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Effect of the Transformed *Lactobacillus* with Phytase Gene on Pig Production Performance, Nutrient Digestibility, Gut Microbes and Serum Biochemical Indexes

Q. Q. Yin*, J. Chang, R. Y. Zuo, L. Y. Chen, Q. X. Chen, X. Y. Wei, Q. F. Guan
J. W. Sun, Q. H. Zheng, X. Yang and G. Z. Ren
College of Animal Science and Veterinary Medicine, Henan Agricultural University, Zhengzhou 450002, China

ABSTRACT : In order to improve the availability of phytase and probiotics together, a phytase gene from *Aspergillus ficuum* has been expressed in *Lactobacillus*. In this study, the transformed *Lactobacillus* with phytase gene was fed to pigs to determine its effect on pig production, feed conversion and gut microbes. Forty eight, 60-day-old, castrated pigs (Duroc×Landrace×Pietrain) were assigned to 6 groups, 8 pigs for each group. Group 1 was the control, group 2 was added with chlortetracycline (500 mg/kg), group 3 was added with the transformed *Lactobacillus* (500 mg/kg) with 20% (w/w) of calcium monohydrogen phosphate (CMP, $CaHPO_4$) removed, group 4 was added with the natural *Lactobacillus* (500 mg/kg) with 20% (w/w) of CMP removed, group 5 was added with the transformed *Lactobacillus* (500 mg/kg) with 40% (w/w) of CMP removed, group 6 was added with phytase (500 mg/kg) with 40% (w/w) of CMP removed. The results showed: i) the average daily gain (ADG) was improved in groups 2, 3 and 4 ($p<0.05$); ii) the diarrhea rates in the groups added with *Lactobacillus* were lower than in the other groups ($p<0.05$), in which the transformed *Lactobacillus* had more effect on reducing digestive disease; iii) the transformed *Lactobacillus* was most effective in improving the digestibilities of crude protein (CP), calcium (Ca), phosphorus (P), compared with the other groups ($p<0.05$); iv) *Lactobacillus* could increase lactic acid bacterium number and ammonia concentrations, and decrease pH values and *E. coli* number in pig feces ($p<0.05$); v) the phytase activity in the feces of pigs fed with the transformed *Lactobacillus* was 133.32 U/g, which was higher than in group 4 (9.58 U/g, $p<0.05$), and was almost the same as group 6 (135.94 U/g); vi) the transformed *Lactobacillus* could increase serum concentrations of IgA, triglyceride, and glutamic oxaloacetic transaminase activity ($p<0.05$), and had no significant effect on other serum indexes ($p>0.05$). (**Key Words :** *Lactobacillus*, Phytase, Pig, Nutrient Digestibility, Gut Microbes, Serum Biochemical Indexes)

INTRODUCTION

Phytic acid exists in the diets of pigs and serves as a P reservoir. It is such a powerful chelating agent that the solubility and digestibility of many nutrients are reduced by the formation of phytate complexes (Selle, 1997). Phytase can catalyze the hydrolysis of phytate and release organic P and phytate-bound nutrients (Wodzinski, 1996; Murry et al., 1997). Because there is little phytase activity in the digestive tracts of the non-ruminant animals (Bitar and Reinhold, 1972), these animals can't use nutrients effectively, specially the phytate-bound P (Sweeten, 1992), resulting in a significant loss of P to the environment. The addition of phytase in animal diet can reduce P excretion by 30-50% for decreasing P pollution as well as reduce the supplementation of inorganic P in animal diets (Selle, 1997).

A lot of antibiotics have been widely used in animal feeds for improving animal production and preventing diseases. Due to the side effects of antibiotics, such as bacterial patience and resistance to antibiotics, and its residue in meat, egg and milk, there is almost universal agreement that animal production must move away from the use of antibiotics and other chemicals (Christensen, 2000). It is imperative to find a product without antibiotic while maintaining or even increasing production efficiency. Researches have shown that *Lactobacillus* is one of the ideal probiotics in improving production, controlling pathogenic microorganisms, and reducing diarrhea in pigs, especially for newly weaned or artificially reared pigs (Pollmann et al., 1980).

At present, phytase produced by the transformed

* Corresponding Author: Q. Q. Yin. Tel: +86-371-63554237, Fax: +86-371-63558998, E-mail: QQZ22@yahoo.com.cn
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microorganisms has been added in almost all the diets of non-ruminant animals as feed additive. How to reduce cost and improve phytase effetiveness in animal gut becomes more and more important. In order to find an alternative economical method to supply phytase for animals, phytase gene has been successfully over-expressed in *Lactobacillus cacei* (*L. cacei*) in our laboratory (Zuo et al., 2009), which was the first report to combine phytase and probiotic together.

The transformed *L. cacei* with phytase gene is able to survive in animal gut and excrete phytase once it is ingested by animals. It will have both functions of phytase and probiotics. The aim of this research was to determine the effect of the transformed *L. cacei* on pig production performance, nutrient digestibility, etc., so as to verify whether it can become a new kind of feed additive for animal feeding in the future.

MATERIALS AND METHODS

Preparation of the transformed *Lactobacillus*

Phytase gene (1.4 kb) was isolated from *Aspergillus ficuum* by PCR, and inserted into the plasmid of pIAβ8 to construct the vector (Feng et al., 2009), which was then transferred into the competent cell of *Lactobacillus casei* by electroporation (Zuo et al., 2009). The positive colonies were picked and incubated in a MRS medium (Difco Laboratory) containing 5 μg/ml chloramphenicol at 37°C for determining phytase activity (Zuo et al., 2009). Five hundreds ml cultures of the transformed *Lactobacillus* were added with 5 g sodium polymannuronate ($C_5H_7O_4COONa$) and mixed, and then dropped into 500 ml 1% (w/v) calcium chloride ($CaCl_2$) to make small pellets for embedding the *L. casei*. The pellets containing *L. casei* were taken out from the solution and mixed with wheat bran (1:1) and air dried at 25°C. The number of the *L. casei* in the product was 1×10^9 cfu/g. The natural *L. casei* preparation was prepared as the same as the above.

The experimental animals, diets and feeding management

Forty eight 60-day-old castrated pigs (Duroc×Landrace ×Pietrain, 17.93±0.98 kg body weight) were assigned to 6 groups, 8 pigs for each group in one pen. Every pig had its own ID code. The basal feed was prepared according to the recommended standard (NRC, 1998). The feed compositions and nutrient levels were listed in Table 1.

The pigs were weighted at initial and terminal experiment, and they were fasted for 12 h before weighting. The experimental period was 70 d, and the pre-trial period was 7 d. The feed and water were given to pigs *ad libitum*. The diarrhea rates were recorded daily, and feed intake in each group was recorded once a week. The temperature in the shed was 16-28°C during the trial.

The experimental design

The diets were mash feed, and the experimental design was shown as the follows:

Group 1: Basal diet
+0.05% (w/w) wheat bran (the control)

Group 2: Basal diet+0.05% (w/w) chlorotetracycline

Group 3: Basal diet (20% (w/w) CMP removed)
+0.05% (w/w) embedded transformed *Lactobacillus*

Group 4: Basal diet (20% (w/w) CMP removed)
+0.05% (w/w) embedded natural *Lactobacillus*

Table 1. Feed compositions and nutrient levels of the basal diet (%)

Feed compositions		Nutrient levels	
Name of raw materials	Percentage	Nutrients	Levels
Corn	69.45	Digestive energy (MJ/kg)	14.13
Soybean meal	25.50	Crude protein	16.82
Soybean oil	2.00	Ca	0.60
L-lysine·HCl	0.20	Total P	0.45
Calcium carbonate	1.00	Available P	0.26
CMP	0.60	Crude fat	4.85
Salt	0.25	Dry matter	87.89
Premix compound	1.00	Lysine	0.95
Total	100	Methionine+crystine	0.57

Premix compound provided per kilogram of diet: 145 mg of Fe (ferrous sulfate); 130 mg of Zn (zinc oxide); 50 mg of Mn (manganese oxide); 15 mg of Cu (copper oxide); 0.9 mg of I (potassium iodate); 0.3 mg of Se (sodium selenite); 13,350 IU of vitamin A; 1,350 IU of vitamin D_3; 100 IU of vitamin E; 3.5 mg of vitamin K; 15 mg of niacin; 10 mg of pantothenic acid; 3.50 mg of riboflavin; 0.025 mg of vitamin B_{12}; 0.35 mg of biotin; 0.3 mg of folacin; 9 mg of pyridoxine; 6 mg of thiamine; and 0.35 mg of ascorbic acid.

Group 5: Basal diet (40% (w/w) CMP removed) +0.05% (w/w) embedded transformed *Lactobacillus*

Group 6: Basal diet (40% (w/w) CMP removed) +0.05% (w/w) commercial phytase (500 U/g)

Note: The 20% (w/w) CMP removed from the basal diet was replaced by adding 12% (w/w) calcium carbonate and 8% (w/w) wheat bran to keep calcium level as the same as the control; 40% (w/w) CMP removed from the basal diet was replaced by adding 24% (w/w) calcium carbonate and 16% (w/w) wheat bran. Even though wheat bran contains about 230 U/kg phytase (Han et al., 1997), the amount in the diet is very low. For example, when 20% (w/w) CMP removed from the basal diet (0.6% CMP) was replaced by adding 12% (w/w) calcium carbonate and 8% (w/w) wheat bran, it means that only 0.048% wheat bran is added in the diet of group 3, and phytase activity in the diet is only 0.11 U/kg, which is very lower than the common diets with phytase additive (250-500 U/kg). As a result, a little wheat bran addition in the diet will have few effects on the experimental results.

Determination of nutrient digestibility

During the middle period of feeding experiment, fresh feces were collected without contamination from each of 5 pigs in each group for 3 d, 3 collecting times daily (35% of the feces were collected each time). The feces samples of each pig from 3 d collections were dried, ground and mixed to determine the concentrations of nutrients and 4 N hydrochloric acid (HCl) insoluble ashes. CP, crude fat, Ca and P in diets and feces were determined with Kjeldahl, ether extract, potassium permanganate ($KMnO_4$) and ammonium molybdate ($(NH_4)_6Mo_7O_{24}$) protocols, respectively (Jurgens, 1997). The nutrient digestibilities were determined by using the endogenous indicator (4 N hydrochloric acid (HCl) insoluble ashes) protocol (Jurgens, 1997). The calculation was made as follows: Nutrient apparent digestibility = 100-(100×indicator content in feed/indicator content in feces×nutrient content in feces/nutrient content in feed). The pH values in feces were measured with pH meter, and ammonia was determined with the former protocol (Webb, 2001). The temperature in the shed was 19-28°C during feces collection.

Determination of the number of *E. coli* and lactic acid bacteria in pig feces

Five grams of fresh feces from each of five pigs were collected sterilely, diluted 10^5-10^9 folds with 0.9% physiological saline (NaCl) for *E. coli* and with anaerobic solution for lactic acid bacteria (Shapton and Board, 1972), and then vortexed completely. The mixtures (0.2-0.3 ml) were dispensed onto the plates with eosin methylene blue agar for determining *E. coli* or into anaerobic roll tubes with MRS agar for determining lactic acid bacteria. The bacteria were incubated for 2 d at 37°C, and then the colonies were counted.

Determination of phytase activity

Five grams of feces were mixed with 45 ml 0.9% physiological saline (NaCl) in a 250 ml conical flask, shaken at 250×g for 30 min, and then filtrated with four-fold gauze. The filtrate was centrifuged at 12,000×g for 15 min. Phytase in the supernatant was determined with the former protocol (Yin et al., 2007). One phytase unit was defined as the activity that released 1 μmol of inorganic phosphorous from sodium phytate per minute.

Determination of serum biochemical indexes

Ten ml samples were withdrawn from the chest veins of 20 pigs in group 1, 2, 3 and 4, five pigs for each group. After the blood was put at room temperature for 30 min, the serum was taken out by transferpettor, and then centrifuged at 13,000×g for 10 min. The biochemical indexes were determined with 7600-020 Automatic Analyzer HITACHI in Biochemical Laboratory of Zhengzhou University, Zhengzhou, China. Titration of IgA levels in pig sera was measured by radioimmunoassay (RIA, Wira et al., 1990). The test kits were purchased from Hua-ying Biotechnical Institute of Beijing, China. Data were obtained through readings on the gamma-discriminating counter, and results were reported as micrograms of antibody protein per liter of serum.

Statistical analysis

Experimental data were expressed as the means and standard errors. The data were analyzed using the ANOVA procedures of Statistical Analysis Systems Institute (SAS 6.0). Duncan's multiple range test was used to compare treatment means. Differences were considered statistically significant at $p<0.05$.

RESULTS

Effect of the transformed *Lactobacillus* on production performance and diarrhea rates of pigs

Table 2 indicated that ADG was increased by adding antibiotics, natural *Lactobacillus* and the transformed *Lactobacillus* with 20% CMP removed from the basal diet, compared with the other groups ($p<0.05$). ADG in the diet with 40% CMP removed was lower than that with 20% CMP removed from the basal diet ($p<0.05$). The diarrhea rates in groups with *Lactobacillus* were lower than that in the other groups ($p<0.05$), indicating that the *Lactobacillus*

Table 2. Production performance in 6 groups

Groups	ADG (g)	ADFI (g)	FC	Diarrhea rate (%)
1	446.40±47.73A	1,183.34	0.38	6.35±0.53A
2	503.60±55.70B	1,212.86	0.42	4.83±0.47B
3	493.60±56.64B	1,195.36	0.41	3.05±0.31C
4	507.10±56.17B	1,212.21	0.42	3.73±0.35C
5	416.80±47.24A	1,175.18	0.35	3.34±0.41C
6	450.00±42.03A	1,197.32	0.38	6.20±0.72A

Each value represents mean±SE of 8 replicates per treatment. In the same column, significant differences at p≤0.05 levels are indicated by the different letters (A, B, C). Data followed by the same letter in the same column are not significantly different from each other (p>0.05).

could replace antibiotics to prevent digestive disease.

Effect of the transformed *Lactobacillus* on nutrient digestibility

Table 3 showed that the digestibilities of protein, Ca and P in the group added with transformed *Lactobacillus* with phytase gene were higher than that in the other groups (p<0.05). It could be concluded that the nutrient digestibilities could be enhanced by the transformed *Lactobacillus* significantly, except for fat digestibility.

The changes of phytase activity, microbes, pH and ammonia content in feces affected by the transformed *Lactobacillus*

Table 4 indicated that the transformed *Lactobacillus* with phytase gene could make the phytase activity get to 133.32 U/g in feces, which was higher than that in group 4 (9.58 U/g, p<0.05), and was almost the same as that in group 6 (135.94 U/g). The natural or transformed *Lactobacillus* in group 3 and 4 had the ability to decrease *E. coli* and increase lactic acid bacterium number in feces better than the antibiotic and control groups (p<0.05). In addition, *Lactobacillus* could decrease pH values and increase ammonia concentrations in pig feces (p<0.05).

Effect of the transformed *Lactobacillus* on serum biochemical indexes

Table 5 showed that the transformed and natural *Lactobacillus* had the same ability as antibiotic to increase serum IgA level, compared with the control (p<0.05). Table 5 also showed that the transformed *Lactobacillus* could increase serum triglyceride contents, compared with the

Table 3. Crude protein, crude fat, Ca and P digestibilities in 6 groups (%)

Groups	Crude protein	Crude fat	Ca	P
1	83.55±5.26AB	93.35±5.20A	70.90±8.01A	68.75±8.77A
2	88.57±4.21A	91.59±3.82A	65.36±9.46A	75.68±9.43B
3	94.73±1.44C	94.34±1.76A	92.01±2.58B	89.64±2.58C
4	87.32±6.55A	82.56±8.97B	77.11±9.60C	74.36±6.53B
5	81.96±6.65AB	85.11±9.06B	62.40±9.59A	66.95±9.16A
6	79.88±3.88B	76.64±4.54C	74.61±5.20C	73.33±5.36B

Each value represents mean±SE of 5 replicates per treatment. In the same column, significant differences at p≤0.05 levels are indicated by the different letters (A, B, C). Data followed by the same letter in the same column are not significantly different from each other (p>0.05).

Table 4. Phytase activity, microbes, pH and ammonia content in feces of 6 groups

Groups	Phytase activity (U/g)	*E. coli* ($\times10^6$ cfu/g)	Lactic acid bacteria ($\times10^9$ cfu/g)	pH values	Ammonia content (μg/g)
1	12.20±2.27A	46.00±3.25A	8.32±0.85A	7.18±0.31A	89.66±1.41A
2	94.98±9.42B	4.11±0.45B	6.30±0.76A	7.32±0.08A	92.13±0.67A
3	133.32±14.69C	1.68±0.20B	63.40±6.29B	6.92±0.13B	95.17±3.12B
4	9.58±1.18A	1.46±0.13B	93.10±8.00B	6.91±0.13B	94.56±0.52B
5	25.27±2.09A	1.52±0.19B	7.49±0.84A	6.52±0.22C	95.60±2.16B
6	135.94±13.23C	0.84±0.15B	32.20±3.18C	7.10±0.16A	98.34±0.89B

Each value represents mean±SE of 5 replicates per treatment. In the same column, significant differences at p≤0.05 levels are indicated by the different letters (A, B, C). Data followed by the same letter in the same column are not significantly different from each other (p>0.05).

Table 5. The changes of serum biochemical indexes in 4 groups

Groups	1	2	3	4
Glutamate-pyruvate transaminase (U/L)	46.75±6.67	43.50±4.85	61.40±7.71	54.00±5.29
Glutamic oxaloacetic transaminase (U/L)	78.00±8.24AC	100.00±10.14A	204.60±27.82B	42.67±12.57C
Glutamyltranspeptidase (U/L)	31.00±3.25	48.50±6.80	39.40±4.29	37.00±4.12
Alkaline phosphatase (U/L)	113.75±15.17	85.75±12.86	115.40±13.00	117.33±11.76
Direct bilirubin (μmol/L)	3.50±0.42	4.25±0.77	4.20±0.47	4.33±0.62
Urea (mmol/L)	5.63±1.32	5.88±0.84	6.12±1.05	4.93±0.76
Creatinine (μmol/L)	100.00±12.41	72.00±7.91	67.00±9.82	103.00±10.61
Uric acid (μmol/L)	10.75±1.42	13.25±2.17	5.20±0.48	4.00± 0.43
Triglyceride (mmol/L)	0.55±0.16AB	0.35±0.20B	0.65±0.07A	0.54±0.10AB
High density lipoprotein (mmol/L)	1.19±0.10	1.06±0.04	1.07±0.07	1.04±0.07
Low density lipoprotein (mmol/L)	1.21±0.14	1.14±0.11	1.25±0.25	1.32±0.09
Lactate dehydrogenase (U/ml)	0.59±0.24	0.82±0.26	1.58±0.95	1.13±0.54
P (mol/L)	4.40±0.83	5.83±1.56	4.86±0.54	4.53±0.73
IgA (g/L)	0.51±0.10 A	0.95±0.14B	1.12±0.14 B	1.11±0.17 B

Each value represents mean±SE of 4 replicates per treatment. In the same row, significant differences at p≤0.05 levels are indicated by the different letters (A, B, C). Data followed by the same letter in the same row are not significantly different from each other (p>0.05).

antibiotic group; and serum glutamic oxaloacetic transaminase activity, compared with the other groups (p<0.05). The most important point was that the transformed *Lactobacillus* could keep almost the same serum P content (4.40 vs. 4.86 mol/L, p>0.05) under the condition of saving 20% (w/w) CMP in pig diet. In addition, serum lactate dehydrogenase and glutamate-pyruvate transaminase were also increased, while serum uric acid content was decreased by the transformed and natural *Lactobacillus*, compared with the control and antibiotic groups (p>0.05).

DISCUSSION

Effect of the transformed *Lactobacillus* on production performance of pigs

This research showed that the natural and transformed *Lactobacillus* had the same effect as antibiotics to improve pig production. Many researches have shown that *Lactobacillus* and phytase are able to improve pig production and feed conversion, respectively (Pollman, 1986; Jendza et al., 2005; Veum and Ellersieck, 2008). The reasons are that *Lactobacillus* or phytase can improve nutrient availability, maintain gut microbial balance and prevent digestive disease, so that pig production will be improved. It is the first report to show that the transformed *Lactobacillus* with phytase gene also has the ability to improve pig production and feed conversion even under the condition of 20% (w/w) CMP removed. When 20% (w/w) CMP was removed from the diet with *Lactobacillus* addition, the production performance of pigs was better than that in the group 1, 5 and 6; but when 40% (w/w) CMP was removed from the diet, it became worse (p<0.05). The above information indicated that the transformed *Lactobacillus* could not keep the common production when P is very deficient. It is very useful to save CMP resources and reduce P pollution to some extents by adding the transformed *Lactobacillus* in pig diets.

The diarrhea rate and microbes in feces

The diarrhea rate in group 3 was the lowest in this study, indicating that the transformed *Lactobacillus* could replace antibiotics to prevent digestive disease, maybe due to the combined functions of probiotic and phytase. Microorganisms in the digestive system of the pig play important roles in nutrient metabolism, restriction of pathogenic microorganisms, maintaining animal health and improving production. In healthy animals, the compositions of the gut microflora remain in a relatively steady state. If the composition of microflora is out of balance, induced by some factors such as food, environment, stress, antibiotic administration, the pathogenic microorganisms may colonize the gut and lead to diarrhea, digestive disorders, poor production and death. The function of *Lactobacillus* is to keep the gut micropopulation in a balanced state and prevent the proliferation of pathogenic microorganisms (Shahani et al., 1977). This is why the pigs in three groups containing *Lactobacillus* have lower diarrhea rate, even lower than the antibiotic group. This result also indicated that *Lactobacillus* could benefit the gastrointestinal tract and animal health by increasing *Lactobacillus* growth and inhibiting *E. coli* proliferation in pig gut, which was corresponding with low diarrhea rate.

Effect of the transformed *Lactobacillus* on nutrient

digestibility of pigs

A large number of experiments showed that *Lactobacillus* can improve nutrient digestibility, so does phytase (Maxwell et al., 1983; Pollman, 1986; Veum and Ellersieck, 2008). This result showed that the transformed *Lactobacillus* possessed the better ability than the natural *Lactobacillus*, phytase or antibiotics to increase most of nutrient digestibilities except for fat digestibility. The reason may be the combined accumulating functions of phytase and probiotic from the transformed *Lactobacillus*. The microbial phytase secreted by the transformed *Lactobacillus* can hydrolyze phytate complexes, reduce the anti-nutrition of phytate and release nutrients; and the lactic acid bacteria can improve nutrient digestion and absorption, too.

Effect of the transformed *Lactobacillus* on phytase activity and chemical indexes in pig feces

This result showed that the transformed *Lactobacillus* with phytase gene could make the phytase activity in feces (133.32 U/g) be the same as group 6 (135.94 U/g) added with phytase in diet, indicating that the transformed *Lactobacillus* had the ability to secrete phytase to make the phytase activity in gut get to the same level as 250 U/kg phytase in diet. From Table 4, it indicated that the transformed *Lactobacillus* secreted less phytase in the diet with 40% (w/w) CMP removed (in group 5) than that with 20% (w/w) CMP removed (in group 3). It may be due to the effect of low P concentration in diet, which will need further study. In addition, *Lactobacillus* could decrease pH values and increase ammonia concentrations in pig feces, due to a large number of lactobacilli surviving in gut to secrete a lot of lactic acid to make gut pH lower. Under the acidic condition of gut, ammonia will become ammonium salt to reduce ammonia discharged to the environment, so the air quality in the shed will increase.

Effect of the transformed *Lactobacillus* on serum biochemical indexes

The result showed that the transformed and natural *Lactobacillus* could increase serum IgA level, which was correspond with the former reports (Marteau et al., 1983; Ya et al., 2008). It was reported that *Lactobacillus* was capable of inducing gut mucosal responses by enhancing the production of secreting IgA as well influencing the systemic immunity via the cytokines released to the circulating blood (Chen et al., 2005). The high level of IgA can increase pig immunity and health to reduce disease and mortality. The transformed *Lactobacillus* could keep serum P concentration almost the same as the control under the condition of saving 20% (w/w) CMP in pig diet because phytase secreted by the transformed *Lactobacillus* increase phytate-P availability. This finding is very useful in saving P resources and reducing P pollution. In addition, the higher serum concentrations of lactate dehydrogenase, glutamate-pyruvate transaminase and glutamic oxaloacetic transaminase induced by the transformed or natural *Lactobacillus* indicated that the metabolisms of lactic acid and amino acid, and glyconeogenesis reaction were increased, resulting in high concentration of triglyceride and low concentrations of uric acid in serum.

It can be concluded that the transformed *Lactobacillus* with phytase gene has phytase function to replace commercial phytase addition as well as probiotic function to regulate gut microbes, reduce diarrhea and replace antibiotics. As a result, nutrient digestibility, immunity, and animal production are increased. The transformed *Lactobacillus* can keep serum P concentration as same as the control under the condition of saving 20% (w/w) CMP in pig diet. It will be a new ideal feed additive for animal production in the future.

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