MANIPULATION OF *LACTOBACILLUS* PROBIOTIC STRAINS TO PRODUCE HETEROLOGOUS β-GLUCANASE FOR CHICKENS

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By

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Application of enzymes as feed additives is common in the livestock industry, especially in poultry, to eliminate the antinutritional factors present in the diets of chickens. However, the efficiency of enzymes seldom achieves their desired effects because of destruction during feed processing and unsuitable conditions in the gastrointestinal tract. Thus, in the present study, investigations were carried out to evaluate the potential of 12 *Lactobacillus* strains as delivery vehicles for a heterologous β-glucanase enzyme in poultry. The 12 *Lactobacillus* strains used were *L. crispatus* I12, *L. acidophilus* I16 and I26, *L. fermentum* I24, I25, C16 and C17, and *L. brevis* I23, I211, I218, C1 and C10. The strains were found to exhibit resistance to chloromphenicol, erythromycin and tetracycline in varying degrees. The erythromycin resistance of *L. acidophilus* I16 and I26, and *L. fermentum* I24 and C17 could be cured by using novobiocin, and *L. brevis* C10 cured by using acriflavin. The chloromphenicol and tetracycline resistances of all the resistant strains were not eliminated even after prolonged curing in sublethal concentrations of individual or mixtures of curing agents such as novobiocin, ethidium bromide, acriflavin or SDS. Electrotransformation efficiency of the *Lactobacillus* strains was affected by growth phase, growth and recovery medium, cell density, electroporation buffer, buffer strength, plasmid concentration and electrical pulse. At optimized conditions, the strains were transformed at 10^3-10^4 transformants/μg plasmid DNA. The erythromycin susceptible wild-type strains (*L. crispatus* I12, *L. brevis* I23, I211 and I218, and *L. fermentum* I25) and cured derivatives (*L. acidophilus* I16C and I26C, *L. brevis*

C10C, and *L. fermentum* I24C and C17C) were then transformed at optimized conditions with plasmid pSA3b6, which carried a β-glucanase gene from *Bacillus amyloliquefaciens*. Five wild-type *Lactobacillus* strains, namely, *L. crispatus* I12, *L. fermentum* I25, *L. brevis* I23, I211 and I218 and a cured derivative, *L. brevis* C10C, which could retain the plasmid at a comparatively higher rate, were used for subsequent studies. The *Lactobacillus* transformants were found to secrete 32-52 U/ml of β-glucanase. Optimum activity of the enzyme was at 39 ^{o}C and pH 5-6. A loss of 0.4-1.6 U/generation of β-glucanase was observed when the strains were grown under non-selective pressure.

PCR analyses of gastrointestinal samples of chickens fed transformed *Lactobacillus* strains revealed that the strains could not persist for more than 24 h in the gut. The β-glucanase activity detected in the jejunum and ileum of chickens fed transformed *Lactobacillus* strains was found to be 2-9.4 folds higher than those obtained from other intestinal sites. In the feeding trial, supplementation of transformed *Lactobacillus* strains to chickens significantly ($P<0.05$) improved the body weight by 2.5 %, and the feed conversion ratio by 1.0-2.6 %. In addition, the apparent metabolizable energy, digestibilities of crude protein and dry matter of feed were improved by 3.4 %, 5.9 % and 3.5 %, respectively. The intestinal fluid viscosity was reduced by 21-46 %. The relative weights of organs and intestinal segments (pancrease, liver, duodenum, jejunum, ileum, cecum and colon) were also reduced by 6-27 %, and the relative length of intestinal segments (duodenum, jejunum, ileum and cecum) was reduced by 8-15 %. Histological examination of the intestinal tissues showed that the jejunal villus height of chickens fed diet supplemented with transformed *Lactobacillus* strains was significantly ($P<0.05$) higher than those of chickens fed other dietary treatments. The transformed *Lactobacillus* strains were also found to reduce the time of feed passage rate by 2.2 h.

The results of the present study showed that the *Lactobacillus* strains have the potential to be used as delivery vehicles for a heterologous β-glucanase enzyme in poultry.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

MANIPULASI STRAIN PROBIOTIK *LACTOBACILLUS* UNTUK PENGHASILAN β-GLUKANASE HETEROLOGUS UNTUK AYAM

Oleh

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Penambahan enzim ke dalam pemakanan haiwan adalah biasa dalam industri penternakan, terutamanya ayam, untuk menyingkirkan faktor antinutrisi dalam pemakanannya. Walau bagaimanapun, efisiensi enzim pada kebiasaannya jarang mencapai kesan yang dikehendaki disebabkan oleh pemusnahan enzim semasa pemprosesan pemakanan dan keadaan yang tidak sesuai dalam usus. Oleh itu, kajian ini dijalankan untuk menilai potensi 12 strain *Lactobacillus* sebagai penghantar alternatif enzim heterologus β-glukanase ke dalam gastrousus ayam. Duabelas strain *Lactobacillus* iaitu *L. crispatus* I12, *L. acidophilus* I16 dan I26, *L. fermentum* I24, I25, C16 dan C17, dan *L. brevis* I23, I211, I218, C1 dan C10 digunakan. Strain-strain tersebut menunjukkan kerintangan terhadap kloromfenikol, eritromisin dan tetrasaiklin pada tahap yang berbeza. Kerintangan eritromisin *L. acidophilus* I16 dan I26, dan *L. fermentum* I24 dan C17 dipulih dengan menggunakan novobiosin dan *L. brevis* C10 dipulih dengan akriflavin. Kerintangan kloromfenikol dan tetrasaiklin kesemua strain yang rintang tidak disingkirkan walaupun proses pemulihan dalam kepekatan sub-kematian agen pemulihan, secara berasingan atau campuran, seperti novobiosin, etidium bromida, akriflavin dan SDS diperpanjangkan. Efisiensi elektrotransformasi strain *Lactobacillus* dipengaruhi oleh fasa pertumbuhan sel, media pertumbuhan dan pemulihan, kepekatan sel, penimbal

elektroporasi, kekuatan penimbal, kepekatan plasmid dan kekuatan elektrik. Pada keadaan optima, strain *Lactobacillus* ditransformasi pada kadar 10^3-10^4 transforman/μg plasmid DNA. Strain asli (*L. crispatus* I12, *L. brevis* I23, I211 dan I218, dan *L. fermentum* I25) dan terbitan yang dipulih (*L. acidophilus* I16C dan I26C, *L. brevis* C10C, dan *L. fermentum* I24C dan C17C) yang sensitif kepada eritromisin ditransformasi pada keadaan optima dengan menggunakan plasmid pSA3b6 yang membawa gen β-glukanase dari *Bacillus amyloliquefaciens*. Lima strain asli *Lactobacillus* iaitu *L. crispatus* I12, *L. fermentum* I25, *L. brevis* I23, I211 and I218 dan satu terbitan yang dipulih, *L. brevis* C10C, yang mampu mengekalkan plasmid pada kadar yang lebih tinggi diguna untuk kajian seterusnya. Transforman *Lactobacillus* merembeskan 32-52 U/ml β-glukanase. Aktiviti optimum enzim diperolehi pada 39 ^oC dan pH 5-6. Pengurangan 0.4-1.6 U/generasi β-glukanase diperhatikan apabila strain ditumbuh di dalam keadaan tanpa tekanan pemilihan.

Analisis PCR sampel gastrousus yang diperolehi dari ayam yang diberi makan strain *Lactobacillus* yang ditransformasi menunjukkan bahawa strain tersebut tidak berkekalan untuk lebih dari 24 jam dalam usus. Aktiviti β-glukanase yang dikesan di dalam jejunum dan ileum adalah 2-9.4 kali lebih tinggi daripada aktiviti di tapak usus yang lain. Penambahan strain *Lactobacillus* yang ditransformasi ke dalam pemakanan ayam meningkat secara signifikan berat badan ayam sebanyak 2.5 %. Kadar penukaran pemakanan juga meningkat 1.0-2.6 %. Selain daripada itu, tenaga yang dimetabolisme, penghadaman protein kasar dan bahan kering pemakanan masing-masing meningkat sebanyak 3.4 %, 5.9 % and 3.5 %. Kelikatan cecair usus juga turun sebanyak 21-46 %. Berat relatif organ dan segmen usus (pankreas, hati, duodenum, jejunum, ileum, cecum dan kolon) turun sebanyak 6-27 % dan ukuran panjang relatif segmen usus (duodenum, jejunum, ileum dan cecum) turun sebanyak 8-15 %. Kajian histologi tisu usus

menunjukkan bahawa ketinggian vilus jejunal ayam yang diberi makanan yang ditambah dengan strain *Lactobacillus* yang ditransformasi adalah lebih tinggi ($P<0.05$) daripada sampel yang diperolehi daripada ayam yang diberi pemakanan lain. Strain *Lactobacillus* yang ditransformasi juga mengurangkan masa untuk kadar laluan pemakanan sebanyak 2.2 jam.

Keputusan kajian ini menunjukkan bahawa strain *Lactobacillus* mempunyai potensi untuk diguna sebagai penghantar alternatif enzim heterologus β-glukanase dalam ayam.

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"And now, a new chapter of life begins…."

I certify that an Examination Committee met on 16 March 2004 to conduct the final examination of Sieo Chin Chin on her Ph.D. thesis entitled "Manipulation of *Lactobacillus* probiotic strains to produce heterologous β-glucanase for chickens" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirements for the degree of Doctor of Philosophy. The members of the Supervisory Committee are as follows:

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DECLARATION

I hereby declare that this thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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SIEO CHIN CHIN

Date:

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