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Evaluation of *Lactobacillus reuteri* Pg4 strain expressing heterologous β-glucanase as a probiotic in poultry diets based on barley

B. Yu [a,*], J.R. Liu [b], F.S. Hsiao [a], P.W.S. Chiou [a]

[a] *Department of Animal Science, National Chung-Hsing University, Taichung, Taiwan*
[b] *Institute of Biotechnology & Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan*

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Abstract

The aim of this study was to evaluate the effectiveness of *Lactobacillus reuteri* Pg4 transformants (TLB) harbouring the heterologous β-glucanase gene as a probiotic supplement in a barley-based poultry diet. Four-hundred broiler chicks were allocated to two treatment groups with or without supplementation with 10^8 CFU/g TLB to investigate growth performance, digesta viscosity and intestinal microflora. Supplementation of the barley-based diet with TLB decreased digesta viscosity and improved body weight gain ($P<0.05$) of broilers from 0 to 37 days of age. Furthermore, a higher total anaerobic and *Lactobacillus* count was observed at 37 days of age in all segments of gastrointestinal tract of chicks fed TLB as compared to control chicks ($P<0.05$). Radial enzyme diffusion methodology was used to demonstrate that the *Lactobacillus* spp. randomly isolated from the GIT of broilers fed TLB possessed β-glucanase secretion capability. The results indicate that the transformed strain survives transit through the stomach and intestine. In conclusion, the transformed *Lactobacillus reuteri* Pg4 can survive and secrete β-glucanase in the broiler GIT, and decrease digesta viscosity and enhance weight gain in birds fed a barley-based diet. Therefore, the transformed *Lactobacillus reuteri* Pg4 harbouring heterologous β-glucanase gene has potential as a poultry probiotic.

Keywords: β-Glucanase; *Lactobacillus reuteri* Pg4; Probiotic; Broiler performance

* Corresponding author. Tel.: +886 4 22840366; fax: +886 4 22860265.
E-mail address: byu@dragon.nchu.edu.tw (B. Yu).

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1. Introduction

Bactericidal additives, including some antibiotics, have been used as growth promoters in broiler feeds for the last 40 years. In many areas of the world, however, antibiotics have been banned due to the risk of residues and the increasing rates of resistance in human population. Probiotics, which contain viable organisms might exert a beneficial effect on animal performance through modification of gastrointestinal tract (GIT) microflora, and might replace antibiotics in feeds (Reid and Friendship, 2002). The genera *Lactobacillus*, a lactic acid bacteria, is used in many probiotic preparations (Tannock, 1998). Currently, most probiotics used are selected from native gut microflora, following methodologies largely empirical. It has been speculated that genetic modification can be used to develop more efficacious probiotics (Sieo et al., 2005).

Barley contains a high proportion of water soluble β-glucan in the aleurone and in the endosperm layers and cell walls of the grain. These non-starch polysaccharides (NSP) have large portions of β-1,4 or β-1,3 glucosidic linkage, and are not hydrolyzed by digestive enzymes of chicks. It has been demonstrated that incorporation of specific enzymes, such as β-glucanase in barley-based diets, may enhance growth and nutrient digestion in broilers (Yu et al., 2002; Jozefiak et al., 2006). In addition, the use of probiotics with high activity of specific enzymes provides additional benefits in terms of reducing the cost of enzyme supplementation. In a previous study, a strain of parent *Lactobacillus reuteri* Pg4 (PLB) was isolated from the GIT of healthy broilers, and it was suggested that the variant had potential as an additive in animal feed (Yu et al., in press). In another study, the *Fibrobacter succinogenes* β-glucanase gene was cloned and expressed into PLB, and it was demonstrated that the transformed *Lactobacillus reuteri* Pg4 (TLB) acquired the capacity to break down β-glucans, without loosing the adhesion efficiency to mucin and mucus and the resistance to bile salts and acid of the parent strain (Liu et al., 2005).

The present study was conducted to evaluate the potential for the transformed *Lactobacillus reuteri* Pg4 as a multifunctional probiotic in a barley-based diet for broilers. The effect of this supplementation on broilers growth performance, digesta viscosity and GIT microflora was also evaluated.

2. Materials and methods

2.1. Experimental bacterial strain

The *Lactobacillus reuteri* Pg4 bacteria strain used in this trial was isolated from the GIT of healthy broilers, and was successfully transformed with the *Fibrobacter succinogenes* β-glucanase gene (Liu et al., 2005; Yu et al., in press). The TLB was kept at −80 °C as a stock culture. After two successive transfers in MRS (De Man, Rogosa, Sharpe) broth (Difco Laboratories, Detroit, Michigan, USA) containing chloramphenicol (10 μg mL^{-1}), the culture solution was inoculated into the broth at 1% (v/v) and incubated at 37 °C for 24 h. Then, skim-milk powder, which was used as a cryoprotector, was added to the incubated culture at a final concentration of 50% (w/v) and the lactobacillus culture preparation was

lyophilized and stored at 4 °C until required. The bacterial count of lyophilized powder was 10^8 CFU g^{-1}.

2.2. Experimental birds and growth trial

A total of 400 1-day-old Arbor Acres broilers was weighed individually and assigned at random to 16 pens of 25 chicks of same sex each. The experiment was randomized with respect to dietary treatment, and each treatment was assigned to four male and four female pens. The experimental diets were based on barley and were offered either non-supplemented or supplemented with 1 g TLB powder/kg.

The composition of the experimental diet is presented in Table 1. During the experimental period (0–37 days), meal feed and water were provided *ad libitum*. Broiler live weight and pen feed consumption were recorded at 21 and 37 days of age.

Table 1
Composition of the experimental diet (g/kg)

Ingredient	Diet	
	0–21 days	22–37 days
Dehulled barley	534.1	553.5
Soybean meal, (440 g crude protein/kg)	235.0	185.0
Full fat soybean meal, (370 g crude protein/kg)	100.0	120.0
Fish meal, (600 g crude protein/kg)	35.0	35.0
Salt	2.5	2.5
Limestone	16.5	14.5
Monocalcium phosphate	9.0	7.2
Tallow	63.2	78.3
DL-methionine	1.2	0.5
Mineral premix[a]	2.5	2.5
Vitamin premix[b]	0.5	0.5
Coccidiostat[c]	0.5	0.5
Calculated value (g/kg)		
ME (MJ/kg)	12.97	13.50
Crude protein	220	200
Lysine	12.4	11.0
Methionine + cystein	8.2	7.2
Calcium	10.0	9.0
Available phosphorus	4.7	4.0
Total phosphorus	7.1	6.5
Analysed value (g/kg)		
Crude protein	215	197
Ether extract	97.5	115.5
Ash	58.0	50.0

[a] Contained per kilogram of diet:Co ($CoCO_3$), 0.255 mg; Cu ($CuSO_4 \cdot 5H_2O$), 10.8 mg; Fe ($FeSO_4 \cdot H_2O$), 90 mg; Mn ($MnSO_4 \cdot H_2O$), 90 mg; Zn (ZnO), 68.4 mg; Se (Na_2SeO_3), 0.18 mg.

[b] Contained per kilogram of diet: Vit. A, 15,000 IU; Vit. D_3, 3,000 IU; Vit. E, 30 mg; Vit. K_3, 4 mg; thiamin, 3 mg; riboflavin, 8 mg; pyridoxine, 5 mg; Vit. B_{12}, 25 μg; Ca-pantothenate, 19 mg; niacin, 50 mg; folic acid, 1.5 mg; biotin, 60 μg.

[c] Contained 120 g/kg of salinomycin.

2.3. Intestinal sample and digesta collection

One chick per pen was sacrificed by immersion in carbon dioxide at 3, 7, 21 and 37 days for measuring microbial population in the digesta from the crop, ileum and caecum. The intestinal microflora colonization in the crop and caecum of birds at 3 and 7 days was also studied using a scanning electronic microscope.

2.4. Determination of viscosity in the duodenal digesta

Samples of the duodenal digesta were collected at 21 and 37 days of ages from the same birds used for viscosity measurement. Samples of duodenal content were taken from 15 cm post-gizzard and were centrifuged at 25 °C and 12,000 × *g* for 3 min. The viscosity of the supernatants was measured according to the method of Fengler et al. (1987) using a Brookfield digital viscometer (Model Lvidv-110P, Middleboro, MA, USA) at 25 °C.

2.5. Analysis of microbial distribution

For bacterial assays, 10-fold serial dilutions were made from 1 g aliquots of intestinal digesta using phosphate buffer saline (PBS) as a diluent. A 100-μL portion of each dilution was spread on the petri dishes containing different growth media. Plate count agar (Difco, Laboratories, Detroit, Michigan, USA), reinforced clostridial agar (Oxoid, Basingstoke, UK), MRS agar (Difco, Laboratories, Detroit, Michigan, USA), and Chromocult agar (Merck, Darmstadt, Germany) were used specific for counting aerobic bacteria, anaerobic bacteria, *Lactobacillus* and coliform bacteria, respectively. Reinforced clostridial and MRS agars were incubated anaerobically at 37 °C for 48 h in a GasPak system (BBL, Cockeysville, MD, USA). Plate count and Chromocult agars were incubated aerobically at 37 °C for 24 h (Jin et al., 1998). The results are expressed as logarithmic colony forming units (log CFU) per gram of wet weight of gastrointestinal digesta.

2.6. Detection of β-glucanase activity of bacterial cells

The β-glucanase activity of the PLB, TLB and randomly selected cultural colonies from the MRS medium which inoculated the digesta of crop, ileum and caecum from the control and supplemented chicks at 3, 7, 21 and 37 days, was determined using enzyme radial 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 *Lactobacillus* spp. cells were inoculated onto the Lichenan 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, v/v with water) for 15 min, and the zones of substrate hydrolysis were evaluated (Wood, 1981).

2.7. Microbial adhesion in crop and caecum

At 3 and 7 day of age, crop and caecum were taken from each 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 caecum (Droleskey et al., 1995). Once pressurized with fixative, two pieces of 1 cm^2 sample from both crop and caecum 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.

2.8. Statistical analysis

Performance data were statistically analyzed on two-way (diet, sex and interaction) and microflora counts were analyzed by three-way (diet, GIT segment, age and their interactions) arrangement using the general linear model procedure of SAS (1999). Statistical analysis of bacteria count was performed after logarithmic conversion of the data. The model is $Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\beta\gamma)_{jk} + (\alpha\gamma)_{ik} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkl.}$ Where Y is the observed response, μ is the overall mean, α_i is the effect of diet ($i = 1, 2$), β_j is the effect of segment ($j = 1, 2,3$), γ_k is the effect of age ($k = 1, 2, 3, 4$), $(\alpha\beta)_{ij}$, $(\beta\gamma)_{jk}$, $(\alpha\gamma)_{ik}$ and $(\alpha\beta\gamma)_{ijk}$ are interactions between the effects of two or three factors, and ε_{ijkl} is the error. The least square mean test was used to detect differences between treatments. The differences were considered to be significant at $P<0.05$.

3. Results

3.1. Growth performance and intestinal viscosity of broilers

Supplementation with TLB improved body weight gain (BWG) from 22 to 37 days of age (1,239 *versus* 1,261 g; $P<0.1$) and from 0 to 37 days of age (1,832 *versus* 1,857 g; $P<0.05$). Furthermore, TLB supplementation decreased intestinal viscosity (4.68 *versus* 3.21and 4.87 *versus* 3.44) at 21 days and 37 days of age, respectively ($P<0.01$) (Table 2).

3.2. Bacterial counts in the contents of different gastrointestinal segments

The composition of the microflora was affected by the dietary supplementation with TLB, the intestinal segment and age ($P<0.01$). Also numerous interactions (diet and segment, diet and age, segment and age) were detected although no interaction diet and segment of the GIT on anaerobic and coliform count was observed. Supplementation with the TLB increased ($P<0.01$) the total aerobic and anaerobic counts in all GIT segments, and *Lactobacillus* spp. count in the ileum and caecum compared to unsupplemented control (Table 3). The counts of the four tested groups of bacteria were higher in the caeca than in the other GIT segments. Coliform count increased ($P<0.01$) with age. The TLB supplementation significantly increased ($P<0.01$) total aerobic, anaerobic and *Lactobacillus* spp. counts at 37 days of age and decreased ($P<0.01$) the coliform count in the GIT, especially in the crop and ileum at 3, 7 and 21 days of age.

Table 2
Effect of transformed *Lactobacillus reuteri* Pg4 strain supplementation on growth performance and digesta viscosity of broilers

	Control diet		Supplemented diet[c]		S.E.M.	Significance[d]		
	Male	Female	Male	Female		1	2	3
0–21 days								
BWG[a], g/bird/day	28.8	27.7	28.9	27.9	0.40	NS	†	NS
Feed intake, g/bird/day	47.9	44.4	47.8	44.2	0.59	NS	**	NS
FCR[b]	1.67	1.61	1.65	1.59	0.042	NS	NS	NS
22–37 days								
BWG, g/bird/day	85.4	69.5	86.4	71.7	1.15	†	*	NS
Feed intake, g/bird/day	138.4	140.4	144.7	136.5	3.69	NS	NS	NS
FCR	1.62	2.02	1.68	1.90	0.127	NS	*	NS
0–37 days								
BWG, g/bird/day	53.3	45.7	53.8	46.8	0.61	*	**	NS
Feed intake, g/bird/day	87.0	85.9	89.7	84.2	1.72	NS	NS	NS
FCR	1.63	1.88	1.67	1.80	0.042	NS	*	NS
Viscosity, cps								
At 21 days	4.7	4.7	3.1	3.3	0.18	**	NS	NS
At 37 days	4.9	4.8	3.5	3.4	0.12	**	NS	NS

NS: non-significant.
† P<0.10.
* P<0.05.
** P<0.01.
[a] Body weight gain.
[b] Feed/gain.
[c] Supplemented with transformed *Lactobacillus reuteri* Pg4 strain.
[d] 1: diet effect; 2: sex effect; 3: interaction of diet and sex.

3.3. β-Glucanase activity of the bacteria isolated from digesta

The PLB strain and the colonies isolated from the control chicks did not show transparent radials, indicating no β-glucanase secretion capability. Conversely, the TLB harbouring heterologous β-glucanase gene acquired the capacity to break down soluble β-glucans and produce prominent transparent radials (data not shown). In addition, the *Lactobacillus* spp. colonies randomly isolated from the GIT of TLB supplemented birds also produced prominent transparent radials indicating that they also possessed β-glucanase secretion capability. However, no transparent radials were founded in the control birds.

3.4. Microbial adhesion in the crop and caecum

The scanning electron micrographs (Fig. 1) of microflora in the crop and caecal mucosa shows more rod type microbes colonizing the crop and caecal mucosa of the TLB supplemented chickens than that in the mucosa of controls at 7 days. However, no differences were found at 3 days.

Table 3
Effect of the supplementation of the transformed *Lactobacillus reuteri* Pg4 strain on the microflora population in different segment of gastrointestinal tract of broilers (log CFU g^{-1})

Days	Control diet				Supplemented diet				S.E.M.	Significant[a]					
	Cr[b]	Il.	Ce.	Ave.	Cr.	Il.	Ce.	Ave.		1	2	3	4	5	6
Total aerobic bacteria									0.153	***	***	***	*	***	***
3	8.70	9.03	11.73	9.82	9.67	9.20	11.89	10.25							
7	8.34	8.47	9.86	8.89	9.67	9.01	10.34	9.67							
21	9.59	9.37	10.36	9.77	9.53	9.34	10.62	9.83							
37	8.69	7.34	9.61	8.55	9.82	9.78	11.02	10.20							
Average	8.83	8.55	10.39	9.26	9.67	9.34	10.95	9.99							
Total anaerobic bacteria									0.246	***	***	***	NS	***	***
3	8.59	9.67	11.79	10.02	9.42	10.66	11.80	10.63							
7	8.07	7.99	9.53	8.53	9.30	8.38	10.40	9.36							
21	9.60	9.64	10.72	9.98	9.88	9.89	10.26	10.01							
37	9.14	9.65	9.97	9.59	9.69	10.19	11.81	10.56							
Average	8.85	9.23	10.50	9.53	9.57	9.77	11.07	10.14							
Lactobacillus spp.									0.330	***	***	NS	***	***	***
3	8.84	8.73	9.42	9.00	8.92	8.61	9.79	9.11							
7	8.34	8.52	9.03	8.63	8.88	9.24	9.34	9.15							
21	9.59	8.24	8.63	8.82	8.54	8.58	9.43	8.85							
37	8.98	7.09	9.16	8.41	9.36	9.15	10.46	9.66							
Average	8.94	8.15	9.06	8.72	8.93	8.90	9.76	9.20							
Total coliforms									0.320	***	***	***	NS	***	***
3	6.62	5.73	6.58	6.31	6.21	5.41	6.02	5.88							
7	5.92	6.94	7.17	6.68	5.44	5.13	6.34	5.64							
21	5.98	6.81	8.42	7.07	5.41	5.99	8.22	5.54							
37	6.66	6.71	7.96	7.11	6.23	6.66	8.32	7.07							
Average	6.26	6.55	7.53	6.78	5.82	5.80	7.23	6.28							

NS: non-significant.
* P<0.05.
*** P<0.001.
[a] 1: Diet effect, 2: segment effect, 3: Age effect, 4: interaction of diet and segment, 5: interaction of segment and age, 6: interaction of diet and age.
[b] Cr: crop: Il: ileum: Ce: caecum. Ave: average.

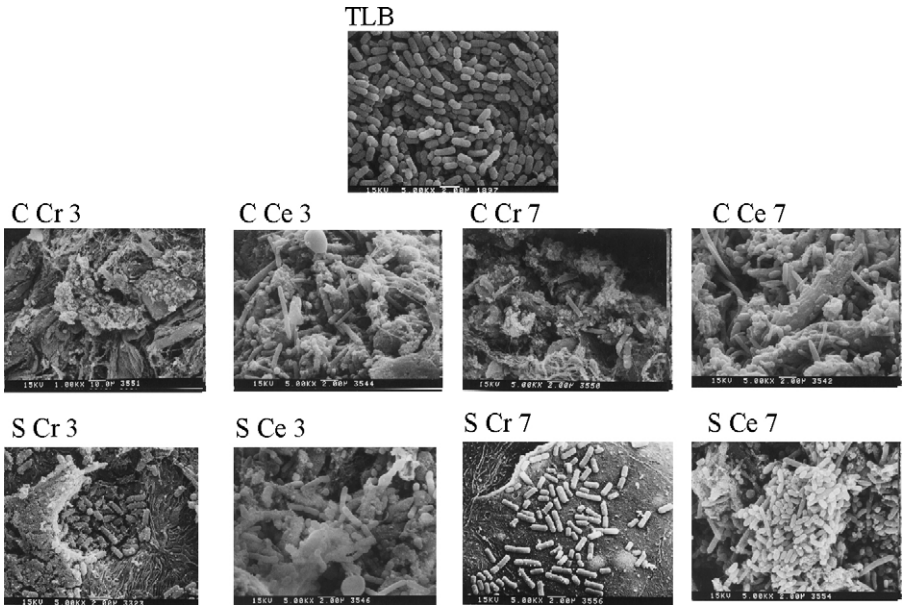

Fig. 1. Scanning electron micrographs of microflora in the crop (Cr) and caecal (Ce) mucosa of control birds (C) and transformed *Lactobacillus reuteri* Pg4 strain supplemented (S) birds at 3 or 7 days (5000×). TLB: transformed *Lactobacillus reuteri* Pg 4 strain.

4. Discussion

The ability of probiotic bacteria to adhere to the intestinal mucus is important for transient colonization, pathogenic antagonism, modulation of the immune system and healing of damaged gastric mucosa (Strompfova et al., 2004). In a previous study, we have demonstrated that the PLB strain can survive transit through the stomach, and that it possesses the ability to survive in the intestine for a protracted period (Yu et al., in press). In addition, more microbes colonized the mucosa of broilers fed the PLB powder compared to the controls. In a previous study, we cloned the rumen microbial β-glucanase gene in PLB and demonstrated that the heterologous gene did not affect bacterial adherence *in vitro* (Liu et al., 2005). In the present investigation, more microbial colonization was observed on the mucosa of TLB supplemented broilers than in the mucosa of control broilers. In addition, the crop and caeca of supplemented birds both harbour high numbers of different lactobacilli, many of them showing a quite similar morphology.

The *Lactobacillus* spp. randomly isolated from the GIT in the TLB supplemented group produced prominent transparent radials in the radial enzyme diffusion assay, indicating that they possessed β-glucanase secretion capability. The TLB exhibits resistance to acid and bile salts *in vitro* condition which mimics GIT of animal (Liu et al., 2005). Therefore, the transformed strain possesses the ability to survive and secrete β-glucanase in the GIT and thus, it might influence microbial ecology of the intestinal environment. Jaskari et al. (1998) reported that β-gluco-oligomers prepared from oat β-glucan by enzymatic hydrolysis enhanced the growth of health-promoting *Lactobacillus* spp. and *Bifidobacterium* spp.

strains. Patterson and Burkholder (2003) also showed that administration of non-digestible oligomers leads to increases of intestinal *Lactobacillus* spp. count in poultry. Therefore, we suggest that the increased in *Lactobacillus* spp. observed in the ileum and caecum of supplemented chickens at 37 days of age may be attributable, at least in part, to the release of β-gluco-oligomers from β-glucan, although it did not affect the count of *Lactobacillus* spp. in the crop at 3, 7 and 21 days.

Increases in intestinal coliform numbers may lead to an imbalance in the ratio of beneficial bacteria to potentially pathogenic bacteria in the GIT resulting in the occurrence of diarrhea (Bazzocchi et al., 2002). The dietary inclusion of *Lactobacillus* spp. has been shown to depress the growth of intestinal coliform bacteria and benefits the host animal (Jin et al., 1998). In this study, we found that the coliform count in the crop, ileum and caecum were significantly lower at 3, 7 and 21 days of broilers fed TLB than the analogous population in control birds. As a result of higher *Lactobacillus* spp. and lower coliform counts in the GIT, a healthier gut environment might be expected in the supplemented birds compared to the control birds.

The use of probiotics in feeds often enhance growth and health and maintains normal intestinal microflora. Jin et al. (1998) showed that addition of either a single *Lactobacillus acidophillus* I-26 strain or a mixture of 12 *Lactobacillus* cultures to broiler diets significantly improved BWG and FCR in broilers from 0 to 6 weeks. However, Watkins and Miller (1983) did not find any significant improvement in growth performance in broiler chickens fed *Lactobacillus* spp. cultures. Probably, the strain and dosage used, the method of preparation and the health status of chicks could be partially responsible for these disparities. The TLB strain used in the current trial might effectively cope with the β-glucan of the diet, due to its capability to secrete β-glucanase (Liu et al., 2005). The NSP in barley consists mainly of soluble β-glucan, which is the major component causing viscous digesta in the GIT of poultry (Almirall et al., 1995). Therefore, addition of β-glucanase to barley-based diets decreases viscosity leading to better production performance (Jozefiak et al., 2006).

Sieo et al. (2005) supplemented a barley-based diet for broilers from 0 to 3 weeks of age with gene transformed strains of *Lactobacillus*, cloned a β-glucanase gene from *Bacillus amyloliquefaciens*. They found that the transformed strains improved BWG and FCR of broilers. In the current study, TLB supplementation to a barley-based diet only improved BWG of broilers from 0 to 37 days of age but not in feed intake and FCR. Furthermore, the FCR of males from 1 to 21 days and from 21 to 37 days of age were very similar which was totally unexpected. However, we do not have any explanation for these results.

5. Conclusion

Supplementation of a barley-based diet with transformed *L. reuteri* Pg4 harbouring heterologous β-glucanase gene enhanced body weight gains in broilers from 0 to 37 days of age. The *Lactobacillus* spp. isolated from the digesta of the crop, ileum and caecum of the supplemented group possessed β-glucanase secretion capability, whereas the analogous control organisms did not. Transformed *Lactobacillus. reuteri* Pg4 survive and colonize the gastrointestinal tract of chickens and secrete β-glucanase into the gastrointestinal tract of chickens and, therefore these variants are potential probiotic feed additives.

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