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Inhibitory effect of lactobacilli supernatants on biofilm and filamentation of *Candida albicans*, *Candida tropicalis*, and *Candida parapsilosis*

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Introduction: Probiotic *Lactobacillus* strains had been investigated for the potential to protect against infection caused by the major fungal pathogen of human, *Candida albicans*. Besides antifungal activity, lactobacilli demonstrated a promising inhibitory effect on biofilm formation and filamentation of *C. albicans*. On the other hand, two commonly isolated non-*albicans* *Candida* species, *C. tropicalis* and *C. parapsilosis*, have similar characteristics in filamentation and biofilm formation with *C. albicans*. However, there is scant information of the effect of lactobacilli on the two species.

Methods: In this study, biofilm inhibitory effects of *L. rhamnosus* ATCC 53103, *L. plantarum* ATCC 8014, and *L. acidophilus* ATCC 4356 were tested on the reference strain *C. albicans* SC5314 and six bloodstream isolated clinical strains, two each of *C. albicans*, *C. tropicalis*, and *C. parapsilosis*.

Results and Discussion: Cell-free culture supernatants (CFSs) of *L. rhamnosus* and *L. plantarum* significantly inhibited *in vitro* biofilm growth of *C. albicans* and *C. tropicalis*. *L. acidophilus*, conversely, had little effect on *C. albicans* and *C. tropicalis* but was more effective on inhibiting *C. parapsilosis* biofilms. Neutralized *L. rhamnosus* CFS at pH 7 retained the inhibitory effect, suggesting that exometabolites other than lactic acid produced by the *Lactobacillus* strain might be accounted for the effect. Furthermore, we evaluated the inhibitory effects of *L. rhamnosus* and *L. plantarum* CFSs on the filamentation of *C. albicans* and *C. tropicalis* strains. Significantly less *Candida* filaments were observed after co-incubating with CFSs under hyphae-inducing conditions. Expressions of six biofilm-related genes (*ALS1*, *ALS3*, *BCR1*, *EFG1*, *TEC1*, and *UME6* in *C. albicans* and corresponding orthologs in *C. tropicalis*) in biofilms co-incubated with CFSs were analyzed using quantitative real-time PCR. When compared to untreated control, the expressions of *ALS1*, *ALS3*, *EFG1*, and *TEC1* genes were downregulated in *C. albicans* biofilm. In *C. tropicalis* biofilms, *ALS3* and *UME6* were downregulated while *TEC1* was upregulated. Taken together, the *L. rhamnosus* and *L. plantarum* strains demonstrated an inhibitory effect, which is likely mediated by the metabolites secreted into culture medium, on filamentation and biofilm formation of *C. albicans* and *C. tropicalis*. Our finding suggested an alternative to antifungals for controlling *Candida* biofilm.

KEYWORDS

Candida, *Lactobacillus*, biofilm, filamentation, gene expression, non-*albicans* *Candida* species

1. Introduction

Candida species are commensal fungi that colonize healthy human skin, gastrointestinal, and genital tracts. They are also opportunistic pathogens, leading to mucosal and systemic infections (Kim and Sudbery, 2011). *Candida* are one of the most important agents of fungal infection worldwide and the most frequently isolated fungal species in healthcare-associated infections (Bongomin et al., 2017). Over 90% of invasive *Candida* infections were caused by five species, *Candida albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei*, with *C. albicans* accounted for 47–66% of cases (Pfaller and Diekema, 2007; Pfaller et al., 2019). Being the predominant cause of fungal infection, *C. albicans* has become the best-studied *Candida* species (Kabir et al., 2012). Although *C. albicans* remains as the most common isolated single species, a progressive shift to increased prevalence of non-*albicans* *Candida* species has been observed over the past decades (Pfaller et al., 2019). In the global SENTRY Antifungal Surveillance Program, the frequency of *C. albicans* was reported to gradually decrease from 57.4% of invasive *Candida* isolates in 1997–2001 to 46.4% in 2015–2016 (Pfaller et al., 2019).

Compared to *C. albicans*, the emerging non-*albicans* *Candida* species exhibit reduced antifungal susceptibility. For example, *C. krusei* is considered as intrinsically resistant to fluconazole. *C. glabrata* has reduced susceptibility to azoles, while *C. parapsilosis* shows elevated minimum inhibitory concentration to echinocandins (Clinical and Laboratory Standards Institute, 2008). Among the isolates collected in 2006–2016 for the SENTRY Program, the fluconazole resistance rate of *C. albicans* was 0.3%, while that of *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* was 8.1, 3.9, and 3.2%, respectively (Pfaller et al., 2019). For echinocandins, the resistance rates were low in *C. albicans* (0–0.1%) and *C. parapsilosis* (0–0.1%) but were higher in *C. tropicalis* (0.5–0.7%), *C. krusei* (0–1.7%), and *C. glabrata* (1.7–3.5%).

Among the four common isolated non-*albicans* *Candida* species, *C. tropicalis* and *C. parapsilosis* are phylogenetically closely related to *C. albicans* (Butler et al., 2009). Similar to *C. albicans*, *C. tropicalis*, and *C. parapsilosis* can undergo yeast-filament transition, which is an important virulence factor of *C. albicans* for invading host epithelial cells and causing tissue damage (Sudbery, 2011). *C. albicans* and *C. tropicalis* produce true hyphae and pseudohyphae, while *C. parapsilosis* produces only pseudohyphae (Lackey et al., 2013). These three species are also capable of forming biofilm composed of both yeast cells and filaments.

Formation of *Candida* biofilm on abiotic surfaces of indwelling medical devices, such as dentures, endotracheal tubes, intragastric balloons, urinary catheters, and intravascular catheters, can lead to device failure and *Candida* infections (Costa et al., 2022). A meta-analysis of 31 studies revealed that the rate of biofilm formation among *Candida* strains isolated from blood culture of hospitalized patients varied from 16 to 100% with a pooled rate of 80.0% (Atencia-Carrera et al., 2022). The pooled attributable mortality of *Candida*-related bloodstream infection was 37.9%, while the mortality rate with biofilm-forming *Candida* strains was 70.0%. Moreover, *Candida* biofilm has shown significantly decreased susceptibility to some commonly used antifungal agents, such as fluconazole and amphotericin B, when compared to the planktonic cells (Silva et al., 2017). Biofilm protects *Candida* cells from antifungal agents, leading to persistent infection that is difficult to eliminate without removal of the infected implant (Lohse et al., 2018).

In view of the emergence of antifungal resistance, the use of probiotic *Lactobacillus* species has been suggested as an alternative therapeutic option against *Candida* infection (Matsubara et al., 2016a). Several clinical

trials reported that the administration of *Lactobacillus* improved the outcome of vaginal candidiasis and reduced colonization of *Candida* in oral cavity of elderly people and in gastrointestinal tract of preterm neonates (Roy et al., 2014; Kovachev and Vatcheva-Dobrevska, 2015; Kraft-Bodi et al., 2015). *In vitro* studies also demonstrated that lactobacilli possess anticandidal ability, inhibitory effect on adhesion to host cells, and inhibitory effect on biofilm formation and filamentation of *C. albicans* (Köhler et al., 2012; Coman et al., 2015; Vilela et al., 2015; Matsubara et al., 2016b; Song and Lee, 2017). Interestingly, as demonstrated with cell-free supernatants and in agar overlay assays, direct interaction of lactobacilli with *C. albicans* cells is unnecessary for the inhibitory effect, suggesting that the effect is exhibited by the metabolites produced by lactobacilli (Matsubara et al., 2016b; Liao et al., 2019).

Although the effect of lactobacilli on *C. albicans* has been extensively studied, there is a relative lack of studies on non-*albicans* *Candida* species. The aim of this study was to investigate the inhibitory effect of *L. rhamnosus*, *L. plantarum*, and *L. acidophilus* on biofilm formation of *C. albicans* and closely related non-*albicans* *Candida* species, namely *C. tropicalis* and *C. parapsilosis*. In addition, we evaluated the change in filamentation and the expression of filamentation- and biofilm-related genes of *C. albicans* and *C. tropicalis* when incubated with cell-free supernatants of *L. rhamnosus* and *L. plantarum*.

2. Materials and methods

2.1. Strains and culture conditions

A laboratory reference strain *C. albicans* SC5314 and six *Candida* clinical strains (*C. albicans* A14 and A69, *C. tropicalis* T18S and T38R, and *C. parapsilosis* P149 and P152) were used in the study. The six clinical strains were initially isolated from blood culture samples, retrieved from our archival laboratory collection and selected because of their strong biofilm-forming ability. All strains were identified with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using a Bruker Biotyper (Bruker, United States) and the manufacturer's database (MBT Compass Library DB-7311) with a score of ≥ 1.7 (Vlek et al., 2014). Prior to experiment, *Candida* cells were first subcultured from glycerol stock culture onto Sabouraud dextrose agar (SDA) and incubated at 35°C for at least 1 day to assess the purity and viability of the culture.

Type strains *L. rhamnosus* GG ATCC 53103 (LGG), *L. plantarum* ATCC 8014 (LP8014), and *L. acidophilus* ATCC 4356 (LA4356) were used. Stock cultures were prepared by resuspending bacterial cells in de Man, Rogosa and Sharpe (MRS) broth with 10% glycerol and stored at –80°C until use. Before experiments, the bacterial cells were first streaked from stock culture onto MRS agar and incubated at 37°C under microaerophilic conditions (5% O₂, provided by CampyGen, Thermo Fisher Scientific, United States) for 2 days.

2.2. Biofilm inhibition

Candida strains were grown in yeast extract peptone dextrose (YPD) broth at 37°C with shaking (200 r.p.m.) for 18 h. The cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS), and resuspended to 10⁶–10⁷ colony-forming unit (CFU)/mL in RPMI 1640 medium (with L-glutamine, buffered with MOPS; Thermo Fisher Scientific, United States). For *Lactobacillus*, *Lactobacillus*

colonies were collected from MRS plates and inoculated into MRS broth. After incubated microaerophilically at 37°C with shaking (200 r.p.m.) for 18 h, the liquid cultures were centrifuged to separate spent media and bacterial cells. Cell-free supernatant (CFS) was prepared by filtering the spent media through a membrane filter with a 0.22 µm pore size (Merck Millipore, United States). MColorpHast pH indicator strip (Millipore, United States) was used to measure the pH of CFS. The harvested *Lactobacillus* cells were washed twice with PBS and resuspended in MRS broth to 1 × 10⁷ CFU/ml.

The incubation of biofilm was divided into two parts, adhesion phase and formation phase (Kuhn et al., 2002). Briefly, in adhesion phase, 100 µl of *Candida* inoculum was seeded into the wells of flat bottom 96-well plates. Plates were prepared in duplicate and then incubated statically at 37°C for 90 min. After incubation, spent medium was aspirated from the well. The wells were washed with PBS to remove non-adherent cells. In the formation phase, 100 µl of RPMI medium and 50 µl of *Lactobacillus* CFS, living cell suspension, or MRS broth as control were added per wells. There were five replicate wells for each condition per plate. The plates were incubated statically at 37°C for an additional 24 h. After incubation, a MColorpHast pH strip was used to check the pH of spent medium. The biofilms were quantified by two methods, XTT [2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay and crystal violet staining.

For XTT reduction assay, 150 µl of XTT (0.5 mg/ml in PBS, Sigma-Aldrich, United States) with 1 µM of menadione (Sigma-Aldrich, United States) was added per well (Pierce et al., 2008). The plate was incubated in dark at 37°C for 2.5 h. The resulting-colored supernatant (120 µl) was transferred to a round bottom 96-well plate and its absorbance was measured at 492 nm using a LED microplate reader (Ledetect 96, Labexim Products, Austria). For crystal violet staining, the biofilm in the well was first fixed with 150 µl of 99% methanol, then stained with 150 µl of 0.0625% w/v crystal violet (CV) solution (Peeters et al., 2008). To solubilize the stain, 225 µl of 33% acetic acid was added per well. The solution (150 µl) was transferred to a round bottom plate and its absorbance was measured at 570 nm (Silva et al., 2009).

To examine whether a lower pH contributed to the inhibitory effect of CFS, the assay was repeated using MRS broth acidified to pH 4.0 with lactic acid, and LGG CFS neutralized to pH 7.0 with sodium hydroxide.

2.3. Filamentation inhibition

The *C. albicans* and *C. tropicalis* strains were tested against the CFSs of lactobacilli LGG or LP8014, which were prepared from 18 h-old culture as previously described and stored at -20°C until use. MRS broth was used as control. The assay was performed following the method described by Wang et al. (2017) with some modifications. In a 1.5 ml Eppendorf tube, 144 µl of *Candida* suspension in RPMI medium at a concentration of 1 × 10⁷ CFU/ml was mixed with 300 µl of *Lactobacillus* CFS or MRS broth and then topped up to a total volume of 900 µl with RPMI. Additional 10% v/v of fetal bovine serum were added for *C. tropicalis* strains to induce filamentation. After incubating statically at 37°C for 4 h, the mixture was vortexed for 20 s and 20 µl of it was loaded into a Fuchs-Rosenthal counting chamber (Marienfeld, Germany). Microphotographs of ten squares with an area of 0.0625 mm² each in the chamber were captured using an AmScope MD500 digital eyepiece camera (AmScope, United States) under a light microscope with a 10× objective lens. The number of filaments within the ten squares was counted from the photographic images.

2.4. Gene expression in biofilm

To determine the effects of *Lactobacillus* CFS on the transcription of biofilm-related genes in *C. albicans* and *C. tropicalis*, the gene expression levels of *ACT1*, *ALS1*, *ALS3*, *BCR1*, *EFG1*, *TEC1*, *RIP1*, and *UME6* of *C. albicans* and the corresponding orthologs in *C. tropicalis* were evaluated using quantitative real-time polymerase chain reaction (qPCR). Except primers for *RIP1*, which were adopted from previous literature, primer for other genes was designed using the NCBI/Primer-BLAST tool¹ based on the gene sequences available in GenBank (Nailis et al., 2006). The specificity of amplification was evaluated *in silico* by performing Basic Local Alignment Search Tool (BLAST) analysis against the NCBI GenBank nucleotide collection (nr) and experimentally by agarose gel electrophoresis of the PCR products. The sequences of primers are listed in Tables 1, 2.

To extract RNA, biofilms of *C. albicans* and *C. tropicalis* were formed in a flat bottom, cell culture-treated 48-well plate (Thermo Fisher Scientific, United States) using the two-phase procedures described for biofilm inhibition, with the volumes of inoculum and CFS (or MRS broth) increased to 200 and 100 µl, respectively. After incubated statically at 37°C for 24 h, RNA was extracted from biofilms using the TRIzol™ Plus RNA Purification Kit (Thermo Fisher Scientific, United States) with homogenization done on a Fastprep-24 5G instrument (MP Biomedicals, United States) with lysing matrix Y 2 ml tubes at a speed of 6.0 m/s for 30 s twice, with a cooling interval on ice for 3 min. The RNA was eluted with 30 µl of RNase-free water preheated to 60°C. RNA samples were DNase treated using the TURBO DNA-free Kit (Thermo Fisher Scientific, United States) according to the manufacturer's instruction. Complementary DNA (cDNA) was reverse transcribed from 500 ng of RNA by the High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Thermo Fisher Scientific, United States). cDNA samples were stored at -80°C until use.

qPCR was performed in 10 µl reaction volume using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, United States) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, United States). Within a single qPCR run (96-well plate), amplification of the eight targeted genes was performed using cDNA samples prepared from the biofilms of a same *Candida* strain grown under three experimental conditions [MRS broth (control), LGG CFS, or LP8014 CFS] in triplicate wells. No-template controls were included for each gene and each qPCR run. The reactions started from a primary denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Specificity of amplification was confirmed by the presence of a single peak in melting curve analysis. *ACT1* and *RIP1* genes were used as reference genes (Nailis et al., 2006; Silva et al., 2011; Lackey et al., 2013). Expression levels of the genes of interest were normalized to the reference genes using the following formula:

$$\text{Normalized relative quantity (NRQ)} = \frac{(E_{GOI})^{\Delta Ct_{GOI}}}{\text{GeoMean}[(E_{REF})^{\Delta Ct_{REF}}]}$$

where E represents the amplification efficiency and ΔCt is the difference in cycle threshold (Ct) values of the treatment and control group (Hellemans et al., 2007).

1 <https://www.ncbi.nlm.nih.gov/tools/primer-blast/>

TABLE 1 Primers used in qPCR for *Candida albicans*.

Species	Target	GenBank accession no.	Primers	Sequence (5' to 3')	Product size (bp)	Reference
<i>C. albicans</i>	ACT1	XM_019475182.1	AACT1 1F	F: TGGTGTACTACACGTTGTTCCA	100	This study
			AACT1 1R	R: GGACAAATGGTTGGTCAAGTCTC		
	ALS1	XM_712984.2	AALS1 2F	F: ACCAGTATCATTCCATCATTTTCCC	71	This study
			AALS1 2R	R: TCAAATGTTGACAAATCGGAGGTT		
	ALS3	XM_705343.2	AALS3 2F	F: ATTCGATCCTAACCGCGACA	119	This study
			AALS3 2R	R: TTGGTGCAGTTTGGTTCAGGT		
	BCR1	XM_707989.2	ABCR1 3F	F: CCTCATCAAATGGGTGGTGGT	110	This study
			ABCR1 3R	R: GCCAATGGTTCGGGTCTTCT		
	EFG1	XM_709144.2	AIEFG1 3F	F: GCACCAATCACCCCAAGTTC	97	This study
			AIEFG1 3R	R: TTTGGCAACAGTGCTAGCTG		
	TEC1	XM_718029.1	AIEC1 2F	F: GATCCAGTGTCTTCTGTTGGC	76	This study
			AIEC1 2R	R: GGACACGTGAATAAGCACCA		
	RIP1	XM_019475318.1	RIP1F	F: TGTACCGTTCACATTATGATATTT	72	Nailis et al. (2006)
			RIP1AR	R: TGGAATTTCCAAGTTCAATGGA		
	UME6	XM_705781.2	AUME6 4F	F: GGTGTCTCTTCTGATGTTGGT	106	This study
			AUME6 1R	R: ACTTCCAGATCCTGTACCACT		

TABLE 2 Primers used in qPCR for *Candida tropicalis*.

Species	Target	GenBank accession no.	Primers	Sequence (5' to 3')	Product size (bp)	Reference
<i>C. tropicalis</i>	ACT1	XM_002549283.1	TACT1 3F	F: TACGCTGGTTTCTCCTTGCC	116	This study
			TACT1 3R	R: GCGGTGGTGAGAAAGTGT		
	ALS1	MK182724.1	TALS1 2F	F: TCTGTTGCCATTCCTGTCGAA	70	This study
			TALS1 2R	R: AAACCAACCCAAGCAAGATCG		
	ALS3	MH753521.1	TALS3 2F	F: TGACTCCTAAGGAAGTATCGGGA	102	This study
			TALS3 2R	R: GCCAAGCTGGATTAGCAGGA		
	BCR1	XM_002545781.1	TBCR1 3F	F: ATCCTTTACCTGCGTTGCGT	146	This study
			TBCR1 3R	R: GGACGAAGCTACTGACTCGG		
	EFG1	KP314278.1	TEFG1 3F	F: TCCTGCAGCTCCACCTATAACC	99	This study
			TEFG1 3R	R: TTGGCTGGGTTTAAACGTGTCT		
	TEC1	XM_002547951.1	TTEC1 3F	F: TTCAAGCAGTGCTCCAAGTTC	103	This study
			TTEC1 3R	R: AGGTGCAGAAATGGAAACCAGT		
	RIP1	XM_002547961.1	RIP1F	F: TGTACCGTTCACATTATGATATTT	72	Nailis et al. (2006)
			RIP1TR	R: TGGGATTTCCAAGTTCAATGGA		
	UME6	XM_002549914.1	TUME6 3F	F: GGTTCAGTCCAACAGAGACA	131	This study
			TUME6 3R	R: CCTGGTTGGCTGTATTCTCCA		

2.5. Statistical analysis

Experiments were repeated in triplicate at different occasions. Results of experiment groups in the biofilm inhibition assay were expressed as a percentage relative to the control (MRS) and compared to the control using Student's *t*-test. Results of filamentation assay were compared to control using one-way analysis of variance (ANOVA) followed by Dunnett's test. Expression levels of genes in biofilm were expressed as NRQ as described. Student's *t*-test was used to compare the log₂-transformed NRQ values of experimental group to control group of the same *Candida* strain. A value of *p* < 0.05 was considered significant

in all tests. Statistical analyses were performed using Prism (Version 9, GraphPad Software, United States) and Microsoft Excel (Office 365, Microsoft, United States).

3. Results

3.1. Biofilm inhibition

The LGG CFS significantly reduced the biomass of the biofilms produced by five *Candida* strains and the metabolic activity of three

strains (Figure 1B). The inhibition was most pronounced (>50% in biomass, $p < 0.01$) in *C. albicans*, followed by *C. tropicalis*. The LP8014 CFS also shown inhibitory effects to some extent and significantly reduced the biomass and metabolic activity of biofilm formed by *C. albicans* SC5314 (Figure 1D, $P < 0.05$). However, living LGG and LP8014 cell suspensions had little to no effect (Figures 1A,C). On the other hand, LA4356's cells significantly reduced both biomass and metabolic activity of the two *C. parapsilosis* strains while its CFS also had similar effect (Figures 1E,F).

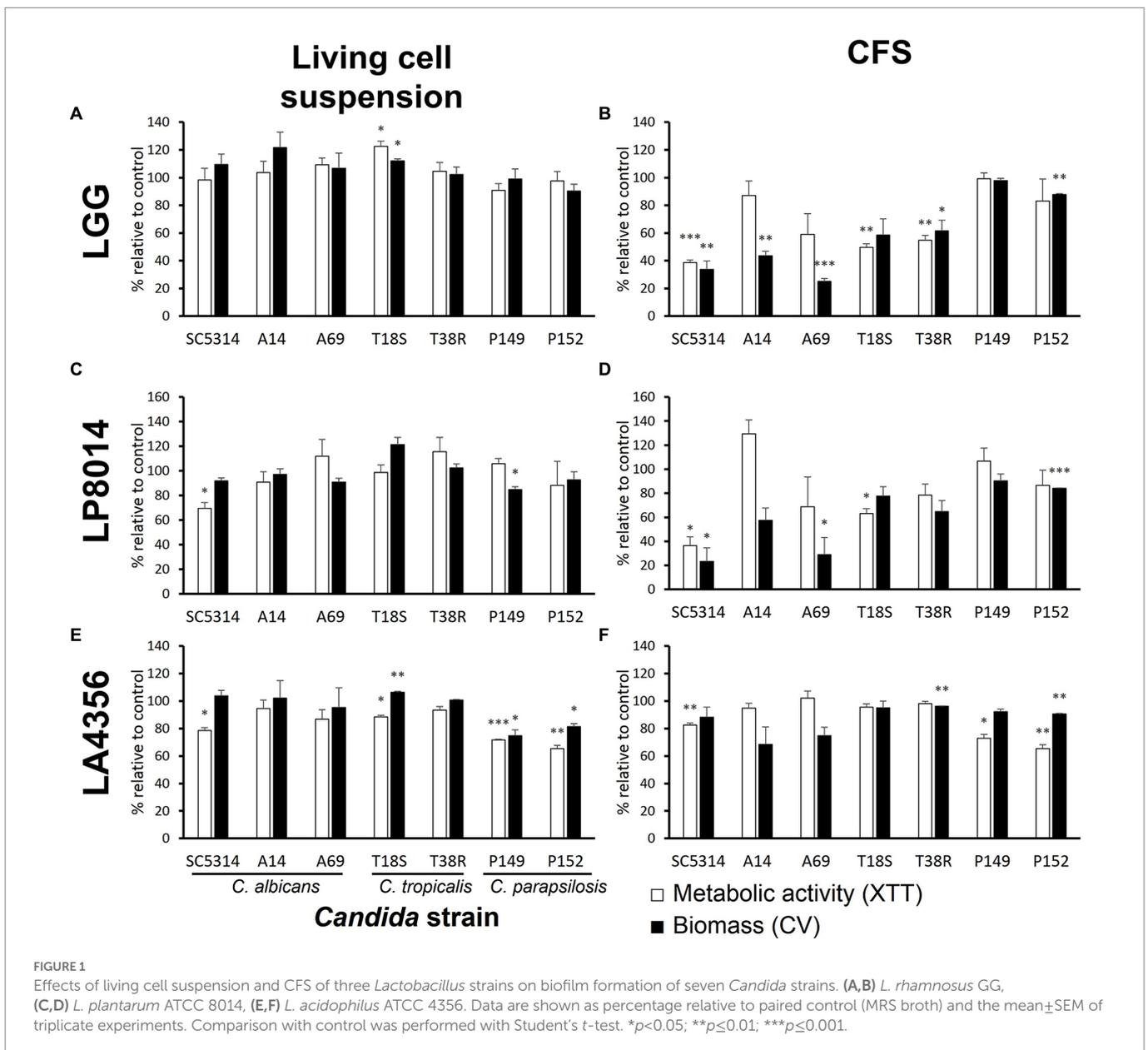
Both LGG CFS and LP8014 CFS had a median pH of 4.0, but LA4356 CFS was less acidic (median pH 5). When used in inhibitory assay, the CFSs lowered the median pH of the spent medium after 24-h incubation from 7.5 (control, MRS broth) to 6.0 (LGG), 6.5 (LP8014), and 7.0 (LA4356), respectively. The medium pH of spent medium was 7.0 in the living cell groups of the three *Lactobacillus* strains. However, when incubated with RPMI medium without *Candida* inoculum, all

three *Lactobacillus* strains were able to reduce the medium pH to 4.5 after 24h.

To examine whether a lower pH contributed to the inhibitory effect of CFS, the assay was repeated with MRS broth acidified to pH 4.0, and LGG CFS neutralized to pH 7.0. As shown in Figure 2, significant inhibition by acidified MRS was only found in biomass of *C. albicans* A14 (35.3% of reduction) and metabolic activity of T18S (12.9%) when compared to MRS control. Neutralized LGG CFS only shown significant reduced inhibitory effect on metabolic activity of *C. albicans* A69 and on biomass of A14 and A69 when compared to untreated LGG CFS.

3.2. Filamentation inhibition

As the CFSs of LGG and LP8014 showed pronounced inhibitory effects on biofilms of *C. albicans* and *C. tropicalis*, their effects on the filamentation of *C. albicans* and *C. tropicalis* were further investigated.



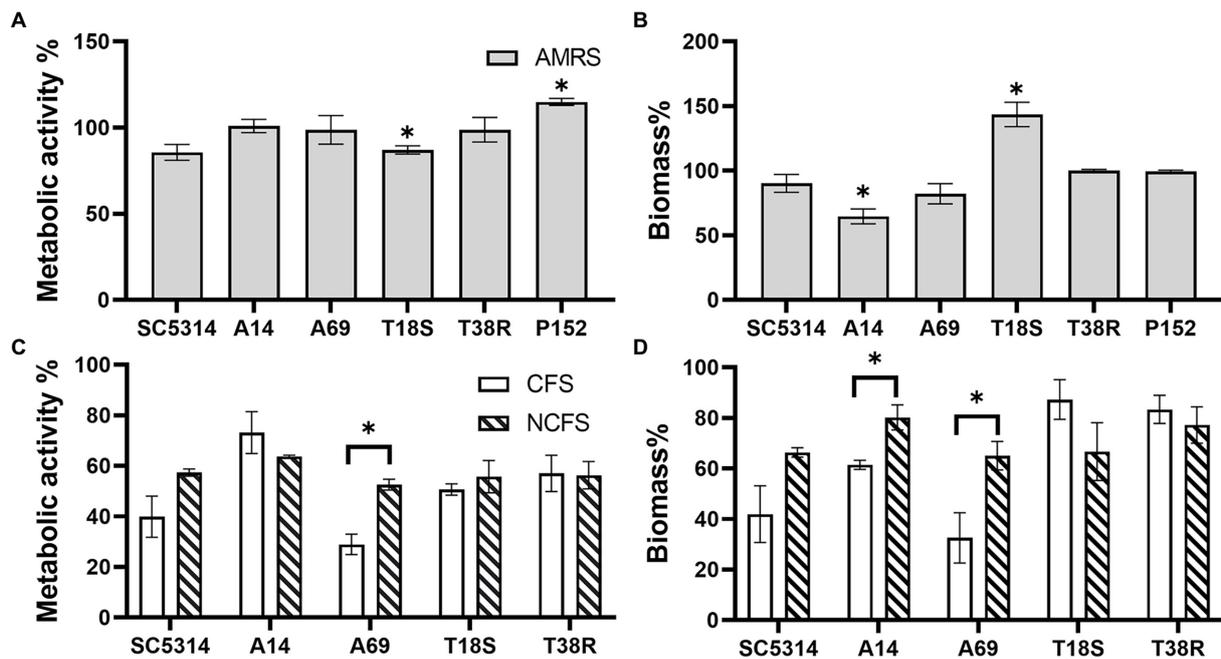


FIGURE 2

Effects of acidified MRS broth (AMRS) and neutralized CFS (NCFS) on *Candida* biofilm. Data are shown as percentage relative to a paired control (untreated MRS) and as mean \pm SEM of triplicate experiments. (A,B) Effects of AMRS on the metabolic activity and biomass of biofilms produced by three *C. albicans* (SC5314, A14, and A69), two *C. tropicalis* (T18S and T38R), and one *C. parapsilosis* (P152) strains. Asterisks indicate data that are significantly different from control (MRS) at $p < 0.05$ as determined by one-sample *t*-test. (C,D) Effects of untreated CFS (pH 4.0) and NCFS (pH 7.0) of LGG on the *C. albicans* and *C. tropicalis* strains. Comparison between CFS, NCFS, and control (MRS) was done with one-way ANOVA with Tukey's test. Asterisks indicate significant difference between CFS and NCFS results at $p < 0.05$.

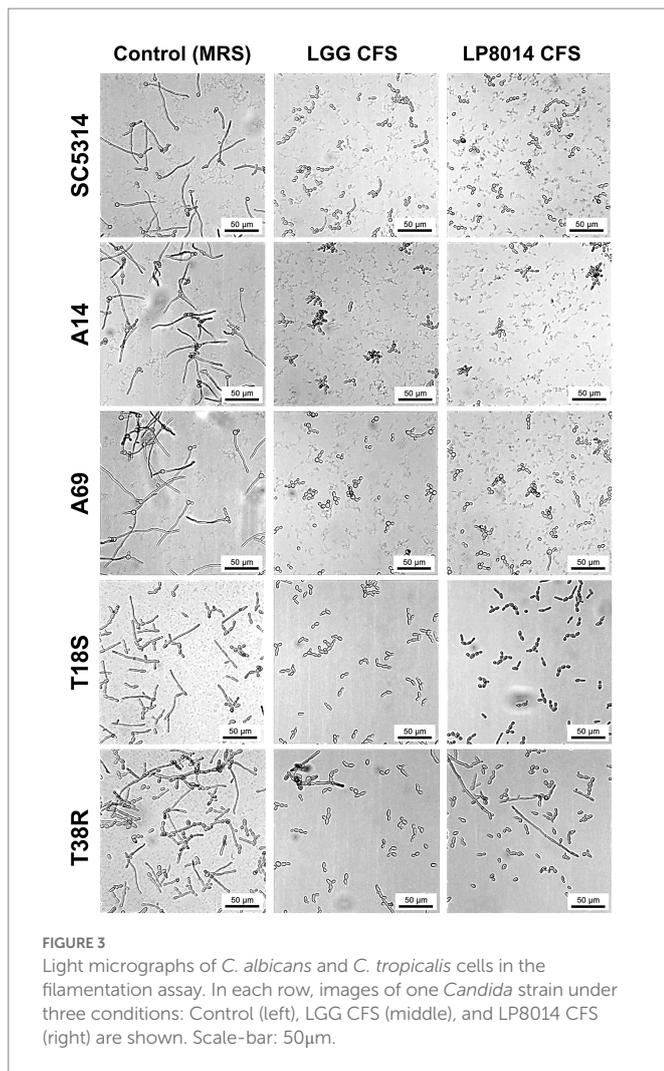
In the filamentation assay, a large number of *Candida* cells developed into filaments after incubating in RPMI medium mixed with MRS broth (control). However, many *Candida* cells remained in yeast form in the presence of *Lactobacillus* CFSs. Figure 3 shows the examples of cell morphology of each tested *Candida* strains under different conditions. Filamentations of *C. albicans* SC5314 and *C. tropicalis* T18S and T38R (Figures 4A,D,E) were statistically significantly inhibited by both LGG CFS and LP8014 CFS ($p < 0.05$). The number of filaments of *C. albicans* A14 was significantly reduced by LGG CFS ($p < 0.05$) but not LP8014 CFS when compared to control (Figure 4B). The filamentation of *C. albicans* A69 when incubated with CFSs was inhibited but not significantly different from the control (Figure 4C).

3.3. Gene expression in biofilm

Expression of *ALS1*, *ALS3*, *BCR1*, *EFG1*, *TEC1*, and *UME6* genes in biofilms of *C. albicans* and *C. tropicalis* strains incubated with LGG or LP8014 CFSs was quantified by qPCR. Although being significant only in some of the strains, there were consistent downregulations in *ALS1*, *EFG1*, and *TEC1* genes caused by LGG CFS, and in *ALS3* and *EFG1* genes by LP8014 CFS in the biofilm of the *C. albicans* strains SC5314, A14, and A69 (Figure 5). On the other hand, *ALS3* and *UME6* genes were downregulated and *TEC1* gene was upregulated by both CFSs in *C. tropicalis* T18S and T38R. Interestingly, the expression levels of *ALS1* in T38R were significantly increased to 1.648- and 1.770-fold by both CFSs (Table 3). The expression level of *UME6* gene was downregulated by LP8014 CFS in SC5314, T18S, and T38R and by LGG CFS in T18S, but the differences were not statistically significant.

4. Discussion

Lactobacillus species have been reported to exhibit an inhibitory effect on biofilm formation and filamentation of *C. albicans*; however, few studies have described their effect on the non-albicans *Candida* species. In the present study, we studied the anti-biofilm effect of cell suspensions and CFSs of three *Lactobacillus* strains on *C. albicans*, *C. tropicalis*, and *C. parapsilosis*. All three of the *Lactobacillus* strains are laboratory reference strains and were shown to have inhibitory effect on *C. albicans* by previous research (Kheradmand et al., 2014; Vilela et al., 2015; Song and Lee, 2017). LGG CFS shown the strongest anti-biofilm activity against *C. albicans* and *C. tropicalis* strains, followed by LP8014 CFS. The *C. parapsilosis* were more susceptible to LA4356 than LGG and LP8014. Previous studies have reported the anti-biofilm activity of *Lactobacillus* cell suspensions on *C. albicans*, however, we detected only a modest effect of cell suspension on *Candida* biofilm in our study (Vilela et al., 2015; Matsubara et al., 2016b; Ribeiro et al., 2017). It is noteworthy that different methods of preparing the cell suspensions were used. In our study, *Lactobacillus* cells were harvested and resuspended in fresh media, while cell suspensions used in other studies were directly diluted from a pre-incubated liquid culture to the desired concentration. Vilela et al. (2015) have tested the anti-biofilm activity of *L. acidophilus* culture at different growth phases (4, 6, 18, and 24h) on *C. albicans* biofilms and found that the reduction of *C. albicans* viable cells in biofilm compared to control was only statistically significant for the 24-h *L. acidophilus* culture, suggesting that inhibitory activity of *Lactobacillus* is possibly associated with the growth stage. In the present study, *C. parapsilosis* was found to be less susceptible to CFSs of LGG and LP8014. Similar *Candida* species-specific manner of the *Lactobacillus* inhibitory activity was



reported in previous research. Tan et al. (2018) observed that biofilm formation of *C. parapsilosis* was less suppressed by the supernatant of *L. gasseri* and *L. rhamnosus* than *C. tropicalis* and *C. krusei*. Parolin et al. (2015) investigated the antifungal activities of CFS of *Lactobacillus* strains isolated from vaginal swabs on *C. albicans* and non-albicans *Candida* species but found that no strains were effective against *C. krusei* and *C. parapsilosis*, even when being effective on other *Candida* species.

Our study showed an inhibitory effect of *L. rhamnosus* (LGG) and *L. plantarum* (LP8014) CFSs on *Candida* biofilm. This finding corroborates the results of numerous studies using other *Lactobacillus* species and strains (Vilela et al., 2015; James et al., 2016; Matsubara et al., 2016b; Ribeiro et al., 2017; Matsuda et al., 2018; Tan et al., 2018). The indirect interaction between *Lactobacillus* cells and *Candida* biofilm suggests that soluble exometabolites secreted into the culture medium by *Lactobacillus* are possibly responsible for the inhibitory effect. Production of lactic acid and the resulted low pH (4.5) environment has been accounted for candidicidal activity of *L. rhamnosus* and *Lactobacillus reuteri* (Köhler et al., 2012). However, in our biofilm assay, the lowest pH of medium measured was 6.0 and caused by the addition of LGG CFS. Moreover, acidified MRS broth showed little inhibitory effect on biofilm, and the inhibitory effect was not diminished in neutralized LGG CFS when compared with MRS control ($p < 0.05$, except for biomass of *C. tropicalis* T18S and T38R) and only reduced when compared with

untreated LGG CFS in *C. albicans* A14 and A69. These results suggested that there are other factors, rather than pH, contributed to the inhibitory effect.

Apart from lactic acid, *Lactobacillus* have been described to produce other exometabolites with antimicrobial and anti-biofilm effects, such as small chain fatty acids, hydrogen peroxide, antimicrobial peptides, and biosurfactants (Gudiña et al., 2010; Nguyen et al., 2011; Rushdy and Gomaa, 2013; Ceresa et al., 2015). Sodium butyrate, a small chain fatty acid produced by *Lactobacillus* species, is a known histone deacetylase inhibitor (Garnaud et al., 2016). Nguyen et al. (2011) demonstrated that sodium butyrate can significantly inhibit biofilm formation of *C. albicans* and *C. parapsilosis*, by 80 and 65%, respectively, at a concentration of 10 mM. Song and Lee investigated the antifungal activity of spent culture medium of *L. rhamnosus* and *L. casei* and found that the growth of *C. albicans* in both yeast- and hyphal-predominant conditions were significantly inhibited with 50% v/v spent culture medium (Song and Lee, 2017). However, proteinase K-treated spent culture medium of both species lost the inhibitory effect, suggesting that the effect was due to antifungal peptides. A biosurfactant produced by a *Lactobacillus brevis* strain CV8LAC was shown to inhibit *C. albicans* biofilm formation by co-incubation and pre-coating the substrate while demonstrated no inhibition on the fungal growth in planktonic and sessile form (Ceresa et al., 2015). Similarly, liposomes and hyalurosomes containing the biosurfactant isolated from a vaginal strain *Lactobacillus crispatus* BC1 were shown to inhibit biofilm development in *C. albicans* and non-albicans *Candida* species (Abruzzo et al., 2021). It is possible that these metabolites and similar compounds also contribute to the inhibitory effect of CFS demonstrated in our study, but further investigation is needed to identify the exact active compounds and their mechanisms.

Filaments are key structural component of mature biofilm of *C. albicans* (Kuhn et al., 2002). Mutants of hyphal-related genes showed defects in forming hyphae and biofilm formation as well (Ramage et al., 2002; Nobile and Mitchell, 2005). Therefore, we investigated the effect of CFSs of LGG and LP8014 on the filamentation of the *C. albicans* and *C. tropicalis* strains. The filamentation of *C. albicans* and *C. tropicalis* cells was greatly reduced when co-incubated with the CFSs. These results are consistent with other studies that demonstrated inhibitory effects of *Lactobacillus* cell suspensions and CFSs on *C. albicans* in filamentation assay of similar designs (Vilela et al., 2015; Ribeiro et al., 2017; Matsuda et al., 2018; Rossoni et al., 2018b). Matsubara et al. (2016b) observed a significant reduction of hyphal elements in *C. albicans* biofilm treated with *L. rhamnosus* supernatant by confocal laser scanning microscopy. In a monolayer model of oral epithelial cells, preincubating the epithelial cells with LGG suspension for 12 h before *C. albicans* infection was shown to significantly reduce hyphal length and invasion of *C. albicans* (Mailänder-Sánchez et al., 2017). In contrast, *Lactobacillus iners*, the abundance of which in the vaginal microbiome was associated with vaginal dysbiosis and bacterial vaginosis, was demonstrated to promote filamentation and biofilm formation, and upregulate the expression of hyphal-specific genes *HWPI* and *ECE1* in *C. albicans* strains with weak to moderate biofilm-forming ability (Sabbatini et al., 2021; Zheng et al., 2021).

Environmental pH is known to induce morphological differentiation in *C. albicans*. Growth in yeast form is favored in acidic conditions, whereas neutral and alkaline conditions prompt hyphal growth (Davis, 2003). We found that the addition of LGG and LP8014 CFSs reduced the pH of the medium from 7.5 of the control group (MRS broth) to 6 and

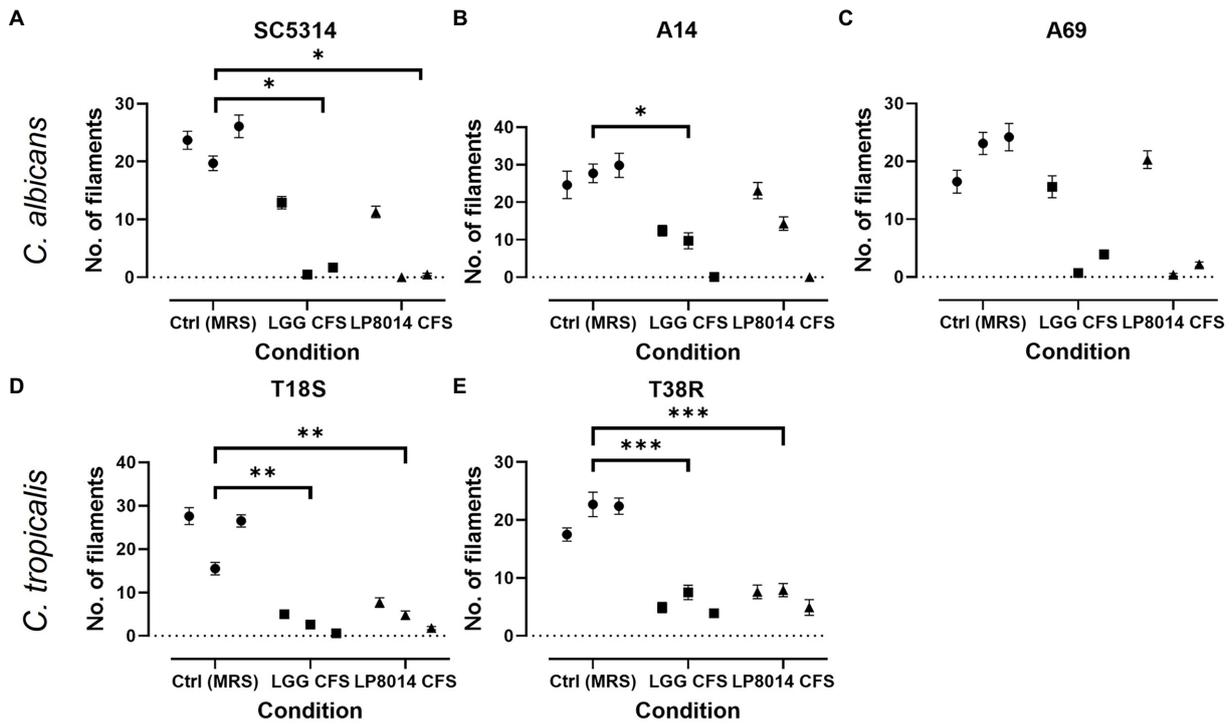


FIGURE 4 Quantification of filament in the filamentation inhibition assays. (A) *C. albicans* SC5314, (B) *C. albicans* A14, (C) *C. albicans* A69, (D) *C. tropicalis* T18S, and (E) *C. tropicalis* T38R. Each data point represents the mean ± SEM of the number of filaments in one experiment. Comparisons with the control group were performed with results of triplicate experiments and Dunnett’s test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

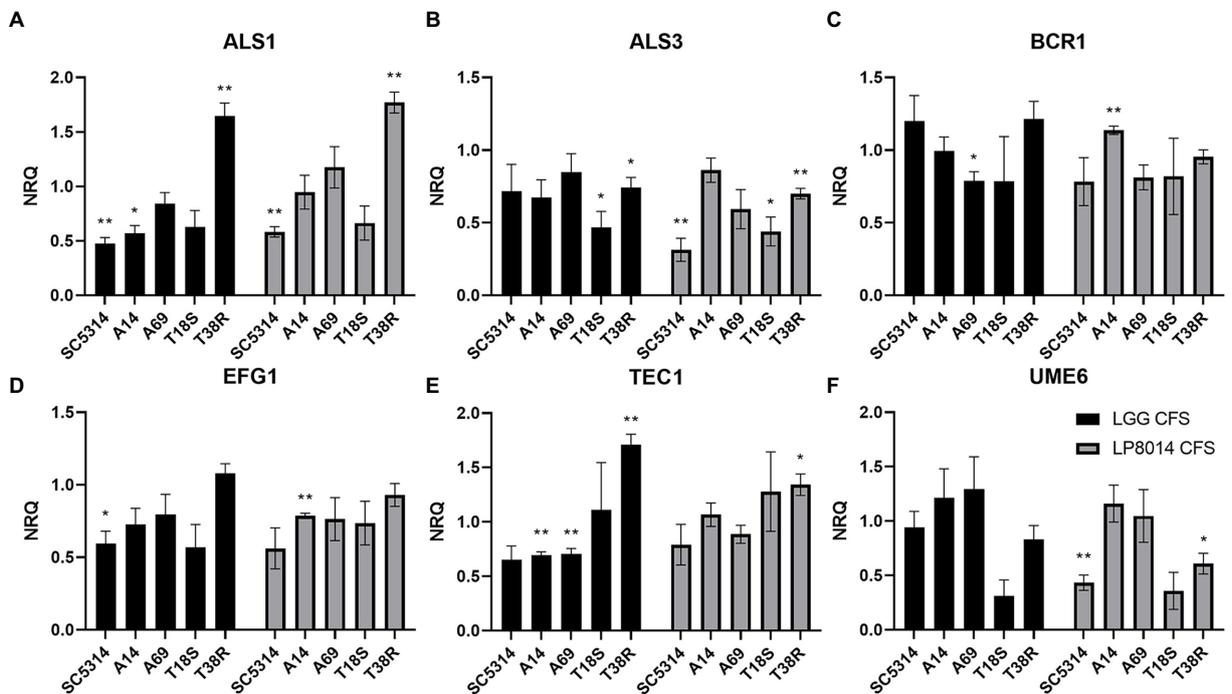


FIGURE 5 Expression levels filamentation and biofilm-related genes in biofilms of *C. albicans* and *C. tropicalis* incubated with LGG or LP8014 CFSs. (A) *ALS1*, (B) *ALS3*, (C) *BCR1*, (D) *EFG1*, (E) *TEC1*, and (F) *UME6*. Expression levels were expressed as the normalized relative quantity (NRQ). NRQ of the control group (MRS) is constantly 1.0 and not shown in the figures. Values represent the mean ± SEM of triplicate experiments. Comparison with control group was performed with the binary logarithm of NRQ by Student’s *t*-test. * $p < 0.05$; ** $p < 0.01$.

TABLE 3 Expression levels filamentation and biofilm-related genes in biofilms of *Candida albicans* and *Candida tropicalis* incubated with LGG or LP8014 CFSs.

Condition	Strain	ALS1		ALS3		BCR1		EFG1		TEC1		UME6	
		NRQ*	P†	NRQ	P								
LGG CFS	SC5314	0.476±0.054	0.0030	0.718±0.184	0.2546	1.202±0.174	0.3590	0.594±0.087	0.0271	0.655±0.124	0.0615	0.943±0.146	0.6223
	A14	0.573±0.068	0.0104	0.675±0.121	0.0697	0.996±0.095	0.8976	0.729±0.109	0.1076	0.694±0.031	0.0012	1.213±0.269	0.5191
	A69	0.843±0.102	0.1887	0.849±0.126	0.2958	0.789±0.064	0.0379	0.796±0.138	0.2115	0.706±0.049	0.0066	1.295±0.296	0.3889
	T18S	0.629±0.150	0.0798	0.469±0.110	0.0406	0.786±0.307	0.4373	0.571±0.156	0.1206	1.113±0.432	0.8952	0.313±0.146	0.0981
LP8014 CFS	T38R	1.648±0.119	0.0027	0.742±0.069	0.0308	1.216±0.119	0.1453	1.080±0.066	0.2885	1.710±0.096	0.0007	0.831±0.129	0.2787
	SC5314	0.583±0.047	0.0029	0.314±0.080	0.0082	0.783±0.165	0.2598	0.562±0.142	0.0584	0.791±0.186	0.3267	0.434±0.070	0.0064
	A14	0.948±0.155	0.6404	0.862±0.084	0.1733	1.137±0.029	0.0071	0.786±0.019	0.0005	1.067±0.108	0.6407	1.161±0.170	0.4365
	A69	1.176±0.190	0.4526	0.593±0.134	0.0629	0.812±0.086	0.1070	0.764±0.148	0.2222	0.887±0.082	0.2243	1.048±0.242	0.9855
LGG CFS	T18S	0.664±0.157	0.1052	0.440±0.100	0.0299	0.819±0.262	0.4574	0.736±0.151	0.2000	1.279±0.365	0.6558	0.360±0.170	0.0956
	T38R	1.770±0.096	0.0004	0.700±0.036	0.0021	0.954±0.048	0.3908	0.930±0.079	0.4156	1.342±0.099	0.0186	0.609±0.095	0.0373

*Expression levels are expressed as mean ± SEM of the normalized relative quantity (NRQ) obtained from triplicate experiments. NRQ of control group (MRS) is constantly 1.0 and not shown. †p-value was obtained by comparing to the control group using Student's t-test. Bold values denote statistical significance at p < 0.05.

7. As Nadeem et al. (2013) have observed that germ tube formation in *C. albicans* at pH 6.4 was moderately lower than at pH 7.4 and further reduced at pH 5.4, we could not exclude the effect of the slightly decreased pH in the inhibition of filamentation observed in our study, but other metabolites of *Lactobacillus* are suggested to contribute. For example, in a recent study, 1-acetyl-β-carboline (1-ABC), a small molecule isolated from the culture supernatant of *Lactobacillus* species, was reported to block *C. albicans* filamentation in a concentration-dependent manner (MacAlpine et al., 2021). It was proposed that 1-ABC inhibits *C. albicans* Yak1 kinase, which is a member of the dual-specificity tyrosine-regulated kinase (DYRK) family and necessary for upregulation of hypha-induced genes in *C. albicans* (Goyard et al., 2008). Purified 1-ABC was also shown to inhibit filamentation of *C. dubliniensis* and *C. tropicalis*, in which Yak1 is conserved (MacAlpine et al., 2021). The authors also demonstrated that acidic pH alone was insufficient to achieve the same degree of filamentation inhibition caused by *Lactobacillus* culture supernatant.

To elucidate the mechanisms of the anti-biofilm activity of *Lactobacillus* CFS, we measured the expression of six biofilm-related genes, namely *ALS1*, *ALS3*, *BCR1*, *EFG1*, *TEC1*, and *UME6*, in the biofilm produced by *C. albicans* or *C. tropicalis* co-incubated with LGG and LP8014 CFSs. *ALS1* and *ALS3*, belonging to the agglutinin-like sequence (*ALS*) gene family, encode cell wall glycoproteins Als1 and Als3, respectively (Hoyer, 2001). Expression of *ALS1* is detectable in both yeast or hyphae form, while *ALS3* transcribed exclusively in germ tubes and hyphae (Green et al., 2005). In *C. tropicalis*, *ALS1*-, *ALS2*-, and *ALS3*- like genes were more upregulated in sessile cells, suggesting their roles in biofilm formation (Galán-Ladero et al., 2019). *BCR1*, encoding a transcription factor Bcr1, is upregulated in *C. albicans* hyphae but not required for normal hyphal development (Nobile and Mitchell, 2005). Bcr1 is required for the full expression of several cell wall proteins, including Als1 and the hyphal-specific Als3, Hwp1, and Hyr1. *EFG1* encodes the transcription factor Efg1, which is essential for yeast-hyphal transition and biofilm formation of *C. albicans* (Stoldt et al., 1997; Ramage et al., 2002). Efg1 is activated by upstream cAMP pathway and regulates expression of hyphal-specific genes and downstream transcription factors such as Tec1 and Eed1 (Sudbery, 2011). Mancera et al. (2015) have reported that *EFG1* ortholog in *C. tropicalis* has conserved function on filamentation and biofilm formation. *TEC1* encodes transcription factor Tec1, deletion of which results in defect of hyphal formation and biofilm formation, and suppression of expression of secreted aspartyl protease (SAP) family proteins Sap4-6 in *C. albicans* (Schweizer et al., 2000; Nobile and Mitchell, 2005). In *C. tropicalis*, deletion mutants of *BCR1*, *BRG1*, *TEC1*, *EFG1*, or *NDT80* homologs produced significantly less biofilm biomass, suggesting the conserved roles of these genes in biofilm formation (Tseng et al., 2020). *UME6*, a common downstream target of regulators Efg1, Chp1, and Ras1, strictly or partially controls the expression of hyphae-specific genes in *C. albicans* (Zeidler et al., 2009). Constitutive high-level expression of *UME6* orthologs in *C. tropicalis* and *C. parapsilosis* also enhances filamentation and biofilm formation (Lackey et al., 2013).

In our study, we observed a downregulation in *EFG1* and *TEC1* genes by CFS in the three *C. albicans* strains, which is in agreement with the findings of previous studies on *C. albicans* biofilm (James et al., 2016; Matsuda et al., 2018; Rossoni et al., 2018a). These studies, however, also reported significant downregulation of *ALS3* and *BCR1* genes in *Lactobacillus*-inhibited biofilm, which was not the case in our results. Interestingly, in our study, the expressions of *ALS1*, *BCR1*, and *TEC1* genes were upregulated in *C. tropicalis* T38R by LGG CFS although LGG

CFS was shown to suppress biofilm formation and filamentation of T38R, suggesting a strain-dependent gene response. The upregulation of *ALS1* in T38R could possibly be a compensatory upregulation due to the suppression of *ALS3* (Liu and Filler, 2011). Such compensatory upregulations had been demonstrated by the upregulation of *ALS2* in *als4/als4* mutant and vice versa, and the upregulation of *SAP5* in *sap1-3* mutant and *SAP2* in *sap 4–6* mutant of *C. albicans* (Zhao et al., 2005; Naglik et al., 2008). The expression of *UME6* in *C. albicans* SC5314, *C. tropicalis* T18S and T38R was downregulated by 0.434-, 0.360- ($p=0.0956$), and 0.609-fold, respectively, suggesting that the mechanism of action of LP8014 CFS involves the repression of *UME6*.

There are a number of limitations to the current study need to be taken into account. First, we used two semiquantitative methods, CV staining and XTT reduction assay, to measure *Candida* biofilm formation. However, neither of the assays provide information about effect of *Lactobacillus* on the three-dimensional architecture of the *Candida* biofilm. Second, the dosage of inhibitory agents was not optimized. Ideally, the experiment should be repeated with a range of concentrations to examine the presence of dose-dependent effect and determine the most effective concentration. Third, the study lacked a characterization of the metabolites in LGG and LP8014 CFSs, which showed strong inhibitory effect. Analyses such as thermal stability, protease stability, activity of hydrogen peroxide, and metabolite profiling by liquid chromatography-mass spectrometry would provide information to identify the active components and elucidate mechanisms of action if performed. Another limitation is that we selected few target genes for the gene expression analysis from the biofilm regulatory network of *Candida*, which involves multiple signaling pathways and more than 50 genes (Finkel and Mitchell, 2011). Other well-known biofilm-related genes, such as *HWPI*, which encodes a cell wall adhesin required in biofilm formation, and *NRG1*, a negative regulator of filamentation, were not included (Braun et al., 2001; Nobile et al., 2006). To unravel the elusive mechanisms of the effect of *Lactobacillus* CFS, genomic-wide transcriptional profiling by technologies such as RNA-seq is a better approach (Chong et al., 2018).

5. Conclusion

The present study evaluated the inhibitory effect of three lactobacilli strains, LGG, LP8014, and LA4356, on *C. albicans*, *C. tropicalis*, and *C. parapsilosis*. Significant inhibitory effects were demonstrated by the CFSs of LGG and LP8014 on filamentation and biofilm formation of both *C. albicans* and *C. tropicalis*. Hypa-related gene expressions in biofilm were also changed in response to the addition of CFSs. However, *C. parapsilosis* biofilms were not

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susceptible to the inhibitory effect of these two lactobacilli strains. The pathogen-specific inhibition suggests a common mechanism of action, presumably mediated by exometabolites of the lactobacilli, on *C. albicans* and *C. tropicalis*. The active components and the potential clinical application of LGG and LP8014 CFSs remain to be investigated.

Author's note

The content of this manuscript has previously been presented in part as a poster at the 29th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), 13–16 April 2019, Amsterdam, Netherlands, and published as part of the doctoral thesis of YP (Poon, 2022). A preliminary version of this manuscript has been released as a preprint at BioRxiv (Poon and Hui, 2022).

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

YP performed the experiments and wrote the manuscript. MH reviewed and revised the manuscript. All authors read and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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