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Lactobacillus helveticus HY7801 ameliorates vulvovaginal candidiasis in mice by inhibiting fungal growth and NF-κB activation

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ABSTRACT

The anti-inflammatory effects of hydrogen peroxide-producing lactic acid bacteria (LAB) against *Candida albicans*-induced vulvovaginal candidiasis in β-estradiol-immunosuppressed mice were examined. Oral and intravaginal treatment with these LABs significantly decreased the level of viable *C. albicans* within the vaginal cavity as well as the quantitated myeloperoxidase activity in the vaginal tissues when compared with control untreated mice. Out of all of the LABs tested, *Lactobacillus helveticus* HY7801 (LH) most potently inhibited vulvovaginal candidiasis. LH also inhibited the expression of the pro-inflammatory cytokines including TNF-α, IL-1β and IL-6, and inflammatory enzymes, COX-2 and iNOS, as well as the activation of NF-κB. However, the addition of LH led to an increase in IL-10 cytokine expression in the vaginal tissues. In addition, the decrease of Lactobacillaceae and the increase of Pasteurellaceae caused by treatment with *C. albicans* were reversed with oral and intravaginal administration of LH, suggesting a potential shift in the vaginal microflora present. Addition of LH was toxic to *C. albicans* in vitro when cultured with HeLa cells. Oral administration of LH inhibited lipopolysaccharide (LPS)-induced TNF-α and IL-1β expressions in β-estradiol-immunosuppressed mice but reversed the expression of anti-inflammatory cytokine IL-10 in comparison to levels observed in the normal control group. LH also inhibited the expression of the pro-inflammatory cytokines, TNF-α and IL-1β, and the activation of NF-κB in LPS-stimulated peritoneal macrophages. Based on these findings, LH may ameliorate vulvovaginal candidiasis by suppressing the NF-κB pathway, as well as through inhibition of the growth of *C. albicans*.

1. Introduction

The human vagina is colonized by microbes. The vaginal microbial flora may play a role in maintaining women's vaginal health [1,2]. Normal healthy vaginal flora is dominated by lactobacilli, which maintain the necessary acidic conditions for growth through the production and secretion of organic acids at times other than menstruation. Disruption of the acidic conditions or of the lactobacilli within the vagina may allow for the potential infection of the pathogenic opportunistic microbes responsible for vulvovaginal candidiasis (VVC).

VVC is a common infection in women. VVC is caused by the *Candida* species, most often *Candida albicans*, which has been isolated from the vaginas of 85 to 95% of women tested [3]. It is estimated that 70 to 75% of women experience VVC during their lifetime. Current treatment options for VVC include topical azole agents or oral fluconazole [4,5]. Nevertheless, the recurrence of VVC is increasing due to the limitations of current antimicrobial therapy, indicating that novel and improved therapeutics are needed.

Lactic acid bacteria (LAB) are gram-positive, non-spore forming, non-respiring cocci or rods that ferment carbohydrates and produce lactic acid as the main product [6–8]. The common LAB genera are *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Pediococcus* sp., *Enterococcus* sp., *Streptococcus* sp., and *Bifidobacterium* sp isolated from fermented foods and intestinal microflora. These LAB are safe microorganisms that improve any disturbances of indigenous microflora [9,10], ameliorate the development of beneficial microflora [7], have anti-inflammatory (anticolitic and antivaginitic) effects [11–13], and induce non-specific activation of the host immune system [10]. Furthermore, some *Lactobacillus* strains isolated from the vaginal cavities of healthy women antagonize *C. albicans* by lowering the pH and/or producing metabolites, such as hydrogen peroxide and lactic acid, and antibacterial molecules, including bacteriocins [14,15]. Nevertheless, the relationship between anti-inflammatory and anti-microbial

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effects of LAB against VVC has not been thoroughly elucidated. Therefore, we isolated H_2O_2-producing LAB and investigated their inhibitory effects against VVC in mice.

2. Materials and methods

2.1. Materials

Lipopolysaccharide (LPS) was purchased from Sigma Co. (St Louis, MO, U.S.A.). Sabouraud dextrose agar (SDA) and broth (SDB) were purchased from BD Co. (Sparks, MD, U.S.A.). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Enzyme-linked immunosorbent assay (ELISA) kits were from Pierce Biotechnology (Rockford, IL, U.S.A.). The protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). The enhanced chemiluminescence (ECL) immunoblot system was from Pierce Co. (Rockford, IL, U.S.A.).

2.2. Microbial strains and selection of hydrogen peroxide-positive lactic acid bacteria

C. albicans KCTC7965 was obtained from the Korean Collection for Type Cultures (KCTC). The KCTC7965 was grown on Sabouraud dextrose agar plates at 37 °C for 48 h. The harvested cells were suspended to 3×10^8 cells/ml in sterilized phosphate buffered saline (PBS, pH 7.4) for vaginal inoculation and 6×10^5 cells/ml in sterilized PBS for in vitro experiment.

The hydrogen peroxide-positive lactic acid bacteria were selected from Korea Yakult Company Collection (Gyunggi prefecture, Korea) of 518 lactic acid bacteria according to the method of Rabe and Hillier [16] by using MRS agar (100 mL) containing 25 mg 3,3′,5,5′-tetramethylbenzidine (Sigma #T2885), 0.5 mg hemin, 0.05 μg vitamin K (Sigma Co. #V3501). Of positive LAB, the most potent four species, *Lactobacillus helveticus* HY7801, *Lactobacillus delbreuckii ssp. bulgaricus* HY7901, *Lactobacillus gasseri* HY7022, and *Lactobacillus fermentum* CS332, were selected.

2.3. Killing activity assay against C. albicans

C. albicans (1×10^8 CFU/mL, 1 mL) was incubated with the *L. helveticus* (1×10^8 CFU/mL, 1 mL) at 37 °C. The incubating medium [BHI broth supplemented with yeast extract (1%), maltose (0.1%), glucose (0.1%) and horse serum (10%), BHIS] was cultured at 35 °C for 24 h. Aliquots were removed at the outset and at predetermined intervals, serially diluted, and plated on SDA to determine the colony counts of *C. albicans*.

2.4. Adhesion assay

Human cervical HeLa cells were cultured at 37 °C in a 5% CO_2–95% air atmosphere in RPMI 1640 supplemented with 10% heat-inactivated (30 min, 56 °C) fetal calf serum (FCS; Boehringer, Mannheim, Germany), as previously described [17]. The culture medium was changed daily. To investigate the adhesion of *L. helveticus*, postconfluent HeLa cells were washed twice with PBS. For each adhesion assay, 500 μL of the *L. helveticus* suspension (2×10^8 CFU/mL) was mixed with Dulbecco's modified Eagle's medium (DMEM) (500 μL), and then added to each well of the tissue-culture plate (24 wells), which was then incubated at 37 °C for 3 h in 10% CO_2–90% air. After incubating, the monolayers were washed five times with sterile PBS, the cells were lysed with sterile H_2O, and appropriate dilutions were then plated on tryptic soy agar to determine the number of viable cell-associated *L. helveticus* by bacterial colony counts. Each cell association assay was performed in triplicate.

2.5. Antagonic activity assay against the adhesion of C. albicans onto HeLa cells

For cell monolayer infection, *C. albicans* was cultured at 37 °C for 18 h in BHIS, as described above. Prior to infection, postconfluent cells prepared in 24-well culture plates were washed twice with PBS. Infecting bacteria were suspended in the culture medium, and a total of 500 μL of DMEM, and 250 μL of cultured *C. albicans* (4×10^8 CFU/mL) or *L. helveticus* (4×10^8 CFU/mL) were added to each well of the tissue culture plate. The plates were incubated at 37 °C in 10% CO_2–90% air for 1 h, then treated with 250 μL of *L. helveticus* (4×10^8 CFU/mL) or *C. albicans* (4×10^8 CFU/mL) for 1 h and washed three times with sterile PBS. Dilutions were inoculated on SDA plates and incubated as described above, to determine the number of viable cell-associated *C. albicans*. Each assay was conducted in triplicate.

2.6. Animals

Female ICR mice (5 weeks-old, 20–25 g) were supplied from Orient Experimental Animal Breeding Center (Seoul, Korea). All animals were housed in wire cages at 20–22 °C and 50 ± 10% humidity, fed standard laboratory chow (Orient Experimental Animal Breeding Center) and allowed water ad libitum. All experiments were performed in accordance with the NIH and Kyung Hee University guidelines for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

2.7. Preparation of VVC model in mice

Previously described VVC model was utilized [18]. Three days prior to infection and on the day of infection, female mice were given 0.5 mg of β-estradiol-3-benzoate subcutaneously to maintain pseudoestrus. On the day of infection, the mice were anesthetized intraperitoneally with 80 mg/kg of ketamine hydrochloride and then inoculated intravaginally with 20 μL of a suspension of *C. albicans* (6×10^6 CFU/mouse). The vaginal cavity of each mouse was swabbed (prior to treatment) 1 day post-infection with *C. albicans* to ensure infection was consistently distributed among animals. LABs were prepared fresh daily in PBS and orally or intravaginally administered once daily for 3 days from the day 1 post-infection. Control mice were infected but received no active treatment; vehicle alone was treated.

On day 4 post-infection, mice euthanized with CO_2 and vaginae were washed with 0.9 mL of sterile saline. Serial 10-fold dilutions were made, and 100 μL of each was placed on SDA agar for *C. albicans* to quantify the number of CFU/mL. After washing, vaginae were excised and stored at −80 °C until required for myeloperoxidase activity assays, immunoblotting, and ELISA.

2.8. Assay of myeloperoxidase activity

The vaginal tissues were homogenized in 300 μL of ice-cold RIPA lysis buffer (3M, Seoul, Korea) containing 1% protease inhibitor cocktail and 1 % phosphatase inhibitor cocktail [19]. The lysate was centrifuged (10,000 ×*g*, 4 °C) for 20 min, and an aliquot (50 μL) of the vaginal supernatant was added to a reaction mixture of 1.6 mM tetramethyl benzidine and 0.1 mM H_2O_2 and incubated at 37 °C; the absorbance was obtained at 650 nm over time. Myeloperoxydase activity was defined as the quantity of enzyme degrading 1 μm/mL of peroxide at 37 °C and expressed as units/mg protein. The protein content was assayed according to the method of Bradford [20].