC 29428, *E. coli* ATCC 25922, and *Salmonella* Typhimurium ATCC 14028 were used as positive controls for PCR

**Table 3.2**. PCR primers used in this study

| Species | Gene | Primer | Sequence (5'-3') | Reference |
|---|---|---|---|---|
| *C. jejuni* | *cadF* | cadF-F2B | TTG AAG GTA ATT TAG ATA TG | (236) |
| | | cadF-R1B | CTA ATA CCT AAA GTT GAA AC | |
| *E. coli* | *tuf* | TEcol553 | TGG GAA GCG AAA ATC CTG | (237) |
| | | TEcol754 | CAG TAC AGG TAG ACT TCT G | |
| *Salmonella* | *invA* | INVA-1 | ACA GTG CTC GTT TAC GAC CTG AAT | (238) |
| | | INVA-2 | AGA CGA CTG GTA CTG ATC GAT AAT | |

*3.2.3 Statistical Analysis*

Bacterial counts were $\log_{10}$ transformed for analysis and reported as the mean ± SEM $\log_{10}$ cfu $g^{-1}$ digestive contents from 9 replicate pens per treatment. Data were analyzed using factorial ANOVA with main effects for AGP, Feed Additive, and AGP ×Feed Additive. A one-way ANOVA was used to determine differences between individual treatment groups. Significantly different means ($P \leq 0.05$) were separated using Duncan's multiple range test. Associations between bacterial counts and feed conversion ratio (**FCR**) were evaluated by pens using Pearson's *r*. Analyses were conducted with IBM SPSS Statistics (V. 24.0, IBM Corp., Armonk, NY).

**3.3 RESULTS**

*3.3.1 Gastrointestinal Microbiota*

*3.3.1.1 Gram-positive Bacteria.* Recovery of total Lactic Acid Bacteria (**LAB**) was greater from broilers treated with virginiamycin (**VM**) and the feed additive (**ADD**) in combination than from the remaining treatment groups on Day 21 and Day 42 (**Figure 3.1 A-B**). On Day 21, recovery of *Clostridium perfringens* was greater from untreated broilers than from the remaining treatment groups (**Figure 3.1 C)**, whereas, on Day 42, recovery of *C. perfringens* was greatest from broilers administered VM alone (**Figure 3.1 D**). Administration of ADD increased counts of total LAB in the cecum of broiler chicks on Day 21 ($P = 0.028$) but had no effect on Day 42 (**Table 3.3**). Whereas no difference was observed on Day 21, administration of Antibiotic Growth Promoters (**AGP**) was observed to have a significant effect on total LAB counts on Day 42 ($P = 0.021$), with the recovery of total LAB being greater from broilers administered VM than from

broilers which were not administered an AGP. Although a significant main effect was not observed for Feed additive administration on Day 21 or Day 42 (**Table 3.3**), recovery of *C. perfringens* in ADD treated broilers was similar to those administered AGPs on Day 21 when compared to untreated broilers (**Figure 3.1 C**). AGP administration was not observed to have a significant effect on Day 21, but fewer *C. perfringens* tended to be recovered from broilers administered VM than from untreated broilers ($P$ = 0.069). On Day 42, more *C. perfringens* were recovered from broilers administered VM than from those administered BMD or untreated broilers ($P$ = 0.014).

**Table 3.3.** Main effect of AGP and Feed Additive administration on gastrointestinal microbiota ($log_{10}$ cfu $g^{-1}$).

| *Main Effect* | Total LAB | | *C. perfringens* | | *Salmonella* | | *Campylobacter* | | *E. coli* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | D 21 | D 42 | D 21 | D 42 | D 21 | D 42 | D 21 | D 42 | D 21` | D 42 |
| *AGP* | | | | | | | | | | |
| Control | 7.67 | 7.67$^{b}$ | 3.11$^{a}$ | 2.16$^{b}$ | 0.24 | 0.00 | 2.01 | 2.05$^{b}$ | 7.10 | 6.48$^{b}$ |
| BMD[1] | 7.85 | 7.88$^{ab}$ | 2.77$^{ab}$ | 2.24$^{b}$ | 0.32 | 0.12 | 1.56 | 2.32$^{ab}$ | 7.48 | 6.40$^{b}$ |
| Virginiamycin[2] | 8.10 | 8.33$^{a}$ | 2.43$^{b}$ | 2.73$^{a}$ | 0.01 | 0.00 | 2.12 | 3.27$^{a}$ | 7.39 | 7.34$^{a}$ |
| Feed Additive | | | | | | | | | | |
| Control | 7.67$^{b}$ | 8.05 | 2.92 | 2.43 | 0.15 | 0.00 | 2.01 | 2.73 | 7.20 | 6.91 |
| Feed Additive[3] | 8.08$^{a}$ | 7.86 | 2.61 | 2.33 | 0.23 | 0.07 | 1.79 | 2.35 | 7.44 | 6.57 |
| ***P*-values** | | | | | | | | | | |
| *AGP* | 0.151 | 0.021 | 0.069 | 0.014 | 0.259 | 0.320 | 0.484 | 0.042 | 0.429 | 0.050 |
| *Feed Additive* | 0.028 | 0.330 | 0.183 | 0.544 | 0.636 | 0.321 | 0.567 | 0.351 | 0.350 | 0.313 |
| *AGP×Feed Additive* | 0.867 | 0.454 | 0.717 | 0.921 | 0.216 | 0.374 | 0.940 | 0.040 | 0.127 | 0.952 |
| ***Pooled SEM*** | 0.094 | 0.102 | 0.119 | 0.085 | 0.080 | 0.037 | 0.196 | 0.222 | 0.126 | 0.176 |

$^{a,b}$ Different superscripts within columns indicate means are significantly different ($P \leq 0.05$)

[1]Bacitracin Methylene Dialicylate; [2]Virginiamycin (20 g $t^{-1}$); [3]DFM + XAP;

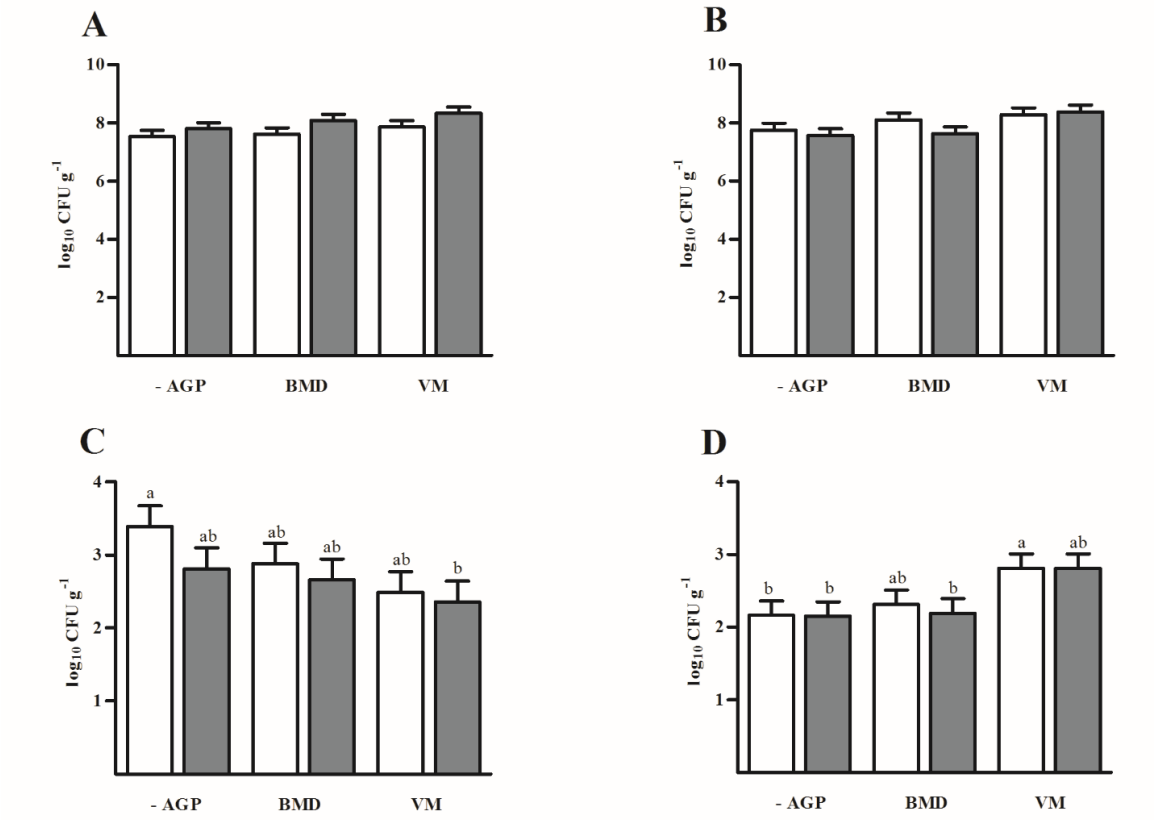


**Figure 3.1. Enumeration of Gram-positive bacteria from broiler chickens.** Total LAB were enumerated from the cecum of broiler chicks at (A) Day 21 and (B) Day 42 post-hatch. *C. perfringens* was enumerated from the small intestine of broiler chicks at (C) Day 21 and (D) Day 42 post-hatch. White bars (control); Gray bars (ADD). Counts are reported as the mean ± SEM $\log_{10}$ CFU $g^{-1}$ digestive contents from 9 broiler chickens per treatment. Different letters above bars indicate means are significantly different ($P \leq 0.05$).

*3.3.1.2 Gram-negative Bacteria.* The administration of AGPs or ADD resulted in no difference in the recovery of *Salmonella* (**Table 3.3**). Indeed, recovery of *Salmonella* was near the limit of detection for all treatment groups (**Figure 3.2 A-B**). Although no significant difference was observed in the recovery of *Campylobacter* on Day 21, a significant main effect for AGP administration was detected with more *Campylobacter* being recovered from broilers administered VM than from untreated broilers ($P = 0.042$) on Day 42 (**Table 3.3**). Additionally, a significant AGP × Feed Additive interaction ($P = 0.04$) was observed on Day 42. In broilers administered BMD, fewer *Campylobacter* were recovered from ADD treated broilers ($P = 0.012$) than from those that did not (**Figure 3.2 D**). Although, no significant difference was observed in the recovery of *E. coli* on Day 21, recovery of *E. coli* was greater from broilers administered VM than from others ($P = 0.05$) on Day 42.

Associations between the relative abundance of microorganisms in the gastrointestinal tract of chickens were also evaluated (not shown). Strong positive associations were detected between counts of total LAB and *E. coli* on Day 21 ($r = 0.599$, $P < 0.001$) and Day 42 ($r = 0.522$, $P < 0.001$). A moderate negative correlation was also detected between LAB and *Salmonella* on Day 42 ($r = -0.290$, $P = 0.034$). Lastly, counts of LAB and *Campylobacter* on Day 21 tended to correlate moderately ($r = 0.263$, $P = 0.055$), whereas LAB and *Campylobacter* counts were found to be correlate moderately ($r = 0.362$, $P = 0.007$) on Day 42. No other significant correlations between groups of microorganisms were observed.

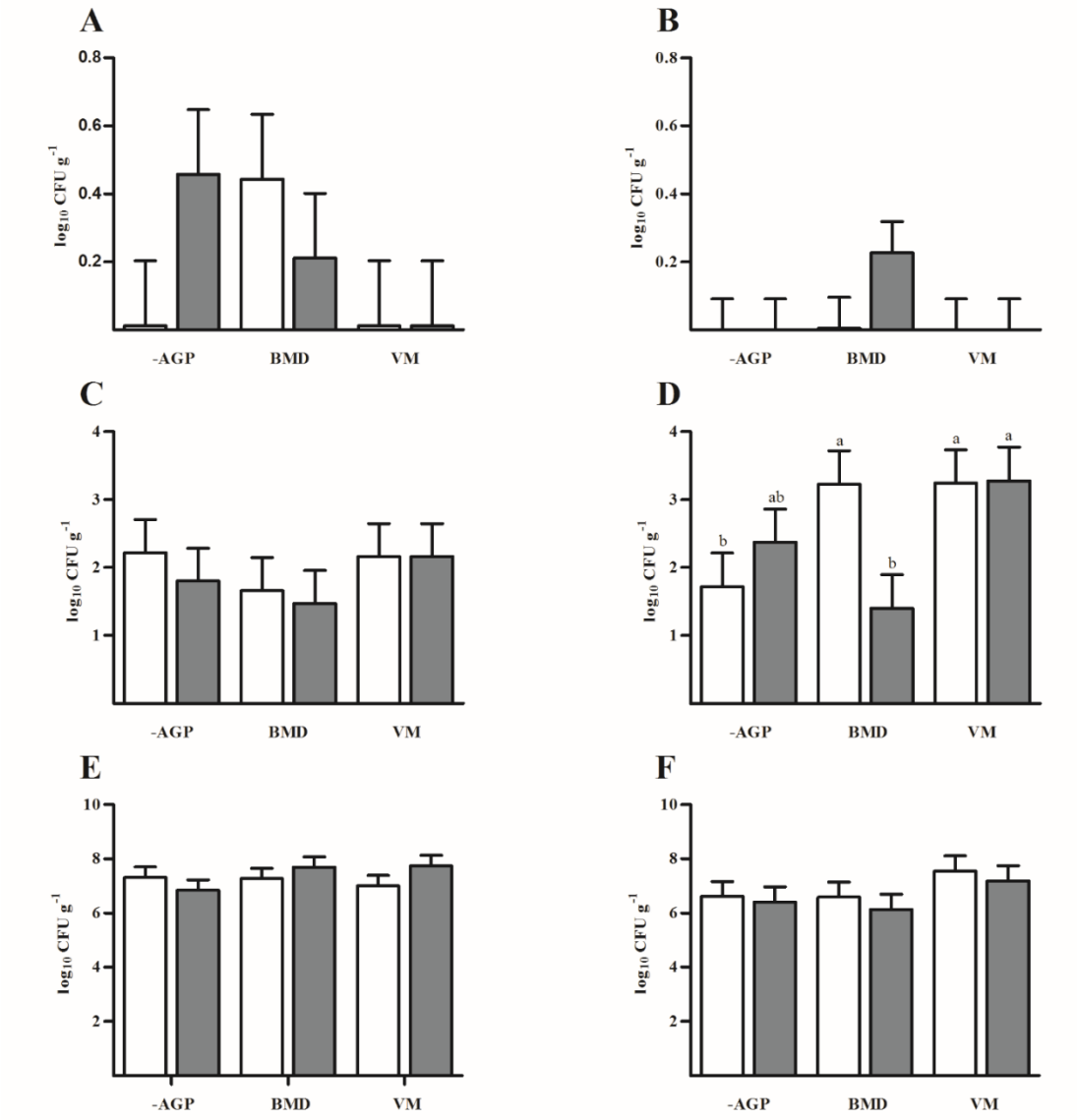


**Figure 3.2. Enumeration of Gram-negative bacteria from broiler chickens.** *Salmonella* were enumerated from the cecum of broiler chicks at (A) Day 21 and (B) Day 42 post-hatch. *C. jejuni* were enumerated from the cecum of broiler chicks at (C) Day 21 and (D) Day 42 post-hatch. *E. coli* were enumerated from the cecum of broiler chicks at (E) Day 21 and (F) Day 42 post-hatch. White bars (control); Gray bars (ADD). Counts are reported as the $\log_{10}$ CFU $g^{-1}$ digestive contents from 9 broiler chickens per treatment. Different letters above bars indicate means are significantly different ($P \leq 0.05$). Different letters above bars indicate means are significantly different ($P \leq 0.05$).

*3.3.4 Feed Conversion*

The effect of the experimental treatments on the growth performance and feed conversion of broiler chickens in this study has been reported comprehensively in a separate publication (235). Feed conversion ratio of broiler chickens reported previously is summarized in **Table 3.1**. Overall, administration of ADD improved early (D 0-21) ($P$ = 0.002), late (D 22-42) ($P$ = 0.005), and cumulative FCR (D 0 -42) ($P < 0.001$) when compared to the control, whereas AGP administration improved only late ($P$ = 0.016) and cumulative FCR ($P$ = 0.015). Administration of ADD improved early feed conversion ($P$ = 0.007) in unmedicated and VM-fed broilers but had no additional effect in broilers administered BMD.

Associations between populations of gastrointestinal microorganisms with feed conversion were evaluated (**Table 3.4**). Negative correlations ($P < 0.05$) were detected between total LAB counts on Day 21 and early FCR (Day 0 – 21) and between total LAB counts on Day 42 and late FCR (Day 21 -42). Additionally, total LAB on Day 21 tended to correlate negatively ($P < 0.1$) with late and cumulative FCR (Day 0 – 42) and total LAB on Day 42 tended to correlate with cumulative FCR. A moderate positive correlation was observed between counts of *C. perfringens* on Day 21 ($P < 0.01$) with late and cumulative FCR. Overall, these data suggest that FCR is lowest in broilers with greater counts of total LAB in the cecum and fewer counts of *C. perfringens* in the ileum. No associations were detected between FCR and *Salmonella* or *E. coli*. However, a strong negative correlation was detected between counts of *Campylobacter* on Day 42 with late and cumulative FCR.

**Table 3.4.** Correlation of bacterial counts with FCR

| Bacterial Counts ($log_{10}$ CFU $g^{-1}$) | | FCR (Feed:Gain) | | |
|---|---|---|---|---|
| | | D 0-21 | D 22-42 | D 0-42 |
| Total LAB | | | | |
| Day 21 | r | -0.287 | -0.237 | -0.247 |
| | *P* | 0.035 | 0.085 | 0.072 |
| Day 42 | r | -0.040 | -0.278 | -0.265 |
| | *P* | 0.773 | 0.042 | 0.053 |
| *C. perfringens* | | | | |
| Day 21 | r | 0.186 | 0.339 | 0.405 |
| | *P* | 0.177 | 0.003 | 0.002 |
| Day 42 | r | 0.213 | -0.019 | 0.014 |
| | *P* | 0.123 | 0.892 | 0.921 |
| *C. jejuni* | | | | |
| Day 21 | r | 0.069 | -0.114 | -0.092 |
| | *P* | 0.621 | 0.410 | 0.509 |
| Day 42 | r | -0.098 | -0.428 | -0.400 |
| | *P* | 0.479 | 0.001 | 0.003 |

## 3.4 DISCUSSION

The objective of this study was to investigate the co-administration of Direct-Fed Microorganisms (**DFM**) and exogenous enzymes in broiler chickens as a potential alternative to and in addition to the use of antibiotic growth promoters (**AGP**). Although AGP have been widely used in the production of poultry and other livestock, the demand for antibiotic free (**ABF**) livestock production has increased due to consumer and regulatory concerns over the development of antibiotic resistant bacteria (199). Because the growth promoting activities of AGPs are a result of their effects on the gastrointestinal microbiota, the microbiota is likely to be an important target for the development of alternatives to antibiotics. The gastrointestinal microbiota is increasingly recognized as an important modulator of human and animal health (239). Additionally, an important role of the microbiota is to augment host metabolism through the conversion of undigested feed components to bioavailable products that can subsequently be utilized by the host (23, 61). The effects of their administration on the gastrointestinal microbiota and in promoting growth performance suggests DFM and exogenous enzyme as potential alternatives to AGPs. The administration of DFMs in livestock has been demonstrated to improve growth performance at levels similar to AGPs (16, 156) and reduce colonization of human food-borne and poultry pathogens in the gastrointestinal tract of poultry (215-217). Exogenous enzymes are used routinely in animal feeds to improve digestibility of poorly digested feed constituents (Zanella, et al., 1999) and reduce their anti-nutritive effects (240). Additionally, the products of their hydrolysis may serve as substrates which

promote the growth or activities of beneficial bacteria (231). Indeed, the potentially synergistic effects of the co-administration of DFM and exogenous enzymes on growth performance have been demonstrated previously (Murugesan and Persia, 2015). In this study, we evaluated the effect of the administration of a feed additive (**ADD**) composed of a DFM product containing spores of three *Bacillus amyloliquefaciens* strains and an XAP enzyme blend on the gastrointestinal microbiota and growth performance of broiler chickens fed diets with and without AGP.

Administration of ADD improved feed efficiency of broiler chickens at levels similar to AGP, suggesting the co-administration of the DFM and enzyme blends may be a potentially important component of an ABF management program. The growth promoting activities of DFM and exogenous enzymes has been widely demonstrated. Despite dramatic reductions in their use, AGPs are still widely administered in poultry production, and administration of products to further improve growth in AGP-fed animals is also of interest. In this study, administration of ADD did further improve feed efficiency in broilers administered VM suggesting co-administration of DFM and enzyme blends may provide additional benefits to growth performance in antibiotic-fed broiler chickens.

In this study, administration of ADD increased counts of Lactic Acid Bacteria (**LAB**) on Day 21 ($P = 0.028$), whereas AGP administration increased LAB counts only on Day 42 ($P = 0.021$). Although the difference was not significant in previously published work, ADD administration has been demonstrated to increase LAB counts in the gastrointestinal tract of broiler chickens (234). Administration of Direct-Fed *B. amyloliquefaciens* (241) and xylanase (242) individually has been demonstrated previously to

increase LAB in the gastrointestinal tract and improve growth performance of broiler chickens. Characterization of gastrointestinal microbiota of broilers fed conventional and ABF diets found no significant difference in total LAB counts between ABF broilers and those fed a diet containing BMD (243), suggesting AGP administration may have only minimal effect on total LAB. LAB isolated from non-animal environments, including starter cultures and fermented foods, are commonly found to be resistant to multiple antibiotics including bacitracin (244-246) and virginiamycin (247, 248), suggesting the resistance determinants are inherent rather than acquired (249, 250).

In this study, the negative correlation of total LAB counts on Day 21 and Day 42 with early (Day 0 – 21) and late (Day 22 – 42) FCR, respectively, suggests an important association between LAB and more efficient feed conversion (**Table 3.4**). The LAB are important inhabitants of the gastrointestinal tract and are generally recognized as beneficial to poultry intestinal health (133, 176, 251). Cultures of LAB, particularly *Lactobacillus* species, have been used widely as probiotics and their administration to broilers has been demonstrated to improve growth performance (94, 157, 176). Administration of probiotic LAB has been shown to reduce colonization of bacterial pathogens, including *Clostridium* (252) and *Salmonella* (73, 253), in the gastrointestinal tract, likely through competition for shared attachment sites in the mucosa (98) and production of anti-microbial metabolites (30, 102). Additionally, measures of improved epithelial barrier function including increased villus height and villus height:crypt depth ratio in the duodenum and ileum (156) and increased mucus production (254) have been observed in broilers administered probiotic LAB (255).

The positive correlation of *C. perfringens* counts on Day 21 with late and cumulative FCR suggests that greater *C. perfringens* counts are associated with less efficient feed conversion (**Table 3.4**). In addition to promoting growth, BMD and VM are administered to control *C. perfringens*, suggesting the reduction of sub-clinical infections of this organism as a specific therapeutic target for the development of alternatives to AGP. Reduced weight gain and increased FCR have been reported when high numbers of *C. perfringens* were recovered from broilers (256, 257), and negative effects on growth performance have been reported when broilers were experimentally infected with *C. perfringens* (258). Necrosis of epithelial tissues mediated by the multiple virulence factors of *C. perfringens*, including collagenolytic enzymes (256), NetB toxin (257), phospholipase C(α-toxin) results in reduced nutrient absorption through the intestinal epithelium (259). Additionally, the subsequent immune response and repair of epithelial tissues further increases the nutritional cost of endogenous losses and results in decreased growth performance (260). Administration of ADD was demonstrated previously to significantly reduce *C. perfringens* in the ileum and cecum of broiler chickens (234). Although a similar reduction was not observed in this study, ADD administration did reduce *C. perfringens* to levels similar to AGP administration. Administration of Direct-Fed-*Bacillus* has been previously demonstrated to reduce *C. perfringens* and improve FCR to levels similar to AGP administration (261, 262). However, xylanase administration was previously demonstrated not to have an effect on the recovery of *C. perfringens* (263).

A negative correlation was observed between *C. jejuni* counts and FCR (**Table 3.4**). However, overall, the treatments evaluated in this study were not observed to affect colonization by *Campylobacter* and *Salmonella*. In the absence of an experimental infection, it is difficult to assess the efficacy of an intervention in reducing colonization by these human food-borne pathogens. Administration of Direct-Fed *Bacillus* has been demonstrated previously to reduce *Campylobacter* (264) and *Salmonella* (213, 216, 265) colonization in experimentally infected broilers. Additionally, co-administration of a DFM and xylanase was previously demonstrated to reduce shedding of *Salmonella* and improve FCR in experimentally infected broilers (232). In the current study, ADD administration reduced *Campylobacter* counts in broilers fed diets containing BMD. Although *C. jejuni* has been widely considered to be a commensal in poultry (266, 267), the understanding of its relationship with the avian host is complicated by reports of its ability to induce intestinal inflammation, reduce intestinal barrier function, and invade intestinal epithelial tissues in poultry (268-270). An improved understanding of the ecological niche filled by *Campylobacter* will inform the development of interventions to reduce colonization of this organism in the gastrointestinal tract of poultry in order to decrease the risk of *Campylobacter*-associated foodborne illness from poultry.

In this study, we investigated the effect of the co-administration of Direct-Fed *Bacillus* and an enzyme blend on the gastrointestinal microbiota and feed efficiency of broiler chickens. We have demonstrated the ability of the feed additive (DFM + XAP) to improve feed efficiency and modify the gastrointestinal microbiota to be similar to the use of antibiotic growth promoters suggesting this and other similar additives may serve

as alternatives to sub-therapeutic use of antibiotics in poultry production. Additionally, we observed a potential additional benefit to growth performance from the co-administration of DFM and enzyme blends in antibiotic-fed broilers. We have observed moderate to strong associations of Lactic Acid Bacteria, *Clostridium perfringens,* and *Campylobacter jejuni* with feed conversion, suggesting potentially important roles of these organisms in gastrointestinal health or in the gastrointestinal fermentation community. Additional research will be required in order to determine the degree to which populations of these organisms should serve as therapeutic targets for the development of products intended to replace AGPs. Although we have not evaluated measures of intestinal barrier function, the effects on the microbiota observed in this study suggest improved intestinal barrier function associated with increased LAB counts and decreased nutritional costs associated with decreased sub-clinical infection by *C. perfringens* may be an important mode of action for the benefits of these antibiotic alternatives. Because of the reliability and effectiveness of antibiotic growth promoters, it is unlikely that a single alternative product will match their efficacy. Thus, the continued development of antibiotic free management programs is likely required to replace AGPs in poultry production.

# 4. COMPARING THE EFFECTS OF TWO PHYTASES ON POPULATIONS OF GASTROINTESTINAL MICROORGANISMS IN BROILERS

## 4.1 INTRODUCTION

Phosphorus is an essential nutrient in poultry production (271) with dietary deficiencies leading to excessive financial losses due to increased mortality (227, 272). Phytic acid (*myo*-inositol hexaphosphate) is an important plant phosphorus storage form and accounts for 50 - 80 % of total phosphorus present in cereal grains and legumes commonly used in livestock animal feeds (82, 83). However, phytate-phosphorus has low bioavailability and is underutilized due to the lack of phytate-degrading enzymes in mono-gastric livestock including poultry (84-86). Additionally, phytic acid exerts anti-nutritive effects (272), sequestering essential cations including calcium, magnesium, iron, and zinc and reducing their bioavailability (273).

Phytases are phosphatases which catalyze the hydrolysis of phytic acid to *myo*-inositol and inorganic phosphate (274). In-feed administration of microbial phytases to improve digestibility of phytic acid is widely used in the production of poultry and other livestock (275, 276). The resulting increases in phytate-phosphorus digestibility (272, 277, 278) and reduction in the anti-nutritive effects (226, 279) of phytic acid are well documented.

The gastrointestinal microbiota of the chicken is recognized to be a complex community that is a potentially important therapeutic target for the promoting health in

the chicken. Its composition has been demonstrated to change in response to many factors including antibiotics (280), gender (281), age (44), and diet (40, 282, 283). The shifts in the composition of microbial communities can potentially produce the beneficial effects including improved feed conversion (284), reduced mortality (69) or have adverse effects, including increase feed intake (285) and proliferation of pathogenic organisms (286). Composition of feed (287), available phosphorous (288, 289), and phytase administration (282, 284) have been demonstrated to affect the composition of the bacterial communities present in the gastrointestinal tract of monogastric animals. Metzler-Zebeli *Et al* (290) demonstrated that increased available calcium and phytate-phosphate from phytase reduced counts of some Lactic Acid Bacteria, while increasing strict anaerobic bacteria. In this study, we investigated the effects of two phytases administered at two inclusion levels on growth performance and populations of gastrointestinal microorganisms in broiler chickens fed a diet with reduced available phosphorus over a 42-day period.

## 4.2. MATERIALS AND METHODS

### *4.2.1 Experimental Design*

Male broilers (Cobb 500, n = 2580) were obtained from a commercial hatchery on day of hatch, randomly assigned to treatment pens with similar starting weights, and provided experimental feed and water ad libitum for the duration of the study. Experimental animals were allocated to 6 experimental treatment groups with 10 replicate pens of 43 broiler chicks arranged as a complete randomized block design. Experimental

treatment groups were fed experimental rations formulated to contain adequate phosphate as a Reference diet (**REF**) (aP%. Starter 0.45; Grower 0.41; and Finisher 0.36), a Reduced phosphate diet (**RED**) (aP%, Starter 0.277; Grower 0.237; and Finisher 0.186), and a Reduced phosphate diet supplemented with one of two phytases. The commercial phytases used in this study are composed of a mutant histidine acid phosphatase derived from *Buttiauxella* sp. (291) expressed in *Trichoderma reesei* **(Phy 1)** and a bacterial 6-phytase from the histidine acid phosphatase family expressed in *Aspergillus niger* **(Phy 2)**, at 500 U $kg^{-1}$ and 1,000U $kg^{-1}$ feed (**Table 4.1**). All animal care and experimental procedures were performed in accordance with protocols approved by the Texas A&M University Institutional Animal Care and Use Committee.

**Table 4.1.** Experimental Treatments of Two Phytases on Broiler Chickens

| Group | Treatments | | |
|---|---|---|---|
| | aP (%) (S/G/F) | Phytase | Inclusion Rate |
| Reference Diet | 0.45/0.41/0.36 | - | - |
| Reduced Diet | 0.277/0.237/0.186 | - | - |
| Phytase 1 – Low | 0.277/0.237/0.186 | Phytase 1 | 500 U $kg^{-1}$ |
| Phytase 2 – Low | 0.277/0.237/0.186 | Phytase 2 | 500 U $kg^{-1}$ |
| Phytase 1 – High | 0.277/0.237/0.186 | Phytase 1 | 1000 U $kg^{-1}$ |
| Phytase 2 – High | 0.277/0.237/0.186 | Phytase 2 | 1000 U $kg^{-1}$ |

*4.2.2 Bacterial Enumeration*

At 21 and 42 days post-hatch, a single chicken of approximately mean pen weight was selected from each replicate pen, euthanized, and necropsied for the collection of tissues for the enumeration of gastrointestinal microorganisms. The ceca and a section (~ 6 cm) of the ileum centered on the midpoint between Meckel's diverticulum and the ileocecal junction were dissected aseptically from each selected chicken. Ileal specimens were homogenized and diluted using Fluid Thioglycolate Medium (**FTM**; BD, Franklin Lakes, NJ), whereas cecal specimens were homogenized and diluted using sterile phosphate buffered saline (**PBS**; Fisher Scientific, Pittsburgh, PA). *Campylobacter jejuni* was enumerated from the ceca using Campy Cefex agar (Hardy Diagnostics, Santa Maria, California) incubated in 10% $CO_2$ at 42 $^{o}$C; total Lactic Acid Bacteria (**LAB**) were enumerated from the ileum and ceca using deMan, Rogosa, and Sharpe agar (**MRS**; BD) supplemented with 100 $\mu g \cdot mL^{-1}$ cycloheximide (Amresco, Solon, OH) incubated in 10 % $CO_2$ at 37 $^{o}$C; and *Clostridium perfringens* from the ileum using Tryptose Sulphite Cycloserine Egg Yolk overlay agar (**TSC-EY**; BD) incubated anaerobically (Coy Laboratory Products, Grass Lake, MI) at 37 $^{o}$C. *C. jejuni* was selectively enriched from cecal specimens using Bolton's Enrichment Broth (**BEB**, Hardy Diagnostic) incubated at 42 $^{o}$C for 24 h followed by isolation using Campy Cefex agar, and *C. perfringens* was selectively enriched from the ileal-FTM homogenate incubated anaerobically at 37°C for 24 h followed by culturing using Iron Milk Medium incubated at 46$^{o}$C for 3 h. Presumptive *C. perfringens* were confirmed using Iron Milk Medium. Specimens for which there were no colonies appearing on enumeration plates but were

positive by selective enrichment were assigned the lower limit of detection, 100 cfu $g^{-1}$ for statistical analysis.

*4.2.3 Statistical Analysis*

Bacterial counts were $log_{10}$ transformed for analysis and reported as the mean ± SEM $log_{10}$ cfu $g^{-1}$ digestive contents from 10 replicate pens per treatment. Data for all treatments were analyzed using one-way ANOVA, while data for phytase-treated groups were also analyzed using factorial ANOVA with main effects for phytase, inclusion rate, and phytase × inclusion rate. Significantly different means ($P \leq 0.05$) were separated using Duncan's multiple range test *post hoc*. Associations between bacterial counts and feed conversion ratio (**FCR**) were evaluated by pens using Pearson's *r*.

## 4.3 RESULTS

*4.3.1 Gastrointestinal Microbiota*

*4.3.1.1 Total Lactic Acid Bacteria.*

*4.3.1.1.1 Ileum*

A significant treatment effect was observed from analysis using One-way ANOVA on counts of total LAB in the ileum at Day 21 ($P = 0.02$) (**Figure 4.1A**) and Day 42 ($P = 0.02$) (**Figure 4.1B**) post-hatch (**Table 4.2**). On Day 21, counts of total LAB were greatest when broilers were fed the Reference diet and diets containing Phy 2. Whereas on Day 42, counts of total LAB were greatest when broilers were fed the Reduced diet and the diet containing Phy 2. Total LAB counts were greater in broilers administered 500 U $kg^{-1}$ Phy 2 as compared to those administered 500 U $kg^{-1}$ Phy 1 on Day 21 ($P = 0.002$), whereas total LAB counts were greater in broilers administered 1000 U

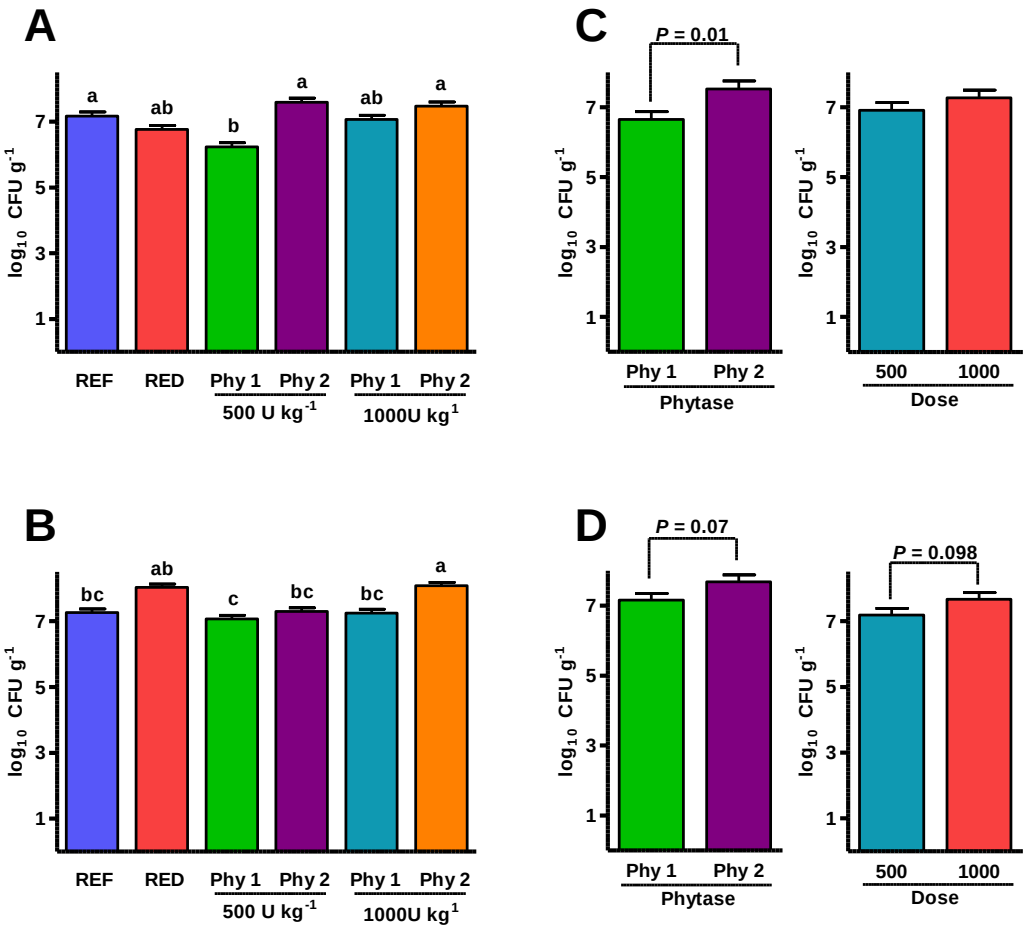


**Figure 4.1. Enumeration of Total LAB in the ileum.** Total LAB were enumerated from the small intestine of broiler chicks at (A) Day 21 and (B) Day 42 post-hatch, followed by main effects between Total LAB and phytase sources; and main effects between Total LAB and dose levels (C/D). Counts are reported as the $\log_{10}$ CFU $g^{-1}$ digestive contents from 10 broiler chickens per treatment. Different letters above bars indicate means are significantly different ($P \leq 0.05$).

$kg^{-1}$ Phy 2 as compared to those fed 1000 U $kg^{-1}$ Phy 1 on Day 42 ($P$ = 0.027) (**Figure 4.1B**).

A significant main effect of phytase on total LAB counts in the ileum was observed on Day 21, with more LAB having been recovered from broilers fed diets containing Phy 2 as compared to those fed diets containing Phy 1 ($P$ = 0.01) (**Figure 4.1C**). A significant main effect of phytase on total LAB counts was not observed for Day 42 post-hatch. However, on Day 42, recovery of total LAB tended to be greater when broilers were fed diets containing Phy 2 ($P$ = 0.07) (**Figure 4.1D**). A significant main effect of the level of phytase inclusion was not observed for LAB counts on Day 21 or Day 42 post-hatch. However, on Day 42, recovery of total LAB tended to be greater when broilers were fed diets containing 1000 U $kg^{-1}$ phytase as compared to those fed diets containing 500 U $kg^{-1}$ phytase ($P$ = 0.098) (**Figure 4.1D**). No significant Phytase × Dose interaction was observed throughout the 42 Day trial.

*4.3.1.1.2 Cecum*

A significant treatment effect was observed from analysis using One-way ANOVA on counts of total LAB in the cecum at Day 21 ($P$ = 0.01) (**Figure 4.2A**). Counts of total LAB were greatest when broilers were fed diets containing 500 U $kg^{-1}$ or 1000 U $kg^{-1}$ of Phy 2 and the Reduced diet, whereas total LAB counts were lower when broilers were fed the Reference diet. A significant treatment effect was not observed on counts of total LAB in the cecum at Day 42 post-hatch ($P$ = 0.36) (**Figure 4.2B**). However, counts of total LAB tended to be greater when broilers were fed diets containing

500 U kg$^{-1}$ Phy 1 ($P$ = 0.077), 1000 U kg$^{-1}$ Phy 1 ($P$ = 0.092), and 1000 U kg$^{-1}$ Phy 2 ($P$ = 0.062) (**Figure 4.2B)** as compared to broilers fed the Reference diet.

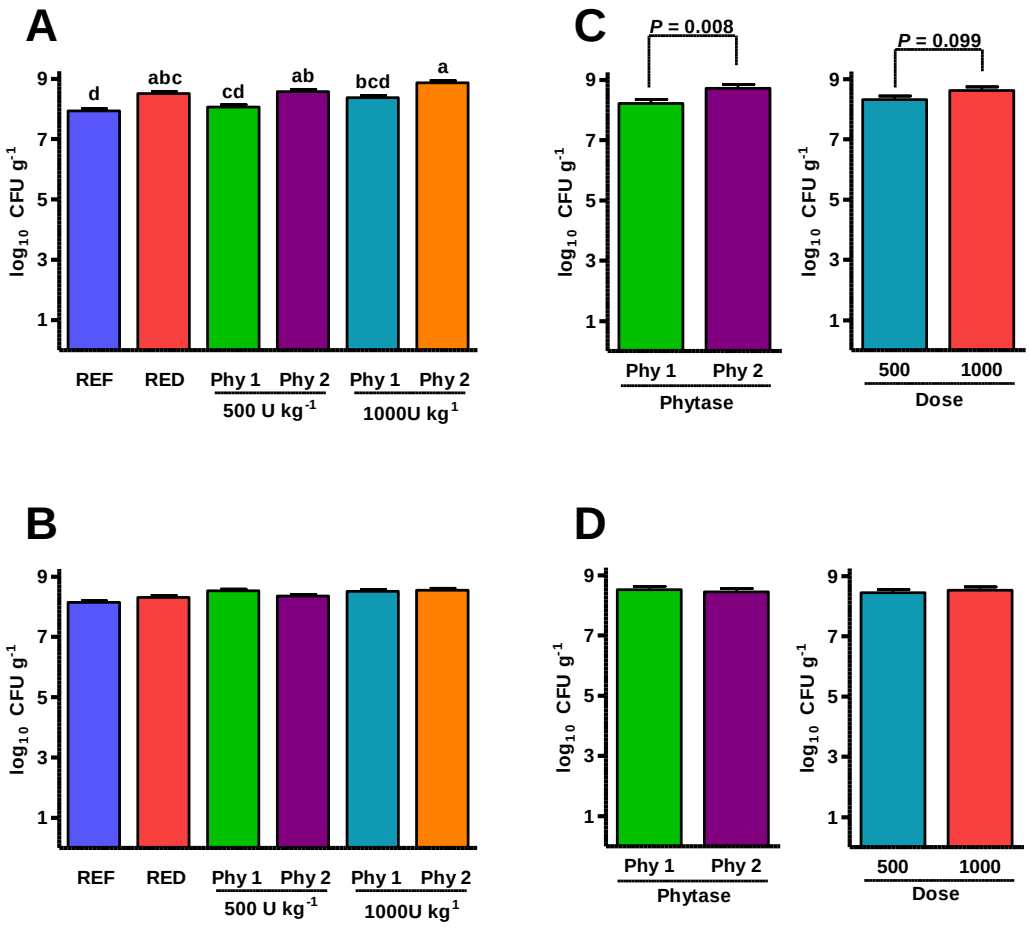


**Figure 4.2. Enumeration of Total LAB in the cecum.** Total LAB were enumerated from the cecum of broiler chicks at (A) Day 21 and (B) Day 42 post-hatch, followed by main effects between Total LAB and phytase sources; and main effects between Total LAB and dose levels (C/D). Counts are reported as the $\log_{10}$ CFU g$^{-1}$ digestive contents from 10 broiler chickens per treatment. Different letters above bars indicate means are significantly different ($P \leq 0.05$).

**Table 4.2.** Effects of phytase on gastrointestinal bacteria ($log_{10}$ cfu $g^{-1}$)

| Treatments | | | Ileum | | | | Cecum | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Total LAB[5] | | *C. perfringens* | | Total LAB | | *C. jejuni* | |
| Diet | Phy[2] | Dose[3] | Day 21 | Day 42 | Day 21 | Day 42 | Day 21 | Day 42 | Day 21 | Day 42 |
| REF[1] | - | 0 | 7.17$^{a}$ | 7.26$^{bc}$ | 2.90 | 3.55 | 7.94$^{a}$ | 8.15 | 3.56 | 6.51 |
| Red[2] | - | 0 | 6.77$^{ab}$ | 8.03$^{ab}$ | 2.55 | 2.50 | 8.52$^{abc}$ | 8.32 | 4.01 | 6.30 |
| Red | 1 | 500 | 6.24$^{b}$ | 7.07$^{c}$ | 2.89 | 2.87 | 8.07$^{cd}$ | 8.53 | 4.28 | 7.00 |
| Red | 2 | 500 | 7.59$^{a}$ | 7.30$^{bc}$ | 2.82 | 2.73 | 8.58$^{ab}$ | 8.36 | 4.50 | 6.28 |
| Red | 1 | 1000 | 7.07$^{ab}$ | 7.25$^{bc}$ | 3.32 | 3.48 | 8.37$^{bcd}$ | 8.53 | 4.44 | 6.40 |
| Red | 2 | 1000 | 7.47$^{a}$ | 8.08$^{a}$ | 3.11 | 3.33 | 8.84$^{a}$ | 8.55 | 4.30 | 6.50 |
| | Pooled SEM | | 0.13 | 0.11 | 0.09 | 0.14 | 0.08 | 0.018 | 0.14 | 0.08 |
| | *P*-value | | 0.02 | 0.02 | 0.22 | 0.20 | 0.01 | 0.36 | 0.32 | 0.17 |

[1] Reference Diet (REF); [2] Reduced Phosphate (RED); [3] Phytase type; [4] Phytase inclusion, U $kg^{-1}$; [5] Lactic Acid Bacteria

[a-d] Different superscripts within columns indicate means are significantly different ($P \leq 0.05$)

A significant main effect of phytase on counts of total LAB in the cecum was observed on Day 21 (P = 0.008) (**Figure 4.2C**), with more LAB recovered from broiler fed diets containing Phy 2 as compared to those fed diets containing Phy 1. However, a significant main effect was not observed on Day 42 post-hatch (*P* = 0.66) (**Figure 4.2D**). A significant main effect of the level of phytase inclusion on counts of total LAB in the cecum was not observed for Day 21 or Day 42 post-hatch (**Figure 4.2C/D**). However, more LAB tended to be recovered from broilers fed diets containing 1000 U $kg^{-1}$ phytase as compared to those fed diets containing 500 U $kg^{-1}$ (P = 0.099) (**Figure 4.2C**). No significant Phytase × Dose interaction was observed throughout the 42 Day trial.

*4.3.1.2 Clostridium perfringens.* A significant treatment effect was not observed from analysis using One-way ANOVA on counts of *Clostridium perfringens* in the ileum of broilers on Day 21 (*P* = 0.22) or Day 42 (*P* = 0.20) (**Table 4.2)** post-hatch. However, *C. perfringens* counts were lowest from broilers fed the Reduced diet as compared to the remaining treatment groups on Day 21 and Day 42 (**Figure 4.3A/B**). Although the overall treatment effect was not significant, more *C. perfringens* were recovered from broilers fed the Reference diet as compared those fed the reduced diet on Day 42 (*P* = 0.039). Additionally, *C. perfringens* counts tended to be greater when broilers were fed diets containing 1000 U $kg^{-1}$ of either Phy 1 (*P* = 0.052) or Phy 2 (*P* = 0.098) (**Figure 4.3B**) as compared those fed the Reduced diet. Significant main effects of phytase on counts of *Clostridium perfringens* in the ileum were not observed on Day 21 or Day 42 (**Figure 4.3C/D**). However, more *C. perfringens* tended to be recovered from broilers fed diets containing 1000 U/kg phytase as compared to those fed diets containing 500

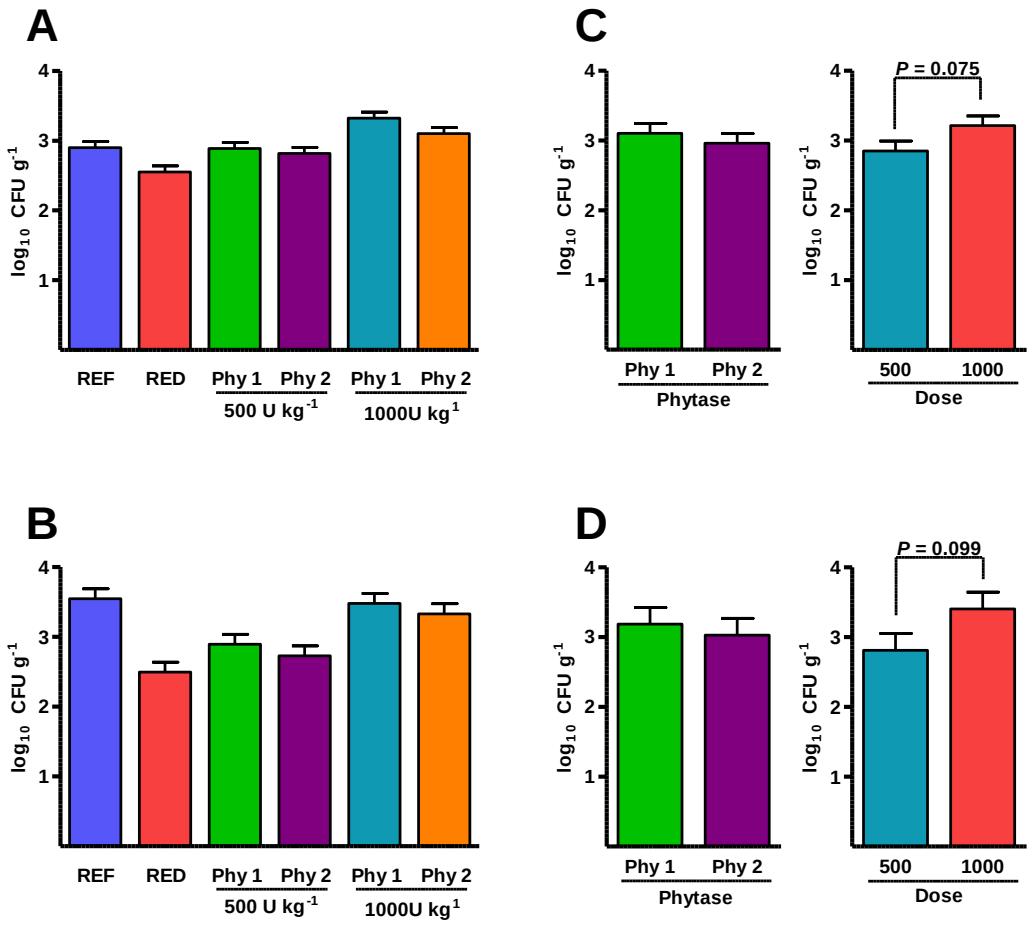


**Figure 4.3. Enumeration of *Clostridium perfringens* in the ileum.** *C. perfringens* were enumerated from the small intestine of broiler chicks at (A) Day 21 and (B) Day 42 post-hatch, followed by main effects between Total *C. perfringens* and phytase sources; and main effects between *C. perfringens* and dose levels (C/D). Counts are reported as the $\log_{10}$ CFU $g^{-1}$ digestive contents from 10 broiler chickens per treatment. Different letters above bars indicate means are significantly different ($P \leq 0.05$).

U/kg phytase on Day 21 ($P$ = 0.075) and Day 42 ($P$ = 0.099) (**Figure 4.3C/D**). No significant Phytase × Dose interaction was observed throughout the 42 Day trial.

*4.3.1.3 Campylobacter jejuni*. A significant treatment effect was not observed from analysis using One-way ANOVA on counts of *Campylobacter jejuni* in the cecum of broilers on Day 21 ($P$ = 0.32) and Day 42 ($P$ = 0.17) (**Figure 4.4A/B**). Although the difference was not significant, fewer *C. jejuni* were recovered when broilers were administered the Reference diet as compared to the other treatments on Day 21. No significant main effects of phytase or level of phytase inclusion on the recovery of *C. jejuni* from the cecum were observed from on Day 21 or Day 42 (**Figure 4.4C/D**).

*4.3.2 Correlation of Bacterial Counts and Growth Performance*

The effect of the experimental treatments on feed conversion and tibia ash of broiler chickens is summarized in **Table 4.3** Overall, administration of Phytase improved early (Day 0-21) ($P$ = 0.007), late (Day22-42) ($P$ < 0.001), and cumulative FCR (cFCR) ($P$ < 0.001) when compared to the Reduced diet, and to levels statistically similar to Reference diet. Administration of 1000 U $kg^{-1}$ Phy 1 had the greatest improvement in feed conversion and recovered Tibia Ash weight when compared to other phytase treatments and the Reference diet. Furthermore Phy 1 was statistically similar to the Reference diet. Likewise, both inclusion rates of Phy 2 improved early (Day 0-21) FCR to similar levels of 1000 U $kg^{-1}$ Phy 1 and the Reference diet.

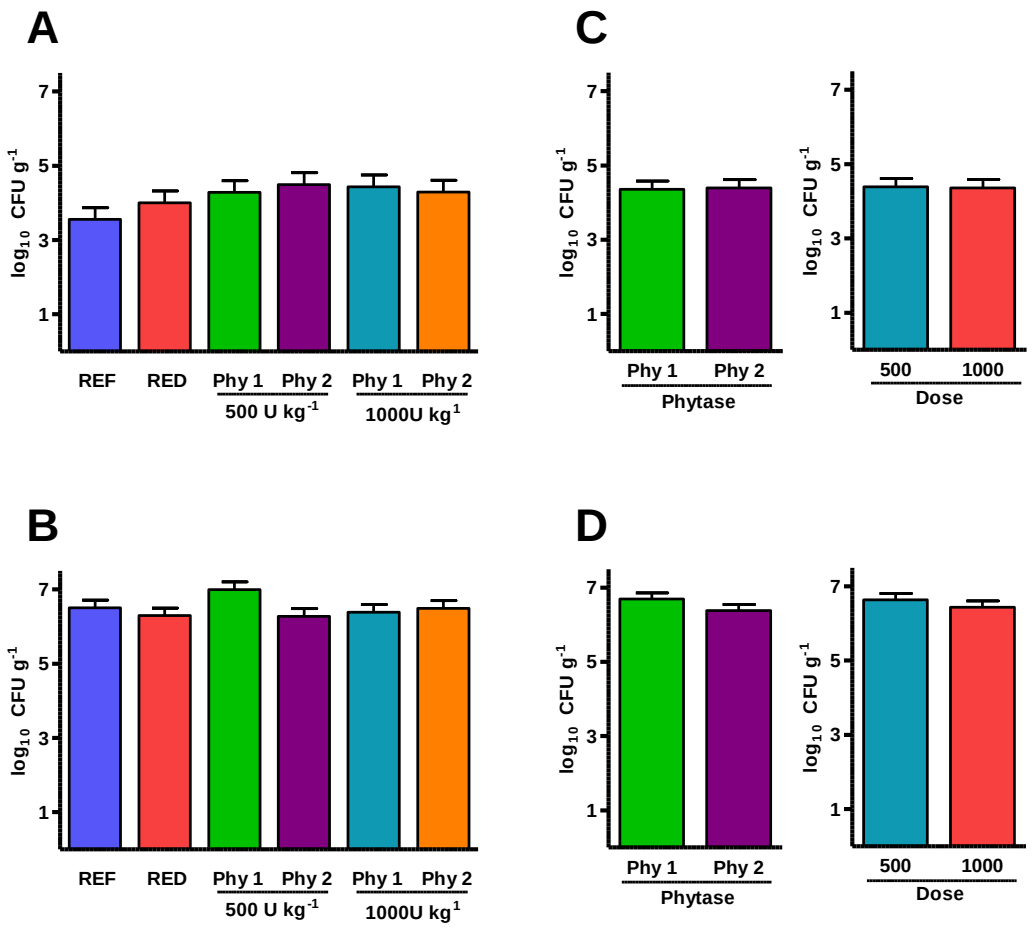


**Figure 4.4 Enumeration of *Campylobacter jejuni* in the cecum.** *C. jejuni* were enumerated from the cecum of broiler chicks at (A) Day 21 and (B) Day 42 post-hatch, followed by main effects between *C. jejuni* and phytase sources; and main effects between *C. jejuni* and dose levels (C/D). Counts are reported as the $\log_{10}$ CFU $g^{-1}$ digestive contents from 10 broiler chickens per treatment. Different letters above bars indicate means are significantly different ($P \leq 0.05$).

**Table 4.3.** Select growth performance measures of broiler chickens

| Treatments | | | FCR (Feed:Gain)[4] | | | Tibia Ash | |
|---|---|---|---|---|---|---|---|
| aP[1] | Phy[2] | Dose[3] | D 00-21 | D 22-42 | cFCR[5] | Ash % | Wt(g) |
| REF | - | 0 | 1.363$^{b}$ | 1.792$^{c}$ | 1.776$^{c}$ | 51.90$^{a}$ | 0.924$^{ab}$ |
| RED | - | 0 | 1.448$^{a}$ | 1.858$^{a}$ | 2.041$^{a}$ | 47.27$^{b}$ | 0.599$^{d}$ |
| RED | 1 | 500 | 1.386$^{ab}$ | 1.818$^{bc}$ | 1.824$^{bc}$ | 51.06$^{a}$ | 0.880$^{bc}$ |
| RED | 2 | 500 | 1.376$^{b}$ | 1.828$^{b}$ | 1.849$^{b}$ | 51.32$^{a}$ | 0.862$^{c}$ |
| RED | 1 | 1000 | 1.372$^{b}$ | 1.797$^{c}$ | 1.776$^{c}$ | 51.65$^{a}$ | 0.947$^{a}$ |
| RED | 2 | 1000 | 1.402$^{ab}$ | 1.804$^{bc}$ | 1.806$^{bc}$ | 51.36$^{a}$ | 0.936$^{a}$ |
| | Pooled SEM | | 0.005 | 0.005 | 0.014 | 0.24 | 0.018 |
| | *P*-value | | 0.007 | <0.001 | <0.001 | <0.001 | <0.001 |

[1] Available phosphate; [2] Phytase type; [3] Phytase inclusion, U kg$^{-1}$; [4] cumulative FCR; [5]Body weight corrected cumulative FCR

[a-d] Different superscripts within columns indicate means are significantly different ($P \leq 0.05$)

Associations between populations of gastrointestinal microorganisms with feed conversion were evaluated (**Table 4.4**). A moderate positive correlation ($P = 0.036$) was detected between total LAB in the ileum on Day 42 and early FCR, in addition to a positive trending correlation with cumulative FCR ($P = 0.082$). Additionally, another weak positive correlation ($P = 0.038$) was detected between total LAB in the cecum on Day 42 and early FCR. Furthermore, total LAB in the ileum on Day 42 tended to correlate negatively ($P < 0.1$) with Tibia Ash % and Tibia Ash weight. Weak negative correlations were detected between total *C. perfringens* counts on Day 21 ($P = 0.030$) and Day 42 ($P = 0.027$) with early FCR (Day 0 – 21) and cumulative body weight corrected FCR, respectively. However, weak positive correlations were detected between total *C. perfringens* counts on Day 21($P = 0.006$) Tibia Ash weight and Day 42 ($P = 0.047$) with Tibia Ash %. No associations were detected between FCR, and *Campylobacter,* and no associations were detected between Tibia Ash and *C. jejuni.*

**Table 4.4.** Correlation of bacterial counts with select growth performance measures

| Bacterial Counts | | FCR (Feed:Gain)[1] | | | Tibia Ash | |
|---|---|---|---|---|---|---|
| ($Log_{10}$ CFU $g^{-1}$) | | D 00-21 | D 22-42 | cFCR[2] | Ash % | Wt(g) |
| *Ileum* | | | | | | |
| Total LAB[3] | | | | | | |
| Day 21 | r | -0.016 | -0.055 | -0.096 | 0.233 | 0.145 |
| | *P* | 0.903 | 0.678 | 0.466 | 0.073 | 0.269 |
| Day 42 | r | 0.272 | 0.016 | 0.226 | -0.232 | -0.237 |
| | *P* | 0.036 | 0.903 | 0.082 | 0.074 | 0.068 |
| *C. perfringens* | | | | | | |
| Day 21 | r | -0.280 | -0.203 | -0.245 | 0.239 | 0.348 |
| | *P* | 0.030 | 0.120 | 0.059 | 0.066 | 0.006 |
| Day 42 | r | 0.110 | -0.226 | -0.286 | 0.257 | 0.167 |
| | *P* | 0.401 | 0.083 | 0.027 | 0.047 | 0.201 |
| *Cecum* | | | | | | |
| Total LAB[3] | | | | | | |
| Day 21 | r | 0.101 | -0.032 | 0.036 | -0.050 | -0.061 |
| | *P* | 0.444 | 0.807 | 0.784 | 0.703 | 0.643 |
| Day 42 | r | 0.268 | 0.055 | -0.009 | 0.129 | 0.053 |
| | *P* | 0.038 | 0.676 | 0.943 | 0.326 | 0.689 |
| *C. jejuni* | | | | | | |
| Day 21 | r | 0.063 | -0.183 | -0.161 | 0.140 | -0.012 |
| | *P* | 0.631 | 0.161 | 0.218 | 0.288 | 0.926 |
| Day 42 | r | -0.015 | -0.056 | -0.135 | 0.168 | 0.152 |
| | *P* | 0.907 | 0.673 | 0.302 | 0.200 | 0.248 |

[1] Mortality corrected FCR; [2] cumulative FCR; [3] LAB, Lactic Acid Bacteria

## 4.4 DISCUSSION

The objective of this study was to compare the effects of two exogenous phytases administered at two inclusion levels on growth performance and the populations of gastrointestinal microorganisms of broiler chickens. In-feed administration of microbial phytases to improve the digestibility of phytic acid is used widely in the production of poultry (276, 292). Although the resulting increase in phytate-phosphorus digestibility and reduction in the anti-nutritive effects of phytic acid are well documented (226, 278), effects of available phosphate and phytase supplementation on the gastrointestinal microbiota have not been widely investigated. The gastrointestinal microbiota is increasingly recognized as an important modulator of human and animal health (239). Additionally, the products of phytate hydrolysis may serve as substrates which promote or limit the growth activities of bacteria (231). It has been suggested that an increase in strict anaerobic bacteria may be associated with greater phosphorous availability in the lumen of the gastrointestinal tract (284, 290). Furthermore, greater calcium and phosphorous availability from the hydrolysis of phytate in the small intestine of swine was demonstrated reduce populations of lactobacilli (290). The concentration of bioavailable phosphorous and calcium has been demonstrated to modulate the microbiota of monogastric animals, including murine (293), porcine (288), and poultry models (294). In this study, we evaluated the effect of the administration of two phytases at two inclusion levels on growth performance, bone ash, and the populations of gastrointestinal microorganisms of broiler chickens fed a diet with reduced available phosphorous over a 42-day growth period.

Administration of phytases improved growth performance of broiler chickens to a level similar to that for those fed a diet adequate in phosphorus. Phosphatases and phytase are commonly used in poultry production (278, 292), and their effects in enhancing performance and nutrient availability is widely known (23, 220, 292) and. There are many sources of microbial phytases (292) including Gram-negative bacteria (295), Gram-positive bacteria (296, 297), and molds (298). The efficacy of phytases may depend on their specificity and mode of action (299, 300). Phytases catalyze the hydrolysis of phosphate groups from specific positions of phytic acid, with preference of the phosphate at the $IP_6$ position, to $IP_1$ in descending order (301, 302). Some bacterial phytases have demonstrated affinity for IP6 and IP5 with high resistance to proteolytic digestion compared to fungal phytases (303). Furthermore, optimal phytase activity differs between microbial species and pH values (304, 305) both *in vitro* (306) and *in vivo* (307). Similarly, a histidine acid-phosphatase from *Aspergillus niger* was demonstrated to release all six phosphates from the *myo*-inositol hexakisphosphate, whereas a histidine phosphatase from *E. coli* only released 5 of the 6 under strict *in vitro* conditions (301). These factors suggest the possibility that there are appropriate phytases for each livestock animal (302), which may further be complicated by dietary-related factors, specifically plant-based feed ingredients (302, 308). In this study, the lowest FCR amongst the phytase treatments was seen with 1000 U $kg^{-1}$ Phy 1 (**Table 4.3).** In actuality, **s**ome phytases may depend on availability of metal-free phytate or calcium-phytate substrates (309), in addition to the previously mentioned factors. Furthermore, similar commercial phytase products have different optimal conditions. Phyzyme® an *E. coli* origin phytase

from Danisco Animal Nutrition has an optimal pH of 4.5 and temperature of 55 ˚C (302), whereas the *Buttiauxella* product Axtra® has an optimal pH range of 3.5-4.5 and temperature of 60 ˚C (302). It is possible that the efficacy of these phytases differ in the small intestine of a broiler chicken pH 6.0-6.5 (310) and 41 ˚C (311). Which could explain the difference between the two histidine phytases used in this current study.In this study, the positive associations with FCR were observed between counts of total LAB in the ileum ($P$ = 0.036) and cecum ($P$ = 0.038) at Day 21 post-hatch (**Table 4.4**). This is contradictory to previous reports reporting LAB improving growth performance in broilers (94, 157, 176). However, LAB make up a significant population of the gastrointestinal tract (44, 294). Furthermore, they are believed to be important inhabitants of the gastrointestinal microbiota, and are generally recognized as beneficial to poultry health (133, 251). It may be possible that specific genera that comprise LAB are responsible for improved growth and general commensalism to the broiler chicken, whereas the remaining genera may not provide a benefit to the host. Additionally, it has been previously demonstrated that the beneficial LAB, lactobacilli were reduced with increased bioavailability of calcium and phytate-P in swine (290). Perhaps this is another reason why reduced growth performance was observed. Also, it may be appropriate to explore if certain lactic acid producing bacteria reduce broiler growth performance, or at least reduce the effectiveness of phytase administration. The understanding of the role of the gastrointestinal microbiota and the role of specific microorganism in animal health is still considered to be in its infancy.  Thus, the role of specific LAB in potentially reducing growth performance is undetermined at this time. Although total LAB was greatest in

broilers administered Phy 2 compared to Phy 1 (**Figure 4.3 and 4.4**), LAB populations were still abundant in the unsupplemented treatment (**Figure 4.1 and 4.2**), suggesting the increased available phosphate from phytase supplementation being the primary factor in growth performance in this study.

The negative correlations of *C. perfringens* counts were observed with early FCR and cumulative FCR (**Table 4.4**). Additionally, positive correlations between *C. perfringens* counts and Tibia ash was detected. Suggesting that 3 $\log_{10}$ CFU $g^{-1}$ of *C. perfringens* is associated with more efficient feed conversion and bone mineralization. Tibia ash % and weight are commonly used indicators of mineral adequacy in poultry, the primary nutrients that make up bone are calcium and phosphorus (312, 313). Poor mineralization is associated with poor nutrient absorption (314). However, reduced weight gain, poor mineralization and increased FCR is commonly reported when high numbers of *C. perfringens* are recovered from both naturally (315, 316) and experimentally infected (258) broilers. Still, *C. perfringens* enumerated from broilers in all experimental treatments was near or below 3 $\log_{10}$ CFU $g^{-1}$, far below *C. perfringens* levels associated clinical infections of necrotic enteritis at 5 $\log_{10}$ CFU $g^{-1}$ (317). Once phytase releases nutrients from phytate, those nutrients become available to both bacteria and broiler chicken (318). It is suggested that both calcium and phosphorus in combination is important to *C. perfringens* proliferation (319, 320) and toxin production (317), not phosphorous alone. Although phytase did not reduce *C. perfringens* levels in these experimental conditions, it was not unexpected. It may be possible that low quantities *C.*

*perfringens* fill an important ecological niche in the gastrointestinal microbiota of poultry.

No correlations were observed between *Campylobacter jejuni* and FCR. Overall treatments evaluated in this study were not observed to affect colonization by *C. jejuni*. Although it is a human food-borne pathogen, *C. jejuni* has been suggested to be naturally occurring (321) and commensal in poultry (266, 267). *C. jejuni* can serve as a hydrogen scavenger (322) potentially accelerating rate-limiting reactions during anaerobic fermentations, suggesting an important ecological role of *Campylobacter* as commensal/mutualistic microorganism in poultry.

In this study, we compared the effects of two phytases administered on growth performance and selected gastrointestinal microbial populations. We observed the efficacy of phytate hydrolysis by different phytase types affected microbial populations. Positive associations were observed between counts of Total LAB in the ileum with FCR and negative associations observed in *C. perfringens* counts with FCR. Although phosphate digestibility was not evaluated in this study, the observed associations suggest that the improved growth performance of broiler chickens was the result, at least in part, of the improved digestibility of phytate-phosphorous released by the phytase enzyme administered to the broiler chickens. Whereas the microbial variations seen were perhaps affected by the released phytate-P and calcium. The effects observed are dependent on feed ingredients, feed composition, enzyme type, and concentration (282). Without a defined microbiome for healthy broiler chickens fed a specific diet and enzyme, associations observed may be indicative of a small group, rather than an entire population.

Thus, the continued development of exogenous enzyme of microbial origin, and their effects on the host microbiota will be important to poultry production.

# 5. DOSE RESPONSE OF DIRECT-FED *CLOSTRIDIUM BUTYRICUM* MIYAIRI 588 ON POPULATIONS OF GASTROINTESTINAL MICROORGANISMS IN BROILERS

## 5.1 INTRODUCTION

Direct-Fed Microorganisms (DFM) are live microorganism fed to livestock animals for some presumed benefit associated with the microorganism (212). When administered to poultry, DFMs have been demonstrated to reduce colonization of poultry-associated pathogens (216, 217) by competitive exclusion (5), promote growth of the host animal at levels similar to antibiotic growth promoters (AGP) (323), and improve measures of intestinal health and function (324). Because of the benefits of their use, DFMs have received significant interest as potential alternatives to AGP (158) in response to increased regulation of antibiotics in livestock animal production (325). Although DFMs are used widely in the production of many livestock species (15), their effectiveness is varied between host species. However, there are several reasons that account for mixed effectiveness, mode of action for many DFMs are not understood. Additionally, DFM species, age, and diet can impact study results (239, 326). These factors promote the need for additional research to identify more effective DFMs for each livestock species.

Spore-forming bacteria, including *Bacillus* spp. and others, are widely used as DFMs in livestock animal production (133, 327). Spore-forming DFMs have greater heat resistance and longer shelf-life compared to the Lactic Acid Bacteria (LAB) which have

been traditionally used as probiotics and DFMs (328). The resulting increased survivability during the feed pelleting process and their prolonged viability in the absence of refrigeration are important advantages to the application of spore-forming bacteria as probiotics and DFMs (329, 330). Additionally, bacterial endospores are highly resilient to the deleterious environmental stresses of low pH and bile (331, 332) than vegetative bacteria in the gastrointestinal tract, allowing for a greater survivability and germination (329). Additionally, Direct-Fed spores have been demonstrated to germinate in the gastrointestinal tract, where they transiently colonize the host and their activities may exert probiotic benefits (333).

*Clostridium butyricum* is a Gram-positive, spore-forming, obligate anaerobe commonly isolated from soil and the human intestine (334, 335). Although pathogenic strains of *C. butyricum* have been characterized (336-338), there is significant interest in the use of non-virulent strains of *C. butyricum* as DFMs in poultry because of their ability to produce butyric acid as the major product of their primary metabolism (339-341). Administration of butyric acid to chickens has been demonstrated to increase villus height and surface area in the intestine (342), which is an important factor in nutrient utilization and growth performance (343, 344). Administration of non-virulent *C. butyricum* has been demonstrated to promote populations of *Lactobacillus* and *Bifidobacterium* in mice (345) and broiler (346). Additionally, administration of *C. butyricum* has been demonstrated to improve measures of growth performance, antioxidation, and immune function in broiler chickens (332, 346).

*Clostridium butyricum* MIYAIRI 588 (CBM 588) is nonpathogenic because it lacks toxin production genes and other virulence factors associated with pathogenic *Clostridium* strains (347). Used as a probiotic culture in humans, it has been demonstrated to reduce *E. coli* O157:H7 infections in mice (348) and antibiotic associated diarrhea caused by *Clostridium difficile* during *H. pylori* eradication therapy in humans (349). In this study, we evaluated the effects of the DFM *C. butyricum* MIYAIRI 588 on the gastrointestinal microbiota and growth performance of broiler chickens in order to determine its potential as a DFM culture for use in poultry production.

## 5.2 MATERIALS AND METHODS

### *5.2.1 Experimental Design*

Male broilers (Cobb 500, n = 2640) were obtained from a commercial hatchery on day of hatch, randomly assigned to treatment pens with similar starting weights, and provided experimental feed and water *ad libitum* for the duration of the study. Experimental animals were allocated to 5 experimental treatment groups with 12 replicate pens of 43 broiler chicks arranged as a complete randomized block design. Experimental treatment groups were fed an Untreated control diet; a diet containing bacitracin methylene disalicylate (**BMD**) (50 g $ton^{-1}$ feed); or diets containing spores of *Clostridium butyricum* MIYAIRI 588 at inclusion levels of 1.25, 2.50, and $3.75 \times 10^8$ cfu $kg^{-1}$ feed. All animal care and experimental procedures were performed in accordance with protocols approved by the Texas A&M University Institutional Animal Care and Use Committee.

*5.2.2 Bacterial Enumeration*

At 14 and 42 days post-hatch, three chickens of approximately mean pen weight were selected from each replicate pen, euthanized, and necropsied. The ceca and a section (~ 6 cm) of the ileum centered on the midpoint between Meckel's diverticulum and the ileocecal junction were dissected aseptically from each selected chicken, and collected specimens were grouped by organ and pooled by pen. Ileal specimens were homogenized and diluted using Fluid Thioglycolate Medium (**FTM**; BD, Franklin Lakes, NJ), while cecal specimens were homogenized and diluted using sterile anaerobic diluent consisting of: 0.45% potassium dihydrogen phosphate (% w/v) (Sigma-Aldrich), 0.6% sodium dihydrogen phosphate dodecahydrate (% w/v) (Sigma-Aldrich), 0.05% L-cysteine hydrochloride monohydrate (% w/v) (Sigma-Aldrich), 0.05% Tween 80 (% v/v) (Sigma-Aldrich), and 0.05% agar (% w/v) (BD). Total Lactic Acid Bacteria (**LAB**), *Bifidobacterium* spp., *C. perfringens*, Total Gram-positive cocci, and total aerobic bacteria were enumerated from the ileum using deMan, Rogosa, and Sharpe agar (**MRS**; BD) supplemented with 100 $\mu g \cdot mL^{-1}$ cycloheximide, *Bifidobacterium* agar, Modified (BD), Tryptose Sulphite Cycloserine Egg Yolk overlay agar (**TSC-EY**; BD) (Amresco, Solon, OH), Sodium Azide Agar (BD), and Trypticase Soy agar (**TSA**, BD) respectively. *Clostridium butyricum* was enumerated from the cecum using BL Agar (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 2% sodium propanoate (% w/v) (Sigma-Aldrich), 0.0002% sodium fluoride (% w/v) (Sigma-Aldrich), 5% defibrinated horse blood (% v/v) (Fisher Sci), 50 $\mu g \cdot mL^{-1}$ Novobiocin, and 100 $\mu g \cdot mL^{-1}$ D-cycloserine. Sodium Azide agar and TSA was incubated aerobically at 37$^{o}$C for 24 h. MRS were incubated in 10%

$CO_2$ at 42ºC and 37ºC for 36 h., respectively. TSC-EY, modified *Bifidobacterium* agar and *Clostridium butyricum* Modified BL agar (Nissui) was incubated at 37ºC anaerobically (Coy Laboratory Products, Grass Lake, MI) for 36 h. *C. perfringens* was selectively enriched from the ileum using FTM homogenate incubated anaerobically at 37°C for 24 h followed by Iron Milk Medium incubated at 46ºC for 3 h. Specimens for which there were no colonies appearing on enumeration plates but were positive by selective enrichment were assigned the lower limit of detection, 100 cfu $g^{-1}$ for statistical analysis. Presumptive *C. perfringens* were confirmed using Iron Milk Medium.

*5.2.3 Statistical Analysis*

Bacterial counts were $\log_{10}$ transformed for analysis and reported as the mean ± SEM $\log_{10}$ cfu $g^{-1}$ digestive contents from 12 replicate pens per treatment. Data was analyzed using ANOVA. significantly different means ($P \leq 0.05$) were separated using Duncan's multiple range test *post-hoc*. Associations between bacterial counts and feed conversion ratio (**FCR**) were evaluated by pens using Pearson's *r*.

## 5.3 RESULTS

### *5.3.1 Enumeration of Gastrointestinal Bacteria*

*5.3.1.1 Clostridium butyricum.* A significant treatment effect was not observed on counts of *C. butyricum* in the cecum of broilers on Day 14 ($P = 0.167$) or Day 42 ($P = 0.095$) (**Table 5.1 and Figure 5.1 A-B**). Although *C. butyricum* MIYAIRI 588 administration was not observed to have a significant effect on Day 42, fewer *C. butyricum* tended to be recovered from broilers administered the BMD diet than remaining treatments ($P < 0.10$). The dose of *C. butyricum* MIYAIRI 588 administered was not observed to affect the recovery of *C. butyricum* on Day 14 or Day 42 (**Figure 5.1 A/B**). However, *C. butyricum* MIYAIRI 588 inclusion rate tended to increase the recovery of *C. butyricum* on Day 42 (**Table 5.1**).

*5.3.1.2 Total LAB.* A significant treatment effect was observed on counts of total Lactic Acid Bacteria in the ileum at Day 14 post-hatch ($P = 0.014$) (**Figure 5.1 C**). Counts of total LAB were greater when birds were fed diets containing *C. butyricum* MIYAIRI 588 and BMD as compared to those fed the Untreated (UNT) diet. A significant treatment effect was also observed on counts of total LAB in the ileum at Day 42 post-hatch ($P < 0.001$) (**Figure 5.1 D**). Fewer total LAB were recovered from broilers fed the diet containing the 1× dose of *C. butyricum* MIYARI 588 than from broilers fed the other diets. The dose of *C. butyricum* MIYAIRI 588 administered was not observed to affect the recovery of total LAB on Day 14. However, a significant effect of *C. butyricum* MIYAIRI 588 inclusion rate on counts of total LAB in the ileum was observed on Day 42 ($P < 0.05$), with more LAB recovered from broiler inclusion levels of 2× and 3×

**Table 5.1.** Effects of *C. butyricum* MIYAIRI 588 administration on gastrointestinal bacteria ($log_{10}$ cfu $g^{-1}$)

| | Cecum | | Ileum | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *C. butryicum* | | Total LAB[2] | | *Bifidobacterium* | | Gram (+) Cocci | | Total Aerobes | | *C. perfringens* | |
| Treatment | D 14 | D 42 | D 14 | D 42 | D 14 | D 42 | D 14 | D 42 | D 14 | D 42 | D 14 | D 42 |
| BMD Control[1] | 7.60 | $6.84^{y}$ | $8.26^{a}$ | $8.34^{a}$ | $6.32^{bc}$ | $7.23^{b}$ | $7.69^{ab}$ | $7.66^{b}$ | $8.01^{xy}$ | $7.76^{b}$ | 2.77 | $3.31^{x}$ |
| Untreated Control (UNT) | 7.80 | $7.31^{x}$ | $7.83^{b}$ | $8.59^{a}$ | $6.26^{c}$ | $7.39^{ab}$ | $7.42^{b}$ | $7.79^{b}$ | $7.53^{y}$ | $8.44^{a}$ | 2.88 | $2.74^{xy}$ |
| CBM 588 $1.25 \times 10^{8}$ cfu $kg^{-1}$ | 8.07 | $7.20^{xy}$ | $8.41^{a}$ | $7.56^{b}$ | $7.03^{a}$ | $6.70^{c}$ | $7.95^{a}$ | $7.27^{c}$ | $8.19^{x}$ | $7.10^{c}$ | 2.55 | $2.53^{y}$ |
| CBM 588 $2.50 \times 10^{8}$ cfu $kg^{-1}$ | 7.65 | $7.14^{xy}$ | $8.14^{ab}$ | $8.56^{a}$ | $6.73^{ab}$ | $7.73^{a}$ | $7.79^{a}$ | $8.23^{a}$ | $8.00^{xy}$ | $8.25^{ab}$ | 2.99 | $2.55^{y}$ |
| CBM 588 $3.75 \times 10^{8}$ cfu $kg^{-1}$ | 7.81 | $7.39^{x}$ | $8.23^{a}$ | $8.70^{a}$ | $6.92^{a}$ | $7.51^{ab}$ | $7.97^{a}$ | $8.17^{a}$ | $8.01^{xy}$ | $8.14^{ab}$ | 3.34 | $2.82^{xy}$ |
| Pooled SEM | 0.065 | 0.068 | 0.055 | 0.076 | 0.080 | 0.088 | 0.054 | 0.072 | 0.072 | 0.101 | 0.135 | 0.093 |
| *P* | 0.167 | 0.095 | 0.014 | <0.001 | 0.004 | <0.001 | 0.004 | <0.001 | 0.060 | <0.001 | 0.479 | 0.064 |
| $r^2$ | 0.294 | 0.321 | 0.339 | 0.556 | 0.338 | 0.591 | 0.429 | 0.550 | 0.270 | 0.453 | 0.168 | 0.295 |

[1]BMD (50 g $ton^{-1}$); [2]Lactic Acid Bacteria

[a-c]Different superscripts within columns indicate means are significantly different, ($P \leq 0.05$); [x-y] Different superscripts within columns indicate means are significantly different, ($P \leq 0.10$)

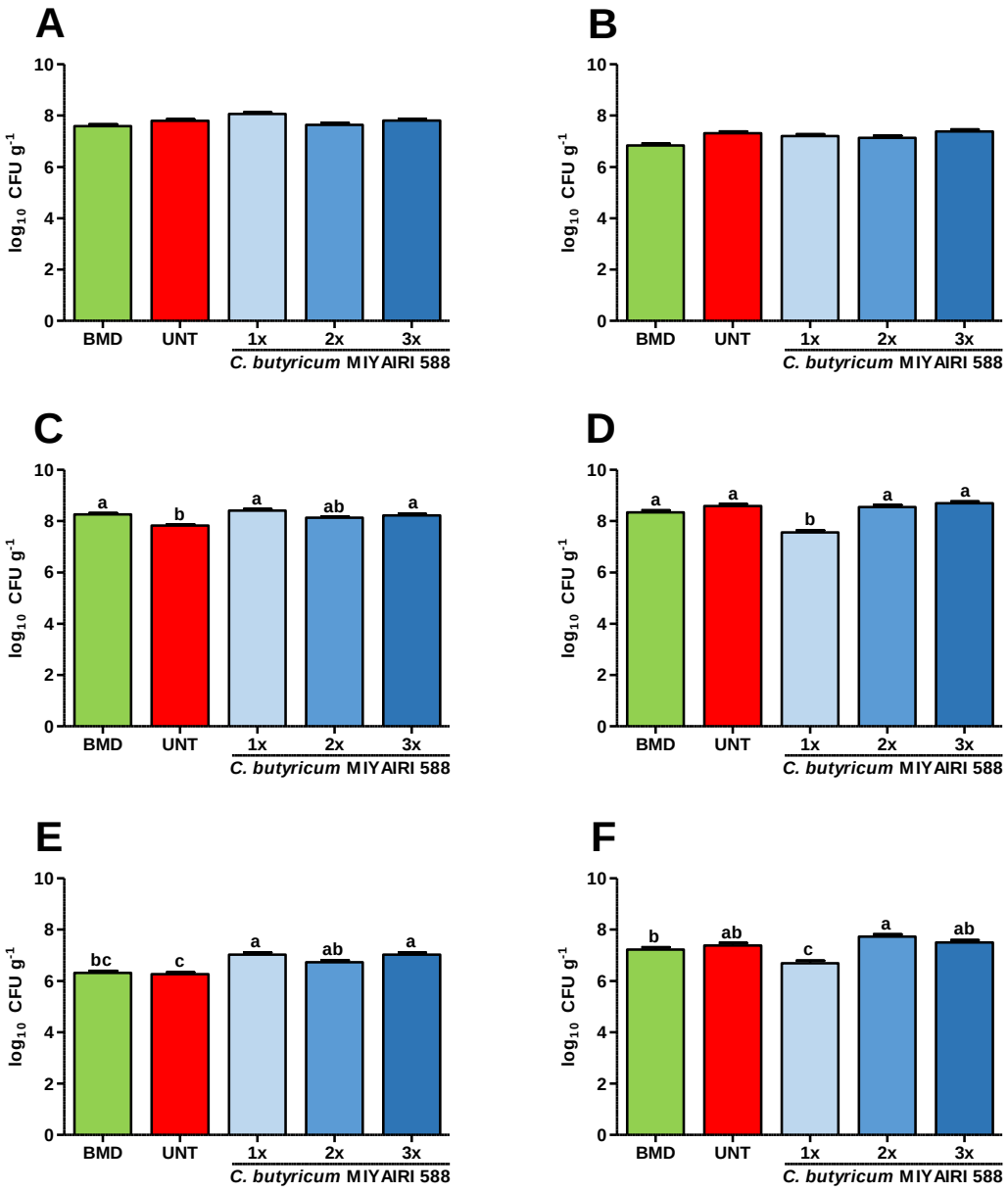


**Figure 5.1. Enumeration of beneficial bacteria from broiler chickens.** *C. butyricum* were enumerated from the cecum of broiler chicks at (A) Day 14 and (B) Day 42 post-hatch. Total LAB was enumerated from the small intestine of broiler chicks at (C) Day 14 and (D) Day 42 post-hatch. *Bifidobacterium* were enumerated from the small intestine of broiler chicks at (E) Day 14 and (F) Day 42 post-hatch. Counts are reported as the $\log_{10}$ CFU $g^{-1}$ digestive contents from 10 broiler chickens per treatment. Different letters above bars indicate means are significantly different ($P \leq 0.05$) and were separated using Duncan's multiple range.

as compared to those fed diets with inclusion levels of 1× containing *C. butyricum* MIYAIRI 588 (**Figure 5.1 D**).

*5.3.1.3 Bifidobacterium.* A significant treatment effect was observed on counts of *Bifidobacterium* in the ileum at Day 14 ($P = 0.004$) and Day 42 ($P < 0.001$) post-hatch (**Figure 5.1 E-F**). On Day 14, counts of total *Bifidobacterium* were greatest when broilers were fed diets containing *C. butyricum* MIYARI 588. Whereas on Day 42, counts of total *Bifidobacterium* were greatest when broilers were fed the Untreated diet and the diet containing 2× and 3× concentrations of *C. butyricum* MIYARI 588. Total *Bifidobacterium* counts were fewer in broilers administered 1× *C. butyricum* MIYARI 588 as compared to those administered BMD ($P = 0.014$) and the remaining treatment groups ($P < 0.001$) on Day 42. The dose of *C. butyricum* MIYAIRI 588 administered was not observed to affect the recovery of *Bifidobacterium* counts on Day 14. However, a significant effect of *C. butyricum* MIYAIRI 588 inclusion rate on counts of *Bifidobacterium* in the ileum was observed on Day 42 ($P < 0.05$), with more *Bifidobacterium* recovered from broiler inclusion levels of 2× and 3× as compared to those fed diets with inclusion levels of 1× containing *C. butyricum* MIYAIRI 588 (**Figure 5.1 F**).

*5.3.1.4 Gram-positive Cocci.* A significant treatment effect was observed on counts of total Gram-positive cocci in the ileum at Day 14 ($P = 0.004$) and Day 42 ($P < 0.001$) post-hatch (**Figure 5.2 A-B**). On Day 14, counts of total Gram-positive cocci were greatest when broilers were fed the 1× *C. butyricum* MIYARI 588 diet, counts of total Gram-positive cocci were lowest when broilers were fed the Untreated diet. On Day 42, Total Gram-positive cocci counts were greater in broilers administered 3× *C.*

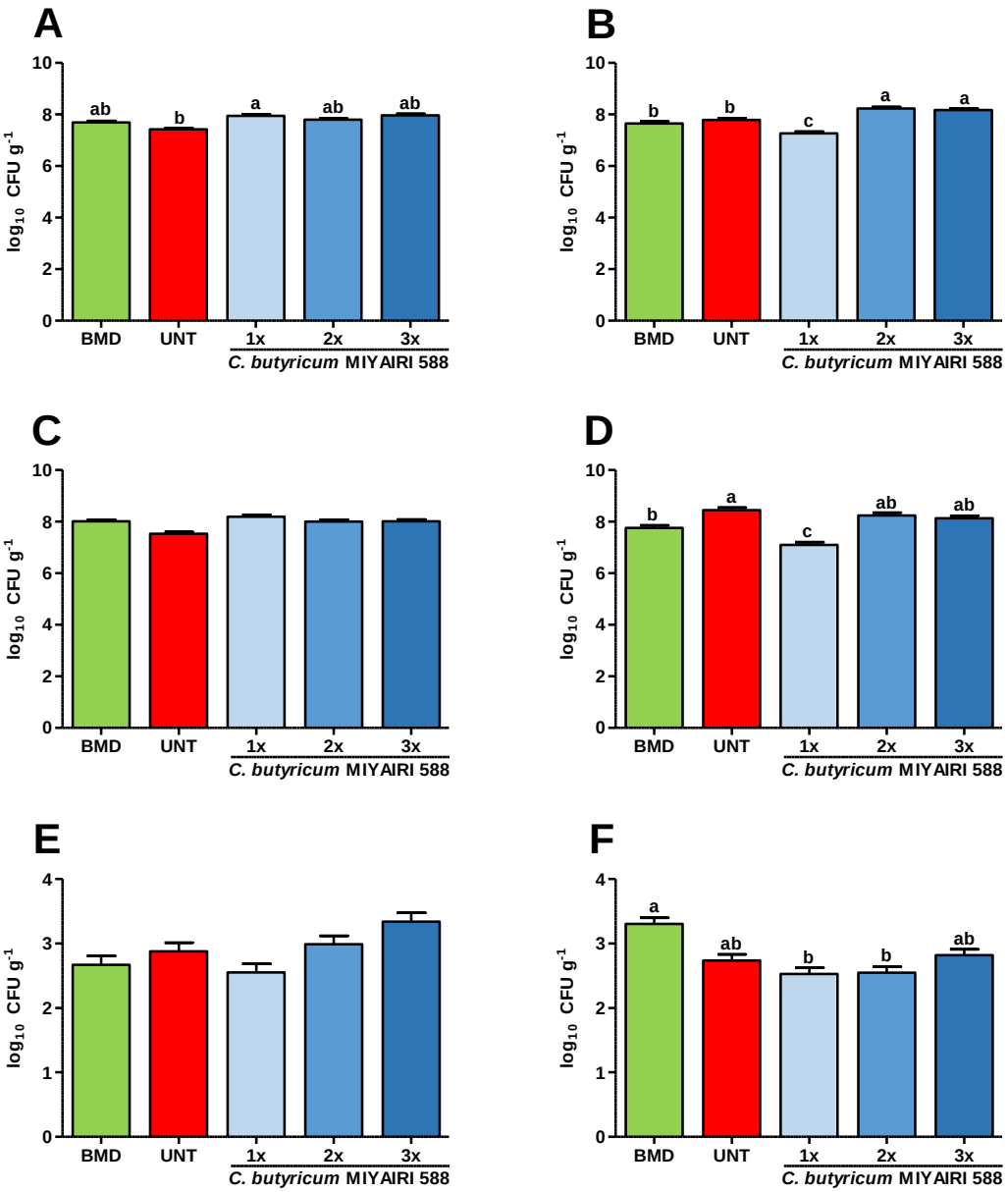


**Figure 5.2. Enumeration of bacteria from broiler chickens.** Gram-positive cocci were enumerated from the small intestine of broiler chicks at (A) Day 14 and (B) Day 42 post-hatch. Total aerobes were enumerated from the small intestine of broiler chicks at (C) Day 14 and (D) Day 42 post-hatch. *C. perfringens* were enumerated from the small intestine of broiler chicks at (E) Day 14 and (F) Day 42 post-hatch. Counts are reported as the $\log_{10}$ CFU $g^{-1}$ digestive contents from 10 broiler chickens per treatment. Different letters above bars indicate means are significantly different ($P \leq 0.05$) and were separated using Duncan's multiple range.

*butyricum* MIYARI 588 diets as compared to the BMD ($P = 0.006$) and Untreated ($P = 0.038$) treatments. Similarly, 2× *C. butyricum* MIYARI 588 was greater than the BMD ($P = 0.002$) and Untreated ($P < 0.001$) diets. Additionally, Total Gram-positive cocci counts were greater in broilers administered 2× and 3× *C. butyricum* MIYARI 588 diets as compared to the 1× *C. butyricum* MIYARI 588 diet ($P < 0.001$). The dose of *C. butyricum* MIYAIRI 588 administered was not observed to affect the recovery of total Gram-positive cocci on Day 14. However, a significant effect of *C. butyricum* MIYAIRI 588 inclusion rate on counts of total Gram-positive cocci in the ileum was observed on Day 42 ($P < 0.05$), with more Gram-positive cocci recovered from broiler inclusion levels of 2× and 3× as compared to those fed diets with inclusion levels of 1× containing *C. butyricum* MIYAIRI 588 (**Figure 5.2 B**).

*5.3.1.5 Total Aerobic Bacteria.* A significant treatment effect was not observed on counts of total aerobic bacteria in the ileum of broilers on Day 14, but fewer aerobic bacteria tended to be recovered from broilers fed the Untreated diet as compared to the remaining treatment groups ($P = 0.060$) (**Table 5.1 and Figure 5.2 C**). However, A significant treatment effect was observed on counts of total aerobic bacteria in the ileum of broilers on Day 42 ($P < 0.001$) (**Figure 5.2 D**). Total aerobic bacteria counts were highest from broilers fed the Untreated diet. While, broiler administered 1× *C. butyricum* MIYARI 588 was significantly lower than the remaining treatment groups. The dose of *C. butyricum* MIYAIRI 588 administered was not observed to affect the recovery of total aerobic bacteria on Day 14. However, a significant effect of *C. butyricum* MIYAIRI

588 inclusion rate on counts of total aerobic bacteria in the ileum was observed on Day 42 ($P < 0.05$), with more aerobic bacteria recovered from broilers dosed with levels of 2× and 3× as compared to those fed diets with inclusion levels of 1× containing *C. butyricum* MIYAIRI 588 (**Figure 5.2 D**).

*5.3.1.6 Clostridium perfringens.* A significant treatment effect was not observed on counts of *C. perfringens* in the ileum of broilers on Day 14 ($P = 0.479$) (**Figure 5.2 E-F**). A significant treatment effect was also not observed on counts *C. perfringens* in the ileum of broilers on Day 42, but greater *C. perfringens* tended to be recovered from broilers fed the BMD diet as compared to broilers administered *C. butyricum* MIYAIRI 588 1× and 2× ($P = 0.064$). A significant effect of *C. butyricum* MIYAIRI 588 inclusion rate on counts of *Clostridium perfringens* in the ileum was not observed on Day 21 or Day 42 (**Figure 5.2 E-F**). However, *Clostridium perfringens* tended to increase with the larger dose levels of *C. butyricum* MIYAIRI 588 on Day 14 and Day 42.

*5.3.2 Feed Conversion*

The effect of the experimental treatments on the feed conversion ratio of broiler chickens is summarized in **Table 5.2.** Overall administration of *C. butyricum* MIYAIRI 588 to broilers improved FCR compared to the Untreated diet and were statistically similar to the BMD treated broilers. Day 0-14 ($P < 0.032$), Day 0-29 ($P < 0.003$), and body weight corrected cumulative FCR ($P < 0.002$) was lower in broilers administered *C. butyricum* MIYAIRI 588 when compared to the Untreated control. Also, the two higher dosages of the DFM were statistically similar to BMD treated broilers.

A moderate negative correlation ($P = 0.013$) was observed between counts of LAB on Day 14 in the ileum with early FCR (Day 0-14), whereas a weak positive correlation ($P = 0.041$) was observed between counts of *C. butyricum* on Day 14 with cumulative FCR **(Table 5.3)**. An additional moderate negative correlation was observed between counts of total aerobes on Day 14 ($P = 0.014$) in the ileum with early FCR (Day 0-14). This data suggesting that increased LAB and total aerobic bacteria were recovered from broilers with lower FCR.

**Table 5.2**. CBM 588: Feed Conversion of broiler chickens

| Treatment | FCR (Feed:Gain)[1] | | | |
|---|---|---|---|---|
| | Days 0-14 | Days 0-29 | Days 0-42 | cFCR[2] |
| BMD Control | 1.338$^{b}$ | 1.656$^{b}$ | 1.772$^{c}$ | 1.778$^{b}$ |
| Untreated Control (UNT) | 1.372$^{a}$ | 1.681$^{a}$ | 1.812$^{a}$ | 1.829$^{a}$ |
| CBM 588 1.25 × $10^{8}$ cfu $kg^{-1}$ | 1.347$^{b}$ | 1.648$^{b}$ | 1.799$^{ab}$ | 1.811$^{a}$ |
| CBM 588 2.50 × $10^{8}$ cfu $kg^{-1}$ | 1.354$^{ab}$ | 1.649$^{b}$ | 1.785$^{bc}$ | 1.777$^{b}$ |
| CBM 588 3.75 × $10^{8}$ cfu $kg^{-1}$ | 1.354$^{ab}$ | 1.677$^{a}$ | 1.786$^{bc}$ | 1.780$^{b}$ |
| P-Value | 0.032 | 0.003 | 0.011 | 0.002 |
| Pooled SEM | 0.004 | 0.004 | 0.005 | 0.007 |

[1] Mortality Adjusted Feed Conversion Ratio (FCR);[2] Body Weight corrected Feed Conversion Ratio FCR (Feed:Gain); [a,b] Means within columns with no common superscript differ significantly using Duncan's Multiple Range Test ($P < 0.05$)

**Table 5.3.** CBM 588: Correlation of bacterial counts with FCR

| Bacterial Counts ($log_{10}$ CFU $g^{-1}$) | | FCR (Feed:Gain) | | | |
|---|---|---|---|---|---|
| | | D 0-14 | D 15-42 | D 0-42 | cFCR |
| Total LAB[1] | | | | | |
| Day 14 | r | -0.320* | -0.168 | -0.055 | -0.077 |
| | *P* | 0.013 | 0.200 | 0.675 | 0.560 |
| Day 42 | r | 0.027 | 0.252 | 0.054 | -0.004 |
| | *P* | 0.837 | 0.052 | 0.683 | 0.974 |
| *Bifidobacterium* | | | | | |
| Day 14 | r | 0.111 | 0.012 | -0.062 | -0.001 |
| | *P* | 0.401 | 0.0926 | 0.639 | 0.996 |
| Day 42 | r | -0.027 | -0.042 | 0.005 | -0.055 |
| | *P* | 0.836 | 0.749 | 0.971 | 0.675 |
| *C. perfringens* | | | | | |
| Day 14 | r | 0.010 | 0.191 | -0.147 | -0.084 |
| | *P* | 0.939 | 0.145 | 0.262 | 0.522 |
| Day 42 | r | -0.107 | 0.110 | -0.098 | -0.009 |
| | *P* | 0.415 | 0.401 | 0.455 | 0.947 |
| Total Gram (+) | | | | | |
| Day 14 | r | -0.157 | -0.225 | 0.009 | -0.040 |
| | *P* | 0.230 | 0.083 | 0.944 | 0.761 |
| Day 42 | r | 0.020 | -0.008 | 0.018 | -0.058 |
| | *P* | 0.880 | 0.952 | 0.889 | 0.658 |
| Total Aerobes | | | | | |
| Day 14 | r | -0.314* | -0.249 | -0.115 | -0.199 |
| | *P* | 0.014 | 0.155 | 0.382 | 0.127 |
| Day 42 | r | 0.135 | 0.160 | 0.145 | 0.038 |
| | *P* | 0.305 | 0.223 | 0.269 | 0.773 |
| *C. butyricum* | | | | | |
| Day 14 | r | 0.051 | -0.009 | 0.216 | 0.264* |
| | *P* | 0.700 | 0.948 | 0.097 | 0.041 |
| Day 42 | r | 0.186 | 0.060 | 0.069 | 0.094 |
| | *P* | 0.154 | 0.646 | 0.600 | 0.475 |

[1]LAB, Lactic Acid Bacteria

## 5.4 DISCUSSION

The objective of this study was to evaluate the dose response of Direct-Fed *C. butyricum* MIYAIRI 588 administration in broiler chickens as a potential alternative to antibiotic growth promoters (AGP). Although AGP have been widely used in production of poultry and other livestock, the demand for ABF livestock production has increased (15, 350) due to consumer concerns and regulatory limitations (325). Because the growth promoting activities of AGP are a result of their effects on the gastrointestinal microbiota (134, 351, 352), the microbiota is likely an important target for the development of alternatives to AGPs. The administration of Direct-Fed Microorganisms in livestock animals has been demonstrated to improve growth performance at levels similar to AGPs (16, 156) and reduce colonization of human food-borne and poultry pathogens in the gastrointestinal tract of poultry (215-217). Although *Clostridium butyricum* MIYAIRI 588 administration has not been previously evaluated in broilers, other *Clostridium butyricum* strains has been evaluated. Administration of non-virulent *C. butyricum* has been demonstrated to promote populations of *Lactobacillus* and *Bifidobacterium* in mice (345) and broilers (346). Additionally, administration of *C. butyricum* has been demonstrated to improve measures of growth performance, antioxidation, immune function (332, 346) and meat quality (353) in broiler chickens. *Clostridium butyricum* MIYAIRI 588 was demonstrated to stimulate mucosal immunity (354), inhibit toxin production, and growth of enterohemorrhagic *E. coli* 0157:H7 (348) in mice. CBM 588 has also been demonstrated to inhibit the human pathogen *Clostridium difficile in vitro*

(355), and promote growth of lactobacilli and bifidobacteria in humans undergoing *Helicobacter pylori* eradication treatment (349). In this study, we evaluate the effect of the administration of Direct-Fed *Clostridium butyricum* MIYAIRI 588 on the gastrointestinal microbiota and growth performance of broiler chickens fed diets without AGP.

In this study, administration of *C. butyricum* MIYAIRI 588 improved FCR when compared to the Untreated broilers and to levels similar to BMD (**Table 5.2**). Although administration of *C. butyricum* MIYAIRI 588 has not been evaluated previously in broiler chickens, other *C. butyricum* strains have been demonstrated to improve growth performance in chickens (332, 346). *Clostridium butyricum* is known to produce butyric acid (339, 356) which is likely an important mechanism responsible for the probiotic benefits of this organism. Administration of butyric acid to broilers has been demonstrated to increase villus height and surface area in the intestine (342), which is an important factor in nutrient utilization and growth performance (343, 344). Butyrate produced by bacteria in the colon of mice have been demonstrated to regulate macrophages, favoring a microbiome with butyrate producing bacteria (357). Additionally butyrate has been demonstrated to protect *in vitro* cells from *C. jejuni* invasion (358), reduce *Salmonella* colonization in layer chickens (359), and down regulates the expression of *Salmonella* pathogenicity island 1 gene (360).

In this study, administration of *Clostridium butyricum* MAYAIRI 588 tended to increase counts of *C. butyricum* recovered from broilers as compared to those fed the BMD diet on Day 14 and Day 42 (**Table 5.1**). Counts of *C. butyricum* were positively correlated with FCR. This is more than likely due to overabundance of non-CBM 588 *C.*

*butyricum* enumerated from broilers fed the Untreated diet, creating a false positive. Although *C. butyricum* were recovered from broilers administered the Untreated diet, it is possible that this wild type *C. butyricum* did not provide any benefits to the host. Furthermore, broilers administered 2× and 3× doses of *C. butyricum* MIYAIRI 588 had the lowest FCR (**Table 5.3**), yet the Untreated had the highest FCR. Additionally, *Clostridium* are predominant members of the cecal microbiota (361-363), which may have added background to our *C. butyricum* selective media, reducing the significance seen between treatments. This could explain why a weak positive correlation was seen between *C. butyricum* MIYAIRI 588 and cFCR on Day 14. Improved weight gain and reduced FCR have been reported previously in broilers administered other strains of *C. butyricum* (346, 364), suggesting the administration of *C. butyricum* MIYAIRI 588 may potentially be an important DFM component of an ABF program.

*Bifidobacterium* and LAB are important inhabitants of the gastrointestinal tract and are generally recognized as beneficial to intestinal health of poultry (133, 176, 251, 365). Administration of the 3× and 2× doses of CBM 588 increased levels of the beneficial *Bifidobacterium* on Day 14 ($P = 0.014$) and Day 42 ($P < 0.001$). Additionally, administration of *Clostridium butyricum* MAYAIRI 588 increased counts of Lactic Acid Bacteria compared to the broilers administered the Untreated control on Day 14 ($P = 0.014$) and 42 ($P < 0.001$). Similar increases in populations of LAB and *Bifidobacterium* was observed in other studies in which *C. butyricum* was administered to broiler chickens (345, 346, 361). A moderate negative correlation ($r = -0.320$, $P = 0.013$) was detected between total LAB counts on Day 14 with early (Day 0-14) FCR, suggesting an

important association between LAB and more efficient feed conversion (**Table 5.3**). LAB cultures, particularly *Lactobacillus* species, have been used widely as DFMs and their administration to broilers has been demonstrated to improve growth performance. (94, 157, 176).

Administration of *Clostridium butyricum* MAYAIRI 588 increased counts of total Gram-positive cocci. Gram-positive cocci have a myriad amount of mechanisms of resistance to antibiotics (366). Commercial poultry production and processing in the United States is known to have antibiotic resistant *Enterococcus* (367) and *Staphylococcus* (368, 369). Due to there being such a broad-spectrum of bacteria that are classified as Gram-positive cocci, inferences between FCR can be difficult to elucidate. Furthermore, total Gram-positive cocci is a broad category that includes *Lactococcus,* which has been demonstrated to reduce colitis in mice (370) and *Pediococcus,* a beneficial microbe known to produce antimicrobial peptides against food-borne pathogens (371). Both of these Gram-positives are LAB and are generally considered beneficial. An increase in Gram-positive LAB in broilers administered the DFM could explain why a significant increase was seen in the enumeration of Total Gram-positives. In future studies, qPCR could be used to observe specific microbial populations.

In this study, a negative correlation ($r$ = -0.314, $P$ = 0.014) was detected between total Aerobic bacteria counts on Day 14 with early (Day 0-14) FCR, suggesting an important association between total Aerobes and more efficient feed conversion (**Table 5.3**). Fewer total aerobic bacteria tended to be recovered from Untreated broilers than broilers administered 1× *C. butyricum* MIYAIRI 588 on Day 14 ($P$ = 0.060). Although

enumeration of total aerobes has been associated with being indicator organisms for human food-borne pathogens (372), the assumed interrelationships between pathogens and total aerobic bacteria may not be appropriate (373), considering aerobic bacteria are mostly commensal microorganism. If pathogen isolation is needed, selectively isolating pathogenic facultative anaerobes *E. coli* (374, 375) and *Salmonella* (376) associated with poultry (377) would constitute for a more suitable target in the future. Additionally, no significant treatment effect was observed on recovery of *C. perfringens*. In this study, *C. perfringens* remained in the low ~$10^2$ CFU $g^{-1}$, which is considered normal (378) and well below the counts normally associated with Necrotic Enteritis (317).

Butyrate produced from *C. butyricum* MIYAIRI 588 may modulate the gut microbiota in broilers and promote integrity of the epithelial barrier (379). Although the butyrate was not measured in this study, administration of butyrate producing DFMs (346, 361) and DFM cocktails with *C. butyricum* (380) have been demonstrated to increase both LAB and *Bifidobacterium* counts and improve growth performance of broiler chickens. VFAs have been confirmed to regulate intestinal adaptive immune response and promote health in mice (381). Furthermore, VFA contribute the maintenance of the intestine and prevention of pathogenic organisms (382). Butyrate generated by microbial fermentation regulates intestinal motility and blood flow (383). Additionally, butyrate is considered to be an alternative to AGPs, demonstrating increased growth rate (384) and reduced fecal shedding and colonization of *Salmonella* infected broilers (359). Mountzouris *et al.* suggest elevated levels of beneficial bacterial populations stimulate

the proliferation and metabolism of bacteria that produce VFAs like butyrate, which may explain for probiotic mediated performance (361).

In this study, we investigated the effect of the dose administration of the DFM *C. butyricum* MIYAIRI 588 on the gastrointestinal microbiota and feed efficiency of broiler chickens. We have demonstrated the ability of the DFM to improve feed efficiency and modify the gastrointestinal microbiota, suggesting this and other DFMs may serve as alternatives to sub-therapeutic uses of antibiotics in poultry. Administration of *C. butyricum* MIYAIRI 588 improved FCR in broilers, while promoting beneficial microorganisms LAB and *Bifidobacterium* in the gastrointestinal tract at Day 14, and reducing *C. perfringens* at day 42 compared to the BMD. We have observed associations with Lactic Acid Bacteria, total aerobes, and *Clostridium butyricum* with feed conversion, suggesting potentially important roles of these organisms in gastrointestinal health or in the gastrointestinal fermentation community. Lastly, an increase in short-chain fatty acids produced by *C. butyricum* MIYAIRI 588 may explain how DFMs mediate host performance and promoted growth of beneficial microbes. Additional research will be required in order to determine the VFA content and the degree to which population of organisms should serve as therapeutic targets for the development of DFM products intended to replace AGPs.

# 6. CONCLUSIONS

## 6.1 CONCLUDING REMARKS

There is a growing body of work investigating the functionality of probiotic and prebiotics in human and livestock animal health. While there is strong evidence to support their efficacy, the complicating factors described in this work provide insight into the questions regarding their overall effectiveness. Host-specific, probiotic strain-specific, and application-specific differences further confound the already complex interactions which occur in the gastrointestinal environment. An understanding of these differences in research studies in humans and livestock animals is necessary for understanding the results of host-specific studies and their broader implications to the science. Additionally, while probiotics and prebiotics are sometimes viewed mistakenly as a universal solution to a wide array of health problems, review of the literature suggests that, similar to small-molecule therapeutics, specific probiotic cultures or prebiotic compounds are only beneficial when used for specific applications in specific host species. Because of the benefits they may provide in both human health and livestock animal production, novel applications for probiotics and prebiotics are being developed. Continued research as described in other chapters is needed to elucidate specific host, microbe, and environmental interactions important in the gastrointestinal tract. An improved mechanistic understanding of probiotic and prebiotic functionality in specific host-species contexts will lead to improved application of probiotics and prebiotics for the benefit of human and animal health and livestock animal production.

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