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Antimicrobial susceptibility

Antagonistic activity against pathogenic bacteria by human vaginal lactobacilli

Supayang Piyawan Voravuthikunchai[a,b,*], Sopa Bilasoi[a], Orawan Supamala[a]

[a]*Department of Microbiology, Faculty of Science, Prince of Songkla University, Hatyai, Songkla 90112, Thailand*
[b]*Natural Products Research Unit, Faculty of Science, Prince of Songkla University, Thailand*

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Abstract

This study attempted to isolate lactobacilli strains from healthy vaginal ecosystem to search for a new effective antibacterial probiotic strain. The strains were identified and characterized for their probiotic properties including bile salt and acid tolerance, growth at acidic pH, their ability to utilize protein, starch, and lipid, the production of hydrogen peroxide and bacteriocin as well as their antibiotic resistance patterns. The antibacterial activity of the culture supernatant of these strains were tested against a wide range of Gram-positive and Gram-negative pathogenic bacteria including *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae. Salmonella typhi*, and *Salmonella typhimurium*. None of the strains inhibited the growth of Gram-negative bacteria. Contrastly, the culture supernatant of strain L 22, identified as *Lactobacillus reuteri*, significantly inhibited all of the clinical isolates of methicillin-resistant *S. aureus* (MRSA). The antibacterial effect of the selected strain L 22 was further investigated. In the presence of L 22, the bacterial growth was assessed in vitro by viable bacterial counting. The numbers of viable cells were significantly lower in L 22-containing broth than those in the control by 6 h. This finding clearly demonstrates that strain L 22 can produce an anti-MRSA effect. The antibacterial ability of the strain L 22 was fundamentally attributed to their bacteriocin production which can cause both cell inhibition and cell death.

Keywords: Probiotic; Bacteriocin; Lactocin; Methicillin-resistant *Staphylococcus aureus*; Antibacterial agent; Lactobacillus; Vaginitis; Gastrointestinal disorder

1. Introduction

It has been well-established that probiotics including *Lactobacillus* spp. [1–5], *Bifidobacterium* spp. [6–8], *Enterococcus* spp. [9] *Escherichia coli* [10], *Leuconostoc* spp. [11], *Pediococcus* spp. [12], *Saccharomyces cerevisiae* [13], and *Streptococcus* spp. [14] can inhibit the growth of a wide range of pathogenic bacteria.

Lactic acid bacteria have a number of properties which render them highly suitable for probiotic therapeutics that are of pharmaceutical interest. They are the predominant micro-organisms isolated from healthy human urogenital ecosystem [15–17]. They play a significant physiological role in the maintenance of the ecological balance mainly because their lactic acid production is responsible for low pH level in the tracts [18,19]. In addition, they also produce many other inhibitory substances such as hydrogen peroxide (H_2O_2) [19], bacteriocins or lactocins [18,19], and some organic acids [18]. Other mechanisms proposed for their microbial antagonism are competition for nutrients [20,21], adhesion inhibition of pathogens to surfaces [21,22], and stimulation of the immune system [23–25].

The rapid emergence of drug-resistant strains and chronic toxicity [26,27] following the widespread use of antibiotics encourages us to study alternative treatment for bacterial infection. We attempted to isolate lactic acid producing strains from healthy human vaginal ecosystem. These strains were identified and initially tested for their probiotic properties. The inhibitory effect of these strains on both Gram-positive and Gram-negative pathogenic

*Corresponding author. Department of Microbiology, and Natural Products Research Unit, Faculty of Science, Prince of Songkla University, Hatyai, Songkla 90112, Thailand. Tel./fax: +66 74 446661.
E-mail address: supayang.v@psu.ac.th (S.P. Voravuthikunchai).

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bacteria was further investigated. In addition, the in vitro antibacterial ability of the selected strain was evaluated against a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA).

2. Materials and methods

2.1. Isolation and identification of lactobacilli from vaginal specimens

Vaginal specimens were obtained from 30 women between the ages of 25 and 45 years with healthy vaginal ecosystems. Lateral vaginal walls were swabbed with sterile cotton-tipped applicators (Copan Diagnostics Inc, Corona, CA, USA). Lactobacilli were isolated by inoculating on de Man-Rogosa Sharpe agar (MRS agar, Difco, Detroit, USA) with 0.3% bile oxgall (Sigma, Lousiana, USA) and 0.2% bromocresol purple (Merck, Darmstadt, Germany), incubated anaerobically at 37 °C for 48 h using an anaerobic jar (BBL, Cockeyville, MD, USA) containing anaerobic pak (Code No. BR 38, Oxoid). Identification of *Lactobacillus* species was performed by phenotypic criteria. All isolates were initially tested for colony morphology, Gram reaction, catalase activity, motilily test, and gas production from glucose. They were further characterized by their carbohydrate fermentation [28].

2.1.1. Detection of hydrogen peroxide

Test was carried out on MRS agar with 5 mg/ml of hemin, 1 mg/ml of vitamin K, 0.01 mg/ml of horseradish peroxide, and 0.25 mg/ml of tetramethylbenzidine (Sigma, LA, USA) [29] as indicator. One loop of each isolate was spotted on MRS agar, incubated anaerobically at 37 °C for 48 h. The culture was subsequently exposed to ambient air at room temperature for 24 h. The horseradish peroxidase oxidized the tetramethylbenzidine in the presence of H_2O_2 to form a blue pigment. Those did not produce H_2O_2 did not form a blue colour in the medium.

2.1.2. Low pH tolerance

Growth was observed in MRS broth (Difco, Detroit, USA) adjusted to different pH (1–5).

2.1.3. Salt tolerance

Test with HCl was performed in MRS broth supplemented with NaCl (1–5%).

2.1.4. Growth at different temperature

Growth was observed in MRS broth after incubated anaerobically at 15 and 45 °C for 48 h.

2.2. Determination of antibiotic resistance

Antibiotic discs (Oxoid, England) were employed to determine the pattern of antibiotic resistance of lactobacilli strains. The discs included ampicillin (10 μg), erythromycin (15 μg), gentamycin (10 μg), kanamycin (30 μg), penicillin G (10 units), and tetracycline (30 μg). Inhibition zones were recorded and compared to standard values [30].

2.3. Indicator strains

Three clinical isolates of Gram-positive MRSA (MRSA 01–03) were obtained from Hatyai hospital, Thailand. *S. aureus* ATCC 25923 was used as a reference strain. Gram-negative bacteria included two strains of *E. coli* O157: H7 (RIMD 05091078 and RIMD 05091083), other Shiga-like toxin-producing strains: *E. coli* 026: H11 (RIMD 05091055), *E. coli* O111: NM (RIMD 05091056), and *E. coli* O22 (RIMD 05091556). These strains were kindly provided by the Research Institute for Microbial Diseases, Osaka University. Other common Gram-negative pathogens used in this study were *Klebsiella pneumoniae* (PSSCMI 0031), *Salmonella typhi* (PSSCMI 0034), and *Salmonella typhimurium* (PSSCMI 0035).

2.4. Detection of bacteriocin production

The antibacterial activity was assayed against of Gram-positive and Gram-negative bacteria. The indicator strains were incubated in Mueller–Hinton broth (MHB, Difco, Detroit, USA) at 37 °C for 24 h. Approximately, 10^4 CFU/ml were then streaked on Mueller–Hinton agar (MHA, Difco, Detroit, USA). For the detection of antibacterial activity, the lactobacilli strains were cultured in MRS broth, incubated anaerobically at 37 °C for 48 h.

A cell-free filtrate was obtained by centrifugation at 5000*g* for 20 min, the supernatant was filtrated through a 0.2 μm pore size cellulose acetate filter (Schleicher and Schull, Germany). The supernatants were neutralized with 1N NaOH to pH 6.5. Inhibition by H_2O_2 was eliminated by the addition of catalase (5 mg/mL). A 20 μL sample of each supernatant was filled on a 6 mm disc and placed on top of the inoculated MHA. Discs filled with sterile MHB were included as controls. Inhibition zones were measured after incubating the plates at 37 °C for 48 h.

2.5. Antagonistic effect of lactic acid bacteria against methicillin-resistant S. aureus (MRSA).

MRSA and *S. aureus* ATCC 25923 were grown in trypticase soy broth (TSB, Oxoid, Basingstoke, England). Equal volumn (2 mL) of lactic acid producing-bacteria and the indicator strain were co-cultured. The tubes were incubated with shaking at 200 rpm at 37 °C. Viable cells were counted by plating on mannitol salt agar (MSA, Oxoid, Basingstoke, England) at different time postincubation. All tests were performed in duplicate.