HAYATI Journal of Biosciences September 2014
Vol. 21 No. 3, p 144-150
EISSN: 2086-4094

Available online at:
http://journal.ipb.ac.id/index.php/hayati
DOI: 10.4308/hjb.21.3.144

Identification of Antibiotic-Resistance Genes from Lactic Acid Bacteria in Indonesian Fermented Foods

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Received January 29, 2014/Accepted May 14, 2014

Lactic acid bacteria (LAB) are known to have an important role in food fermentation and are thought to have health-promoting abilities such as probiotic properties. In this study, LAB were isolated from Indonesian fermented foods such as dadih (fermented buffalo milk), tempoyak (fermented durian), bekasam (fermented meat), and tape ketan (fermented glutinous rice). Those isolates were investigated for their resistance to two antibiotics: chloramphenicol and erythromycin. Recent efforts in food science have sought to identify genetic markers for antibiotic resistance within LAB strains, so that these genes can be selected for genetic modification. Such research is currently being directed toward the development of food-grade vectors (plasmid). The aim of this study was to screen LAB isolated from Indonesian traditional fermented foods, for chloramphenicol and erythromycin resistance. In this study, a total of 120 LAB samples were taken from traditional Indonesia fermented foods, and were tested for resistance to chloramphenicol and erythromycin. The results show that three LAB strains remained resistant to doses of up to 5 µg/mL chloramphenicol, while the LAB strain *Lactobacillus plantarum* showed resistance to the antibiotic erythromycin up to a concentration of 15 µg/mL.

Key words: antibiotic resistance, fermented food, food grade vectors, lactic acid bacteria

INTRODUCTION

Antibiotic resistance (AR) has become a global concern as a result of increasing use of antibiotics in humans as well as animals. Compounding this risk is the possibility of horizontal transfer among bacteria in nature so that resistant bacteria may spread zoonotically from animals to humans or between animal species and populations. Studies on selection and dissemination of AR have focused mainly on species with medical clinical relevance (Tjaniadi *et al*. 2003; Deashinta *et al*. 2007). However, interest is lately expanding to include studies on food microbes, with greater attention to probiotic lactic acid bacteria (LAB). LAB are of considerable economic significant because of their use in industrial food fermentation processes, especially those that have probiotic properties and are promoted as a "healthy food". Moreover, interest in antibiotic resistance traits as selectable markers for the genetic modification of LAB, is being directed toward generating food grade vectors (Mathur & Singh 2005; Pan *et al*. 2011).

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Fermented foods may be important vehicles for large amounts of living LAB to enter the human body. Although LAB are generally recognized as safe (GRAS), it is necessary to evaluate the antibiotic resistance of these microbes in different fermented foods (Pan *et al*. 2011). Some LAB strains showed acquired transferable antibiotic resistance traits. They may represent a potential source for the spread of AR genes when added to different kinds of probiotic products (D'Aimmo *et al*. 2007). Several LAB species isolated from different fermented foods have been investigated for their resistances to antibiotics, including erythromycin (Tannock *et al.* 1994; Fons *et al.* 1997; Lin & Chung 1999; Gfeller *et al*. 2003; Sudhamani *et al.* 2007) and chloramphenicol (Biet *et al*. 1999; Park *et al*. 2004; Sudhamani *et al*. 2007).

Fermented foods common in Indonesia include dadih (fermented buffalo milk), tempoyak (fermented durian), bekasam (fermented meat), and tape ketan (fermented glutinous rice) as described in Table 1. These have been consumed for centuries, but no investigation has been conducted yet to assess the genes that determine antibiotic resistance in LAB isolated from those traditional foods. The aim of this study, therefore, is to screen antibiotic resistance

Table 1. Indonesian traditional fermented foods and their processing

Fermented foods	Processing
Bekasam	Flesh was mixed with 10-20% salt (w/v) and ground, roasted rice, then fermented (in sealed container) for 14 days
Tempoyak (Durian meat)	Durian (*Durio zibethinus*) flesh was mixed with 2.5% salt (w/v) and placed in a sealed container to ferment for about 7 days
Dadih	Fermented from fresh raw buffalo milk placed in bamboo tubes capped with banana leaves
Tape ketan	Glutinous rice was steamed, followed by inoculation with ragi tape, then fermented for about 1-2 days until an acid-alcoholic taste was achieved

(in this case erythromycin and chloramphenicol) in LAB strains from Indonesian fermented foods, and to identify the genes that can serve as a selectable marker for such resistance. Our results are not only useful for fermented food safety assessment and evaluation, but will also be advantageous in providing basic information to develop and generate LAB cloning vectors.

MATERIALS AND METHODS

Bacterial Strain and Growth Condition. A total of 120 samples of LAB isolated from dadih, tempoyak, bekasam, and tape ketan were obtained from stock cultures taken from the Research Center for Biotechnology Indonesian Institute of Sciences (LIPI). Samples were stored at -70 °C in de Mann Rogosa and Sharpe (MRS) broth in the presence of 20% glycerol. All LAB were maintained by subculturing in MRS (Oxoid, UK) broth medium supplemented with 0.02% (w/v) sodium azide from 1% (v/v) inoculum and incubated overnight at 37 °C.

Determination of Antibiotic Resistance and Bacterial Sensitivity. To determine the phenotypic antibiotic resistance, 1% (v/v) inoculum LAB was grown in MRS broth medium containing 0.02% sodium azide and 1 µg/mL erythromycin (Sigma, USA), and/or 2 µg/mL chloramphenicol (Gold Biotechnology, USA). The culture was incubated overnight (16 h) at 37 °C. LAB which acquire antibiotic resistance are able to grow in medium, and the presence of such resistant LAB will cause a change in the color of medium.

Bacterial sensitivity to antibiotics was also determined by the viable cell count technique. MRS broth medium was treated in different dishes with different concentrations of erythromycin (Sigma, USA) and/or chloramphenicol (Gold Biotechnology, USA). Each dish was then inoculated with 1% (v/v) of each sample of LAB and incubated overnight at 37 °C. The LAB cultures were serially diluted in a 1:10 ratio. Next, 100 µL of appropriate dilutions were spread on agar plates and the plates were incubated at 37 °C for 24-48 h. If colonies appeared on an agar plate, that LAB sample was classified as antibiotic resistant.

DNA Extraction. LAB isolates from the strains classified as antibiotic-resistant were cultured in MRS broth (pH 7.0) for 24 h. Bacterial cells were collected by centrifugation at 6,000 rpm for 10 min. The genomic or chromosome DNA was extracted as previously described with modification (Zhu *et al*. 1993). The pellet was resuspended with TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) containing 60 mg/mL lysozyme and then incubated at 37 °C for 1 h. After incubation, 200 µL of 10% sodium dodecyl sulfate, 100 µL of 5 M NaCl and 80 µL of 10% CTAB were added. The mixture was then incubated at 68 °C for 30 min and an equal amount of chloroform (1:1, v/v) was added. Centrifugation was conducted at 13,000 rpm for 10 min. The supernatant was collected and 1:1 (v/v) ethanol was added. The mixture was inverted and then centrifuged at 13,000 rpm for 10 min. After being air-dried, the DNA was dissolved in TE buffer containing 10 µg/mL RNAse and was stored at -20 °C until use.

Plasmid DNA was extracted from the LAB samples using plasmid MiniKit (Qiagen, USA) according to manufacture's protocol. However, lysis cells where modified using lysozyme as mentioned above.

PCR Amplification for 16S rRNA Identification. For 16 S rRNA sequencing, primers 8F (5-AGAGTTT GATCATGGCTCAG-3; positions 8 to 27) and 15R (5-AAGGAG GTG ATC CAA CCG CA-3; positions 1541 to 1522) were used to amplify the full length of bacterial 16S rRNA (Chao *et al.* 2008). For each 25 uL PCR, we used 400 nM of each primer, 1 U of Taq polymerase, and 10 ng of the genomic DNA template. The PCR conditions were 96 °C for 5 min; 35 cycles consisting of 96 °C for 1 min, 55 °C for 3 min, 72 °C for 1 min, 72 °C for 7 min. The PCR products were subjected to gel electrophoresis in 1% agarose gel, followed by ethidium bromide staining.

PCR Detection of Antibiotic Resistance Genes. Chromosomes and plasmid DNA were used