เป็นหนังสือภาษาอังกฤษ

รายงานวิจัยฉบับสมบูรณ์

โครงการ การต้านแบคทีเรีย และ/หรือการผลิตโปรตีเนสของแบคทีเรียแลกติกแอซิดและแบคทีเรีย ชนิด purple nonsulfur ที่แยกได้จากมูลไก่

(Antibacterial activity and/or proteinase production of lactic acid bacteria and purple nonsulfur bacteria isolated from chicken faeces)

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ชื่อเรื่อง

การต้านแบคทีเรีย และ/หรือการผลิตโปรตีเนสของแบคทีเรียแลกติกแอซิดและแบคทีเรียชนิด purple nonsulfur ที่แยกได้จากมูลไก่

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หัวหน้าโครงการวิจัย

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บทคัดย่อ

จากการแยกเชื้อจากตัวอย่างมูลไก่ 108 ตัวอย่าง โดยไก่มีอายุ 5-6 เดือน พบว่าได้แบคทีเรียที่ไม่สร้างแคทาเลสและเป็นแกรมบวกจำนวน 490 สายพันธุ์ และแบคทีเรียที่เป็นแกรมลบและมีสีแดงจำนวน 58 สายพันธุ์ ภายหลังการศึกษาการยับยั้งแบคทีเรียก่อโรค ได้แก่ *Staphylococcus aureus* ATCC 28955, *Salmonella* Typhimurium, *Escherichia coli* ATCC 25922 and *Bacillus cereus* โดยวิธี agar diffusion พบว่า แบคทีเรียที่ไม่สร้างแคทาเลสและเป็นแกรมบวกจำนวน 10 สายพันธุ์ คือ LB_2, LB_4, LB_{13}, LB_{28}, LB_{30}, LB_{90}, LB_{91}, LB_{95}, LB_{107} และ LB_{108} สามารถยับยั้งแบคทีเรียก่อโรคดังกล่าวได้ โดยเห็นเป็นบริเวณใสขนาด 18-20 มม. และยังสามารถผลิตเอนไซม์โปรตีเนสออกนอกเซลล์ได้ในช่วง 0.001-0.2 units นอกจากนี้ยังพบว่าแบคทีเรียที่เป็นแกรมลบและมีสีแดงไม่สามารถยับยั้งแบคทีเรียก่อโรค แต่มี 15 สายพันธุ์จาก 58 สายพันธุ์ที่สามารถผลิตเอนไซม์โปรตีเนสออกนอกเซลล์ได้ในช่วง 0.2-0.8 units โดยสายพันธุ์ P_{41} มีกิจกรรมของโปรตีเนสสูงสุดคือ 0.8 units และสามารถเทียบเคียงเชื้อชนิดนี้ได้กับ *Enterobacter* sp. sb-3 แบคทีเรียที่ไม่สร้างแคทาเลสและเป็นแกรมบวกจำนวน 10 สายพันธุ์นี้สามารถเจริญได้ที่ pH 3 และที่ที่มีเกลือน้ำดี 0.3% การต้านแบคทีเรียของสารละลายส่วนใสจากแบคทีเรียทั้ง 10 สายพันธุ์นี้ พบว่าเป็นผลเนื่องจากสภาพความเป็นกรด โดยแบคทีเรียสายพันธุ์ LB_2, LB_{13}, LB_{90}, LB_{95}, และ LB_{107} เทียบเคียงได้กับ *Lactobacillus* sp. strain 9D10, LB_{91} เทียบเคียงได้กับ *Lactobacillus* sp. strain P23, LB_{28} เทียบเคียงได้กับ *Enterococcus thermitidis* และ LB_4 เทียบเคียงได้กับ *Bacillus* sp. SXQ-2004 นอกจากนี้ LB_{30} และ LB_{108} เทียบเคียงได้กับ *Corynebacterium glutamicum* strain CICC10117 และ *C. glutamicum* strain CICC10178 ตามลำดับ

Abstract

A total of 490 strains of Gram-positive and catalase-negative bacteria, and 58 strains of Gram-negative and red-pigmented bacteria were isolated from 108 samples of faeces of 5-6 months-old chicken. The inhibition activity against pathogenic bacteria *Staphylococcus aureus* ATCC 28955, *Salmonella* Typhimurium, *Escherichia coli* ATCC 25922 and *B. cereus* were assayed by agar diffusion. The results showed that 10 strains of Gram-positive and catalase-negative bacteria, LB_2, LB_4, LB_{13}, LB_{28}, LB_{30}, LB_{90}, LB_{91} LB_{95}, LB_{107} and LB_{108} could produce inhibition zones, 18-20 mm, against all of the pathogenic bacteria. These 10 strains gave proteinase activity in the range of 0.001-0.2 units. In addition, all strains of Gram-negative and red-pigmented bacteria were found not to produce inhibition zones against any pathogenic bacteria, but 15 out of 58 strains gave proteinase activity in the range of 0.2-0.8 units. Among them, strain P_{41} gave the highest proteinase activity, 0.8 units, which was identified as *Enterobacter* sp. sb-3. All the 10 strains of Gram-positive and catalase-negative bacteria were able to grow at pH 3 and in the presence of 0.3% bile salt. The antibacterial activity of all supernatant from the 10 strains were due to acidity. LB_2, LB_{13}, LB_{90}, LB_{95} and LB_{107} strains were identified as *Lactobacillus* sp. strain 9D10. LB_{91} was identified as *Lactobacillus* sp. strain P23. LB_{28} was identified as *Enterococcus thermitidis*, and LB_4 was identified as *Bacillus* sp. SXQ-2004. LB_{30} and LB_{108} were identified as *Corynebacterium glutamicum* strain CICC10117 and *C. glutamicum* strain CICC10178, respectively.

Author Keywords: antibacterial activity; proteinase; lactic acid bacteria; purple nonsulfur bacteria; chicken faeces

1. Introduction

Probiotics are live microorganisms used as food supplement for human and animal hosts. They are able to protect the host from the toxin and infection of bacteria, and may improve enzymatic activity (Fuller, 1989). Various kinds of antibiotics are used in the poultry industry in order to prevent poultry pathogens and disease, but continued use of dietary antibiotics has resulted in common problems, such as the development of drug-resistant bacteria (Sorum and Sunde, 2001), imbalance of normal flora (Andremont, 2000), and drug residues in the bird body (Burgat, 1991). For these reasons, the use of probiotics as chicken feed supplement will be beneficial. Positive effects resulted in increased body weight, better feed conversion and decreased mortality (Kralik *et al.*, 2004). The protective effects of probiotics against intestinal infections were shown both with animals and with in vitro cell culture models (Asahara *et al.*, 2004; Fernandez *et al.*, 2003; Filho-Lima *et al.*, 2000; Lievin-Le Moal *et al.*, 2002; Silva *et al.*, 1999; Todoriki *et al.*, 2001). In vivo assays, it takes time and needs labors for evaluation of probiotics properties (Koenen *et al.*, 2004). Hence, in vitro assays have been developed. Also selection methods have been described for probiotics that express antimicrobial activity (Abee *et al.*, 1994, Mcauliffe *et al.*, 1998, Blom and Mortvedt, 1991). There are reports on purple nonsulfur bacteria used as livestock feed. Cells of *Rhodobacter capsulatus*, a purple nonsulfur bacterium, contain a lot of protein with a good balance of amino acids, vitamins and other effective substances, which will increase the egg-laying rate of chickens by 15-20% (Kobayashi and Kobayashi, 1995). There have been proposed mechanisms of proteases, which modulate the immune system, in order to control and eliminate tumors. Some studies indicate that proteases are able to remove some adhesion molecules such as CD4, CD44, B7-1, ICAM-1, B7-2, CD45RA, CD6, CD7, E2/MIC2, and Leu81/LAM 1 from cell surfaces. The removal of these surface molecules has markedly enhanced CD2 mediated T-cell activity (http://www.transformationenzymes.com.html/products/ tpp_protease.html, 5/7/2006). Moreover, a protease from *Saccharomyces boulardii* used as probiotics could digest toxin A and B of *Clostridium difficile* (Herbrecht and Nivoix, 2005), and a protease from *L. delbrueckii* subsp. *bulgaricus* strains might also increase growth of bifidobacteria by increasing the amounts of valine, glycine and histidine (Saarela *et al.*, 2000). Thus, proteases not only modulate the immune system, but enhance the hydrolysis of food proteins for enhanced bio-availability of amino acids, which could ultimately supply needed amino acids to the host too.

The aim of this study was to screen the lactic acid bacteria and purple nonsulfur bacteria which have antibacterial activity and/or produce proteinase outside the cells.

2. Materials and methods

2.1 Materials

MRS (Man Rogasa and Sharpe) and Mueller-Hinton broth were from Difco (Becton, Dickinson and Company, USA). Proteinase K and catalase were from SIGMA, USA. All purchased chemicals were analytical grades.

2.2 Isolation of lactic acid bacteria and purple nonsulfur bacteria

The strains used for screening of antibacterial activity of lactic acid bacteria and purple nonsulfur bacteria were isolated from fresh chicken faeces at the chicken-egg farm of Department of Animal Science, Faculty of Natural Resources of the Prince of Songkla University, Hat Yai, as no antibiotics are used in the farm. A total of 108 faeces samples were collected separately from 5-6 months-old chicken. Cultures producing acid on MRS plate were tested for catalase. The Gram-positive and catalase-negative colonies were selected, as were isolates of Gram-negative bacteria with red pigment on GM (Glutamate-malate) plate under micro-aerobic condition with light.

2.3 Pathogenic bacteria

The pathogenic bacteria *Staphylococcus aureus* ATCC 28955, *Salmonella* Typhimurium, *Escherichia coli* ATCC 25922 and *B. cereus* used in this study were from the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok.

2.4 Cultivation of lactic acid bacteria

MRS medium was used for cultivating lactic acid bacteria. The Gram-positive and catalase-negative bacteria were inoculated 1% (v/v) in MRS broth and incubated at 35°C for 18 hr. The culture broth was centrifuged at 12,000 rpm for 5 min. The supernatant was used for antibacterial activity and proteinase assay.

2.5 Cultivation of purple nonsulfur bacteria

Glutamate-malate (GM) medium was used for cultivating purple nonsulfur bacteria. The Gram-negative with red pigment bacteria were inoculated 5% (v/v) in a 15-ml screw-cap tube containing 13 ml of GM medium and incubated under micro-aerobic condition with light (3,000 Lux) at 35°C for 48 hr. The culture broth was centrifuged at 12,000 rpm for 5 min. The supernatant was used for antibacterial activity and proteinase assay.

2.6 Screening of bacteria with antibacterial activity

The antibacterial activity assay was modified from the agar diffusion method of Forbes (Forbes *et al.*, 1990) as follows. An individual active culture of pathogenic bacteria was cross streaked on the MHA to get a single colony. Three to four single colonies of the bacteria were transferred into MHB (Mueller-Hinton broth) and incubated at 37°C for 4-6 hr. The turbidity of cells was then compared with Mc Farland No. 0.5 (about 1.5 x 10^8 cells/ml). One hundred microliter of cell suspension was pipetted into 9.9 ml of melted MHA. The inoculated medium was then overlaid on top of 15 ml of NA (Nutrient agar) plate. The plate was left to dry in laminar flow for 10 min. An 8 mm diameter sterile metal cup was placed on the NA plate, and then 200 μl of the culture supernatant of Gram-positive and catalase-negative bacteria, and Gram-negative bacteria with red pigment each was pipetted into the metal cup and incubated at 37° C for 18 hr. An inhibition zone around the metal cup was measured. This treatment was carried out in duplicate.

2.7 Screening of bacteria producing proteinase outside the cells

Bacteria which could produce proteinase outside the cells were screened by the agar plate method as follows. The Gram-positive and catalase-negative bacteria with antibacterial activity and Gram-negative bacteria with red pigment were both stabbed on the MRS and GM overlaid with skim milk, respectively. Each of them was incubated under different conditions for 24-48 hr. A clear zone around the colony was determined.

2.8 Proteinase assay

The supernatant from culture broth of lactic acid bacteria and purple nonsulfur bacteria mentioned in 2.4 and 2.5 were used for proteinase assay. Proteinase activity was measured by the casein Folin-Ciocalteau assay (Oda and Murao, 1974). Casein solution was first prepared by dissolving 4/3% of casein in 0.1 M Tris-HCl buffer at pH 8.0. Both of 350 μl-enzyme solution and 300 μl-casein solution was warmed at 35°C for 5 min. Duplicate and blank reactions were done. The warmed casein solution was added with 100 μl of the warmed enzyme solution. The reaction mixture was incubated at 35°C for 60 min. Proteianase reaction was stopped by adding 400 μl of 0.44 M TCA. The precipitrated mixture was then centrifuged at 12,000 rpm for 5 min, and 500 μl of supernatant was pipetted into 2.5 ml of 0.44 M Na_2CO_3. The mixture was then added with 500 μl of diluted phenol solution 1:2 ml (v/v) with deionized water, and mixed well. The mixture was incubated in water bath at 35°C for 20 min. One unit of enzyme activity was defined as the enzyme quantity that liberates 1 μg of tyrosine per ml of the reaction mixture per min.

2.9 Screening of acid-resistant strains

The bacterial strains with antibacterial activity and proteinase activity were separately subcultured in MRS broth, in which the pH value of the media was adjusted to 2, 3 and 4 with 1 N HCl and incubated for 3, 12 and 24 hr. The growth of bacteria was determined by measuring the absorbance at 660 nm.

2.10 Screening of bile-resistant strains

The bacterial strains with antibacterial activity and proteinase activity were each subcultured in MRS broth containing 0.3% ox-bile. The MRS broth without ox-bile was used as control. They were incubated for 0, 1, 2, 3, 4 and 24 hr, and determined the growth capability by measuring the absorbance at 660 nm.

2.11 Investigation of the type of inhibitory substances

To investigate the type of inhibitory substances, the effect of temperature, enzymes, pH and organic solvent on the activity of the culture supernatant were determined as follows.

Two ml of culture supernatant of each strains were heated at 70 and 100°C for 10 min, cooled and assayed for antibacterial activity.

The enzymes, proteinase K (EC 3.4.21.14) and catalase (EC 1.11.1.6) were added in the supernatant at a concentration of 1 mg/ml and 10 mg/ml, respectively. Instead of enzyme, the phosphate buffer was used as the control. Both were incubated at 35°C for 80 min and assayed for antibacterial activity.

The culture supernatants of the strains, with the volume of 4.5 ml, were adjusted with 0.5 N HCl and/or 0.5 N NaOH to pH 7.0 to obtain the volume of the culture supernatant, namely 4.75 ml, after which the activity of the samples was determined.

One molar of acetic acid was used instead of culture supernatant in order to determine the effect of organic acid on the antibacterial activity.

2.12 Biochemical and molecular identification of selected strains

Some taxonomic characteristics of bacterial strains were identified according to Kandler and Weiss (1986). In addition, 16S rDNA sequence analysis was carried out for identification of the strains.

Genomic DNA was extracted by using the standard method (Maniatis *et al.*, 1989) and then amplified by GeneAmp PCR System 9600. Universal primers were used: position 27 for forward and 1389 for reverse. The PCR product was 1.3 kb. A partial DNA sequencing was performed by starting at the region of 520 bp. An amplified DNA was sequenced by using API 377 DNA Sequencer. The 16S rDNA sequence had the highest similarity when compared with the NCBI database.

3. Results

3.1 Isolation

A total of 490 strains of Gram-positive and catalase-negative bacteria, and 58 strains of Gram-negative bacteria with red pigment could be isolated from 108 samples of faeces of 5-6 months-old chicken. They were named LB for Gram-positive and catalase-negative bacteria and P for Gram-negative bacteria with red pigment. These were stocked in glycerol and kept at -80°C for further study.

3.2 Screening of bacteria with antibacterial activity

The agar diffusion method was carried out for antibacterial activity assay. The results showed that 167 out of 490 strains of the Gram-positive and catalase-negative bacteria had antibacterial activity against *Staphylococcus aureus* ATCC 28955, *Salmonella* Typhimurium, *Escherichia coli* ATCC 25922, and *B. cereus*. They showed the diameter of clear zone in the range of 18-20 mm (data not shown). The 58 strains of Gram-negative bacteria with red pigment could not produce inhibition zones against any pathogenic bacteria.

3.3 Screening of bacteria producing proteinase outside the cells

Only 10 out of 167 strains of Gram-positive and catalase-negative bacteria which had antibacterial activity were able to produce proteinase outside the cell. All of the 10 strains showed a 2-4 times larger diameter of haloes than those of the colonies (data not shown), but there is none of the Gram-negative bacteria with red pigment showed the haloes. The casein Folin-Ciocalteau assay was carried out for proteinase assay. The 10 lactic acid bacterial strains gave proteinase activity in the range of 0.001-0.2 units. In addition, 15 out of 58 strains of purple nonsulfur bacteria

gave proteinase activity in the range of 0.2-0.8 units. One of them, named P_{41}, purple nonsulfur bacteria, gave the highest proteinase activity, 0.8 units.

3.4 Screening of acid-resistant strains

The ability of the 10 strains to grow in MRS medium with the pH of 2, 3 and 4 was investigated. All strains were able to grow at pH 3 and 4 for 3, 12 and 24 hr, but they could not grow at pH 2 (data not shown).

3.5 Screening of bile-resistant strains

The growth capability of 10 strains in MRS medium containing 0.3% ox-bile was monitored by measuring the absorbance at 660 nm at several times of incubation. The results showed that all 10 strains could grow in the presence of 0.3% bile salt after incubating from 0 to 24 hr (data not shown).

3.6 Investigation of the type of inhibitory substances

The supernatant from 10 strains retained more than 80% of antibacterial activity after heating at 70 and 100 °C for 10 min (data not shown).

The antibacterial activity of the supernatant from 10 strains were lost completely when treated with catalase or adjustment of pH to 7.0 (date not shown).

Treatment with proteinase K did not cause any apparent loss of antibacterial activity (data not shown).

Acetic acid could inhibit the growth of *Staphylococcus aureus* ATCC 28955, *Salmonella* Typhimurium, *Escherichia coli* ATCC 25922 and *B. cereus*.

3.7 Biochemical and molecular identification of selected strains

Some taxonomic characteristics of bacterial strains were identified according to Kandler and Weiss (1986). The taxonomic characteristics of the potent strains, LB_2, LB_4, LB_{13}, LB_{28}, LB_{30}, LB_{90}, LB_{91}, LB_{95}, LB_{107}, and LB_{108}, were practically the same (Table 1). They were positive for all characteristics, but catalase, xylose (sugar fermentation) and H_2S production for all 10 bacterial strains were negative (Table 1). Regarding the 16S rDNA sequence analysis, the results revealed that some of them showed a high homology of 100% correlation to the type strains: LB_2, LB_{13}, LB_{90}, LB 91, LB_{95}, and LB_{107}, *Lactobacillus* sp. 9D10 (Breidt and Plengvidhya, 2006); LB_{30}, *Corynebacterium glutamicum* strain CICC10117 (Liu *et al.*, 2005) (Table 2). In addition, LB_4 and LB_{108} have a 99% high homology to *Bacillus* sp. strain SXQ-2004 (Qiu *et al.*, 2004), and *Corynebacterium glutamicum* strain CICC10178 (Liu *et al.*, 2004), respectively (Table 2). LB_{28} has a 97% high homology to *Enterococcus termitidis* (Svec *et al.*, 2006) (Table 2). The Gram-negative and red-pigmented bacteria named P_{41}, which gave the highest proteinase activity, was identified according to Richard (1984) (Table 3) and 16S rDNA sequence analysis (Table 2). It was as *Enterobacter* sp. sb-3 (Sun, 2006).

4. Discussion

In addition to the Gram-positive and catalase-negative bacteria, Gram-negative bacteria with red pigment could be isolated from 108 samples of chicken faeces. Ten strains out of 167 strains are capable of producing proteinase and amylase (data not shown) as well. The 10 strains out of 490 strains of the lactic acid bacteria had antibacterial activity against *Staphylococcus aureus* ATCC 28955, *Salmonella* Typhimurium, *Escherichia coli* ATCC 25922, and *B. cereus*. They had an inhibitory effect against Gram positive and Gram negative bacteria. Lactic acid bacteria are capable of producing and excreting inhibitory substances to a wide spectrum of microorganisms (De Vuyst and Vandamme, 1994). Therefore the type of the substance needs to be investigated further. The 58 strains of Gram-negative bacteria with red pigment could not produce inhibition zone against any pathogenic bacteria which have not been reported so far.

There were 10 strains of Gram-positive, catalase-negative bacteria with antibacterial activity, and 15 out of 58 strains of Gram-negative bacteria with red pigment which could produce proteinase outside the cells. Many kinds of bacteria can produce various types of extracellular proteinases to degrade proteins in their surroundings (Branden and Tooze, 1999). The present study suggests that all 10 strains of lactic acid bacteria had antibacterial activity and could produce proteinase outside the cells, but 15 strains of Gram-negative bacteria with red pigment had no antibacterial activity, although they could produce proteinase outside the cells.

The 10 strains of Gram-positive and catalase-negative bacteria could grow in the presence of 0.3% ox-bile and at pH 3 for 24 hours. The acid and bile tolerance are two fundamental properties that indicate the ability of a probiotic microorganism to survive the passage through the gastrointestinal tract, resisting the acidic conditions in the stomach and the bile acids at the beginning of the small intestine (Hyronimus *et al.*, 2000; Prasad *et al.*, 1998). It has been reported that the alimentary tract of chicken can let feed pass through within 2.5 hr, which is shorter than in the human and domestic animals' tract (Duke, 1977). Regarding the investigation of the type of inbibitory substances, the results emphasize that antibacterial activity of all supernatant from 10 Gram-positive and catalase-negative bacteria were due to acidity.

Five strains LB_2, LB_{13}, LB_{90}, LB_{95} and LB_{107} out of 10 antibacterial strains were identified as *Lactobacillus* sp. strain 9D10. LB_{91} was identified as *Lactobacillus* sp. strain P23. These are the typical lactic acid bacteria, which could be isolated from a variety of habitats, including manure, humans and animals (Kandler and Weiss, 1986). Only *Lactobacillus* spp. could be isolated from chicken faeces (Niamsup *et al.*, 2003). LB_{28} was identified as *Enterococcus thermitidis*, which is also one of the lactic acid bacteria. In a paper, Pollmann *et al.* reported on the effects of an *Enterococcus faecium* probiotic strain (NCIMB 10415), as a representative of the autochthonous gut flora of pigs, on the rate of chlamydial infection in swine as a possible means to decrease infections of newborn piglets (Pollmann *et al.*, 2005). However, *E. termitidis* used as probiotics or found in chicken faeces is not mentioned.

LB_4 was identified as *Bacillus* sp. SXQ-2004. It is not one of the lactic acid bacteria, but it could produce lactic acid, without forming endospore, which contrasts to the other spore-forming and lactic acid producing bacteria of the genera *Bacillus* (Fritze and Claus, 1995). It could not grow in nutrient broth at pH 6.5 but could grow at a temperature of 37°C (data not shown). LB_{30} and LB_{108} were identified as *Corynebacterium glutamicum* strain CICC10117 and *C. glutamicum* strain CICC10178, respectively. They are not lactic acid bacteria, but they are still Gram-

positive bacteria. There have been reports on glutamic acid (Nakazawa *et al.*, 1996; Bona and Moser, 1997; Fudou *et al.*, 2002), glutamicin CBII-a bacteriocin-like substance produced by *C. glutamicum*- (Pátek *et al.*, 1986), and bacteriocin produced by *Corynebacterium* spp. (Gross and Vidaver, 1979; Abrehem and Zamiri, 1983). Moreover, *C. glutamicum* does produce organic acids such as lactic acid from glucose, which has been already reported (Inui *et al.*, 2004; Okino *et al.*, 2005). Thus, the *Corynebacterium glutamicum* strain CICC10117, which showes antibacterial activity, produces lactic acid or other organic acids and may produce glutamic acid might be a good probiotics for chickens or other avians and useful for food and feed production.

Regarding the biochemical test for the identification of LB_4, LB_{30} and LB_{108}, the catalase was negative, which is opposed to that of Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986). Therefore, the 16S rDNA sequence analysis may be not a good method for all probiotic strains. Molecular typing methods such as plasmid profiling, restriction enzyme analysis (REA), pulse-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), or ribotyping may be alternatives for specific characterization and identification of these strains.

Strain P_{41}, which gave the highest proteinase activity, was identified as *Enterobacter* sp. sb-3. This was an accidental experiment because the strain was Gram-negative and red-pigmented bacteria incubated in GM medium under micro-aerobic conditions with light. Carotenoid gene clusters of *Enterobacteriaceae* strains have been reported recently (Sedkova *et al.*, 2005; Lehner *et al.*, 2006). Although it is not a purple nonsulfur bacterium as we expected, it has red pigment in GM medium under conditions mentioned above, which is opposed to the report on yellow pigment of *Enterobacter sakazakii* (Lehner *et al.*, 2006), and the pathogenesis of the strain is not reported. Hence, this bacterium will be another carotene source, and may possibly be used as food supplement for chickens or other poultry or fowl. In addition, this strain secretes proteinase outside the cell. Therefore, to increase the digestability of food proteins, it might be applicable in chickens food. However, two of the Gram-negative and red-pigmented bacteria without proteinase activity screened in this study, namely P_{12} and P_{72}, were identified as *Rhodopseudomonas palustris strain* NCIB8288 (Accession no. AF416661) and *Rhodopseudomonas palustris* strain DCP3 (Accession no. AF416663) (data not shown), respectively. These purple nonsulfur bacteria are widely distributed in aquatic and some terrestrial habitats naturally (Kobayashi *et al.*, 1967). Thus, these two strains might be used as carotene source as well.

In conclusions, the data obtained in this study about antibacterial activity, proteinase production, acid-resistance and bile-resistance, indicate that all 10 strains of bacteria can be used as a feed supplement for chickens with the additional advantage of controlling infections in chicks. Mono- or mixed cultures of live microorganisms applied to chicken, beneficially affect them by controlling their health. The amount of protein or various kinds of amino acid, vitamins and other substances in the P_{41}, P_{12} or P_{72} will be investigated in a future study. These strains might be applicable for other avians as well.

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Table 1. Some taxonomic characteristics of the bacterial strains

Characteristics	All 10 bacterial strains
Gram stain	+
Catalase	-
Cell shape	rod
Sugar fermentation	
Lactose	+
Maltose	+
D-Galactose	+
Sucrose	+
Raffinose	+
Arabinose	+
Sorbitol	+
Trehalose	+
Glucose	+
Fructose	+
Esculin	+
Xylose	-
Melibiose	+
Mannitol	+
Rhamnose	+
Ribose	+
Growth	
at 20°C	+
at 45°C	+
in 4% NaCl broth	+
H_2S production	-

Symbols: +, positive; -, negative

Table 2. Partial 16S rRNA sequence analysis

Strains	Closet sequence	% similarity	Accession number
LB_2	*Lactobacillus* sp. 9D10	100	DQ682970
LB_4	*Bacillus* sp. SXQ-2004	99	AY590138
LB_{13}	*Lactobacillus* sp. 9D10	100	DQ682970
LB_{28}	*Enterococcus termitidis*	97	AM039968
LB_{30}	*Corynebacterium glutamicum* C1CC10117	100	DQ234080
LB_{90}	*Lactobacillus* sp. 9D10	100	DQ682970
LB_{91}	*Lactobacillus* sp. P23	100	EF100969
LB_{95}	*Lactobacillus* sp. 9D10	100	DQ682970
LB_{107}	*Lactobacillus* sp. 9D10	100	DQ682970
LB_{108}	*Corynebacterium glutamicum* C1CC10178	99	AY794054
P_{41}	*Enterobacter* sp. sb-3	100	EF152284

Table 3. Comparison of the characteristics of *Enterobacter* sp. and P_{41} strain

Characteristics	***Enterobacter* sp.***	**P_{41} strain**
Gram staining	negative	negative
Cell shape	rod	rod
Motility	+	+
Pigmentation	yellow	yellow, red**
Photoautotrophic growth	ND	+
Citrate utilization (Simmons')	+	+
Urease	-	+
H_2S	-	-
Acid from sucrose	+	+
Acid from lactose	$\pm$	-
Indole test	-	-
Methyl red test	$\pm$	+
Voges-Proskauer test	$\pm$	-

Symbols: *, Richard, 1984

**, in GM medium under micro-aerobic condition with light

+, positive; -, negative; $\pm$, positive in some strains but negative in other strains; ND, not determined