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The Probiotic and Adherence Properties of *Lactobacillus reuteri* Pg4 Expressing the Rumen Microbial β-Glucanase

Yu, B.[a], J. R. Liu[1, a], F. S. Hsiao, T. T. Lee[2] and P. W. S. Chiou[3, *]

Department of Animal Science, National Chung-Hsing University, 250 Kuo-Kuang Rd, Taichung 402, Taiwan

ABSTRACT : This study was conducted to evaluate the potential of the transformed *Lactobacillus reuteri* Pg4 (T-Pg4) harboring the β-glucanase gene as a poultry probiotic. The probiotic properties of the T-Pg4 strain were evaluated *in vitro* by their adherence capability and acid and bile salt tolerance, and were evaluated *in vivo* by their survival and adhesion in the gastrointestinal tract (GIT) of specific-pathogen-free (SPF) chickens. The results showed that the T-Pg4 strain exhibited resistance to acidic conditions and contact with bile salt, and adhered efficiently to the crop and intestinal epithelial cells of chickens *in vitro*. The T-Pg4 strain also could survive and colonize the gastrointestinal epithelium of the experimental SPF chickens *in vivo*. In addition, radial enzyme diffusion was used to demonstrate that the *Lactobacillus* spp. randomly isolated from the GIT of the SPF chickens fed T-Pg4 possessed β-glucanase secretion capability. These findings have demonstrated that the transformed *L. reuteri* Pg4 survives transit through the stomach and intestine, and may secrete β-glucanase in the chicken GIT. Therefore, it is suggested that this organism could be used as a multifunctional poultry probiotic. (**Key Words :** β-Glucanase, *Lactobacillus reuteri* Pg4, Probiotic, *In vitro* Evaluation)

INTRODUCTION

For years, bactericidal additives, including some antibiotics, have been used for treatment of diseases and for growth promotion. However, with mounting public concerns associated with antibiotic residues and increasing rates of antibiotic resistance, antibiotics have been strictly banned in some areas of the world. Addition of probiotics to feed is one of the alternatives to be used as a replacement for antibiotics. Probiotics, which contain viable organisms and exert a beneficial effect on animal performance through modification of gastrointestinal microflora, offer great potential both as feed additives and as a replacement for antibiotics (Reid and Friendship, 2002). There is sufficient evidence to show that probiotics are effective for enhancement of the immune system, increasing body weight gain, reducing diarrhea, and improving feed-conversion efficiency (Patterson and Burkholder, 2003). Currently used or researched probiotics are mostly selected from native gut microflora, with the selection of optimal strains often largely empirical. It has been speculated that genetic modification can be used to develop more efficacious probiotics. Examples of such genetically modified probiotics include strains that produce antibodies, detoxification enzymes and cytokines for immune intervention (Steidler, 2003).

Cereals are a major component of diets fed to mono-gastric animals. In European countries, barley is one of the major feed grains. The endosperm cell wall of barley contains a high proportion of indigestible fiber known as non-starch polysaccharide (NSP). The NSP in barley are mainly β-glucan, which has large portions of β-1,4 or β-1,3 glucosidic linkage. The animals do not synthesize the enzyme β-glucanase, which is capable of degrading this structural polysaccharide, and as a result, the undigested β-glucan can often be problematic for mono-gastric animals being fed such a barley-based diet, causing intestinal disturbances, typified by sticky droppings and poor growth in young animals (Almirall et al., 1995). In addition, the sticky droppings also reportedly create management problems in poultry farming. Therefore, it is not surprising

* Corresponding Author: P. W. S. Chiou. Tel: +886-4-22860799, Fax:+886-4-22860265, E-mail: wschiou@dragon.nchu.edu.tw
[1] Institute of Biotechnology and Department of Animal Science and Technology, National Taiwan University, Taipei 106, Taiwan.
[2] Department of Life Science, Mingdao University, 369 Wenhua Road, Peetow, Changhua 523, Taiwan.
[3] Chung-Jen College of Nursing, Health Science and Management, 217 Hung-Mao-Pi, Chia-Yi, Taiwan.
[a] These two authors contribute equally to this work.
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that addition of β-glucanase into barley-based diets for non-ruminant animals decreases viscosity and consequently reduces the anti-nutritional effect of β-glucan, leading to better production performance (Yu et al., 2002). However, enzyme supplementation is not only expensive but is typically only ever used as a short-term solution for enhancing the digestion of cereals. An alternative and less-expensive analogous strategy might be to develop probiotics with the capacity to digest plant cell wall structural carbohydrates by introduction of heterologous genes encoding polysaccharide-degrading enzymes. In a previous study, we isolated a strain of *Lactobacillus reuteri* Pg4 from the GIT of healthy broilers, and suggested that it had potential as an additive in animal feed (Yu et al., 2007). In another study, we cloned the *Fibrobacter succinogenes* β-glucanase gene into parent *L. reuteri* Pg4, and demonstrated that the transformed *L. reuteri* Pg4 (T-Pg4) acquired the capacity to break down β-glucan (Liu et al., 2005).

In the present investigation, we have investigated the probiotic characteristics such as adherence capability, acid tolerance, and bile salt tolerance of the T-Pg4 strain *in vitro*. We also examined β-glucanase secretion capability and the survival of the transformed lactobacilli in the GIT of specific pathogen-free chickens fed with these *L. reuteri* Pg4 transformants.

MATERIALS AND METHODS

Experimental bacteria strain

The *Lactobacillus reuteri* Pg4 strain used in this study was isolated from the gastrointestinal tract of healthy broilers, and it has been successfully transformed with the *Fibrobacter succinogenes* β-glucanase gene (Liu et al., 2005; Yu et al., 2007). *Lactobacillus reuteri* Pg4 and its transformant (T-Pg4) were kept at -80°C as a stock culture. After two successive transfer and grow at 37°C in MRS (De Man, Rogosa, Sharpe) broth (Difco Laboratories, Detroit, Michigan, USA.) and MRS broth containing chloramphenicol (10 μg/ml), respectively.

Evaluation of the probiotic properties of the transformed *L. reuteri* Pg4 *in vitro*

Acid and bile salt tolerance of the transformed Lactobacillus reuteri Pg4 : One ml aliquot of the overnight cultures of the parental *L. reuteri* Pg4 or the T-Pg4 strains were centrifuged (7,000 rpm, 5 min.), the pellets were re-suspended in adjusted pH 2.0 sterile phosphate buffered saline (PBS) and incubated at 37°C. After 0, 1, and 3 h, aliquots of 1 ml of the suspension were removed, serially diluted with PBS (pH 7.4), and portions were spread onto MRS agar plates for the determination of the presence of colony forming units. In order to test the *Lactobacillus* resistance to bile salts, after being treated with acid the surviving cells were collected by centrifugation and washed once with PBS. They were resuspended (1%) into MRS broth, with and without 0.3% oxgall (Bile, Sigma B-3883) and incubated at 37°C. Aliquots of 1 ml were removed from the cultures after 0, 2, 4, 6, 8, 10, 12, and 24 h for determining the absorbance at a wavelength of 570 nm. The tolerance to bile salts was calculated as (%) = (the slope of the absorbance curve in MRS broth with 0.3% oxgall)/(the slope of the absorbance curve in MRS broth without 0.3% oxgall)×100 (Gilliland and Walker, 1990; Toit et al., 1998).

Adherence capability of the transformed Lactobacillus reuteri Pg4 : The epithelial cells were isolated from the crop and small intestinal mucosa of chickens according to the method reported by Annika et al. (1983), and suspended in PBS buffer to a cell concentration of 10^4-10^5 cells/ml. Cells from overnight cultures of the parental *L. reuteri* Pg4 or the transformed *L. reuteri* Pg4 cells were harvested by centrifugation and resuspended in PBS buffer to a cell concentration of 1×10^8 CFU/ml. The epithelial cell suspension was mixed with the same volume of bacterial suspension and incubated at 37°C, 80 rpm rotation, for 30 min. Finally, the adhesion of the lactobacilli to the epithelial cells was observed by microscopy. The strain in which more than 15 bacterial cells adhered per epithelial cell was considered possessed the adherent ability (Pedersen and Tannock, 1989).

Evaluation of the survivability of the transformed *Lactobacillus reuteri* Pg4 in SPF chicken

Experimental design : A total of 20 newly hatched SPF chicken were given from Animal Drugs Inspection Branch, Animal Health Research Institute, Council of Agriculture, Taiwan. There were negative for the antibody detection to bursal disease (IBD), infectious bronchitis (IB), Mycoplasma gallinarum (MG) and Mycoplasma synovitis (MS). The chickens were assigned at random to probiotic and control groups. Chicks of the probiotic group (n = 10) were orally administered with 1 ml (1×10^8 CFU/ml) of T-Pg4 broth on the 1, 2 and 3 d of age, while those of control group (n = 10) were orally administered with 1 ml of sterile water on these days. All birds had access to sterile feed and water *ad libitum* and were housed separately in two laminar flow cabinets under pathogen-free conditions. On days 1, 3, 5, and 7, fecal samples of each group were collected for measurement of the *Lactobacillus spp.* counts. On days 3 and 7, five chickens from each group were sacrificed for measurement of the *Lactobacillus spp.* population in the digesta and tissue of jejunum and cecum, for detection of β-glucanase activities of the *Lactobacillus* spp. in the GIT, and for observation of the microbial adhesion on the crop and cecal mucosa by scanning electronic microscopy.

Viable counts of Lactobacillus spp. in the feces and in

Table 1. Survival count of *L. reutri* Pg4 and transformed *L. reutri* Pg4 (T-Pg4) strains after incubation in pH 2.0 PBS and the activity of bile tolerance

Strains	Viable counts after incubation time			Bile tolerance[1]
	0 h	1 h	3 h	
	log cfu/ml			(%)
L. reutri Pg4	8.76[2]	8.75	8.49	89.8
T-Pg4	9.53	9.37	8.6	87.7

[1] The comparing slope of increase of OD in MRS broth with and without 3% oxgall.
[2] Mean of three duplicate.

the gastrointestinal tract : The feces, the digesta and tissue of crop, jejunum, ileum and cecum of the chicks were serially diluted with PBS, and portions were spread onto MRS agar containing chloramphenicol (10 μg/ml). The agar plates were incubated under anaerobic conditions (95% CO_2 and 5% O_2) at 37°C until colonies appeared (usually 48 h). The results are expressed as logarithmic colony forming units (log CFU) per gram of wet weight of the digesta, tissues and feces.

Detection of β-glucanase activity of Lactobacillus isolated from the feces and gastrointestinal contents by radial enzyme diffusion : The culture colony randomly selected from the chloramphenicol including MRS medium which inoculated the feces and the digesta from supplemented chicks, and detected the β-glucanase activity by radial enzyme diffusion. Lichenan (1 g/L) was dissolved in 100 mM sodium acetate buffer (pH 5.0) with heat, and then mixed with sterilized melted MRS agar and poured into petri dishes to a depth of 4 mm and allowed to solidify. The culture colony and parental *L. reuteri* Pg4 cell as a control were then inoculated onto the plates and incubated at 37°C for 24 h, and stained with 3 g/L of congo red for 20 min at 25°C. After removal of the residual dye by rinsing the agar surface thoroughly with water, the stain was fixed by flooding the plate with dilute acetic acid (1:9 vol/vol with water) for 15 min, and the zones of substrate hydrolysis were observed (Wood, 1981).

Observation of microbial adhesion in crop and cecum using scanning electronic microscopy : At 7 days of age, crop and cecum were taken from each group chick and ligated. Then, a fixative solution containing 30 g/kg glutaraldehyde, 50 mM phosphate buffer (pH 7.4), 50 mM sucrose and 120 g/kg picric acid was injected into the lumen of crop and cecum (Droleskey et al., 1995). Once pressurized with fixative, two pieces of 1 cm^2 sample from both crop and cecum were immersed in an additional fixative solution for 60 min at room temperature. After post-fixation with 1% osmium tetroxide, the samples were dehydrated, critical-point dried, mounted on aluminum stubs, coated with gold, and placed in the scanning electronic microscope (Nanolab 2100; Bausch & Lomb Inc., Rochester, NY, USA) for evaluation.

Statistical analysis

All results were analyzed using the Statistical Analysis System software package (version 6.1; SAS, 1999). Statistical analysis of bacteria count was performed after logarithmic conversion of the data. Results are given as means±SD. The t-test was used to detect difference between treatment means.

RESULTS AND DISCUSION

Acid and bile salt tolerance of the transformed *Lactobacillus reuteri* Pg4 strains

Both the *L. reuteri* Pg4 strains tested herein survived after 3 h incubation at pH 2.0 (Table 1). The parental *L. reuteri* Pg4 strain almost survived after 3 h incubation at pH 2.0, while T-Pg4 strain showed 0.90 to 1.00-log reduction in counts after the same treatment. Therefore, the acid tolerance of the T-Pg4 strain was lesser than that of the parental strain. In addition, we also noted that both of the parental and transformed *L. reuteri* Pg4 strains survived at an incubation period of 24 h in MRS broth containing 0.3% oxgall (Table 1). The bile salt tolerance of the parental *L. reuteri* Pg4 strain and T-Pg4 strain were 89.8% and 87.7%, respectively.

Lactobacillus used as probiotic adjuncts are commonly delivered in a food and/or feed system and, thus, begin their journey to the lower intestinal tract via the mouth. Therefore, probiotic bacteria should be resistant to the digestion process in the stomach and intestinal tract (Kim et al., 2006). In comparison to humans and domestic animals, the alimentary tract of chicken is shorter. The time required for feed to pass through the upper alimentary canal is as short as 1-2 h (Chou and Weimer, 1999). Therefore, acid tolerance for bacterial strains in chickens is not as crucial as for those in other animals where the feed passage rate is much longer. Both of the *L. reuteri* Pg4 strains tested in this study survived after an incubation period of 3 h at pH 2.0. This indicated that transformed *L. reuteri* Pg4 strain would have the potential to survive transit through the stomach and might possess the ability to reach the intestinal environment in which they may effectively work.

Once the bacteria reach the intestinal tract, their ability to survive depends on their resistance to bile salt (Gilliland and Walker, 1990). Bile salt entering the duodenal section of the small intestine has been found to reduce survival of bacteria. This is probably due to the fact that all bacteria have cell membranes consisting of lipids and fatty acids that are very susceptible to destruction by bile salt (Jin et al., 1998). Hence, tolerance to bile salt is an important screening criterion for probiotics. Both of the *L. reuteri* Pg4 strains survived at an incubation period of 24 h in MRS broth containing 0.3% oxgall. Therefore, it is likely that T-Pg4 strain is able to survive in the intestine and is thus can

Table 2. Effect of oral T-Pg4 strain supplement on *Lactobacillus spp.* cells in different segments of GIT of the Specific Pathogenic Free chicken (log cfu/g)

Segment	*Lactobacillus* cells			
	3 day		7 day	
	Control	T-Pg4	Control	T-Pg4
Digesta				
Crop*	0	8.01±0.02	0	8.12±0.03
Jejunum*	0	7.08±0.04	0	7.84±0.01
Ileum*	0	7.44±0.01	0	8.42±0.01
Cecum*	0	9.72±0.10	0	10.14±0.02
Intestinal tissue				
Jejunum*	0	6.89±0.11	0	6.59±0.02
Cecum*	0	8.66±0.12	0	8.58±0.04

* Means are significantly different in the same age ($p<0.05$).

potentially be important to the microbial ecology of the intestinal environment.

Adherence capability of the T-Pg4 strain

Adhesiveness is another important screening criterion for probiotics. Ability of probiotic bacteria to adhere to the intestinal mucus is considered important for transient colonization, antagonism against pathogens, modulation of the immune system and enhanced healing of damaged gastric mucosa (Jin et al., 1997; Strompfova et al., 2004). The difficulties in assessing adherence of probiotic strains *in vivo* have led to the development of *in vitro* adherence assays. Various cultured cells, such as Int 407, Caco-2, and HT-29, and isolated epithelial cells from crop and intestinal tract, have been proposed to test the adhesion of probiotics (Bouzaine et al., 2005). In this study, we used the chicken crop and intestinal epithelial cells to evaluate the adherence ability of the *L. reuteri* Pg4 strains. Both of the parental *L. reuteri* Pg4 strain and T-Pg4 adhered efficiently to both crop and intestinal epithelial cells *in vitro*, and there appeared to be no difference between the adherence ability of the transformed *L. reuteri* Pg4 strain and that of the parental *L. reuteri* Pg4 strain.

Lactobacillus reuteri frequently occurs in the intestinal microflora of various animals and has been widely regarded as potential probiotic. When present in sufficient numbers within gastrointestinal tract, *L. reuteri* is believed to be able to create a healthy equilibrium between beneficial and potentially harmful microflora in the gut. Havenaar et al. (1992) indicated that the adherent *Lactobacillus* strains only colonize the intestine temporarily and might disappear within days or weeks. Therefore, the probiotic strains must proliferate rapidly in order to prolong adhesion and colonization on the intestinal mucosal surfaces. In the present study, both of the *L. reuteri* Pg4 strains reached the stationary growth phase at 6 h of incubation in MRS broth at 37°C, with a concentration of 10^9 CFU/ml (data not shown). Savage (1983) reported that the capacity of

Table 3. Effect of oral T-Pg4 strain supplement on *Lactobacillus* cell of excreta in SPF chicken at different age (log cfu/g)

Age (day)	Control group	T-Pg4 group
0	0	0
1*	0	6.48±0.02
3*	0	6.51±0.03
5*	0	6.23±0.01
7*	0	6.11±0.05

* Means are significantly different ($p<0.05$).

Lactobacillus spp. using enzymes to digest mucinous glycoproteins and utilize the degradation products as carbon, energy and nitrogen sources to grow may be important for them to colonize on the gastric or epithelial surfaces. Factors including fast prolification, short generation time, producing antimicrobial substances, adaptation to new substract, syntrophic and cross feeding in addition to adhension may also affect the *in vivo* colonization. Ouwehand et al. (1999) suggested that the normal microflora may not greatly affect the adhesion of the probiotic bacteria.

Based on the results above, the transformed *L. reuteri* Pg4 strain as similar parental strain have the prerequisites to resist to acidic pH and bile salts, to adhere to the gastrointestinal epithelial cells, to proliferate rapidly and therefore, represent potential candidates for probiotic strain.

Distribution of *Lactobacillus* spp. in the gastrointestinal tract and the feces of experimental SPF chicks

Table 2 and 3 show the effect of the supplementation with or without the T-Pg4 in terms of the *Lactobacillus spp.* population in different segments of GIT and the feces of the experimental SPF chicks, respectively. The counts of lactobacilli were determined on MRS agar containing chloramphenicol (10 μg/ml). Chloramphenicol is a selective antibiotic marker for the transformed lactobacilli in this study (Liu et al., 2005). At the ages of 3 and 7 d, no bacteria were found in the intestinal contents or on the intestinal tissue of the chickens in the control group (Table 2). On the other hand, the supplementation of the T-Pg4 had resulted in significant increases in the populations of *Lactobacillus* spp. in the contents of crop, jejunum, ileum and cecum, and on the tissue of jejunum and cecum, compared to the control chickens ($p<0.05$). The similar result of *Lactobacillus spp.* population in the feces was also found. The T-Pg4 supplemented group had significant higher populations of *Lactobacillus spp.* in the feces (Table 3). These indicate that the T-Pg4 strain can survive transit through the GIT and possesses the ability to survive in the GIT of chicks.

Identification of β-glucanase activity of the *Lactobacillus spp.* from the intestine by radial enzyme diffusion

The β-glucanase activity of the parental and

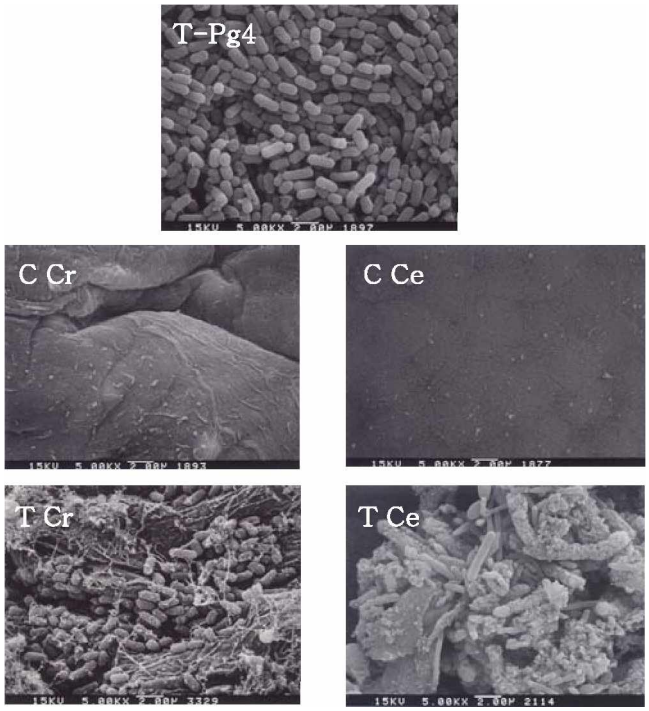

Figure 1. Scanning electronic micrographs of microflora in the crop (Cr) and cecal (Ce) mucosa of control (C) and T-Pg4 strain treatmented (T) SPF chickens at 7 days old (5,000×). T-Pg4: transformed *L. reutri* Pg4.

transformed variants of *L. reuteri* Pg4, and randomly selected cultural colonies isolated from the crop, jejunum, ileum and cecum digesta of the chickens in probiotic group as determined using enzyme radial diffusion. The interaction of the direct dye Congo red with intact lichenan, which contains contiguous β-(1→4)- and β-(1→3)-linked D-glucopyranosyl units, provides the basis for a rapid and sensitive assay system for bacterial strains possessing β-glucanase activities (Wood, 1981). The parental *L. reuteri* Pg4 strain did not show the transparent radials, indicating that it did not possess β-glucanase secretion capability. Conversely, the T-Pg4 harboring β-glucanase gene acquired the capacity to break down soluble lichenan and produce prominent transparent radials. In addition, the *Lactobacillus spp.* isolated from the crop, jejunum, ileum or cecum in the probiotic group also produced prominent transparent radials, indicating that they also possessed β-glucanase secretion capability. This indicates that the *Lactobacillus* isolated from the gastrointestine of broilers should be the transformed *L. reuteri* Pg4 strain.

Microbial adhesion in the crop and cecum

The scanning electronic micrographs of microflora in the crop and cecal mucosa shows more rod type microbes colonizing the mucosa of the probiotic group chickens at the ages of 7 d. Conversely, it shows scarce microbes colonizing the crop and cecal mucosa of the control chickens (Figure 1). These results were consistent with the results shown in Table 2 and Table 3, i.e. no *Lactobacillus spp.* bacteria were found in the feces, intestinal contents or on the intestinal tissue of chickens of the control group.

In conclusion, it was demonstrated that the introduction of *Fibrobacter succinogenes* β-glucanase gene into *L. reuteri* Pg4 cells did not appear to affect probiotic properties of such bacterial cells. It was also demonstrated that the *Lactobacillus spp.* isolated from the digesta of the crop, ileum and cecum of the probiotic group possessed β-glucanase secretion capability, while the analogous control organisms did not. This proves that the transformed *L. reuteri* Pg4 survive and secrete β-glucanase into the gastrointestinal tract of the supplemental SPF chickens. This also proved that the probiotics could be used as a multifunctional probiotics and a useful carrier to transfer beneficial genes for poultry production. For practical application in broiler production, the effect of this transgenic *L. reuteri* Pg4 strain on broiler growth performance and GIT microflora in conventional feeding environment needs to be further evaluated in the future.

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