EFFECT OF PHYTATE-DEGRADING PROBIOTICS ON BROILER PERFORMANCE

A Thesis

by

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ABSTRACT

Probiotics have been demonstrated to promote growth, stimulate immune responses, and improve the microbial food safety of poultry. While widely used, their effectiveness is mixed and the mechanisms through which they contribute to poultry production are not well understood. Phytases isolated from microorganisms are increasingly supplemented in feed to improve digestibility and reduce anti-nutritive effects of phytate. The microbial origin of these enzymes suggests a potentially important mechanism of probiotic functionality.

Our objective was to investigate phytate degradation as a novel probiotic mechanism using recombinant *Lactobacillus* cultures expressing *Bacillus subtilis* phytase. *B. subtilis phyA* was codon optimized for expression in *Lactobacillus* and cloned into the expression vector, pTRK882. The resulting plasmid, pTD003, was transformed into *Lactobacillus acidophilus*, *Lactobacillus gallinarum*, and *Lactobacillus gasseri*. SDS-PAGE revealed an approximately 44 kDa protein in the culture supernatants of *Lactobacillus* pTD003 transformants corresponding to the predicted molecular weight of *B. subtilis* phytase. The phytate degrading ability of these cultures was evaluated by determining the amount of inorganic phosphate released from sodium phytate. Expression of *B. subtilis* phytase increased phytate degradation of *L. acidophilus*, *L. gasseri*, and *L. gallinarum* approximately 4-, 10-, and 18-fold over the background activity of empty vector transformants.

The effect of administration of recombinant phytase-expressing *L. gallinarum* and *L. gasseri* was evaluated in broiler chicks fed a phosphorous deficient diet (0.25% aP). Phytase-expressing *L. gasseri* improved weight gain ($P < 0.05$) of broiler chickens to a level comparable to chickens fed a phosphorous adequate control diet (0.40% aP) demonstrating proof of-principle that administration of phytate-degrading probiotic cultures can improve performance of livestock animals. Additionally, this will inform future studies investigating whether probiotic cultures are able to combine the performance benefits of feed enzymes with the animal health and food safety benefits traditionally associated with probiotics.

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CHAPTER I

INTRODUCTION OF ALTERNATIVES TO ANTIBIOTIC GROWTH PROMOTERS

1.1 INTRODUCTION

Antibiotics have been used to promote the growth of broiler chickens and other livestock animals in the United States for more than 50 years (1-3). Antibiotics are known to increase weight gain (1), improve feed efficiency (4, 5), and reduce mortality in livestock animals (6, 7). However, the use of antibiotic growth promoters (AGPs) is in decline because of consumer preferences and regulatory concerns (8). The development of antibiotic resistant microorganisms is of particular concern (9). In 1969 the British Government issued a report detailing the discovery of *Salmonella enterica* Typhimurium resistance to the antibiotic oxytetracycline (10). The committee recommended halting the use of streptomycin, oxytetracycline, and penicillin for sub-therapeutic use in order to reduce the occurrence of resistant pathogens (10). In 1986, Sweden became the first country to ban AGPs after consumer confidence dropped due to the abundant use of antibiotics and concerns over food safety (11, 12). Denmark followed Sweden in banning AGPs in 1998 (8).

After bans in Sweden and Denmark, the European Union (EU) banned use of antibiotic growth promoters starting with Avoparcin in 1997. All non-therapeutic antibiotic use in livestock was eventually banned across the EU by 2006 (13). Consequences of the AGP ban has been reported in Denmark (4, 14). The Danish

poultry industry experienced a significant increase in necrotic enteritis, from one flock in 1997 to 25 flocks the following year after the food animal industries decided to voluntarily ban AGPs in order to be completely AGP free by 1999 (14). Feed conversion ratio has also increased after removal of AGPs (4). The reduction of AGP use in the swine industry has led to dietary zinc deficiencies (15).

Several alternatives to AGPs are currently being used by livestock producers. Some of these alternatives include non-starch polysaccharide-degrading enzymes known as NSPases (16) and the phytate degrading enzymes, phytases (17). These enzymes are administered to livestock animals to aid in the digestion of indigestible feed constituents (18). Other alternatives to AGPs are probiotic microorganisms, including *Lactobacillus, Bacillus,* and *Enterococcus* species (19). Additional alternatives include prebiotic oligosaccharides and organic acids (19). These AGP alternatives alter the gastrointestinal microbiota of livestock animals, promoting immune development and reducing pathogen colonization (19).

1.2 ANTIBIOTIC GROWTH PROMOTERS

1.2.1 AGP Use in Poultry

The growth promotion effects of antibiotics on broiler chickens were first demonstrated by Moore *et al.* (1) Broiler chicks were fed showed streptomycin increased growth when compared to broilers not fed streptomycin (1). Because of the evidence of growth promotion by antibiotics in poultry, the United States Food and Drug Administration has allowed the non-therapeutic use of antibiotics for growth promotion in animal feed since 1951 (20, 21). AGPs have helped the poultry industry to produce

healthy broiler chickens while reducing costs of therapeutic treatments and economic loss from massive flock mortality (22). While AGPs promote growth of livestock animals, they are also known to select for antibiotic resistant variants of microorganisms. Coliform bacteria were observed to mutate and become resistant in poultry where streptomycin was administered (23, 24). The fear of bacterial infections becoming more resistant to treatment has resulted in the banning of AGPs in Europe (8). Furthermore, resistant strains of human pathogens have developed due to the use of AGPs for the past 50 years (9). These pathogenic strains are more difficult to treat due to the fact that they are currently highly resistant to an array of antibiotics and the patients must undergo longer periods of antibiotic treatments to ensure destruction of the pathogen (9).

1.2.2 Mechanisms of Antibiotics in Growth Promotion

The gastrointestinal microbiota is thought to decrease host performance through reduced intestinal absorption of nutrients, direct competition for nutrients with the host, production of growth depressing metabolites, and sub-clinical infection (25, 26). AGPs have been shown to modify the gastrointestinal microbiota of livestock animals (27, 28), reducing its negative effect on host performance.

When AGPs are introduced to the intestine, availability of nutrients increases. It is believed that antibiotics also reduce the overall microbial population, which induces the thinning of the small intestine and increase in the thickness of the muscosa. The reduction of pathogens and their antimetabolites results in better nutrient absorption, which improves the growth and feed efficiency of the livestock animal (9, 29).

Colonization of poultry by pathogens including *Clostridium perfringens* is known to depress growth rates in chickens reducing the overall productivity of broiler flocks (30). Inflammation in response to sub-clinical infection with *C. perfringens* causes the release of catabolic hormones, resulting in a reduction of muscles mass (31). AGPs reduce pathogen populations with little impact to commensal bacteria (32) reducing growth depression due to sub-clinical infection. Additionally, *C. perfringens* has been implicated in necrotic enteritis (NE) pathology in chickens (33). The disease can decimate broiler flock populations (33) result in mortality of at least 30%.

By altering the microbiota, AGP reduce the production of growth suppressing microbial metabolites. Organisms like *Bacillus* spp., *Salmonella* spp., *Campylobacter* spp., *Pseudomonas*, and *Escherichia coli* while not pathogenic in chickens produce toxins that are strong chelating agents. Siderophores produced by both Gram-positive and Gram-negative microorganisms including *Salmonella*, *Staphylococcus aureus* and *E. coli (34-36)* are highly destructive compounds produced by bacteria and possess powerful binders have a high affinity for the metal ion iron (37). Once iron is sequestered from the host, cellular function is disrupted, resulting in apoptosis and necrosis of host cells. Pyocyanine, pyoverdin produced from *Pseudomonas (38)*. AGPs known to inhibit pathogens like *Staphylococcus* and *Campylobacter* are administered to the livestock animal, theoretically reducing the amount of undesirable harmful bacteria in the microbiota population of the animal.

As a result, the remaining microbial population in the animal has a smaller impact in reducing available nutrients to the animal. The reduced competition for

nutrients from microorganism in the gastrointestinal tract ideally results in animals that are healthier and better able to absorb nutrients previously utilized by undesirable microorganisms. With the reduction of undesirable microbes in the chicken's intestinal tract, the intestinal villi of the gut wall is altered (39, 40) by thinning the small intestinal barrier, allowing for improved nutrient absorption. With the improvement on nutritional uptake, improved feed efficiency occurs, producing chickens that grow faster.

1.2.3 AGP Resistant Microorganisms

Antibiotics that are commonly used in treating bacterial infections found in humans are sometimes used as AGPs in poultry. These shared antibiotics are penicillin, tetracycline, and erythromycin (20). This is a cause for concern because the antibiotic resistant bacteria found in livestock animals can be zoonotic. *Campylobacter (41, 42)* and *Salmonella* (43, 44) are pathogens found in poultry reservoirs, they are also two of the most common foodborne pathogens infecting humans (45).

Even though the United States has yet to ban AGPs, the European Union has banned AGPs in livestock for years. The first country to do so was Sweden in 1986 (12), Sweden lead Europe in the removal of AGPs due to the findings of antibiotic resistant pathogens. In the early 90's other European countries like Germany, Denmark and Finland followed Sweden's stance on AGPs and banned antibiotics used in animals feeds, particularly the antibiotics involved in human medicine. These countries banned glycopeptides, spiramycin and virginiamycin use as AGPs due to their significance in treating disease in humans.

1.3 FEED ADDITIVE ENZYMES

Used in feed more than 80% of poultry feed the United States, feed additive enzymes are the most widely used alternative to AGPs (46). The inability of monogastric animals to fully digest plant-based feeds is well known (47). Exogenous enzyme increase available nutrients to the animal by hydrolyzing indigestible feed constituents, while reducing the anti-nutritive effects of plant based feeds constituents. It is accepted that enzymes reduce the viscosity in the gastrointestinal tract, allowing for an increase rate of passage (48-51).

Feed enzymes in poultry were used in Europe well before they were used in the United States. Interest in feed enzymes resulted from changing attitudes towards drug and antimicrobial use in food and the abundance of cheap feed ingredients (46). The majority currently used feed enzymes come from *Bacillus* spp. and *Aspergillus* spp (52). Poor nutrient uptake and feed conversion from the presence of indigestible feed constituents is improved with the addition of exogenous enzymes. Reducing the undesirable effects of indigestible feed constituents reduces the amount of money and resources exhausted on producing an animal, while at the same time providing a ample and healthier animal (53).

1.3.1 NSPases

The nutritional benefit impact of feed made up of oats, barley, rye, and wheat have been underutilized because of the indigestible feed constituent, non-starch polysaccharides (NSP) (54). Rye and barley based diets are known to exert anti-nutritive effects and alter the microbiota in early growth of poultry (55-57). These non-

digestible feed constituents interfere with nutritional value, restricting vitamins and other nutrients essential in development. With the supplementation of NSPase to poultry diets, an improvement to the nutritional value and digestibility of grain based feeds containing complex polymers was seen (17, 58). The particular aim of the enzyme use was to hydrolyze with specific non-starch polysaccharides, producing a digestible energy source. Monogastric animals cannot produce enzymes capable of breaking down the non-starch polysaccharides like cellulose, arabionoxylan, and β-glucans which sequester desired nutrients (59). With the addition of enzymes in feed, the anti-nutrient properties of oats, barley, and wheat are diminished. The reduction of anti-nutritive properties and the improved intestinal viscosity allows for of improved nutrient absorption. Improved growth can be seen in broilers due to the increase of digestible energy along with the reduction of sequestered nutrients, (49, 60, 61).

The latest enzymes used in the feed industry are β-Glucanases and Xylanases (59). These NSPase enzymes break down the complex polysaccharides glucan and xylan found in plant cell walls and turn otherwise indigestible polysaccharides into digestible carbohydrates for energy. Though these enzymes are still used, the focus of enzymatic use in the feed industry has shifted to phosphatases.

1.3.2 Phytases

Phosphorous is an essential nutrient in poultry production (62) with dietary deficiencies leading to excessive financial losses due to increased mortality (63, 64). 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 (65, 66). However, phytate-phosphorous has low bioavailability and is underutilized due to the indigestibility of phytic acid in non-ruminant livestock, including poultry (67, 68) and swine (69). Additionally, phytic acid exerts anti-nutritive effects (63), sequestering essential cations including calcium, magnesium, iron, and zinc, reducing their bioavailability (70).

Phytase is a particular phosphatase enzyme originally derived from *Aspergillus fumigates*, with the ability to increase the inorganic phosphate absorption by the hydrolysis of phytic acid found in plant based feeds (71).Phytases are phosphatases which catalyze the hydrolysis of phytic acid to *myo*-inositol and inorganic phosphate (72). In-feed administration of microbial phytases to improve digestibility of phytic acid is widely used in the production of poultry and other livestock (73, 74). There are several classes of phytases including histidine acid phosphatases, β-propeller phytases, purple acid phosphates, and cysteine phosphatases (75). Most commercial phytases used currently in livestock agriculture are histidine acid phosphatases from *E. coli* and fungi (18).

Bacterial β -propeller phytases from the *Bacillus* species are an alternative to the enzymes used from the histidine acid phosphatase group (18). *Bacillus* phytase is a β-propeller phytase containing a six-bladed propeller fold architecture (76). The *Bacillus* phytases have a high thermal stability, and a calcium phytate complex with a neutral pH range. Phytase activity is dependent on the pH range of the host's gastrointestinal tract, which is why not all phytases are suitable for every animal in agriculture. *Bacillus* phytases are being closely examined for application in the pelleting processes of feed,

due to their high heat tolerance (77). Another desirable quality of *Bacillus subtilis* phytase is some strains, like *Bacillus subtilis* 168, are already used in fermentation of food (78) and is generally recognized as safe.

1.4 OTHER ALTERNATIVES

1.4.1 Prebiotics

Prebiotics are non-digestible compounds that benefit the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving the host's health (79). In order to be considered a prebiotic, the feed ingredient cannot be hydrolyzed or absorbed in the upper gastrointestinal tract. It must be a selective substrate to a limited amount of commensal organisms, and must alter the microbiota of the host towards a healthy composition (80). Oligosaccharides including galacto-oligosaccharide, inulin, and lactulose have been found to stimulate beneficial intestinal microbes (81, 82).

One of the steps in the infection process is when pathogenic bacteria colonize the mucosal tissue. Prebiotics can be used in chickens to reduce colonization of pathogenic bacteria. When administered to *Salmonella* Typhimurium challenged broilers, a 25-fold decrease in was observed in broilers administered prebiotic mannan oligosaccharide compared to broilers not given the prebiotic (83). This prebiotic is believed to help in the prevention of *Salmonella* Typhimurium adherence to the intestinal wall, while not affecting the adherence of *Lactobacillus* and *Enterococcus.*

1.4.2 Organic Acids

Another alternative to AGPs is the use of organic acids in feed. Organic acid is known to increase energy contribution to feed and improve protein digestion by the increasing pepsin activity (48, 84). Organic acids have been able to reduce levels of *Salmonella (85)* and *Campylobacter jejuni (86)* in chickens. Although the mechanisms of organic acids are not completely understood, organic acids are able to exhibit bactericidal properties to certain microorganisms (87). It is believed that these acids lower the gastrointestinal pH of the animal allowing organic acids to penetrate across the lipid membrane and reduce bacterial growth by changing the internal pH and interfering with cellular function (87).

1.5 PROBIOTICS

Probiotics are living microorganisms that when administered in adequate amounts, confer beneficial health effects upon the host (88). Most of these probiotic microorganisms fall under the category of Lactic acid bacteria (LAB), some LAB can aid in the physiological functions of the gastro intestinal tract, including immune system stimulation, improve nutritional bioavailability, and restoration of mucosal layer (89).

The objective of these alternatives to AGPs is to improve the gastro intestinal health by influencing the commensal microflora in intestine of the livestock animal (48). Microflora of the animal can be successfully manipulated to inhibit or competitively exclude pathogens, while at the same time providing enhanced growth performance by enabling the additional probiotic mechanisms that increase nutritional absorption within the gastro intestinal tract.

1.5.1 Probiotic Benefits

Humans and animals alike have key inhabitants in the gastrointestinal tract that play essential roles in health. The probiotic *Lactobacillus* has multiple functions that are not just limited to food fermentation. *Lactobacillus* also provides pathogen exclusion, bioactive materials, and regulations of gastro intestinal health. The ability of *Lactobacillus* to be used in functional foods as a health related product is growing. The breakthrough of the nomenclature of *Lactobacillus* has allowed for the broadening of the amount of *Lactobacillus* available in research. This allows for the ability to pinpoint certain strains to perform more ideally for *in vitro* research. It is critical to understand the roles, mechanisms and interactions of each unique species of *Lactobacillus* used as probiotics. Genetic characterization and the ability to manipulate the functions of *Lactobacillus* allow for the use of expression vectors to provide additional benefits to the host, and expand the potential uses of probiotic organisms (90).

1.5.2 Probiotic Benefits to Poultry Health

LAB Probiotics like *Lactobacillus*, *Enterococcus* and *Streptococcus* can be considered beneficial and can be used as health-promoting functional food ingredients. Intestinal pathogens are susceptible to many of the defenses LAB probiotics produce. LAB production of lactic acid and bacteriocins inhibits the growth of pathogens found in poultry, such as *Salmonella (91)* and *Campylobacter* (92). The lactic acid produced by LAB effectively lower the pH of the gastrointestinal environment, slowing the growth of non-commensal organisms competing for nutrients. The ability to increase resistance to

disease and effectively reduce the need of antibiotics in livestock has made LAB an ideal probiotic organism (19).

1.5.3 Probiotic Mechanisms

The mechanisms behind these physiological functions are not fully understood; nevertheless specific benefits are connected with probiotics' ability to modify the gut microbiota within the host. This prevents pathogen adherence by producing bacteriocins in order to keep healthy intestinal function (93). Probiotics introduced to chickens early in rearing, should edge out, or at least reduce the amount of undesirable organisms. The reduction of pathogens could lessen the chance of human contact with these pathogens, while at the same time producing a healthier chicken. Prior to hatch, the chick's intestinal tract is sterile, however, it is quickly colonized by facultative anaerobes like *Listeria* and *Salmonella* (94). For best results, probiotics are introduced early in the rearing process as possible. Even with early administration, undesirable microbes could possibly colonize a different part of the GI tract. Because of this, a cocktail of multiple probiotic strains known to colonize in different parts of the GI tract may be needed in order to successfully reduce undesirable microbes. This method would be instrumental in providing three key benefits to the host animal. One would be the resistance to pathogenic and nonindigenous microbes by competitive exclusion (95). The second would be the stimulation of the host's defenses, by developing the mucosal layer, epithelial layer, and the lamina propria throughout the GI tract. A healthy mucosal layer will segregate normal healthy microbes and assist in keeping pathogenic microbes away from animal tissue. Within the epithelium tissue and lamina propria, a healthy

abundance of immune cells will provide defenses against pathogens that get past the mucosal layer (96). The third benefit would be microflora-secreted nutrients. These nutrients excreted by the beneficial microflora can provide amino acids, vitamins and short chain fatty acids. These fatty acids will help provide a energy supply for the growing broiler and will reduce the amount of undesirable microbes in the ceca of the broiler (9, 97).

New uses of probiotics are constantly being examined. Recent studies pertaining to identifying the possible mechanisms of probiotics suggest the ability to improve immune system of hosts (98, 99), with the possibility of treatments for chronic bowl disease diseases like Crohn's and ulcerative colitis (100, 101), colon cancer (102) and bladder cancers (93, 103). Additional novel mechanisms of probiotics is targeted enzyme delivery for improved feed utilization (104, 105), and vaccine delivery (106) in prevention of diseases.

The probiotic functionality that results in the benefits that increase overall health of broilers and monogastric animals may not be new, but it is certainly of great significance. The ability to introduce or control the microflora of agricultural animals would not only benefit the development of the animal, but also it would benefit marketing the animal to consumers. To be able to tell consumers that the animal product they purchase at the super market had healthy microbiota, and that it was raised antibiotic free would be a huge selling point. The ability to administer microbes as a simple probiotic could possibly solve many nutritional and industrial needs.

1.6 CONCLUSION

It is possible that the United States may follow the European Union and ban non-therapeutic use of antibiotics. The development of zoonotic pathogenic from animal reservoirs through the use of AGPs is of great concern. Pathogenic bacteria are already becoming resistant to vancomycin which regularly used in hospitals used for antibiotic chemotherapy (107, 108). The risk associated with drug resistant strains due to the AGP use will continue to be debated in United States.

Alternatives to AGPs including feed additives enzymes, organic acids, prebiotics and probiotics will be important for the poultry industry future in order to keep performance promotion levels to the equivalence of AGPs. The ability to provide alternatives to AGPs can be a reasonable solution to the hurdles facing the poultry industry. Feed utilization, anti-nutritional properties, pathogenic bacteria and gastrointestinal health are all problems that can be seemly solved with the introduction feed additives, prebiotics, and probiotic cocktails. These alternatives are able to deliver additional enzymes that can degrade non-starch polysaccharides and other indigestible feed constituents that have anti-nutritional properties. While at the same time, keep pathogens from adhering to intestinal tract of the host. The utility that probiotics bring is incredibly advantageous; the ability to solve multiple problems with one organism that is recognized as safe is of utmost value.

Probiotics are expected to be a particularly important alternative to AGPs. These microorganisms could possibility provide all the needed healthy mechanisms needed for growth promotions in livestock. The ability to improved digestion, competitive

exclusion, and immune response makes probiotics a viable alternative to antibiotics. This will allow for the host to be able to utilize nutrients that were not regularly available prior to ingesting probiotics. Some probiotics like *Lactobacillus* are capable of delivering vectors, vaccines and biotherapeutics to chickens and other animals (109). It is possible that these probiotics could have the ability to synthesize digestive enzymes. *Lactobacillus* and other LAB are ideal organisms to be used in future research due to these synergetic benefits. The potential of being able to provide probiotics with the capability of hydrolyzing non-starch polysaccharides and phytic acid found in feed into a useable source of nutrition would greatly improve feed utilization, without sacrificing the health of the animal.

CHAPTER II

ADMINISTRATION OF PHYTATE-DEGRADING *LACTOBACILLUS* IMPROVES GROWTH OF BROILER CHICKENS

2.1 INTRODUCTION

Lactobacillus species are important inhabitants of the gastrointestinal tract of humans and animals and are increasingly being used as probiotic microorganisms due to their health promoting properties (82, 110). Probiotics, sometimes called Direct Fed Microbials (DFM) when used in animals (111), are live microorganisms administered to confer a health benefit upon the host (112). Administration of probiotic *Lactobacillus* to poultry has been demonstrated to promote growth at levels similar to antibiotics (113, 114) and reduce gastrointestinal colonization of human food borne pathogens including *Campylobacter* (115, 116), *Clostridium* (117), and *Salmonella (118, 119).* Because of concern over antibiotic resistant pathogens and pressure from both consumers and regulatory agencies, probiotics have received increased interested as potential alternatives to antibiotic growth promoters (120). While probiotics are used widely across the livestock industry (121), their effectiveness is varied and, the mechanisms responsible for their benefits are not well understood.

Phosphorus is an essential nutrient in poultry production (62) with dietary deficiencies leading to excessive financial losses due to increased mortality (63, 64). 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 (65, 66). However, phytate-phosphorus has low bioavailability and is underutilized due to the poor digestibility of phytic acid in non-ruminant livestock including poultry (67, 68) and swine (69). Additionally, phytic acid exerts anti-nutritive effects (63), sequestering essential cations including calcium, magnesium, iron, and zinc and reducing their bioavailability (70).

Phytases are phosphatases which catalyze the hydrolysis of phytic acid to *myo*-inositol and inorganic phosphate (72). In-feed administration of microbial phytases to improve digestibility of phytic acid is widely used in the production of poultry and other livestock (73, 74). The resulting increases in phytate-phosphorus digestibility (17, 63, 122) and reduction in the anti-nutritive effects (123, 124) of phytic acid are well documented. The microbial origin of phytases used in livestock production suggests degradation of phytic acid may be a potentially important mechanism of probiotic functionality. Combining the nutritional and performance benefits of phytase with the food safety and animal health benefits traditionally associated with probiotics is of great interest to livestock producers. In this study, we investigated phytate degradation as a novel mechanism of probiotic functionality. Recombinant *Lactobacillus* cultures expressing *B. subtilis* phytase were constructed and, the effect of their administration on growth performance was evaluated in broiler chicks fed a phosphorus deficient diet. We demonstrate proof-of-principle that administration of a phytate-degrading probiotic culture can improve the performance of livestock animals

2.2 MATERIAL AND METHODS

2.2.1 Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used or constructed in this study are listed in Table 1. *Lactobacillus* strains were cultured using deMan, Rogosa, and Sharpe (MRS) medium (Difco, Franklin Lakes, NJ) and incubated in 10% CO_2 at 37°C with 5 µg/ml erythromycin (Erm; EMD Chemicals, Inc., San Diego, CA) added when appropriate. *Escherichia coli* strains were cultured using Luria-Bertani (LB) medium (Difco, Franklin Lakes, NJ) aerobically at 37°C with 150 µg/ml Erm, when appropriate.

2.2.2 DNA Isolation, Manipulation, and Transformation

E. coli plasmid DNA was isolated using the QIAprep Spin Miniprep kit (QIAGEN Inc., Valencia, CA), while DNA was isolated from *Lactobacillus* according to the method of Walker and Klaenhammer (125). DNA restriction fragments were purified from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN, Germantown, MD). All manipulations were performed using standard molecular cloning techniques (126). Restriction enzymes, T4 ligase, and *Taq* DNA polymerase were used according to the manufacturer's instructions (NEB, Ipswich, MA). PCR primers are listed in Table 2. Electocompetent *E. coli* MC1061 and TOP10 were prepared and transformed according to standard methods (127). *L. acidophilus* and *L. gasseri* were transformed using the method of Luchansky *et al* (128), while *L. gallinarum* was transformed a using the method of Beasley *et al* (129).

2.2.3 Recombinant Phytase Expression in Lactobacillus

The *phyA* gene from *B. subtilis* (130) was codon optimized for expression in *L. acidophilus* using the OPTIMIZER web server (131) and commercially synthesized with EcoRI and NotI restriction sites to facilitate cloning. The synthetic DNA sequence was provided by the manufacturer (Life Technologies, Inc. Carlsbad, CA) in a plasmid (pTD001). The synthetic *phyA* gene was isolated from pTD001 and ligated into pTRK882 (109) for constitutive high-level expression in *Lactobacillus*. The resulting plasmid, pTD003, was transformed into and subsequently propagated in *E. coli* MC-1061. The plasmids pTD003 and pTRK882were introduced into *Lactobacillus* species by electrotransformation. Transformations were confirmed by PCR using gene specific primers (Table 2).

2.2.4 SDS-PAGE

Supernatants from overnight *Lactobacillus* cultures were concentrated and purified by dialysis using Microsep advanced centrifugal devices (Pall Corporation, Ann Arbor, MI). Total protein was precipitated using 100 % (w/v) trichloroacetic acid (TCA) (Sigma-Aldrich) and pelleted by centrifugation. Protein pellets were washed 3 times using 80 % (w/v) acetone and resuspended in PBS. Protein concentration was determined using the Bradford method (132). Protein was separated by SDS-PAGE using Mini-PROTEAN TGX Precast protein gels (any kD) (Bio-Rad Laboratories, Hercules, CA) in Tris-Glycine-SDS Buffer (Bio-Rad) with a low range protein standard (Bio-rad). Wells were loaded with 3.5 µg of protein in Laemmli buffer (133). Gels

were stained with GelCode Blue Safe Protein Stain (Thermo Scientific, Waltham, MA) for visualization of protein.

2.2.5 Phytate Hydrolysis

Phytate hydrolysis by *Lactobacillus* transformants was observed using a modification of the method of Bae *et al* (134). *Lactobacillus* colonies were selected and aseptically transferred onto the surface of MRS agar plates (5µg/mL Erm) and incubated for 36 hours. Plates were then overlaid with modified MRS (135), in which is 0.5% (w/v) sodium phytate (Pfaltz & Bauer, Waterbury, CT) was the sole phosphorus source and incubated for an additional 24 hours. Plates were stained with cobalt chloride solution and counterstained with an ammonium molybdovanadate solution. Phytate hydrolysis is indicated by zones of clearing.

2.2.6 Phytase Enzyme Activity Assays

Phytase activity from cell free extracts (CFE) of recombinant *Lactobacillus* cultures was assayed by determining the amount of inorganic phosphate released from sodium phytate in phytase reaction buffer (6.4 mM sodium phytate, 2 mM $CaCl_2$, 100 mM Tris-HCl, pH 7.0) at 55°C. Enzyme reactions were terminated by the addition of an equal volume of 5 % (w/v) TCA and free phosphate was determined colorimetrically (620 nm) using the ammonium molybdate method (136) with a sodium phosphate standard. CFEs were prepared (109) in phytase extract buffer (2 mM $CaCl_2$, 100 mM Tris-HCl, pH 7.0) as described previously. Protein concentrations were determined using the Bradford method (132). Phytase specific activity was reported as U mg^{-1} total

protein (μmol $^{-}PO_4$ released min^{-1} mg^{-1}). Data were analyzed using ANOVA and significantly different means were determined using Duncan's multiple range test.

2.2.7 Broiler Chickens

On day-of-hatch, male broiler chicks (Ross × Ross) were obtained from a commercial hatchery, individually weighed, wing banded, and assigned to pens based on body weight to ensure all treatment groups began with statistically similar weights. Broiler chicks were housed in battery brooders and given access to water and experimental rations *ad libitum*. All experimental procedures were performed in accordance with protocols approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC).

2.2.8 Broiler Performance Trial

A total of 144 broiler chicks were separated into 6 treatment groups of 24 birds each. Four experimental treatment groups were fed a phosphorus deficient diet (0.25% aP) and administered recombinant *Lactobacillus* cultures in Maximum Recovery Diluent (MRD) by oral gavage. Chicks were administered *L. gallinarum* TDCC 63 ($rPhyA^{+}$), *L. gallinarum* TDCC 62 (empty vector), *L. gasseri* TDCC 65 ($rPhyA^{+}$), and *L. gasseri* TDCC 64 (Empty Vector). Control groups were administered a mock inoculation (sterile MRD) and fed a phosphorus adequate diet (0.40% aP) (positive control) or the phosphorus deficient diet (0.25% aP) (negative control). Broiler chicks were weighed individually at Day 0,7,14, and 21 post-hatch. Data were analyzed using ANOVA and significant difference between treatment groups determined using Duncan's multiple range test using individual birds as the experimental unit.

2.2.9 Experimental Diets

A phosphorus deficient basal starter diet was formulated with 0.25 % available phosphate (aP) and all other nutrients meeting or exceeding or exceeding industry type broiler diet requirements for market broilers for Days 0-21 post-hatch (Table 3). The positive control phosphorus adequate diet was formulated by increasing aP to 0.40 % with the addition of KH_2PO_4 to the basal diet. Feed samples were analyzed by an independent laboratory for total phosphorus, calcium, and protein to confirm nutrient profile.

2.3 RESULTS

2.3.1 Recombinant Phytase Expression in Lactobacillus

The 1,149 bp *phyA* (BSU19800) gene encoding a phytase (76) from *B. subtilis* (130) was selected for recombinant expression in *Lactobacillus*. Protein domain analysis of the 382 amino acid sequence predicted the presence of a Gram-positive signal peptide (Amino Acids 1 - 26) suggesting the protein would likely be secreted via the *sec* pathway (137). *B. subtilis phyA* was codon optimized for expression in *Lactobacillus* using OPTIMIZER (131). Before optimization, the codon adaptation index of the native *phyA* sequence was 0.27 and improved to 1.00 after optimization. The optimized sequence was commercially synthesized and subcloned into pTRK882. The resulting plasmid, pTD003 (Figure 1), and the empty vector, pTRK882 , were transformed into *L. acidophilus* NCFM, *L. gallinarum* ATCC 33319^T, *L. gasseri* ATCC 33323^T. Transformations were confirmed by PCR to detect *ermC* and recombinant *phyA* (*rphyA*) (data not shown). Amplification of both *phyA* and *ermC* indicated successful

transformation by pTD003 and, amplification of *ermC* alone indicated successful transformation by pTRK882.

2.3.2 SDS-PAGE

Total protein in culture supernatants from *Lactobacillus* cultures was separated using SDS PAGE (Figure 2). A protein with a molecular weight approximately 44 kDa was present in supernatants of *L. acidophilus* TDCC 61, *L. gallinarum* TDCC 63, and *L. gasseri* TDCC 65. While a faint protein band of similar molecular weight did appear in the supernatant of *L. gasseri* TDCC 64, this protein was not detected in supernatants of the empty vector controls, *L. acidophilus* TDCC 60 and *L. gallinarum* TDCC 62. The molecular weight of the secreted mature phytase from *B. subtilis* is 44kDa (76). These data suggest that recombinant PhyA phytase (rPhyA) is expressed and secreted by *Lactobacillus* cultures transformed with pTD003.

2.3.3 Phytate Hydrolysis

Phytate hydrolysis by *Lactobacillus* cultures was evaluated qualitatively (Figure 3). Zones of clearing appeared around colonies of pTD003 transformed cultures, *L. acidophilus* TDCC 61, *L. gallinarum* TDCC 63, and *L. gasseri* TDCC 65. However, little to no clearing appeared around colonies of the empty vector control cultures, *L. acidophilus* TD 60, *L. gallinarum* TDCC 62, and *L. gasseri* TDCC 64.

2.3.4 Phytase Activity of Recombinant Lactobacillus Cultures

Phytase activity from cell pellets of recombinant *Lactobacillus* cultures was evaluated (Table 4). Phytase activity of *L. acidophilus* TDCC 61, *L. gallinarum* TDCC 63, and *L. gasseri* TDCC 65 was approximately 4-, 18-, and 10-fold, greater than the

respective empty vector control cultures. *Lactobacillus* empty vector (pTRK882) transformants are wild-type for phytase activity and account for background phytate degradation by non-specific phosphatases. Phytase activity of *L. gallinarum* TDCC 63 and, *L. gasseri* TDCC 65 was approximately 2 and 3 times greater, respectively, than *L. acidophilus* TDCC 61.

2.3.5 Broiler Performance Trial

The effect of rPhyA producing *Lactobacillus* cultures on the performance of broiler chicks was evaluated (Figure 4). There were no differences in body weight between the treatment groups at Day 0 and Day 7 post-hatch. For mock inoculated treatment groups, the body weight of chicks fed a phosphorus adequate diet (positive control) was greater than those fed a phosphorus deficient diet (negative control) at Day 14 and 21 post-hatch ($P<0.05$). The body weight of chicks administered PhyA producing *L. gallinarum* (TDCC 63) and *L. gasseri* (TDCC 65) was not significantly different than those administered the respective empty vector control cultures, *L. gallinarum* TDCC 62 and *L. gasseri* TDCC 64, or the negative control group. However, the body weight of chicks administered *L. gasseri* TDCC 65 was not significantly different than the positive control group. While performance was not significantly increased compared to the negative control or relevant empty vector control the administration of rPhyA producing *L. gasseri* improved weight gain of broiler chickens to a level statistically comparable to chicks fed a phosphorus adequate diet.

2.4 DISCUSSION

The objective of this study was to investigate phytate degradation as a novel mechanism of probiotic functionality. An important role of the gastrointestinal microbiota is to indirectly augment host metabolism by utilizing undigested food and producing short chain fatty acids and micronutrients which can be utilized by the host (79). The microbial origin of exogenous enzymes used in livestock production, including phytases, suggests that direct augmentation of host metabolism through the production and delivery of these enzymes *in situ* may be an important mechanism of probiotic functionality. While these enzyme activities have been suggested as selection criteria for probiotic cultures (135, 138), biocatalysis by probiotics in the gastrointestinal tract has not been explored.

Phytate-degrading activity has been reported in *Lactobacillus* species and has been suggested to improve nutritional quality of fermented cereal grains (139-141). De Angelis *et al.* (142) reported the purification of a phytase from *Lactobacillus sanfranciscensis*. However, the significantly greater substrate specificity of this enzyme towards *p*-nitrophenyl phosphate over phytate suggests this enzyme would more appropriately be classified as a non-phytate specific acid phosphatase. Phytate degradation has been attributed to non-specific acid phosphatases in other lactobacilli (143, 144). Additionally, a phytase gene has not yet been identified in a *Lactobacillus* species.

Because true phytase-producing *Lactobacillus* cultures have not yet been identified, recombinant cultures were used to model phytate degradation by probiotic

microbes. Recombinant expression of exogenous phytase in *Lactobacillus* has been previously investigated (145, 146). However, our approach to the expression of exogenous phytase is novel in that we have maximized expression using pTRK882, which is under control of the constitutive high-expressing promoter P_{pgm} from *L. acidophilus* NCFM (109), and codon optimized the sequence of our recombinant phytase gene for expression in *Lactobacillus* species. This expression system was previously demonstrated to be effective in enzyme expression (109), the production and delivery of immune modulating cytokines (147), and an anthrax vaccine (106). Additionally, its wide host range allowed the transformation of *L. acidophilus, L. gallinarum*, and *L. gasseri.*

The *phyA* gene from *B. subtilis* (130) encodes a β-propeller phytase with high specificity for phytic acid and activity over broad pH and temperature ranges (76). Analysis of the amino acid sequence using SignalP (137) predicted the presence of a Gram-positive secretion signal suggesting that heterologous expression of this protein in *Lactobacillus* will result in production of a secreted protein. Thus, we selected the *B. subtilis phyA* for expression in *Lactobacillus*. Interestingly, the popularity of probiotic and DFM products containing spore-forming bacteria including *B. subtilis* has increased (148-151). *Bacillus* species are workhorse bacteria in microbial fermentations and highly prized as producers of industrially important enzymes (152). Heterologous expression of *B. subtilis* phytase using *Lactobacillus* in this study not only demonstrates biocatalytic phytate degradation as a general mechanism of probiotic functionality but

will guide future studies investigating this specific mechanism in *Bacillus* species further supporting their use in probiotic and DFM products.

Phytase was expressed recombinantly using *L. acidophilus* NCFM, *L. gallinarum* ATCC 33319, and *L. gasseri* ATCC 33323. Both *L. acidophilus* and *L. gasseri* cultures used in this study were originally isolated from the human gastrointestinal tract (153, 154). They are commonly used as model organisms in research investigating mechanisms of probiotic functionality because they are readily transformed (155, 156), genetically tractable (109, 157), and the availability of the complete genome sequences (158, 159) for these microorganisms. *L. gallinarum* was originally isolated from the crop of a chicken (160) and has been demonstrated to reduce gastrointestinal colonization of *Campylobacter jeuni* in experimentally challenged broiler chickens (116). Transformation and heterologous protein expression in *L. gallinarum* ATCC 33319 has not been reported previously.

SDS-PAGE revealed the presence of a protein with a molecular weight similar to *B. subtilis* phytase (76) in the supernatants of *L. acidophilus* TDCC 61, *L. gallinarum* TDCC 63, and *L. gasseri* TDCC 65, which was likely to be recombinant PhyA expressed using pTD003. Additionally, a protein of similar molecular weight was also present in the supernatant of the empty vector control culture *L. gasseri* TDCC 64. The LAB-Secretome DB (161) predicted three secreted proteins expressed by *L. gasseri* ATCC 33323 with molecular weights between 39 kDa and 51 kDa, which may be the protein present.

Differential media containing phytate are commonly used to detect phytase activity (134, 135, 162). Phytase activity is indicated by zones of clearing around colonies cultured using phytate containing media. However, reduced pH around colonies of acid producing bacteria may also cause the appearance of zones of clearing. False positive detection of phytase activity can be reduced by staining with aqueous cobalt chloride and ammonium molybdovanadate solutions (134). Staining of differential screening plates requires colonies to be washed from the plate surface prior to detection of enzymatic activity (134, 135). In this study, an overlay medium (104) containing phytate was used to remove the need to wash colonies from the plate surface. This modification is expected to facilitate future screening for phytate-degrading *Lactobacillus* cultures by allowing isolates to be picked through the overlay agar for subculture.

Recombinant expression of phytase in *Lactobacillus* cultures has previously been reported (145, 146). However, comparison with previously reported results was impossible because activity was not evaluated (146) or specific activity was not reported (145). Comparison with published studies of wild-type *Lactobacillus* cultures was also complicated because specific activity was not reported (135) or reported in non-standard units (142, 144, 163). Nonetheless, we have determined our recombinant cultures produce 10-50 fold greater activity than previously reported for wild-type lactobacilli (135, 142, 144, 163).

L. gallinarum TDCC 63 and *L. gasseri* TDCC 65 were selected for administration to broiler chicks because they produced greater phytase activity than *L.*

acidophilus TDCC 61(Figure 4). Because colonization by allochthonous lactobacilli is transient, the probiotic cultures were administered daily (164) in order to maintain high levels of administered lactobacilli in the gastrointestinal tract of the experimental animals. While the probiotic potential of phytate-degrading *Lactobacillus* cultures has been explored previously (135, 138, 145), this is the first study to evaluate the effect of their administration *in vivo*. Supplementation with commercial phytase improved 3-week weight gain of broilers fed a phosphorus deficient diet (0.27 % aP) to a level similar to broilers fed a phosphorus adequate diet (0.47 % aP) (165). It is generally accepted that the aP content of broiler chicken rations supplemented with commercial phytases can be reduced by 0.1% or more without a significant decrease in weight gain (18, 166). Body weight gain of chicks administered *L. gasseri* TDCC 65 ($rPhyA^{+}$) was not significantly greater than other groups fed a phosphorus deficient diet. However, weight gain was improved to a level statistically comparable to the phosphorus adequate control group. Similar results were seen in early pilot studies investigating supplementation with a crude phytase preparation (167). While the increased performance of chicks administered *L. gasseri* TDCC 65 over negative and empty vector control groups was not significant, this study demonstrates proof of principle of *in situ* phytate-degradation as probiotic functionality. Further improvement of increased growth performance is expected in future studies.

CHAPTER III

CONCLUSION

Using recombinant expression of *B. subtilis* phytase in *Lactobacillus* cultures, we have demonstrated that administration of phytate-degrading probiotic cultures can improve performance of non-ruminant livestock animals fed a phosphorus deficient diet. While phytate-degradation by *Lactobacillus* reported previously was attributed to non-specific phosphatases, a sufficiently large screen may identify *Lactobacillus* cultures expressing this desired activity. Alternatively, true specific phytase activity may not be critical if sufficient phytate-degradation can be produced from non-specific phosphatases. The use of recombinant microorganisms allowed us to investigate this novel mechanism and inform future studies to identify and investigate wild-type probiotic microorganisms able to improve utilization of phytate and other indigestible feed constituents. We have demonstrated proof of principle of *in situ* degradation of indigestible feed constituents in the gastrointestinal tract as a novel mechanism of probiotic functionality. Feed additive enzyme supplementation and probiotic administration are widely used in livestock management programs. The development of probiotic cultures able to provide the nutritional and performance benefits of feed additive enzymes and the animal health and food safety benefits traditionally associated with probiotics is expected to benefit livestock production.

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APPENDIX A

Table 1. Bacterial Strains and Plasmids Used in This study

Strain or plasmid	Relevant Characterisitcs	Source or Reference
L. acidophilus		
NCFM	Human intestinal isolate	(158)
TDCC 60	NCFM with pTRK882	This study
TDCC 61	rPhyA^{+}, NCFM with pTD003	This study
L. gallinarum		
ATCC 33319^{T}	Chicken crop isolate, type strain	ATCC
TDCC 62	ATCC 33319 with pTRK882	This study
TDCC 63	rPhyA^{+}, ATCC 33319 with pTD003	This study
L. gasseri		
ATCC 33323^{T}	Human isolate, type strain	(159)
TDCC 64	ATCC 33323 with pTRK882	This study
TDCC 65	rPhyA^{+}, ATCC 33323 with pTD003	This study
E. coli		
MC1061	Str^{r}, *E.coli* transformation host	(124)
TOP10	Str^{r}, *E.coli* transformation host	Invitrogen
NCK1814	MC1061 with pTRK882	(109)
TDCC 33	TOP10 with pTD001	This study
TDCC 66	MC1061 with pTD003	This study
Plasmids		
pTRK882	4.4kb, Erm^{r}, constitutive expression vector, P_{pgm}	(109)
pTD001	3.5kb, Amp^{r}, pMAT::*phyA*	This study
pTD003	5.6kb, Erm^{r}, pTRK882::*phyA*	This study

ATCC =American Type Culture Collection; T= Type Culture

Table 2. PCR Primers

Target Gene	Primer	Sequence (5'→ 3')
*erm*C	pGK12_ermF	ATTCTCTTGGAACCATAC
	pGK12_ermR	ACTGCCATTGAAATAGAC
phyA	phy_1258F	ATTATCAACTGCTGCTGGTT
	phy_1976R	ATCAACAACTTGACCCTTTG

Table 3. Ingredient Profile and Nutrient Concentration of the Basal Starter Diet

Ingredient	Percentage
Corn	60.03
Soybean Meal (48% Crude Protein)	34.14
Limestone	1.70
Sodium Chloride	0.46
Fat (Animal/Vegetable Blend)	2.24
L-Lysine HCl	0.17
DL-Methionine (99%)	0.26
Vitamins[1]	0.25
Minerals[2]	0.05
Monocalcium PO_4	0.60
L-Threonine	0.03
Calculated Nutrient Concentration	
Crude Protein (%)	22.00
Metabolizable Energy (kcal/kg)	3,050
Methionine (%)	0.58
Total Sulfur Amino Acids (%)	0.95
Lysine (%)	1.30
Threonine (%)	0.85
Tryptophan (%)	0.26
Calcium	0.85
Sodium	0.20
Total Phosphorus	0.50
Available Phosphorus	0.25

[1] Vitamin premix added at this rate yields 11,023 IU vitamin A, 3,858 IU vitamin D_3, 46 IU vitamin E, 0.0165 mg B_{12}, 5.845 mg riboflavin, 45.93 mg niacin, 20.21 mg d-pantothenic acid, 477.67 mg choline, 1.47 mg menadione, 1.75 mg folic acid, 7.17 mg pyroxidine, 2.94 mg thiamine, 0.55 mg biotin per kg diet. The carrier is ground rice hulls.

[2] Trace mineral premix added at this rate yields 149.6 mg manganese, 125.1 mg zinc, 16.5 mg iron, 1.7 mg copper, 1.05 mg iodine, .25 mg selenium, a minimum of 6.27 mg calcium, and a maximum of 8.69 mg calcium per kg of diet. The carrier is calcium carbonate and the premix contains less than 1% mineral oil.

Table 4. Phytase Activity of Recombinant *Lactobacillus* Cultures

Cultures	Specific Activity (U/mg)[1]		Activity Increase[2]
	pTD003	pTRK882	
L. acidophilus	0.168±0.019[c]	0.046±0.029[d]	4.04±2.46[c]
L. gallinarum	0.556±0.077[a]	0.034±0.011[d]	18.61±5.80[a]
L. gasseri	0.387±0.041[b]	0.038±0.003[d]	10.68±0.33[b]

[1] Units; International Units, µmol $^{-}$PO4 released min^{-1} mg^{-1}total protein;[-2] Fold increase between pTD003 ($rPhyA^{+}$) and pTD882 (empty vector) transformed cultures; Error bars represent the SEM of replicate reactions from three independent assays.[a-c] different superscripts within columns indicate the means differ significantly ($P<0.05$)

Figure 1. Plasmid Map of pTD003

Black arrows, replication determinants; light gray arrows, erythromycin resistance marker, *ermC*; black boxes, transcriptional terminators; white arrow, P_{pgm} promoter; dark gray arrow, codon optimized phytase gene, *phyA.*

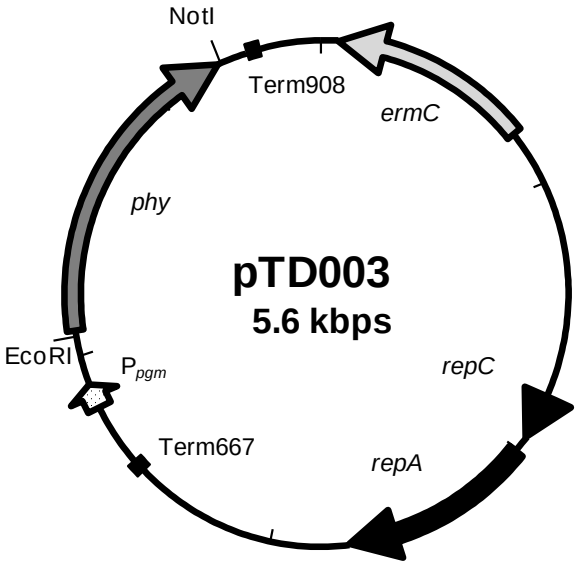


Figure 2. SDS-PAGE

Supernatants from *Lactobacillus* cultures were analyzed using SDS-PAGE. Lane 1, *L. acidophilus* TDCC 61; Lane 2, *L. acidophilus* TDCC 60, Lane 3, *L. gallinarum* TDCC 63; Lane 4, *L. gallinarum* TDCC 62, Lane 5, *L. gasseri* TDCC 65, Lane 6, *L. gasseri* TDCC 64; M, low range molecular weight marker.

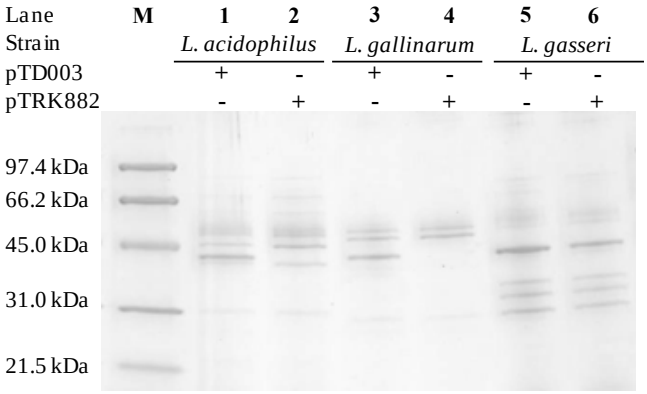


Figure 3. Phytate Hydrolysis

Lactobacillus cultures were spotted onto MRS agar and incubated 36 hours. Plates were overlaid with modified MRS agar with 0.5% sodium phytate and incubated 24 hours. Plates were stained with cobalt chloride solution and counterstained with ammonium molybdovanadate solution. Zones of clearing indicate phytate hydrolysis.

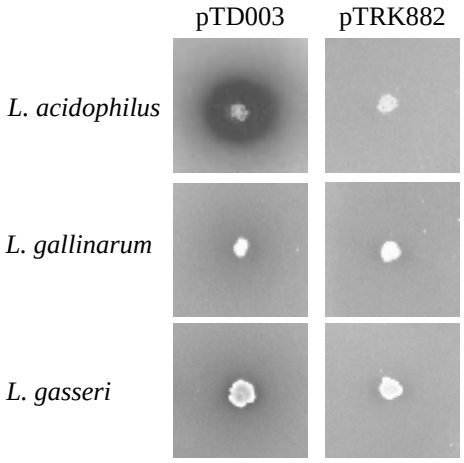
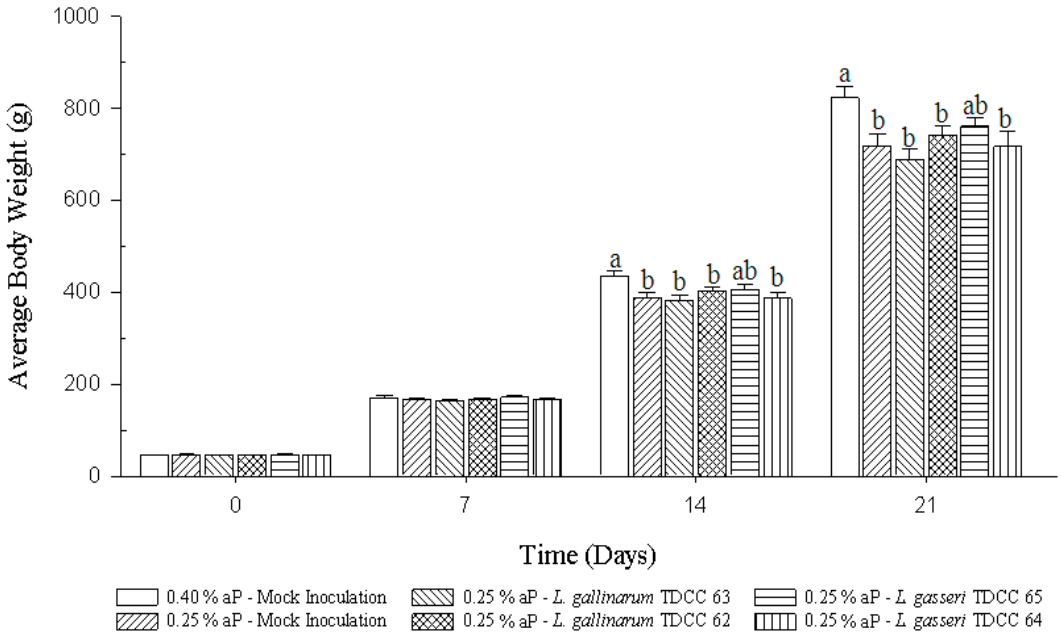


Figure 4. Average Body Weights of Broiler Chicks

Male broiler chicks were divided between six treatment groups and fed either a phosphorus adequate control diet (0.40% aP) or a phosphorus deficient diet (0.25% aP) and administered either a mock inoculation (MRD) or cultures of *L. gallinarum* and *L. gasseri* by oral gavage daily. Broiler chicks were weighed individually at Day 0, 7, 14, and 21 post-hatch. Data are shown as the mean body weight for each treatment group and error bars represent the SEM. Different letters indicate means are significantly different.



APPENDIX B

Supplemental Table 1. Average Body and Tibia Weights of Broiler Chicks

Male broiler chicks were divided between six treatment groups and fed either a phosphorus adequate control diet (0.40% aP) or a phosphorus deficient diet (0.25% aP) and administered either a mock inoculation (MRD) or cultures of *L. gallinarum* and *L. gasseri* by oral gavage daily. Broiler chicks were weighed individually at Day 0, 7, 14, and 21 post-hatch. Right tibia was collected from each bird for bone ashing. Data are shown as the mean body weight and ash weight for each treatment group and error bars represent the SEM. Different letters indicate means are significantly different.

Supplemental Table 1. Performance of Male Broiler Chicks Administered Recombinant *Lactobacillus* Cultures

Diet	P-Adequate	P-Deficient	P-Deficient	P-Deficient	P-Deficient	P-Deficient
aP%[1]	0.40%	0.25%	0.25%	0.25%	0.25%	0.25%
Strain[2]	-	-	*L. gallinarum*		*L. gasseri*	
			pTD003	pTRK882	pTD003	pTRK882
Weight (g)						
Day 0	46.0±2.2	47.0±2.0	46.0±3.2	46.3±3.2	46.8±2.4	45.7±2.4
Day 7	170.8±20.2	166.4±14.6	164.3±13.9	166.8±16.8	170.9±15.8	165.7±14.7
Day 14	434.1±55.5[a]	388.1±57.8[b]	382.4±49.7[b]	402.1±42.6[b]	406.1±36.9[a,b]	386.5±54.4[b]
Day 21	822.6±120.0[a]	719.3±120.7[b]	688.4±109.5[b]	741.3±90.3[b]	760.7±77.2[a,b]	716.5±154.4 [b]
Bone Ash (%)	45.90±0.62[a]	40.11±0.34[b]	40.32±0.17[b]	40.41±1.76[b]	39.99±1.59[b]	38.72±2.60[b]

[1]Available phosphate; [2]Bacterial strain administered; [a-b] Different superscripts within rows indicates means differ significantly ($P<0.05$)