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Evaluation of Phytate-Degrading *Lactobacillus* Culture Administration to Broiler Chickens

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Probiotics have been demonstrated to promote growth, stimulate immune responses, and improve 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. Microbial phytases are increasingly supplemented in feed to improve digestibility and reduce antinutritive effects of phytate. The microbial origin of these exogenous enzymes suggests a potentially important mechanism of probiotic functionality. We investigated 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 a protein in the culture supernatants of *Lactobacillus* pTD003 transformants with a molecular weight similar to that of the *B. subtilis* phytase. 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, respectively. Phytase-expressing *L. gallinarum* and *L. gasseri* were administered to broiler chicks fed a phosphorus-deficient diet. Phytase-expressing *L. gasseri* improved weight gain of broiler chickens to a level comparable to that for chickens fed a control diet adequate in phosphorus, demonstrating proof of principle that administration of phytate-degrading probiotic cultures can improve performance of livestock animals. This will inform future studies investigating whether probiotic cultures are able to provide both the performance benefits of feed enzymes and the animal health and food safety benefits traditionally associated with probiotics.

Lactobacillus species are important inhabitants of the gastrointestinal tracts of humans and animals and are increasingly being used as probiotic microorganisms due to their health-promoting properties (1, 2). Probiotics, sometimes called direct-fed microbials (DFM) when used in animals (3), are live microorganisms administered to confer a health benefit upon the host (4). Administration of probiotic *Lactobacillus* to poultry has been demonstrated to promote growth at levels similar to antibiotics (5, 6) and to reduce gastrointestinal colonization of human foodborne pathogens, including *Campylobacter* (7, 8), *Clostridium* (9), and *Salmonella* (10, 11). Because of concern over antibiotic-resistant pathogens and pressure from both consumers and regulatory agencies, probiotics have received increased interest as potential alternatives to antibiotic growth promoters (12). While probiotics are used widely in livestock production (13), their effectiveness is varied, and the mechanisms responsible for their benefits are not well understood.

Phosphorus is an essential nutrient in poultry production (14), with dietary deficiencies leading to excessive financial losses due to increased mortality (15, 16). Phytic acid (*myo*-inositol hexaphosphate) is an important plant phosphorus storage form and accounts for 50 to 80% of total phosphorus present in cereal grains and legumes commonly used in livestock animal feeds (17, 18). However, phytate phosphorus has low bioavailability and is underutilized due to the lack of endogenous phytate-degrading enzymes in nonruminant livestock, including poultry (19, 20) and swine (21). Additionally, phytic acid exerts antinutritive effects (15), sequestering essential cations, including calcium, magnesium, iron, and zinc, and reducing their bioavailability (22).

Phytases are phosphatases which catalyze the hydrolysis of phytic acid to *myo*-inositol and inorganic phosphate (23). In-feed administration of microbial phytases to improve digestibility of phytic acid is widely used in the production of poultry and other livestock (24, 25). The resulting increases in phytate phosphorus bioavailability (15, 26, 27) and reduction in the antinutritive effects (28, 29) of phytic acid are well documented. The microbial origin of phytases used in livestock production suggests that degradation of phytic acid may be a potentially important mechanism of probiotic functionality. Combining the nutritional and growth 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 *Bacillus 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.

MATERIALS AND METHODS

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 de Man, 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)

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid[a]	Relevant characteristics	Source or reference
Strains		
L. acidophilus		
NCFM	Human intestinal isolate	66
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[a]
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	62
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	29
TOP10	Str^r, *E. coli* transformation host	Invitrogen
NCK1814	MC1061 with pTRK882	37
TDCC 33	TOP10 with pTD001	This study
TDCC 66	MC1061 with pTD003	This study
Plasmids		
pTRK882	4.4 kb, Erm^r, constitutive expression vector, P_{pgm}	37
pTD001	3.5 kb, Amp^r, pMAT::*phyA*	This study
pTD003	5.6 kb, Erm^r, pTRK882::*phyA*	This study

[a] ATCC, American Type Culture Collection.

TABLE 2 PCR primers

Target Gene	Primer	Sequence (5′→3′)
ermC	pGK12_ermF	ATTCTCTTGGAACCATAC
	pGK12_ermR	ACTGCCATTGAAATAGAC
phyA	phy_1258F	ATTATCAACTGCTGCTGGTT
	phy_1976R	ATCAACAACTTGACCCTTTG

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.

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 (30). DNA restriction fragments were purified from agarose gels using the QIAquick gel extraction kit (Qiagen, Germantown, MD). All DNA manipulations were performed using standard molecular cloning techniques (31). 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. Electrocompetent *E. coli* MC1061 and TOP10 were prepared and transformed according to standard methods (32). *Lactobacillus acidophilus* and *Lactobacillus gasseri* were transformed using the method of Luchansky et al. (33), while *Lactobacillus gallinarum* was transformed using the method of Beasley et al. (34).

Recombinant phytase expression in *Lactobacillus*. The *phyA* gene from *B. subtilis* (35) was codon optimized for expression in *L. acidophilus* using the OPTIMIZER web server (36) 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 (37) for constitutive high-level expression in *Lactobacillus*. The resulting plasmid, pTD003, was transformed into and subsequently propagated in *E. coli* MC1061. The plasmids pTD003 and pTRK882 were introduced into *Lactobacillus* species by electrotransformation. Transformations were confirmed by PCR using gene specific primers (Table 2).

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% (wt/vol) trichloroacetic acid (TCA) (Sigma-Aldrich) and pelleted by centrifugation. Protein pellets were washed 3 times using 80% (wt/vol) acetone and resuspended in phosphate-buffered saline (PBS). Protein concentrations were determined using the Bradford method (38). Protein was separated by SDS-PAGE using Any kD Mini-PROTEAN TGX Precast protein gels (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 (39). Gels were stained with GelCode Blue Safe protein stain (Thermo Scientific, Waltham, MA) for visualization of protein.

Phytate hydrolysis. Phytate hydrolysis by *Lactobacillus* transformants was observed using a modification of the method of Bae et al. (40). *Lactobacillus* colonies were selected, aseptically transferred onto the surfaces of MRS agar plates (5 μg/ml Erm), and incubated for 36 h. Plates were then overlaid with modified MRS (41), in which 0.5% (wt/vol) sodium phytate (Pfaltz & Bauer, Waterbury, CT) was the sole phosphorus source, and incubated for an additional 24 h. Plates were stained with cobalt chloride solution and counterstained with an ammonium molybdovanadate solution. Phytate hydrolysis was indicated by zones of clearing.

Phytase enzyme activity assays. Phytase activity from cell extracts 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% (wt/vol) TCA, and free phosphate was determined colorimetrically (620 nm) using the ammonium molybdate method (42) with a sodium phosphate standard. Cell extracts were prepared by bead beating (37) as described previously in phytase extract buffer (2 mM $CaCl_2$, 100 mM Tris-HCl, pH 7.0). Protein concentrations were determined using the Bradford method (38). Phytase specific activity was reported as U mg^{-1} total protein (μmol PO_4^{3-} released min^{-1} mg^{-1}). Data were analyzed using analysis of variance (ANOVA), and significant differences between strains were determined using Duncan's multiple-range test.

Broiler chickens. On the day of hatch, male broiler chicks (Ross × Ross) were obtained from a commercial hatchery, weighed individually, wing banded, and assigned to pens based on body weight to ensure that 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).

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% available phosphate [aP]) and administered recombinant *Lactobacillus* cultures (10^8 CFU) in 1 ml maximum recovery diluent (MRD) (Difco) by oral gavage daily. 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 fed a diet adequate in phosphorus (0.40% aP) (positive control) or the phosphorus-deficient diet (0.25% aP) (negative control) and administered a mock inoculation of 1 ml sterile MRD by oral gavage daily. Broiler chicks were weighed individually at days 0, 7, 14, and 21 posthatch. Data were analyzed using ANOVA, and significant differences between treatment groups were determined using Duncan's multiple-range test with individual birds as the experimental unit.

TABLE 3 Ingredient profile and nutrient concentrations for the basal starter diet

Parameter	%
Ingredients	
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[a]	0.25
Minerals[b]	0.05
Monocalcium PO_4	0.60
L-Threonine	0.03
Calculated nutrient concn	
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[c]	0.50
Available phosphorus	0.25

[a] 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, and 0.55 mg biotin per kg diet. The carrier is ground rice hulls.

[b] 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, 0.25 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.

[c] Analyzed total phosphorus was 0.67% for the phosphorus-deficient basal diet and 0.81% in the positive-control diet with adequate phosphorus.

Experimental diets. A phosphorus-deficient basal starter diet was formulated with 0.25% available phosphate (aP) and all other nutrients meeting or exceeding industry-type broiler diet requirements for market broilers for days 0 to 21 posthatch (Table 3). The positive-control diet adequate in phosphorus was formulated by increasing the aP to 0.40% with the addition of KH_2PO_4 to the basal diet. Feed samples were analyzed by an independent commercial laboratory for total phosphorus content (43).

RESULTS

Recombinant phytase expression in *Lactobacillus*. The 1,149-bp *phyA* (BSU19800) gene, encoding a phytase (44) from *B. subtilis* (35), 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 to 26), suggesting that the protein would likely be secreted via the *sec* pathway (45). *B. subtilis phyA* was codon optimized for expression in *Lactobacillus* using OPTIMIZER (36). The codon adaptation index of the native *phyA* sequence was 0.27, and this improved to 1.00 after optimization. The optimized sequence was commercially synthesized and subcloned into pTRK882. The resulting plasmid, pTD003 (Fig. 1), and the empty vector, pTRK882, were transformed into *L. acidophilus* NCFM, *L. gallinarum* ATCC 33319^T, and *L. gasseri* ATCC 33323^T. Transformations were confirmed by PCR to detect *ermC* and recombinant *phyA* (r*phyA*) (data not shown). Amplification of both *phyA* and *ermC* indicated successful transformation by pTD003, and amplification of *ermC* alone indicated successful transformation by pTRK882.

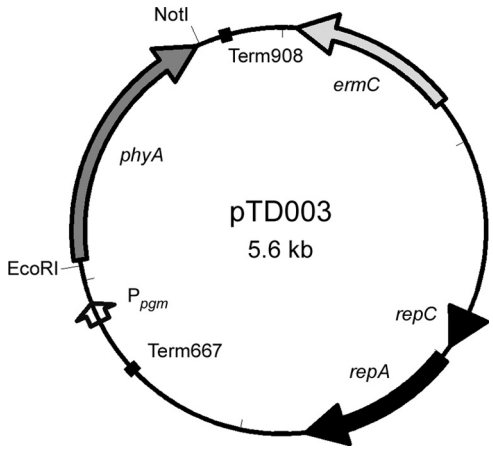

FIG 1 Plasmid map of pTD003. Black arrows, replication determinants; light gray arrow, erythromycin resistance marker, *ermC*; black boxes, transcriptional terminators; white arrow, P_{pgm} promoter; dark gray arrow, codon-optimized phytase gene, *phyA*.

SDS-PAGE. Total protein in culture supernatants from *Lactobacillus* cultures was separated using SDS-PAGE (Fig. 2). A protein with a molecular mass of 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 mass 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 mass of the secreted mature phytase from *B. subtilis* is 44 kDa (44). These data suggest that recombinant PhyA phytase (rPhyA) is expressed and secreted by *Lactobacillus* cultures transformed with pTD003.

Phytate hydrolysis. Phytate hydrolysis by *Lactobacillus* cultures was evaluated qualitatively (Fig. 3). Zones of clearing appeared around colonies of pTD003-transformed cultures, *L. acido-*

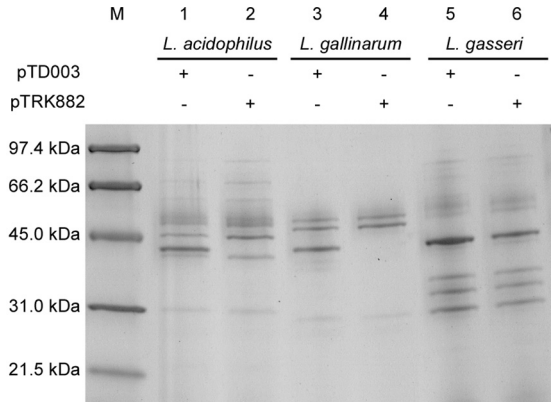

FIG 2 SDS-PAGE. Supernatants from *Lactobacillus* cultures were analyzed using SDS-PAGE. Lane 1, *L. acidophilus* TDCC 61; lane 2, *L. acidophilus* TDCC 60; lane 3, *L. gallinarum* TDCC 63; lane 4, *L. gallinarum* TDCC 62; lane 5, *L. gasseri* TDCC 65; lane 6, *L. gasseri* TDCC 64; lane M, molecular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

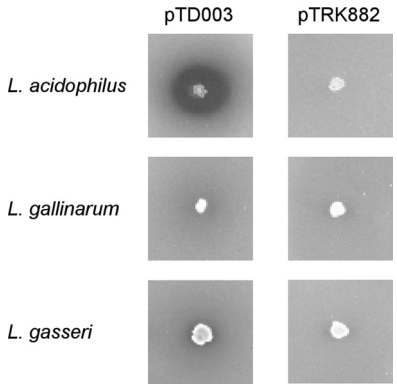

FIG 3 Phytate hydrolysis. *Lactobacillus* cultures were spotted onto MRS agar and incubated for 36 h. Plates were overlaid with modified MRS agar containing 0.5% sodium phytate, incubated for 24 h, and stained with cobalt chloride and ammonium molybdovanadate solutions. Zones of clearing indicate phytate hydrolysis.

philus 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.

Phytase activity of recombinant *Lactobacillus* cultures. Phytase activity from cell pellets of recombinant *Lactobacillus* cultures was evaluated (Table 4). The phytase activities of *L. acidophilus* TDCC 61, *L. gallinarum* TDCC 63, and *L. gasseri* TDCC 65 were approximately 4-, 18-, and 10-fold greater than those of the respective empty-vector control cultures, respectively. *Lactobacillus* empty-vector (pTRK882) transformants are wild type for phytase activity and account for background phytate degradation by nonspecific phosphatases. The phytase activities of *L. gallinarum* TDCC 63 and *L. gasseri* TDCC 65 were approximately 3- and 2-fold greater, respectively, than that of *L. acidophilus* TDCC 61.

Broiler performance trial. The effects of rPhyA-producing *Lactobacillus* cultures on the performance of broiler chicks were evaluated (Fig. 4). There were no differences in body weight between the treatment groups at days 0 and day 7 posthatch. For mock-inoculated control groups, the body weight of chicks fed a diet adequate in phosphorus (positive control) was greater than that of those fed a phosphorus-deficient diet (negative control) at days 14 and 21 posthatch ($P < 0.05$). The body weight of chicks administered rPhyA-producing *L. gallinarum* (TDCC 63) and *L. gasseri* (TDCC 65) was not significantly different from that of 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 from that of the positive-control group ($P > 0.05$). While performance was not significantly increased compared to that with 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 that of chicks fed a diet adequate in phosphorus.

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 (46). The microbial origin of exogenous enzymes used in livestock production, including phytases, suggests that direct augmentation of host metabolism through the *in situ* production and delivery of these enzymes by microorganisms in the gastrointestinal tract may potentially be an important mechanism of probiotic functionality. While these enzyme activities have been suggested as selection criteria for probiotic cultures (41, 47), 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 the nutritional quality of fermented cereal grains (48–50). De Angelis et al. (51) reported the purification of a phytase from *Lactobacillus sanfranciscensis*. However, the significantly greater substrate specificity of this enzyme toward *p*-nitrophenyl phosphate over phytate suggests that this enzyme would more appropriately be classified as a non-phytate-specific acid phosphatase. Phytate degradation has been attributed to nonspecific acid phosphatases in other lactobacilli (52, 53). 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. The *phyA* gene from *B. subtilis* (35) encodes a β-propeller phytase with high specificity for phytic acid and activity over broad pH and temperature ranges (44). Analysis of the amino acid sequence using SignalP (45) predicted the presence of a Gram-positive secretion signal, suggesting that heterologous expression of this protein in *Lactobacillus* would 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 (54–57). *Bacillus* species are workhorse bacteria in microbial fermentations and are highly prized as producers of industrially important enzymes (58). Heterologous expression of *B. subtilis* phytase using *Lactobacillus* in this study not only demonstrates biocatalytic phytate degradation as a 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.

B. subtilis phyA was codon optimized and cloned into pTRK882, under the control of the constitutive high-expressing P_{pgm} promoter from *L. acidophilus* NCFM (37), in order to maximize expression in *Lactobacillus* species. This expression system

TABLE 4 Phytase activities of recombinant *Lactobacillus* cultures

Culture	Sp act (U/mg)[a]		Activity increase[b]
	pTD003	pTRK882	
L. acidophilus	0.168 ± 0.019 c	0.046 ± 0.029	4.04 ± 2.46 c
L. gallinarum	0.556 ± 0.077 a	0.034 ± 0.011	18.61 ± 5.80 a
L. gasseri	0.387 ± 0.041 b	0.038 ± 0.003	10.68 ± 0.33 b

[a] International units, μmol PO_4^{3-} released min^{-1} mg^{-1} total protein. Data are means ± SEMs for replicate reactions from three independent assays. Different letters within columns indicate that the means differ significantly ($P < 0.05$).
[b] Fold increase between pTD003 ($rPhyA^+$)- and pTD882 (empty-vector)-transformed cultures. Data are means ± SEMs for replicate reactions from three independent assays. Different letters within columns indicate that the means differ significantly ($P < 0.05$).

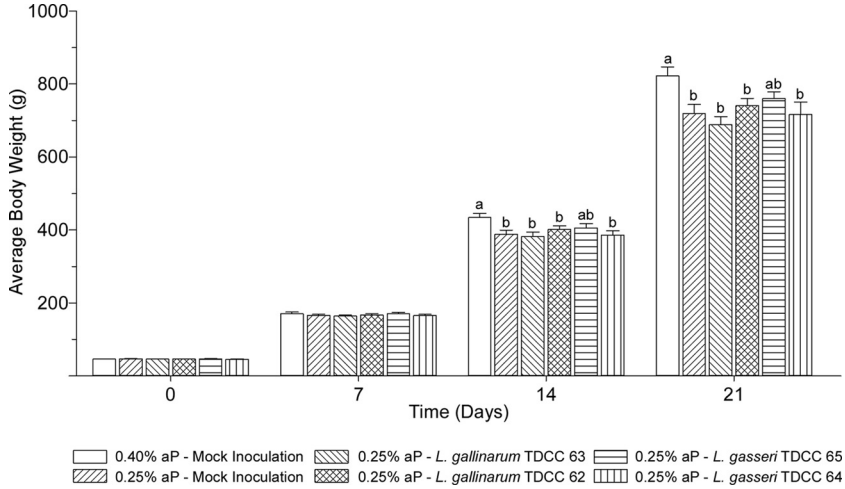

FIG 4 Average body weights of broiler chicks. Male broiler chicks were divided between six treatment groups ($n = 24$ broiler chickens in each treatment) and either fed a control diet adequate in phosphorus (0.40% aP) and administered a mock inoculation (MRD) or fed a phosphorus-deficient diet (0.25% 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) by oral gavage daily. Broiler chicks were weighed individually at days 0, 7, 14, and 21 posthatch. Data shown are the mean body weight for each treatment group, and error bars represent the standard error of the mean (SEM). Different letters indicate that means are significantly different ($P < 0.05$).

has been previously demonstrated to be effective in enzyme expression (37), the production and delivery of immune modulating cytokines (59), and an anthrax vaccine (60), and its wide host range allowed the transformation of *L. acidophilus*, *L. gallinarum*, and *L. gasseri*. *L. acidophilus* NCFM and *L. gasseri* ATCC 33323 were originally isolated from the human gastrointestinal tract (61, 62). These cultures are commonly used as model organisms in research investigating mechanisms of probiotic functionality because they are readily transformed (63, 64) and genetically tractable (37, 65) and because a complete genome sequences is available for these microorganisms (62, 66). *L. gallinarum* was originally isolated from the crop of a chicken (67) and has been demonstrated to reduce gastrointestinal colonization of *Campylobacter jejuni* in experimentally challenged broiler chickens (8). Plasmid transformation and heterologous protein expression in *L. gallinarum* ATCC 33319 have not been reported previously.

SDS-PAGE revealed the presence of a protein with a molecular mass similar to that of *B. subtilis* phytase (44) in the supernatants of *L. acidophilus* TDCC 61, *L. gallinarum* TDCC 63, and *L. gasseri* TDCC 65, which was likely to be recombinant rPhyA expressed using pTD003. Additionally, a protein of similar molecular mass was also present in the supernatant of the empty-vector control culture of *L. gasseri* TDCC 64. The LAB-Secretome database (68) predicted three secreted proteins expressed by *L. gasseri* ATCC 33323 with molecular masses between 39 kDa and 51 kDa, which may be the protein present.

Differential media containing phytate are commonly used for detection and qualitative evaluation of phytase activity (40, 41, 69). 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 (40). Staining of differential-screening plates requires colonies to be washed from the plate surface prior to detection of enzymatic activity (40, 41). In this study, an overlay medium (70) 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* has been demonstrated previously (71, 72). However, comparison with these studies was impossible because activity was not evaluated quantitatively (72) or because specific activity was not reported (71). Comparison with published studies of wild-type *Lactobacillus* cultures was also complicated because specific activity was not reported (41) or was reported in nonstandard units (51, 53, 73). Nonetheless, we have determined that our recombinant cultures produce 10- to 50-fold-greater activity than previously reported for wild-type lactobacilli (41, 51, 53, 73).

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 (Table 4). Broiler chicks were inoculated daily with 10^8 CFU *Lactobacillus* by oral gavage. Broiler chicks have been administered 10^8 CFU *Lactobacillus* by oral gavage in studies investigating their administration in poultry (8, 74, 75) in order to maximize detection of any potential beneficial effects. Because colonization by allochthonous lactobacilli is transient, the probiotic cultures were administered daily (76) in order to maximize the presence of administered lactobacilli in the gastrointestinal tracts of the experimental animals. While the probiotic potential of phytate-degrading *Lactobacillus* cultures has been explored previously (41, 47, 71), this is the first study to evaluate the effect of their administration *in vivo*.

Nutritional models using phosphorus-deficient corn soybean meal rations are widely used to investigate phytate phosphorus metabolism in poultry (77, 78). Body weight gain is depressed in broiler chicks fed rations deficient in aP relative to those fed a diet adequate in aP. A decrease in the growth depression caused by aP deficiency is an effective and commonly used measure of the abil-

ity of exogenous phytase and other feed additives to improve bioavailability of phytate phosphorus (79–81).

Yi et al. (82) demonstrated that supplementation with commercial exogenous phytase improved the 3-week weight gain of broiler chicks fed a phosphorus-deficient diet (0.27% aP) to a level similar to that for those fed a diet adequate in phosphorus (0.47% aP). 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 (83, 84). The body weight gain of chicks administered *L. gasseri* TDCC 65 (rPhyA$^+$) was not significantly greater than those of other groups fed a phosphorus-deficient diet (Fig. 4). However, weight gain was improved to a level statistically comparable to that for the control group fed a diet adequate in phosphorus. Similar results were seen in early studies investigating supplementation with crude exogenous phytase preparations (78). Additionally, weight gain was improved only in chicks administered rPhyA$^+$ *L. gasseri* (TDCC 65) and not in those administered the empty-vector *L. gasseri* (TDCC 66). These *L. gasseri* ATCC 33323-derived cultures are isogenic strains which are either *phyA*$^+$ or wild type for phytase expression, indicating that that improved weight gain was due to increased bioavailability of phytate phosphorus mediated by phytase expression in *L. gasseri* TDCC 65 (rPhyA$^+$).

While the *in vitro* phytase activity of *L. gallinarum* TDCC 63 was greater than that of *L. gasseri* TDCC 65, the *in vivo* effectiveness of probiotic cultures is multifactorial. Other factors potentially affecting the behavior of these cultures when administered to poultry include the ability of these organisms to adhere and persist in various locations in the gastrointestinal tract (85, 86), their ability to survive or tolerate acid and bile (86), and the efficiency with which they are able to produce and secrete these enzymes in the gastrointestinal tract.

Recombinant expression of *B. subtilis* phytase in *Lactobacillus* has allowed us to demonstrate that administration of phytate-degrading probiotic cultures can increase the bioavailability of phytate phosphorus and improve the performance of nonruminant livestock animals fed a phosphorus-deficient diet. While phytate degradation by *Lactobacillus* reported previously was attributed to nonspecific 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 nonspecific phosphatases. While it is unlikely that regulatory agencies would approve the use of recombinant microorganisms in commercial livestock production, their use has allowed us to investigate this novel mechanism and inform future studies which will identify and investigate the potential of wild-type probiotic microorganisms able to improve utilization of phytate and other indigestible feed constituents. We have demonstrated proof of principle of *in situ* enzyme production and degradation of indigestible feed constituents by microorganisms in the gastrointestinal tract as a novel mechanism of probiotic functionality. Although administration of exogenous enzymes is currently relatively inexpensive, the identification of probiotic cultures able to increase the bioavailability of phytate phosphorus at levels similar to those with exogenous enzymes may reduce the need for isolation and purification of enzymes from industrial fermentations. Alternatively, rather than being a replacement for exogenous enzymes, the identification of probiotic organisms producing phytase or other important enzymes may offer a value-added benefit in addition to the food safety and animal health benefits traditionally associated with probiotic administration.

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