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Antimicrobial Activity of Mesenchymal Stem Cells (MSCs)-Conditioned Media and Mitochondria Against Clinically Relevant Pathogens

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RESUMEN

Ante el aumento de la resistencia antimicrobiana y la dificultad de tratar infecciones se han explorado opciones terapéuticas alternativas. Una posibilidad que llama la atención son las células madre mesenquimales (MSCs) cuyos medios condicionados han demostrado propiedades antimicrobianas, atribuidas a los factores secretados por estas células. Estos factores aún no han sido estudiados completamente, pero podrían incluir mitocondrias. Por ende, este estudio profundizó la actividad antimicrobiana de medios condicionades de MSCs y exploró la contribución de mitocondrias a dicho efecto, que incluyeron ensayos de biomasa y tinción vivas/muertas bajo microscopía de fluorescencia. En el caso de ensayos de biomasa se decidió utilizar medios activados con LPS y medios sin activar. Se seleccionaron patógenos clínicamente relevantes como *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli* y *Candida albicans*. La concentración de 0.5 ng/µl proporcionó los mejores resultados para las especies bacterianas, siendo los medios condicionas activados con LPS los que tuvieron un mejor efecto en *E. coli* y *P. aeruginosa*. En *S. aureus*, el medio no activado tuvo mayor efecto y en *C. albicans* la concentración que mejor inhibió fue 0.3 ng/µl. Por otro lado, los resultados de ensayos de biomasa para mitocondrias mostraron un mejor efecto sobre *S. aureus* con inhibición en un rango de 20 a 27%. Los resultados de tinción vivas/muertas bajo microscopía de fluorescencia mostraron que existió una gran disminución de células totales y mayor porcentaje de mortalidad a una concentración de 0.5 ng/µl para todos los microorganismos. Estos resultados muestran que tanto los medios condicionados como las mitocondrias por si solas presentan actividad antimicrobiana que no solo inhibe el crecimiento de biomasa, sino que mata las células microbianas.

Palabras Clave: Células Madre Mesenquimales (MSCs), medios condicionados, mitocondrias, actividad antimicrobiana, infecciones, ensayos de biomasa, tinción vivo/muerto, patógenos clínicamente relevantes, microscopía de fluorescencia.

ABSTRACT

Faced with the global rise in antimicrobial resistance and the challenges associated with infections, alternative therapeutic options have been explored. One intriguing possibility involves mesenchymal stem cells (MSCs), whose conditioned media have demonstrated antimicrobial properties attributed to factors secreted by these cells. Although these factors, possibly including mitochondria, have not been fully studied, this research delves into the antimicrobial activity of MSC-conditioned media and explores the potential contribution of mitochondria to this effect. Biomass assays and live/dead staining under fluorescence microscopy were employed. For biomass assays, both lipopolysaccharide (LPS)-activated and non-activated media were utilized. Clinically relevant pathogens, such as *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli,* and *Candida albicans,* were selected. The results confirm the antimicrobial activity of conditioned media previously reported, now directed towards four microorganisms. A concentration of 0.5 ng/µl exhibited the best results for bacterial species, with LPS-activated media showing a superior effect on *E. coli* and *P. aeruginosa*. In *S. aureus*, non-activated media had a greater effect, and the optimal inhibitory concentration for *C. albicans* was 0.3 ng/µl. Additionally, biomass assay results for mitochondria demonstrated the best effect on *S. aureus*, with inhibition ranging from 20 to 27%. Live/dead staining results indicated a significant reduction in total cells and a higher percentage of mortality at a concentration of 0.5 ng/µl for all microorganisms. These findings highlight that both conditioned media and mitochondria alone exhibit antimicrobial activity, not only inhibiting biomass growth but also causing microbial cell death.

Keywords: Mesenchymal Stem Cells (MSCs), conditioned media, mitochondria, antimicrobial activity, infections, biomass assays, live/dead staining, clinically relevant pathogens, fluorescence microscopy.

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1. INTRODUCTION

Around the world, sepsis continues to be the main cause of death related to infections with a prevalence higher than 40% of mortality (Napolitano, 2018). Currently, the diagnosis and treatment of sepsis are derived from evidence and previous clinical criteria (Napolitano, 2018). However, optimizing different parameters to treat sepsis is beneficial in the early phase. Other clinical trials have suggested that reducing the host inflammatory response could help during the acute phase, but this has failed so far (Cangui-Panchi et al., 2022; Uhle et al., 2015). So, a proper understanding of the pathogenesis of impaired organ function could help find new solutions (Edwards, 2022) Furthermore, the increase in antimicrobial resistance (AMR) worldwide has led to several studies evaluating alternative treatments against clinically relevant pathogens (Cabezas-Mera et al., 2023; Fernandez-Soto et al., 2023; Machado et al., 2023; Reyes et al., 2019).

Mesenchymal stem cell (MSC)-conditioned media has shown antibacterial properties in recent studies (Alcayaga-Miranda et al., 2017; Maxson et al., 2012), so it could be an ideal alternative treatment to fight infections. For example, canine bone marrow mesenchymal stem cell-conditioned media showed antibacterial, antibiofilm, and anti-quorum sensing properties against *Escherichia coli* JM109 pSB1142 and *Staphylococcus aureus* 14 (*hla, isdA, sdrE* PCR positive) and 11 (*hla, isdA, isdB* PCR positive) (Bujňáková et al., 2020). Moreover, the proteomic analysis demonstrated the ability of MSC-conditioned media to promote a reduction in cell membrane hydrophobic properties in *S. aureus* 14 and modulation of the bacterial communication through avoiding bioluminescence in *E. coli* JM109 pSB1142, indicating N-acyl-L-homoserine lactone (AHL) degradation (Bujňáková et al., 2020). Similar results were obtained from another study realized by McCarthy and colleagues, which investigated the effect of MSC-conditioned media from MSC of bone marrow and umbilical cord against relevant clinical pathogen isolates more exactly *E. coli*, *S. aureus*, and *Klebsiella pneumoniae*

(McCarthy et al. 2020). In addition, Kouhkheil and colleagues showed a decrease in colony-forming units (CFU) over infected rats wounds with methicillin-resistant *S. aureus* (MRSA)(Kouhkheil et al., 2018).

MSCs have become a focal point in regenerative medicine, notably for their role in secreting microvesicles (MVs) and extracellular vesicles (EVs) that carry mitochondria. These MVs and EVs are pivotal in intercellular communication and tissue regeneration, particularly in their ability to transfer mitochondria to target cells. This transfer process plays a significant role in altering the metabolic activities and functional state of the recipient cells, both under normal and pathological conditions (Balcázar et al., 2020; Caicedo et al., 2021).

However, little is still known about the bioactive principles of MSC-conditioned media being postulated that the release of mitochondria could be partially responsible for these antimicrobial properties. Therefore, the present study aims to explore the *in vitro* antimicrobial activity of MSC-conditioned media and mitocondria against several biofilm-forming microorganisms in their planktonic growth mode thus establishing a comparison between their antimicrobial properties and a baseline for future evaluation (i.e., an initial characterization of the potential of mitochondria extracts as antimicrobial agents against well-known pathogens in different modes of growth) through *in vivo* assays. Regarding published literature, the most identified microbial species found in bacterial sepsis course were: *Pseudomonas aeruginosa, Staphylococcus epidermidis, S. aureus, K. pneumoniae, E. coli, Enterococcus faecium, Enterococcus faecalis,* and *Enterobacter cloacae* (Cangui-Panchi et al., 2022; Uhle et al., 2015). To explore the potential of antimicrobial activity of mitochondria extracts and compared to MSC-conditioned media, we selected the following reference microorganisms (American Type Culture Collection, ATCC): *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231.

2. MATERIALS AND METHODS

2.1 Bacterial Isolates and Growth Conditions

S. aureus ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231 isolates from the Institute of Microbiology at Universidad San Francisco de Quito (IM-USFQ) were selected for the present study. Strains were stored at -80 °C, and, 24 hours before each assay, a new culture in Trypticase Soy agar (TSA) at 37°C for 24h was prepared. After growth culture, bacterial cells were harvested and suspended in phosphate-buffered saline (PBS) to obtain a cellular density equal to 1E+8 colony-forming units (CFU) per mL using 0.5 McFarland turbidity standard and then a dilution 1:200 was realized to obtain the final concentration of 5E+5 CFU/mL in 96-well plates for antimicrobial activities evaluation (see Supplemental Material).

2.2 Microbial Growth Assays

The antimicrobial activity of previously isolated MSC-conditioned media (LPS-activated and non-activated) and mitochondria extract, as illustrated in Fig. 1, were then evaluated against the several pathogens (*P. aeruginosa* ATCC27853, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *C. albicans* ATCC 10231) through the Microdilution method assays, as described by Wiegand, Hilpert & Hancock (2008), under the Clinical Laboratory Standards Institute (CLSI) guidelines. For antimicrobial evaluation assays, serial dilutions were realized starting with 10 ng/µL of MSC-conditioned media and mitochondria on Mueller Hinton broth (MHB), and 10 µL of MSC-conditioned media or mitochondria was added in 96-well plates(Patel et al., 2021), achieving the final concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 ng/µL. Subsequently, 190 µL of each pathogenic species within MHB was added at a final concentration of $5x10^5$ CFU/mL in the total volume of MHB in each well and the 96-well plate was incubated for 18-24 h at 37 °C (Wiegand et al., 2008). Finally, the lowest concentration of MSC-conditioned media or mitochondria without growth of pathogenic species was classified as the minimum

inhibitory concentration. In the case of microbial growth at all concentrations, the microbial biomass growth percentages were calculated as described in Equation 1. Several controls were prepared as follows: wells with strictly medium were used as sterility controls; wells with medium and MSC-conditioned media previously activated with LPS-activated and non-activated, as well as mitochondria extract but without microbial inoculum were used as negative controls; and, finally, wells with medium plus microbial inoculum were used as positive controls. The optical density (OD) was measured by spectrophotometry in the ELISA Elx808 spectrophotometer (BioTek, Winooski, GU, USA) at an optical density of 570 nm. The microbial growth percentage was calculated accordingly as the following equation:

EQUATION 1: % Microbial Biomass Growth = $\left[\left(\frac{OD\ treated\ sample - OD\ negative\ control}{OD\ positive\ control - OD\ sterility\ control}\right) \times 100\right]$

The assays were performed in triplicate in three independent experiments, using the same negative, sterile, and positive controls previously described.

2.3 Fluorescence Microscopy using Live/Dead Staining

All control and mitochondria-treated samples at 0.1, 0.3, and 0.5 ng/μL were further analyzed by fluorescence microscopy (FM) using live/dead staining. After each microbial growth assay in 96-well plates at the same experimental settings previously described was further evaluated for the total cells and live/dead cells count. A working solution of fluorescent stains was prepared by adding 1.0 mL of SYTO® 9 stain and 10 μL of propidium iodide (PI) stain (FilmTracer™ LIVE/DEAD® Biofilm Viability Kit), mixed in the proportion 1:100 of PI/SYTO-9, into 10 mL of filter-sterilized water in a foil-covered container. About 100 μL of the live/dead working solution was added onto control and mitochondria-treated samples and were then incubated for 15-30 min at room temperature, protected from light. Finally, FM analysis was carried out using an Olympus BX50 microscope (Olympus Corporation, Tokyo,

Japan) equipped with a 100x oil immersion objective. Images were captured with AmScope Digital Camera MU633-FL (AmScope, California, USA) and digitalized with AmScope software version 1.2.2.10. As previously described (Rosenberg et al., 2019), at least 12 images were taken per sample for counting purposes on the 22-mm diameter glass coverslip at random locations. These results were expressed as the number of cells ± standard deviation per cm^2 (N. of cells/cm^2 ± SD). The percentages of dead and live cells within images were measured through ImageJ version 1.57 by Fiji (Schindelin et al., 2012) using the macros Biofilms Viability checker (Mountcastle et al., 2021).

2.4 Statistical Analysis

All data of the present study were evaluated by statistical analyses. Due to the non-normal distribution of the data set, a non-parametric test was applied, more exactly the Wilcoxon nonparametric test was used for pairwise comparison between control and treated samples in both microbial growth inhibition and FM using live/dead staining assays. Statistical analyses were realized in R studio version 4.0 (https://www.rstudio.com/products/rstudio/download/) using several R packages ("ggpubr", "rstatixs", "openxlsx" and the "tidyverse" set of packages) (Kassambara, 2021; Wickham et al., 2019). Finally, all p-values <0.05 were considered significant.

3. RESULTS

3.1 Microbial Growth Inhibition by Mesenchymal Stem Cell-Conditioned Media

To validate the inhibitory activity of MSC-conditioned media against clinically relevant pathogens, two types of media were tested in a 96-well U-shaped microplate, more exactly a LPS-activated MSC-conditioned media and a non-activated LPS MSC conditioned media. Five concentrations were examined for each conditioned media, and the results are shown in Table 1. Statistical analyses were performed using the Mann-Whitney Wilcoxon test. No significant differences were observed between the positive control and cell media control for all bacteria and MSC-conditioned media treated samples exhibited superior growth inhibition at higher conditioned media concentrations.

In the case of *S. aureus* ATCC 25923 the percentage of inhibition was higher when compared to other bacteria, as illustrated in Fig 2. A). Notably, the non-activated LPS MSC-conditioned media demonstrated greater efficiency in inhibiting *S. aureus* ATCC 25923 growth with 83.66% inhibition (p-value <0.0001 when compared to cell media control), while LPS-activated MSC-conditioned media showed 57.37% of growth inhibition (p-value <0.0001), both media with the optimal concentration identified as 0.5 ng/μl. For *E. coli* ATCC 25922, the most effective inhibitory concentration was LPS-activated MSC-conditioned media at 0.5 ng/μl, with 18.98% inhibition (p-value =0.0002) against 10.38% for non-activated LPS MSC conditioned media (p-value =0.0005), as illustrated in Fig. 2. B. Moreover, LPS-activated MSC-conditioned media at 0.5 ng/μl exhibited an inhibition percentage of 20.88% (p-value <0.0001) in *P. aeruginosa* ATCC 27853, revealing a substantial difference between LPS-activated and non-activated conditions, where non-activated LPS MSC-conditioned media exhibited 6.43% inhibition (p-value =0.5893), as shown in Fig. 2. C. In the case of *C. albicans* ATCC 10231 (the only fungus examined in the present study), a significant difference was observed between the positive control and the cell media control (Fig. 2. D). The treated

samples on different concentrations demonstrated similar inhibitory growth values against *C. albicans* ATCC 10231, showing the best inhibitory activity for LPS-activated MSC-conditioned media at 0.3 ng/µl with 17.66% of growth inhibition (p-value <0.0001) against 12.65% for non-activated LPS MSC-conditioned media (p-value =0.0002). Interestingly, no discernible pattern emerged for any concentration, indicating a distinct *Candida* growth pattern for each concentration.

3.2 Microbial Growth Inhibition by Mitochondria

Furthermore, mitochondria extract demonstrated a lower inhibition rate compared to MSC-conditioned media (see Fig. 3). The same concentrations were tested for antimicrobial activity through mitochondria extracts exhibiting different growth inhibition percentages (Table 2). For the three bacteria analyzed in these experiments, no significant differences were noted between the positive control and control cell media. Consequently, subgroup statistical analyses were performed comparing the results with control cell media. *S. aureus* ATCC 25923 showed the optimal concentration of 0.5 ng/µl for biomass growth inhibition. However, in comparison with other bacteria, growth inhibition was higher for this gram-positive bacterium across all concentrations, as illustrated in Fig. 3. A. In fact, *S. aureus* ATCC 25923 evidenced a range of inhibition from 21.49 % to 26.39% (all p-values were <0.05), which means a great significance between the cell media control and treatments. Meanwhile, *E. coli* ATCC 25922 displayed lower growth inhibition across the five concentrations, evidencing biomass inhibition in the range from 3% to 9.06% (Fig. 3. B) despite statistical significance through Wilcoxon test was obtained (all p-value <0.05), which also represent a great significance against cell media control. Remarkably, the most effective concentration for inhibition in *E. coli* ATCC 25922 was 0.4 ng/µl with 9.06% inhibition and p-value <0.0001, and all concentrations presented an inhibition range from 3.78 to 9.06 %. In the case of *P. aeruginosa* ATCC 27853, the biomass inhibition mediated by mitochondria extract at 0.3 ng/µl demonstrated the least efficiency

across all concentrations when compared to the previous pathogens, showing biomass inhibition percentages ranging from 0% to 1.74% (Fig. 3. C). Finally, *C. albicans* ATCC 10231 exhibited a significant difference between the control cell media and the positive control with a p-value <0.0001 (Table 2), aligning with the previous observed results with MSC-conditioned media (Table 1). The percentage of biomass inhibition for mitochondria concentrations ranged from 4.25% (p-value <0.0001) at 0.5 ng/μl to the highest at 7.12% (p-value <0.0001) for concentration 0.4 ng/μl (Fig. 3. D).

3.3 Fluorescence Microscopy (FM) using Live/Dead Staining by Mitochondria

To understand the biomass composition, we conducted live/dead staining and subsequently visualized the results through fluorescence microscopy. The findings are shown in Table 3. Notably, all pathogens exhibited significant differences in the number of total cells and dead cell percentages across various mitochondria concentrations. The most noteworthy outcome emerged at 0.5 ng/μl. At this concentration, *S. aureus* ATCC 25923 displayed a 51% reduction in total cell numbers (p-value <0.0001) and nearly 10% higher mortality compared to the positive control (p-value =0.0919). For *E. coli* ATCC 25922, a 47.68% reduction in total cell numbers (p-value <0.0001) and 24% additional mortality were observed compared to the positive control (p-value =0.0006). Regarding *P. aeruginosa* ATCC 27853, the results indicated a 35.4% reduction in total cells (p-value =0.0004) and a 22.5% augmentation in mortality when compared to the positive control (p-value <0.0001). Finally, *C. albicans* ATCC 10231 demonstrated the most substantial reduction in total cells with a 58.8% diminution (p-value <0.0001) and an additional 8% mortality when compared to the positive control (p-value =0.0315).

4. DISCUSSION

MSCs constitute part of the natural regenerative pathway in humans or animals being still characterized in several studies (Campagnoli et al., 2001; Chen et al., 2008; Gao et al., 2016). Gnecchi and colleagues evidenced the presence of bioactive factors in patients after transplantation (Gnecchi et al., 2008). However, little is still unknown about these bioactive factors and their regenerative as well as antimicrobial activities. Some studies have tried to understand the intrinsic antibacterial effect of MSCs, and findings showed that one of the main bioactive factors was the human cathelicidin antimicrobial peptide, also known as hCAP-18/LL-37, against gram-negative bacteria like *P. aeruginosa* and *E. coli* (Krasnodembskaya et al., 2010). In the present study, we hypothesized that the partially antimicrobial properties could be associated with the release of mitochondria outside of the MSCs. Therefore, we first confirmed the antimicrobial activity of the MSC-conditioned media against several clinically relevant pathogens, where the MSC-released bioactive factors evidenced different antimicrobial activities in our pathogen group set. In 2010, Krasnodembskaya and colleagues showed that MSC-conditioned media had an antimicrobial effect against *E. coli*, suggesting the LL-37 cationic antimicrobial peptide as the principal antimicrobial mechanism (Krasnodembskaya et al., 2010). Another study showed a total cell viability inhibition of *S. aureus* through colony-forming unit (CFU) counting assays using MSC-conditioned media with or without cells and evidencing a similar antimicrobial effect in both cases (Yagi et al., 2020). In the present study, we only worked with MSC-conditioned media without cells showing biomass growth inhibition on *S. aureus* and *E. coli*, as previously described in the literature (Krasnodembskaya et al., 2010; Yagi et al., 2020). However, other well-known pathogens were also evaluated, such as *P. aeruginosa* and *C. albicans*. In the case of *P. aeruginosa*, MSC-conditioned media showed an increment of biomass suggesting *P. aeruginosa*'s ability to efflux bioactive factors and being able to proliferate among the

evaluated media (Oliveira et al., 2020). On the other hand, the *P. aeruginosa* biofilm also demonstrates an ability to diminish the viability of MSCs (Ward et al., 2015), highlighting that the use of MSC-conditioned media without cells is the optimal approach to directly evaluating the antibacterial effect of MSC bioactive factors onto this bacterium. Nonetheless, activated-LPS MSC-conditioned media demonstrated biomass growth inhibition (see Fig 2. C) suggesting that the previous stimulation with the usual gram-negative endotoxin was able to improve the antimicrobial properties of the bioactive factors from MSCs. Moreover, Krasnodembskaya and colleagues reported better antimicrobial activity when exposing their bone marrow MSCs to *E. coli* before performing the antibacterial evaluation when compared to MCS non-exposed *E. coli* control. In agreement, our results evidenced the same scenario when evaluating *P. aeruginosa* biomass growth.

In recent years, mitochondria have been hypothesized as one of the bioactive factors present in the extracellular secretion of MSCs (Abuaita et al., 2018; Jackson et al., 2016a; Levoux et al., 2021; Miliotis et al., 2019). Several studies have demonstrated the ability of MSCs to transfer mitochondria from healthy MSCs to other MSCs and other cell types (Jackson et al., 2016b; Levoux et al., 2021; Wang et al., 2018) such as macrophages, epithelial cells, and other MSCs. This phenomenon revealed the possibility of MSCs excreting mitochondria as a bioactive factor. Most mitochondria are transferred through microtubules, facilitating intercellular interchange and modulating cellular metabolism. Recent studies demonstrated that this type of transference enhanced macrophage phagocytic activity against external pathogens (Jackson et al., 2016c; Kidwell et al., 2023). Consequently, mitochondria may play by itself a role in the antimicrobial activity of MSCs. To explore this hypothesis, we evaluated five concentrations of mitochondria, as previously shown in Figure 3. Biomass results revealed a biomass inhibition range of 20 to 26% for *S. aureus* ATCC 25923, which exhibited the most significant inhibition compared to other pathogens. *P. aeruginosa* ATCC 27853 exhibited a lower biomass

inhibition ranging from 0 to 1.74%, while *E. coli* ATCC 25922 and *C. albicans* ATCC 10231 displayed inhibition ranges between 4 to 9%. However, the exact metabolic mechanisms associated to its antimicrobial activity remain unknown. Nonetheless, the present study was able to demonstrate a direct antimicrobial effect of mitochondria against several clinically relevant pathogens. To the authors best knowledge, this study is also the first report to evidence the total cell reduction and dead cell increment caused by mitochondria against gram-negative and gram-positive bacteria, as well as *C. albicans*. Until now, no studies reported the antimicrobial activity induced by mitochondria through live/dead staining using fluorescence microscopy. Our results demonstrated total cell reduction up to 58.8% and dead cells increment up to 24%. Existing literature indicates that mitochondria play a role in infections by generating reactive oxygen species (ROS) (Andrieux et al., 2021), suggesting a potential direct involvement in biomass reduction.

In summary, our results highlight the ability of mitochondria to inhibit biomass growth and live/dead staining using fluorescence microscopy demonstrated significant statistical differences between control and treated samples. From the evaluated mitochondria concentrations in our group set, the concentration of 0.5 ng/µl emerged as the most effective, demonstrating the highest reduction in total cells and a notable increase in the dead cell percentage across all pathogens. This underscores that mitochondria not only affected biomass reduction but also reduced total cells and induced cell mortality within biomass growth of all clinically relevant pathogens. Future perspectives should focus on identifying the precise molecular patterns involved in this antimicrobial effect. Additional assays, such as colony-forming units (CFU) counting assays, and evaluation of higher mitochondria concentrations should be conducted to further elucidate the antimicrobial potential of mitochondria.

5. CONCLUSIONS

The antimicrobial activity previously documented in literature was reaffirmed by MSC-conditioned media results in the present study. Moreover, the activation with LPS showed better growth inhibition for Gram-negative bacteria, as previously mentioned in the literature. A novelty in the antimicrobial activity of MSC-conditioned media was its application against *Candida albicans* allowing it to be compared with bacterial pathogens. Regarding the mitochondria evaluation, our results displayed significant antimicrobial effects, contributing to the reduction of microbial biomass and total cell counts while increasing the dead cell percentages. Notably, the inhibitory impact on growth biomass was most pronounced in *S. aureus*, yet the highest reduction in total cells and an increased rate of dead cells were observed against *C. albicans* and *E. coli*, respectively. From our initial group set, the concentration of 0.5 ng/µl was the optimal mitochondria concentration showing the most efficient mitigation of pathogens. Further studies should evaluate the cell viability of treated samples, the metabolic pathways associated with the antimicrobial activity induced by mitochondria, and its antibiofilm properties through inhibition and eradication assays.

6. TABLES

Table 1. Overall results of microbial biomass growth inhibition by LPS-activated and non-activated LPS MSC-conditioned media against clinically relevant pathogens.

	MSC +LPS extract				MSC extract				MSC +LPS extract versus MSC extract P-value
	Concentration	Growth Inhibition (%)	SD (%)	P value		Biomass growth Inhibition (%)	SD (%)	P-value	
S. aureus ATCC 25923	Cell Media control	-2	5.77	0.5964	Cell Media control	-2	5.77	6.01E-01	5.67E-06
	0.5 ng/µL	57.37	5.2	<0.0001	0.5 ng/µL	83.66	0.59	<0.0001	1.80E-04
	0.4 ng/µL	52.62	4.023	<0.0001	0.4 ng/µL	77.71	4.15	<0.0001	8.23E-05
	0.3 ng/µL	43.66	4.35	<0.0001	0.3 ng/µL	69.36	5.22	<0.0001	0.000407
	0.2 ng/µL	32.15	3.06	<0.0001	0.2 ng/µL	39.15	5.071	<0.0001	0.006
	0.1 ng/µL	14.27	8.43	<0.0001	0.1 ng/µL	44.82	5.83	<0.0001	8.23E-05
E. coli ATCC 25922	Cell Media control	-6.37	9.08	0.102	Cell Media control	-6.37	9.08	0.102	1.75E-04
	0.5 ng/µL	18.98	5.2	0.0002	0.5 ng/µL	10.38	5.98	0.0005	3.80E-02
	0.4 ng/µL	4.31	4.690	0.0254	0.4 ng/µL	1.54	3.85	0.093	0.372
	0.3 ng/µL	1.31	4.69	0.1195	0.3 ng/µL	-5.73	8.31	0.963	0.074
	0.2 ng/µL	-21.065	2.65	0.0006	0.2 ng/µL	-20.32	6	0.0053	0.524
	0.1 ng/µL	1.5	6.14	0.0789	0.1 ng/µL	2.14	6.32	0.0106	0.744
P. aeruginosa ATCC 27853	Cell Media control	-2.24	7.1	6.01E-01	Cell Media control	-2.24	7.1	6.01E-01	5.67E-06
	0.5 ng/µL	20.88	7.7	<0.0001	0.5 ng/µL	1.52	6.43	0.5893	1.80E-04
	0.4 ng/µL	10.31	7.83	0.0013	0.4 ng/µL	1.02	6.48	0.5658	8.23E-05

	0.3 ng/µL	3.83	5.43	0.0339	0.3 ng/µL	5.48	5.57	0.0105	0.000407
	0.2 ng/µL	4.54	7.08	0.0389	0.2 ng/µL	10.77	9.51	0.0034	0.006
	0.1 ng/µL	6.49	5.72	0.002	0.1 ng/µL	14.69	6.46	<0.0001	8.23E-05
C. albicans ATCC 10231	Cell Media control	3.952	4.4	0.0001	Cell Media control	3.952	4.4	0.000447	7.80E-02
	0.5 ng/µL	7.61	3.52	0.0747	0.5 ng/µL	9.06	2.62	0.0085	3.97E-01
	0.4 ng/µL	13.09	1.6	<0.0001	0.4 ng/µL	12.61	2.09	<0.0001	7.43E-01
	0.3 ng/µL	17.66	5.69	<0.0001	0.3 ng/µL	12.65	2.78	0.0002	0.012
	0.2 ng/µL	16.78	4.28	<0.0001	0.2 ng/µL	14.93	3.84	<0.0001	0.462
	0.1 ng/µL	14.19	6.34	<0.0001	0.1 ng/µL	15.87	4.98	<0.0001	3.74E-01

Legend- Evaluated concentrations of LPS-activated and non-activated MSC-conditioned medium, which are indicated in the table above. Microbial growth values of each sample and control were calculated as the percentage of microbial growth through the optical density comparison between each treatment concentration (MSC conditioned media and pathogen) and cell media control. The positive control was also evaluated (pathogen growth in only medium culture) and it was considered 100% when compared to Cell media control and samples in the assay. All sterility control of pathogens (Negative control) showed no growth and were considered as 0.00%. All statistical analyses (p-values) were analyzed using a non-parametric Wilcoxon test (95% confidence interval) for comparison between microbial growth % values. The inner p-values obtained when comparing cell media control and treated samples for each LPS-activated and non-activated conditioned media. The general p-values obtained when comparing samples from LPS-activated and non-activated MSC-conditioned media at the same concentration.

Table 2. Overall results of microbial biomass growth inhibition by mitochondria extract concentrations against clinically relevant pathogens.

Mitochondria extract				
	Concentration	Biomass growth inhibition (%)	SD (%)	P-value
S. aureus ATCC 25923	Cell Media control	-22.7	18.96	0.672
	0.5 ng/µL	26.39	4.093	0.0002
	0.4 ng/µL	21.97	3.219	0.0006
	0.3 ng/µL	24.24	4.059	0.0007
	0.2 ng/µL	22.49	4.013	0.0008
	0.1 ng/µL	21.4	1.954	0.0012
E. coli ATCC 25922	Cell Media control	-4.4	5.76	0.0684
	0.5 ng/µL	5.49	3.355	0.0002
	0.4 ng/µL	9.06	4.289	<0.0001
	0.3 ng/µL	5.53	3.931	0.0015
	0.2 ng/µL	6.72	4.961	0.0002
	0.1 ng/µL	3.78	1.649	0.0005
P. aeruginosa ATCC 27853	Cell Media control	-5.5	4.487	0.0575
	0.5 ng/µL	1.5	5.827	0.0096
	0.4 ng/µL	1.32	5.814	0.0021
	0.3 ng/µL	0.01	3.515	0.0027
	0.2 ng/µL	-0.8	4.739	0.0224
	0.1 ng/µL	1.74	4.328	0.0006
C. albicans ATCC 10231	Cell Media control	-36.3	19.63	<0.0001
	0.5 ng/µL	4.25	3.060	<0.0001
	0.4 ng/µL	7.12	1.928	<0.0001
	0.3 ng/µL	6.98	2.344	<0.0001
	0.2 ng/µL	4.87	3.63	<0.0001
	0.1 ng/µL	4.65	3.797	<0.0001

Legend- Evaluated concentrations of Mitochondria, which are indicated in the table above. Microbial growth values of each sample and control were calculated as the percentage of microbial growth through the optical density comparison between each treatment concentration (mitochondria and pathogen) and cell media control. The positive control was also evaluated (pathogen growth in only medium culture) and it was considered 100% when compared to cell media control. All sterility control of pathogens (Negative control) showed no growth and were considered as 0.00%. All statistical analyses (p-values) were analyzed using a non-parametric Wilcoxon test (95% confidence interval) for comparison between microbial growth % values. The p-values obtained when comparing cell media control and treated samples.

Table 3. Overall results of fluorescence microscopy using live/dead staining by mitochondria extract concentrations against clinically relevant pathogens.

		***S. aureus* ATCC 25923**					***E. coli* ATCC 25922**				
Samples	n[c]	Mean of cells/frame (SD)[c]	Mean of cells/ml (SD)	Dead % (SD)	Live % (SD)	Wilcoxon test *p*-value	Mean of cells/frame (SD)[c]	Mean of cells/mL (SD)	Dead % (SD)	Live % (SD)	Wilcoxon test *p*-value
Control	2	6.35E+03 (1.13E+03)	7.62E+05 (1.35E+05)	6.925 (5.024)	93.07 (5.024)		4.53E+03 (1.22E+03)	5.43E+05 (1.47E+05)	3.065 (5.734)	96.94 (5.734)	
0.5 ng/µL	2	3.14E+03 (8.88E+02)	3.77E+05 (1.06E+05)	16.15 (20.93)	83.85 (20.93)	<0.0001[a] 0.0919[b]	2.37E+03 (1.33E+03)	2.84E+05 (1.60E+05)	27.15 (54.84)	97.26 (3.846)	<0.0001[a] 0.0006[b]
0.3 ng/µL	2	4.84E+03 (1.46E+03)	5.80E+05 (1.75E+05)	7.25 (4.173)	92.72 (4.173)	0.005[a] 0.7526[b]	3.50E+03 (1.32E+03)	4.20E+05 (1.58E+05)	10.7 (10.99)	89.3 (10.99)	0.0205[a] 0.0014[b]
0.1 ng/µL	2	4.32E+03 (1.25E+03)	5.18E+05 (1.49E+05)	4.25 (3.69)	95.74 (3.69)	<0.0001[a] 0.05[b]	3.07E+03 (8.57E+02)	3.69E+05 (1.03E+05)	2.738 (3.846)	72.85 (54.84)	<0.0001[a] 0.8252[b]
		***P. aeruginosa* ATCC 27853**					***C. albicans* ATCC 10231**				
Samples	n[c]	Mean of cells/frame (SD)[c]	Mean of cells/mL (SD)	Dead% (SD)	Live % (SD)	Wilcoxon test *p*-value	Mean of cells/frame (SD)[c]	Mean of cells/mL (SD)	Dead % (SD)	Live % (SD)	Wilcoxon test *p-value*
Control	2	4.41E+03 (1.62E+03)	5.29E+05 (1.95E+05)	1.005 (1.342)	99 (1.342)		6.43E+03 (1.34E+03)	7.71E+05 (1.60E+05)	2.622 (3.626)	97.38 (3.626)	
0.5 ng/µL	2	2.85E+03 (5.94E+02)	3.42E+05 (7.12E+04)	23.57 (29.37)	76.43 (29.37)	0.0004[a] <0.0001[b]	2.65E+03 (1.26E+03)	3.18E+05 (1.51E+05)	10.2 (12.46)	89.8 (12.46)	<0.0001[a] 0.0315[b]
0.3 ng/µL	2	4.11E+03 (1.03E+03)	4.94E+05 (1.24E+05)	38.74 (12.16)	61.26 (12.16)	0.5682[a] <0.0001[b]	6.47E+03 (2.00E+03)	7.76E+05 (2.40E+05)	8.739 (7.881)	91.26 (7.881)	0.7369[a] 0.0013[b]
0.1 ng/µL	2	3.58E+03 (7.01E+02)	4.30E+05 (8.41E+04)	37.25 (22.29)	62.75 (22.29)	0.0549[a] <0.0001[b]	6.38E+03 (1.12E+03)	7.66E+05 (1.35E+05)	8.602 (4.298)	91.4 (4.298)	0.7369[a] <0.0001[b]

Legend – Results obtained from image analysis, we chose 3 concentrations for the mitochondria treatment against each pathogen to compare the microbial growth against positive control using the dead/live assay by epifluorescence microscopy to obtain the total cell count and live/dead percentages. All images were processed in ImageJ by Fiji version 1.5 using the macros Biofilms Viability checker (see methods), and then images were processed by a sequence of modules forming a pipeline in Cell Profiler software. The applied pipeline is previously described (Atiencia-Carrera et al., 2022; Cabezas-Mera et al., 2023). [a] Wilcoxon test was realized in cells/mL results between positive control

and treated samples. [b] Wilcoxon test was realized in dead cell results between positive control and treated samples. [c] number of assays on different days, in each assay, we collected at least 12 photographs for counting. cell/mL was calculated by the following formula: average of pathogen cells (SD)* (1E+08 μm^2/12880μm^2)*(1000µL/100µL).

7. FIGURES

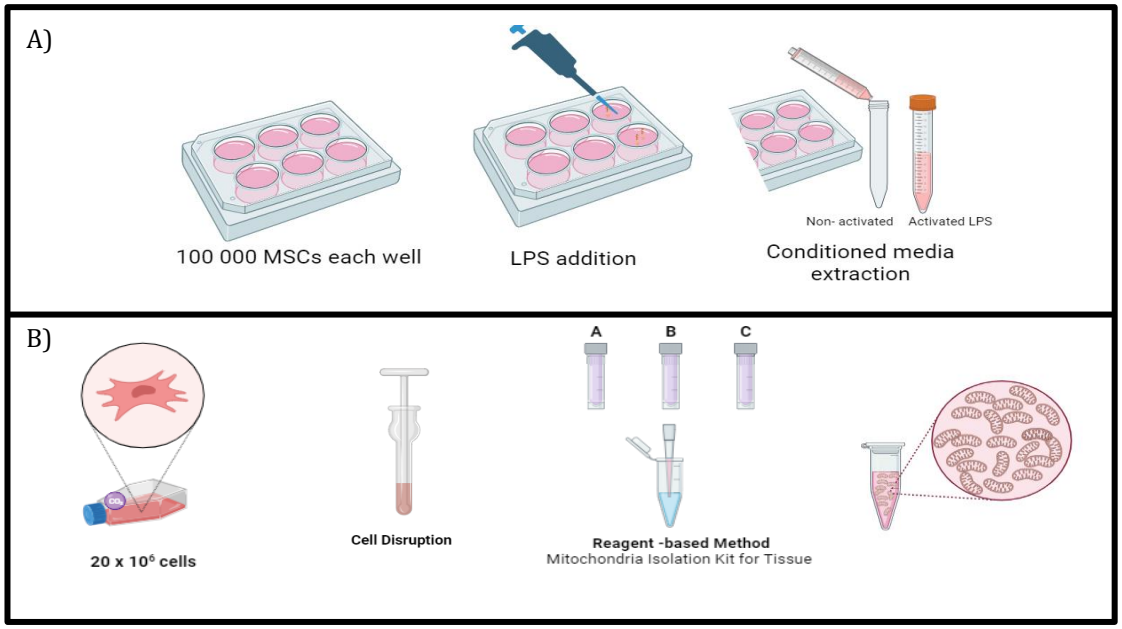

Figure 1. Illustration of MSC-conditioned media extraction and mitochondria extraction.

The figure summarizes the extraction process of MSC-conditioned media (A). The process consisted of the first MSCs growth in 6-well plate for one day and after some washes with LPS was added into some wells in order to LPS-activated the MSCs to excrete factors against gram-negative bacteria. Finally, after incubation, the LPS-activated and non-activated MSC-conditioned media were extracted. For mitochondria extraction (B), a commercial kit was used, more exactly the Mitochondria Isolation kit for tissue by Thermo Fisher Scientific. This kit consists in 3 reagents, which allow outer membrane lysis and separation between mitochondria with the rest of the membrane through weight.

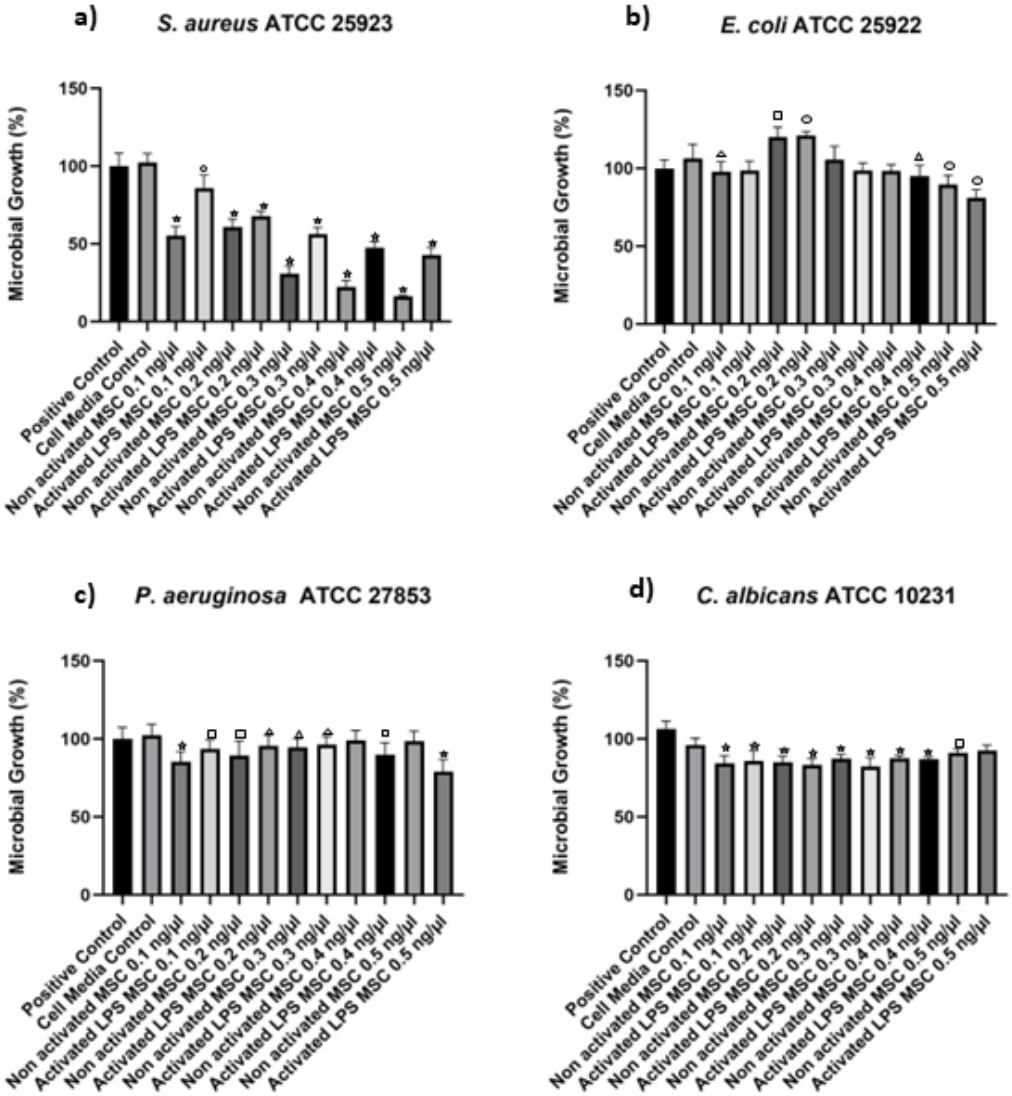

Figure 2. Representative illustration of the antimicrobial assays by MSC-conditioned media.

(A) Evaluation of the antimicrobial activity against *S. aureus* ATCC 25923. (B) Evaluation of the antimicrobial activity against *E. coli* ATCC 25922. (C) Evaluation of the antimicrobial activity against *P. aeruginosa* ATCC 27853. (D) Evaluation of the antimicrobial activity against *C. albicans* ATCC 10231. All statistical analyses (p-values) were analyzed using a nonparametric Mann–Whitney test (95% confidence interval) for comparison between Microbial growth values, where: △p-values < 0.05; □p-values < 0.001; ○p-values < 0.0001; ★p-values < 0.00001.

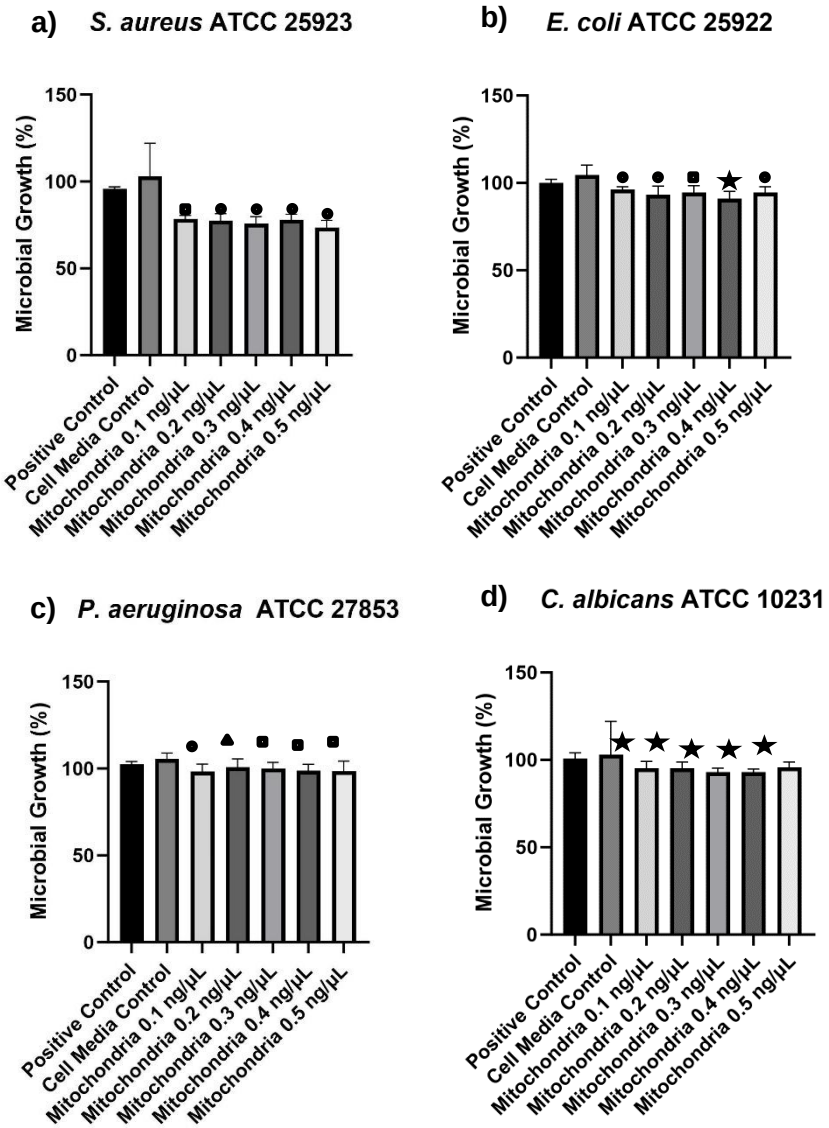

Figure 3. Representative illustration of the antimicrobial assays by mitochondria extract concentrations.

(A) Evaluation of the antimicrobial activity against *S. aureus* ATCC 25923. (B) Evaluation of the antimicrobial activity against *E. coli* ATCC 25922. (C) Evaluation of the antimicrobial activity against *P. aeruginosa* ATCC 27853. (D) Evaluation of the antimicrobial activity against *C. albicans* ATCC 10231. All statistical analyses (p-values) were analyzed using a nonparametric Mann–Whitney test (95% confidence interval) for comparison between Microbial growth values, where: △p-values < 0.05; □p-values < 0.001; ○p-values < 0.0001; ★p-values < 0.00001.

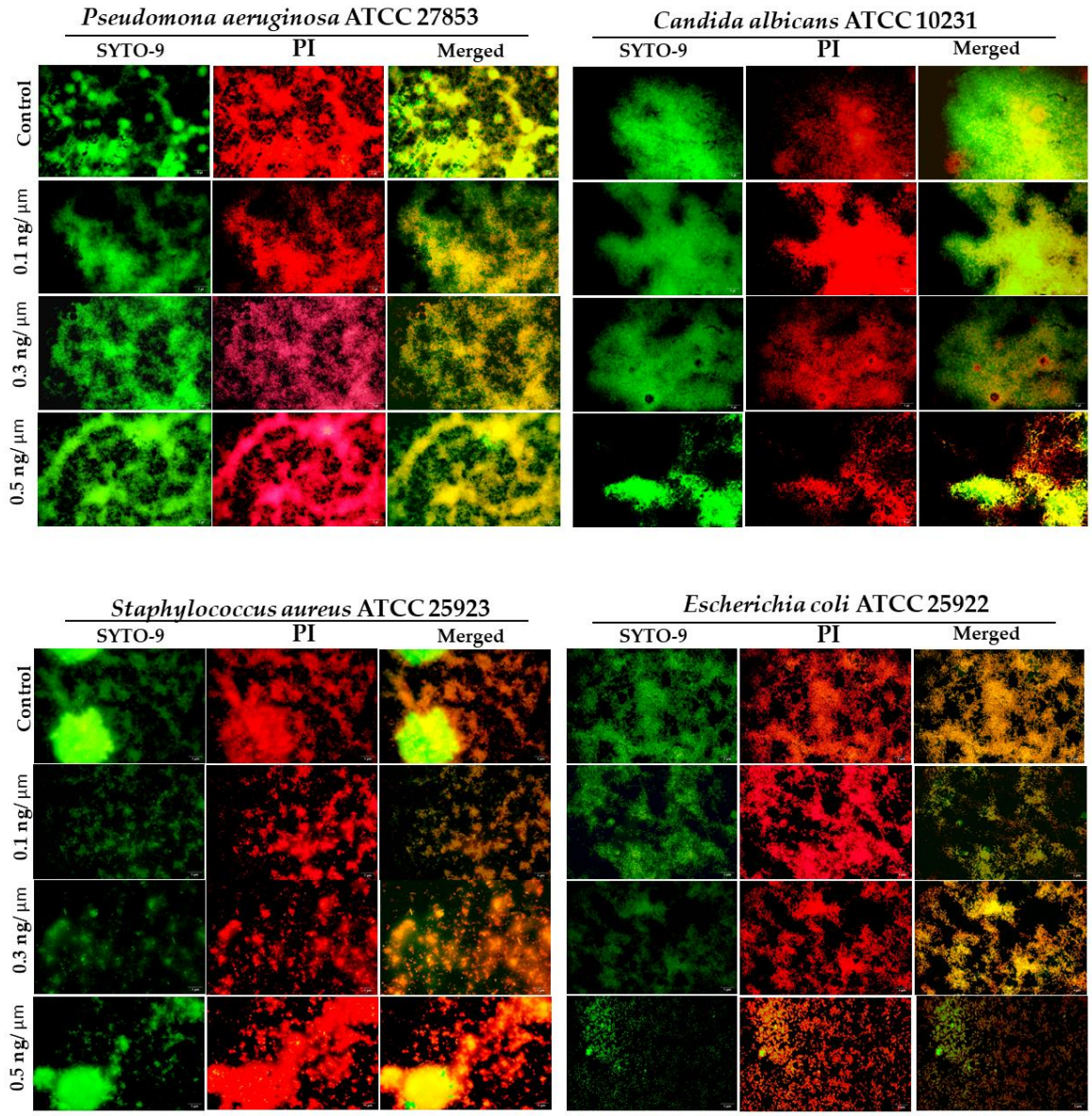

Figure 4. Illustration of fluorescence microscopy analysis in Microbial Growth with Mitochondria treatments.

Microbial growth of *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 under different Mitochondria treatments by fluorescence microscopy using Live/Dead staining assays. The pictures illustrated control and treated samples being evaluated for the total cell count and the live/ dead cells. An Olympus BX50 microscope with a total 1000X magnification was used, images were obtained with AmScope software, and images were merged with Fiji-ImageJ software.

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