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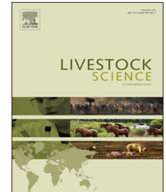

In vitro screening of lactic acid bacteria for multi-strain probiotics

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ABSTRACT

The aim of this research was to screen and evaluate some probiotic properties of lactic acid bacteria strains isolated from 150 animal fecal samples, such as cows, pigs, chickens, and ducks. To achieve an objective, 81 isolates were tested for antimicrobial activity, tolerance to bile salts and acid, potential adhesion to cell surface, and *in vitro* adhesion. The results of the antimicrobial activity against pathogenic bacteria showed that, out of 81 isolates, 61, 59, 43, 78, and 79 isolates had antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella* sp., *Shigella* sp., and *Kiebsiella* sp., respectively. Most lactic acid bacteria (LAB) isolates were tolerant to 1.0% bile salts and were able to survive at pH 3.5 for 3 h. Twelve isolates were then evaluated on their hydrophobic character. Five isolates with probiotic properties were subsequently selected. Sequence analysis of 16S rDNA demonstrated that 2 isolates belong to *Lactobacillus reuteri* (strains P8 and P30), 2 to *Lactobacillus plantarum* (strains P6 and P31), and another one to *Lactobacillus paraplantarum* (strain P25). To assess their viability in broiler chicken diets, 3 strains, *L. plantarum* (strain P6), *L. paraplantarum* (strain P25), and *L. reuteri* (strain P30), containing 10^8 cfu/g were inoculated into a commercial chicken diet in the form of single strain and multi-strain preparations. We found that in all of the treatments, the numbers of viable cells of LAB (cfu/g of diet) were decreased below 10^7 cfu/g after 3 d of incubation. Therefore, based on our study, the single- and multi-strains may have a potential use as novel probiotics in broiler chicken diets.

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1. Introduction

FAO/WHO (2002) defined probiotic as live microorganisms, which when administered in adequate amounts, confer a health benefit to the host. Among those microorganisms, lactic acid bacteria are one of the major groups of probiotics. They are non-pathogenic, belong to the indigenous microflora in the gastrointestinal tract (GIT) in most animals, and maintain an effective balance between beneficial bacteria and harmful bacteria. Currently, widely used probiotic bacteria include lactobacilli and bifidobacteria; however, the use of other strains of lactic acid bacteria, such as *Lactococcus*, *Enterococcus* (Dunne et al., 1999; Salminen et al., 1998), *Propionibacterium* (Grant and Salminen, 1998), *Streptococcus*

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thermophilus (Collins et al., 1998), and *Lactobacillus delbrueckii* subsp. *bulgaricus*, has been reported (Bezkorovainy et al., 1997; Naidu et al., 1999).

The benefits of probiotic bacteria have been reported elsewhere. The production of organic acids and antimicrobial compounds prevents the growth of pathogenic bacteria *via* competitive exclusion (Erkkilä and Petäjä, 2000; Fuller, 1989; Mogensen, 1995). Other health-promoting effects attributed to probiotics are related to their ability to adhere to the intestinal mucosa to modulate the host immune response (Lebeer et al., 2008, 2010; Marco et al., 2006).

Several characteristics have been used to select for potential probiotics. One of the basic requirements is the ability to survive acid and bile conditions in the GIT, and adhesion potential to the intestinal epithelium (Dunne et al., 2001). However, the most common criterion for the selection of probiotic strains is the ability to colonize to epithelial cells and mucosal surfaces of the human/animal GIT. The adhesion properties of probiotic strains are accessed *via* their ability to adhere to human colon carcinoma, as well as *in vitro* hydrophobicity. Lastly, probiotic strains have been evaluated in animal studies to determine whether they are beneficial to their host by its incorporation as dietary adjuncts.

A recent study revealed that the beneficial effects of probiotics are strain-dependent (Galdeano et al., 2010). Thus, the combination of different probiotic strains with selected functions may be more effective than single strain probiotics (Timmerman et al., 2004). However, few studies have investigated the use of multi-strain probiotics as dietary supplements in animal studies (Timmerman et al., 2004). In the present work, resistance against environmental stress (acid and bile salts), antimicrobial activities, adhesion potential to the cell surface in human colon carcinoma, and the survival of probiotic bacteria in chicken feed were investigated.

2. Materials and methods

2.1. Bacterial strains and culture media

The bacterial strains used for the inhibitory tests were *Escherichia coli, Staphylococcus aureus, Shigella* sp., *Salmonella* sp. and *Klebsiella* sp., which were propagated in a Brain-heart infusion (BHI) agar slant (Difco Laboratories, Detroit, MI). All of the strains of lactic acid bacteria (LAB) stock cultures were stored at −20 °C in de Man, Rogosa, and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI) containing 20% (v/v) glycerol, where as other bacterial strains were maintained as frozen stocks at −20 °C in BHI broth containing 10% (v/v) glycerol.

2.2. Lactic acid bacteria isolation

A total of 150 fecal samples from cows, pigs, and chickens from various farms in Thailand were collected for isolation of lactic acid bacteria strains. All of the fecal samples were serially diluted with a sterile 0.85% NaCl solution and were mixed thoroughly. Subsequently, 0.1 mL of each dilution was spread onto MRS with 0.1% $CaCO_3$ and incubated anaerobically for 24 to 48 h at 37 °C. Transparent halo-surrounding colonies were selected and re-streaked onto MRS agar plates to obtain pure cultures. Each pure culture was tested for cell morphology, Gram reaction, and catalase formation by dropping a 3% H_2O_2 solution directly onto each plate. All isolates including the Gram-positive and catalase-negative strains were selected. The other biochemical tests used were cytochrome oxidase and production of acid and gas from 1% glucose (tested in MRS broth at 37 °C for 48 h) (Schillinger and Lücke, 1987). The 16S rDNA was amplified using a standard PCR protocol and universal primers as previously described by Pringsulaka et al. (2011). The PCR products were detected using 0.6% (w/v) agarose gel electrophoresis and visualized under UV light after staining with ethidium bromide (1 μg/mL). The 16S rDNA fragments were purified using gel extraction kit (MinElute Gel Extraction kit; Qiagen, Valencia, CA, US) and sequenced. A similarity search was then carried out in GenBank using BLAST (www.ncbi.nlm.nih.gov/blast). A phylogenetic analysis of the members of *Lactobacillus* was conducted using PAUP* 4.0b (Swofford, 1998) based on the neighbor joining method. The reliability of internal branches of the tree was assessed using the bootstrapping (BS), a statistical method provides assessments of confidence for each clade of an observed tree, with 1000 replicates. Purified cultures were maintained in MRS broth for daily use.

2.3. Evaluation of the probiotic potential

2.3.1. Detection of antimicrobial activity

Escherichia coli, S. aureus, Shigella sp., *Salmonella* sp., and *Klebsiella* sp. (Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand) were used as indicator strains for the detection of antimicrobial activity. Antibacterial activity was determined using the agar spot test as previously described by Schillinger and Lücke (1989). Overnight cultures of the strains to be tested for antagonistic activity were spotted onto the surface of MRS agar plates containing 1.5% agar and incubated for 24–48 h at 30 °C and 37 °C until colony development. Approximately 10^7 cfu/mL of indicator strains were inoculated into soft MRS agar containing 0.5% agar and immediately poured onto previously prepared MRS agar plates. The plates were incubated at 30 and 37 °C for 24 h in a jar under anaerobic conditions. Growth inhibition was then measured. The isolates displaying the largest inhibitory halos were selected for further study.

2.3.2. Growth under unfavorable conditions

To determine growth under unfavorable conditions, strains were cultured in MRS broth supplemented with 0.3 and 1% (w/v) of oxgall to detect growth under high bile-salt conditions. To detect the growth of isolates under low pH, the MRS broth was adjusted to pH 4.0 and 3.5 (by the addition of 1 M HCl). Growth was monitored by measuring the absorbance at $OD_{600\ nm}$ after culturing at 37 °C for 24 h. Standard MRS broth was used as a control (Guo et al., 2010). Data were obtained from 3 independent experiments.

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