보건학석사 학위논문

Inhibitory effect of *Lactobacillus fermentum* SNUV175 and *Lactobacillus crispatus* SNUV220 isolated from healthy Korean women on vulvovaginal candidiasis

한국인 여성 유래 락토바실러스 퍼멘텀 SNUV175 및 락토바실러스 크리스파투스 SNUV220 배양액의 질염모델에서 칸디다 알비칸스 저해 연구

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Abstract

Inhibitory effect of *Lactobacillus fermentum* SNUV175 and *Lactobacillus crispatus* SNUV220 isolated from healthy Korean women on vulvovaginal candidiasis

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Lactobacillus spp. is commensal microbiota in vaginal environment known to inhibit pathogen growth by producing anti-microbial compounds. we isolated *Lactobacillus fermentum* SNUV175 and *Lactobacillus crispatus* SNUV220 strains from Korean healthy women vagina. In this study we investigated how *Lactobacillus* species interact with vaginal pathogen *C. albicans*. We used *Lactobacillus* cell-free supernatant (LCS) to demonstrate growth inhibiting effect. Interestingly, we found that not only LCS effectively inhibited *C. albicans* growth *in*

vitro and *in vivo* level, but also yeast-hyphae transition known as virulence factor of vulvovaginal candidiasis. To know how LCS affect *C. albicans* growth and hyphae transition, we analyzed gene expression level of *C. albicans* in hyphal inducing condition. Such as *SAP5, HWP1, ECE1, ALS3* gene related to hyphal and toxin production were downregulated significantly in mRNA level. Through the metabolomic analysis using UPLC-QTOF, we investigated in *Lactobacillus* cell-free supernatant were significantly increased.

Our study suggests that *L. fermentum* and *L. cristpatus* cell-free supernatant result in suppression of *C. albicans* growth and decreased virulence of *C. albicans* in vitro and in vivo level. Further, we performed mass spectrometric analysis to identify potential inhibitory molecule. In conclusion, we confirm that *Lactobacillus* cell-free supernatant prevent infection and inhibit *C. albicans* overgrowth.

Keywords: *Lactobacillus fermentum*, *Lactobacillus crispatus*, *Candida albicans*, vulvovaginal candidiasis

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CONTENTS

List of Tables

List of Figures

I. Introduction

The infection caused by *Candida* spp affects 75% of women at least, 6-9% of women experienced recurrent vulvovaginal candidiasis (1). In most case, *C. albicans* infection is not life-threatening disease, but lowering the quality of life in women with *C. albicans* infection. General treatment of *C. albicans* infection is use of anti-fungal medication such as Clotrimazole, Fluconazole (2). But there are many drug interaction, allergic response and resistance. Further, azole class of drug tends to be shown resistance and liver toxicity (3). Managing the fungal infection is remaining concerns for public health.

Lactobacillus spp. is a commensal microbiota of vaginal environment known as inhibiting pathogen growth by producing lactic acid, hydrogen peroxide and anti-microbial compound. (4-6). We isolated vaginal-derived microbiota from Korean healthy women who doesn't have any clinical symptoms such as bacterial vaginosis, vulvovaginal candidiasis and any sexually transmitted disease (7, 8). *L. rhamnosus* GR-1 and *L. fermentum RC-14* known as suppressing the growth of *C. albicans* and kill the fungal cell. (9) especially, low pH levels like healthy vaginal environment was more effect. also, it alleviated inflammation induced by C. albicans infection(9). Also *L. rhamnosus* GR-1 and *L. fermentum*

RC-14 cell-free supernatant shown antagonistic effect against *C. glabrata(10).* Recently published papers, *L. crispatus* cell-free supernatant shown strong inhibition of *C. albicans* growth and virulence(11). In animal model, *Lactobacillus* spp shown effect of *C. albicans* and other pathogens growth inhibition.(12, 13)

It suggests that vaginal microbiota-derived *L. fermentum* and *L. crispatus* are promising candidate to reduce burden of *C. albicans.* We isolated Korean healthy women vagina-derived *L. fermentum* SNUV175 and *L. crispatus* SNUV220. In this research, we investigated the inhibition of *C. albicans* growth and virulence by using two types of vaginal lactobacilli using *in vitro* and *in vivo* model.

Table1. microbial, cell and mouse strains used in this study

Strains	**Source**
Lactobacillus fermentum SNUV175	Isolated strains
Lactobacillus crispatus SNUV220	Isolated Strains
Candida albicans MYA-4788	ATCC
HeLa cell	ATCC
C57BL/6J 6-weeks old	Orient

Table2. Resources used in this study

Reagent and Resource		
D-glucose	Sigma-aldrich	G8270-1KG
Agar powder	Duksan	9002-18-0
Peptone	BD	211677
Yeast extract	BD	212750
L-cysteine hydrochloride	Sigma-aldrich	52-89-1
Difco Lactobacilli MRS broth	BD	288130
Tryptone	BD	211705
Glycerol	Amresco	0854-1L
Anaerobic pack	MGC	A-06
Low melting Agarose	Lonza	50100
DMEM	GIBCO	11965-092
Characterized FBS	Hyclone	SH30919.03
HPLC grade Methanol	JT Baker	9093-03
HPLC grade Water	JT Baker	4128-03
Estradiol 17-valerate	Sigma-aldrich	E1631
Sesame oil	Sigma-aldrich	S3547
RNA later	Invitrogen	AM7024
Universial qPCR master mix (SYBR green)	Kapabio Systems	KM4101
High-Capacity RNA-to-cDNA Kit	Applied biosytems	4387406
Easy-spin™ Total RNA Extraction Kit	Intronbio	17221
YeaStar RNA Kit	Zymo Research	R1002
LDH Cytotoxicity Assay Kit	Pierce	88953
Thermocylcer	Qiagen	rotor gene Q
High resolution mass spectrometry	Waters	SYNAPT G2-Si
Microsplate reader	Tecan	Infinite M200

Table 3. Primer sequences used in real-time PCR assay

Gene	Sequence
ACT1-F	TCAGACCAGCTGATTTAGGTTTG
ACT1-R	GTGAACAATGGATGGACCAG
ALS3-R	CCTGAAATTGACATGTAGCA
ALS3-F	CTAATGCTGCTACGTATAATT
ECE1_F	GCTGGTATCATTGCTGATAT
ECE1_R	TTCGATGGATTGTTGAACAC
HWP1-F	TGGTGCTATTACTATTCCGG
HWP1-R	CAATAATAGCAGCACCGAAG
SAP5_F	CAGAATTTCCCGTCGATGAGA
SAP5_R	CATTGTGCAAAGTAACTGCAACAG
mHPRT-F	TTATGGACAGGACTGAAAGAC
mHPRT-R	GCTTTAATGTAATCCAGCAGGT
mIL1β-F	GAAATGCCACCTTTTGACAGTG
mIL1β-R	CTGGATGCTCTCATCAGGACA
mIL-6-F	CTGCAAGAGACTTCCATCCAG
mIL-6-R	AGTGGTATAGACAGGTCTGTTGG

F represents sequences of a forward primer

R represents sequences of a reverse primer

II. Materials and Methods

1. Microbial strains and culture condition

Lactobacillus spp. were isolated from vaginal swab specimens of The Healthy Twin Study Korea, (7, 8) *C. albicans* ATCC® MYA-4788 was purchased from American Type Culture Collection. *C. albicans* was routinely grown in Yeast Extract-Peptone-Dextrose (YPD) medium (yeast extract 10 g, peptone 20 g, glucose 20 g) in aerobic condition at 30°C for 18 h. All *Lactobacillus* spp. were routinely growth in De Man, Rogosa and Sharpe (MRS) medium (Becton, Dickinson and Company, MD, USA) with 0.05% L-cysteine hydrochloride anaerobically at 37°C. All stock cultures of all strains were stored at -80°C with 17% glycerol as cryoprotectant.

2. Preparation of *Lactobacillus* cell-free supernatant

Lactobacilli were growth in MRS broth anaerobically at 37°C for 48 h. *Lactobacillus* culture was centrifuged at 4,000×g for 10 min at 4°C, and supernatant was collected. Then, pH of supernatant was adjusted to 6.9 with 5 M sodium hydroxide. Supernatant was filtered through 0.22-μm nitrocellulose filter (Advantec Manufacturing Inc, USA). Filtrate was stored at -20°C until used.

3. Mammalian cell culture

Cell culture was performed using HeLa human cervix epithelial cell. Cells were cultured using Dulbecco's Modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated Fetal bovine serum (FBS; Hyclone, USA) and 100 U/mL penicillin-streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and 5% CO_2

4. Epithelial cell damage assay

Epithelial cell damage caused by *C. albicans* was measured by assessing the release of lactate dehydrogenase (LDH) into the medium. Epithelial cells were grown in a 96-well culture plate and infected with 2×10^4 *C. albicans* cells without FBS. Uninfected cell culture medium was used to assess basal LDH release during an incubation period. Triton X-100 (0.1%) was used as a positive control to assess maximal level of LDH release during cell lysed following incubation period. After incubation at 37°C for 24 h with 5% CO_2, LDH activity was measured using Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions. Briefly, 50 μL of medium and 50 μL assay reagents were mixed and incubated for 20 min and stop solution added. absorbance of the dye was measured spectrophotometrically at 490 nm using Infinite M200 microplate reader

(Tecan, Männedorf, Switzerland). Cytotoxicity was calculated following equation $(\text{Test sample} - \text{uninfected control})/(\text{positive control} - \text{uninfected control}) \times 100$

5. *C. albicans* growth inhibition assay

C. albicans growth inhibition assay was performed to assess antimicrobial activity of *Lactobacillus* cell-free supernatant. *C. albicans* was cultured in YPD broth for 18 h at 30°C in a shaking incubator at 180 rpm. *C. albicans* was washed twice in phosphate buffered saline (PBS) and adjusted to concentration of 1×10^5 colony forming unit (CFU)/well in PBS. One hundred microliters of *Lactobacillus* cell-free supernatant, 100 μL of YPD broth and 50 μL of *C. albicans* suspension were added to each well of 96-well culture plate (SPL Life Sciences Co., Ltd., Korea). MRS broth was used as a growth control, Fluconazole was used as an inhibition control. The culture plate was incubated aerobically at 30°C for 24 h. The growth of *C. albicans* was measured spectrophotometrically at 600 nm.

6. Hyphal growth assay

Hyphal growth assay was performed based on the assay from (14). *C. albicans* was cultured in YPD broth at 30°C for 18 h in a shaking incubator at 180 rpm. *C. albicans* was washed twice in PBS and adjusted to a concentration of 5×10^4 cells/mL in serum-free Roswell Park

Memorial Institute (RPMI) 1640 medium. One milliliter of *C. albicans* cell suspension was added to 24-well culture plate. The plate was incubated at 37°C, 5% CO_2 for 3h. After incubation, medium was eliminated and *C. albicans* cells were fixed in 4% paraformaldehyde and stained with Calcofluor white (Sigma-Aldrich, St. Louis, MO, USA). Hyphal Images were taken from 200 hyphae using EVOS FL cell imaging system (Thermo Fisher Scientific, Waltham, MA, USA). Hyphal length was measured using the software ImageJ (National Institutes of Health, Bethesda, USA). All of hypha branches were included in the length measurement.

7. RNA extraction and quantification of gene expression

C. albicans was cultured in YPD medium at 30°C for 16 h in a shaking incubator (180 rpm). *C. albicans* cells were washed with PBS twice and resuspended with serum-free RPMI 1640 medium. *C. albicans* 1×10^7 were inoculated in 100-mm^2 culture dish and incubated at 37°C and 5% CO_2 for 3 h. After incubation, medium was eliminated, and then *C. albicans* was rinsed with cold PBS and collected using cell scraper. harvested *C. albicans* was washed with 1 mL of cold PBS and centrifuged (3,000×g) at 4°C for 2 min. Supernatant was removed, and cell pellet was stored in -80°C until RNA extraction. RNA extraction was

performed using Yeastar^{TM} RNA Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. Using 500 μg RNA as a template, cDNA was synthesized using High Capacity RNA-to-cDNA Kit (Applied biosystems, USA) according to manufacturer's instruction. cDNA samples were used for quantitative PCR with KAPA SYBR® FAST qPCR Kit Master Mix (Kapa biosystems, MA, USA). Amplification were performed using a Rotor Gene-Q (Qiagen, USA). The $2^{(-\triangle\triangle C_T)}$ method ($\triangle\triangle C_T=(C_{T,Target}-C_{T,Ref})_{treated}-(C_{T,Target}-C_{T,Ref})_{control}$) was used (where C_T is the threshold cycle) for calculating relative gene expression

8. Murine model of vaginal *C. albicans* infection

Murine vulvovaginal candidiasis model study was performed according to Seoul National University IACUC guidelines (SNU-170801-7). Animal model of *C. albicans* infection based on (15, 16) with minor modification. Briefly, 0.5 mg of β-estradiol 17-valerate (Sigma-Aldrich, St Louis, MO, USA) in 100 μL sesame oil was administrated intraperitoneally for 72 h prior to *C. albicans* infection. It was continuously administrated weekly. Lactobacillus cell-free supernatant (20 μL) was administrated intravaginally for 14-day pre/post-infection with anesthetics. C. albicans (5×10^6 CFU) in 10 μL PBS with 1% low-melting agarose (Lonza, Basel, Switzerland) was prepared and

administrated intravaginally. After 6-day post infection, all mice were anesthetized and sacrificed, and then vaginal fluid and organs were collected.

9. Mass spectrometric analysis using UPLC-QTOF

Two *Lactobacillus* stains (*L. fermentum* SNUV175 and *L. crispatus* SNUV220) were cultured anaerobically at 37℃ for 48 hr. *Lactobacillus* cells were removed by centrifugation and filtration using 0.22 μm syringe filter (Adventec, USA). Two hundred milliliter of *Lactobacillus* cell-free supernatant and eight hundred milliliter of ethanol were mixed to extract soluble compound. supernatant-methanol mixtures were sonicated for 3 min and centrifuged at 13,000×g for 2 min. Supernatants were transferred into new tube and add 1 mL of methanol was added to solid pellet and sonicated for 3 min, vortexed for 5 min and centrifuged at 13,000xg for 2 min. supernatants were added to previous extracted sample. Methanol extracts were evaporated using centrifugal vacuum concentrator (Eppendorf, USA) at 30 ℃ and reconstituted with 55 %/45 % methanol/water(v/v). all samples were filtrated with 0.22 μm syringe filter and store at -20℃ until analysis. Supernatant metabolites separated using an Acquity UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 m) and Acquity UPLCTM system (Waters, MA, USA). The analysis conditions for chromatography were as follows: mobile phase A, water

with 0.1% formic acid; mobile phase B, acetonitrile with 0.1% formic acid; injection volume, 10 μl. The time gradient of mobile phase B was from 5% to 100% in 15 min and the flow rate was 0.4 ml/min. Qualitative metabolome analysis was conducted using Synapt G2-Si Q-TOF mass spectrometer (Waters, MA, USA) equipped with an electrospray (ESI) probe and negative ionization mode with tofMRM mode. For the Synapt G2-Si QTOF, the following mass spectrometer parameters were applied: capillary, 25 kV; source temperature, 100°C; sampling cone, 40; source offset, 80; desolvation temperature, 250°C; cone gas flow, 50 L/h; desolvation gas flow, 600 L/h; nebuliser gas flow, 6.5 Bar. The measurement of supernatant metabolites was performed in positive ionization mode and MSE scan mode using the same column and UPLC conditions. The mass range was set from 50 to 1200 Da and the scan time was set to 0.1 sec. The following mass spectrometer parameters were used: capillary, 2 kV; source temperature, 120°C; sampling cone, 40; source offset, 80; desolvation temperature, 400°C; cone gas flow, 50 L/h; desolvation gas flow, 600 L/h; nebuliser gas flow, 6.5 bar. All systems were controlled by MassLynxTM software 4.1 (Waters, MA, USA). Intergroup metabolism analysis was carried out by transferring MassLynxTM software to the Progenesis QI software (Waters, MA,

USA). A retention time window of 0.20 min and mass tolerance of 1.0 ppm were set to align the compounds. Then, ANOVE p-value and max fold change were applied to filter compounds and annotated using ChemSpider database including BioCyc, *E.coli* metabolome database, Human metabolome database, KEGG, Yeast metabolite database.

10. Statistical analysis

All data were analyzed with Prism 5 (GraphPad Software, San Diego, CA) and R version 3.4.4 (http://www.R-project.org). Statistical significance was measured using Mann-Whitney test when comparing two groups, One-way ANOVA with Tukey's post-tests comparing each group. In all graphs, data were presented as mean ± sem. Statistical significance was given as * *P*-value <0.05, ** *P*-value < 0.01, *** *P*-value <0.001.

III. Results

1. Effect of *Lactobacillus*-cell free supernatant on *C. albicans* growth.

Lactobacillus fermentum and *Lactobacillus crispatus* cell-free supernatant were assayed for their *C. albicans* growth inhibition activity. All *Lactobacillus* cell-free supernatant pH was adjusted to neutral pH (pH 6.9) and filtrated to prevent potential *Lactobacillus* or other contamination could affect assay results. Firstly, we measured activities of cell-free supernatant at stationary phase of *Lactobacillus* growth. 12 h, 24 h, 48 h and 72 h after inoculation, *Lactobacillus* cell-free supernatants were harvested and subjected for *C. albicans* growth inhibition assay. *Lactobacillus fermentum* cell-free supernatant shown anti-microbial activity at early time and *Lactobacillus crispatus* cell-free supernatant shown activity after 24 h inoculation (Figure1a). After 24 hr and 48 h incubation, *Lactobacillus* cell-free supernatant shown optimal inhibition activity robustly. (Figure 1b). *L. fermentum* supernatant and *L. crispatus* supernatant shown growth inhibition activity significantly versus MRS media control (*L. fermentum*, p=0.0006; *L. crispatus*, p=0.0008) equivalent to Fluconazole 250 ng/mL (p=0.0002), one of anti-fungal drug. These results suggest that *L. fermentum* and *L. crispatus* supernatant limit *C. albicans* growth. But, other species such as *L.*

jensenii and *L. gasseri* only weak activity on inhibition of *C. albicans* growth (Figure 1c). In this study, we confirmed *C. albicans* growth inhibition potency of *Lactobacillus* cell-free medium in vitro and in murine vulvovaginal *C. albicans* infection model

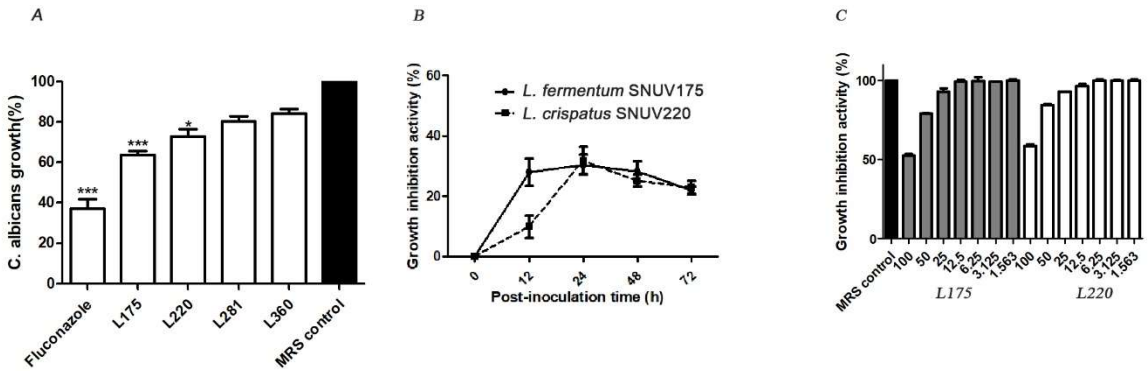

Figure 1. Anti-fungal compound production of *Lactobacillus* cell-free supernatant

Candida albicans $5x10^3$ cells in YPD broth with *Lactobacillus* cell-free supernatant were incubated at 30℃ for 24 h. *C. albicans* growth was measured. (a) *Lactobacillus* spp. effect on *C. albicans* growth. Fluconazole, an antifungal agents as a positive control, L175, L220, L281, L360 represent *L. fermentum* SNUV175, *L. crisptatus* SNUV220, *L. gasseri* SNUV281, *L. jensenii* SNUV360 respectively. (b) *C.albicans* growth activity of *Lactobacillus* cell free-supernatant at different growth stage (incubation time). (c) *C. albicans* growth inhibition activity of Lactobacillus cell free supernatant at different concentration. represent as the means with standard error of mean(SEM) of four independent experiments. Statistical significance was calculated using Kruskal-Wallis test with a post hoc Dunn comparison test. ***, $p<0.005$, **, $P<0.001$, *, $P<0.05$, ns, not significant.

2. The effect of *Lactobacillus* cell-free supernatant on *C. albicans* hyphae growth.

C. albicans hyphal growth were reported as a virulent factor of *C. albicans* infection (17-21). We investigate effect of *Lactobacillus* cell-free supernatant under hyphae inducing conditions. *C. albicans* was incubated with Lactobacillus cell-free supernatant shown shorten hyphal length versus MRS control group. *L. fermentum* SNUV175 ($p<0.0001$) and L. crispatus SNUV220 ($p<0.0001$) cell-free supernatant significantly decreased hyphal length. Averagely hyphal length (Mean ± SD) of MRS control (26.13 ± 26.13) compared to *L. fermentum* (17.98 ± 10.07) and *L. crispatus* (18.74 ± 10.44). Hyphae-state of *C. albicans* were known as a virulent form. *ECE1* gene, coding candidalysin peptide which peptide secreted and has potent to lysis mammalian cell membrane.(22) this peptide represent virulence. Only hyphae form of *C. albicans* can secrete and induce cellular damage. Average hyphal length decreased but some population of *C. albicans* still longer in same group. Namely, hyphal length of total population decreased but no all individuals decreased. Non-response *C. albicans* were shown.

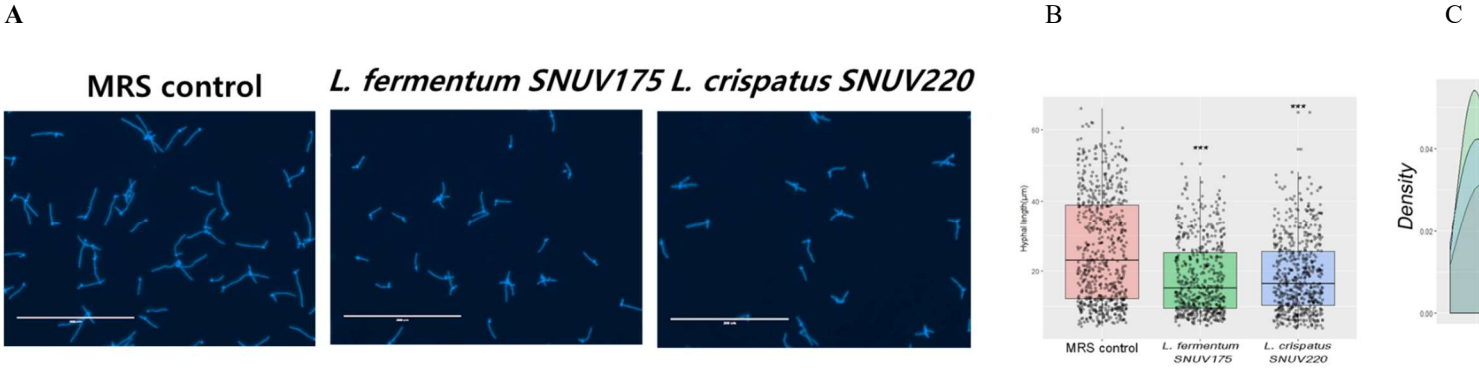

Figure 2. Effect of *Lactobacillus* cell-free supernatant on yeast-to-hypha transition.

C. albicans $1x10^4$ were incubated in serum-free RPMI1640 with *Lactobacillus* cell-free supernatant at 37℃ with 5% CO_2 condition for 3
formation. (a) Hypha was stained with Calcofluor White and hyphal length of *C. albicans* was manually measured, (b) represent as the mec
boxplot with plotted distribution. (c) distribution of hyphal lengths were overlapped in a histogram. Statistical significance was calcula
Wallis test with a post hoc Dunn comparison test. ***, p<0.005, **, P<0.001, *, P<0.05. Scale bar 200μm

3. *Lactobacillus* cell-free supernatant modulate *C. albicans* gene expression.

To confirm Lactobacillus cell-free supernatant effect in *C. albicans* gene expression, we performed qPCR assay to quantification of virulence gene expression. The expression levels of the hyphae-specific genes (*ALS3, HWP1, ECE1, EAP1* and *SAP5*) transcriptional regulator genes were quantified in *C. albicans* after *Lactobacillus* cell-free supernatant treatment using real time PCR. (Figure 4) shown *L. crispatus* and *L. fermentum* cell-free supernatant down-regulated the expression of hyphal-specific gene significantly. Gene expression level of *ALS3* (0.44-fold; 0.4-fold)*, ECE1 (*0.33-fold; 0.42-fold*), HWP1* (0.60-fold; 0.60-fold) and *SAP5* (0.44-fold; 0.4-fold) were significantly downregulated. The results suggested human vagina-derived *Lactobacillus* cell-free supernatant modulate *C. albicans* at gene expression level. This virulence genes were reported that patients with *C. albicans* infection with symptom shown significantly increased these gene expression (23).

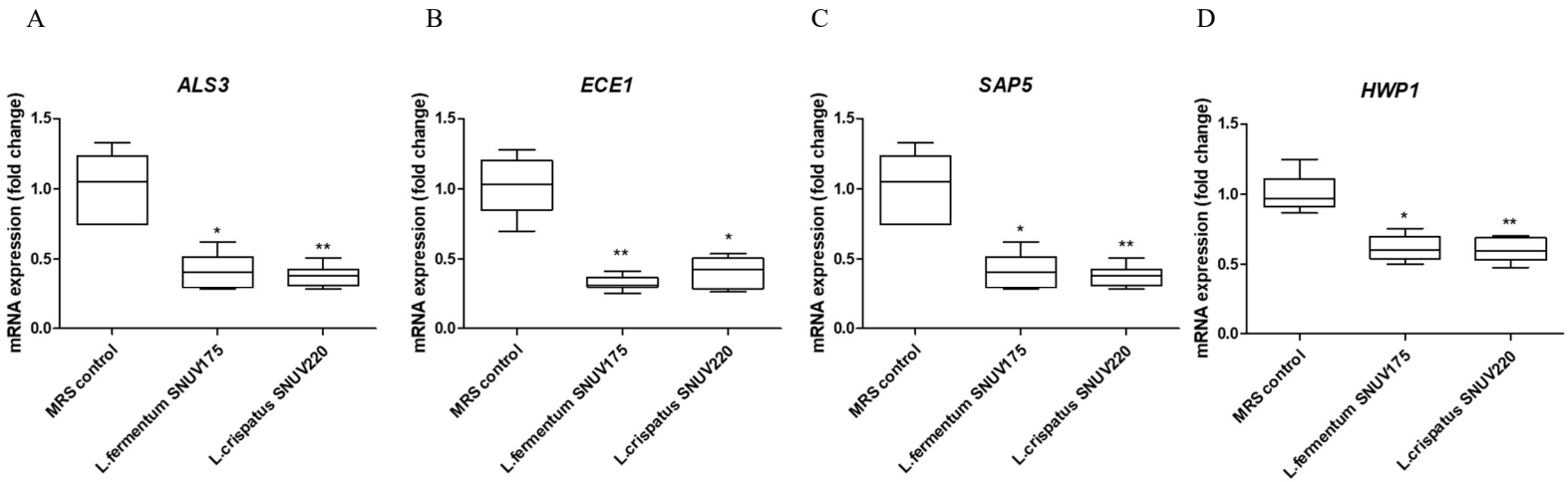

Figure 3. Effect on Lactobacillus cell-free supernatant on virulence gene expression of *C. albicans*

Candida albicans 1x10^7 cells in RPMI 1640 with *Lactobacillus* cell-free supernatant were incubated at 37°C for 3h, harvested and subjected to qPCR analysis. Expression level of *(a)ALS3, (b)ECE1, (c)SAP5, (d)HWP1* genes were measured, represent as the median and quartile on boxplot of three independent experiments and two experimental replications. Statistical significance was calculated using Kruskal-Wallis test with a post hoc Dunn comparison test. ***, $p<0.005$, **, $P<0.001$, *, $P<0.05$

4. *Lactobacillus* cell-free supernatant effect on HeLa cell infection model

Next, we investigate the effect of *Lactobacillus* cell-free supernatant on cytotoxicity of *C. albicans* A cytotoxicity assay was performed in HeLa cell was co-incubated with *C. albicans* cell and *lactobacillus* cell-free supernatant for 24h and we analyzed secreted Lactate dehydrogenase (LDH) as a marker for cell damage by infection. It was found that treatment of *lactobacillus* cell-free supernatant increased cell damage significantly. But in these cell-based model, *Lactobacillus* supernatant doesn't show any effect on *C. albicans* infection. Cytotoxicity is slightly increased

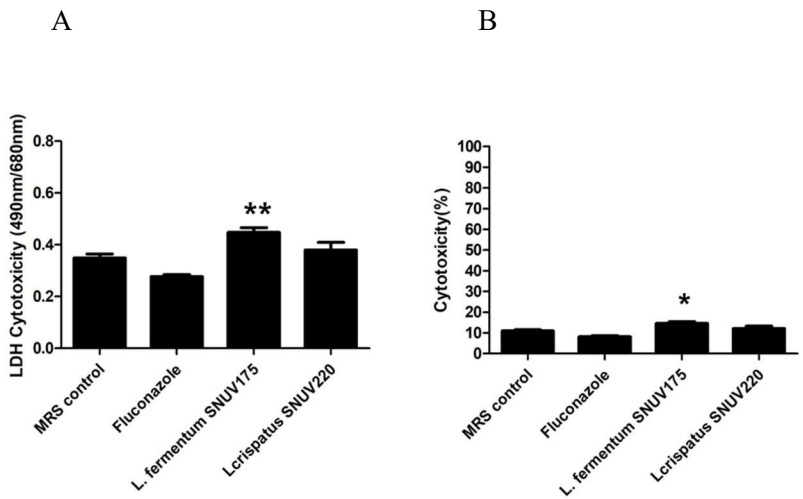

Figure 4. Effect on *Lactobacillus* cell-free supernatant on cell line model of *C. albicans* infection

Hela cell was infected with *C. albicans* 5×10^3 cells and *Lactobacillus* cell-free supernatant. Infected cell was incubated at 37 ℃ for 24 h, cytotoxicity was measured, represent as the means with standard error of mean(SEM) of four independent experiments and two experimental replications. (a) LDH level at 24hr after *C. albicans* infection. (b) percent of cytotoxicity at 24hr after *C. albicans* infection. Statistical significance was calculated using Kruskal-Wallis test with a post hoc Dunn comparison test. ***, $p<0.005$, **, $P<0.001$, *, $P<0.05$

5. *Lactobacillus* cell-free supernatant alleviate *C. albicans* burden of in vivo vaginal infection model

To confirm an effect of *Lactobacillus* cell-free supernatant on mouse vaginal tissue of *C. albicans* infection model, mouse vaginal fluid and vaginal tissues were harvested and analyzed. In *Lactobacillus* cell-free supernatant treatment group, the burden of *C. albicans* was significantly decreased (CFU/mL, p=0.026) compared to control group (Figure 6a) and increased *C. albicans* clearance in histological evaluation (Figure 6b). further, we analyzed mRNA expression level of pro-inflammatory cytokine. IL-1β and IL-6 level shown tendency to decrease but, not statistically significantly (IL-1β, p=0.6088; IL-6, p=0.2768) in our animal study. Both of IL-1β and IL-6 were known as pro-inflammatory cytokine when *C. albicans* infected vaginal tissue(24, 25). this cytokine was induced by Candidalysin(26), one of virulence factors of vulvovaginal candidiasis.

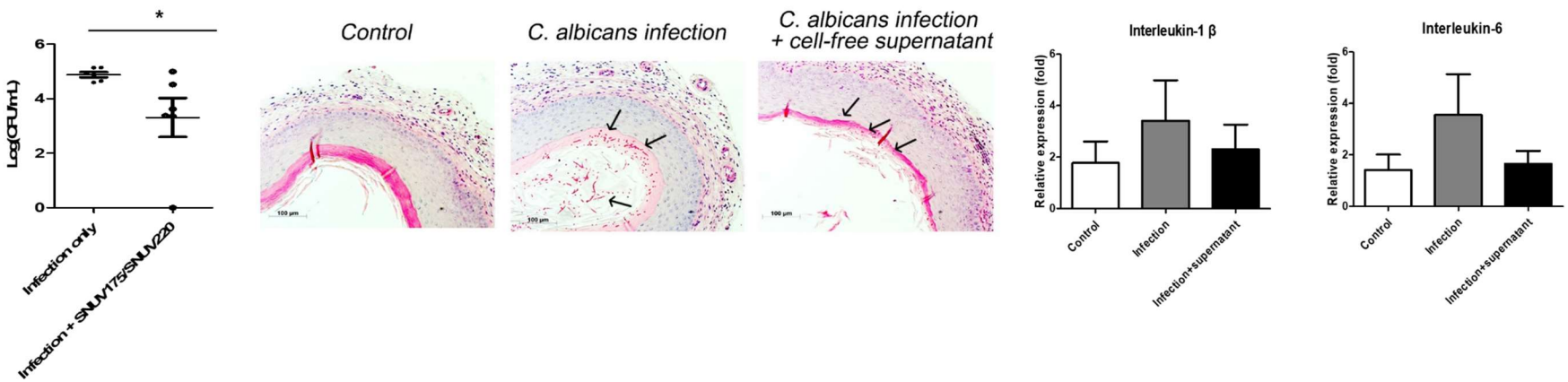

Figure 5. Effect on Lactobacillus cell-free supernatant on Candida albicans growth in murine model of vulvovaginal candidiasis.

C57BL/6 mice were infected intravaginally with *C. albicans* 5x10^6 cells and *Lactobacillus* cell-free supernatant 20 uL was treated daily for 2 weeks (pre-/post-infection). (a) *C. albicans* colony forming unit (CFU) in vaginal fluid were counted on SDAC agar. (b) histological evaluation of vaginal infected with *candida albicans* with period acid-Schiff (PAS) staining. (c) expression level of IL-1β and IL-6 gene were measured, represent as the means with standard error of mean(SEM) of control group(n=6), infection group(n=6) and infection and *Lactobacillus* cell-free supernatant group(n=7). Statistical significance was calculated using Kruskal-Wallis test with a post hoc Dunn comparison test. ***, p<0.005, **, P<0.001, *, P<0.05. Scale bar 100μm. black arrow: *C. albicans* in vaginal tissue.

Table 4. Metabolomic analysis of *Lactobacillus* cell-free supernatant using UPLC-qTOF-MS/MS

Mass per charge(m/z)	Charge	Retention time (min)	*p*-value	*q*-value	Fold Change	Average abundance
Lactobacillus fermentum SNUV175						
517.5723466	1	6.120017	4.95E-08	0.000542	Infinity	130.1074
883.2651114	2	3.67995	5.94E-07	0.002009	Infinity	36.70314
1342.748325	1	7.67545	6.53E-07	0.002009	Infinity	51.62812
219.9822541	1	3.901317	9.20E-07	0.002009	Infinity	97.55688
580.7084937	3	12.63508	1.10E-06	0.002009	1530.049	185.2886
1386.097293	2	7.754	3.54E-06	0.004839	130708.1	49.77158
1220.216238	1	7.56835	6.24E-06	0.007586	160.1513	10.28973
475.5790112	1	6.041467	9.65E-06	0.010171	Infinity	37.45057
682.6260621	1	12.67078	1.26E-05	0.011454	3.247389	296.8317
318.0013168	2	0.775967	3.17E-05	0.024576	Infinity	30.75518
590.9672037	1	6.262833	3.37E-05	0.024576	Infinity	75.85158
583.2077756*	2	13.17013	4.23E-05	0.025745	1303.648	278.8606
1494.474737	3	6.420083	4.82E-05	0.027289	Infinity	27.71424
211.9473149	1	13.2344	5.70E-05	0.027289	Infinity	122.2817
1222.103358	3	6.83355	6.98E-05	0.031829	Infinity	59.35631
127.1128305	1	13.44078	7.67E-05	0.032465	Infinity	38.76407
1212.81816	2	8.039133	0.000112	0.039098	Infinity	11.30567
1165.844086	1	0.548167	0.000115	0.039098	Infinity	268.0162
398.5529646	3	13.31295	0.000118	0.039098	Infinity	131.9507
1127.96031	3	8.410733	0.000164	0.049836	105.7636	28.76904
Lactobacillus crispatus SNUV220						
583.2077756*	2	13.17013	5.76E-06	0.02338	485.2573	103.8004

* denote same mass per charge (m/z) of *L. fermentum SNUV175* and *L. crispatus SNUV220.*

6. Metabolomic analysis of *Lactobacillus* cell-free supernatant

We investigate *Lactobacillus* cell-free supernatants to identify secreted compound produced *L. fermentum* and *L. crispatus.* Mass spectrometric analysis shown *L. crispatus* and *L. fermentum* specific metabolites increased significantly after incubation in MRS media. We prioritize metabolites according to fold change and statistical comparison using ANOVA. We found that compounds were highly increased versus control. we applied q-value <0.05 cutoff to prioritize detected compound (Table 4.). This metabolomic analysis suggest that these compounds are potential candidate of anti-*candida* compound.

IV. Discussion

In this study, we investigate how *Lactobacillus* cell-free supernatant inhibit *C. albicans* growth and virulence. Interestingly, we found that not only inhibition of *C. albicans* growth was inhibited but also yeast-hyphae transition known as virulent factor significantly using *in vitro* and *in vivo* model. In previous study*, L. rhamnosus, L. reuteri* are well-studied in vaginal probiotics that inhibit pathogen growth((9, 10, 27). Also, recent study shown *L. crispatus* cell-free supernatant suppress *C. albicans* growth and hyphal growth in vitro level(11). *L. fermentum* shown broad inhibitory effect on *Candida* spp(28). Most of studies focused on in vitro level to confirm inhibitory effect on *C. albicans* growth, biofilm formation. But here, we confirmed *C. albicans* inhibition activity, hyphal growth, and we established animal model for vaginal *C. albicans* infection model to test effect of *Lactobacillus* cell-free supernatant.

But this mouse vaginal infection model does not mimic human vaginal environment completely(29). The vaginal environment of humans maintains a pH of around 4, but in mice it exhibits neutral pH. For this reason, It was difficult to colonize in vagina of *Lactobacillus* however, the experiment using *Lactobacillus* cell free supernatant

showed a successful decrease of CFU.

To know how *Lactobacillus* cell-free supernatant affect *C. albicans* growth and hyphae transition, we analyzed gene expression level of *C. albicans* in hyphal inducing condition. Such as *SAP5, HWP1, ECE1, ALS3* gene coding hyphal and toxin related proteins(30, 31) were downregulated significantly in gene expression level. Especially ECE1 gene known as coding 31a.a Candidalysin, a toxin, that can cause cell damage and inflammasome activation (26, 32). Inhibition of ECE1 gene expression could be alleviate inflammation and prevent cell barrier disruption in vaginal tissue.

However, the exact mechanism regulating the growth and virulence-related gene expression is not fully understood. There is still unidentified compound that modulate *C. albicans* gene expression and *C. albicans* growth. Through the metabolomic analysis using UPLC-qTOF. We confirmed that specific compound *Lactobacillus* cell-free supernatant were significantly increased. We thought that these chemicals are potential biomarker and candidate compound inhibiting *C. albicans* growth and further reduce virulence. but we failed to identify these chemicals using known LC-MS/MS database in this research.

The limitation of our research is firstly, that mouse model of

vulvovaginal candidiasis could reflect human vaginal physiology. Especially vaginal pH is typically 4.5 in human, but any other non-human mammals shown pH 5.4-7.8 range and *Lactobacillus* spp. is not dominant microbiota in non-human mammals(33). In non-target metabolomic analysis, we confirmed compound increased in *Lactobacillus* cell-free supernatant, but we couldn't identify these chemical form *L. crispatus* and *L. fermentum.* Thus, we did not validate whether these chemicals inhibit C. *albicans* growth or not. It still remains our follow up study. In future studies are needed to elucidate the mechanism of action at detailed molecular level what molecule suppress growth and hyphal growth. Wang reported antifungal compound that can inhibit hyphal growth would be protein (11) but, growth inhibitory molecule seems to be different to that protein. A lot of compound could be contributing C. albicans growth and hyphal growth to maintain physiology of vagina.

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국문초록

한국인 여성 유래 락토바실러스 퍼멘텀 SNUV175 및 락토바실러스 크리스파투스 SNUV220 배양액의 질염모델에서 칸디다 알비칸스 저해 연구

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장 성 재

지도교수 고 광 표

락토바실러스균은 여성의 질내 환경에 서식하는 균으로 병원균의성장을 저해하고 항생물질을 분비하는 것으로 알려져 있다.

건강한 한국인 여성의 질에서 분리한 *Lactobacillus fermentum SNUV175* 및 *Lactobacillus crispatus SNUV220*을 이용해 칸디다성 질염에 적용하기 위한 연구를 진행하였다. 여성의 질유래 락토바실러스와 질염 원인균인 *C. albicans* 간의 상호작용을 확인하기 위한 연구 수행하였다. 락토바실러스 배양상층액을 이용하여 칸디다균의 성장저해를 확인하였고, 질염동물모델을 통해 질내 환경에서도 락토바실러스 유래물질이 칸디다의 성장을 저해하고 면역을 조절함을 확인하였다. 흥미롭게도 락토바실러스의 배양상층액은 칸디다의 성장을 저해할 뿐 아니라, 칸디다성질염의 원인으로 알려져 있는 균사성장을 저해하는 것을 확인했다. 또한 균사성장 및 독성인자에 대한 유전자수준에서의 변화를 학인하기 위해 독성유전자로 알려진 *ALS3, ECE1, HWP*, *SAP5*에 대한 유전자발현량 변화를 확인하였으며 유의적인 수준으로 감소하는 것을 확인하였다. 또한 락토바실러스 배양상층액을 질량분석기를 이용해 대사체를 분석을 수행하였다. 요약하자면 락토바실러스 배양상층액이 칸디다 알비칸스의 성장을 저해하고 독성을 감소시키는 것을 확인했으며, 질량분석기를 통한 칸디다성장 및 독성을 억제하는 잠재적인 후보물질을 확인했다. 결론적으로 여성 질내 서식하는 락토바실러스균이 항진균물질을 분비해 칸디다의 성장과 독성을 저해함을 확인하였다. 이 락토바실러

스 유래의 항진균물질은 칸디다성 질염의 발병원인을 설명하고 잠재적으로 칸디다 감염의 저해함으로써 질병에 대한 부담을 줄여줄 수 있을 것으로 보인다

주요 단어: 락토바실러스 퍼멘텀, 락토바실러스 크리스파투스, 칸디다 알비칸스, 칸디다성질염

학번: 2016-28127

감사의글

졸업논문을 위해 실험을 수행하고 작성되어 이곳까지 오기까지 도움을 주신 교수님들과 연구실 선후배님, 친구, 함께 다녔던 대학원동기들 그리고 가족들에게 감사를 드립니다. 특히나 졸업논문을 심사해주신 백도명 교수님, 고광표 교수님, 유현주 교수님께 감사합니다. 보건대에 들어온 지 어느덧 2년이 지나고 졸업을 하게 되었습니다. 보건대학원에 들어오기 전부터 졸업까지 우여곡절도 많고, 저에겐 의미 있는 장소이기에 석사과정의 시간은 많은 추억이 남을 듯합니다. 처음으로 보건학과 환경보건학이라는 분야의 새로운 학문도 접해볼 수 있었고, 특히나 마이크로비옴과 같이 흥미로운 분야에 대해서도 새로 접해보고 공부할 수 있는 값진 시간이었습니다.

대학교를 졸업해 대학원을 다니는 친구들과, 직장인이 된 친구들 그리고 새로운 길을 찾아가는 친구들까지 만날 때마다 재미있는 이야기, 좋은 이야기 많은 도움이 되었습니다. 그리고 대학교를 졸업하고, 대학원까지 다닌다고 말은 많으시지만 격려해주시는 부모님과 가족들에게 감사합니다.

대학원에 입학해 졸업하기까지 여러 길을 돌아왔지만 결국 이제 와서야 어릴 적 생각했던 길을 다시 찾아가는 것 같습니다. 아직은 모자란 점도 많고 아쉬움도 남아 보건대학원에 박사과정에 남게 되었지만 그동안 연구실의 교수님들과, 선후배님들, 졸업하는 대학원동기들, 친구들 그리고 가족들의 많은 도움과 응원에 다시 한번 감사합니다.