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Original Article

Effect of postbiotics on biofilm formation and gene expression in *Candida* spp. isolates from patients with recurrent vulvovaginal candidiasis

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Abstract

First-line treatment for vulvovaginal candidiasis (VVC) has reduced efficacy in patients with recurrent VVC (RVVC), prompting the search for therapeutic alternatives such as probiotics. Recently, postbiotics (cellular components and metabolites derived from probiotics) have gained relevance because they offer similar benefits to probiotics without the risks associated with live microorganisms. This study aimed to evaluate the anti-biofilm effect of cell-free supernatants (CFSs) derived from probiotic strains against *Candida* spp. isolates from patients with RVVC, as well as their impact on the expression of genes associated with biofilm formation. Therefore, the anti-biofilm activity of CFSs from *Lacticaseibacillus rhamnosus* DM-UFMG 63, *Bifidobacterium longum* 5,[1A] and *Lactobacillus acidophilus* NCFM SD 5221 was evaluated in 40 *Candida* spp. isolates. The CFSs from *L. acidophilus* NCFM SD 5221 and *Lc. rhamnosus* 63 were analyzed using the XTT assay, and their effect on gene expression was measured by qPCR. At an estimated protein concentration of ∼200 μg/ml, these CFSs significantly inhibited biofilm formation by ∼ 50% ($P < .01$)). This phenotypic effect correlated with the downregulation of key biofilm-associated genes (*ALS3*, *HWP1*, *EFG1*, *TEC1*, and *UME6*), alongside a concomitant upregulation of the transcriptional repressor *NRG1*. The findings of this study demonstrate that CFSs derived from *Lc. rhamnosus* DM-UFMG 63 and *L. acidophilus* NCFM SD 5221 exhibit significant antagonistic activity against biofilm formation in clinical isolates from patients with RVVC. Consequently, these CFSs represent a promising therapeutic and prophylactic alternative for the management of RVVC.

Lay summary

In some cases, conventional therapies are ineffective against recurrent vulvovaginal candidiasis. This study demonstrates that cell-free supernatants derived from probiotics can inhibit *Candida* biofilm formation and modulate associated genes, supporting their therapeutic and prophylactic potential.

Keywords *Candida* spp., RVVC, biofilm, postbiotic, cell-free supernatant

Introduction

Vulvovaginal candidiasis (VVC) is a debilitating infection caused by yeasts of the genus *Candida*, with *Candida albicans* being the most common etiological agent associated with this condition.[1] *Candida albicans* is part of the mucosa-associated microbiota that, under certain conditions, can cause disease[2,3]; in this sense, the ability of some species of the genus *Candida* to undergo the morphological transition from blastoconidia to hypha or pseudohyphae is crucial for promoting the conversion from commensal to pathogen.[4]

It is estimated that VVC affects ∼ 75% of the female population at least once in their lifetime[5]; however, ∼ 10% of women who suffer from VVC present recurrences (RVVC), which is defined as the occurrence of at least three episodes of VVC in 1 year.[6] Factors such as pregnancy, hormone replacement therapy, use of oral contraceptives, diabetes, genetic predisposition, fungal virulence factors, and the use of broad-spectrum

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antibiotics have been reported to be associated with the occurrence of RVVC.[7]

The use of broad-spectrum antibiotics can generate dysbiosis in the vaginal microenvironment due to the decrease or eradication of lactic acid bacteria such as *Lactobacillus crispatus*, *L. gasseri*, and *L. jensenii*, which are part of the vaginal microbiota and have the ability to inhibit the excessive growth of *C. albicans* through various mechanisms, including the production of metabolites such as lactic acid, bacteriocins, antimicrobial peptides, hydrogen peroxide (H_2O_2) and biosurfactants, in addition to competition for nutrients and epithelial attachment sites.[8,9]

Probiotics are live microorganisms that, when administered in adequate concentrations, could potentially confer a health benefit to the host.[9] Based on this, some species of the genera *Lactobacillus* and *Bifidobacterium* are commonly used as vaginal probiotics and are administered orally or vaginally.[10] However, recent evidence has shown that the use of probiotics could cause opportunistic systemic and local infections, especially in individuals with predisposing immunological conditions.[11] Thus, in recent years, it has been recognized that, like probiotics, the use of cellular components of microorganisms and their metabolites can generate health benefits for the host. In this sense, the term "postbiotic" has been coined, which is defined as "a preparation of inanimate microorganisms and/or their components (proteins, short-chain fatty acids, vitamins, and amino acids, among others) that confer a health benefit".[12]

Despite the recent surge in probiotics as beneficial agents for human health, few studies have evaluated their role in VVC/RVVC. *In vitro* studies have reported that *C. albicans* exhibits decreased proliferation when co-cultured with bacteria such as *L. crispatus*, *L. jensenii*, *L. gasseri*, *L. reuteri*, *L. acidophilus*, and *Lacticaseibacillus rhamnosus*.[13–16] Additionally, to date, there are no reports evaluating the anti-*Candida* capacity of cell-free supernatants (CFSs) derived from probiotics against *Candida* spp. isolates from patients with RVVC.

Based on the above, the objective of this study was to evaluate the anti-biofilm effect of CFSs derived from the probiotic strains *Lc. rhamnosus* 63, *Bifidobacterium longum* 5^{1A}, and *L. acidophilus* NCFM SD 5221 (Probiac ®) against *Candida* spp. isolates from patients with RVVC, as well as to determine the expression of genes associated with biofilm formation in response to these postbiotics.

Materials and methods

Ethical aspects

This study was conducted in accordance with the Declaration of Helsinki, and both the isolates and the clinical information of the participating individuals were obtained from a previous study approved by the Bioethics Committee in Human Research (Act No. 21-28-935) of the University Research Headquarters (SIU) of the University of Antioquia, Medellín, Colombia.

Microorganisms and culture conditions

A total of 40 *Candida* spp. clinical isolates—comprising 37 *Candida albicans* and three *Clavispora lusitaniae* (formerly *Candida lusitaniae*)—were evaluated. These isolates were recovered during a previous study by our research group[17] where clinical samples were obtained exclusively from patients diagnosed with RVVC. Within this specific cohort, criteria were applied to exclude any isolates originating from patients who presented with co-existing vulval pathologies, diabetes, HIV, cancer, autoimmune diseases, or pregnancy. Furthermore, isolates were excluded if the source patient had a history of antibiotic, antifungal, or corticosteroid therapy within 7 days prior to sample collection. On the other hand, the reference strains *C. albicans* SC5314 (ATCC MYA-2876) and *C. parapsilosis* CLIB214 (ATCC 22019) were used. Prior to use, both the strains and the *Candida* isolates were stored at -80°C in BHI broth (HIMEDIA, M210I) supplemented with 10% glycerol. After thawing the isolates and strains, they were cultured on Sabouraud Glucose Agar (SDA) (Merck, 103 873) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, P0781) and incubated at 37°C for 24 h.

Additionally, three previously characterized probiotic strains donated by the Laboratory of Biotherapeutic Agents of the Department of Microbiology at the Institute of Biological Sciences of the Federal University of Minas Gerais (UFMG) were used: *Lacticaseibacillus rhamnosus* DM-UFMG 63,[18] *Bifidobacterium longum* 5^{1A},[19] and *Lactobacillus acidophilus* NCFM SD 5221 (Probiac®). For the assays, five isolated colonies were inoculated into 5 ml of de Man Rogosa and Sharpe (MRS) broth (Becton Dickinson, 288 210) and incubated at 37°C with shaking for 48 h under aerobic conditions for *Lc. rhamnosus* 63 and *L. acidophilus* NCFM SD 5221, and under anaerobic conditions for *B. longum* 5^{1A}.

Obtaining CFSs from Probiotics

CFSs derived from the aforementioned probiotics were obtained after 48 h of incubation in MRS broth (Becton Dickinson, 288 210) until a bacterial concentration of 1.5 × 10 CFU/ml was reached. Subsequently, the cultures were centrifuged at 3500 rpm for 10 min at 4°C. The resulting supernatants were sterilized using 0.22 μm filters (Minisart®, 16 541). Sterility controls were performed by incubating 1 ml of each CFS at 37°C for 24 h to assess turbidity, followed by plating aliquots of the CFSs onto BHI agar (HIMEDIA, M211) and incubating them for 48 h at 37°C.[20] Furthermore, the pH of each CFS was determined using a pH meter, and all supernatants were adjusted to a pH range of 7.0–7.2. Additionally, an exploratory estimation of the protein concentration in each CFS was performed via spectrophotometric analysis at 280 nm (A_{280}), after which the samples were stored at −20°C until further use

Determination of minimum biofilm inhibitory concentrations and evaluation of the pH effect of CFSs on *Candida* spp. biofilm

To determine the minimum biofilm inhibitory concentration (MBIC) and the pH effect of CFSs, the *C. albicans* SC5314 strain was used. This strain was cultured in BHI broth (HIMEDIA, M210I) for 24 h at 37°C. Subsequently, an inoculum of 1 × 10^6 cells/ml was adjusted, and 100 μl of this inoculum was added to 96-well microplate wells (NEST Scientific, 701 001). Different v: v concentra-

Table 1. List of genes and primers sequences used for qPCR analyses.

Phase	Gene	Primer	Nucleotide sequence	Reference
Adhesion	*BCR1*	BCR1-F	AGGCATTCCCTGTTGTGGTT	*de novo* synthesis
		BCR1-R	ACAAGCCCAAGTTCACATGC	
	ALS3	ALS3-F	GTTGGGTTTGGCAGTGGAAC	*de novo* synthesis
		ALS3-R	TACTACCGCTGTGACCACCT	
	HWP1	HWP1-F	GACCGTCTACCTGTGGGACAGT	[22]
		HWP1-R	GCTCAACTTATTGCTATCGCTTATTACA	
Maduration	*EFG1*	EFG1-F	CATCACAACCAGGTTCTACAACCAAT	[22]
		EFG1-R	CTACTATTAGCAGCACCACCC	
	TEC1	TEC1-F	ACTATCGGGGCTTGACTCGT	*de novo* synthesis
		TEC1-R	CCAGAAAACGCATCACCCAC	
Dispertion	*UME6*	UME6-F	GCAGCAGCACTAACACTGAC	*de novo* synthesis
		UME6-R	AGATGCAGCAAACCCATCATC	
	NRG1	NRG1-F	ATCAGACCATCTCCAGGGCT	*de novo* synthesis
		NRG1-R	TGGGTCTTTGCTTTGGGTGT	
Housekeeping	*ACT1*	ACT1-F	TGGAAGCTGCTGGTATTGACC	[23]
		ACT1-R	TGGATGGACCAGATTCGTCG	

tions of CFSs were then added to each well at both pH 7 and pH 4, in triplicate, as follows: 6.75%, 12.5%, 25%, and 50%. In addition, a growth control at pH 7 and pH 4 (without supernatant) and an inhibition control with amphotericin B (AmB, 2%) were included. Biofilm formation was evaluated after 48 h at 37°C. Subsequently, metabolic activity in each well was evaluated using the XTT (Invitrogen, X6493) assay, following the methodology proposed by Pierce *et al.* [21]

Evaluation of the anti-biofilm effect of different CFSs

The biofilm-forming capacity of different *Candida* isolates from patients with RVVC was evaluated. An inoculum of each *Candida* spp. isolate or strain was prepared at a concentration of 1×10^6 cells/ml in RPMI 1640 medium (Sigma-Aldrich, R6540) supplemented with 4-morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, M1254). The *C. albicans* SC5314 and *C. parapsilosis* ATCC 22019 strains were used as positive and negative controls, respectively, for biofilm formation; in addition, a cell-free control and a total inhibition control using amphotericin B (2%) as treatment were also included. In a 96-well microplate (NEST Scientific, 701 001), 100 μl of each inoculum and 25 μl of the different treatments and controls were seeded: CFSs, MRS medium, or AmB 2%. All wells were brought to a final volume of 200 μl using RPMI medium supplemented with MOPS. The plates were incubated at 37°C for 48 h. After this time, each well was vigorously washed twice with 200 μl of PBS, and the excess PBS was discarded. Subsequently, 100 μl of XTT solution (Invitrogen, X6493) supplemented with menadione was added to each well. The plates were incubated for 3 h at 37°C in the dark. Consequently, 80 μl of the XTT/menadione solution was extracted and transferred to a new 96-well plate. Finally, the metabolic activity of the *Candida* spp. biofilms was analyzed by measuring the absorbance at 490 nm using a Multiskan FC spectrophotometer (Thermo Scientific, 11 500 695).

RNA Extraction and cDNA Synthesis

RNA was extracted from isolates and strains subjected to CFS treatments, as well as from isolates and strains not treated with CFSs, using the TRIzol reagent (Invitrogen, 15 596 026) and following the manufacturer's instructions. For each treatment, this extraction was performed at 2, 24, and 48 h, corresponding to the biofilm development phases (adhesion, maturation, and dispersion, respectively). This was done to specifically evaluate gene expression during the corresponding biofilm development phase. Furthermore, the integrity, quantity, and quality of the extracted RNA were evaluated by agarose gel electrophoresis and spectrophotometry using the NanoDrop One (Thermo Fisher Scientific), taking into account the 260/230 and 260/280 absorbance ratios to assess sample purity. Subsequently, the samples were treated with DNase using DNase I Amplification Grade (1 unit/μl) (Sigma-Aldrich, 89 836), and then cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, 4 368 814) according to the manufacturer's instructions. Finally, the quality and quantity of the cDNA samples were determined using the NanoDrop One instrument (Thermo Fisher Scientific) under the same conditions described above.

Determination of the expression of genes associated with *Candida* spp. biofilm formation in response to CFSs

To determine the effects of CFSs on the expression of *Candida* spp. genes related to biofilm formation in the different developmental phases, the expression of the following genes was evaluated by qPCR: *BCR1, ALS3,* and *HWP1*, related to the adhesion phase; *EFG1, TEC1*, related to the maturation phase; and *UME6* and *NRG1*, which are related to the biofilm dispersion phase (Table 1).

Gene expression was evaluated using qPCR with the ExcelTaq 2 × Fast Q-PCR Master Mix kit, SYBR, without ROX (SMOBIO, TQ1200), and the CFX96 Touch Real-Time PCR system (Bio-Rad). A final volume of 15 μl was used for each reaction, along with the

following amplification protocol: an initial polymerase activation cycle for 20 s at 95°C, followed by 40 cycles at 95°C for 15 s with a 30 s melt-and-extension phase. A dissociation protocol was also included to determine the melting curve of each primer, considering a temperature range of 58°C–95°C, in order to verify that each primer pair produced only one PCR product. The relative expression of the genes of interest was calculated using the formula $2^{-\Delta\Delta Ct}$, considering the *ACT1* gene as the constitutive or normalizing gene.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 9.0 (GraphPad Software, LLC) and R version 4.5.0. Data normality was assessed using the Shapiro–Wilk test. For non-normally distributed datasets, differences were analyzed using the Kruskal–Wallis test followed by Dunn's post-hoc multiple comparison test. Conversely, normally distributed data were evaluated via one-way analysis of variance followed by the Holm–Šidák multiple comparison test. Relative gene expression from qPCR assays was calculated using the $2^{-\Delta\Delta Ct2}$ method. Data are expressed as mean ± standard error of the mean or as median with interquartile range from three independent experiments. Statistical significance was strictly defined at $P < .05$.

Results

Estimated protein content in CFSs via spectrophotometric analysis

To provide a preliminary baseline of the secretome composition, the protein-associated content of the CFSs was estimated spectrophotometrically at 280 nm (A_{280}). Based on the standard analytical premise that an absorbance of 1.0 correlates to ∼ 1000 μg/ml of total protein, the equivalent concentrations were determined. The analyses revealed comparable protein yields across the strains, with estimated concentrations of 1570 μg/ml for *Lacticaseibacillus. rhamnosus* DM-UFMG 63, 1580 μg/ml for *Lactobacillus acidophilus* NCFM SD 5221, and 1640 μg/ml for *Bifidobacterium longum* 51A.

pH does not alter the anti-biofilm effect of CFSs against *Candida albicans*

The MBIC of the evaluated probiotic CFSs was determined to be 12.5%, which corresponds to an estimated protein concentration of ∼ 200 μg/ml. The effect of pH on the anti-biofilm effect of the CFSs against *Candida* spp. isolates was also evaluated, and it was observed that at pH 4 or 7, there was no alteration in the anti-biofilm effect of the CFSs against *C. albicans* (Fig. 1).

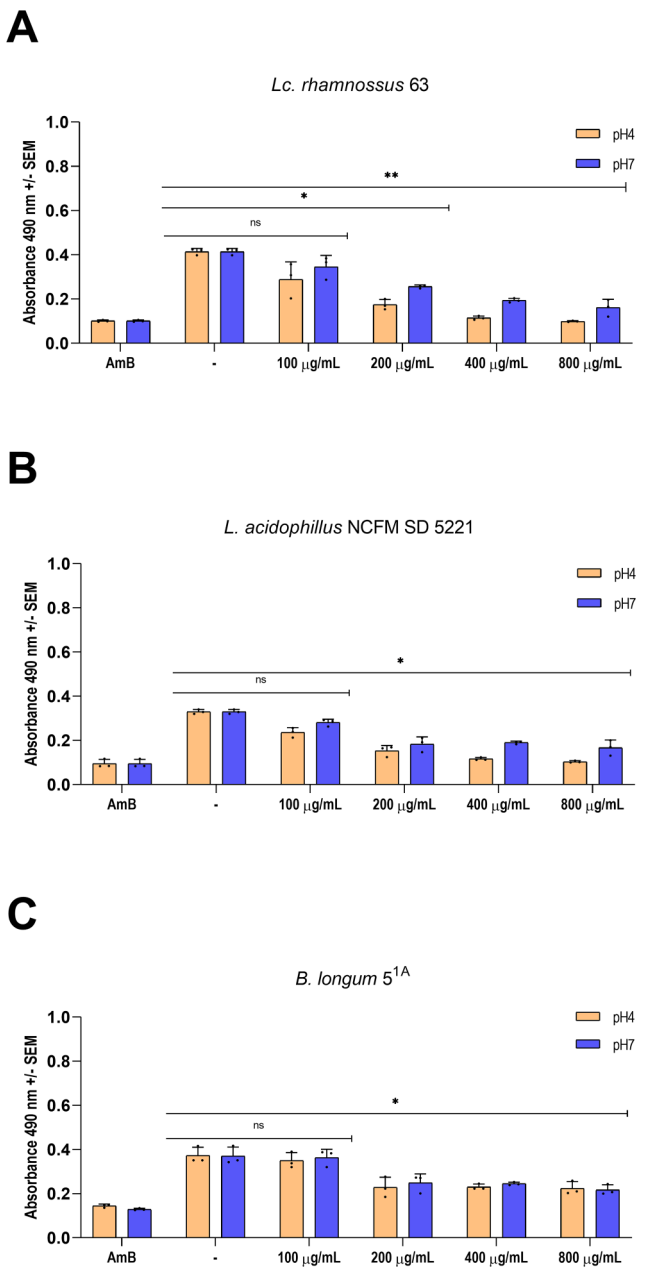

Figure 1. Effect of CFSs (CFSs) obtained from probiotic strains on biofilm-forming capacity of *Candida albicans* SC5314 at different pH conditions. (A) *Lacticaseibacillus rhamnosus* DM-UFMG 63, (B) *Lactobacillus acidophilus* NCFM SD 5221, and (C) *Bifidobacterium longum* 5^{1A}. Biofilm-forming capacity was quantified by measuring absorbance at 490 nm using the XTT reduction assay, after treatment with increasing concentrations of CFSs (∼100 μg/ml–∼800 μg/ml), adjusted to pH 4 or pH 7. Amphotericin B (AmB) was included as a positive control, and untreated wells (–) as growth controls. Data are expressed as the mean ± standard error of the mean (SEM). $*P < .05$; $**P < .01$; ns, not significant.

CFSs derived from different probiotics inhibit biofilm formation in *Candida* spp. isolates from patients with RVVC

The effect of the CFSs derived from *Lc. rhamnosus* 63, *L. acidophilus* NCFM SD 5221, and *B. longum* 51A on *Candida* spp. biofilm formation was evaluated using the XTT reduction assay. The CFS from *Lc. rhamnosus* 63 exerted a robust inhibitory effect compared to untreated controls, significantly reducing biofilm viability in both *C. albicans* (95% Confidence Intervalo (CI): −0.2061 to −0.1417; $P < .01$) and *C. lusitaniae* (95% CI: −0.4040 to −0.2400;

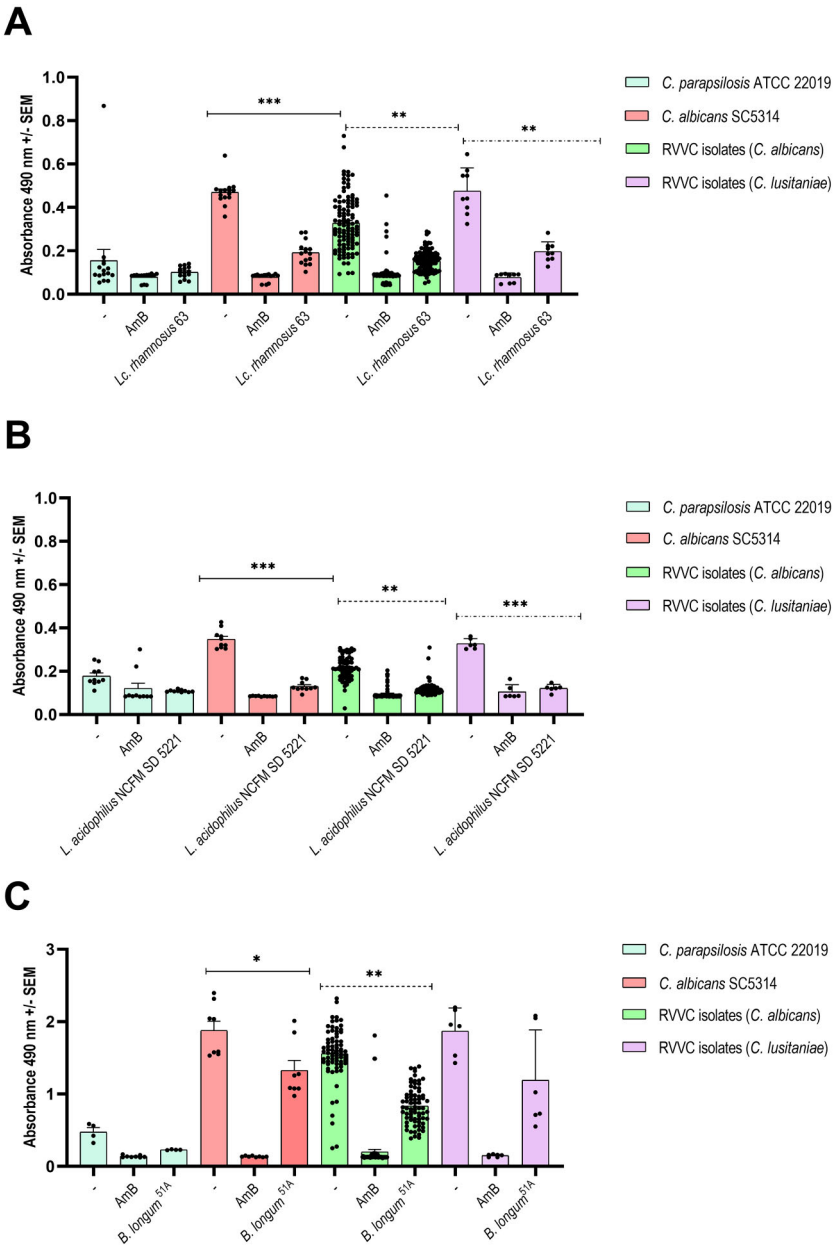

Figure 2. Effect of cell-free supernatants (CFSs) on biofilm-forming capacity of RVVC *Candida* spp. isolates. CFS derived from: (A) *Lacticaseibacillus rhamnosus* DM-UFMG 6363, (B) *Lactobacillus acidophilus* NCFM SD 5221, and (C) *Bifidobacterium longum* 5^{1A}. Biofilm-forming capacity was quantified by measuring absorbance at 490 nm using the XTT reduction assay. A total of 40 *Candida* spp. isolates (37 *Candida albicans*) and 3 *Clavispora lusitaniae* were evaluated. Amphotericin B (AmB) was included as a positive control, and untreated wells (–) were used as growth controls. Data are expressed as mean ± standard error of the mean (SEM). $*P < .05$; $**P < .01$; $***P < .001$.

$P < .01$). Similarly, the *L. acidophilus* NCFM SD 5221 CFS induced a significant decrease in biofilm-associated metabolic activity for both *C. albicans* (95% CI: −0.2540 to −0.1639; $P < .01$) and *C. lusitaniae* (95% CI: −0.2664 to −0.1443; $P < .01$) (Fig. 2A, B). In contrast, the CFS from *B. longum* 51A exhibited a more limited antagonistic profile, showing a significant but smaller inhibitory effect exclusively against *C. albicans* isolates (95% CI: −1.7510 to −0.9040; $P < .01$), with no significant activity observed against *C. lusitaniae* (Fig. 2C).

CFSs derived from *Lc. rhamnosus* 63 and *L. acidophilus* NCFM SD 5221 induce a decrease in the expression of genes associated with the adherence phase during biofilm formation

Considering the lesser anti-biofilm effect observed with the postbiotic derived from *B. longum* 5^{1A}, subsequent gene expression

analyses in *Candida* spp. isolates from patients with RVVC were performed in the presence or absence of CFSs derived from *Lc. rhamnosus* 63 and *L. acidophilus* NCFM SD 5221. Two hours after biofilm formation, a significant decrease in *ALS3* gene expression was observed regardless of the CFS evaluated in all the isolates analyzed. Likewise, a decrease in *HWP1* expression was observed in *C. albicans* isolates in the presence of the CFS derived from *Lc. rhamnosus* 63. With respect to the *BCR1* gene, no differences in its expression were observed between the treatments evaluated (Fig. 3A–C).

CFSs derived from *Lc. rhamnosus* 63 and *L. acidophilus* NCFM SD 5221 induce a decrease in the expression of genes associated with the maturation phase during *Candida* biofilm formation

During the biofilm maturation phase (24 h), the CFSs from both probiotics induced a significant decrease in the expression of the *TEC1* gene in both *C. albicans* and *C. lusitaniae* isolates. For the *EFG1* gene, only the CFS from *Lc. rhamnosus* 63 significantly decreased its expression in *C. albicans* isolates (Fig. 4A–B).

CFSs from *Lc. rhamnosus* 63 and *L. acidophilus* NCFM SD 5221 altered the expression of genes associated with the dispersion phase during *Candida* biofilm formation

Expression analyses of the *NRG1* and *UME6* genes, associated with the dispersion phase during *Candida* spp. biofilm formation, were evaluated at 48 h. In the presence of the CFSs derived from *Lc. rhamnosus* 63 and *L. acidophilus* NCFM SD 5221, an increase in *NRG1* gene expression was observed in both *C. albicans* and *C. lusitaniae* isolates. Interestingly, the *L. acidophilus* NCFM SD 5221 CFS (Probiac®) induced the highest expression of this gene in all isolates (Fig. 5A).

Finally, regarding the expression of the *UME6* gene, it was observed that only the CFSs derived from *Lc. rhamnosus* 63 induced a significant decrease in the gene in the *C. albicans* isolates (Fig. 5B).

Discussion

RVVC affects ∼ 10% of women with VVC. The first-line treatment for VVC/RVVC is azole antifungals[24] However, in some cases, fluconazole does not allow for complete resolution of the disease. Consequently, probiotics have recently been explored as adjunct or alternative therapies[25] Nevertheless, it has been reported that the use of probiotics presents some disadvantages, mainly associated with the intrinsic risk they may pose to the host, especially in immunocompromised populations.[26,27] This has shifted attention toward postbiotics—defined as bioactive compounds derived from probiotics[28]

CFSs were harvested after 48 h of incubation in MRS broth. This specific time point was selected based on literature indicating that bioactive metabolite production and accumulation by *Lacticaseibacillus rhamnosus*, *Lactobacillus acidophilus*, and *Bifidobacterium longum* peak during the late exponential and stationary growth phases, with robust metabolomic profiles already well-established after 24 h.[29,30] Furthermore, growth curve kinetics performed in this study confirmed that all evaluated probiotic strains reached cell densities in the order of 10^9 CFU/ml at 48 h, a yield comparable to commercial probiotic formulations[31] Thus, this incubation period ensured standardized conditions for both bacterial biomass and metabolite accumulation across all strains.

Following harvest, a spectrophotometric estimation of the protein-associated components in the CFSs was conducted at 280 nm (A_{280}). The analysis revealed highly comparable protein profiles among the secretomes, yielding estimated concentrations of 1,70 μg/ml for *Lc. rhamnosus* 63, 1580 μg/ml for *L. acidophilus* NCFM SD 5221, and 1640 μg/ml for *B. longum* 51A. However, these values represent relative estimations of total protein content rather than absolute quantifications of purified proteins or specific bioactive fractions. Consequently, the precise temporal characterization of metabolite secretion throughout the distinct growth phases was outside the scope of this preliminary report and remains an important avenue for future mechanistic investigations.

Moreover, given that the low pH induced by lactic acid bacteria has been reported as one of the main protective factors against the proliferation of *Candida* spp. in the vaginal microenvironment[32] this study first evaluated the effect of pH on the efficacy of CFSs derived from *Lc. rhamnosus* 63, *B. longum* 5^{1A}, and *L. acidophilus* NCFM SD 5221 (Probiac®) against the biofilm formation of *Candida albicans* SC5314. However, the CFSs evaluated at both pH 4 and pH 7 showed significant anti-biofilm activity. Thus, contrary to y Boahen *et al.* [33] our findings suggest that the pH associated with the organic acids produced by the evaluated probiotics is not the mechanism related to the inhibition of *Candida* spp. biofilm. This suggests that, at least for the CFSs evaluated in this study, factors other than pH may be exerting a significant anti-biofilm effect. These findings are similar to those obtained in another study that evaluated the effect of pH on CFSs derived from various probiotics against *C. parapsilosis* ATCC 22019, and in which it was observed that the evaluated CFSs exhibited a similar inhibitory effect at both acidic and neutral pH[20] Likewise, a recently study evaluated the CFSs from *Lc. rhamnosus* ATCC 53103, *Lactiplantibacillus plantarum* ATCC 8014, and *L. acidophilus* ATCC 4356 against *C. albicans* SC5314 and six clinical isolates (comprising two strains each of *C. albicans*, *C. tropicalis*, and *C. parapsilosis*); in alignment with our observations, the authors reported that the *Lc. rhamnosus* CFS sustained its inhibitory capacity even at a neutralized pH (pH 7.0), strongly suggesting that bioactive metabolites other than organic acids contribute to the observed antifungal activity[34] This phenomenon is further supported by Garcia-Gamboa *et al.* [35] who demonstrated that CFSs derived from *L. plantarum* modulated the growth of *C. albicans* and *Candida kefyr*. Notably, and mirroring our own findings, a CFS concentration of 200 μg/ml was sufficient to significantly inhibit the growth of both *Candida* species, reinforcing the potent anti-candidal profile of these probiotic secretomes.

Interestingly, the CFSs derived from *Lc. rhamnosus* 63 and *L. acidophilus* NCFM SD 5221 (Probiac ®) showed a greater anti-biofilm effect than the CFS derived from *B. longum* 5^{1A}. This aligns with evidence that, unlike *B. longum*, both *Lc. rhamnosus* and *L. aci-*

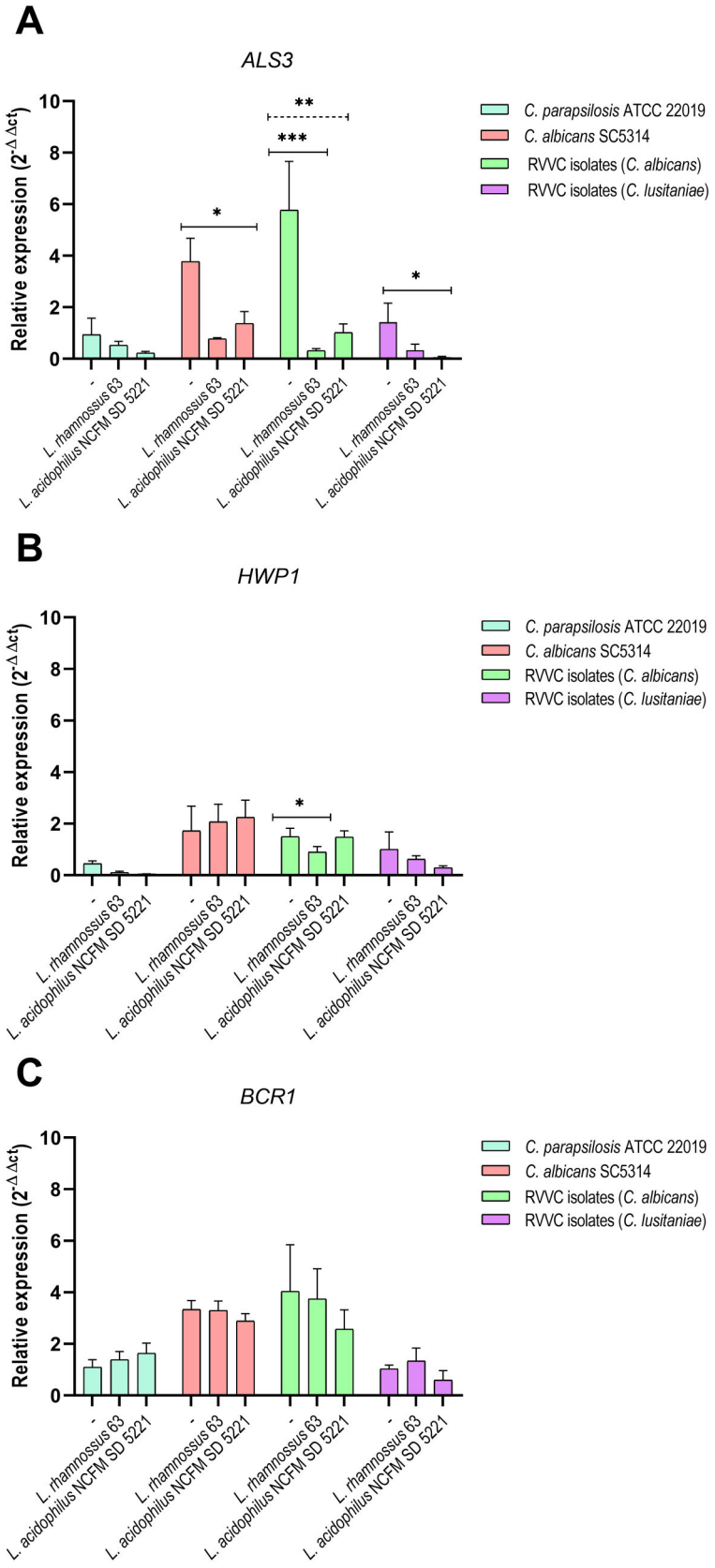

Figure 3. Effect of cell-free supernatants (CFSs) on the relative expression of adhesion phase biofilm-associated genes in RVVC *Candida* spp. isolates. (A) *ALS3*, (B) *HWP1*, and (C) *BCR1* genes. Gene expression was quantified by Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) and expressed as $2^{-\Delta\Delta Ct}$ following exposure to *Lacticaseibacillus rhamnosus* DM-UFMG 63 and *Lactobacillus acidophilus* NCFM SD 5221 CFSs. A total of 40 *Candida* spp. isolates (37 *Candida albicans*) and 3 *Clavispora lusitaniae* were evaluated. (–) Untreated control wells. Data are expressed as median and interquartile range (IQR). Adjusted *P*-values are indicated as follows: $*P < .05$; $**P < .01$; $***P < .001$.

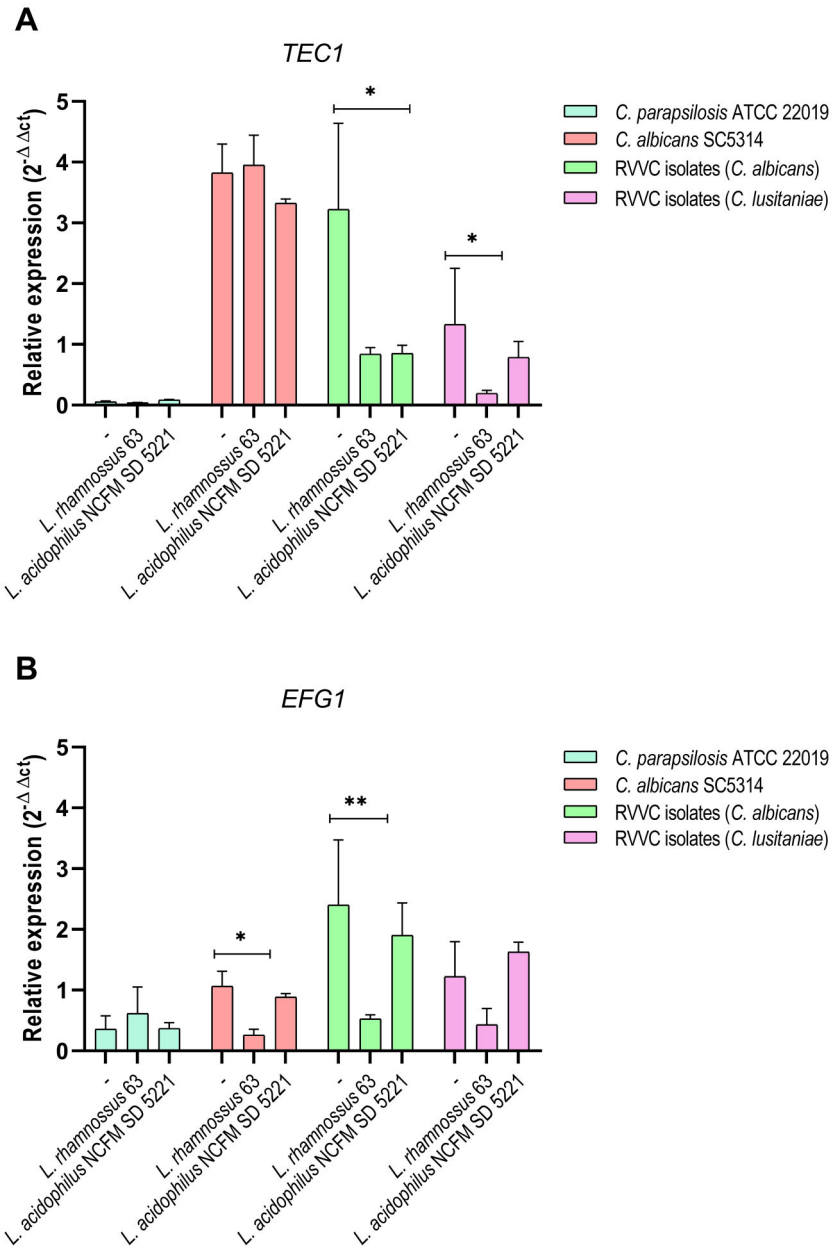

Figure 4. Effect of cell-free supernatants (CFSs) on relative expression of maturation-phase biofilm-associated genes in RVVC *Candida* spp. isolates. (A) *TEC1* and (B) *EFG1* genes. Gene expression was quantified by RT-qPCR and expressed as $2^{-\Delta\Delta Ct}$ following exposure to *Lacticaseibacillus rhamnosus* DM-UFMG 63 and *Lactobacillus acidophilus* NCFM SD 5221 CFSs. A total of 40 *Candida* spp. isolates (37 *Candida albicans*) and 3 *Clavispora lusitaniae* were evaluated. (–) untreated control wells. Data are expressed as median and interquartile range (IQR). Adjusted *P*s are indicated as follows: $*P < .05$; $**P < .01$.

dophilus are bacterial species with a great capacity to produce secondary metabolites such as biosurfactants and antimicrobial peptides, which can reduce microbial adhesion by altering the membrane structure, which can result in inadequate protein interactions and cell lysis, thus preventing pathogen biofilms from developing successfully.[36–39]

Candida biofilm development is mainly divided into adhesion, maturation, and dispersion.[40] To elucidate the possible molecular mechanisms related to the inhibition of biofilm formation in the presence of the evaluated CFSs, this study, for the first time, assessed the expression of genes related to biofilm formation at three different time points, considering the phases of biofilm development. Thus, 2 h after biofilm formation, the expression of genes related to the adhesion phase—*ALS3*, *HWP1*, and *BCR1*—was evaluated. These genes encode proteins essential for *Candida* adhesion, morphogenesis, and biofilm formation, and therefore play an important role in *Candida* pathogenesis. Regarding *ALS3* and *HWP1*, it was observed that in the presence of the CFSs from *Lc. rhamnosus* 63 and *L. acidophilus* NCFM SD 5221, these genes showed a significant decrease in their expression. These results are interesting, considering that the *ALS3* gene encodes an adhesion protein expressed in hyphae, which is important for the initial interaction between *Candida* spp. and the extracellular matrix, whether of epithelial cells or abiotic surfaces; therefore, the de-

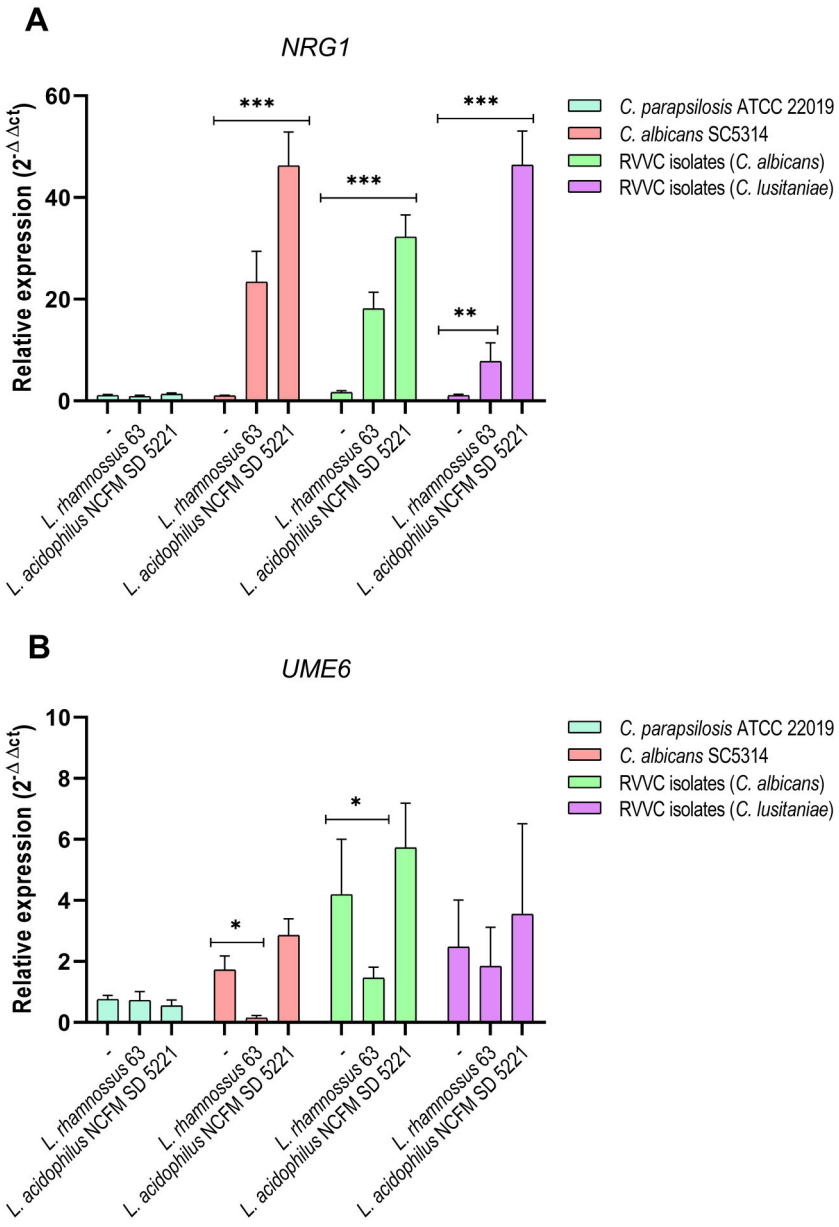

Figure 5. Effect of cell-free supernatants (CFSs) on relative expression of dispersion-phase biofilm-associated genes in *Candida* isolates. (A) *NRG1* and (B) *UME6* genes. Gene expression was quantified by RT-qPCR and expressed as $2^{-\Delta\Delta Ct}$ following exposure to *Lacticaseibacillus rhamnosus* DM-UFMG 63 and *Lactobacillus acidophilus* NCFM SD 5221 CFSs. A total of 40 *Candida* spp. isolates (37 *Candida albicans*) and 3 *Clavispora lusitaniae* were evaluated. (–) Untreated controls. Data are expressed as median and interquartile range (IQR). Adjusted *P*-values are indicated as follows: $*P < .05$; $**P < .01$; $***P < .001$.

crease in *ALS3* expression could be related not only to a decrease in adhesion capacity, but also to a decrease in the ability to capture essential microelements such as iron, since it has been reported that the Als3 protein also binds to ferritin of host cells.[41] Additionally, with respect to *HWP1*, this gene encodes a hyphal cell wall protein, which is essential for proper filamentation. In this regard, reduced *HWP1* expression likely contributes to the inhibition of biofilm formation in *Candida* observed in our study, since *Candida* hwp1Δ strains have been reported to exhibit structurally defective biofilms.[42] Furthermore, similar to our findings, a study by Wang *et al.* using the *C. albicans* ATCC 10231 strain determined the expression of *ALS3* and *HWP1* in the presence of CFSs derived from *L. crispatus* and showed a decrease in their gene expression.[43]

Additionally, 24 h after biofilm formation, the expression of genes related to the maturation phase was evaluated in the presence of CFSs, and a decrease in the expression of the *EFG1* and *TEC1* genes was observed. These genes are important transcription factors associated with the biofilm formation process, as they directly regulate the expression of genes such as *ALS3* and *HWP1*. Therefore, this finding is consistent with the decreased gene expression observed for *ALS3* and *HWP1* in the presence of CFSs derived from *Lc. rhamnosus* 63 and *L. acidophilus* NCFM SD 5221. From this, it could be inferred that the effect of CFSs is not isolated, but rather that they possibly affect the entire regulatory cascade of biofilm formation. Consistent with our findings, in a study by Poon *et al*., it was shown that both *EFG1* and *TEC1* were negatively

regulated in *C. albicans* isolates subjected to CFS treatment from *Lc. rhamnosus* and *L. plantarum* .[34]

Finally, 48 h after biofilm formation, the expression of the *UME6* and *NRG1* genes was evaluated in the presence of CFSs. These genes are related to the final phase of biofilm dispersion. The assays showed an increase in *NRG1* gene expression in the isolates in the presence of the CFSs; this finding is similar to that reported by Wang *et al*., who evaluated the expression of this gene in the presence of CFSs derived from *L. crispatus, L. gasseri*, and *L. jensenii*, and observed that, regardless of the CFS evaluated, *NRG1* expression was positively regulated.[43] These findings are important because *NRG1* has been reported as a negative regulator of genes such as *HWP1* and *ALS3*, which are essential for proper biofilm development. This could be relevant *in vivo*, since an increase in *NRG1* could significantly decrease the virulence capacity of *Candida*, as this gene favors the maintenance of the blastoconidia morphotype as the main morphological state of *Candida* .[44] Similarly, Li *et al.* [45] evaluated the anti-*Candida* activity of CFSs derived from *Lacticaseibacillus paracasei* CPU-Lps0708 against *C. albicans* ATCC 14053. In alignment with our observations, the authors demonstrated that the CFS upregulated the expression of the *NRG1* gene, resulting in an ∼ 35% inhibition of biofilm formation. These convergent lines of evidence strongly support the notion that a key regulatory mechanism mediated by *Lacticaseibacillus* species—such as *Lc. rhamnosus* and *Lc. paracasei*—involves the upregulation of *NRG1*, thereby restricting filamentation and subsequent biofilm assembly in *Candida* spp.

Additionally, a decrease in *UME6* expression was observed in the presence of the CFS derived from *Lc. rhamnosus* 63, which is relevant considering that this gene is necessary for *Candida* to maintain the hyphal morphotype stably. This finding is related to the findings of Poon *et al*., who observed that in the presence of CFS derived from *L. plantarum* ATCC 8014 (LP8014), *UME6* expression was downregulated in *C. albicans* SC5314[34]. Additionally, in another study where the effect of co-culture of *C. albicans* with *Lactobacillus paracasei* 28.4 was evaluated, a reduction in the expression of *UME6* was reported, which was associated with inadequate hyphal development and a higher survival rate in an *in vivo* model of *Caenorhabditis elegans*.[46]

Limitations of the study

Despite the relevance of the findings, this exploratory study has some limitations that should be acknowledged. First, CFSs demonstrated a robust anti-biofilm effect; their active components were not chemically or biochemically characterized. Therefore, while the observed effects are likely associated with metabolites such as antimicrobial peptides, biosurfactants, or other secreted compounds, the specific molecules and mechanisms involved remain to be elucidated. Second, biofilm inhibition was primarily assessed using the XTT reduction assay, which reflects metabolic activity but does not allow for direct discrimination between fungistatic, fungicidal, or purely metabolic effects. In addition, although many clinical isolates were evaluated, the small number of *Clavispora lusitaniae* isolates limits species-specific inferences, and these results should be interpreted as exploratory. Finally, the experiments were performed under *in vitro* conditions, which do not fully reproduce the complexity of the vaginal microenvironment, including host immune responses, epithelial interactions, and microbiota dynamics. Future studies combining biochemical characterization and extraction-based quantification methods of postbiotic components and *in vivo* infection models will be essential to confirm the translational potential of these findings.

In conclusion, the findings of this study demonstrate that the CFSs derived from *Lacticaseibacillus rhamnosus* DM-UFMG 63 and *Lactobacillus acidophilus* NCFM SD 5221 exhibit a significant antagonistic effect against biofilm formation in *Candida albicans* and *C. lusitaniae* isolates from patients with RVVC. Furthermore, contrary to reports by other authors, the anti-*Candida* effect of the evaluated CFSs was shown to result from the action of metabolites such as bacteriocins and biosurfactants, rather than from an acidic pH. These metabolites may directly influence the regulation of genes essential for biofilm formation.

Specifically, the evaluated CFSs significantly reduced the expression of the genes *ALS3, HWP1*, *EFG1, TEC1*, and *UME6,* while increasing the expression of the negative regulator *NRG1*. These interesting findings indicate that the use of CFSs could represent both a therapeutic and prophylactic approach for the management of RVVC. However, further studies are needed to specifically elucidate the molecular mechanisms by which CFSs derived from *Lc. rhamnosus* DM-UFMG 63 and *L. acidophilus* NCFM SD 5221 exert their anti-biofilm effects on *Candida*. This should also include the use of an *in vivo* infection model to evaluate the effects of these CFSs in humans.

Author contributions

Jeiser Marcelo Consuegra-Asprilla (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing—review & editing), Mariana González-Idarraga (Formal analysis, Investigation, Methodology, Writing—review & editing), Santiago Montoya-Carrascal (Formal analysis, Investigation, Methodology, Writing—review & editing), Dulce Xiomara Tello-Tobón (Formal analysis, Investigation, Methodology, Writing—review & editing), Andrés Abril Gómez (Formal analysis, Investigation, Methodology, Writing—review & editing), Flaviano Santos Martins (Formal analysis, Investigation, Validation, Visualization, Writing—review & editing), Ángel González (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing).

Declaration of interest

The authors declare that they have no financial conflict of interest.

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