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Article

Inhibitory Effects of Vaginal Lactobacilli on *Candida albicans* Growth, Hyphal Formation, Biofilm Development, and Epithelial Cell Adhesion

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Abstract: Antifungal agents are not always efficient in resolving vulvovaginal candidiasis (VVC), a common genital infection caused by overgrowth of *Candida* spp., including *Candida albicans*, or preventing recurrent infections. Although lactobacilli (which are dominant microorganisms constituting healthy human vaginal microbiota) are important barriers against VVC, the *Lactobacillus* metabolite concentration needed to suppress VVC is unknown. Therefore, we quantitatively evaluated *Lactobacillus* metabolite concentrations to determine their effect on *Candida* spp., including 27 vaginal strains of *Lactobacillus crispatus*, *Lactobacillus jensenii*, *Lactobacillus gasseri*, *Lacticaseibacillus rhamnosus*, and *Limosilactobacillus vaginalis*, with inhibitory abilities against biofilms of *Candida* clinical isolates. *Lactobacillus* culture supernatants suppressed viable bacteria by approximately 24%-92% relative to preformed *Candida* biofilms, but their suppression differed between strains, not species. Lactate production was necessary to suppress preformed biofilms and hyphal elongation of *C. albicans*, whereas hydrogen peroxide was not always essential. Both lactic acid and hydrogen peroxide were required to suppress *Candida* planktonic cell growth. *Lactobacillus* strains that significantly inhibited biofilm formation in culture supernatant also inhibited *Candida* adhesion to epithelial cells in an actual live bacterial adhesion competition test. Healthy human microflora and their metabolites may play important roles in the development of new antifungal agent against VVC caused by *C. albicans*.

Keywords: *Candida albicans*; Lactobacillus species; biofilm; probiotics

1. Introduction

Fungal diseases cause considerable morbidity and mortality, resulting in a high economic burden [1,2]. Vulvovaginal candidiasis (VVC), a common genital infection, is commonly caused by *Candida albicans*, with a lifetime prevalence of up to 78% in women [3,4]. Eight percent of women with VVC experience recurrent VVC (RVVC), which relapses more than four times a year due to the low response to antifungal treatment, including azoles such as fluconazole [5,6]. The highest prevalence of RVCC occurs among 25-34-year-olds, and it has an annual economic burden of US$14-39 billion in developed countries because it reduces the quality of life [5]. The emergence and spread of antimicrobial resistance (AMR) have become a global concern, and fungal infections have

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been excluded from the AMR program [7]. However, as with bacterial infections, the use of antifungal drugs is strongly implicated in the occurrence of pathogenic fungi, and new methods of prevention or treatment of RVVC that are not dependent on antifungals are required [8].

C. albicans is a dimorphic fungus that can transform from yeast to an invasive filamentous hyphal form [4,9]. Biofilm formation by *C. albicans* commonly consists of four major stages: yeast cells adhere to a substrate to form a yeast basal layer; initiation of propagating cells where the hyphae are formed; hyphae are formed and extracellular matrix accumulates, such as extracellular polysaccharides, structural proteins, cell debris, and nucleic acids; and dispersion of yeast cells from the biofilm to initiate biofilms at new locations [10]. These biofilm structures are intrinsically resistant to antifungals, making VVC difficult to combat [11].

The vaginal microbiota of humans is known to be less complex than the intestinal microbiota and is usually dominated by the genus *Lactobacillus* [12]. The disruption of these vaginal microbiota promotes colonization by pathogenic microorganisms that leads to bacterial vaginosis and subsequent VVC [13]. With recent progress in sequencing technology, the presence of certain lactobacilli has been found to be associated with vaginal health. *Lactobacillus crispatus*-and *Lactobacillus jensenii*-dominated vaginal microbiota, are strongly associated with health [14]. Furthermore, the *Lactobacillus iners*-dominated environment could be affected by vaginal dysbiosis [14]. These species contribute to vaginal homeostasis mainly by producing metabolites, including lactic acid and hydrogen peroxide, although their production abilities vary among isolates of the same species [15].

The effects of lactobacilli on hyphal and biofilm formation by *C. albicans* have previously been explored using a single specie [8,16–19]. However, these previous studies have evaluated lactate production indirectly using the pH or qualitatively using hydrogen peroxide production; therefore, quantitative evaluation of the metabolite is lacking. The aim of this study was to quantitatively evaluate the metabolites of lactobacilli to determine the effects of lactobacilli on *Candida* growth, hyphal formation, biofilm development, and epithelial cell adhesion.

2. Materials and Methods

2.1. Strains

Forty-five *C. albicans* strains, which were clinically isolated from the vagina and provided by Microskylab Inc. (Tokyo, Japan), were used in this study. All 27 *Lactobacillus* strains were previously obtained from vaginal swabs of healthy Japanese women at Aichi Medical University [12]. These strains belonged to five species: *Lactobacillus crispatus*, *Lactobacillus jensenii*, *Lactobacillus gasseri*, *Lacticaseibacillus rhamnosus*, and *Limosilactobacillus vaginalis*. The characteristics of these bacterial strains are listed in Table S1.

2.2 Biofilm formation and viability assay

A 96-well microtiter plate-based method was used in this study [20]. *Candida* strains were cultured overnight in Yeast Peptone Dextrose (YPD) agar (Difco, Detroit, United States) at 30 °C under aerobic conditions. A single colony was inoculated into the YPD medium and incubated at 30 °C, shaking at 160 rpm under aerobic conditions. The cells were centrifuged at 3,500 ×*g* for 10 min and re-suspended in RPMI 1640 medium buffered with morpholinepropanesulfonic acid (RPMI-MOPS) at a concentration of 10^7 cells/mL, and 100 μL of the inoculum was seeded into a 96-well microtiter plate. The biofilms formed on the surface of the wells were gently washed twice with phosphate-

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buffered saline (PBS) after 48 h of incubation. Total biofilm mass and cell viability were quantified using crystal violet (CV) (Merck KGaA, Darmstadt, Germany) and water-soluble tetrazolium salts (WST-1) (TaKaRa, Shiga, Japan), respectively [21,22]. To quantify total biomass, washed biofilms were stained with 0.1% (w/v) CV solution for 1 min. Each well was washed twice with PBS and dried for 30 min. The bounded CV was eluted using 99.5% (v/v) ethanol. The viability of microbial cells inhabiting the biofilm population was analyzed using WST-1, based on the reduction of tetrazolium salt. To each well, we added 100 µL of PBS and 10 µL of premix WST-1, then they were incubated at 37 °C for 3 h under shade conditions. The absorbance (Abs) of CV and WST-1 was measured at 595 nm and 440 nm, respectively.

2.3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

ISOGEN II (Nippon Gene, Co., Ltd., Tokyo, Japan) was used for total RNA extraction from *C. albicans* HB-10 strain. The RNA concentrations were measured using a Qubit® RNA Assay Kit (Promega, WI, USA). To prepare complementary DNA (cDNA), the PrimeScript™ RT reagent Kit (TaKaRa, Shiga, Japan) was used in accordance with the manufacturer's instructions. Quantitative RT-PCR analysis was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa, Shiga, Japan) in accordance with the manufacturer's protocol. Briefly, PCR was performed in a reaction mixture of TB Green Premix Ex Taq II (2 ×) 12.5 µL, PCR forward primer 1 µL, PCR reverse primer 1 µL, and RNase free dH_2O 8.5 µL added to 2 µL of each reverse transcription reaction solution. Primers used in this study are listed in Table S2. The amplification conditions were as follows: 40 cycles under heat treatment at 95 °C for 30 s, heat denaturation at 95 °C for 5 s, and annealing at 55 °C for 30 s, which is the optimum temperature for the primer. Melting curves were used to verify the quality of qPCR, and the fold expression was calculated using the delta-delta Ct method.

2.4. Supernatants produced by Lactobacillus

Cell-free culture supernatants were extracted from *Lactobacillus* species. A single strain each was inoculated in de Man, Rogosa, and Sharpe (MRS) broth (Merck KGaA, Darmstadt, Germany) and incubated at 37°C for 72-h under anaerobic conditions (10% H_2, 10% CO_2, and 80% N_2) in an anaerobic chamber. Growth at the sampling point (72-h) was determined by measuring the optical density (OD) at 600 nm using a microplate reader (SH-9000Lab, HITACHI). The culture medium was then centrifuged at 3,500 ×*g* for 10 min and filtered through a 0.22-µm membrane filter (Sarutorius AG, Gettingen, Germany). Each collected culture supernatant was stored at −80 °C until use.

2.5. High-performance liquid chromatography (HPLC) analysis of culture supernatants

HPLC (SHIMADZU, Kyoto, Japan) equipped with a conductivity detector was used to measure lactate and short-chain fatty acids, such as acetate, propionate, and butyrate, in culture supernatants, as previously described [23]. Briefly, the mobile phase required 5 mM p-toluenesulfonic acid (KANTO Chemical, Tokyo, Japan). The reaction buffer was 5 mM p-toluenesulfonic acid, 100 µM ethylenediaminetetraacetic acid (KANTO Chemical, Tokyo, Japan), and 20 mM bis (2-hydroxyethyl) aminotris (hydroxymethyl) methane (Tokyo Chemical Industry, Tokyo, Japan). The flow rate, oven temperature, and detector cell temperature were set at 0.8 mL/min, 40 °C, and 48°C, respectively. The samples

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contained in 1.0 mL disposable vials (SHIMADZU Co., Kyoto, Japan) were held at 4°C in a sample cooler (SHIMADZU, Kyoto, Japan), and 10 μL was applied to tandemly-arranged two columns (SHIMADZU, Kyoto, Japan) to measure lactate. The calibration curve solution adjusted with lithium DL-lactate (FUJIFILM Wako Pure Chemical, Co., Ltd., Osaka, Japan) was dissolved in deionized water. The quantification analyses for HPLC were performed using LabSolutions version 5.90 (SHIMADZU Co., Kyoto, Japan), and the peak area was used as the signal intensity.

2.6. Detection of hydrogen peroxide in culture supernatants

Hydrogen peroxide production was estimated using a hydrogen peroxide assay kit (ab102500, Abcam, MA, USA) according to the manufacturer's instructions. The stored culture supernatant was neutralized to pH 7.0, and 100 μL of the adjusted supernatants were reacted for 10 min in the presence of horseradish peroxidase. Duplicate wells were measured at a wavelength of 595 nm using a microplate reader.

2.7. Effect of Lactobacillus culture supernatants on Candida biofilm

The efficacy of the anti-biofilm activities of lactobacilli was determined by adding the culture supernatant of *Lactobacillus* to the biofilm. *C. albicans* HB-10, which formed a mature biofilm in the assay described above, was selected and used for subsequent inhibition assays. A mature biofilm of *C. albicans* HB-10 was formed in a 96-well microtiter plate after 24 h of incubation under the same conditions as the biofilm formation and viability assay. The planktonic cells were aspirated from each well and washed twice with PBS. Cell-free supernatant extracted from a single *Lactobacillus* strain was added to each well at a final concentration of 8% (v/v) and incubated for 24 h. Culture supernatants were aspirated from each well and washed twice with PBS. Biofilm formation was quantified using CV and WST-1, as described above.

2.8. Effect of Lactobacillus culture supernatants on Candida hyphal formation

Candida yeast-to-hyphal transition in the presence of *Lactobacillus* culture supernatants was estimated as previously described [19]. Lactobacilli with strong inhibition of biofilm formation and those with low inhibition were selected. *C. albicans* HB-10 cells from overnight culture were washed with PBS and re-suspended at approximately 10^7 CFU/mL in YPD broth. The yeast cell suspensions were then incubated with or without *Lactobacillus* culture supernatant at 37°C for 4 h. Quantification of the inhibitory effect of *Lactobacillus* on hyphal formation was observed using a light microscope (AxioCam MRc5; Carl Zeiss, Jena, Germany). The percentage of hyphal formation was calculated by obtaining the ratio of total *Candida* with hyphae to the total number of *Candida* counted.

2.9. Adhesion assay of Candida and Lactobacilli

Human cervical cancer HeLa cells (RCB0007; Riken BRC Cell Bank, similar to ATCC CCL2) were grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, MA, USA) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher

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Scientific, MA, USA) and 1% (v/v) penicillin and streptomycin (FUJIFULM Wako Pure Chemical, Co., Ltd., Osaka, Japan) at 37°C under 5% CO_2 and humidity. HeLa cells were seeded into a 12-well plate (AGC Techno Glass Co., Ltd., Shizuoka, Japan) at approximately 1.0×10^5 cells per well and grown to confluency. After 90% confluency, each well was washed twice with PBS. *C. albicans* HB-10 and lactobacilli were grown under the conditions described above. Briefly, a single colony of *C. albicans* HB-10 and lactobacilli was inoculated into YPD and MRS broth, respectively. After 48 h of incubation, the bacterial cultures were centrifuged at 3,500 ×*g* for 10 min and re-suspended in DMEM. The suspension of *C. albicans* HB-10 and lactobacilli contained approximately 1.0×10^7 CFU/mL. To the HeLa cell culture well, 100 µL of 10-fold serial dilutions of Lactobacilli suspensions was added and incubated at 37 °C for 4 h under 5% CO_2. Subsequently, 100 µL of 10-fold serial dilutions of *C. albicans* HB-10 suspensions was added to each well and incubated for 6 h under the same conditions to allow *C. albicans* HB-10 to adhere to cells. After incubation, each well was washed twice with PBS to remove non-adherent *Candida* and then treated with 0·05% trypsin-EDTA (Nacalai Tesque, Inc., Kyoto, Japan). To determine whether lactobacilli prevent *C. albicans* HB-10 adhesion, the cell lysates were 10-fold serially diluted and spread on MRS and YPD agar, respectively. The inhibitory rate of adhesion was calculated as the number of *Candida* that adhered to HeLa cells with lactobacilli pre-treatment, per the number of *Candida* that adhered to HeLa cells in the absence of lactobacilli.

2.10 Statistical analysis

Statistical analyses were performed using R and RStudio (versions 4.0.3 and 1.4.1106, respectively). Mann–Whitney *U* test was used to determine significant differences between DMEM control and different *Lactobacillus* cells. One-way analysis of variance was used to compare multiple groups. Statistical significance was set at $p < 0.05$ Correlations between growth and metabolites (lactate and hydrogen peroxide) were determined using Spearman's rank correlation coefficient.

3. Results

3.1. Biofilm formation abilities of C. albicans

Biofilm formation by *C. albicans* clinical isolates was assessed using the WST-1 formazan dye (Figure 1a).

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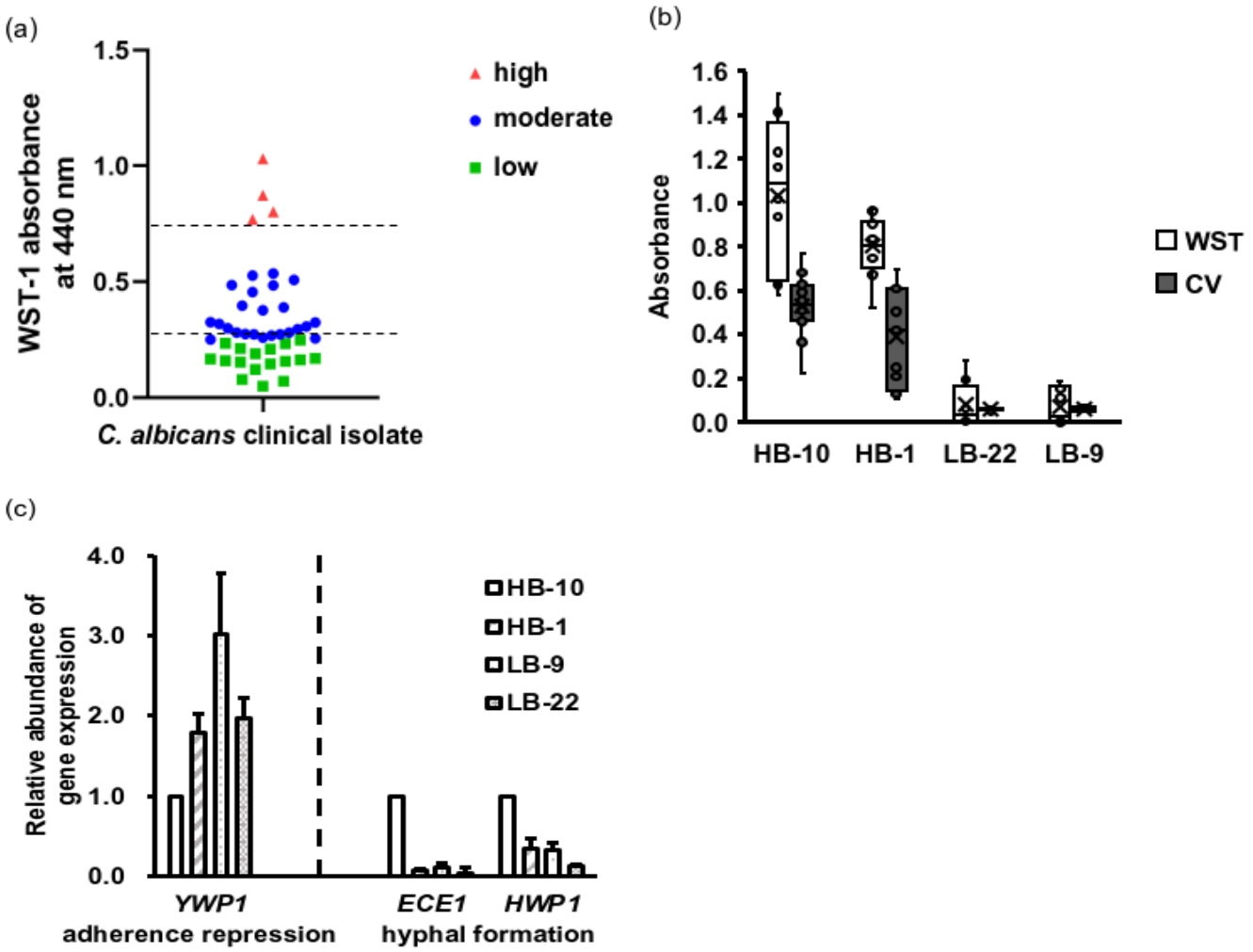

Figure 1. Clinical isolates of *Candida albicans* with different abilities to form biofilms. CV, crystal violet; WST, water-soluble tetrazolium salts.

(a**)** Biofilm biomass of 45 *C. albicans* clinical isolates were measured by WST-1 using the 96 well plate assay. Eight wells were used for each isolate. The experiment was performed on three separate occasions, with the mean of each represented. (b) Biofilm formation by different *Candida* strains was estimated using both WST-1 reduction and CV staining. (c) Relative quantitation of genes associated with adherence repression (*YWP1*), or hyphal formation (*HWP1* and *ECE1*) normalized to the β-actin gene. The *C. albicans* HB-10 strain was used as a reference to depict the difference among 4 *C. albicans* clinical isolates.

Strains were classified as 4 (8.89%) high biofilm producers ($Abs_{440} \geq 0.75$), 24 (53.33%) moderate producers ($0.25 < Abs_{440} < 0.75$), and 17 (37.78%) poor or non-biofilm producers ($Abs_{440} \leq 0.25$). Two representative strains were selected from each class of high and poor biofilm-forming strains and renamed *C. albicans* HB-1 and HB-10 and *C. albicans* LB-9 and LB-22, respectively. The biofilm-forming abilities of these four strains were also evaluated using ancillary CV. As shown in Figure 1b, consistent with the WST results, high and low biofilm-forming strains were clearly distinguished using CV, which stained the entire biofilm structure. The expression levels of the three genes, *ECE1*, *HWP1*, and *YWP1*, which regulate different stages of biofilm formation in *C. albicans*, were determined using qRT-PCR (Figure 1c).

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Four *C. albicans* strains with gene expression of *HWP1*, *ECE1*, *and YWP1* exhibited bar plot normalized by *C. albicans* HB-10 gene expression levels. These gene expression levels were calculated according to *ACT1* gene expression levels. Similar to the phenotypic biofilm-forming analysis, the relative gene expression levels of *ECE1* and *HWP1* in the HB-10 strain were significantly higher than those in the LB-9 (10.53-fold and 3.21-fold, respectively) and LB-22 strains (31.21-fold and 8.72-fold, respectively) ($p < 0.05$). Interestingly, the HB-1 strain, which was a high biofilm producer and did not show such a large difference in biofilm-forming ability, had significantly lower expression levels of these genes than the HB-10 strain. In contrast, *YWP1*, which suppresses initial adhesion, was the lowest in the HB-10 strain.

3.2. Characterization of culture supernatant

Vaginal lactobacilli produce various metabolites that exhibit antifungal activity. Cell-free culture supernatants extracted from 27 strains of *Lactobacillus* belonging to five species were characterized (Figure 2).

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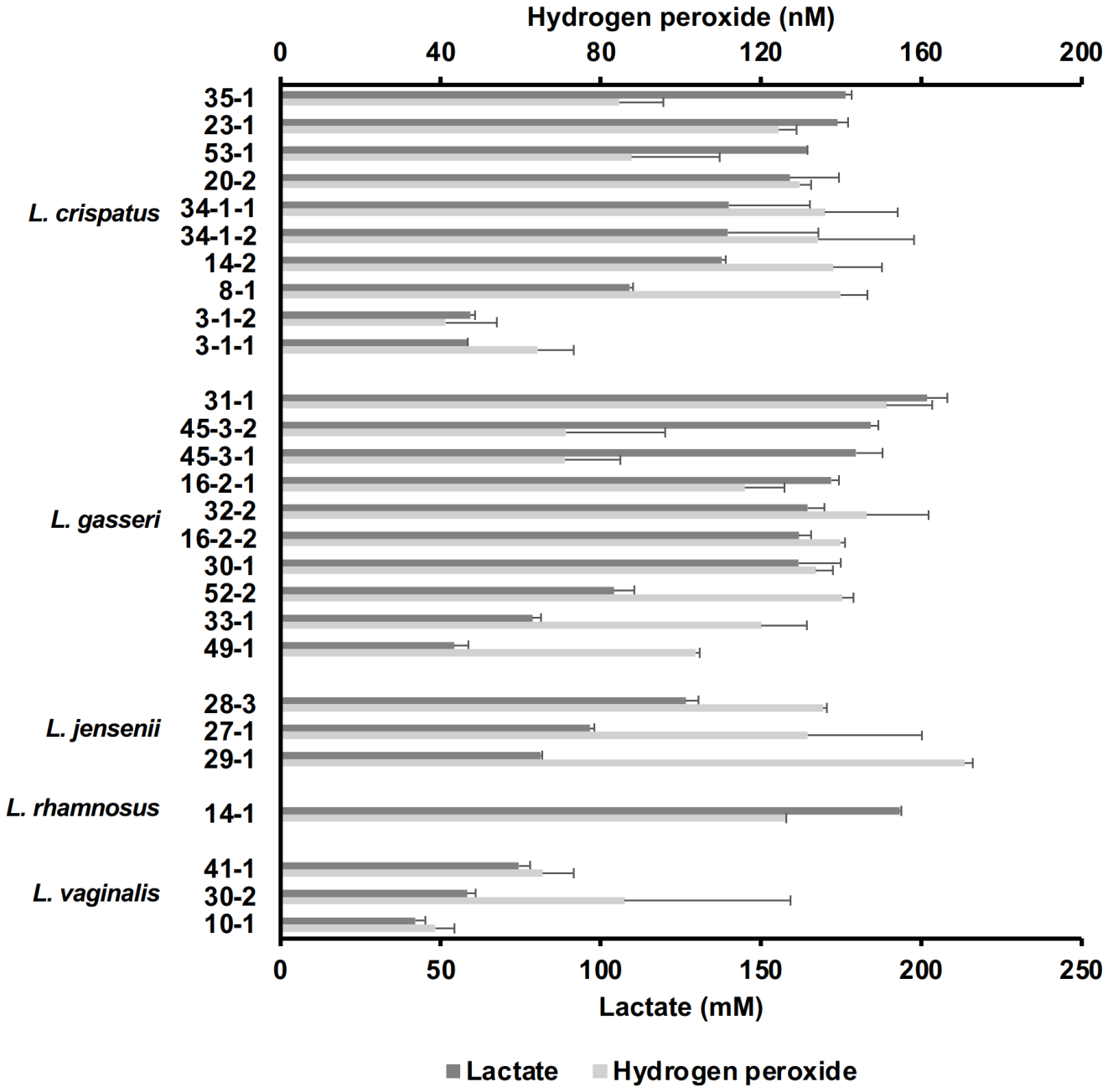

Figure 2. Lactate and hydrogen peroxide production by 27 *Lactobacillus* clinical isolates

Twenty-seven *Lactobacillus* clinical isolates were cultured in de Man, Rogosa, and Sharpe (MRS) broth for 72 h, and cell-free culture supernatants were collected. Lactate was measured quantitatively by HPLC, and hydrogen peroxide was measured quantitatively using a hydrogen peroxide assay kit. Data are represented as the mean across three replicates.

The lactic acid and hydrogen peroxide production of lactobacilli used in this study ranged from 42.1 to 201.7 mM and 38.7 to 170.8 nM, respectively. There was no correlation between lactate production and hydrogen peroxide production ($r = 0.426$; $p = 0.217$). In contrast, OD and lactate at the 72-h sampling point showed moderately positive correlations ($r = 0.667$; $p < 0.001$), while OD and hydrogen peroxide showed no correlation ($r = 0.265$; $p = 0.181$). In *L. jensenii*, the average hydrogen peroxide production

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was the highest compared to other species (mean 146.0 nM), although lactic acid production was not as high (ranged from 81.3 to 126.5 mM).

3.3. Effect of Lactobacillus culture supernatant on preformed biofilm

In a typical experiment using 96 well plates, biofilm formation takes 24 h to reach confluency. We investigated the effects of *Lactobacillus* culture supernatants on preformed biofilms (Figure 3).

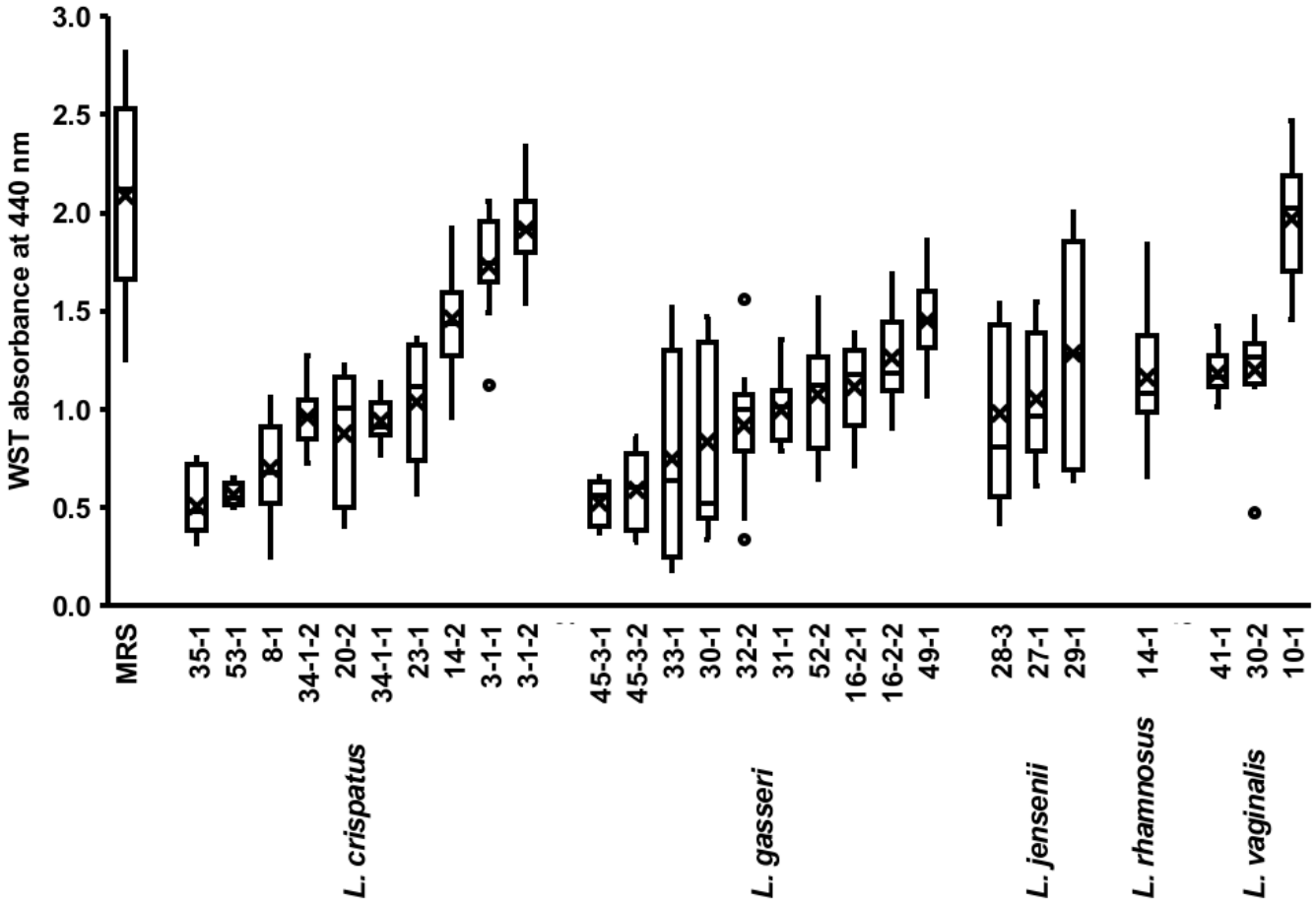

Figure 3. Biofilm formation of *C. albicans* HB-10 treated with culture supernatants of 10 different *Lactobacillus* clinical isolates.

Biofilm formation of *C. albicans* HB-10 treated with culture supernatants of 10 different *Lactobacillus* clinical isolates was measured using the WST-1 reduction reaction. MRS broth was used as a control. WST, water-soluble tetrazolium salts; MRS, de Man, Rogosa, and Sharpe. Box plot shows the median (horizontal thick blank line), mean (cross), and first and third quartiles (box).

The addition of *Lactobacillus* culture supernatant resulted in 24.3%-91.8% relative WST-1 readings compared to those of non-added control. The culture supernatants of *L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. vaginalis* with average relative WST-1 readings were approximately 52.5%, 43.1%, 57.2%, and 58.9%, respectively. No significant differences in fixation rates were observed between the species in the *Lactobacillus* culture

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supernatant. A moderate negative correlation was found between *Lactobacillus* lactate production and WST-1 readings ($r = -0.625$; $p < 0.001$), but no correlation was observed between hydrogen peroxide production and WST-1 readings. We added several concentrations of the standards to the biofilm to reproduce lactate and hydrogen peroxide as metabolites in the culture supernatant (Figure S1). The results showed that the values were lower than those of the control at all lactate concentrations, with a concentration-dependent effect on WST values ($r = -0.930$; $p = 0.001$). Hydrogen peroxide, however, showed significantly lower WST values than the control, but not in a concentration-dependent manner. Lactate and hydrogen peroxide further showed no additive synergistic effects on the preformed biofilms. A strong effect on the preformed biofilm was observed when the culture supernatant of *the L. crispatus* 35-1 strain was added (final concentration 14.1 mM), which was consistent with the WST-1 values for the biofilm when the 16 mM lactate standard was added.

3.4. Effect of Lactobacillus culture supernatant on the growth of planktonic cultures

The inhibitory effect of *Lactobacillus* spp. on *Candida* yeast cell growth was also evaluated. Significant growth inhibition was shown in 4/27 (14.8%) of the strains with the addition of culture supernatant of each *Lactobacillus* as follows: *L. crispatus* strain 23-1 and 20-2, *L. gasseri* strain 31-1 and *L. rhamnosus* strain 14-1. Interestingly, three of these strains showed lactic acid and hydrogen peroxide production above 165 mM and 120 nM, respectively.

3.5. Effect of Lactobacillus culture supernatant on the hyphal formation

The effect on the rate of hyphal formation was investigated for Lactobacillus culture supernatants that exhibited significant differences in their fixation to the preformed biofilm and for those that did not (Figure 4).

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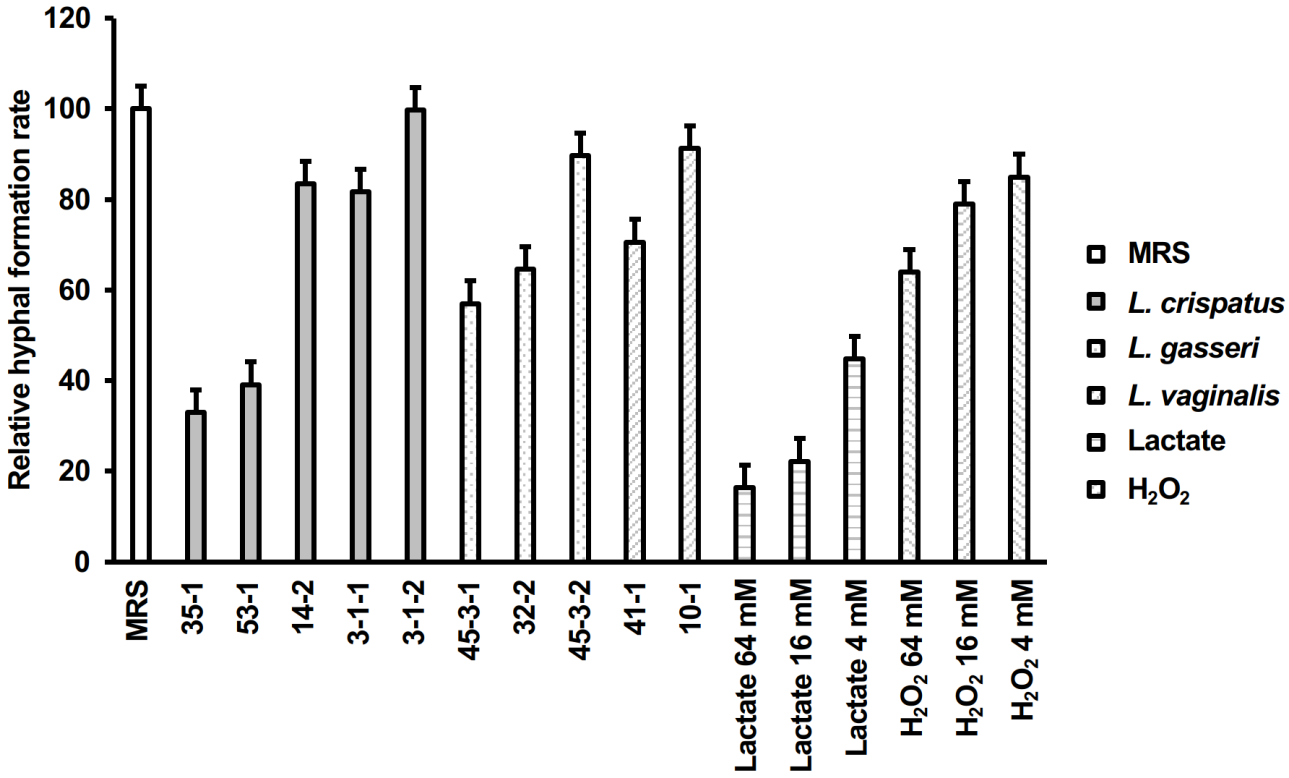

Figure 4. Hyphal formation rate of *C. albicans* HB-10 strain treated with *Lactobacillus* culture supernatants, lactate, or hydrogen peroxide.

Relative hyphal formation in *C. albicans* HB-10 treated with culture supernatants of 10 different *Lactobacillus* clinical isolates, lactate, or hydrogen peroxide. Bars represent the standard deviation from the mean values. MRS broth was used as a control. MRS, de Man, Rogosa, and Sharpe.

Yeast, hyphae, and pseudohyphae were identified under a microscope (Figure S2). The addition of MRS medium control resulted in 54.95 ± 9.61% of the hyphae and pseudohyphae were identified after 3 h of incubation. Compared to MRS control alone, lactate standards showed a concentration-dependent decrease in hyphal formation at 16 to 64 mM (p<0.05), while only 64 nM of hydrogen peroxide showed a significant difference. The percentage of hyphal formation by Lactobacillus culture supernatants ranged from 18.11 to 54.77%. In terms of the percentage of a hyphal formation relative to untreated MRS, significant decreases were observed with the addition of culture supernatant in *L. crispatus* strain 35-1 (32.97 ± 13.29%) and 53-1 (39.10 ± 7.40%), *L. gasseri* strain 45-3-1 (56.99 ± 6.90%) and 32-2 (70.75 ± 4.15%), and *L. vaginalis* strain 41-1 (70.60 ± 8.12%). All of these had final lactate concentrations > 50 mM. In contrast, although there were no differences in lactic acid and hydrogen peroxide metabolite profiles between *L. gasseri* strains 45-3-1 and 45-3-2, only strain 45-3-1 showed significantly lower hyphal formation than the MRS control.

3.6. Effect of Lactobacillus bacterial cell on the initial adhesion

The inhibition of *C. albicans* yeast adhesion to human epithelial cells by *Lactobacillus* bacterial cells was assessed (Figure 5).

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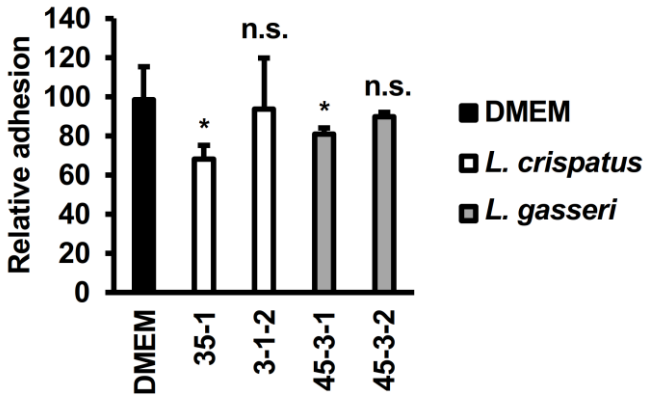

Figure 5. Adhesion of *C. albicans* HB-10 strain to HeLa cells according to the presence or absence of Lactobacilli.

Relative adherence of *the C. albicans* HB-10 strain to HeLa cells pretreated with DMEM or different *Lactobacillus* cells. Bars represent the standard deviation from the mean values. *, p <0.05 by *U*-tests. DMEM, Dulbecco's modified Eagle's medium.

L. crispatus strain 35-1 showed more efficient inhibition of *C. albicans* HB-10 (adhesion rate: 68.29 ± 6.90%). In contrast, *L. crispatus* strain 3-1-2 showed no statistically significant difference (adhesion rate: 93.90 ± 25.87%). Interestingly, *L. gasseri* strain 45-3-1, which showed inhibition of hyphal formation, also showed a significant reduction in *C. albicans* HB-10 adhesion (80.95 ± 3.17%), whereas strain 45-3-2 showed no inhibitory effect on initial adhesion (89.68 ± 2.38%).

4. Discussion

In this study, quantitative evaluation of lactobacilli metabolite (lactate and hydrogen peroxide) concentrations revealed that the inhibitory effects of lactate and hydrogen peroxide on *Candida* act through multiple stages, such as *Candida* growth, hyphal formation, biofilm development, and adhesion to epithelial cells.

Of the two *C. albicans* strains with the highest capacity for biofilm formation used in this study (HB-1 and HB-10), only HB-10 had high expression levels of *HWP1* and *ECE1*, genes important for epithelial cell adhesion and invasion [24,25]. In contrast, HB-1 formed high biofilms, but the gene expression levels of *HWP1* and *ECE1* were low, indicating a dissociation between phenotype and gene expression. This may be because hyphal formation and invasion of epithelial cell, which is important for biofilm formation, are composed of multiple signal transduction pathways [9].

In the human vagina, the presence of hydrogen peroxide-producing lactobacilli stabilizes the vagina with *Lactobacillus*-dominant microbiota [26]. However, it is unclear how hydrogen peroxide concentration affects *Candida* growth and biofilm formation. In this study, lactate inhibited biofilm formation in a concentration-dependent manner, while hydrogen peroxide did not. Moreover, because inhibition of biofilm formation was observed even with hydrogen peroxide at a concentration of 4 nM, it may be important for hydrogen peroxide to be present at a concentration of 4 nM or higher to inhibit biofilm formation. It was also confirmed that lactate and hydrogen peroxide did not exhibit synergistic effects. Furthermore, the concentrations of lactate and hydrogen peroxide in the supernatant of lactobacilli that were found to inhibit the growth of *Candida* yeast cells were greater than 165 mM and 120 nM, respectively. In this and

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previous studies, *Lactobacillus* could produce hydrogen peroxide of more than 4 nM [27,28]. Therefore, in addition to lactate production, it is important to determine whether *Lactobacillus* produces hydrogen peroxide which has inhibitory effect on biofilm formation. Furthermore, the difference in strains and species of *Lactobacillus*, which leads to differences in the amount of lactate production, may be important in terms of the inhibitory effect on *Candida* growth.

Hyphal formation and growth are associated with *Candida* virulence [29,30]. HB-10 used in this study expresses the *ECE1* gene; Ece1p, a protease encoded by *ECE1*, causes inflammation in epithelial cells and allows *Candida* hyphae to adhere to and invade the cell epithelium [31]. To inhibit biofilm formation, it is important to prevent *Candida* adhesion to the epithelial cells. In the present study, lactate inhibited hyphal formation in a concentration-dependent manner. Despite the similar metabolic profiles of lactate and hydrogen peroxide in *L. gasseri* strains 45-3-1 and 45-3-2, only strain 45-3-1 significantly inhibited hyphal formation. Similarly, among the *L. gasseri* strains 45-3-1 and 45-3-2, only strain 45-3-1 significantly inhibited *C. albicans* adhesion to epithelial cells. This suggests that metabolites other than lactate and hydrogen peroxide inhibit hyphal formation. Indeed, recent studies have suggested that small molecules produced by *Lactobacillus* may inhibit *Candida* biofilm formation and growth as antimicrobial compounds [29,32].

A worrisome trend is that VVC caused by non-*albicans Candida species* (NAC), *C. tropicalis, C. krusei*, and *C. glabrata*, has been increasing [33,34]. In particular, *C. tropicalis* is frequently isolated in Asia and is known to have high hyphal budding ability and to form strong biofilms that are resistant to treatment [33,35,36]. Although *C. tropicalis* has good in vitro drug susceptibility to azoles, candins, and polyenes, the poor clinical prognosis may be related to biofilm formation [37,38]. Visualization of biofilm formation has shown that candin- and polyene-based drugs are suitable for biofilm-forming NAC [36,39]. In the actual human vaginal environment, glycogen is digested by α-amylase to produce maltose, maltotriose, and maltotetraose [40]. *Lactobacilli* are known to consume glycogen-breakdown products to produce lactate. However, in vitro experiments have not fully mimicked the vaginal environment with respect to nutrient sources for *lactobacillus* development, which may have affected their growth and metabolite production [40,41]. The results of this study suggest that *lactobacillus* metabolites other than lactate and hydrogen peroxide may also affect *Candida*, but they have not been evaluated in detail. In future, the effects of various metabolites produced by *Lactobacillus* on *Candida* need to be evaluated under conditions that are more similar to the human vaginal environment.

This study revealed that metabolites of lactobacilli greatly inhibited *Candida* biofilm development. Therefore, combining antifungal drugs with lactobacilli as a live biotherapeutic product, which has anti-biofilm development activity, may lead to the development of new treatment strategies. Future work evaluating how lactobacilli affect both *C. albicans* and NAC is needed to promote its global use.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: The biofilm formation of *C. albicans* HB-10 treated with lactate, hydrogen peroxide, or a combination of lactate and hydrogen peroxide; Figure S2: The hyphal formation observed under light microscope; Table S1: Characteristics of *Lactobacillus*; Table S2: Primers used in this study.

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