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In vivo study on the persistence of transformed β-glucanase-producing *Lactobacillus* strains in the gastrointestinal tract of chickens

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ABSTRACT

The persistence and ability of transformed *Lactobacillus* strains to function and produce β-glucanase in the gastrointestinal tract of chickens was investigated. Chickens were fed diets supplemented with 10^9 cells/kg of transformed *Lactobacillus* strains which harboured plasmid pSA3 carrying a gene encoding β-glucanase. The transformed β-glucanase-producing *Lactobacillus* strains were *L. brevis* C10CpSA3b6, *L.crispatus* I12pSA3b6, *L. brevis* I23pSA3b6, *L. fermentum* I25pSA3b6, *L. brevis* I211pSA3b6 and *L. brevis* I218pSA3b6. At days 14 to 18, digesta samples from the gizzard, duodenum, jejunum, ileum and caecum were collected daily and the presence of the respective β-glucanase-producing *Lactobacillus* strains was determined by a PCR-based method. Results of the study showed that the β-glucanase-producing *Lactobacillus* strains could persist in the gastrointestinal tract of the chickens for about two days. The strains were found to be alive and active *in vivo*, in which β-glucanase activity was significantly ($P<0.05$) higher in chickens fed diets supplemented with β-glucanase-producing *Lactobacillus* strains. Higher enzyme activities were located in the jejunum and ileum, especially in birds fed β-glucanase-producing *L. brevis* C10CpSA3b6 and *L. brevis* I211pSA3b6. The results indicated that the *Lactobacillus* strains could be a potential vehicle to deliver enzymes into the gastrointestinal tract of chickens.

KEY WORDS: chickens, β-glucanase, *in vivo* enzyme activity, persistence, β-glucanase-producing *Lactobacillus*

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INTRODUCTION

Application of probiotics has been recognized as an alternative method of improving livestock nutrition and health. Probiotic preparations very often contain lactic acid bacteria, which are common bacteria occurring throughout the alimentary tract. Of all the lactic acid bacteria, *Lactobacillus* species are the most commonly used probiotic bacteria. Probiotics are expected to influence the health of the host by improving the intestinal balance and thus preventing and correcting the microbial dysfunctions (Salminen et al., 1999). In order to be an efficacious probiotic, the probiotic strains should be host specific, non-pathogenic and non toxic, tolerant to acid and bile in the digestive tract of the host, able to adhere and establish itself rapidly in the gut, able to inhibit growth of pathogens, able to stabilize the intestinal microflora, able to modulate immune responses, capable of withstanding manufacturing processes such as freezing or exposure to high temperatures and able to retain their viability under storage and field conditions (Bomba et al., 2002).

In recent years, attempts have been made to use probiotic strains as a delivery vehicle for biological compounds (Rastall and Maitin, 2002). Of interest is the delivery of enzyme into the gut of the host to enhance digestion. This is particularly useful for monogastric animals, which lack digestive enzymes to digest the main fibrous components of the feed. The development of strains with a capacity to digest plant structural carbohydrates by the introduction of heterologous genes encoding polysaccharide-degrading enzymes would be able to assist the animals in feed digestion. However, before the transformed strains could be applied for their desired applications, the ability of the strains to survive and reproduce in the given environment must be determined (Lucchini et al., 1998).

In order to assess the performance of the transformed strains *in vivo*, a rapid and accurate detection method is required. Conventional methods which identify and type bacteria, especially *Lactobacillus* isolates, often require several days and variable amount of work, are time-consuming and often ambiguous. Current molecular techniques based on polymerase chain reaction (PCR) are increasingly being used in the field of bacterial identification in environmental, health and food microbiology. In fact, PCR has become an invaluable tool due to the speed and simplicity in which specific DNA segments can be amplified from a background of complex genomes (Arnheim and Erlich, 1992). Thus, in the present study, the *in vivo* (in chickens) performance of six *Lactobacillus* strains transformed to produce β-glucanase was evaluated. The persistence of the β-glucanase-producing *Lactobacillus* strains in the gastrointestinal tract of chickens fed diets supplemented with these strains was determined by PCR-based method and the β-glucanase activities at the different gastrointestinal sites were also analysed.

MATERIAL AND METHODS

Transformed β-glucanase-producing Lactobacillus strains

The six transformed β-glucanase-producing *Lactobacillus* strains used were *L. brevis* C10CpSA3b6, *L. crispatus* I12pSA3b6, *L. brevis* I23pSA3b6, *L. fermentum* I25pSA3b6, *L. brevis* I211pSA3b6 and *L. brevis* I218pSA3b6. The wild-type strains of these transformants were isolated from the gastrointestinal tract of local broiler chickens and were the same as those described by Jin et al. (1996). All the transformants harbored plasmid pSA3 carrying a *bgl*A gene from *Bacillus amyloliquefaciens* (Thompson and Collins, 1991). The *bgl*A gene encodes β-glucanase (E.C. 3.2.1.73; 1,3-1,4-β-glucan-4-glucanohydrolase) which specifically hydrolyses glucans containing a mixture of β-1,3 and β-1,4 linkages, such as lichenan and barley β-glucan (Borris et al., 1985). All the strains were maintained anaerobically at 39°C in Man Rogosa Sharpe (MRS) broth containing 50 μg ml^{-1} erythromycin.

Preparation of β-glucanase-producing Lactobacillus strains for in vivo study

Each transformed β-glucanase-producing *Lactobacillus* strain was inoculated at 1% (v/v) into MRS broth containing 50 μg ml^{-1} erythromycin and incubated for 16-20 h at 39°C under anaerobic conditions. The cells were then harvested by centrifugation at 1,880 × *g* for 20 min at 4°C. The pellet was collected and stored at -20°C before it was freeze-dried for 12-24 h. The freeze-dried cells were then ground through a 0.2 mm sieve. Viability of the freeze-dried strain was determined by plating appropriate dilutions on MRS agar containing 50 μg ml^{-1} erythromycin. Enumeration of colonies was carried out after 48 h incubation. The final concentration of the freeze-dried strain was then adjusted to 1×10^9 cells g^{-1} by using maize starch and stored at -20°C until use.

Feeding experiment

One hundred and five one-day-old male broiler chickens (Avian 43) were used in the experiment. The chickens were assigned randomly to seven wired-floor cages of 15 chickens each. The cages were housed at different places to minimize cross contamination of the transformed β-glucanase-producing *Lactobacillus* strains. The chickens were fed from days 1 to14 with one of seven diets. The diets were: 1. basal diet; 2. basal diet containing 0.1% (w/w) *L. brevis* C10CpSA3b6; 3. basal diet containing 0.1% (w/w) *L. crispatus* I12pSA3b6; 4. basal diet containing 0.1% (w/w) *L. brevis* I23pSA3b6; 5. basal diet containing

0.1% (w/w) *L. fermentum* I25pSA3b6; 6. basal diet containing 0.1% (w/w) *L. brevis* I211pSA3b6; and 7. basal diet containing 0.1% (w/w) *L. brevis* I218pSA3b6. The composition of the basal diet is shown in Table 1. The transformed β-glucanase-producing *Lactobacillus* strains were mixed daily into the basal diet. Sampling started on day 14. Two h after the chickens were fed with their respective dietary treatments, the chickens were removed from their cages and the cages were cleaned. When the chickens were returned to their cleaned cages, fresh faecal droppings were collected from each group of chickens and three chickens, randomly selected from each treatment group, were euthanized by severing the jugular vein (day 0 sample). Digesta samples from the gizzard, duodenum, jejunum, ileum, and caecum were then collected and kept at -20°C until use. This process was repeated for another 4 consecutive days. During this period, all the birds were fed with the basal diet only.

Table 1. Composition of the basal diet

Item	%
Ingredient	
maize	54.05
soyabean meal	36.2
fish meal	3.0
palm oil	4.0
choline chloride (60%)	0.25
vitamin-mineral premix*	0.1
NaCl	0.2
DL-methionine	0.18
limestone	0.89
dicalcium phosphate	1.35
Calculated analysis	
crude protein (N × 6.25)	21.04
metabolizable energy, MJ kg^{-1}	12.78

* vitamin premix provided the following per kilogram of diet: mg: Fe, 100; Mn, 110; Cu, 20; Zn, 100; I, 2; Se, 0.2; Co, 0.6; sanoquin, 0.6; retinal, 2000; cholecalciferol, 25; α-tocopherol, 23,000; menadione, 1.33; cobalamin, 0.03; thiamin, 0.83; riboflavin, 2; folic acid, 0.33; biotin, 0.03; pantothenic acid, 3.75; niacin, 23.3; pyridoxine, 1.33

Detection by polymerase chain reaction (PCR)

Preparation of template from pure cultures of transformed β-glucanase-producing Lactobacillus strains. One ml of pure culture of transformed β-glucanase-producing *Lactobacillus* strain was centrifuged at 1,880 × *g* for 10 min at 4°C. The pellet obtained was washed once with dH_2O and resuspended in 100 µl of dH_2O. The suspension was then heated at 95°C for 10 min, and 2 µl of the heated suspension was used for PCR amplification.

Preparation of template from intestinal samples. Total DNA was extracted from faeces or digesta using the QIAamp DNA Stool Mini Kit (Qiagen Ltd., Crawley, UK). The extraction was carried out according to the manufacturer's protocol. Details of the protocol are given at http://www1.qiagen.com/literature /protocols/QIAampDNAStool Mini.aspx. The cells in the gut samples (0.1 g) were lysed in buffer at 95°C for 10 min. PCR inhibitors present in the sample were adsorbed on 'InhibitEX", proteins were digested using proteinase K, and DNA was purified on the QIAamp spin columns. The DNA was eluted in a final volume of 100 µl, and 2 µl was used for PCR amplification.

Development of primers. The oligonucleotides designed and used for this study were: bglAF1 (5'ATGAAAGCGC TTTCCTCGTATTAATTG3') and bglAR1 (5'GGATTT GATGCAGCCAATGCCT ATCA3') which amplified the *bgl*A gene from positions 258 to 977; and pACYC184F (5'GCCGCGGCAAAG CCGTTTTTCCATA3') and pACYC184R (5'GATTTAGAGGAGTTAGTCT TGAAGT3') which amplified the component of pSA3, pACYC184, from positions 831 to 1220.

PCR amplification. Simultaneous amplifications of the *bgl*A β-glucanase gene and cloning vector of pure cultures of transformed β-glucanase-producing *Lactobacillus* strains or intestinal samples using primer pairs bglAF1/bglAR1 and pACYC184F/pACYC184R were conducted. Amplification was carried out with the 2400 GeneAmp PCR thermal cycler (Perkin Elmer, USA) using the following protocol: pre-incubation at 94°C for 5 min, followed by a 35-cycle amplification at 94°C for 30 s, 60°C for 30 s and 72°C for 60 s, and a single cycle of primer extension at 72°C for 4 min. The programme finished with a 4-min incubation at 72°C. The PCR reactions were performed with 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 1 µM of each primer, 0.625 U TaqDNA polymerase and 2 µl of template. The PCR products were electrophoresed in a 0.8% agarose gel, then stained with ethidium bromide and visualized using UV illumination.

PCR sensitivity test

The sensitivity of the PCR method was evaluated for the six transformed β-glucanase-producing *Lactobacillus* strains. The transformed *Lactobacillus* cells from 1 ml of overnight culture were harvested by centrifugation at 1,880 × *g* for 10 min at 4°C. The cells were then serially diluted in 0.1 M sodium phosphate buffer (pH 7.0) and enumerated by plating appropriate dilutions on MRS agar containing 50 µg ml^{-1} erythromycin. One ml of sample was obtained from each dilution and processed for PCR amplification.

β-glucanase assay of gastrointestinal samples

The intestinal samples were diluted 10 times with ice-cold phosphate buffer (pH 7.0) based on sample weight, and vortexed thoroughly for 60 s. The samples were then centrifuged at 6,000 × g for 20 min at 4°C. The supernatant recovered was used

to determine β-glucanase activity (Waffenschnidt and Jaenicke, 1987). An enzyme control tube containing the supernatant incubated in a waterbath at 100°C for 15 min to inactivate the β-glucanase was prepared for every test sample. The difference in reducing sugar concentration between the tested tube and control tube was used to calculate the β-glucanase activity in the fresh gut contents. One unit of β-glucanase activity was defined as the amount of enzyme required to liberate 1 μmole of glucose equivalent of reducing sugar per h per mg of intestinal protein under the given assay conditions. The intestinal and faecal protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, USA).

Statistical analysis

Data for β-glucanase activity of gastrointestinal samples obtained were analysed by the general linear models (GLM) procedure for analysis of variance (SAS Institute, 1997). Significant differences among the treatment means were separated by Duncan's new multiple range test (Duncan, 1955) at 5% level of probability.

RESULTS

Amplification profiles

Simultaneous amplification by primer pairs bglAF1/bglAR1 and pACYC184F/pACYC184R successfully produced two fragments of approximately 720 bp (fragment amplified from *bgl*A gene) and 390 bp (fragment amplified from cloning vector) in all the transformed β-glucanase-producing *Lactobacillus* strains (Figure 1). The intensity of the smaller fragment (390 bp) was much higher and more distinct than the larger fragment (720 bp).

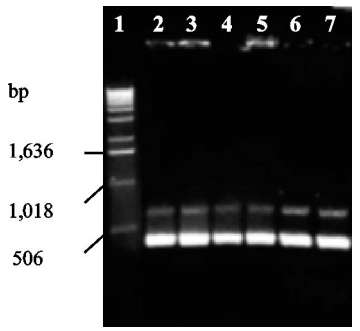

Figure 1. *Simultaneous amplification of bglA gene and cloning vector by primer pairs* pACYC184F/pACYC184R and bglAF1/bglAR1. Lane 1, 1 kb DNA ladder; lane 2, *L. brevis* C10CpSA3b6; lane 3, *L. crispatus* I12pSA3b6; lane 4, *L. brevis* I23pSA3b6; lane 5, *L. fermentum* I25pSA3b6; lane 6, *L. brevis* I211pSA3b6; and lane 7, *L. brevis* I218pSA3b6

PCR sensitivity test

The PCR sensitivity with simultaneous amplification of the *bgl*A gene and cloning vector of pure cultures of the six transformed β-glucanase-producing *Lactobacillus* strains using primer pairs bglAF1/bglAR1 and pACYC184F/ pACYC184R was strain dependent. Representative gel of PCR sensitivity for *L.brevis* I23pSA3b6 is shown in Figure 2. Amplification of both the large (720 bp) and small (390 bp) fragments together, was only detected with at least 10^5 cells ml^{-1} for *L. brevis* C10CpSA3b6, *L. fermentum* I25pSA3b6 and *L. brevis* I218pSA3b6, and 10^4 cell ml^{-1} for *L. crispatus* I12pSA3b6, *L. brevis* I23pSA3b6 and *L. brevis* I211pSA3b6. However, amplification of the smaller fragment (390 bp) alone was detected even when there were 10 cells ml^{-1} for all the strains, and for *L. brevis* I23pSA3b6, the fragment was detected with as few as 2 cells ml^{-1}.

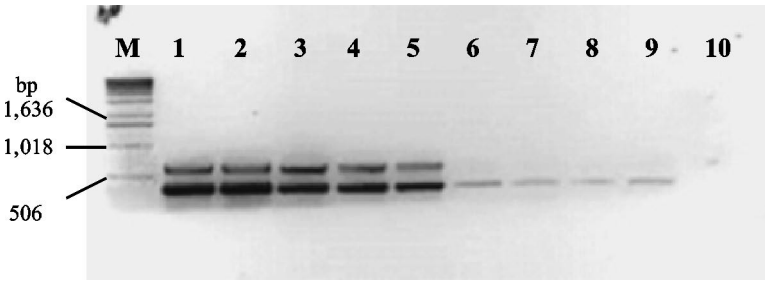

Figure 2. PCR sensitivity test for pure culture of *L. brevis* I23pSA3b6. The PCR products were amplified from the following amounts of cells in per ml sample: lane 1, 10^8 cells; lane 2, 10^7 cells; lane 3, 10^6 cells; lane 4, 10^5 cells; lane 5, 10^4 cells; lane 6, 10^3 cells; lane 7, 10^2 cells; lane 8, 10 cells; lane 9, 2 cells; lane 10, 0 cell (negative control). Lane M, 1 kb DNA ladder

Amplification from intestinal samples

PCR amplification was not detected in the intestinal samples of chickens fed a basal diet only. However, strong PCR amplifications of the *bgl*A gene and cloning vector were observed in the upper gastrointestinal tract samples (gizzard, duodenum, jejunum and ileum) and weaker amplifications were observed in the caecal and faecal samples 2 h after the chickens were fed diets with their respective transformed β-glucanase-producing *Lactobacillus* strains (day 0 samples) (a representative gel is shown in Figure 3).

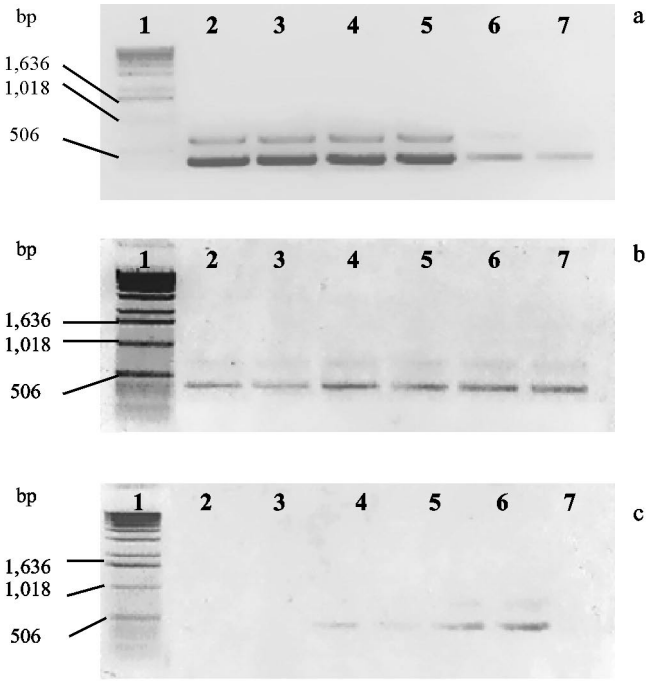

Figure 3. Amplification of the *bgl*A gene and cloning vector from various gastrointestinal samples obtained at day 0 (a), day 1 (b), and day 2 (c) after removal of dietary treatment supplemented with *L. brevis* C10CpSA3b6. Lane 1, 1 kb DNA ladder; lane 2, gizzard; lane 3, duodenum; lane 4, jejunum; lane 5, ileum; lane 6, caecum and lane 7, faeces

The amplification profiles of samples obtained after day 0 varied. Amplifications were either at lower levels or not detected at all in certain parts of the gastrointestinal tract. For instance, in chickens fed a diet supplemented with *L. brevis* C10CpSA3b6 (Figure 3) or *L. brevis* I23pSA3b6, amplifications of day 1 samples (24 h after the removal of the respective strains of transformed β-glucanase-producing *Lactobacillus* from the diets) were detected at lower levels (based on the intensity of amplified bands) in all parts of the gastrointestinal tract as compared to day 0 samples, and amplifications of day 2 samples (48 h after the removal of transformed β-glucanase-producing *Lactobacillus* strains from the diets) were mostly detected in the mid and lower gastrointestinal tract regions (jejunum, ileum, caecum) and faeces.

For chickens fed *L. crispatus* I12pSA3b6, amplifications were observed in the jejunum and faeces of day 1 samples and in the faeces of day 2 samples. Amplifications were observed in the jejunum, ileum, caecum and faeces of day 1 samples of chickens fed diet supplemented with *L. fermentum* I25pSA3b6, *L. brevis*

I218pSA3b6 or *L. brevis* I211pSA3b6, but in day 2 samples, weak amplifications were detected in the jejunum, ileum, caecum and faeces of chickens fed *L. brevis* I218pSA3b6, and faeces of those fed *L. brevis* I211pSA3b6 but not in those fed *L. fermentum* I25pSA3b6. No PCR amplifications were detected in all samples of day 3 (72 h after the removal of the respective strains of transformed β-glucanase-producing *Lactobacillus* from the diet) and day 4 (96 h after the removal of the transformed β-glucanase-producing *Lactobacillus* strains from the diet).

β-glucanase activity of gastrointestinal samples

The β-glucanase activities in all the gastrointestinal samples of chickens fed different dietary treatments are shown in Figure 4. The results showed that

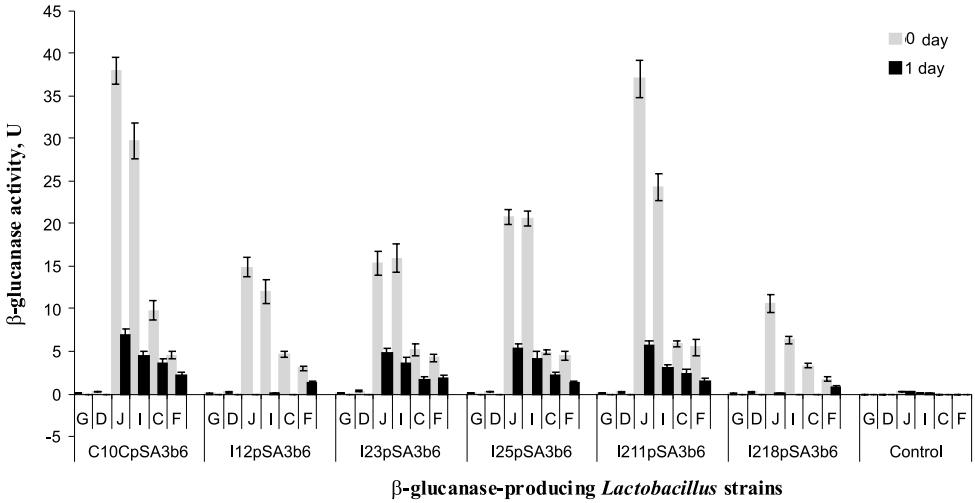

Figure 4. β-Glucanase activity in different parts of the gastrointestinal tract of chickens fed diets supplemented with various β-glucanase-producing *Lactobacillus* strains. G = gizzard; D = duodenum; J = jejunum; I = ileum; C = caecum; and F = faeces. Bar = ±SE

β-glucanase activity (0.05-40 U) was detected in all parts of the gastrointestinal tract 2 h after removal of the transformed β-glucanase-producing *Lactobacillus* strains from the diets (day 0 samples). The enzyme activities detected in the jejunum and ileum were approximately 2-9.4 folds higher when compared to the other parts of the gastrointestinal tract, regardless of *Lactobacillus* strain. In terms of strains, the enzyme activities located in the jejunum and ileum of birds fed *L. brevis* C10CpSA3b6 or *L. brevis* I211pSA3b6 were significantly ($P<0.05$) higher than those of birds fed other strains. In the control chickens (fed basal diet), no β-

glucanase activity was detected in the gizzard, duodenum, caecum and faeces and only very low activities (0.18-0.2 U) were detected in the jejunum and ileum.

A drastic decrease (51-100%) in enzyme activity was observed in the chickens of all the treatment groups 24 h after removal of the transformed β-glucanase-producing *Lactobacillus* strains from the diets (day 1 samples). Only 0.05-7.0 U of β-glucanase was detected in the jejunum, ileum and caecum. Enzyme activity was not detected at all in the gizzard and duodenum of chickens of all dietary treatment groups. Two days after removal of the transformed β-glucanase-producing *Lactobacillus* strains from the diet (day 2 samples), enzyme activity was not detected in any parts of the gastrointestinal tract of chickens in all treatment groups.

DISCUSSION

In order to detect and locate the presence of the transformed β-glucanase-producing *Lactobacillus* strains in the gastrointestinal tract of chickens, a rapid, sensitive and specific detection method is needed. In the present study, a PCR-based method was used for this purpose. As specific probes to detect the transformed β-glucanase-producing *Lactobacillus* strains were not available during the course of the present study, primers which specifically amplified the cloned gene (*bgl*A gene) and cloning vector were employed. This was carried out with the assumption that successful amplification indicates the presence of the transformed β-glucanase-producing *Lactobacillus* strains. Although horizontal transfer of gene may be a concern, it has been reported that the possibility of it occurring is relatively rare (Lorenz and Wackernagel, 1994). The gene transfer in the environment is 10^{1}- to 10^{5}-fold lower than the rates measured under laboratory conditions (Schlundt et al., 1994; Marcinek et al., 1998). In an *in vitro* forced filter mating study, Netherwood et al. (1999) found that gene transfer occurred at a rate of less than 10^{-8}. In order for horizontal transfer to occur, DNA must first be released from the donor organism in a form which can be incorporated into the genome of a putative recipient bacterium and must remain intact for a sufficient time for the transfer to occur (Chambers et al., 2002).

The two pairs of designed primers used simultaneously for amplification, successfully amplified the target sequences from the transformed β-glucanase-producing *Lactobacillus* cells, producing PCR products of expected sizes, i.e. a 720 bp fragment amplified from the *bgl*A gene and a 390 bp fragment amplified from the cloning vector. Preferential amplification of the smaller fragment (390 bp) occurred during simultaneous amplification by the two primer pairs. This was shown by the higher intensity and more distinct band of the 390 bp

fragment as compared to the larger 720 fragment. Castellanos et al. (1996) also found that when two fragments were simultaneously amplified, the quantity of the larger fragment (200 bp) decreased noticeably and, in some cases, was not detectable, when compared to the smaller fragment (80 bp). Preferential amplification was found to be more evident when the concentration of template was low. This was observed when the sensitivity of the PCR method using pure cultures of the six transformed β-glucanase-producing *Lactobacillus* strains was evaluated. Amplification of both fragments (720 and 390 bp) was observed only when the number of cells was at least 10^5 cells ml^{-1} for *L. brevis* C10CpSA3b6, *L. fermentum* I25pSA3b6 and *L. brevis* I218pSA3b6, and 10^4 cells ml^{-1} for *L. crispatus* I12pSA3b6, *L. brevis* I23pSA3b6 and *L. brevis* I21pSA3b6. Below these levels, only the smaller fragment (390 bp) was amplified. Although Wang et al. (1996) obtained amplification with as few as two cells of *Bifidobacterium longum*, they suggested that PCR sensitivity for other species varied and was dependent upon the species, e.g., PCR sensitivity was 4 cells for *Fusobacterium prausnitzii,* 40 cells for *Peptostreptococcus productus* and 10,000 cells for *Bifidobacterium adolescentis*. In the present study, amplification was observed with at least 10 cells for all the strains except for *L. brevis* I23pSA3b6 where amplification was obtained with as few as 2 cells.

The results on the amplification obtained from the gastrointestinal samples showed the presence of the transformed β-glucanase-producing *Lactobacillus* strains *in vivo*. All the transformed β-glucanase-producing *Lactobacillus* strains were detected throughout the gastrointestinal tract (gizzard, duodenum, jejunum, ileum, caecum) and faeces 2 h after the chickens were fed the dietary treatments (day 0 samples). Based on the intensity of the amplified fragments of day 0 samples, it was found that most of the strains were in the upper and mid gastrointestinal tract (gizzard, duodenum, jejunum and ileum). As simultaneous amplification of both fragments was observed, it was estimated that at least 10^5 cells were present in these segments. However, with the removal of the transformed β-glucanase-producing *Lactobacillus* strains from the feed, gradual disappearance of the strains from the gastrointestinal tract occurred. Amplification was at a lower level or not detected at all in certain parts of the gastrointestinal tract. Two days after the removal of the treatment diets, the strains were mostly detected at a low level in the mid or lower gastrointestinal tract. Amplification was not successful 3 and 4 days after the removal of the transformed β-glucanase-producing *Lactobacillus* strains from the diets. These results showed that the transformed β-glucanase-producing *Lactobacillus* strains were not able to establish in the gastrointestinal tract of the chickens. In an *in vivo* experiment carried out by Kobayashi et al. (2000) to evaluate the performance of *Butyrivibrio fibrisolvens* OB156 recombinant in the rumen, they observed that the number of recombinant bacteria decreased rapidly,

from 10^6 to 10^2 ml^{-1}, over the first 24 h. The number of cells persisted at this level for 72 h after inoculation and only became undetectable at 144 h. They proposed that amplification during this period (72-144 h) might be from bacterial cells which had lost their cultural viability. In another study, the number of externally introduced *L. lactis* in a simulated gut ecosystem showed a decline from 1 x 10^9 to 5 × 10^3 ml^{-1} in 48 h (Scott et al., 2000). Although rapid decrease of externally introduced microorganisms to undetectable level has been reported, successful establishment has also been demonstrated (Gregg et al., 1998). The inability of the strains to establish in the gut environment is probably the result of nutritional competition among the microorganisms or outflow of the bacteria from the gut (Kobayashi et al., 2000; Gory et al., 2001).

Nevertheless, positive amplification of the transformed β-glucanase-producing *Lactobacillus* strains, especially in the faecal material, indicated that the strains survived the passage through the gastrointestinal tract. It is generally required that bacteria survive the passage through the gastrointestinal tract to exert their effect (Yuki et al., 1999). Although the PCR method is considered more superior than the culture method for detecting bacteria as the former detects *in situ*, whereas the latter detects bacteria after enrichment, Wang et al. (1996) pointed out that amplification could be from dead bacterial cells as well.

In order to determine that the transformed β-glucanase-producing *Lactobacillus* strains were alive and active in the gastrointestinal tract, β-glucanase activities in various gastrointestinal segments were determined. Enzyme activity was detected throughout the gastrointestinal tract 2 h after the chickens were fed the dietary treatments, but gradually disappeared thereafter. This observation corresponded with the amplification profile. Even though there was a drastic drop of enzyme activity in all parts of the gastrointestinal tract 24 h after removal of the transformed β-glucanase-producing *Lactobacillus* strains from the diet, it indicated that the strains were alive and active for at least 24 h. Higher amounts of enzyme were located in the ileum and jejunum when compared to the other segments. This could be an advantage as the process of absorption occurs mostly in the jejunum and ileum (Sturkie, 1965a,b).

In conclusion, the results of the present study showed that the transformed β-glucanase-producing *Lactobacillus* strains could persist in the gastrointestinal tract of chickens for about two days. The strains were alive and active in the chickens and were able to express the *bgl*A β glucanase gene. As the transformed β-glucanase-producing *Lactobacillus* strains could only persist in the gastrointestinal tract for about two days, it would be necessary to supplement the transformed strains in the diet of the chickens daily in order to obtain positive effects of the strains.

REFERENCES

Arnheim N., Erlich H., 1992. Polymerase chain reaction strategy. Annu. Rev. Biochem. 61, 131-156

Bomba A., Nemcova R., Mudronova D., Guba P., 2002. The possibilities of potentiating the efficacy of probiotics. Trends Food Sci.Technol. 13, 121-126

Borris R., Baumlein H., Hofemeister J., 1985. Expression in *Escherichia coli* of a cloned β-glucanase gene from *Bacillus amyloliquefaciens*. Appl. Microbiol. Biotechnol. 22, 63-71

Castellanos M.I., Chauvet A., Deschamps A., Barreau C., 1996. PCR methods for identification and specific detection of probiotic lactic acid bacteria. Curr. Microbiol. 33, 100-103

Chambers P.A., Duggan P.S., Heritage J., Forbes J.M., 2002. The fate of antibiotic resistance marker genes in transfenic plant feed material fed to chickens. J. Antimicrob. Chemother. 49, 161-164

Duncan D.B., 1955. Multiple range and multiple F tests. Biometrics 11, 1-42

Gory L., Montel M.-C., Zagorec M., 2001. Use of green fluorescent protein to monitor *Lactobacillus sakei* in fermented meat products. FEMS Microbiol. Lett. 194, 127-133

Gregg K., Hamdorf B., Henderson K., Kopecny J.,Wong C., 1998. Genetically modified ruminal bacteria protect sheep from fluoroacetate poisoning. Appl. Environ. Microbiol. 64, 3496-3498

Jin L.Z., Ho Y.W., Ali M.A, Abdullah N., Ong K.B., Jalaludin S., 1996. Adhesion of *Lactobacillus* isolates to intestinal epithelial cells of chicken. Lett. Appl. Microbiol. 22, 229-232

Kobayashi Y., Forster R.J.,Teather R.M., 2000. Development of a competitive polymerase chain reaction assay for the ruminal bacterium *Butyrivibrio fibrisolvens* OB156 and its use for tracking an OB156-derived recombinant. FEMS Microbiol. Lett. 188, 185-190

Lorenz M.G., Wackernagel W., 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol. Rev. 58, 563-602

Lucchini F., Kmet V., Cesena C., Coppi L., Bottazzi V., Morelli L., 1998. Specific detection of a probiotic *Lactobacillus* strain in faecal samples by using multiplex PCR. FEMS Microbiol. Lett. 158, 273-278

Marcinek H., Wirth R., Muscholl-Silberhorn A., Gauer M., 1998. *Enterococcus faecalis* gene transfer under natural conditions in municipal sewage water treatment plants. Appl. Environ. Microbiol. 64, 626-632

Netherwood T., Bowden R., Harrison R., O'Donnel A. G., Parker D.S., Gilbert H.J., 1999. Gene transfer in the gastrointestinal tract. Appl. Environ. Microbiol. 65, 5139-5141

Rastall R.A., Maitin V., 2002. Genetic engineering: threat or opportunity for the dairy industry? Int. J. Dairy Technol. 55, 161-165

Salminen S., Ouwehand A., Benno Y., Lee Y.K., 1999. Probiotics: how should they be defined? Trends Food Sci. Technol. 10, 1107-1110

SAS Institute, 1997. SAS® Users Guide: Statistics: Release 6.12. Edition, SAS Institute Inc. Cary, NC

Schlundt J., Saadbye P., Lohmann B., Jacobsen B.L., Nielsen E. M., 1994. Conjugal transfer of plasmid DNA between *Lactococcus lactis* strains and distribution of transconjugants in the digestive tract of gnobiotic rats. Microb. Ecol. Health Dis. 7, 59-69

Scott K.P., Mercer D.K., Richardson A.J., Melville C.M., Glover L.A., Flint H.J., 2000. Chromosomal integration of the green fluorescent protein gene in lactic acid bacteria and the survival of marked strains in human gut simulations. FEMS Microbiol. Lett. 182, 23-27

Sturkie P.D., 1965a. Alimentary canal: Anatomy, prehension, deglutition, appetite, passage of ingesta, motility. In: P.D. Sturkie (Editor). Avian Physiology. Cornstock Publishing Associates, pp. 272-312

Sturkie P.D., 1965b. Digestion, absorption, secretion of gastric juices, pH, liver. In: P.D. Sturkie (Editor). Avian Physiology. Cornstock Publishing Associates, pp.. 288-312

Thompson K., Collins M.A., 1991. Molecular cloning in *Lactobacillus helveticus* by plasmid pSA3: pVA797 co-integrate formation and conjugal transfer. Appl. Microbiol. Biotechnol. 35, 334-338

Wang R.F., Cao W.W., Cerniglia C.E., 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol. 62, 1242-1247

Waffenschmidt S., Jaenicke L., 1987. Assay of reducing sugars in the nanomole range with 2-2'-bicinchoninate. Anal. Biochem. 165, 337-340

Yuki N., Watanabe K., Mike A., Tagami Y., Tanaka R., Ohwaki M., Morotomi M., 1999. Survival of a probiotic, *Lactobacillus casei* strain Shirota, in the gastrointestinal tract: Selective isolation from faeces and identification using monoclonal antibodies. Int. J. Food Microbiol. 48, 51-57