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Influence of Bacterial Presence on Biofilm Formation of *Candida albicans*

Su Jung Park,[1*] Kyoung-Hee Han,[2*] Joo Young Park,[1] Sun Ju Choi,[1] and Kyoung-Ho Lee[1]

Departments of [1]Microbiology and [2]Obsterics and Gynecology, Yonsei University Wonju College of Medicine, Wonju, Korea.

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Corresponding author: Dr. Kyoung-Ho Lee,
Department of Microbiology,
Yonsei University Wonju College of Medicine,
20 Ilsan-ro, Wonju 220-701, Korea.
Tel: 82-33-741-0324, Fax: 82-33-742-5034
E-mail: leekh@yonsei.ac.kr

*Su Jung Park and Kyoung-Hee Han contributed equally to this work.

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Purpose: *Candida albicans* is an opportunistic pathogen that is commonly found in human microflora. Biofilm formation (BF) is known as a major virulence factor of *C. albicans*. The aim of this study was to examine the influence of bacterial presence on biofilm formation of *C. albicans*. **Materials and Methods:** The BF of *Candida* was investigated when it was co-cultured with *C. albicans* (*C. albicans* 53, a yeast with a low BF ability, and *C. albicans* 163, a yeast with high BF ability) and bacteria. BF was assessed with XTT reduction assay. A scanning electron microscope was used to determine the structure of the biofilm, and real-time reverse transcriptase polymerase chain reaction was used to amplify and quantify hyphae-associated genes. **Results:** Co-culturing with two different types of bacteria increased the BF value. Co-culturing with *C. albicans* 53 and 163 also increased the BF value compared to the value that was obtained when the *C. albicans* was cultured individually. However, co-culturing with bacteria decreased the BF value of *C. albicans*, and the BF of *C. albicans* 163 was markedly inhibited. The expression of adherence and morphology transition related genes were significantly inhibited by co-culturing with live bacteria. **Conclusion:** Bacteria have a negative effect on the formation of biofilm by *C. albicans*. This mechanism is the result of the suppression of genes associated with the hyphae transition of *C. albicans*, and bacteria particles physically affected the biofilm architecture and biofilm formation.

Key Words: *Candida albicans*, biofilm, co-culture, bacteria

INTRODUCTION

Most microorganisms in their natural form, as driven by the environmental conditions of their habitats, stay as free-floating cells or exist in a planktonic state or as biofilms that adhere to and grow on extracellular surfaces.[1] When nutrients are depleted, the response is transformation into the state of biofilm.[2] In addition, a number of physical, biological and chemical processes are involved in the formation of biofilm.

The biological significance of the biofilm in *C. albicans* is that it protects the bacteria by delaying its physical destruction and acts as a protective barrier against

host immune substances and the penetration of anti-fungal agents.[3] The formation of biofilm in *C. albicans* occurs in three stages. The first stage is adherence to a suitable temperament basal layer; this is initiated after the start of incubation. This initial adherence stage is regulated by non-specific factors, such as hydrophobic or electrostatic interaction, and specific factors through specific receptors like fibrinogen and fibronectin.[4,5] The second stage is the process in which the adhered *C. albicans* continuously multiply and the yeast cells transform into hyphae that then form three-dimensional structures. The three-dimensional structures of biofilm are generally comprised of yeast, pseudohyphae, and hyphae.[6,7] The last maturation stage is the process of quantitative increase of extracellular substances; the mature biofilm enables the *Candida* yeast to fix biofilm on-to the extracellular surface, and the hyphae form a cross-sectional structure with structural frames.[8] As described, the dimorphism of *C. albicans* serves as a major factor that influences the formation of biofilm.[9,10]

In the human body, a variety of bacteria and fungi interact and coexist regardless of whether the host is in a healthy or diseased state.[11-13] Most interactions among complex strains comprise a colony by one microorganism, and the colony is either commensal in which the existence of the other microorganisms is beneficial or antagonistic in which microorganisms with differing microbial products inhibit, wound, or kill the growth of other microorganisms.[2,14,15] Recognition of other populations by bacteria or fungi can be explained by Quorum Sensing, and bacteria are known to control the formation of microfilm with respect to the density and growth rates of the other surrounding populations.[16-19]

The *C. albicans* infection that appears on inserted medical devices is caused by the formation of *C. albicans* microfilm. The fact that biofilm is observed in many cases when *Candida* and other various bacteria are mixed together warrants attention. Previous studies on biofilm development have been focused on the interactions of bacterial species and have not included the study of interactions between bacteria and fungi. In this study, attempts were made to determine how the existence of bacteria affected the formation of the microfilm, by first identifying the biofilm that is formed when bacteria and fungi are incubated individually. The second aim was to identify the biofilm that is formed when bacteria-and-bacteria or fungi-and-fungi incubations occur. The third aim was to identify the biofilm that is formed by complex strains of co-cultured bacteria and fungi.

MATERIALS AND METHODS

Organisms

Clinical isolates of *Candida albicans* were obtained; one commensal strain was isolated from the oral cavity of a healthy volunteer and the other was isolated from the blood of a patient. *Escherichia coli*, *Pseudomonas aeruginosa*, and *Proteus vulgaris* were isolated from urine of a patient. In addition, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus salivarius* were isolated from blood of a patient. The identity of all strains was confirmed using the API 20E identification systems (BioMerieux, Marcy I'Etoile, France) for *P. aeruginosa*, *P. vulgaris* and *E. coli*, the API 20STREP identification system for streptococci and the API STAPH identification system for staphylococci.

Culture conditions

Prior to each experiment, *C. albicans* isolates were cultured at 30°C for 18 h on Sabouraud's dextrose agar (Difco™, Becton Dickinson, Spark, MD, USA), and one colony of bacteria was inoculated into yeast nitrogen base (Difco™) medium supplemented with 50 mM glucose. The two streptococcal species used were *S. pyogenes* and *S. salivarius*, which were first sub-cultured at 37°C for two days on blood agar. One colony of bacteria was inoculated into brain heart infusion (Difco™) medium and incubated at 37°C in a 5% CO_2 incubator for two days. *E. coli*, *P. aeruginosa*, *P. vulgaris*, and *S. aureus* were first subcultured at 37°C for 18 h on tryptic soy agar. One colony of each bacterium was then inoculated into tryptic soy broth (TSB, Difco™) and incubated at 37°C for 18 h.

XTT reduction assay

Biofilm formation was quantified using the method developed by Ramage, et al.[14] Biofilms were allowed to form on commercially available pre-sterilized, polystyrene, flat-bottom 96-well microtiter plates (Costar, Cambridge, MA, USA). *C. albicans* (OD. 0.2) was cultured alone. *C. albicans* (OD_{600}=0.1) and each strain of bacteria (OD_{600}=0.1) were prepared and transferred into selected wells of a microtiter plate. The plate was incubated for 90 min at 37°C in an orbital shaker at 75 rpm. After the initial adhesion phase, the cell suspensions were aspirated, and each well was washed twice with phosphate-buffered saline (PBS) to remove loosely adherent cells. A volume of 200 μL of me-

dium was added to each well, and the plate was then incubated for another 72 h. After biofilm formation, the medium was aspirated, and non-adherent cells were removed by thoroughly washing the biofilm three times with PBS. A quantitative measure of biofilm formation was calculated using XTT [2,3-bis(2-methyoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide]-reduction assay. A 200 μL aliquot of XTT (1 mg/mL, Sigma, St. Louis, MO, USA) and menadione (0.4 mM, Sigma) solution was then added to each well containing the prewashed biofilm and the control well. The plates were then incubated in the dark for up to 3 h at 37°C. A colorimetric change resulting from XTT reduction was measured using a microtiter plate reader (Emax, Molecular Devices, Sunnyvale, CA, USA) at 490 nm.

Scanning electron microscopy

We developed biofilms from single species as well as candidal biofilms that were co-cultured with bacteria on polystyrene coverslips as described. The coverslips were washed twice with PBS and placed in PBS with a fixative of 2.5% glutaraldehyde (Sigma) for 20 h. After, they were washed for 5 min in PBS and then placed in 1% osmium tetroxide for 30 min. After a series of alcohol washes, a final drying step was performed using the critical point drying method. Biofilms were then mounted and gold coated. Samples were imaged with a scanning electron microscope (TM-1000, Hitachi, Tokyo, Japan) in high-vacuum mode at 15 kV.

Relative quantification by real-time reverse transcriptase polymerase chain reaction

RNA was isolated from *C. albicans* 163 cells using the MasterPure Yeast RNA Extraction kit (Epicentre Biotechnologies, Post Rd, Madson, WI, USA). RNA was treated with amplification grade DNase I (Epicentre) and used for cDNA synthesis with random hexamer primer (Invitrogen Life Technologies, Carlsbad, CA, USA) using Superscript II reversetranscriptase reagents (Invitrogen Life Technologies). Each reaction contained 1 μg of total RNA, 1 μL hexamer, 50 μM and 1 μL dNTP 10 mM in a final volume of 10 μL. Reactions were incubated at 65°C for 5 min and cooled on ice. To each reaction tube, 10 μL of the following mixture was added: 4 μL of 5 X First-Strand Buffer, 2 μL $MgCl_2$ 10 mM, 2 μL DTT 0.1 M, 1.4 μL RNAse inhibitor and 1 μL Superscript II. Reactions were incubated at 42°C for 50 min and then at 70°C for 15 min.

Real-time polymerase chain reaction (PCR) contained 10 μL of Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) as well as a forward and reverse primer (1 μL of each) (Table 1)[17,18] and sterile water to make a final volume of 20 μL. The PCR was run on MicroAmp® Optical 384-well reaction plates in an ABI 7900 Real-Time PCR system (Applied Biosystems). Real-time PCR reactions were performed at 95°C for 5 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Dissociation curves were analyzed for all reactions to verify single peaks/products. Expression levels were analyzed using ABI 7900 System SDS software (Applied Biosystems). Real-time PCR data were normalized with the geometric mean of two reference genes. *ACT1* and *PMA1* were used for this purpose.

Statistical analysis

All experiments were performed in triplicate on three different occasions. All data were expressed as mean values with corresponding standard deviations. Student's t-tests and Mann-Whitney U tests were used to compare the differences between *Candida* only and *Candida* co-cultured with bacteria

Table 1. The Primers Used in the Real-Time Reverse Transcriptase Polymerase Chain Reaction

Gene	Primer	Product size
Hwp1	Forward 5'-TCAGCCTGATGACAATCCTC-3' Reverse 5'-GCTGGAGTTGTTGGCTTTTC-3'	105 bp
Pma1	Forward 5'-ACTGCTGCCACAGTCAATGAAGC-3' Reverse 5'-GCCATGTCACCACCACCGGA-3'	161 bp
Sap5	Forward 5'-CCAGCATCTTCCCGCACTT-3' Reverse 5'-GCGTAAGAACCGTCACCATATTTAA-3'	71 bp
Als3	Forward 5'-CAACTTGGGTTATTGAAACAAAAACA-3' Reverse 5'-AGAAACAGAAACCCAAGAACAACCT-3'	80 bp
Ece1	Forward 5'-CCAGAAATTGTTGCTCGTGTTG-3' Reverse 5'-CAGGACGCCATCAAAAACG-3'	138 bp
Act1	Forward 5'-ATGTGTAAAGCCGGT TTTGCCG-3' Reverse 5'-CCATACGTCCAGTTGGAAAC-3'	202 bp

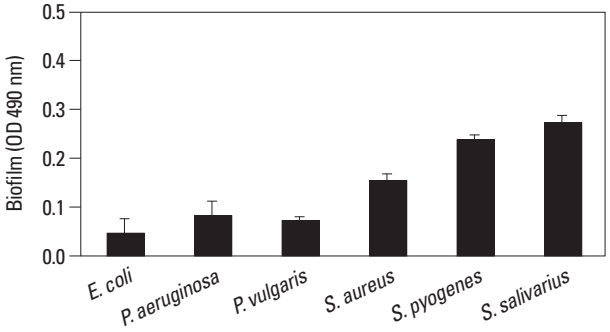

Fig. 1. Biofilm formation was monitored for the different types of bacteria. Suspensions of each type of bacteria (OD. 0.2) were added to wells in a 96-well microtiter plate. The plate was incubated for 1.5 h at 37°C in an orbital shaker at 75 rpm. After the initial adhesion phase, the cells suspensions were aspirated, and each well was washed twice with PBS to remove loosely adherent cells. Each well had 200 µL of fresh TSB added to promote biofilm growth and was incubated at 37°C for 72 h. The amount of biofilm formed was measured using the XTT assay. Absorbance at 490 nm was measured following a 3 h incubation with XTT (1 mg/mL)-Menadion (0.4 mM). Presented values are mean±SD of three independent experiments. PBS, phosphate-buffered saline; TSB, tryptic soy broth.

Table 2. Comparison between Single Microorganism Culture and Mix Species Culture on Biofilm Formation

Biofilm organisms	XTT values*
C. albicans 53	0.392±0.064†
C. albicans 163	2.406±0.064
C. albicans 53+C. albicans 163	3.680±0.058
E. coli	0.060±0.011
P. aeruginosa	0.120±0.006
E. coli+P. aeruginosa	0.125±0.03
S. pyogenes	0.239±0.023
S. salivarius	0.276±0.001
S. pyogenes+S. salivarius	0.518±0.027

*Values represent absorbance using XTT reduction assay.
†Data are means±SD of three independent experiments done in triplicate.

and a *p*-value of <0.05 was considered statistically significant.

RESULTS

Formation of bacterial biofilm

The biofilm value generated when each type of bacteria was incubated separately was 0.048±0.026 for *E. coli*, 0.081±0.030 for *P. aeruginosa*, 0.072±0.008 for *P. vulgaris*, 0.155±0.011 for *S. aureus*, 0.239±0.008 for *S. pyogenes*, and 0.276±0.014 for *S. salivarius* (Fig. 1). These values were confirmed, and we hypothesized that Gram-positive bacteria formed a microfilm more often than that of Gram-negative bacteria.

Comparison of biofilm formations in complex-strain and single-strain incubations

C. albicans 53 is a strain isolated from normal oral flora, and the biofilm formation OD value was 0.392±0.064 when it was incubated alone. *C. albicans* 163 is a strain isolated from blood, and the biofilm formation OD value was 2.406±0.064 when it was incubated alone. When the two aforementioned *C. albicans* strains were co-incubated, the biofilm value increased sharply to 3.680±0.058. When the Gram-negative bacteria *E. coli* and *P. aeruginosa* were incubated separately, the biofilm value was 0.06±0.011 and 0.120±0.006, respectively, and when they were co-incubated, the biofilm value was 0.125±0.03. When the Gram-positive bacteria *S. pyogenes* and *S. salivarius* were incubated separately, the value was 0.239±0.023 and 0.276±0.001, respectively, and when they were co-incubated, the value was 0.518±0.027 (Table 2). In each of these three cases, the amount of biofilm formed when two bacteria were incubated together was equal to the sum of the two biofilm values when the bacteria were incubated separately. However, in the case of fungi, the value was much higher when incubated together than the sum of these values when they were incubated separately. In other words, bacteria-bacteria incubation has an additive effect, whereas, fungi-fungi incubation has a synergistic effect.

Effects of multiple strain incubation of bacteria and *C. albicans* on the formation of biofilm by *C. albicans*

The formations of biofilm for *C. albicans* incubated alone and when it was incubated along with six kinds of bacteria were compared. The biofilm value when *C. albicans* 53 was incubated alone was 0.392±0.064, and when it was incubated with *E. coli*, the biofilm value was 0.177±0.040. When the *C. albicans* was incubated with *P. aeruginosa*, *P. vulgaris*, *S. aureus*, *S. pyogenes*, *S. salivarius*, the biofilm values were 0.120±0.050, 0.079±0.006, 0.162±0.015, 0.298±0.026, and 0.198±0.053, respectively (Fig. 2A). *C. albicans* 163, which has a high biofilm formation when incubated alone, had a biofilm formation as measured using OD of 2.406±0.064. When incubated with each of the six different types of bacteria separately, the value of the biofilm when incubated with *E. coli* was 0.182±0.033, with *P. aeruginosa* 0.197±0.029, with *P. vulgaris* 0.143±0.051, with *S. aureus* 0.237±0.044, with *S. pyogenes* 0.349±0.024, and with *S. salivarius* 0.157±0.032 (Fig. 2B). Both the *Candida* with low biofilm formation and the *C. albicans* with high biofilm formation had lower biofilm formation when incubated with other types of bacteria than when incubated alone. The strains with high biofilm formation also showed pronouncedly low biofilm formation in the presence of bacteria.

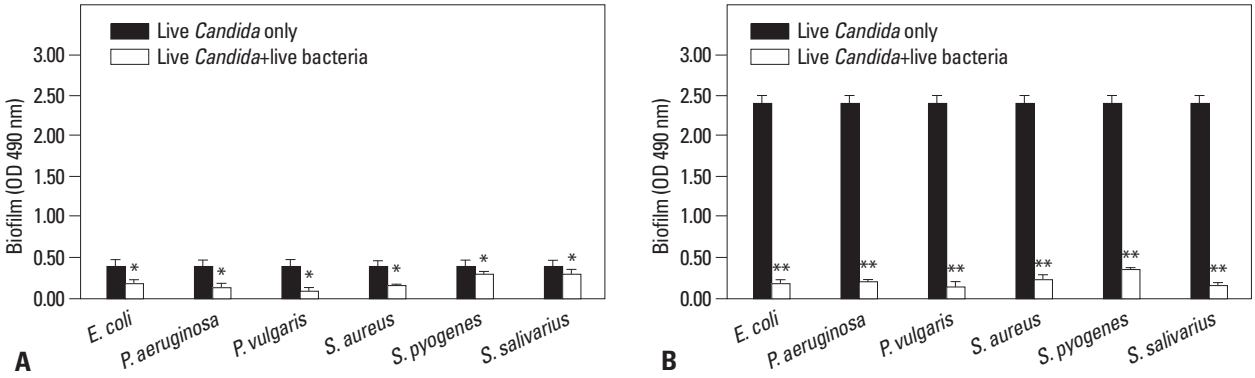

Fig. 2. The effect of co-culture of bacteria and *C. albicans* on biofilm formation. (A) Biofilm formation ability of *C. albicans* 53 was low. (B) Biofilm formation ability of *C. albicans* 163 was high. *C. albicans* (OD. 0.2) was cultured alone. Suspensions of bacteria (OD. 0.1) and *C. albicans* (OD. 0.1) were added to wells in a 96-well microtiter plate. The plate was incubated for 1.5 h at 37°C in an orbital shaker at 75 rpm. After the initial adhesion phase, the cells suspensions were aspirated, and each well was washed twice with PBS to remove loosely adherent cells. The plate was incubated for 72 h at 37°C in an orbital shaker at 75 rpm. The amount of biofilm formed was measured using the XTT assay. Absorbance at 490 nm was measured following incubation with XTT (1 mg/mL)-Menadion (0.4 mM) for 3 h. Open bar: live *C. albicans*+live bacteria, black bar: *C. albicans* alone. Presented values are mean±SD of three independent experiments. $p<0.05$ was considered statistically significant. *$p<0.05$, **$p<0.01$. PBS, phosphate-buffered saline.

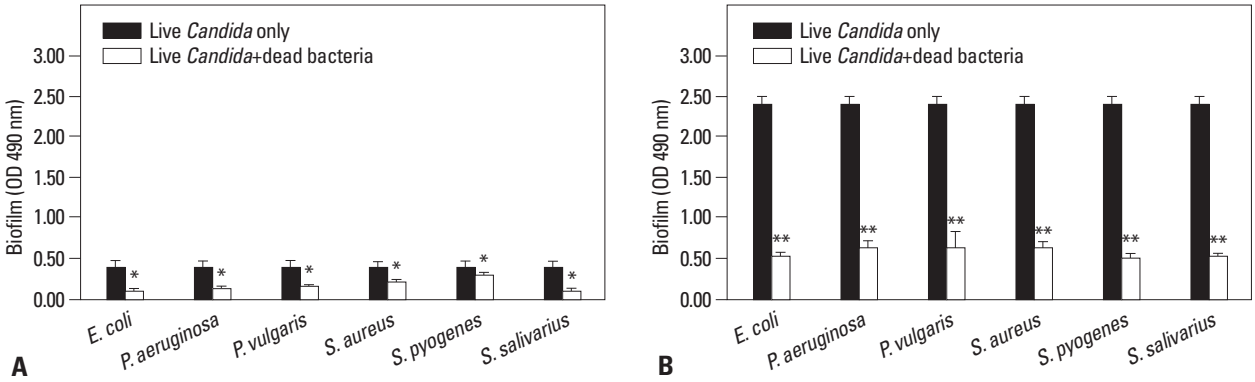

Fig. 3. The effect of the presence of dead bacteria on biofilm formation of *C. albicans*. (A) Biofilm formation ability of *C. albicans* 53 was low. (B) Biofilm formation ability of *C. albicans* 163 was high. The bacteria were killed by an incubation of 100°C for 30 min. Suspensions of bacteria (OD. 0.1) and *C. albicans* (OD. 0.1) were added to wells in a 96-well microtiter plate. The plate was incubated for 1.5 h at 37°C in an orbital shaker at 75 rpm. After the initial adhesion phase, the cells suspensions were aspirated, and each well was washed twice with PBS to remove loosely adherent cells. The plate was incubated for 72 h at 37°C in an orbital shaker at 75 rpm. The amount of biofilm formed was measured using the XTT assay. Absorbance at 490 nm was measured following incubation with XTT (1 mg/mL)-Menadion (0.4 mM) for 3 h. Open bar: live *C. albicans*+dead bacteria, black bar: live *C. albicans* alone. Presented values are mean±SD of three independent experiments. $p<0.05$ was considered statistically significant. *$p<0.05$, **$p<0.01$. PBS, phosphate-buffered saline.

Effects of incubation of dead bacteria and complex strains on the formation of *C. albicans* biofilm

To assess the effect of dead bacteria on the biofilm formation of *C. albicans*, *C. albicans* were incubated with each type of tested bacteria that had been heat-treated for 30 min at 100°C. The value of the biofilm formation when the *C. albicans* 53 strain was incubated with heat-treated *E. coli* was 0.105±0.013. This value was 0.118±0.019 when this strain was incubated with heat-treated *P. aeruginosa*, 0.153±0.007 when incubated with heat-treated *P. vulgaris*, 0.215±0.028 when incubated with heat-treated *S. aureus*, 0.085±0.012 when incubated with heat-treated *S. pyogenes* and 0.111±0.017 when incubated with heat-treated *S. salivarius* (Fig. 3A). The value of the biofilm formation when *C. albicans* 163 and heat-treated *E. coli* were co-incubated was 0.519±0.067. This value was 0.631±0.100 when this strain of *C. albicans* was incubated with heat-treated *P. aeruginosa*, 0.643±0.204 when incubated with heat-treated *P. vulgaris*, 0.637±0.079 when incubated with heat-treated *S. aureus*, 0.512±0.037 when incubated with heat-treated *S. pyogenes*, and 0.534±0.033 when incubated with heat-treated *S. salivarius* (Fig. 3B). Thus, our findings were able to verify that the biofilm formation of *C. albicans* is reduced by 50% to 80% when incubated with dead bacteria.

The effect of dead *C. albicans* on the formation of biofilm generated by bacteria

Changes were observed in the biofilm formation of bacteria incubated alone and that co-cultured with *C. albicans* heat-treated at 100°C for 30 min in order to provide a basal layer. When heat-treated *C. albicans* was incubated with *E. coli*, the value of the biofilm was 0.033±0.011, 0.100±0.030

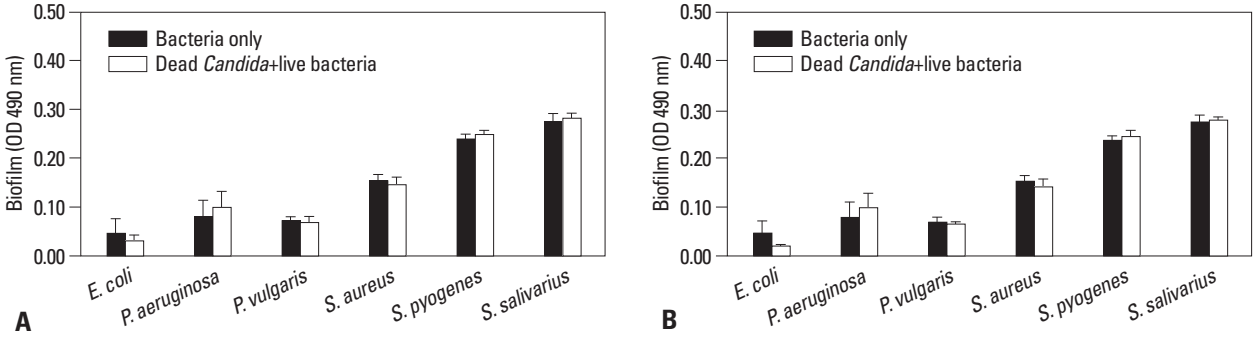

Fig. 4. The effect of the presence of dead *C. albicans* on the biofilm formation of bacteria. (A) Biofilm formation ability of *C. albicans* 53 was low. (B) Biofilm formation ability of *C. albicans* 163 was high. The *C. albicans* isolates were killed by incubation at 100°C for 30 min. Suspensions of bacteria (OD. 0.1) and *C. albicans* (OD. 0.1) were added to wells in a 96-well microtiter plate. The plate was incubated for 1.5 h at 37°C in an orbital shaker at 75 rpm. After the initial adhesion phase, the cells suspensions were aspirated, and each well was washed twice with PBS to remove loosely adherent cells. The plate was incubated for 72 h at 37°C in an orbital shaker at 75 rpm. The amount of biofilm formed was measured using the XTT assay. The absorbance at 490 nm was measured following a 3 h incubation with XTT (1 mg/mL)-Menadion (0.4 mM). Open bar: dead *C. albicans*+live bacteria, black bar: live bacteria alone. Presented values are mean±SD of three independent experiments. PBS, phosphate-buffered saline.

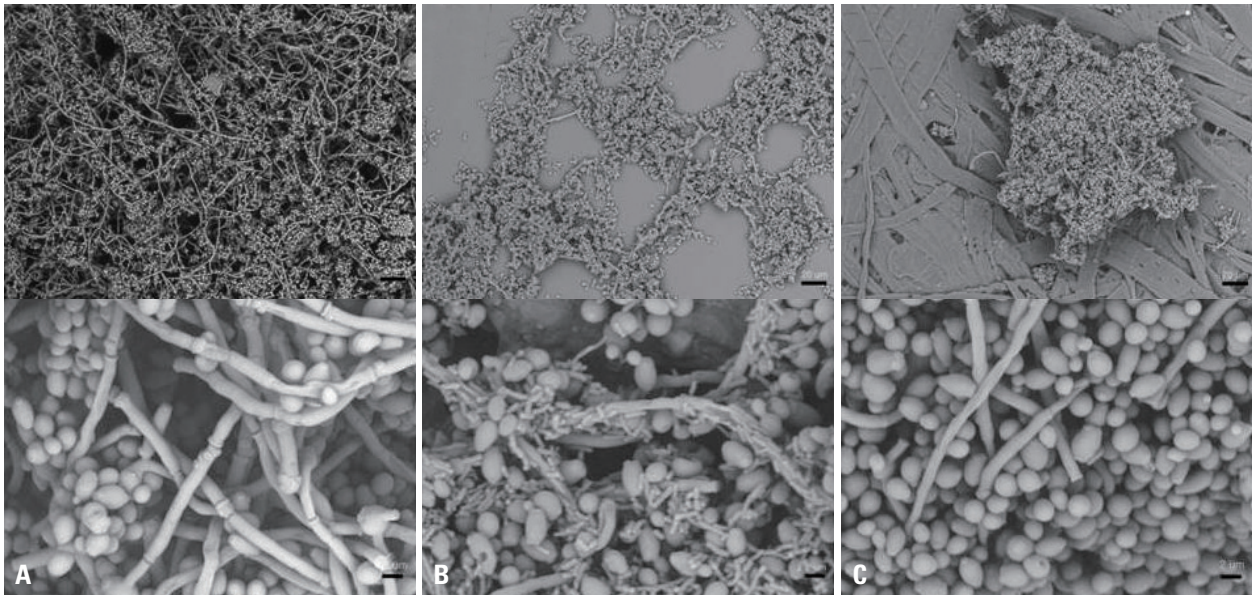

Fig. 5. Scanning electron micrograph images of monospecies (*C. albicans* or *E. coli*) and dual species (*C. albicans* and *E. coli*). (A) *C. albicans* 163 monospecies biofilm. (B) Live *C. albicans* 163 and live *E. coli* dual species biofilm. (C) Live *C. albicans* 163 and dead *E. coli* dual species biofilm. Magnifications are 500× (scale bar; 20 μm) and 4000× (scale bar; 2 μm), respectively.

when incubated with *P. aeruginosa*, 0.063±0.010 when incubated with *P. vulgaris*, 0.146±0.012 when incubated with *S. aureus*, 0.249±0.005 when incubated with *S. pyogenes* and 0.189±0.012 when incubated with *S. salivarius*. The cases in which the biofilm formation of the suspension with the dead *C. albicans* were similar to the suspensions containing only bacteria demonstrates that dead bacteria affected *C. albicans* biofilm formation, but that dead *C. albicans* did not affect bacteria biofilm formation (Fig. 4).

Structures of the formed biofilm when complex strains were co-incubated or when strains are incubated individually

When biofilm is formed by adhering to the extracellular surface of a microorganism, it grows from a single-layer structure to a multi-layer three-dimensional structure. Structural differences exist depending on whether the formed biofilm develops in *C. albicans* 163 strain and whether bacteria is incubated individually or together, and this difference has been confirmed by scanning electron microscopy. The biofilm of *C. albicans* when incubated alone appeared to be high in density and was a multi-layer solid (Fig. 5A). The biofilm formed when *C. albicans* and bacteria were incubated together showed that the bacteria were attached to hyphae of the *C. albicans* and inserted between the *C. albicans* cells (Fig. 5B). The thickness of the biofilm in these cases also appeared to be thin and low in density. This also confirmed that biofilm formation was low in cases where *C.*

albicans were incubated with heat-treated bacteria (Fig. 5C).

Effects of multiple strain incubation of bacteria and *C. albicans* on expression of hyphae-specific genes in *C. albicans*

We performed quantitative reverse transcriptase PCR to assess the expression of hyphae-specific genes on during biofilm development of *C. albicans*. Over time, the expression of hyphae-specific genes (*Als3*, *Ece1*, *Hwp1*, *Sap5*) were strongly enhanced (Fig. 6). To clarify the inhibitory effect of mixed cultures on biofilm formation, we analyzed changes in the gene expression levels of *C. albicans* in biofilm co-cultured with indicated bacteria. The transcription levels of hyphae-specific genes were remarkably reduced by *C. albicans* co-cultured with bacteria during the early stage of biofilm development (Fig. 7). The transcription levels of hyphae-specific genes were further reduced by *C. albicans* co-cultured with gram-negative bacteria (*P. aeruginosa*, *P. vulgaris* and *E. coli*) than with gram-positive bacteria (*S. aureus*, *S. pyogenes*, and *S. salivarius*). This was the most

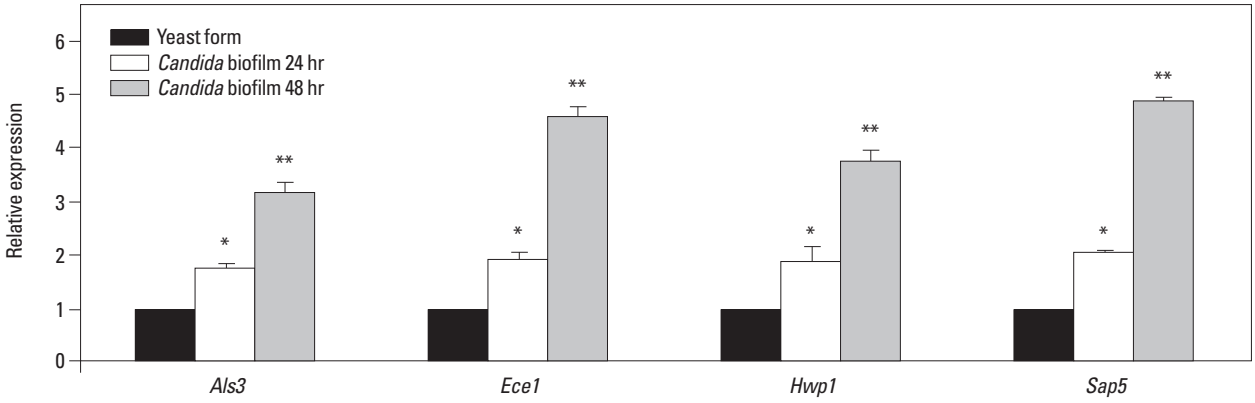

Fig. 6. Relative quantitation of hyphae-specific genes (*Als3*, *Ece1*, *Hwp1*, *Sap5*) expression. The expressions of mRNA were evaluated via quantitative real-time reverse transcriptase polymerase chain reaction in *C. albicans* 163 biofilm formation. Data represent the mean±SD of three separate cultures. $p<0.05$ was considered statistically significant. *$p<0.05$, **$p<0.01$.

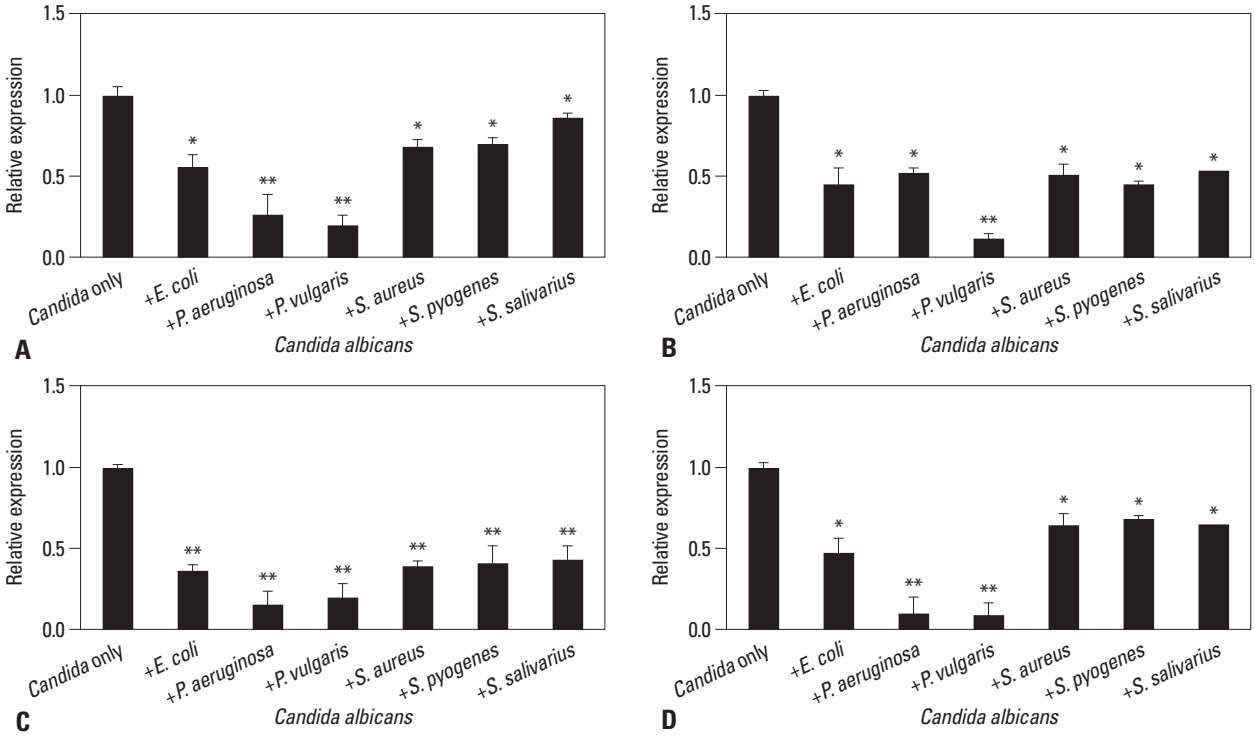

Fig. 7. Effect of live bacteria presence on expression of hyphae related gene on *C. albicans*. Relative quantitation of *Als3* (A), *Ece1* (B), *Hwp1* (C), and *Sap5* (D) gene expression was evaluated. *C. albicans* 163 cells were co-cultured with bacteria for 24 h and the target genes were determined by quantitative real-time reverse transcriptase polymerase chain reaction. Housekeeping gene *Act1* and *Pma1* were used for normalization. The data represent the average and standard deviation of three separate cultures. $p<0.05$ was considered statistically significant. *$p<0.05$, **$p<0.01$.

significant reduction of mRNA expression of hyphae-specific genes when *C. albicans* cultured with *P. vulgaris*.

DISCUSSION

Previous studies on biofilm have been based on only a single type of microorganism and, in particular, have focused mostly on bacteria that are associated with pathogenicity.[19] However, biofilms that are discovered outside laboratories are not only produced by various bacteria, but also are comprised of forms that include eukaryotes. Nearly 60% to 70% of recent hospital infections have been associated with medical devices that are inserted into the body, and it has been verified that bacteria and fungi interact together to form such biofilms.[20] *C. albicans* is the fungus that is most often associated with biofilm formation on medical devices, and the resulting *C. albicans* is associated with a high fatality rate. The first stage of *C. albicans* biofilm formation is the adherence to a surface, after which, along with bacterial proliferation, the biofilm is mixed with various extracellular polymers to form colonies with a solid structure. In order for *C. albicans* to adhere to a surface, non-specific factors, such as cell-surface hydrophobicity and electrostatic force, and specific factors, such as adhesins and receptors, are required.[4,5]

In situations where *C. albicans* and bacteria are mixed and incubated together, the architecture of the biofilm and the consequential biological functions have not been accurately identified because the interactions among the strains created from a mixed-incubation environment are not well understood.

In this study, when two different types of bacteria were incubated together, the amount of biofilm that was formed showed an additive effect in that it was the sum of the biofilms formed by the two bacteria incubated separately. However, when two different *C. albicans* strains were incubated together, the resulting amount of biofilm was much greater than the sum of the biofilms when the two strains were incubated separately, and this may be attributable to the two types of *C. albicans* fungi that provide structural assistance to the formation of biofilm when in culture.

The biofilm formation of *C. albicans* was reduced when *C. albicans* suspension was incubated with each of the six different types of bacteria compared to the biofilm formed when the *C. albicans* was incubated alone. The reduction in biofilm formation was more pronounced in the strains with high biofilm formation. When *P. aeruginosa* was incubated with *C. albicans*, it inhibited the biofilm formation of *C. albicans*, consistent with the known reports of the ability of *P. aeruginosa* to inhibit the biofilm formation of *C. albicans* by suppressing the hyphae formation of *C. albicans* and eventually inducing death by secreting neurotransmitters.[21,22] These results were equivalent to the results of the studies about *E. coli*, a bacteria that is present in the intestines, being able to suppress biofilm formation of *C. albicans* by strongly inhibiting it from adhering to an extracellular surface.[23]

Biofilm formation of *C. albicans* was also reduced when incubated with heat-treated bacteria. The inhibiting action of bacteria was thought to induce not only a metabolite that synthesizes with the bacteria during the phase of multiplication and growth, but also structural changes in the formation of the biofilm, which thereby reduce the biofilm formation of *C. albicans*. Although the biofilm of *C. albicans* is inhibited by bacteria, the mere presence of *C. albicans* does not have an effect on bacterial biofilm formation. *C. albicans* biofilm is formed through many steps and has a complex structure, while bacterial biofilms have more simplistic structures.

In a scanning electron microscope, it was shown that the *C. albicans* biofilm was made up of several layers, was highly dense, and was developed evenly throughout the surface, while the *E. coli* biofilm consists of a single layer, was of low density and was concentrated in one location. When the complex strains of *C. albicans* and *E. coli* were co-cultured together, the magnified image of the biofilm, showed that the *E. coli* strains were located between the *C. albicans*, and that the density of the biofilm was decreased compared with *Candida* alone.

Adhesins, such as *Hwp1* and *Als3*, were required on the surface of yeast-form cells for biofilm formation and appear to directly interact with each other during the biofilm formation process.[24,25] Moreover, the expression of *Ece1* correlated with *Candida* hyphal elongation,[26] and the *Sap5* gene was expressed in biofilm associated with mucosal surfaces.[27] Using real-time PCR, we accessed the expression of adherence and morphology transition related genes such as *Sap5*, *Als3*, *Ece1*, and *Hwp1*. These genes were up-regulated as biofilm formation progressed. Interestingly, expression of the *Candida* genes were significantly inhibited by co-culturing with live bacteria, but were not changed by co-culturing with dead bacteria (data not shown).

In this study, gram-negative bacteria (*P. aeruginosa*, *P. vulgaris* and *E. coli*) reduced *C. ablicans* biofilm more than that of gram-positive bacteria (*S. aureus*, *S. pyogenes*, and *S.*

salivarius). Interestingly, gram-negative bacteria are motile while gram-positive bacteria are non-motile. In particular, *P. vulgaris*, with the highest motile activity,[28,29] remarkably inhibited the biofilm formation of *C. albicans*. Moreover, these results correspond to the mRNA expression levels of hyphae-specific genes. This suggests that gram-negative cell wall constitutions and/or motile activity of bacteria may inhibit biofilm formation of *C. albicans*.

In conclusion, the growth of *C. albicans* was inhibited by bacteria (data not shown) and the formation of *Candida* biofilms was also inhibited. Additionally, these results were obtained with not only true of live bacteria, but also of dead bacteria. Bacterial mass may inhibit *C. albicans* biofilm formation as structural discordance. Bacterial growth may influence the down-regulation of hyphal transition-associated gene expression. Future studies are needed to identify the differences in biofilm formation in cases of mixed-incubation by classifying the bacteria into particular types and should enable the bacteria to coexist with the *C. albicans* fungus. Future studies should also aim to identify the factors that affect biofilm formation due to the interaction between the *C. albicans* fungus and different types of bacteria.

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