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Original article

# Characterization of *Candida albicans* and *Staphylococcus aureus* polymicrobial biofilm on different surfaces

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ABSTRACT

*Background:* *Staphylococcus aureus* and *Candida albicans* have been co-isolated from biofilm-associated diseases such as denture stomatitis, periodontitis, and burn wound infections, as well as from medical devices. However, the polymicrobial biofilm of both microorganisms has not been fully characterized.
*Aims:* To characterize the polymicrobial biofilm of *C. albicans* and *S. aureus* in terms of microbial density, synergy, composition, structure, and stability against antimicrobials and chemical agents.
*Methods:* Crystal violet assay was used to measure the biofilm formation. Scanning electron microscopy and confocal microscopy were used to analyze the structure and chemical composition of the biofilms, respectively.
*Results:* Supplemented media with fetal bovine serum (FBS) decreased the biofilm formation of *S. aureus* and the polymicrobial biofilm. For *C. albicans*, depending on the culture media, the addition of glucose or FBS had a positive effect in biofilm formation. FBS decreased the adhesion to polystyrene wells for both microorganisms. Supplementing the media with glucose and FBS enhanced the growth of *C. albicans* and *S. aureus*, respectively. It seems that *C. albicans* contributes the most to the adhesion process and to the general structure of the biofilms on all the surfaces tested, including a catheter model. Interestingly, *S. aureus* showed a great adhesion capacity to the surface of *C. albicans* in the biofilms. Proteins and β-1,6-linked polysaccharides seem to be the most important molecules in the polymicrobial biofilm.
*Conclusions:* The polymicrobial biofilm had a complex structure, with *C. albicans* serving as a scaffold where *S. aureus* adheres, preferentially to the hyphal form of the fungus. Detection of polymicrobial infections and characterization of biofilms will be necessary in the future to provide a better treatment.



## Caracterización de la biopelícula polimicrobiana de *Candida albicans* y *Staphylococcus aureus* sobre diferentes superficies

*Palabras clave:*
*Candida albicans*
*Staphylococcus aureus*
Infecciones polimicrobianas
Biopelículas

RESUMEN

*Antecedentes:* *Staphylococcus aureus* y *Candida albicans* son aislados conjuntamente de infecciones asociadas a la formación de biopelículas, tales como periodontitis, estomatitis e infecciones provenientes de quemaduras, así como en dispositivos médicos. Sin embargo, la biopelícula formada por ambos microorganismos no ha sido completamente caracterizada.

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*Objetivos:* Caracterizar la biopelícula de *C. albicans* y *S. aureus* en cuanto a densidad microbiana, sinergismo, composición, estructura y estabilidad frente a agentes químicos y antimicrobianos.
*Métodos:* El análisis de la formación de biopelícula se realizó mediante el ensayo de cristal violeta. Se analizó la composición química y la estructura de las biopelículas mediante microscopio confocal y microscopio electrónico de barrido, respectivamente.
*Resultados:* La adición al medio de suero bovino fetal (SBF) redujo la biopelícula mono- y polimicrobiana de *S. aureus*. En *C. albicans*, con la adición de glucosa o SBF, se incrementó la formación de biopelícula. La adhesión de los microorganismos a las placas de poliestireno se redujo en presencia de SBF. La suplementación del medio con glucosa y SBF favoreció la proliferación de *C. albicans* y *S. aureus*, respectivamente. *C. albicans* mostró una mejor adhesión y contribuyó más a la densidad total de la biopelícula en diferentes superficies probadas, incluyendo un modelo de catéter. De manera interesante, *S. aureus* mostró una mejor adhesión a la superficie de *C. albicans* en la biopelícula. Las proteínas y los polisacáridos con enlaces β-1,6 parecen ser las moléculas más abundantes en la biopelícula.
*Conclusiones:* La biopelícula formada por ambas especies resultó ser más compleja, con *C. albicans* formando una superficie sobre la que *S. aureus* se adhiere, preferentemente a la forma miceliar de la levadura. El estudio de las biopelículas asociadas a infecciones polimicrobianas será necesario en el futuro con el fin de proporcionar un mejor tratamiento.


*Candida albicans* is the most common fungal species in the human microbiota colonizing the oral cavity, skin, and gastrointestinal and reproductive tract of healthy individuals. Changes in the local environment, host immunity, and the microbiota of each person may induce the proliferation of *C. albicans* and a subsequent infection that may turn from a superficial skin or mucosal infection into a candidemia,[17] a bloodstream infection with high mortality rates.[19,30] It has been shown that *C. albicans* is the third most common pathogen from intravascular catheter-associated infections and the fourth most common microorganism isolated from nosocomial bloodstream infections.[6,32] Also, it is the most frequent fungal species isolated from medical devices such as catheters, prostheses, implants, pacemakers, dental materials, etc.[14] It has been suggested that the ability to colonize and infect these surfaces is related to its capacity to form biofilms.[7,8]

In a similar way, *Staphylococcus aureus* is a commensal bacterium found on the skin and mucous membranes of healthy individuals. *S. aureus* colonizes the nasal cavity of around 30% of the human population.[15,31] However, this microorganism is also considered a human pathogen and causes community-acquired and hospital-acquired infections, which in some cases are difficult to treat due to the presence of MRSA (methicillin-resistant *S. aureus*).[27] *S. aureus* causes a wide range of infections, from mild skin and soft-tissue infections to bacteremia, endocarditis, osteomyelitis, etc.,[28] and has also the capacity to adhere to medical devices, resulting in biofilm formation and infection.[16,18]

Polymicrobial infections have been associated with higher mortality rates when compared with monomicrobial infections.[9,20,25] In this regard, *S. aureus* and *C. albicans* have been co-isolated from biofilm-associated diseases.[1] For example, it has been reported that 27% of nosocomial bloodstream infections caused by *C. albicans* were polymicrobial, with *S. aureus* as the third most common pathogen isolated along with the fungus.[13] Furthermore, the lethality of *S. aureus* in an intraperitoneal infection model in mice was much higher in a co-infection with *C. albicans*.[3] For this reason, the relevance of polymicrobial infections has been recognized as a major problem.[2] The aim of this work was to characterize the polymicrobial biofilm of *C. albicans* and *S. aureus* on different surfaces, including a catheter model. We also analyzed the stability and composition of the biofilm using destabilizing chemical compounds and antimicrobials.

## Materials and methods

### *Growth conditions of* S. aureus *and* C. albicans

*S. aureus* ATCC 29213 was seeded on brain heart infusion (BHI) agar plates and incubated at 37 °C for 24 h. Then, a colony was inoculated into BHI broth and incubated at 37 °C with shaking at 50 rpm for 24 h. In order to know the bacterial colony forming units (CFU), we generated a growth curve correlating the optical density of the cultures at 600 nm with CFU on BHI agar plates. *C. albicans* ATCC 10231 was seeded on Sabouraud dextrose (SD) agar plates, and incubated at 37 °C for 24 h. Then, one colony was inoculated into SD broth at 30 °C with shaking at 50 rpm for 24 h. The number of yeast cells was obtained by means of the trypan blue exclusion test using a hemocytometer.

### *Mono- and polymicrobial biofilm formation with* C. albicans *and* S. aureus

To standardize the optimal concentration of *C. albicans* and *S. aureus* that yield a mono- or polymicrobial biofilm in 96-well cell culture plates (Costar, NY, USA), we used *S. aureus* in concentrations of $1 \times 10^7$ CFU, $1 \times 10^8$ CFU, and $1 \times 10^9$ CFU per well; the concentrations for *C. albicans* were $1 \times 10^6$ CFU and $1 \times 10^7$ CFU per well. A final volume of 200 μl of RPMI-1640 medium (Gibco, NY, USA) per well was used, with pH 7 adjusted with MOPS (3-(N-morpholine)propanesulfonic acid, Sigma, Saint Louis, USA). Microplate wells with a mono- or polymicrobial suspension were incubated at 37 °C in a humidified chamber for 24 h. Then, the excess of cells was removed and the plate was washed three times by submersion in a recipient containing tap water. In order to stain the cells, we added 125 μl of a 0.4% solution of crystal violet per well, and the plate was incubated at room temperature for 15 min. Afterwards, the excess of colorant was discarded, the plate was rinsed in tap water three times by submersion, and the excess of liquid was removed using paper towels. At this time, it was possible to photograph the plate for a qualitative analysis. Finally, in order to quantify the biofilm formed, we added 125 μl of a 30% solution of acetic acid per well, and the content of each well was transferred after 15 min of incubation at room temperature to a new microplate to assess the absorbance at 595 nm in a microplate reader. The absorbance value of the negative control containing

only RPMI-1640 medium was subtracted from that obtained for each well. In order to compare biofilm formation in different media, Dulbecco's modified eagle's medium (DMEM) was used as DMEM low glucose concentration (Gibco), and DMEM high glucose concentration (Gibco). RPMI-1640 containing 0.2% glucose (low) was adjusted to 2% glucose (high).

*Adherence and growth kinetics of* S. aureus *and* C. albicans

Adhesion experiments testing *S. aureus* ($1 \times 10^8$ CFU) or *C. albicans* ($1 \times 10^6$ CFU) were carried out in 12-well plates (Costar) containing the different culture media with or without fetal bovine serum (FBS). After 90 min of incubation at 37 °C, the cell suspension was discarded and the adhered cells were washed twice with phosphate-buffered saline (PBS). The attached cells were detached with a cell scraper (CellTreat, Pepperell, USA) and collected in 1 ml of PBS. The suspensions of cells were diluted and plated on BHI agar (*S. aureus*) or SD agar (*C. albicans*) in order to count CFU. For the growth kinetics analysis a colony of *S. aureus* was picked up and diluted in 1 ml of RPMI-1640. One hundred microliters of the suspension were transferred to 900 μl of RPMI-1640 supplemented or not with glucose and/or FBS. The resulting suspensions were incubated with shaking at 37 °C. For *C. albicans*, a 100 μl suspension containing $1 \times 10^5$ cells was added to 900 μl of RPMI-1640 supplemented or not with glucose and/or FBS. The suspensions were incubated with shaking at 37 °C. The optical density was checked at 595 nm at different times.

*Biofilm inhibition and destabilization using chemical agents and antimicrobials*

In order to study the inhibition of biofilm formation we used proteinase K (Sigma) at 100 μg/ml, sodium (meta)periodate (Sigma) at 40 mM, or DNase I (Thermo Fisher Scientific, Waltham, USA) at 50 μg/ml in the following manner: together with a suspension of *S. aureus* ($1 \times 10^7$ cells) and/or *C. albicans* ($1 \times 10^6$ cells) in polystyrene 96-well microplates, or on pre-formed biofilms, after a washing step with PBS to remove the excess of cells, for three hours. In a different experiment, the polymicrobial biofilm destabilization was also analyzed adding vancomycin (Sigma) at 10, 100, and 1000 μg/ml, and/or amphotericin B (Thermo Fisher Scientific) at 0.25, 2.5, and 25 μg/ml for 24 h into the wells containing the pre-formed biofilms after a washing step with PBS. Biofilm formation and quantification was carried out as aforementioned in this section.

*Analysis of biofilms by confocal laser scanning microscopy*

Lab-Tek II system (Thermo Fisher Nunc, NY, USA) has been used for studying biofilm formation with confocal microscopy, as it consists of a slide with a removable chamber that keeps sterile conditions for cell culture.[22] Two hundred microliters of *S. aureus* ($1 \times 10^7$ cells) and/or *C. albicans* ($1 \times 10^6$ cells) were added into the wells of Lab-Tek chamber slides. Incubation for biofilm formation was carried out in a humidified chamber for 24 h at 37 °C. Non-adhered cells were removed and the slides were stained with FilmTracer FM 1–43 Green Biofilm Cell Stain, Ex/Em 472/580 nm (Thermo Fisher Scientific), FilmTracer SYPRO Ruby Biofilm Matrix stain, Ex/Em 450/610 nm (Thermo Fisher Scientific), wheat germ agglutinin, Alexa Fluor 488 conjugate, Ex/Em 495/519 nm (Thermo Fisher Scientific), or BOBO-3 Iodide, Ex/Em 570/602 nm (Thermo Fisher Scientific) following the manufacturer's instructions. The removable cells were discarded, and ProLongGold Antifade Mountant (Thermo Fisher Scientific) was added to the slide. Three images per treatment in triplicate were obtained using a confocal microscope (Zeiss, LSM700) with Zen Black software, selecting one as representative. This experiment was repeated twice.

*Analysis of biofilms by scanning electron microscopy (SEM)*

*S. aureus* ($1 \times 10^7$ cells) and/or *C. albicans* ($1 \times 10^6$ cells) were added to the wells of Lab-Tek humidified chamber slides, letting biofilm to be formed for 24 h at 37 °C. For the catheter model, a catheter section of around 1 cm in length was placed into a 1.5 ml tube containing 1 ml of RPMI-1640 medium with *S. aureus* ($1 \times 10^7$ cells) and/or *C. albicans* ($1 \times 10^6$ cells). The tubes were placed in a rotating mixer at 25 rpm and incubated for 24 h at 37 °C. Afterwards, the non-adhered cells were removed and the samples were fixed with 2.5% glutaraldehyde. The samples were then dehydrated with increasing concentration of ethanol (60%, 70%, 80%, 90%, 96%, and 100%), for 10 min with each concentration. Finally, the samples were dried at 37 °C/5% $CO_2$ for 24 h and coated with 10 Å of gold, using Denton Vacuum Desk II. At least three images per treatment in triplicate were obtained and analyzed under JEOL JSM 5900LV scanning electron microscope (JEOL, Akishima, Tokio, Japan), selecting one image as representative. This experiment was repeated twice.

*Statistical methods*

GraphPad Prism 8.0 was the software used to evaluate the quantitative data in this study. Tukey's multiple comparison test (two-way ANOVA) was used to analyze the differences in biofilm formation according to the media used, as well as the growth kinetics ($p \leq 0.05$). Tukey's multiple comparison test (one-way ANOVA) was used to evaluate the adhesion of the microorganisms in different culture conditions ($p \leq 0.05$). Dunnett's multiple comparison test (one-way ANOVA) was used to analyze biofilm inhibition and destabilization using chemical reagent and antimicrobials ($p \leq 0.05$).

## Results

*Biofilm formation by* S. aureus *and* C. albicans *in different culture media*

In order to study the association of *S. aureus* and *C. albicans* through biofilm formation, we analyzed the microbial density necessary to establish an *in vitro* polymicrobial biofilm in 96-well microplates, and the biofilm formed was analyzed with the crystal violet method. A CFU value of $1 \times 10^6$ per well for *C. albicans* was better than $1 \times 10^7$, whereas for *S. aureus* concentrations from $1 \times 10^7$ to $1 \times 10^9$ bacteria per well did not result in a significant change (data not shown). Then, the incubation in different culture media supplemented or not with FBS showed that the monomicrobial biofilm density was higher in *C. albicans* (inoculum of $1 \times 10^6$ CFU per well) than in *S. aureus* (inoculum of $1 \times 10^7$ CFU per well). The addition of FBS affected both the biofilm formed by *S. aureus* and the polymicrobial biofilm (Fig. 1A). In the case of *C. albicans*, biofilm formation was not altered by FBS, but glucose addition increased the biofilm density in DMEM. In RPMI, the presence of glucose and FBS increased considerably the biofilm density.

*Adherence and growth of* S. aureus *and* C. albicans

Crystal violet assay analyzes only the overall density of the biofilm, considering neither microbial viability nor microbial growth. Using the different media supplemented or not with dextrose and FBS we found that FBS highly promoted the proliferation of *S. aureus*, while bacterial growth was limited in RPMI or null in DMEM without FBS (Fig. 1B, Suppl. Fig. 1A). For *C. albicans*, the optimal growth was observed with the addition of dextrose, while

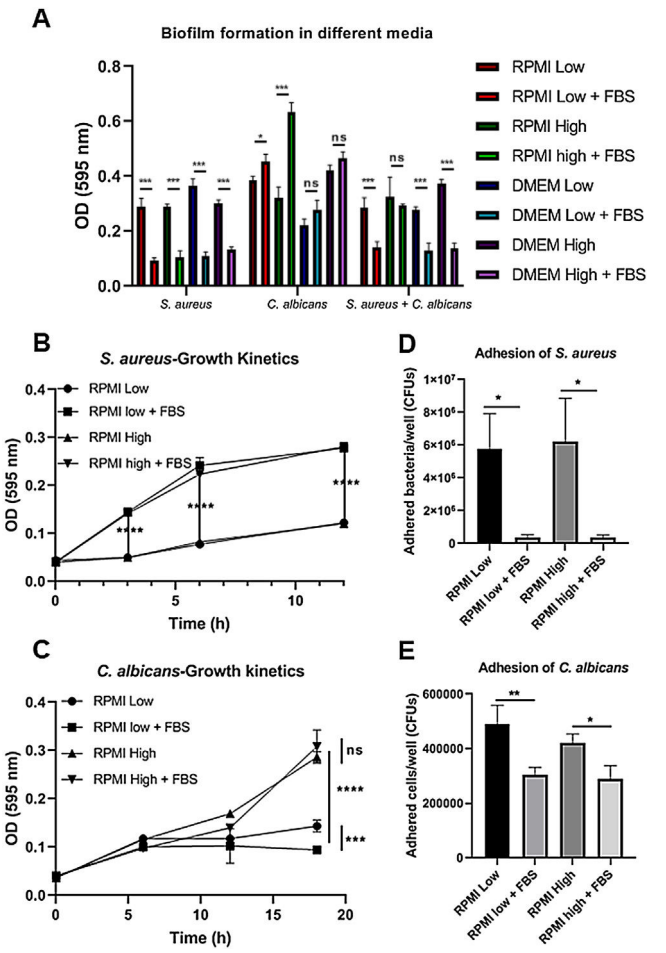


**Fig. 1.** Biofilm formation in different culture media. (A) Biofilm formation by *S. aureus* ($1 \times 10^7$ bacteria per well) and/or *C. albicans* ($1 \times 10^6$ cells per well) was carried out in 96-well plates using different media supplemented or not with FBS: RPMI low and high glucose concentrations, and DMEM low and high glucose concentrations. (B and C) Growth kinetics analysis for *S. aureus* and *C. albicans* in RPMI low and high glucose concentrations, supplemented or not with FBS. Two-way ANOVA with multiple comparisons (Tukey test) was used to compare among treatments ($p \leq 0.05$). (D and E) Adhesion of *S. aureus* or *C. albicans* in RPMI low and high glucose concentrations, supplemented or not with FBS, was evaluated on 12-well plates. The different media were compared by means of one-way ANOVA with multiple comparisons (Tukey test, $p \leq 0.05$). This experiment was repeated twice in triplicate.

the addition of FBS increased slightly that growth at 37 °C (Fig. 1C, Suppl. Fig. 1C). Analyzing the *in vitro* adhesion in 12-well plates, we observed that the addition of FBS affected significantly the adhesion of both microorganisms (Fig. 1D and E, Suppl. Fig. 1B and 1D).

### *Analysis of biofilm formation on different surfaces with scanning electron microscopy*

Concerning biofilm formation in Nunc Lab-Tek II chamber slides, we observed that *C. albicans* formed a more structured biofilm if compared with that of *S. aureus* (Fig. 2A, B). The polymicrobial biofilm was more complex, with *C. albicans* forming an outwards scaffold with hyphae and pseudohyphae where *S. aureus* attached (Fig. 2C, D). It is worth to mention that the adhesion of the biofilms to this surface (Permanox™ plastic slides) was not as good as to the polystyrene surface, since the biofilms were easily detached in the washing step.

As *S. aureus* has been isolated along with *C. albicans* from bloodstream infections, we analyzed the biofilm formation of both microorganisms in a catheter model. We used a rotating mixer to accomplish a more dynamic system simulating a contaminated fluid passing through the catheter. In contrast with the static model in the Lab-Tek system aforementioned, the monomicrobial biofilm formation was visually lower in the catheter model for both microorganisms. However, *C. albicans* seemed to have better adherence and a more homogeneous biofilm-like structure in comparison with *S. aureus*, that adhered mainly as individual or grouped cocci, and biofilm-like structures were only observed in very few places throughout the catheter (Fig. 3A–F). On the other hand, the polymicrobial biofilm was more complex and structured, with *C. albicans* adhered to the internal surface of the catheter and *S. aureus* attached on the different morphological forms of the fungus (Fig. 3G–I).

### *Analysis of biofilm composition through fluorescent dyes and confocal microscopy*

To further analyze the biofilms, both microorganisms were incubated again in Lab-Tek II chamber slides. After the biofilms were formed, the slides were stained with different fluorescent reagents and observed by confocal microscopy. With FilmTracer 1–43, a dye that stains cell membranes, we observed a more complex biofilm when both microorganisms were present, with *C. albicans* having a better adherence to the slide surface and *S. aureus* attaching on the hyphal phase of the fungus (Fig. 4A–C). FilmTracer SYPRO Ruby stains most types of proteins; interestingly, unlike the monomicrobial biofilms, in the polymicrobial one the dye stained preferentially *C. albicans* rather than the bacteria (Fig. 4D–F, Supplemental Fig. 2A, 2B). In contrast, *S. aureus* was preferentially stained in the polymicrobial biofilm with WGA (Fig. 4G–I, Supplemental Fig. 2C, 2D) and BOBO-3 (Fig. 4J–L), fluorescent reagents that stain glycoproteins and extracellular DNA, respectively.

### *Biofilm inhibition and destabilization using chemical agents and antimicrobials*

We characterized the biofilms using destabilizing chemical agents and antimicrobials, and found that when the reagents were used in the RPMI-1640 medium during the biofilm formation (24 h), proteinase K strongly inhibited biofilm formation of both *C. albicans* and *S. aureus*. Sodium (meta)periodate (MPI) resulted in an inhibition of the *C. albicans* monomicrobial biofilm, but surprisingly had no effect on the biofilm of *S. aureus* (Fig. 5A–C). Interestingly, DNase I treatment clearly affected the monomicrobial *S. aureus* biofilm (Fig. 5A), but had no effect on the *C. albicans* biofilm or the polymicrobial one (Fig. 5B, C). We also found that the polymicrobial biofilm was inhibited by proteinase K and MPI (Fig. 5C). In order to rule out the cytotoxicity of the compounds or the inhibition of the microbial adhesion, we used them for three hours in the pre-formed biofilms. Destabilization of the monomicrobial and polymicrobial biofilms occurred with the treatment of proteinase K and MPI only, while the treatment with DNase I did not lead to biofilm destabilization (Fig. 5D–F). Finally, we tested the stability of the pre-formed polymicrobial biofilm using vancomycin and/or amphotericin B. A destabilization of the biofilm was observed when amphotericin B was present, while the treatment with vancomycin did not result in a clear destabilization of the biofilm (Fig. 6A–C).

## Discussion

*S. aureus* and *C. albicans* are common microorganisms in several locations of our body that may also be responsible for many hospital-acquired infections. These microorganisms have been co-isolated from several biofilm-associated diseases such as denture stomatitis, periodontitis, burn wound infections, and also from medical devices.[1,4,11,13] It is known that *S. aureus* does not produce biofilms so easily on abiotic surfaces.[5] In our study the density of

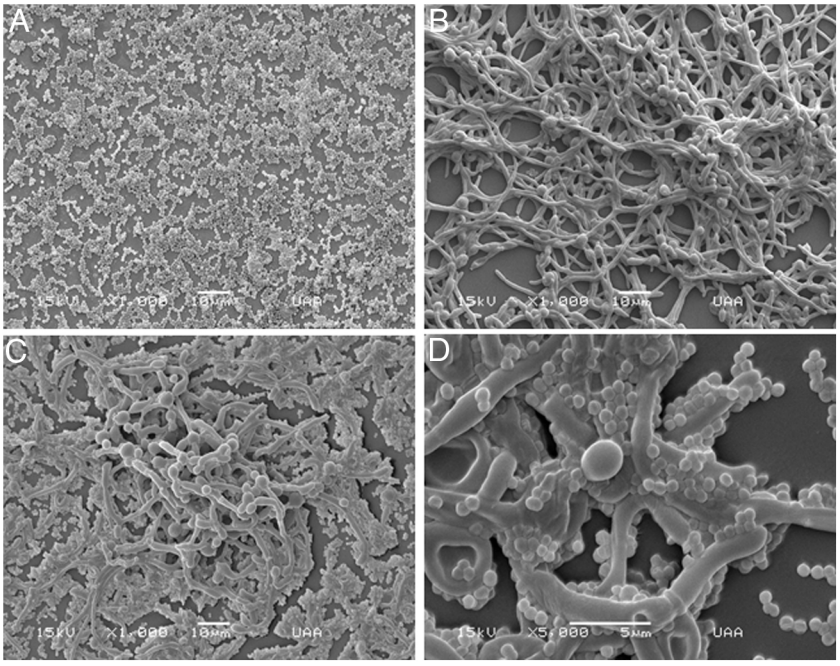


**Fig. 2.** Analysis of biofilms by SEM. Biofilm formation in Lab-Tek II Chamber Slide System for: (A) *S. aureus* (1000×), (B) *C. albicans* (1000×), (C) *C. albicans* and *S. aureus* (1000×), and (D) *C. albicans* and *S. aureus* (5000×). This experiment was repeated twice in triplicate.

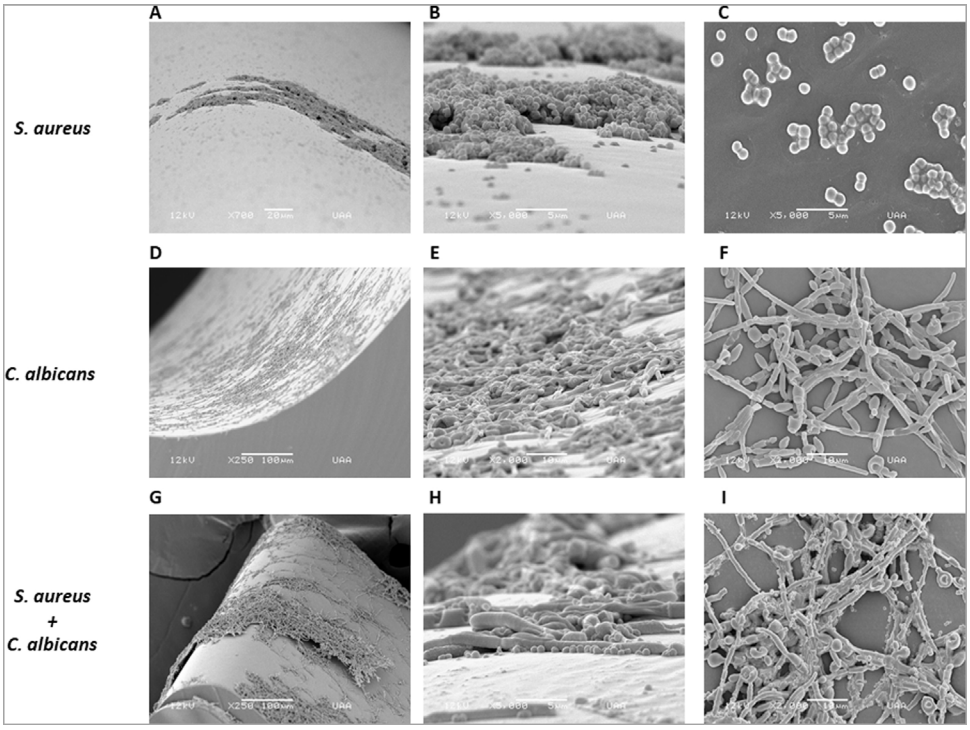


**Fig. 3.** Analysis by SEM of the biofilm formed by *S. aureus* and/or *C. albicans* in a catheter model. (A, B and C) *S. aureus* monomicrobial biofilm at 700×, 5000×, and 5000×. (D, E and F) *C. albicans* monomicrobial biofilm at 250×, 2000×, and 2000×. (G, H and I) *C. albicans* and *S. aureus* polymicrobial biofilm at 250×, 5000×, and 2000×. This experiment was repeated twice in triplicate.

*S. aureus* biofilm was lower when compared with that of *C. albicans* biofilm in the different media tested. RPMI medium has been used to study the interaction between *S. aureus* and *C. albicans*.[23,33] However, there is no information regarding the use of other culture media to study the changes in biofilm formation by these microorganisms. It has also been reported that *C. albicans* biofilm development is influenced by carbon sources.[21] This may have a clinical relevance as the presence of *C. albicans* was found to be higher in the oral cavity of patients with diabetes in comparison with non-diabetic individuals.[26] Also the presence of glucose in relation to *C. albicans* biofilm development has been analyzed using RPMI medium.[12,29]

Thus, we tested the biofilm formation capacity of both *S. aureus* and *C. albicans* using RPMI and DMEM supplemented or not with glucose and/or FBS. In terms of biofilm formation, the addition of FBS reduced the capacity of biofilm formation in *S. aureus* and

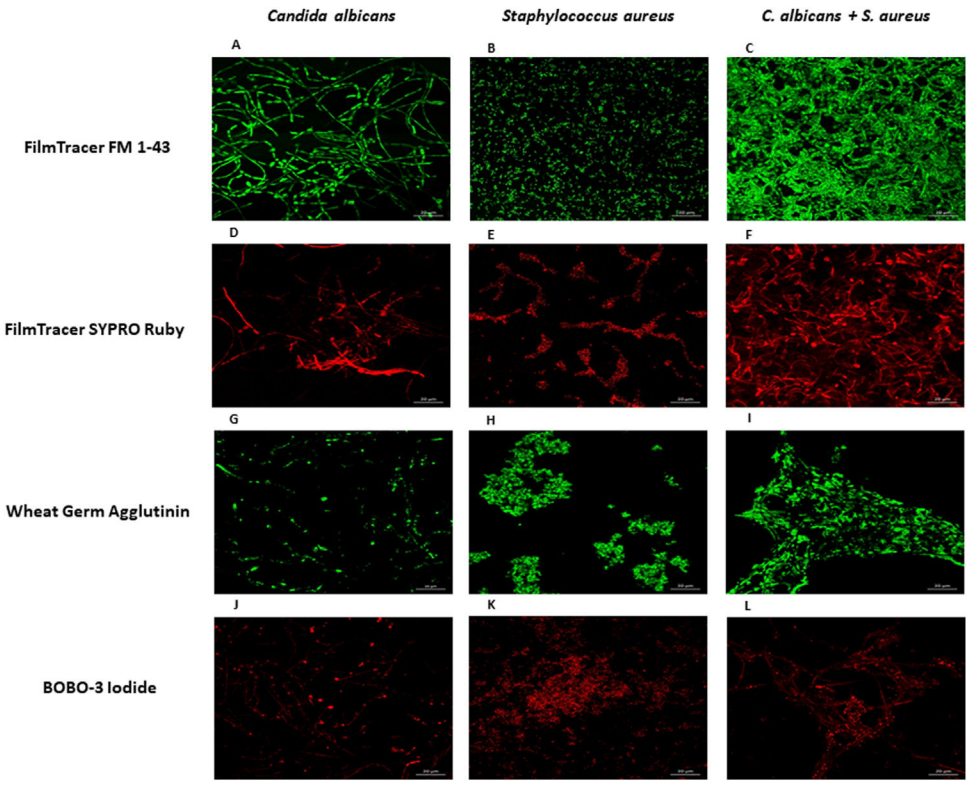


**Fig. 4.** Analysis by confocal microscopy of the *S. aureus* and *C. albicans* mono- and polymicrobial biofilms. Biofilms were stained with the next reagents: (A, B, and C) FilmTracer FM 1-43 Green Biofilm cell stain. (D, E, and F) FilmTracer SYPRO Ruby biofilm matrix stain. (G, H, and I) wheat germ agglutinin, Alexa Fluor 488 Conjugate. (J, K, and L) BOBO-3 Iodide. This experiment was repeated twice in triplicate.

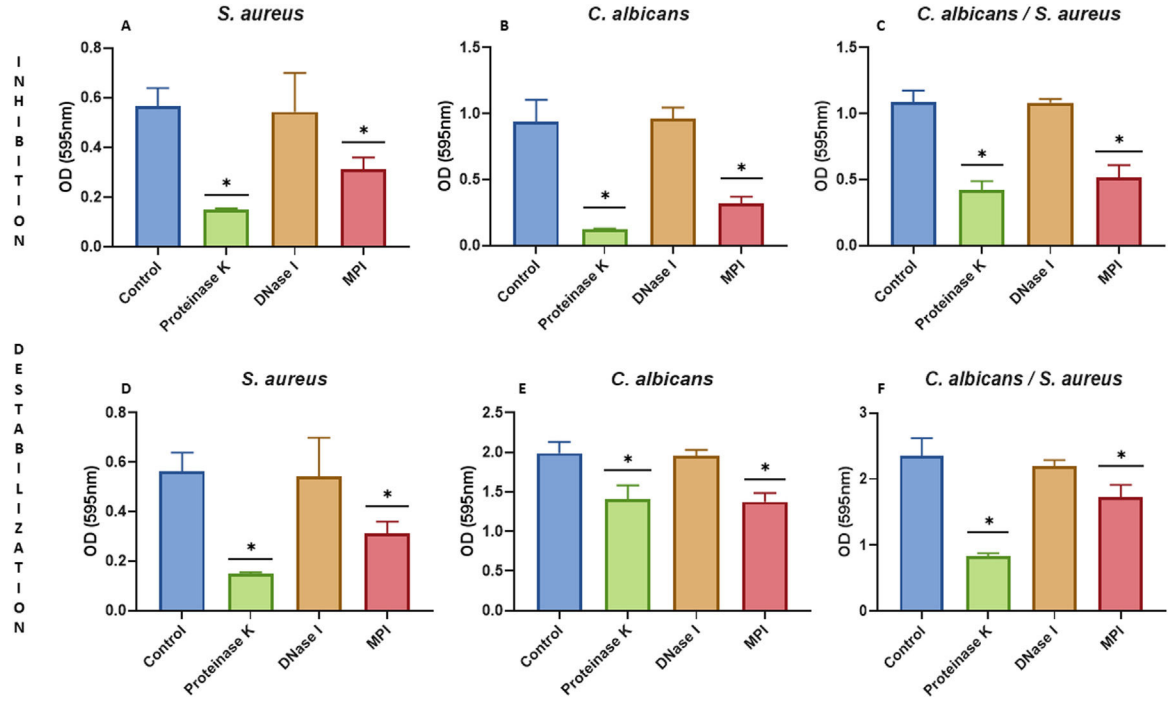


**Fig. 5.** Inhibition or destabilization of the mono- and polymicrobial biofilm formed by *S. aureus* and *C. albicans* using proteinase K, DNase I, and sodium (meta)periodate. Biofilm formation was inhibited for: (A) *S. aureus* monomicrobial biofilm, (B) *C. albicans* monomicrobial biofilm, and (C) *S. aureus* and *C. albicans* polymicrobial biofilm. Preformed biofilms were destabilized for three hours for: (D) *S. aureus* biofilm, (E) *C. albicans* biofilm, and (F) *S. aureus* and *C. albicans* polymicrobial biofilm. Dunnett's multiple comparison test (one-way ANOVA) was used to analyze statistical significance (comparison with an untreated control, $p \leq 0.05$). This experiment was repeated three times in triplicate.

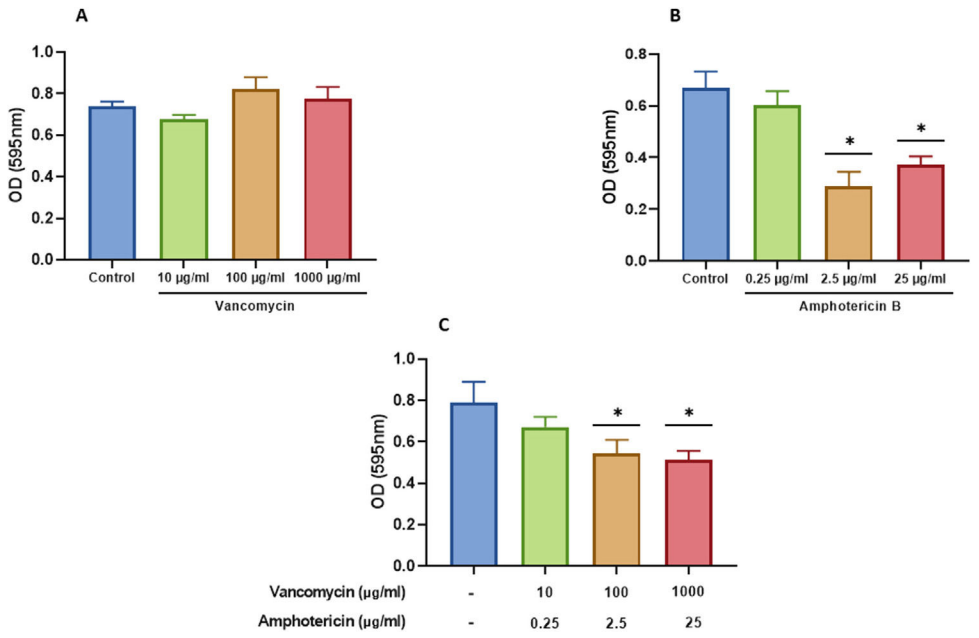


**Fig. 6.** Destabilization of the *S. aureus* and *C. albicans* polymicrobial biofilm with amphotericin B and vancomycin. Polymicrobial biofilms were treated for 24 h with: (A) vancomycin at 10, 100, and 1000 µg/ml; (B) amphotericin B at 0.25, 2.5, and 25 µg/ml; and (C) amphotericin B and vancomycin at the aforementioned concentrations. Dunnett's multiple comparison test (one-way ANOVA) was used to find statistically significant differences ($p \leq 0.05$). This experiment was repeated three times in triplicate.

the polymicrobial biofilm in all media. Bacterial adhesion and cell growth, important biological processes involved in biofilm formation, were analyzed. The ability of *S. aureus* to adhere to the polystyrene wells was severely reduced in the presence of FBS. This may be the reason behind the reduction in biofilm formation, even when FBS highly promoted the *in vitro* proliferation of the bacteria. We suggest that the adhesion of *S. aureus* to the wells was altered by the binding to serum proteins such as albumin. It has been shown that the ability of *S. aureus* to adhere to central venous catheters is drastically reduced in the presence of human blood plasma or serum albumin.[10] In blood stream infections plasma proteins could act as an interface, dragging bacterial cells adhered to catheters and releasing them to the bloodstream.

In the case of *C. albicans* biofilm, even when FBS also decreased the adhesion of *C. albicans*, biofilm density seemed to be not affected. We found that a concentration of $1 \times 10^6$ cells per well was better than $1 \times 10^7$ cells. Thus, after reaching a certain level of adhesion, cell growth is a key matter for biofilm development. In this regard, glucose addition increased the biofilm density only with DMEM, while with RPMI supplementation with glucose and FBS increased significantly biofilm density. It is worth mentioning that glucose promoted a higher cell growth rate than FBS in *C. albicans*. Differences in *C. albicans* biofilm formation in both culture media may come from differences in the content of some chemical compounds. It is known that the development of *C. albicans* biofilm involves several steps, including adhesion, proliferation, morphological changes, extracellular matrix production, and cell dispersion.[17] We need to further characterize the influence of the microbial density and the effect of nutrients over the steps involved in *C. albicans* biofilm formation.

Polymicrobial biofilm was similar in optical density to the monomicrobial biofilm of *C. albicans*, and with some media it was slightly lower. However, the analysis with SEM showed a complex polymicrobial biofilm, with *C. albicans* adhered to the surface of the Lab-Tek II slides and *S. aureus* adhered preferently to *C. albicans*. This complexity in the polymicrobial biofilm was also observed in our catheter model, in which *C. albicans* seemed to have a better capacity to adhere to this abiotic surface. This relation between both microorganisms unveiled by SEM could be analyzed in terms of the mechanisms the bacteria display to adhere to the fungal layer.

Fluorescent staining methods, along with confocal microscopy, enable us to observe that *S. aureus* attached to the hyphal phase of the fungus. Interestingly, SYPRO Ruby, that stains most classes of proteins, stained preferentially *C. albicans* while WGA, that stains glycoproteins, stained preferentially *S. aureus*. It has been shown that the production of an adhesin named polysaccharide intercellular adhesin (PIA) or poly-N-acetylglucosamine (PNAG) encoded in the *icaADBC* operon by methicillin-sensitive *S. aureus* (MSSA) is the main mechanism of adhesion and biofilm formation in these bacteria.[16,18] We suggest that in the polymicrobial biofilm the adhesion to the slide surface comes mainly from *C. albicans*, where proteins play a major role on this process, while the contribution of glycoproteins in the biofilm formation could be more important for *S. aureus*. Of further interest is the role of the adhesin PIA of *S. aureus* in the adhesion to the hyphal form of *C. albicans*.

The composition of *C. albicans* biofilm in the dry weight extracellular matrix includes proteins (55%), carbohydrates (25%), lipids (15%), and extracellular DNA, as well as mannans, the most abundant polysaccharides, associated with linear β-1,6 glucans.[24] Thus, it is not surprising that proteinase K and MPI, a reagent used to oxidize hydroxyl groups of polymeric hexosamines such as those of the PNAG, inhibit the monomicrobial and polymicrobial biofilms of *C. albicans*. However, in the case of *S. aureus*, MPI did not inhibit the monomicrobial biofilm. As PIA (PNAG) has been proposed as the main mechanism of adhesion in MSSA, we suggest that *S. aureus* may use a different adhesion mechanism when MPI is present in the RPMI medium; in this respect, other mechanisms of biofilm formation in MSSA have been described.[16] The stability of the *C. albicans* preformed biofilm was also greater than that of *S. aureus* biofilm. This result was expected as the biofilm of *C. albicans* showed a much higher optical density in the crystal violet assay; however, preformed biofilms were also affected by proteinase K and MPI. Thus, proteins and β-1,6-linked polysaccharides seem to have a prominent role in the biofilm structure.

Finally, destabilization of the polymicrobial biofilm was only evident when using amphotericin B, while vancomycin had no

effect. Once again, we suggest that *C. albicans* contributes the most to the final structure and stability of the polymicrobial biofilm.

## Conclusions

We characterized the *in vitro* polymicrobial biofilm of *C. albicans* and *S. aureus* as they have been found together in biofilm-related infections. We found interesting the fact that FBS affects the adhesion of both microorganisms, as well as *S. aureus* biofilm formation and the polymicrobial biofilm formation. We suggest that plasma proteins, such as albumin and fibrinogen, may act as an interface, binding to biofilm-derived microorganisms in colonized catheters. *C. albicans* contributed the most to the whole density of the biofilm, in which β-1,6-linked polysaccharides seem to be important components. Finally, *C. albicans* adhered preferentially to all the surfaces tested, while *S. aureus* adhered mainly to the surface of *C. albicans*. The requirement of nutrients that may be involved in all the steps of biofilm formation, such as adhesion, cell growth, extracellular matrix formation, and dispersion, should be analyzed in the future.

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## Conflict of interest

The authors declare that they have no conflict of interest.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.riam.2022.04.001.

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