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The effect of *Lactiplantibacillus plantarum* and *Lacticaseibacillus rhamnosus* cell-free supernatant on mature polymicrobial biofilm mass of *Candida albicans* and *Escherichia coli*

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Abstract Biofilm is one of the leading causes of prolonged infection known currently with higher treatment failure if caused by polymicrobial biofilm. *Lactobacillus* strain is known to produce antibiofilm substances that could disrupt biofilm by fungi and bacteria. This study aims to evaluate two probiotic strains, *L. plantarum* and *L. rhamnosus'* ability as single and multistrain to disrupt the biofilm of *Candida albicans* and *Escherichia coli*. This study employed a true experiment post test only control group design, using *C. albicans* and *E. coli* clinical isolate as subjects. The subjects in this study are divided into control and treatment groups. Treatment groups were subdivided into those receiving *L. plantarum, L. rhamnosus* and both probiotics for 24 h. Pathogens were incubated for 48 h in a 96-well microplate to reach a mature state before treatment groups were exposed to probiotics suspension for 24 h. Then, biomass was monitored using Crystal Violet (CV) staining and read using an ELISA reader to obtain the Optical Density (OD) values. The biofilm mass between treatments and control groups demonstrated a meaningful difference ($p < 0.05$), further post hoc analysis demonstrated multistrain cell-free supernatant of *L. plantarum and L. rhamnosus* demonstrated the highest antibiofilm between treatment groups in lowering biomass of single and dual-species biofilm *C. albicans* and *E. coli* (*C. albicans* = 0.215 ± 0.086; *E. coli* = 0.218 ± 0.099; dual-species biofilm = 0.123 ± 0.025). With the exposure of 24 h, the combination of *L. plantarum and L. rhamnosus* can decrease the biomass of single or dual-species biofilm formed by *C. albicans* and *E. coli*.

Keywords: Polymicrobial biofilm, *L. rhamnosus, L. plantarum, C. albicans, E. coli*

1. Introduction

The impact of biofilms on health is a serious issue because of their ability to escape host immune responses, display antibiotic resistance up to a thousand times greater than that of planktonic cells, and survive on both biotic and abiotic surfaces (Bjarnsholt, 2011). Pathogen-producing biofilms are able to form three-dimensional architectures with self-produced extracellular polymeric substances (EPSs) that provide protection against external threats and commonly comprise multiple microorganisms (Bjarnsholt, 2011). This ability leads to persistent infections, increased risk of treatment failure, high recurrence rates, increased healthcare costs, decreased quality of life, and even mortality (Azevedo et al., 2017). According to the National Institutes of Health, 80% of infections are biofilm-related, making them a significant threat (Azevedo et al., 2017). Urinary tract infections (UTIs) rank as the second most prevalent infection globally, with 60-80% of the pathogens reported to be capable of forming biofilms, and 39% of cases are polymicrobial-related infections. The predominant pathogen in UTIs is *Escherichia coli*, whereas species such as *Candida* sp. and *Enterococcus* sp. have been found to be more common in complicated UTIs than other species, such as *Proteus mirabilis, Klebsiella pneumoniae, Streptococcus epidermidis,* and *Staphylococcus aureus* (Gaston et al., 2021; Medina & Castillo-Pino, 2019).

Biofilm formation is suspected to be the cornerstone of UTI pathogenesis, making it challenging to eliminate (Ahmed et al., 2019; Delcaru et al., 2016; Lila et al., 2023; Maione et al., 2023; Rishpana & Kabbin, 2015). The presence of bacteria is a known risk factor for fungal infections, with polymicrobial fungal-bacterial infections resulting in increased morbidity and mortality rates (Pinto et al., 2021). Interkingdom interactions between *C. albicans* and *E. coli* are regarded as one of the clinically significant fungal-bacterial combinations. Previous studies have revealed a synergistic effect between the two; when cultured together, *C. albicans* affects *E. coli* antibiotic tolerance by producing a biofilm matrix (Eshima et al., 2022). Moreover *E. coli*

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increases *C. albicans* virulence factors, leading to increased biofilm formation (De Brucker et al., 2015). This synergistic effect was shown by a decreased survival rate in a *G. mellonella* model (Farrokhi et al., 2021).

Antibiotics remain the cornerstone of many infection treatments, including UTIs. However, the increasing trend of resistance to multiple antibiotics has led many researchers to explore alternative treatments, such as medicinal plant extracts, probiotics, or other substances (Ervianti et al., 2023; Murtiastutik et al., 2022). Among these alternatives, *Lactobacillus* strain have shown promising results against biofilm-producing pathogens. During sugar fermentation, lactobacilli synthesize antimicrobial compounds that can directly combat bacteria or fungi or interfere with vital components of the microorganisms, including proteins, cell membranes, or metabolic pathways (Hill et al., 2014; Vagios et al., 2020; Vazquez-Munoz & Dongari-Bagtzoglou, 2021; Zeise et al., 2021). *Lactobacilli* possess various compounds known to be antibacterial and antifungal, including the most dominant ones: lactic acid, exopolysaccharides, bacteriocins or plantaricins, hydrogen peroxide, short-chain fatty acids (including butyrate), organic acids, and reuterin (Vazquez-Munoz & Dongari-Bagtzoglou, 2021; Zeise et al., 2021). *Lactiplantibacillus plantarum*, formerly recognized as *Lactobacillus plantarum*, and *Lacticaseibacillus rhamnosus*, previously identified as *Lactobacillus rhamnosus*, have shown efficacy against uropathogens, including both fungi and bacteria (Vagios et al., 2020). This study aims to evaluate the ability of *L. plantarum* and *L. rhamnosus*, both as single- and multistrain treatments, to disrupt polymicrobial biofilms of *Candida albicans* and *Escherichia coli*.

2. Materials and methods

2.1. Organism and isolate preparation

This experiment was conducted at the Medical Laboratory Sudharma and the Institute of Tropical Disease (ITD), Universitas Airlangga, Surabaya, East Java, Indonesia. The pathogen models used in this study included clinical isolates of *Candida albicans* and *Escherichia coli* obtained from the Clinical Microbiology Laboratory at RSUD Dr. Soetomo, Surabaya, Indonesia. The two probiotic strains used in this study, *L. plantarum* and *L. rhamnosus*, were obtained from Universitas Surabaya, Indonesia. Before being prepared into suspensions, the isolates were recultured in selected growth media. *C. albicans* was cultured on Sabouraud dextrose agar (Oxoid, UK), and *E. coli* was cultured on MacConkey agar (Oxoid, UK) for 24 hours at 37 °C. Subsequently, 3-4 colonies of *C. albicans* and *E. coli* were collected and suspended in 5 mL of Tryptic Soy Broth (Oxoid, UK) each and then incubated for another 18 hours at 37 °C before being standardized to 0.5 McFarland (Stepanović et al., 2007).

2.2. Probiotic Cell-Free Supernatant Preparation

To retrieve the cell-free supernatant (CFS), the bacterial colonies of both probiotic strains were first transferred into 20 mL of De Man Rogosa and Sharpe (MRS) broth (Himedia, India) and incubated anaerobically at 37 °C for 48 hours to establish active cultures. Following incubation, the cultures were adjusted to a concentration of 1×10^8 colony-forming units per milliliter, subjected to centrifugation at 3,000 rpm at 4 °C for 15 minutes, and filtered through a 0.22 µm membrane filter (Millex-GV Filter, Germany) to generate the CFS (Matsuda et al., 2018; Pertiwi et al., 2023).

2.3. Biofilm Cultivation

The prepared suspensions of *C. albicans* (20 µL) and *E. coli* (20 µL) were added to a 96-well microplate (Biologix, China, flat bottom, sterile) with TSB (130 µL) and 1% glucose (20 µL). For biofilm cultivation, each well was added in the following order: *C. albicans* suspension (20 µL) + TSB (130 µL) and 1% glucose (20 µL); *E. coli* (20 µL) + TSB (130 µL) and 1% glucose (20 µL); and a mixture of *C. albicans* and *E. coli* (40 µL) + TSB (130 µL) and 1% glucose (20 µL). The process was repeated, and a total of 72 wells were filled to separate the control group from the treatment groups. Under static conditions, the microplate was incubated at 37 °C for 48 hours to reach the mature biofilm state.

2.4. Probiotic effect of the cell-free supernatant on biofilms

After 48 hours of incubation, the same microplate was treated with single-strain and multistrain CFS probiotics: *L. plantarum* (25 µL), *L. rhamnosus* (25 µL), and multistrain probiotics (50 µL). This treatment was repeated four times for the groups treated with single-strain CFS probiotics and eight times for the multistrain probiotics, resulting in a total of 36 wells. The microplate was then covered and incubated again for 24 hours at 37 °C under static conditions.

2.5. Crystal Violet Assays

After the incubation was complete, to remove planktonic cells, the microplate was rinsed three times with phosphate-buffered saline (300 µL, pH 7.2, room temperature). The mixture was then saturated with methanol (150 µL) and dried overnight. Next, crystal violet (150 µL, 0.1% wt/vol) was pipetted into the wells to stain the adherent cells, which were incubated for 15 minutes. After the plates were washed with water, 95% ethanol (150 µL) was added to fix the adherent cells, and the absorbance at 595 nm was measured by an ELISA reader to obtain the optical density (OD).

2.6. Biofilm mass analysis

The overall biofilm mass produced by the control groups was obtained through crystal violet staining and measured via an ELISA to obtain the OD. The results were categorized into several categories (Stepanović et al., 2007):

$$\boldsymbol{OD_C = \bar{X}OD_{Negative} + 3SD_{Negative}} \quad (1)$$

$\boldsymbol{\bar{X}OD_{Negative}}$ = OD of the negative control group with no pathogens or probiotics, consisting only of growth media
$\boldsymbol{SD_{Negative}}$ = standard deviation generated by the same group

The results were interpreted via the following categories:
1. OD ≤ ODc = Nonbiofilm Forming (0)
2. OD ≤ 2 × ODc = Weak biofilm forming (+)
3. 2 × ODc < OD < 4 × ODc = Moderate biofilm forming (++)
4. OD ≥ 4 × ODc = High biofilm forming (+++)

2.7. Antibiofilm activity of probiotics

The evaluation of antibiofilm activity produced by the CFS probiotics was presented as a percentage (%) and determined via the following formula (Pertiwi et al., 2023):

$$\boldsymbol{Antibiofilm}\ \% = \frac{(\bar{x}\boldsymbol{OD\ control} - \bar{x}\boldsymbol{OD\ treatment})}{\bar{x}\boldsymbol{OD\ control}} \times \mathbf{100} \quad (2)$$

$\boldsymbol{\bar{x}OD\ control}$ = mean optical density of the control group
$\boldsymbol{\bar{x}OD\ treatment}$ = mean optical density of the treatment groups

The calculated antibiofilm activity was categorized into three groups: high antibiofilm activity (> 50%), moderate antibiofilm activity (25%-50%), and minimal antibiofilm activity (< 25%).

2.8. Statistical analysis

The results are presented as the mean ± standard deviation (SD) for the overall biofilm mass and percentages (%) for antibiofilm activity. The OD values, which represent the results of the crystal violet assay, were analyzed statistically via SPSS (version 26.0) to identify significant differences between groups (control and treatment groups). Homogeneity of variances and normality tests were performed prior to statistical analysis, with $p > 0.05$ indicating that the data were normally distributed and homogeneous. Nonparametric analysis with the Kruskal-Wallis test was employed to analyze statistically significant differences between groups, followed by a post hoc test (Mann-Whitney test) to assess further significant differences between treatment groups, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Biofilm Formation

In this study, single- and dual-species fungal-bacterial biofilms (*C. albicans* and *E. coli*) were cultivated for 72 hours at 37 °C and labeled as the control groups, with the OD values presented as the means ± standard deviations (Table 1). Before the ability of the microorganisms to produce biofilms was categorized, a cutoff OD value was determined by calculating the average OD of the negative control and adding it to three times the standard deviation of the negative control. On the basis of these values, each microorganism's ability to produce biofilms can be categorized as non-biofilm-forming, weak biofilm-forming, moderate biofilm-forming, or high biofilm-forming. Overall, all the microorganisms in this study produced biofilms at levels two times greater than the cutoff value (0.210). *C. albicans* was categorized as a moderate biofilm with an average OD three times greater than the cutoff value but less than four times greater than the cutoff value. In contrast, *E. coli* and the dual-species biofilms were grouped as high biofilm formers, producing biofilms four times greater than the cutoff OD value (Table 1).

Table 1 Optical density biofilm of the control group.

Species	Label	Mean ± SD	Category
C. albicans Clinical Isolate	Control Group	0.674 ± 0.364	MBF
E. coli Clinical Isolate	Control Group	0.944 ± 0.470	HBF
C. albicans + *E. coli*	Control Group	1.125 ± 0.693	HBF
Negative control	TSB	0.117 ± 0.030	-

Notes: MBF = moderate biofilm formation, HBF = high biofilm formation.

3.2. Effects of L. plantarum and L. rhamnosus Cell-Free Supernatants on Mature Biofilms

There were noticeable differences in the overall OD between the control and treatment groups (Figure 1). Nonparametric analysis via the Kruskall-Wallis test on the species groups (compared with the control and treatment groups) yielded a p-value of 0.006 for *C. albicans* and *E. coli* and 0.001 for the dual-species biofilms, indicating significant differences (Table 2). A further Mann-Whitney post hoc test was conducted on each species group. For single-species biofilms, only the groups treated with multistrain CFS probiotics (G10 and G11) presented statistically significant differences, with p = 0.000 for *C. albicans* and p = 0.001 for *E. coli*. Single-strain CFS probiotics were not considered statistically significant. There was also a significant difference between G3 and G12, where the dual-species biofilms exposed to multistrain CFS probiotics presented the lowest average OD (0.123) of all the groups, with p = 0.000, indicating a highly significant difference between the control and treatment groups.

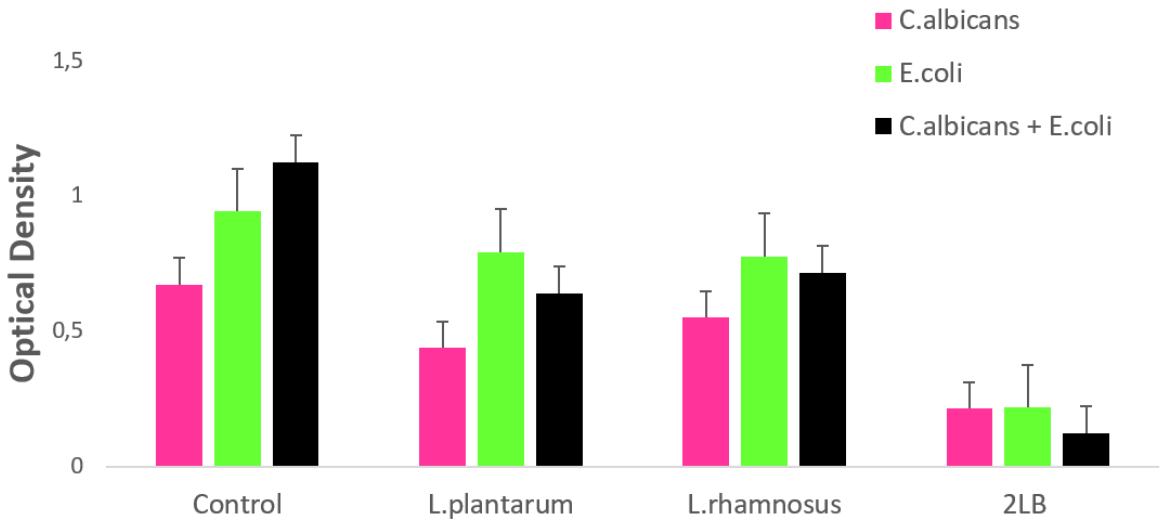

Figure 1 Histogram of optical density comparison between the control and treatment groups. The control group was incubated for 72 hours, whereas the treatment group was incubated for 48 hours before exposure to single-strain CFS probiotics (*L. plantarum* or *L. rhamnosus*) and 2LB (two *Lactobacillus*) or multistrain CFS probiotics (*L. plantarum* and *L. rhamnosus*) for 24 hours.

The antibiofilm activity of each probiotic was calculated by subtracting the mean OD of the control group from the mean OD of the treatment groups and then dividing the result by the mean OD of the control group. On the basis of this calculation, antibiofilm activity was categorized as minimal, moderate, or high. Overall, the multistrain CFS probiotics demonstrated high antibiofilm activity against *C. albicans* (68%), *E. coli* (79%), and dual-species biofilms (89%) (Table 2). *L. plantarum* exhibited moderate antibiofilm activity (36%) against *C. albicans*, while *L. rhamnosus* showed minimal antibiofilm activity (17%). For *E. coli*, both single-strain CFS probiotics (*L. plantarum* and *L. rhamnosus*) demonstrated minimal antibiofilm activity (19% and 17%, respectively). Additionally, both single-strain CFS probiotics demonstrated moderate antibiofilm activity against dual-species biofilms, although these differences were not statistically significant in the post hoc test. These results highlight the importance of multistrain probiotics in disrupting mature fungal-bacterial biofilms.

Table 2 Data comparison between the control and treatment groups.

Species	Treatment	Mean ± SD	Antibiofilm	Kruskal–Wallis	Mann–Whitney
C. albicans	Control Group (G1)	0.674 ± 0.364	-	0.006	
	CFS *Lp* (G4)	0.440 ± 0.242	36% (Moderate)		0.461
	CFS *Lr* (G7)	0.553 ± 0.226	17% (Minimal)		0.683
	CFS *Lp & Lr* (G10)	0.215 ± 0.086	68% (High)		0.000*
E. coli	Control Group (G2)	0.944 ± 0.470	-	0.006	
	CFS *Lp* (G5)	0.792 ± 0.620	19% (Minimal)		0.808
	CFS *Lr* (G8)	0.776 ± 0.283	17% (Minimal)		0.570
	CFS *Lp & Lr* (G11)	0.218 ± 0.099	79% (High)		0.001*
C. albicans+ E. coli	Control Group (G3)	1.125 ± 0.693	-	0.001	
	CFS *Lp* (G6)	0.640 ± 0.222	43% (Moderate)		0.283
	CFS *Lr* (G9)	0.718 ± 0.194	36% (Moderate)		0.283
	CFS *Lp & Lr* (G12)	0.123 ± 0.025	86% (High)		0.000*

Notes: G1 = *C. albicans* control group; G4-G7-G10 = *C. albicans* treatment group; G2 = *E. coli* control group; G5-G8-G11 = *E. coli* treatment group; G3 = *C. albicans* + *E. coli* control group; G6-G9-G12 = *C. albicans* + *E. coli* treatment group. *Lp (L. plantarum), Lr (L. rhamnosus),* CFS = cell-free supernatant.

4. Discussion

An increase in biofilm mass often correlates with prolonged infection and treatment failure, which can lead to increased morbidity and mortality. The results of this study revealed that when *C. albicans* and *E. coli* were cultured together, the biofilm mass produced a greater OD (1.125 ± 0.693) than single-species biofilms did, indicating a synergistic relationship between the two. This finding is consistent with the findings of Farrokhi et al., who also reported an increase in the biomass of *C. albicans* and *E. coli* when cultured together. Similarly, Yang et al. reported upregulation of Agglutinin-like sequence 3 (ALS3) and Hyphal Cell Wall Protein 1 (HWP1) when *C. albicans* was cocultured with enterohemorrhagic *E. coli* (EHEC) (Farrokhi et al., 2021; Yang et al., 2016). ALS3 in *C. albicans* is related to biofilm formation, mediating attachment to biotic or abiotic surfaces, and HWP1 is a surface protein essential for maintaining biofilm architecture and integrity in *C. albicans* in vitro (Gulati & Nobile, 2016; Nobile et al., 2008). Both ALS3 and HWP1 are specifically expressed by the pseudohyphal and hyphal forms of *C. albicans*, which are its virulent forms, suggesting that *E. coli* induces the virulence factors of *C. albicans* for biofilm formation (Liu & Filler, 2011).

This study aimed to evaluate the effects of the cell-free supernatant (CFSs) of *L. plantarum* and *L. rhamnosus*, which are both single-strain and multistrain probiotics, on the disruption of mature polymicrobial biofilms of *C. albicans* and *E.coli*. The results revealed a significant reduction in optical density ($p < 0.05$) in the treatment groups exposed to the single- and multistrain CFS probiotics for 24 hours compared with the control group that did not receive any treatment. Crystal violet, which is commonly used for biofilm staining, was used to represent the number of viable cells, with the remaining dye measured by an ELISA reader (Gulati et al., 2018). The group treated with multistrain CFS probiotics (*L. plantarum* and *L. rhamnosus*) presented the lowest average optical density among all the groups: 0.123 ± 0.025 ($p = 0.000$) for the dual-species biofilm, 0.215 ± 0.086 ($p = 0.000$) for the *C. albicans* biofilm, and 0.218 ± 0.099 ($p = 0.001$) for the *E. coli* biofilm. Although the optical density also decreased in the single-strain CFS probiotic (*L. plantarum* or *L. rhamnosus*) groups, the differences were not statistically significant. Furthermore, the combination of *L. plantarum* and *L. rhamnosus* had greater antibiofilm activity (> 50%) than single-strain CFS probiotics. On the basis of this statistical analysis, it can be concluded that, compared with single--strain probiotics, multistrain probiotics significantly reduce the number of viable *C. albicans* and *E. coli* biofilms.

This finding aligns with the findings of Carvalho et al., who reported a significant reduction in viable preformed *E. coli* biofilms after 6 hours (69% and 61%) and 24 hours (70% and 76%) of exposure to *L. plantarum* and *L. rhamnosus CFS*, respectively, compared with the untreated group (Carvalho et al., 2021). Similarly, Song et al. (2019) demonstrated significant disruption of *E. coli* biofilms after 2 and 4 hours of exposure to microencapsulated *L. rhamnosus* (LGG) (Song et al., 2019). *Lactobacillus* sp., which are part of the lactic acid bacteria (LAB) group, produce compounds such as lactic acid and acetic acid through sugar fermentation, which act as antibiofilm agents and antimicrobial agents (Zeise et al., 2021). In addition, *Lactobacillus* sp. displays antagonistic relationships with fungal and bacterial pathogens through mechanisms involving bacteriocins, reuterin, biosurfactants, fatty acids, hydrogen peroxide (H_2O_2), plantaricin, and short-chain fatty acids (SCFAs) (Vagios et al., 2020; Zeise et al., 2021). Lactic acid, produced by *Lactobacillus*, acts as an antibacterial agent against *E. coli* by permeating and disrupting cell membranes, causing protein leakage and potentially inhibiting protein synthesis (Wang et al., 2015). Bacteriocins also disrupt cell membranes, whereas hydrogen peroxide exhibits bactericidal properties that directly kill *E. coli* (Vagios et al., 2020).

A study by Santos et al. revealed that among several tested *Lactobacillus strain*, *L. rhamnosus* significantly inhibited *C. albicans* biofilm growth and exhibited antiadhesion and antibiofilm activities through its biosurfactants (Itapary Dos Santos et al., 2019). The antagonistic interactions between *Lactobacillus* and *C. albicans* involve both direct and indirect antifungal activities. Direct antifungal activity is related to the production of active antimicrobial compounds, whereas indirect antifungal activity involves interactions between the host, *Lactobacillus*, and *C.albicans* (Vazquez-Munoz & Dongari-Bagtzoglou, 2021; Zeise et al., 2021). Biosurfactant antiadhesion and antibiofilm properties against *C. albicans*, through changes in electrical properties and hydrophobicity, inhibit *C. albicans* attachment to epithelial or abiotic surfaces (Zeise et al., 2021). Bacteriocins are also believed to disrupt cell membrane integrity, causing depolarization and essential ion leakage, whereas plantaricin increases reactive oxygen species (ROS), leading to cell apoptosis (Vazquez-Munoz & Dongari-Bagtzoglou, 2021). Organic acids, such as lactic acid, affect sugar fermentation in yeast, whereas benzoic acid inhibits glycolysis, leading to a decrease in ATP. Benzoic acid can also cause intracellular acidification, resulting in increased cell turgor, oxidative stress, and a decrease in molecules, including RNA (Vazquez-Munoz & Dongari-Bagtzoglou, 2021).

Research by Poon and Hui revealed that CFS from *L. plantarum* and *L. rhamnosus* significantly inhibited *C. albicans* biofilm growth. Compared with that of the untreated control, the expression of *C. albicans* biofilm-related genes (ALS3) was downregulated. The cell-free supernatant (CFS) of probiotics is a clear liquid consisting of growth media and antimicrobial metabolites harvested through filtering after at least 24–48 hours of incubation. This form has broader antimicrobial activity than pure extracts of probiotic antimicrobial compounds do (Mani-López et al., 2022). Among the CFS probiotics, lactic acid is predominant and acts as an antimicrobial agent against fungi and bacteria. On the basis of the results of the present study and previous findings, the combination of CFS from *L. plantarum* and *L. rhamnosus* works synergistically and has the potential to disrupt mature polymicrobial fungal-bacterial biofilms of *C. albicans* and *E. coli*.

The potential of probiotics in targeting fungal-bacterial interactions has yet to be widely studied. On the basis of this study, further research employing a broader range of evaluation times and assays to assess the three-dimensional effects of *lactobacilli* on *C. albicans* and *E. coli* is needed to provide more representative results.

5. Conclusions

The increased optical density of *C. albicans* and *E. coli* when cultured together demonstrated their synergistic relationship. Compared with those in the control groups, the average optical density in the treatment groups was lower, indicating that the number of viable cells in the treatment groups was lower than that in the control groups. The administration of multistrain CFS probiotics with 24-hour exposure significantly decreased the overall optical density of both single-species and dual-species biofilms. In conclusion, this study revealed the ability of *L. plantarum* and *L. rhamnosus* cell-free supernatants to disrupt mature polymicrobial biofilms of *C. albicans* and *E. coli* on abiotic surfaces.

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Ethical considerations

Ethical approval for this study was obtained from the Health Research Ethical Committee of the Faculty Medicine, Universitas Airlangga, Surabaya (No. 345/EC/KEPK/FKUA/2023).

Conflict of interest

The authors declare that they have no conflicts of interest.

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