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# Culture Supernatants of *Lactobacillus gasseri* and *L. crispatus* Inhibit *Candida albicans* Biofilm Formation and Adhesion to HeLa Cells

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## Abstract

**Purpose**: Vulvovaginal candidiasis (VVC) is a common superficial infection of the vaginal mucous membranes caused by the fungus *Candida albicans*. The aim of this study was to assess the mechanisms underlying the inhibitory effects of the culture supernatants of *Lactobacillus gasseri* and *Lactobacillus crispatus*, the predominant microbiota in Asian healthy women, on *C. albicans* biofilm formation. The inhibition of *C. albicans* adhesion to HeLa cells by *Lactobacillus* culture supernatant was also investigated.

**Methods**: *C. albicans* biofilm was formed on polystyrene flat-bottomed 96-well plates, and the inhibitory effects on the initial colonization and maturation phases were determined using the XTT reduction assay. The expression levels of biofilm formation-associated genes (*HWP1*, *ECE1*, *ALS3*, *BCR1*, *EFG1*, *TEC1*, and *CPH1*) were determined by reverse transcription-quantitative polymerase chain reaction. The inhibition of *C. albicans* adhesion to HeLa cells by *Lactobacillus* culture supernatant was evaluated by enumerating viable *C. albicans* cells.

**Results**: The culture supernatants of both *Lactobacillus* species inhibited the initial colonization and maturation of *C. albicans* biofilm. The expression levels of all biofilm formation-related genes were downregulated in the presence of *Lactobacillus* culture supernatant. The culture supernatant also inhibited *C. albicans* adhesion to HeLa cells.

**Conclusions**: The culture supernatants of *L. gasseri* and *L. crispatus* inhibited *C. albicans* biofilm formation by downregulating biofilm formation-related genes and *C. albicans* adhesion to HeLa cells. These findings support the notion that *Lactobacillus* metabolites may be useful alternatives to antifungal drugs for the management of VVC.


**Key words**: Biofilm; *Candida albicans*; HeLa cells; *Lactobacillus*; vulvovaginal candidiasis.

# Introduction

Vulvovaginal candidiasis (VVC) is a common infection of the vaginal mucosa caused by *Candida* species (*Candida albicans* in 85–95% of cases) [1]. VVC affects 75% of women at least once during their lifetime and manifests as a thick vaginal discharge and pruritus [1, 2]. Clotrimazole or miconazole is commonly used for the treatment of VVC [1]. 5% of women suffer from recurrent disease, defined as four or more episodes within 1 year [3]. Quality of life is diminished for women who experience recurrent VVC [4].

*C. albicans* exists as a yeast and/or hyphal form in the vagina. Yeast cells undergo the budded-to-hyphal form transition (BHT) under certain temperature or pH conditions, or combinations of several factors [5, 6]. The hyphal form plays an important role in disease by invading epithelial cells and damaging tissues [7]. *Candida* species in humans may also form biofilms. Biofilm formation is an important virulence attribute of *Candida*; cells in biofilms are more resistant to both antifungals and host defenses than are their planktonic or suspended counterparts [8].

A healthy vaginal microbiota, which plays a key protective role in the prevention of gynecological diseases, is predominated by *Lactobacillus* species [9, 10]. VVC occurs frequently in patients after taking antibiotics, indicating that the vaginal microbiota may be related to the colonization of *Candida* [11]. Morales et al. [12] described how the normal vaginal microbiome protects against *C. albicans* infection; the bacteria inhibit *Candida* adhesion to epithelial cells by outcompeting fungal cells for adhesion sites, secreting biosurfactants, which physically reduce fungal binding, and releasing hydrogen peroxide and lactic acid that inhibit *C. albicans* proliferation and the formation of invasive hyphae [12]. Therefore, *Lactobacillus* species play important roles in the prevention and the treatment of VVC.

*Lactobacillus gasseri* and *L. crispatus* are predominant in the vaginal microbiota of Asian women [9]. In the present study, we evaluated the inhibitory effects of the culture supernatants of *L. gasseri* and *L. crispatus* on biofilm formation by *C. albicans* and on *C. albicans* adhesion to HeLa cells.

# Materials and Methods

## Microorganisms, growth media, and culture conditions

*C. albicans* SC5314, and *L. gasseri* JCM 1131, and *L. crispatus* JCM 1185 were routinely and anaerobically grown on Sabouraud Dextrose Agar (SDA) and de Man-Rogosa-Sharpe (MRS) broth (Oxoid, UK), respectively.

## Preparation of culture supernatants of *Lactobacillus*

*L. gasseri* and *L. crispatus* were incubated anaerobically in 20 mL MRS broth for 24 h at 37°C, and then

bacterial cell numbers were adjusted to $1 \times 10^8$/mL in MRS broth. The cell suspension was incubated for an additional 48 h at 37°C. The culture supernatant was collected by centrifugation and filtered through a 0.22 μm membrane.

## Formation and inhibition of *C. albicans* biofilm

The effects of *Lactobacillus* culture supernatant on the initial colonization and maturation of biofilm formation by *C. albicans* were investigated according to the method described by Matsubara et al. [8] (Fig. 1). A *C. albicans* suspension (100 μL, $1 \times 10^7$ cells/mL) in RPMI 1640 (Life Technologies, USA) was added to polystyrene flat-bottomed 96-well plates and incubated for 1.5 h at 37°C. Subsequently, the cell suspensions were removed, and the wells were washed twice with phosphate-buffered saline (PBS) to remove non-adherent cells. In the initial colonization assay, 100 μL RPMI 1640 and the *Lactobacillus* culture supernatant diluted in MRS broth (to final concentrations of 7.5, 15, and 30% [v/v]) were transferred to wells, and the plates were subsequently incubated at 37°C for 24 h (total, 25.5 h). For the maturation phase, 100 μL RPMI 1640 were added to biofilms after a 1.5 h incubation and then incubated for another 24 h. All wells were washed twice with PBS, and 100 μL RPMI 1640 and *Lactobacillus* culture supernatant diluted with MRS broth (adjusted to final concentrations of 7.5, 15, and 30% [v/v]) were added to the biofilms after the 25.5 h incubation (1.5 h plus 24 h). The plates were then incubated for an additional 24 h (total, 49.5 h). Plates containing MRS broth only served as controls. Biofilm formation was evaluated using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay [8, 13], a method commonly utilized for quantitative measurement of *Candida* biofilm mass and growth [14]. The XTT assay is an eminent tool due to its ability to detect live yeast and hyphal organisms in the biofilm [14].

## Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

RNA extracted from maturation-phase biofilms was synthesized into cDNA using PrimeScript RT Master Mix (TaKaRa Bio, Japan) according to the manufacturer's instructions. RT-qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, USA) and the primers listed in Table 1 [15].

## Inhibition of *C. albicans* hyphal formation by *Lactobacillus* culture supernatant

A hyphal formation assay of *C. albicans* was performed in RPMI 1640 medium according to the method of Wang et al. [16], with slight modifications. *C. albicans* grown on SDA was washed with PBS, suspended in RPMI 1640, and adjusted to $1 \times 10^6$ cells/mL. Culture supernatants of *L. gasseri* or *L. crispatus*, at final concentrations of 2% and 8% (v/v), were added to 3 mL of the *C. albicans* suspension. Changes in cell morphology were observed microscopically after incubation for 3 h or 24 h at 37°C.

### Inhibition of *C. albicans* adhesion to HeLa cells by *Lactobacillus* culture supernatant

HeLa cells (JCRB Cell Bank, Japan) were grown overnight in 96-well flat-bottomed plates at 37°C under 5% (v/v) $CO_2$ to 100% confluence in Dulbecco's Modified Eagle's Medium (DMEM; Nacalai Tesque, Japan) supplemented with 10% (v/v) fetal bovine serum. The inhibition of *C. albicans* adhesion to HeLa cells by *Lactobacillus* culture supernatant was evaluated by the method of Coman et al. [17] with slight modifications. After removing DMEM, 100 µL *C. albicans* cell suspensions ($1 \times 10^9$/mL) diluted with *Lactobacillus* culture supernatant (adjusted to final concentrations of 0 and 100% [v/v]) were added to each well, and the plate was incubated for 1.5 h at 37°C under 5% (v/v) $CO_2$. After incubation, the cell suspensions were removed, and the wells were washed five times with PBS to remove non-adherent *C. albicans* cells. Ten-fold serial dilutions were spread onto SDA plates for the enumeration of yeasts adhering to HeLa cells.

### Statistical analysis

Statistical comparison between two groups was performed by Welch's two sample t-test. A P-value < 0.05 was considered statistically significant.

## Results

### Effects of *Lactobacillus* culture supernatant on *C. albicans* biofilm formation

The *L. gasseri* and *L. crispatus* culture supernatants (15, 30% [v/v]) significantly inhibited biofilm formation by *C. albicans* (Fig. 1). The two culture supernatants exerted almost identical inhibitory effects; *L. gasseri* and *L. crispatus* culture supernatants at 30% reduced the initial colonization phase to 56.9 ± 14.0% and 49.5 ± 14.7% of the control, respectively, and the maturation phase to 28.5 ± 8.1% and 35.5 ± 10.1% of the control, respectively.

### Gene expression during biofilm formation by *C. albicans*

The expression of BHT- and biofilm-associated genes was investigated by RT-qPCR (Fig. 2). Both *Lactobacillus* culture supernatants significantly suppressed the expression of *HWP1* and *ECE1* (hypha-specific genes), *ALS3* (adhesion-related gene), *BCR1* (biofilm-related gene), and *EFG1*, *TEC1*, and *CPH1* (hypha-related transcription factors) by *C. albicans* [5, 6, 18, 19]. The expression of *BCR1* and *CPH1* was only suppressed under certain conditions (Fig. 3).

### Effects of *Lactobacillus* culture supernatant on hyphae formation by *C. albicans*

The effects of the *Lactobacillus* culture supernatants on hyphae formation by *C. albicans* were evaluated. Medium without *Lactobacillus* culture supernatants induced hyphae formation by *C. albicans* after 3 h of

incubation. The *L. gasseri* culture supernatant, at a 2% concentration, reduced hyphae formation after 3 h of incubation and, at an 8% concentration, hyphae were not observed microscopically after 3 h and 24 h of incubation. The *L. crispatus* culture supernatant, at 2%, did not inhibit hyphae formation after 3 h of incubation, whereas the culture supernatant at 8% reduced hyphae formation after 3 h and 24 h of incubation (Fig. 4, a-j).

### Inhibition of *C. albicans* adhesion to HeLa cells by *Lactobacillus* culture supernatants

The effects of *Lactobacillus* culture supernatants on *C. albicans* adhesion to HeLa cells were investigated (Fig. 5). *Lactobacillus* culture supernatants inhibited the adhesion of *C. albicans* to HeLa cells (Fig. 5, a, b). The *L. gasseri* culture supernatant reduced *C. albicans* adhesion to 0.03 compared with the control (MRS medium), while the *L. crispatus* culture supernatant reduced *C. albicans* adhesion to 0.10 compared with the control ($p < 0.05$) (Fig. 5, b).

## Discussion

Many studies on the use of probiotics for the treatment of bacterial vaginosis and VVC have been reported. Attasi et al. [20] reported that human vaginal isolates, the supernatant of *L. gasseri* reduced the viability of the vaginosis-associated pathogens *Gardnerella vaginalis* and *Prevotella bivia.* The *in vitro* activity of lactobacilli against *Candida* species had also been examined in several studies on the development of new therapeutic agents based on probiotics [21, 22, 23]. Based on these works, we aimed to evaluate the inhibitory activity of metabolites of *L. gasseri* and *L. crispatus* against *C. albicans* for the supplemental treatment of VVC.

*L. gasseri* and *L. crispatus* are predominant in the vaginal microbiota of healthy Asian women [9]. Therefore, we hypothesized that it was clinically reasonable that the predominant species, *L. gasseri* and *L. crispatus*, inhibit the growth of, or adhesion to, vaginal epithelial cells by *C. albicans.*

Fungal biofilm formation involves the initial colonization phase and the maturation phase [18, 19, 24]. During the initial colonization phase, yeast cells adhere to appropriate surfaces, undergo morphogenesis, aggregate, and produce extracellular materials (cell wall polysaccharides and proteins) [24]. In mature biofilms, hyphal growth and extracellular material production continue, and the yeast and hyphal elements form a complex network [24]. In the present study, the culture supernatants of two *Lactobacillus* species suppressed both phases of biofilm formation. *C. albicans* biofilm formation is associated with the BHT, and the *C. albicans* BHT pathway has been investigated extensively [5, 6, 18] (Fig. 2). The transcription factor Bcr1 plays important roles in biofilm formation. Bcr1 is required for the expression of genes related to cell surface adherence and hyphal formation, such as *ALS3* and *HWP1* [18, 19]. Under biofilm-inhibitory conditions, the expression levels of *HWP1*, *ECE1*, *ALS3*, *BCR1*, *EFG1*, *TEC1*, and *CPH1* are downregulated [5, 6, 18, 19]. Therefore, inhibition

of *C. albicans* biofilm formation by the *Lactobacillus* culture supernatants was likely mediated by the BHT pathway. Hyphae formation was also inhibited by the *Lactobacillus* species culture supernatants (Fig. 4). Studies on the inhibitory effects of the culture supernatants of *Lactobacillus* species against *Candida* cells have been previously reported [8, 13, 16, 25], and the *Lactobacillus* supernatants are reportedly capable of inhibiting *C. albicans* hyphae formation by modulating gene expression [16, 25]; however, little is known about the expression of these genes in *C. albicans* biofilm. Therefore, this is the first study to report that the predominant *Lactobacillus* species in the vaginal microbiome inhibit *C. albicans* biofilm formation and suppress the expression of genes in the BHT pathway.

Vaginal isolates of *L. gasseri* and *L. crispatus* were reported to inhibit *C. albicans* adhesion via the following mechanisms: 1) modification of polar lipid organization and physical properties and 2) modulation of α5β1 integrin exposure [26]. Indeed, the culture supernatants of the two *Lactobacillus* species reduced the adherence of *C. albicans* to HeLa cells (Fig. 5).

Several clinical trials were performed to evaluate the ability of intravaginally administered lactobacilli to inhibit *Candida* colonization. However, evidence of the efficacy of probiotics against VVC is not entirely clear [2]. The administration of metabolites may be more useful than that of viable microorganisms, rendering unnecessary *Lactobacillus* adhesion to, and colonization of, the vaginal mucosa in competition with other microorganisms in clinical settings. As women can self-administer metabolite solutions [27], our results will facilitate the development of better management strategies for VVC.

In conclusion, our data indicate that culture supernatants of *L. gasseri* and *L. crispatus*, which are commonly found in the vagina, inhibit *C. albicans* biofilm formation and adhesion to HeLa cells.

## Compliance with Ethical Standards

Conflict of Interest: We declare no conflict of interest.

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# Tables

**Table 1** Primers used for RT-qPCR

| Primer | Sequence (5'–3') | Reference |
|---|---|---|
| HWP1-F | CGGAATCTAGTGCTGTCGTCTCT | Samaranayake et al. [28] 2013 |
| HWP1-R | CGACACTTGAGTAATTGGCAGATG | Samaranayake et al. [28] 2013 |
| ECE1-F | CCAGAAATTGTTGCTCGTGTTG | Bassilana et al. [29] 2005 |
| ECE1-R | CAGGACGCCATCAAAAACG | Bassilana et al. [29] 2005 |
| ALS3-F | GTGATGCTGGATCTAACGGTATTG | Uppuluri et al. [30] 2009 |
| ALS3-R | GTCTTAGTTTTGTCGCGGTTAGG | Uppuluri et al. [30] 2009 |
| BCR1-F | CACTAACGCCGACATTAACC | Kurakado et al. [15] 2017 |
| BCR1-R | GAACTTGATGCCGACGATTC | Kurakado et al. [15] 2017 |
| EFG1-F | CACTTCTGCTTCGGCTCCTC | Kurakado et al. [15] 2017 |
| EFG1-R | CAGCCTTGGTATTTACCGGACTA | Kurakado et al. [15] 2017 |
| TEC1-F | TGGATTCATACCGTATTGGTCATTA | Bassilana et al. [29] 2005 |
| TEC1-R | TCGGGCAATCCTTTGAATAAA | Bassilana et al. [29] 2005 |
| CPH1-F | CTCCACTACCCCCAATATCATC | Kurakado et al. [15] 2017 |
| CPH1-R | CTTCTCCACCATTAGCACTCTG | Kurakado et al. [15] 2017 |
| 26S-F | GAGTCGAGTTGTTTGGGAATGC | Hierro et al. [31] 2006 |
| 26S-R | TCCATCACTGTACTTGTTCGC | Kurakado et al. [15] 2017 |

Abbreviations: F, forward primer; R, reverse primer

# Figure Captions

**Fig. 1** Inhibition of *C. albicans* biofilm formation by the culture supernatants of *Lactobacillus* species.

The inhibitory effects of *L. gasseri* and *L. crispatus* culture supernatants were evaluated on biofilms after a 1.5 h incubation (initial colonization phase) (a) and 25.5 h incubation (maturation phase) (b).

Inhibitory effects are presented as ratios relative to the control (without culture supernatant).

*, $P < 0.05$, compared with the control

**Fig. 2** Regulation of *C. albicans* budded-to-hyphal form transition.

The Cph1-mediated Cek1 MAPK and Efg1-mediated cAMP pathways play major roles in dimorphic regulation [6]. *HWP1* and *ECE1*, hypha-specific genes; *ALS3*, adhesion-related gene; *EFG1*, *TEC1*, and *CPH1*, hypha-related transcription factors; *TUP1*, repressors of hypha-specific genes; *NRG1*, *MIG1*, and *RFG1*, negative regulators

**Fig. 3** Gene expression levels in *C. albicans* biofilms in the presence of culture supernatants of *Lactobacillus* species.

Gene expression levels were evaluated in the presence of 15% and 30% *Lactobacillus* culture supernatants and are presented as fold changes relative to the control.

*, $P < 0.05$, compared with the control

**Fig. 4** Morphology of *C. albicans* cells in the presence of culture supernatants.

Cell morphology was observed after 3 h and 24 h of incubation with culture supernatants.

(a–e) 3 h of incubation; (f–j) 24 h of incubation. (a and f) medium control; (b and g) 2% *L. gasseri* culture supernatant; (c and h) 8% *L. gasseri* culture supernatant; (d and i) 2% *L. crispatus* culture supernatant; (e and j) 8% *L. crispatus* culture supernatant

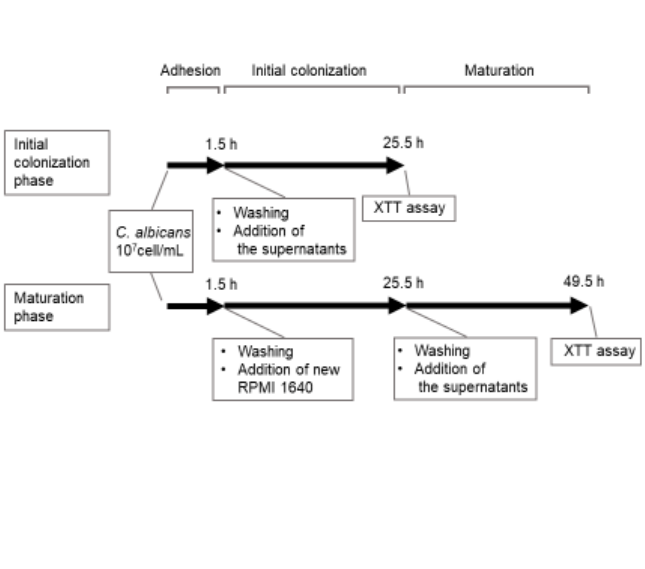
**Fig. 5** Adherence of *C. albicans* to HeLa cells.

(a) May-Giemsa-stained cells. A, control (without culture supernatant); B, culture supernatant of *L. gasseri*; C, culture supernatant of *L. crispatus*. (b) Colony-forming units.

*, $P < 0.05$, compared with the control

# Figures

Fig. 1



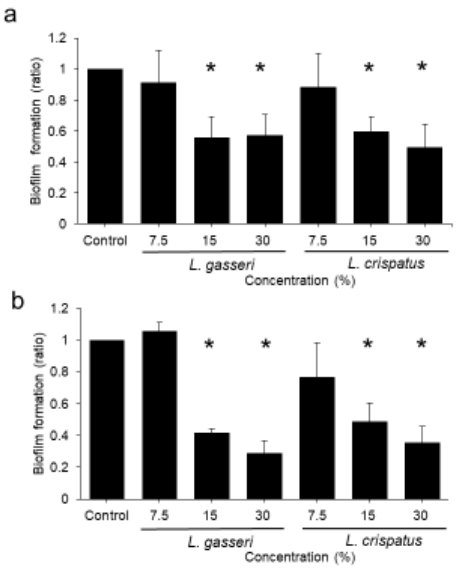



Fig. 2

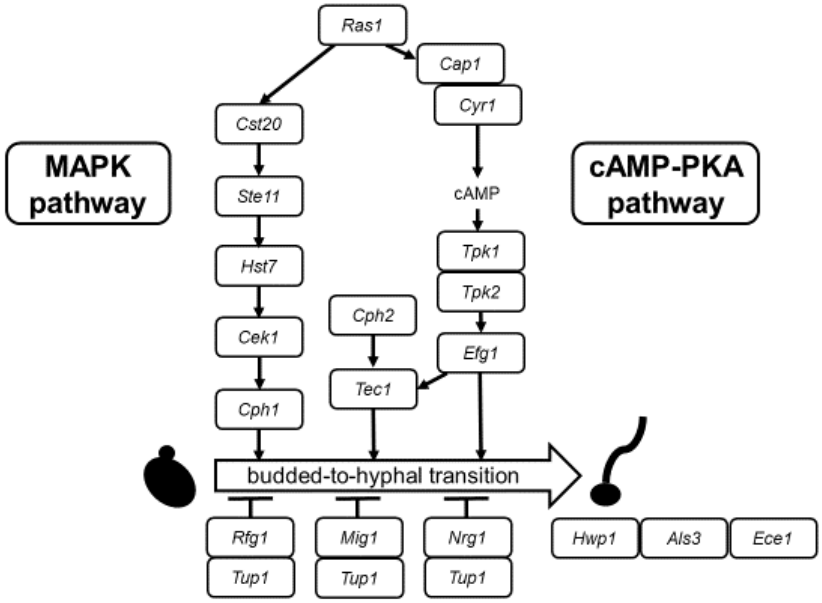

Ras1
Cap1
Cyr1
Cst20
MAPK pathway
cAMP-PKA pathway
Ste11
cAMP
Tpk1
Hst7
Tpk2
Cph2
Cek1
Efg1
Tec1
Cph1
budded-to-hyphal transition
Rfg1
Mig1
Nrg1
Hwp1
Als3
Ece1
Tup1
Tup1
Tup1

Fig. 3

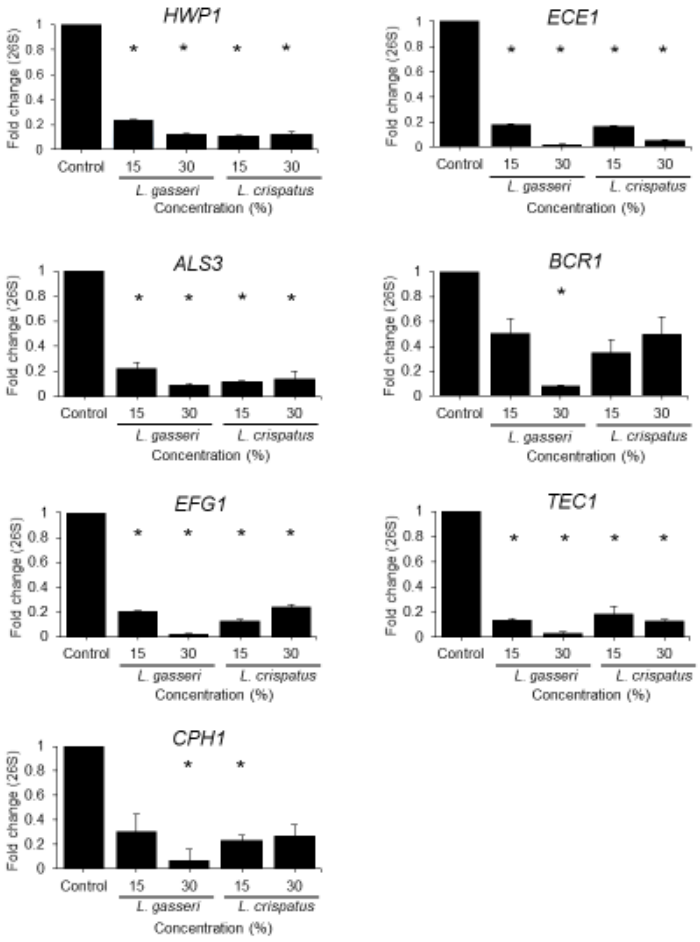

HWP1
ECE1
ALS3
BCR1
EFG1
TEC1
CPH1
Fold change (26S)
0
0.2
0.4
0.6
0.8
1
Control
15
30
15
30
L. gasseri
L. crispatus
Concentration (%)
*

Fig. 4

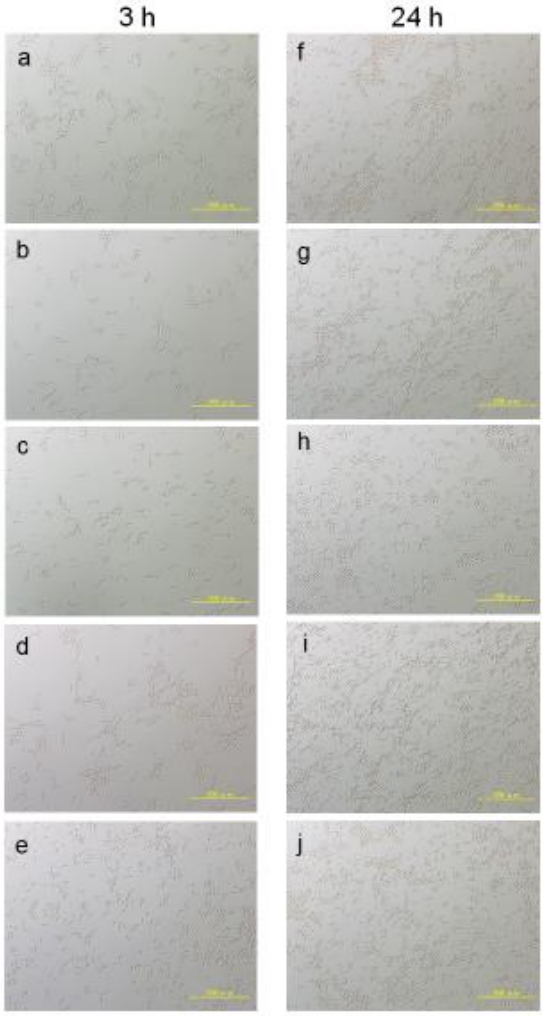
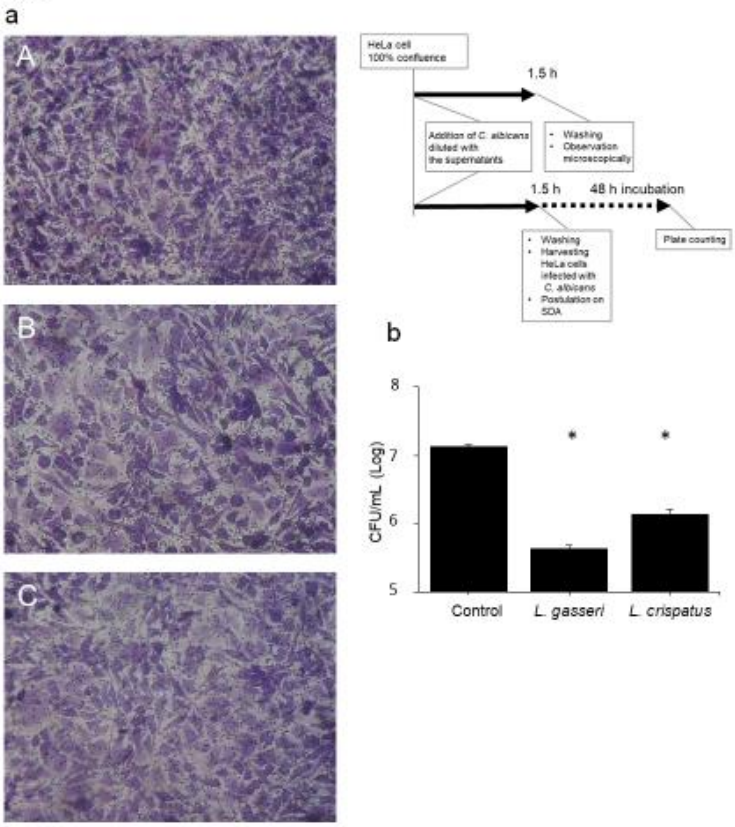


Fig. 5


a
A
B
C
HeLa cell
100% confluence
1.5 h
Addition of C. albicans
diluted with
the supernatants
- Washing
- Observation
microscopically
1.5 h
48 h incubation
Plate counting
• Washing
• Harvesting
HeLa cells
infected with
C. albicans
• Postulation on
SDA
b
CFU/mL (Log)
8
7
6
5
*
*
Control
L. gasseri
L. crispatus