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Weissella confusa Strain PL9001 Inhibits Growth and Adherence of Genitourinary Pathogens

LEE, YEONHEE*

Department of Biology, Seoul Women's University, Seoul 139-774, Korea

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Abstract The capability of lactic acid bacteria (LABs) to adhere to intestinal epithelial cells and vaginal epithelial cells is an important factor in the formation of a barrier to prevent the colonization of pathogenic bacteria. In addition, the ability to coaggregate with pathogens and production of antimicrobial agents also allow LABs to fight against pathogens. In this work, *Weissella confusa* PL9001 was tested for its ability to inhibit the growth and adherence of genitourinary pathogens, including *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*, and vancomycin-resistant *Enterococcus faecium* (VRE), isolated from the urine of hospitalized female patients. *W. confusa* PL9001 was found to coaggregate with the four pathogens, as observed with a light microscope and scanning electron microscope. In competition, exclusion, and displacement tests, the adherence of the pathogens to T24 bladder epithelial cells was also inhibited by *W. confusa* PL9001. Accordingly, these results suggest that *W. confusa* PL9001 is potentially useful for both preventive and therapeutic treatment of genitourinary infections.

Key words: Adherence, bladder cells, coaggregation, urinary tract infection, *Weissella confusa*

Urinary tract infections (UTIs) include infections of the kidneys, urethra, and bladder and are mainly caused by bacteria. These infections are also more common in women, as women have a shorter duct than men for draining their bladder proximal to the vagina that acquires pathogens from the rectum. Also, UTIs are especially common among hospitalized patients and post-menopausal women [14]. UTIs are usually treated with antibiotics for three to seven days and occasionally one day of therapy. However, such antibiotic treatment has many side effects and should not be used for prophylaxis or health maintenance. Thus, since the emergence of virulent and multi drug resistant pathogens [30], safer and more effective prophylactic methods for UTIs are urgently needed.

The best candidate to replace antibiotics is probiotics, lactic acid producing bacteria (LABs) [23, 27, 28]. Recently, several researchers have shown that LABs exhibit an inhibitory activity towards voice prostheses [9], surgical wounds [10], and stomach ailments [24]. *Lactobacillus acidophilus* [7], *L. casei* [2, 32], and *L. fermentum* [11] have all produced successful results in preventing and treating UTIs in humans and model animals. Yet, these abilities are strain dependent [11] and their exact mechanisms have not been fully identified.

In the present work, *Weissella confusa* PL9001, originally isolated from baby feces and recently developed as a probiotic with inhibitory activity towards *Helicobacter pylori* [24], was tested as a possible probiotic for UTIs.

Materials and Methods

Microbial Strains

W. confusa PL9001 [24] was cultured in MRS (Difco, Sparks, U.S.A.). *Candida albicans* (CCARM14020), *Escherichia coli* (CCARM 1360), *Staphylococcus aureus* (CCARM 3703), and vancomycin-resistant *Enterococcus faecium* (VRE; CCARM 5100) were all isolated from the urine of female patients hospitalized in Guro Hospital, Korea University, Seoul, Korea and cultured in a Brain Heart Infusion (BHI; Difco) medium.

Overlay Method to Detect Inhibitory Activity Towards Pathogen Growth

W. confusa PL9001 (1×10^8 CFU) and each pathogen (1×10^7 CFU) were co-cultured in 50 ml of BHI medium. After 6 h incubation, the number of each pathogen was calculated using the most probable number (MPN) method based on inoculating the mixture onto MacConkey agar, mannitol salt agar, or Enterococcosal agar for selective growth of *E. coli*, *S. aureus*, or VRE, respectively. Meanwhile,

*Corresponding author
Phone: 82-2-970-5664; Fax: 82-2-970-5901;
E-mail: yhlee@swu.ac.kr

W. confusa PL9001 (1×10^8 CFU) and *C. albicans* (1×10^6 CFU) were co-cultured in 50 ml of yeast extract peptone dextrose broth and the CFU of *C. albicans* counted on a potato dextrose agar.

Ethyl Acetate Extraction of Culture Supernatant

To avoid the effect of a low pH, organic acid, and hydrogen peroxide, an aliquot (10 µl) of an ethyl acetate extract of the culture supernatant of *W. confusa* PL9001 was laid on each pathogen inoculated onto Muller Hinton solid medium (Difco). After culturing overnight, the growth inhibition zone was observed. The ethyl acetate extract was prepared by extracting the culture supernatant with the same volume of ethyl acetate, evaporating, and dispersing in distilled water (1/500 volume of the original volume).

Coaggregation Test

After culturing overnight, *W. confusa* PL9001 and pathogens were collected by centrifugation and dispersed in saline, making A_{600}=0.6. *W. confusa* PL9001 and each pathogen (1:1 v/v) were then incubated in an orbital shaker at 100 rpm for 4 h at 37°C. A drop of the mixture was placed on a glass slide and observed under a light microscope after Gram staining or observed with a scanning electron microscope (SEM; JSM-5200, Jeol, Tokyo, Japan). To induce hyphal forms (H-form), *C. candida* was incubated in bovine serum albumin (FBS) overnight.

Cell Culture

T24 human bladder epithelial cells, purchased from the Korean Cell Line Bank (Seoul, Korea), were incubated in RPMI 1640 (GIBCO BRL, Grand Island, U.S.A.) containing 10% fetal bovine serum (GIBCO BRL) and 1% antibiotic-antimycotics (GIBCO BRL) on a 30-mm plate under 5% CO_2 at 37°C. The cells were only used in experiments when the plate became 70-80% confluent with cells. The medium was changed every other day.

Adherence Assay

To test the adherence, *W. confusa* PL9001 (1×10^8 CFU) or each pathogen (1×10^7 CFU) suspended in 100 µl saline was added to the confluent T24 cells and incubated for 30 min at 37°C. The cells were then washed with phosphate-buffered saline to remove any unbound bacteria, fixed with absolute methanol for 10 min, Gram stained, and observed under a light microscope.

Interference Assay

For the exclusion test, *W. confusa* PL9001 (1×10^8 CFU) in 100 µl saline was added to the confluent T24 cells and incubated for 30 min. Then, each pathogen (1×10^7 CFU) in 100 µl saline was added to the mixture and incubated for an additional 30 min. For the competition test, each pathogen and *W. confusa* PL9001 were added to the T24 cells at the same time and incubated for 30 min. For the displacement test, each pathogen was incubated with the T24 cells 30 min before *W. confusa* PL9001 was added.

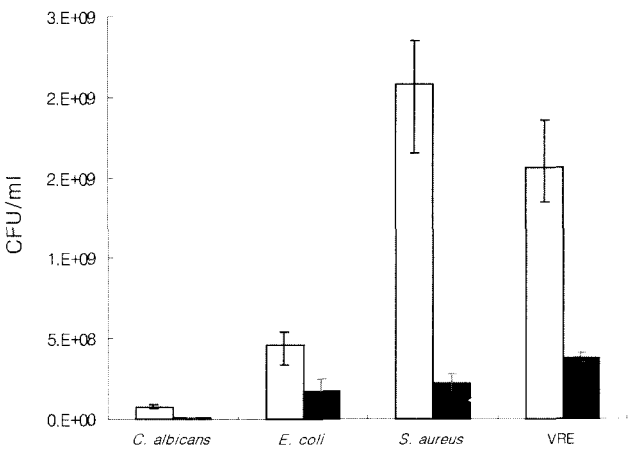

Fig. 1. Growth inhibition of genitourinary pathogens by *W. confusa* PL9001.
W. confusa PL9001 was co-cultured with UTI pathogens. After 6 h, the CFU for each UTI pathogen was calculated using the MPN method. The experiments were performed in triplicate.

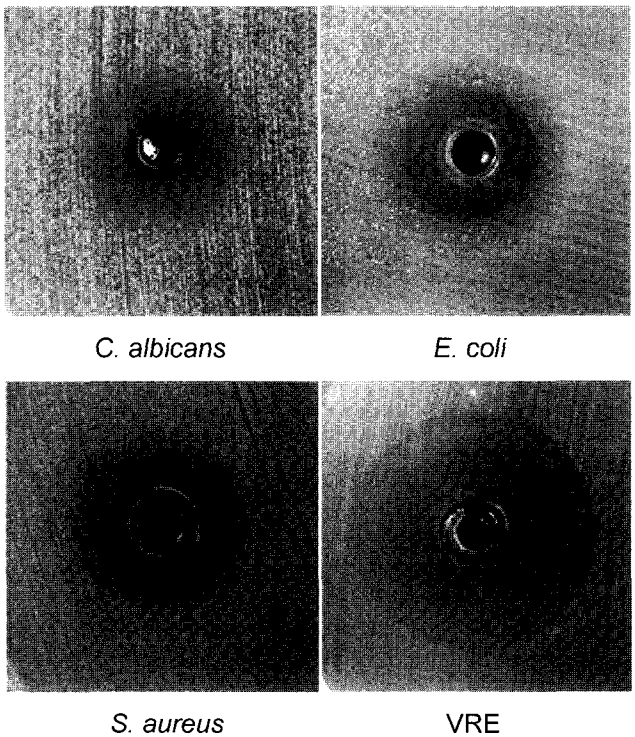

Fig. 2. Growth inhibition zone of UTI pathogens formed by *W. confusa* PL9001.
An aliquot of an ethyl acetate extract of the culture supernatant of *W. confusa* PL9001 was laid on *C. albicans*, *E. coli*, *S. aureus*, and vancomycin-resistant *E. faecium* inoculated onto BHI solid media, and the growth inhibition zone observed was after culturing overnight.

Table 1. Numbers of UTI pathogens adhering to T24 cells in 20 fields in adherence, exclusion, displacement, and competition tests.

	Adherence	Exclusion	Displacement	Competition
Candida albicans	31.44±4.24	3.63±0.71	23.26±4.84	10.17±4.08
Escherichia coli	42.86±6.55	3.68±2.07	38.22±11.23	1.36±0.37
Staphylococcus aureus	908.5±92.78	532.27±40.71	819.25±134.15	394.54±17.42
Enterococcus faecium	23.23±3.26	8.80±1.79	15.00±3.8	2.81±0.58

After an additional 30 min of incubation, the cells were treated and observed as described above.

Statistical Analysis

Twenty fields were randomly chosen in each culture plate, and the number of bacterial cells in each field was counted under a light microscope and averaged. The standard deviation was obtained using a Sigma Plot (SPSS Inc., Chicago, U.S.A.).

RESULTS

Growth Inhibition of UTI Pathogens by *W. confusa* PL9001

The growth of each pathogen in broth media was strongly inhibited by *W. confusa* PL9001 (Fig. 1). When the inhibition activity was expressed as a percentage of the controls, the growth of *C. albicans* was most inhibited, 8% of the control, while that of *E. coli* was least inhibited, 38% of the control. The sizes of the growth inhibition zones formed by the ethyl acetate extract of *W. confusa* PL9001 were 28.6 mm on VRE, 20 mm on *S. aureus*, 17 mm on *C. albicans*, and 16 mm on *E. coli* (Fig. 2).

Coaggregation of *W. confusa* PL9001 with Genitourinary Pathogens

W. confusa PL9001 coaggregated with each pathogen. Large purple cells of *C. albicans* in a yeast form (Y-form) as well as a hyphal form (H-form) surrounded with *W. confusa* PL9001 were easily observed under a light microscope (Figs. 3A and 4). In the case of Gram-negative *E. coli*, pink *E. coli* were clearly discerned from the purple Gram-positive *W. confusa* PL9001. The coaggregation of *C. albicans* or *E. coli* with *W. confusa* PL9001 was also easily observed with SEM due to the differences in size and morphology. In the case of Gram-positive coccus *S. aureus* and *E. faecium*, it was difficult to distinguish them from *W. confusa* PL9001 under a light microscope. However, the typical club-shaped morphology of *W. confusa* PL9001 was easily differentiated from *S. aureus* or *E. faecium* with SEM (Fig. 3B).

Adherence Assay

The average number of *W. confusa* PL9001 adhering to T24 cells in a field was 326.88±63.68 cells/field. The average number of UTI pathogens adhering to T24 cells was as shown in Table 1: *C. albicans*, 31 cells/field; *E. coli*, 43 cells/field; *S. aureus*, 909 cells/field; VRE, 23 cells/field.

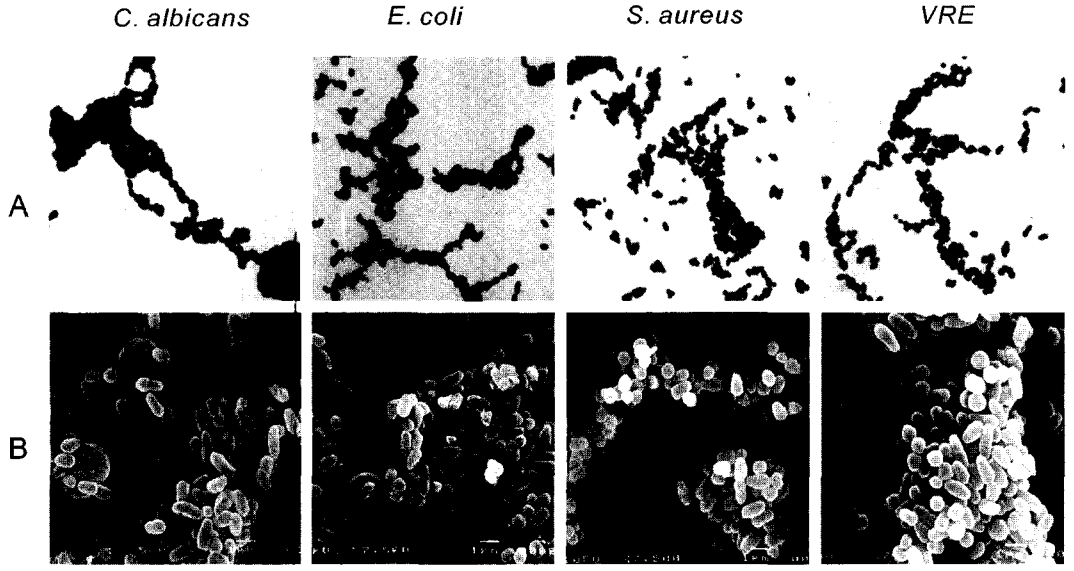

Fig. 3. Coaggregation of *W. confusa* PL9001 with various genitourinary pathogens.
After co-incubation, *W. confusa* PL9001 and various genitourinary pathogens were observed under a light microscope after Gram-staining (A, 1,000×) or SEM (B, 7,500×).

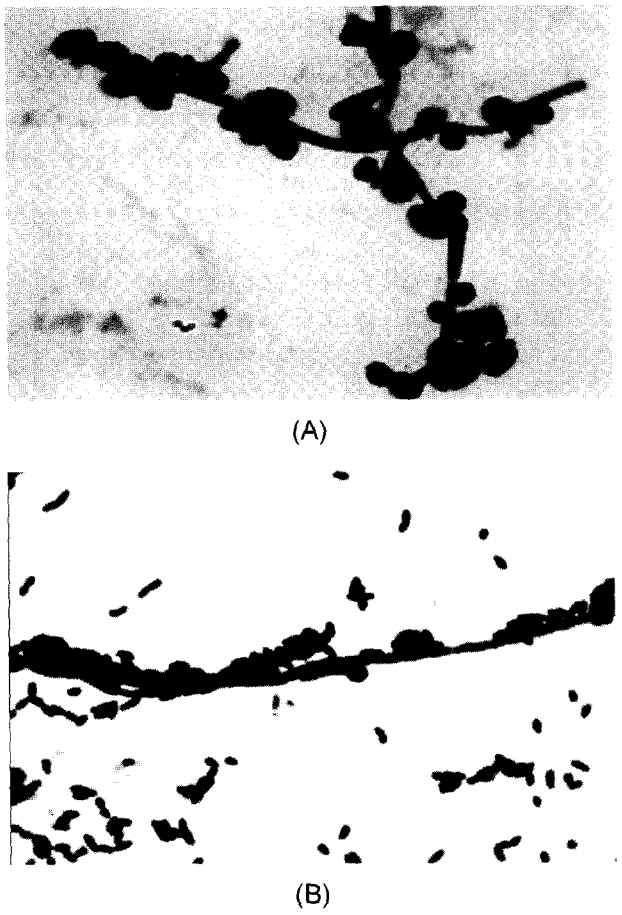

Fig. 4. Coaggregation of *W. confusa* PL9001 with *C. candida* in H-form.
The H-form of *C. candida* induced by growing in the presence of FBS was co-cultured with *W. confusa* PL9001. (A) *C. candida*; (B) *C. candida* with *W. confusa* PL9001.

Interference Assay

When compared to the controls (Fig. 5A), the number of each pathogen adhering to T24 cells in the absence of *W. confusa* PL9001 decreased in the exclusion (Fig. 5B), displacement (Fig. 5C), and competition (Fig. 5D) tests. The average numbers of each pathogen adhering to T24 cells in the exclusion, displacement, and competition tests are shown in Table 1. When *W. confusa* PL9001 was added before the pathogen (exclusion), the number of pathogens adhering to T24 cells decreased to 11.54% (*C. albicans*), 8.47% (*E. coli*), 58.59% (*S. aureus*), and 37.88% (VRE). When *W. confusa* PL9001 and each pathogen were added to the T24 cells at the same time (competition), the numbers of adhered pathogens decreased to 32.35% (*C. albicans*), 3.17% (*E. coli*), 42.43% (*S. aureus*), and 12.1% (VRE). When the pathogens were added to the T24 cells before *W. confusa* PL9001 (displacement), although the number of adhered pathogens decreased, the reduction rate was relatively low.

DISCUSSION

It has been well documented that specific strains of LABs exhibit antagonistic activity towards various human intestinal pathogens [29]. However, the capability of LABs to adhere to intestinal epithelial cells and vaginal epitheial cells is also an important factor in the formation of a barrier to prevent the colonization of pathogenic bacteria and viruses at these sites [3, 15, 21, 25]. Also, the coaggregation of LABs with pathogens appears to prevent the adherence of pathogens to epithelial cells [26]. In addition to these mechanisms, certain LABs synthesize antimicrobial compounds that are related to the bacteriocin family [16]. Other such compounds synthesized by LABs are well-known metabolic end products of lactic acid fermentation, such as lactic and acetic acids and hydrogen peroxide, or are as yet unidentified [4].

W. confusa (previously known as *L. confusus* or *L. coprophilus*) is present in the normal microflora of human intestines [12, 13, 34, 35] and has been isolated worldwide from foods [1, 6, 17, 18, 19]. The genus *Weissella* was recently separated from the genus *Lactobacillus* due to developments in DNA technology [31], and *W. confusa* PL9001 is the only strain in the *Weissella* genus that has been developed as a probiotic. This strain shows inhibitory activity towards *Helicobacter pylori* via inhibiting its growth and adherence to gastric epithelial cells [24]. In the present study, *W. confusa* PL9001 exhibited inhibitory activity towards all four UTI pathogens by several mechanisms: coaggregation, production of antimicrobial materials, and inhibition of the adherence of UTI pathogens. In particular, *W. confusa* PL9001 showed the strongest inhibitory activity towards the growth of *E. coli*, which causes the majority of UTIs [30].

Adherence is the first and most important step of infection. The number of adhered bacterial cells to bladder cells was less than 10 cells/field in the case of non-UTI isolates (data not shown), whereas UTI pathogens used in this work showed a much stronger adherence. A decrease in the adherence of pathogens can be accomplished by competition with the same strain genetically modified to be nonvirulent [13] or by administering probiotics. In the case of intestinal infection, the adherence of intestinal pathogenic bacteria is decreased by probiotics that coaggregate with the pathogens [29]; also, during coaggregation, antimicrobial products can affect the pathogens directly and prevent them from contacting cells.

In the case of *C. albicans*, the hyphal form (H-form) is commonly thought of as being more invasive than the yeast form (Y-form), and is frequently identified in infected tissue [5, 8]. The pathogenesis of disseminated candidosis involves the adhesion and penetration of hyphal cells from a colonized mucosal site to internal organs. Hypha-deficient mutants are also known to be avirulent in systemic infections

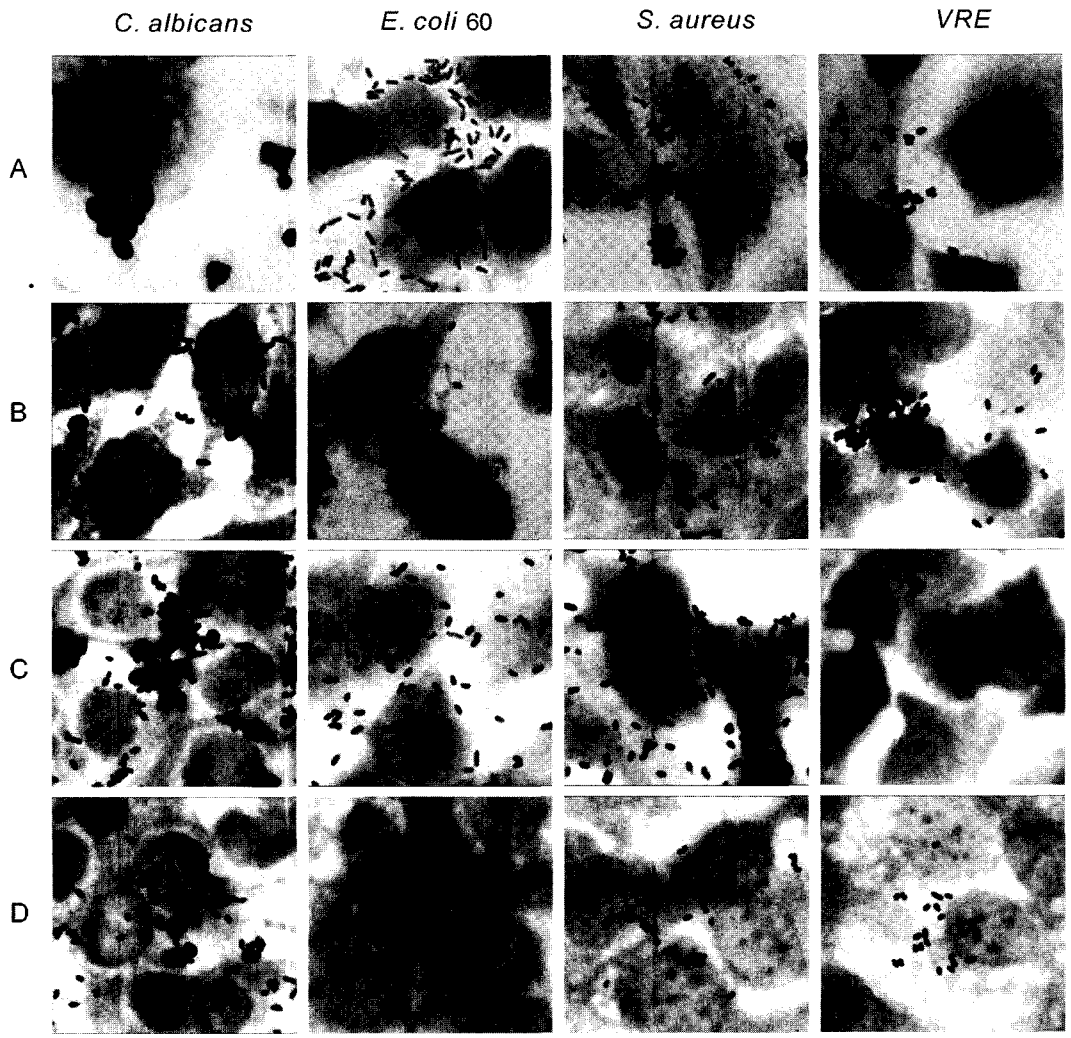

Fig. 5. UTI pathogens adhering to T24 cells after exclusion, displacement, and competition with *W. confusa* PL9001.
UTI pathogens adhering to T24 cells were observed under a light microscope after Gram-staining (1,000×). Adhered pathogens: (A) controls; (B) after exclusion test; (C) after displacement test; (D) after competition test.

[20]. *W. confusa* PL9001 was found to coaggregate with both the Y-form and H-form, as observed with SEM. Thus, *W. confusa* PL9001 is expected to efficiently prevent *C. albicans* infections. It has already been shown that orally consumed LABs can be isolated from the intestines and vagina [22, 28]; therefore, *W. confusa* PL9001 could be delivered to the urinary tract by oral consumption as well as direct insertion into the bladder or perhaps the vagina.

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