Characterization of *Weissella kimchii* PL9023 as a potential probiotic for women

Yeonhee Lee *

*Culture Collection of Antimicrobial Resistant Microbes, Department of Biology, Seoul Women's University, Seoul 139-774, Korea*

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Abstract

From more than 100 lactic acid bacteria, *Weissella kimchii* PL9023 was selected as producing the most hydrogen peroxide and was characterized as a probiotic for women. *W. kimchii* PL9023 inhibited the growth and adherence of vaginal isolates of *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus agalactiae*. The surface components involved in adherence of *W. kimchii* PL9023 to vaginal epithelial cells appeared to be glycoproteins, as determined by susceptibility to proteinase K, heat, or periodate. *W. kimchii* PL9023 did not produce harmful metabolites or enzymes. The results of this study suggest that *W. kimchii* PL9023 is a potential probiotic for vaginal health.

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1. Introduction

Lactic acid-producing bacteria (LAB) have long been considered to constitute the primary microbiological barrier to infection by urogenital pathogens. They exert a protective role mainly through a combination of steric exclusion and inhibitory substance production [1]. An average healthy premenopausal woman has $10^7$–$10^8$ CFU of LAB in 1 g vaginal fluid. *Lactobacillus crispatus*, *L. fermentum*, *L. gasseri*, *L. iners*, *L. jensenii*, *L. pentosus*, *L. plantarum*, *L. vaginalis*, and *Weissella viridescens* have been detected in Western women [2–4]. Most of these species of LAB have rarely been found in Korean women suggesting regional differences in vaginal microflora. To develop a probiotic for Korean women, it was hypothesized that LAB isolated from Korean women would easily colonize in Korean women. Since hydrogen peroxide-producing LAB are predominant in the vaginal tract of healthy women [5,6], a vaginal isolate from Korean women that produced the most hydrogen peroxide was selected and characterized in terms of various activities pertinent to its application as a probiotic for vaginal health.

2. Materials and methods

2.1. Isolation of hydrogen peroxide-producing LAB

Hydrogen peroxide-producing LAB were detected using a modified version of the method developed by Rabe and Hillier [7]. LAB were inoculated on TMB-Plus agar containing horseradish peroxidase and 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB)
and incubated at 37 °C for 48–72 h in an anaerobic environment. After incubation, the plate was exposed to air for 30 min; then, the oxidation of TMB by peroxidase in the presence of hydrogen peroxide produced blue pigments on the colonies. Blue colonies were selected, and the amount of hydrogen peroxide produced by each colony was measured. After culture overnight in Man–Rogosa–Sharpe (MRS) broth (Difco, Sparks, MD), bacterial cells were collected by centrifugation, washed with Brucella broth (Difco), and suspended in Brucella broth to an OD of 4 (at 600 nm). After incubation for 4 h, the amount of hydrogen peroxide produced by 50 μl of the culture supernatant was assayed using an Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes, Eugene, OR). The isolate (PL9023) producing the most hydrogen peroxide was selected and characterized as a potential probiotic.

2.2. Identification of Weissella kimchii PL9023 and preparation of bacterial cells

W. kimchii PL9023 was originally isolated from the vagina of a healthy premenopausal woman. This isolate was identified as L. coprophilus (73.3%) according to Bergy's manual of systematic bacteriology using an API50CH kit (Bio-Merieux, Marcy-l’Etoile, France). Sequencing of its 16S rDNA was performed using unieus (CCARM 3707), and preparation of bacterial cells Candida albicans following the procedure of others [11]. Vaginal isolates of S. aureus (CCARM 1428), Staphylococcus aureus (CCARM 3707), and Escherichia coli (CCARM 1428) were grown in potato dextrose agar (Difco), mannitol salt agar (Mast Group, Merseyside, UK), and MacCONKEY agar (Mast Group), respectively. Vaginal isolates of Streptococcus agalactiae (CCARM 4506) were cultured in blood agar (Difco) in a 10% CO2 incubator.

2.3. Overlay method to detect the inhibitory activity on growth of vaginal isolates

An aliquot (10 μl) of an extract of ethyl acetate of the culture supernatant of W. kimchii PL9023, avoiding the effect from pH and hydrogen peroxide, was laid on each pathogen in the early stationary phase, after inoculation by a swab with Muller Hinton agar (Difco). After culture overnight, the growth-inhibition zone was measured. The extract was prepared by extracting equal volumes of culture supernatant and ethyl acetate, which then underwent evaporation and dispersion in distilled water (1/100 of the original volume).

2.4. Preparation of vaginal epithelial cells

Vaginal epithelial cells were prepared as described by Wood et al. [12]. In brief, vaginal epithelial cells were obtained from three consecutive scrapings from the lateral vaginal wall, using a new pop brush for each scraping, from a healthy premenopausal woman. Cells were dispersed in 10 ml minimal essential medium (MEM; GIBCO BRL, Grand Island, NY) at various pHs. Indigenous bacteria were removed from cells by shaking in 10 ml MEM for 30 s and by centrifugation (750g) for 5 min three times. Then, cells were filtered through a membrane filter (8 μm, mixed cellulose acetate and nitrate; Millipore Corporation, Bedford, MA) and dispersed in MEM, at a final concentration of 2 × 10⁴ cells ml⁻¹.

2.5. Adherence of W. kimchii PL9023 to vaginal cells

Adherence of W. kimchii PL9023 to vaginal cells was assayed as described by Boris et al. [13]. The same volumes of W. kimchii PL9023 cells (10⁶ cells ml⁻¹ in MEM) and vaginal cells (2 × 10⁴ cells ml⁻¹ in MEM) were mixed in an orbital shaker (100 rpm) for 30 min at 37 °C. The mixture was passed through a membrane filter (8 μm) and washed with the same volume of MEM. Cells retained on the filter were placed on an albumin-coated microscope slide (Fisher) and were fixed with 97% methanol. Cells were observed under a light microscope after Gram staining. The number of bacterial cells bound to one vaginal cell was calculated by averaging the numbers of bacterial cells that had adhered to >60 vaginal cells. To observe the effect of pH on adherence, the pH of the medium was adjusted to 4.4, 6.0 and 7.2 with 0.1 N HCl.

2.6. Hydrophobicity determination

The hydrophobicity and ionic characteristics of the cell surface of W. kimchii PL9023 were determined by the bacterial adhesion to hydrocarbons (BATH) method [14] and the microbial adhesion to solvents (MATS) method [15]. In brief, bacterial cells at the stationary phase were harvested by centrifugation at 5000g for 10 min and were washed twice and suspended in 0.1 M KNO₃ (pH 6.2) to an OD of 1.0 (at 600 nm) (~×10⁸ CFU ml⁻¹). The cell suspension (1.2 ml) was mixed with 0.2 ml of each solvent for 2 min. After phase separation (~15 min), the OD of the aqueous phase was measured at 600 nm. The relative hydrophobicity (RH) of cells was calculated using the following equation:

\[ \text{RH} (%) = (1 - \frac{\text{OD}_{\text{after}}}{\text{OD}_{\text{before}}}) \times 100 \]

(OD_after indicates the OD at 600 nm after extraction, and OD_before indicates the OD at 600 nm before extraction).
2.7. Biochemical treatment to determine the cell-adhesion factor

W. kimchii PL9023 cells were treated for 1 h at 37 °C with 1 mg ml⁻¹ (final concentration) proteinase K to remove proteins or with 1 mg ml⁻¹ (final concentration) lipase to remove lipids and then were washed twice with PBS (pH 7.2). Alternatively, cells were treated with 100 mM (final concentration) sodium periodate in the dark at 4 °C overnight to block carbohydrate moieties, and then washed three times with phosphate buffered saline (PBS, pH 7.2). Bacterial cells were heated in boiling water for 10 min and were washed twice with PBS to denature the proteins. Treated bacterial cells were added to vaginal cells, and the number of adhered W. kimchii PL9023 cells was counted as described above.

2.8. Aggregation test

W. kimchii PL9023 and C. albicans were prepared as described above and suspended in pH-adjusted MEM. Incubation was in an orbital shaker (100 rpm) at 37 °C. After 4 h, bacterial cells were Gram-stained and observed under a light microscope.

2.9. Interference assay

After W. kimchii PL9023 (10⁸ CFU ml⁻¹) and vaginal epithelial cells (2 × 10⁶ cells ml⁻¹) were mixed for 30 min, each pathogen (1–2 × 10⁶ CFU ml⁻¹) was added to the mixture, which was incubated for another 30 min to assay the interference activity of W. kimchii PL9023 on pathogen binding to vaginal epithelial cells. To assay competition activity, W. kimchii PL9023, each pathogen, and vaginal epithelial cells were mixed at the same time and incubated for 30 min. To assay displacement activity, each pathogen was incubated with vaginal epithelial cells 30 min before W. kimchii PL9023 was added. After incubation, vaginal epithelial cells were treated and observed as described above.

2.10. Statistical analysis

Each experiment was performed in triplicate and statistical analyses were performed using SPSS, version 10.0 (SPSS Inc., Chicago, IL).

2.11. In vitro safety test of W. kimchii PL9023

Published methods were used to detect various virulence factors, such as the production of amine [16], ammonia [17], and indole [18], the degradation of gelatin [19] and the presence of β-glucuronidase [20] and nitroreductase [21].

3. Results

3.1. Isolation and identification of W. kimchii PL9023

Of >100 LAB isolates, several produced blue-colored colonies on TMB-Plus medium. Of these, every vaginal isolate of W. kimchii produced hydrogen peroxide in the quantitative assay and W. kimchii PL9023 produced the largest amount of hydrogen peroxide (96.86 μM) while the strain type (W. kimchii CHJ3 KCTC3746) produced 38.71 μM. W. kimchii PL9023 was originally isolated from a healthy premenopausal woman’s vagina and was identified as L. coprophilus with API 50CH (bio-Mérieux, Marcy l’Etoile, France). The sequence of 16S rRNA matched 100% with W. kimchii (GenBank accession no. AY190629) and was deposited in the Korea Agriculture Culture Collection as KACC 91139. L. coprophilus was a synonym of L. confusus [22] and was then renamed as Weissella confusa [23], which was recently branched into W. kimchii [10].

3.2. Biochemical properties of W. kimchii PL9023

An extract of the spent culture supernatant of W. kimchii PL9023 inhibited the growth of various pathogens, including E. coli, S. aureus, Strep.agalactiae, and C. albicans (data not shown).

When the RH of the bacterial cell surface was assayed by the BATH method, RH values were 95.77 ± 0.32% in hexadecane and 85.34 ± 4.79% in xylene, suggesting high hydrophobicity of the bacterial cell surface. When the ionic characteristics were determined by the MATS method, a value of 94.42 ± 0.92% in chloroform showed that W. kimchii PL9023 has a strong affinity to chloroform-acidic electron acceptors. A small RH value (45.70 ± 2.27%) in ethyl acetate confirmed the non-acidic and electron donor properties of the cell surface of W. kimchii PL9023.

W. kimchii PL9023 was negative for gelatin liquefaction, ammonia production, indole production, phenylalanine degradation and the presence of β-glucuronidase and nitroreductase.

3.3. Adhesion properties of W. kimchii PL9023 to vaginal cells

W. kimchii PL9023 bound to vaginal epithelial cells at various pHs. The number of adhered bacterial cells (dark purple color) to vaginal cells (light pink color with a dark pink colored nucleus) increased at a lower pH (Fig. 1). Lipase treatment did not affect the binding of W. kimchii PL9023 to vaginal epithelial cells. When protein was removed by proteinase K or by denaturation with heat, the binding decreased significantly. Periodate treatment to modify carbohydrate moieties also
decreased binding, suggesting that glycoprotein is the cell-adhesion factor (Fig. 2).

3.4. Aggregation test

Self-aggregation has been suggested to be one of the factors in the constitution of the microflora [24]. As shown in Fig. 3(a), W. kimchii PL9023 showed the best self-aggregation at pH 4.0. When W. kimchii PL9023 was mixed with various uropathogenic pathogens in cell culture medium at various pHs, it aggregated with C. albicans at all three pHs. Large purple cells of C. albicans in yeast form, as well as hyphal form, that were surrounded by W. kimchii PL9023 were easily observed under the light microscope (Fig. 3(b)). Whereas W. kimchii PL9023 did not aggregate significantly with other uropathogenic pathogens.
3.5. Interference assay

As shown in Fig. 4, W. kimchii PL9023 significantly decreased (P < 0.001) the adherence of C. albicans to vaginal cells at pH 4.4, 6.0 and 7.2 in all three tests. Regarding the adherence of E. coli and S. agalactiae, a significant decrease induced by W. kimchii PL9023 was observed only at pH 6.0. Since neither E. coli nor S. agalactiae bound well to vaginal cells at pH 4.4, it was difficult to observe any change. W. kimchii PL9023 significantly decreased (P < 0.001) the adherence of S. aureus at pH 4.4 in the competition and exclusion tests. W. kimchii PL9023 significantly decreased (P < 0.001) the adherence of S. agalactiae at pH 6.0 in the exclusion and displacement tests.

4. Discussion

It has been well documented that specific strains of LAB exhibit antagonistic activity toward various human intestinal pathogens, producing antimicrobial compounds such as lactic and acetic acids, hydrogen peroxide and bacteriocins, as well as compounds not yet identified [25]. The capability of LAB to adhere to vaginal epithelial cells is also an important factor in the formation of a barrier that prevents the colonization of pathogenic bacteria at these sites [26,27]. The binding of LAB to cells involves non-specific factors, especially hydrophobicity [13], surface charge [14], and specific moieties on the surface such as protein and carbohydrates [28]. The basic properties of the cell surface can create Lewis acid-based interactions with acceptors on vaginal epithelial cells [15].

Hydrogen peroxide producing activity has been suggested as the main protective factor from vaginal pathogens [29]. Every vaginal isolate of W. kimchii in this study produced hydrogen peroxide suggesting hydrogen peroxide producing activity may be a common characteristic of W. kimchii. In addition to the hydrogen peroxide producing activity, W. kimchii PL9023 produced bacteriocin-like substances which inhibited the growth of various vaginal pathogens. W. kimchii PL9023 aggregated with C. albicans at various pHs and prevented C. albicans from binding to vaginal cells. Coaggregation must be advantageous for W. kimchii PL9023 to exert a direct inhibitory effect on C. albicans.

High RH and basic characteristics of the cell surface of W. kimchii PL9023 could have an advantage in binding to vaginal epithelial cells, especially at low pH. The adhesion factor for W. kimchii PL9023 is thought to be glycoprotein, since binding decreased when protein was removed or denatured and carbohydrate moieties were modified.

Since W. kimchii PL9023 can inhibit the growth of various vaginal pathogens while producing no harmful metabolites or enzymes, it can be included in a formula for local application in the vaginal tract [29] or in an oral probiotic [30].

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References


