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MICROBIOLOGY

Coculturing *Streptomyces* sp. with *Acanthamoeba polyphaga* enhances the antimicrobial effectiveness of its crude extract against multidrug-resistant *Pseudomonas aeruginosa* and *Escherichia coli*

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Abstract: Bacterial infections stand as prominent contributors to global mortality and morbidity rates. Harnessing the potential antimicrobial activity of secondary metabolites derived from natural sources holds promise for developing novel therapeutic drugs. *Streptomyces* spp. represents pivotal microorganisms in the synthesis of these compounds. *Acanthamoeba* spp. serves as natural virulence amplifiers for a wide range of bacterial pathogens. This study evaluates the antimicrobial efficacy of crude extracts of *Streptomyces* sp. cocultured trials with *Acanthamoeba polyphaga* against multidrug-resistant *Pseudomonas aeruginosa* and *Escherichia coli*. The production of crude extracts from *Streptomyces* sp. was monitored over 28 days. The antimicrobial activity against *P. aeruginosa* and *E. coli* was evaluated by measuring the inhibitory halos. Viability amoebae and bacteria were assessed. A slight decrease in the viability of *A. polyphaga* was noted during the coculture. Conversely, coculture promoted bacterial growth and facilitated the synthesis of extracts that showed antimicrobial effects against *P. aeruginosa* and *E. coli*, while showing no impact *on* amoebae. The extracts were active mainly against *P. aeruginosa*. The findings show that the interaction between *A. polyphaga* and *Streptomyces* sp. modulates the production of antimicrobial secondary metabolites by bacteria. Further investigations are needed to characterize the nature of this modulation, and the bactericidal components.

Key words: *Acanthamoeba polyphaga*, *Streptomyces* sp., coculture, crude extract, antimicrobial secondary metabolites.

INTRODUCTION

Bacterial infections stand as one of the foremost contributors to mortality and morbidity across all human diseases worldwide (GBD 2018). Bacterial strains that are not susceptible to one or more antimicrobials from different classes are defined as multidrug-resistant (MDR) (Magiorakos et al. 2012).

The rapid and relentless spread of MDR strains poses a significant threat to the effective treatment of a growing range of infectious diseases with conventional antibiotics. Furthermore, the increasing use of broad-spectrum antibiotics contributes to high rates of MDR, thus perpetuating the cycle (WHO 2014, Khameneh et al. 2016, Muhie 2019, Duin & Paterson 2020). Furthermore, the emergence of MDRs in both community and hospital acquired infections has outpaced the speed of development and delivery of new drugs to clinical settings (Devasahayam et al. 2010).

In this context, the study of secondary metabolites from microbial sources with potential antimicrobial activity has emerged as a promising alternative for the development of new therapeutic drugs against MDRs. Among microorganisms, actinobacteria, especially *Streptomyces* spp., are of great importance, as they are known producers of many secondary compounds with diverse biological and antimicrobial activities (Manivasagan et al. 2014, Riahi et al. 2019, She et al. 2020). The production of secondary metabolites in *Streptomyces* spp. is initiated especially when sporulation and the development of aerial hyphae begin (Rehan et al. 2021).

Acanthamoeba spp. are opportunistic protozoa belonging to the group of free-living amoebae (FLA) found widely distributed in the environment (Otero-Ruiz et al. 2022, Zhang et al. 2023, Chaúque et al. 2023, da Silva et al. 2024). These species have two developmental stages: trophozoites, the vegetative feeding forms, and cysts, which are resistant to a wide range of biocides and exhibit minimal metabolic activity (Zhang et al. 2023, Dos Santos et al. 2024).

Acanthamoeba spp. including *Acanthamoeba polyphaga*, are versatile organisms that modulate microbial communities (Rønn et al. 2002, Greub & Raoult 2004, Rosenberg et al. 2009). Besides utilizing bacteria as a food source, *Acanthamoeba* spp. also serve as vectors/reservoirs for bacterial pathogens (Siddiqui & Khan 2012), as well as natural amplifiers of the virulence of a wide range of bacterial pathogens, making them virulent or enhancing their pathogenicity (Evstigneeva et al. 2009, Dey et al. 2012, 2019 Mou & Leung 2018). This group of bacteria is known as Amoeba-Resistant Bacteria (ARB) because they can resist post-phagocytosis amoebic digestion (Thomas et al. 2010) and can survive inside the amoebic cell, altering its virulence repertoire (Guimarães et al. 2016, Gomes et al. 2018, Denet et al. 2018).

The production of secondary metabolites by bacteria depends on environmental factors, such as growing conditions, including stresses of both biotic and abiotic nature (Liu et al. 2017). Strategies that mimic natural habitats and promote the coexistence of microorganisms sharing the same space could be explored. *A. polyphaga* and *Streptomyces* spp., which co-occur in soil, may be utilized to enhance the production of secondary metabolites *in vitro* (Sandland et al. 2007, Beck et al. 2012). The production of bioactive compounds in coculture assays of *Streptomyces* spp. with other microorganisms is documented in the literature (Cho & Kim 2012, Yu et al. 2015).

Therefore, in this study, coculture of *Streptomyces* sp. and *A. polyphaga* was used as an alternative approach for the production of crude extract with secondary metabolites of *Streptomyces* sp. and its antimicrobial potential was evaluated against multidrug-resistant strains of *Pseudomonas aeruginosa* and *Escherichia coli*.

Our findings demonstrated that the contact with *A. polyphaga* induced increase in the number of *Streptomyces* sp. cells, which produced secondary metabolites in the crude coculture extract. The extract showed antimicrobial activity against *P. aeruginosa* and *E. coli* but had a negligible effect on amoeba.

MATERIALS AND METHODS

Organisms and culture conditions

Acanthamoeba polyphaga

The experiments were conducted using trophozoites of *Acanthamoeba polyphaga* (ATCC 30872), an environmental isolate, which was cultured as previously documented

(Schuster 2002, Chaúque & Rott 2021). Briefly, the trophozoites were cultured axenically in PYG medium (2% peptone proteose, 0.2% yeast extract, and 1.8% glucose), supplemented with antibiotics (10,000 IU/mL of penicillin and 10,000 µg/mL of streptomycin), and incubated at 30°C for 7 days.

Streptomyces sp.

We utilized the endophytic actinomycete R18(6), belonging to the genus *Streptomyces*, previously isolated from tomato roots (*Lycopersicon esculentum*) (De Oliveira et al. 2010). The actinobacteria were cultured on Petri dishes containing starch casein agar (SCA: 10.0 g starch, 2.0 g casein, 0.034 g K_2HPO_4, 2.0 g NaCl, 2.0 g KNO_3, 0.05 g $MgSO_4$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4$, 15.0 g agar to 1000 mL of distilled H_2O) to a final concentration of 10^7 spores/mL. The plates were then incubated at 30°C for 7 days.

Pseudomonas aeruginosa and Escherichia coli

Multidrug-resistant clinical isolates of *P. aeruginosa* and *E. coli* were utilized, graciously provided by Ana Lúcia Souza Antunes/ Laboratório de Análises Clínicas da Faculdade de Farmácia-Universidade Federal do Rio Grande do Sul. The bacteria were cultured in TSB medium (tryptic soy broth: 17.0 g casein peptone, 3.0 g soy peptone, 2.5 g glucose, 5.0 g sodium chloride, 2.5 g dipotassium phosphate per 1000 mL of distilled H_2O) at 37°C for 24 and 48 hours.

Coculture assays of *Streptomyces* sp. and *A. polyphaga*

The coculture assays were performed as previously described in literature (Yli-Pirilä et al. 2006). Briefly, 5 mL of phosphate buffer solution (PBS) containing 10^6 cells/mL of *A. polyphaga* trophozoites were added to cell culture flasks (25 cm^2) and allowed to adhere for 30 minutes under incubation at 30°C. Subsequently, 5 mL of PBS containing 10^7 spores/mL of *Streptomyces* sp. were added, resulting in a final coculture volume of 10 mL.

A total of 84 cell culture flasks were utilized, of which 18 bottles were used as control of *A. polyphaga* and 33 bottles were used as control of *Streptomyces* sp., while 33 flasks were allocated for coculture assays. Both controls, *A. polyphaga*, and *Streptomyces* sp., were independently cultured under identical conditions as the coculture trials. In summary, all 84 cell culture flasks underwent incubation at 30°C for varying durations, ranging from 24 to 672 hours, based on the specific experimental objectives, such as assessing cell viability or producing crude extracts.

The cell viability of both amoebae and bacteria was assessed at 24, 48, 72, 96, 120, and 144 hours of incubation. Viability assays were conducted in triplicate for each incubation period, (resulting in a total of 54 bottles out of the initial 84). Amoebae, and bacterial viability following coculture were determined via light microscopy examination of 1 mL pellet aliquots stained with 0.4% trypan blue vital exclusion dye (Sigma-Aldrich, USA), and 0.4% acridine orange (Sigma-Aldrich, USA), respectively.

Two dilutions (10^{-1}, 10^{-2}) of each coculture sample were also plated on SCA medium and subsequently incubated at 30°C for 5 days to determine the number of bacterial colony-forming units (CFU).

Crude extract production from coculture assays

The remaining 30 cell culture flasks (15 for Streptomyces sp. control and 15 for coculture assays) were designated for crude extract production following the coculture experiments. These flasks were maintained at 30°C and assessed weekly at five time points (T) in terms of incubation days, specifically: T1 = 24h, T7 =

168h, T14 = 336h, T21 = 504h, and T28 = 672 h, totaling 28 days. All experiments were conducted in triplicate.

In each analysis, 6 cell culture bottles (3 bacterial controls and 3 coculture samples) were obtained, and the cell content was lysed by pulling the suspension through a 27 G needle for 7 times (Steenbergen et al. 2004). After lysis, the resulting cell content was seeded on SCA medium and incubated in Petri dishes at 30°C for 5 days.

The originated colonies were inoculated into 50 mL of casein starch broth (SCB) stored in 250 mL erlenmeyer flasks and incubated at 30°C for 48 h under shaking at 100 rpm. Five milliliters of this pre-inoculum were transferred to new erlenmeyer flasks containing 50 mL of SCB and incubated at 30°C for up to 10 days under shaking at 100 rpm to produce the crude extract. The crude extract, prepared from the SCB medium containing actively growing bacteria previously cocultivated with *A. polyphaga*, will henceforth be referred to as coculture crude extract (CCE). The antimicrobial activity of the CCE was assessed daily. On each day (from day 1 to 10), the cellular culture was centrifuged for 10 minutes at 5,000 rpm to separate the cell mass and the supernatant. An aliquot of supernatant was removed for an additional centrifugation for 10 min at 13,000 rpm in order to obtain a cell-free extract.

Evaluation of the antimicrobial activity resulting of the coculture assays

Antibiosis by well diffusion

The antibiosis assay was adapted from Devillers et al. (1989). A bacterial suspension of *P. aeruginosa* or *E. coli* (density 10^8 cells/mL) was seeded on plates containing 20 mL of TSA medium (tryptone soy agar). Wells were performed on agar using cylinders with 9 mm diameter on equidistant positions along the plate and filled with 100 µL of the CCE. The plates were incubated at 4°C for 16 hours to facilitate the diffusion of the extract from the wells into the culture medium. Subsequently, the plates were incubated at 37°C for 24 and 48 h for the bacterial growth. The antimicrobial activity against *P. aeruginosa* and/or *E. coli* was assessed by monitoring the formation of inhibitory halos and measuring their size. All assays were performed in independent triplicates.

Evaluation of the amoebicidal activity from coculture crude extract

To assess the amoebicidal activity of extract against *A. polyphaga*, trophozoites and cysts (1.6×10^5 cells/mL) were exposed to CCE and the control extract produced during T1 (24 h) and T21 (504 h) of coculture for 24 and 48 hours. Dilutions of 1:1, 1:2, and 1:4 were prepared and tested in a 96-well plate. The T1 and T21 extract samples were chosen due to their demonstrated high bactericidal activity in preliminary assessments. Viability was evaluated using the 0.4% trypan blue exclusion staining test under light microscopy and quantified using a Fuchs Rosenthal counting chamber.

Statistical analysis

The results obtained with the well-diffusion assays were assessed by analysis of variance (ANOVA) multifactorial and Tukey's test to 95% of confidence interval (after verifying the normality using Shapiro-Wilk test, 5%) using the BioEstat 12.0 software. Graphs were generated using the GraphPad Prism 8.02 software to visualize the data.

RESULTS

Evaluation of the cellular viability after coculture assays

A slight progressive decrease in the viability of *A. polyphaga* trophozoites was observed during the coculture times analyzed (24 to 144 h) compared to the control (Figure 1).

A. polyphaga viability in coculture with *Streptomyces* sp. over time. The viability of amoebae after coculture was evaluated in 1 mL pellet aliquots stained with exclusion trypan blue vital dye 0.4%. The assays were performed in triplicates

On the other hand, the viability of *Streptomyces* sp. spores was not affected by coculture assays with *A. polyphaga* compared to control by staining with acridine orange dye. A positive effect of the amoebae on the growth of *Streptomyces* sp. was observed with a significant increase in the amount of bacterial spores after the coculture assays, hampering the count of the CFU at two serial dilutions (10^{-1}, 10^{-2}). Furthermore, the *Streptomyces* sp. spores developed into hyphae after 24 hours of coculture, unlike of the control where the organisms remained in the spores form.

Evaluation of the antimicrobial activity of coculture crude extract by well diffusion

The CCE showed antimicrobial activity against *P. aeruginosa* and *E. coli*. The analysis of the antimicrobial activity of the CCE was performed daily for 10 days after each incubation period (5 periods) by measuring of inhibitory halos formed against *E. coli* and *P. aeruginosa*. On the period 1 (24 h) of coculture assays, the CCE and extract control showed activities starting at the second day of incubation (Figure 2).

Antibacterial activity of the crude extract from *Streptomyces* sp. previously cocultured with *A. polyphaga* for 24 hours. The evaluation of the antimicrobial activity of the crude extract against *Escherichia coli* and *Pseudomonas aeruginosa* was performed by analysis of the formation of inhibitory halos, measured over 10 days. The control used was the extract from *Streptomyces* cultured individually. The assays

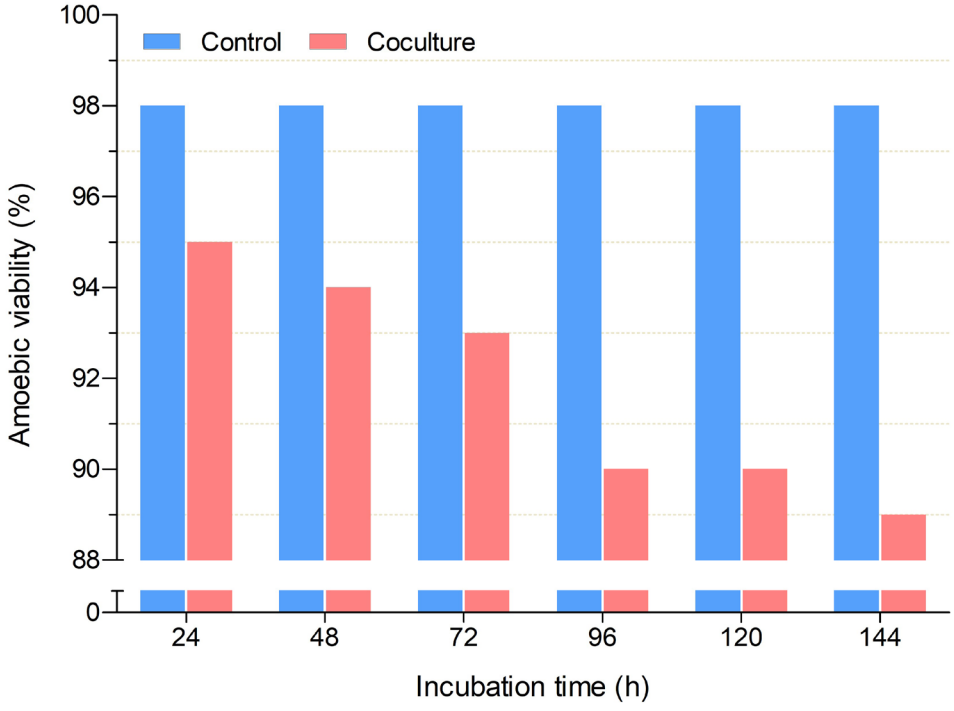

Figure 1. ***Acanthamoeba polyphaga*** **viability in coculture with** ***Streptomyces*** **sp. over time. The viability of amoebae after coculture was evaluated in 1 mL pellet aliquots stained with exclusion trypan blue vital dye 0.4%. The assays were performed in triplicates.**

were performed in triplicates. (*) - Statistically different from the corresponding control ($p < 0.05$).

The CCE exhibited significantly higher antimicrobial activity compared to the control ($p < 0.05$) starting from the second day for both *E. coli* and *P. aeruginosa*, with a decline observed on the sixth day against *E. coli*. Days 4 to 8 revealed the peak antimicrobial activity rates of CCE against *P. aeruginosa* (Figure 2). Figure 3 displays the inhibition halos observed on the eighth day of assessing the crude extract's effects against *E. coli* and *P. aeruginosa*.

Visualization of inhibition halos by the effect of the crude control extract (a and c) and co-culture (b and d), against *E. coli* (a, b) and *P. aeruginosa* (c, d). The figure refers to the eighth day of monitoring the effect of the extract produced through co-cultivation of *Streptomyces* sp. and *A. polyphaga* for 24 hours.

The CCE, produced through the coculture of *Streptomyces* sp. for 7 days (168 h) with *A. polyphaga*, demonstrated antimicrobial activity against *P. aeruginosa* and *E. coli* on days 9 and 10, while the control exhibited no activity during this period. Notably, while the antimicrobial activity of CCE surpassed that of the control on days 3 and 5 against *P. aeruginosa* ($p < 0.05$), it either remained similar or, in certain instances, significantly decreased against both bacteria on the other days (Figure 4a).

The CCE obtained after 14 days (336 hours) of cocultivation exhibited significantly higher antimicrobial activity ($p < 0.05$) compared to the corresponding control on days 2, 3, 4, and 8 against *P. aeruginosa*, and on day 6 against *E. coli*. Furthermore, on day 10, only the CCE displayed activity against both bacteria (Figure 4b).

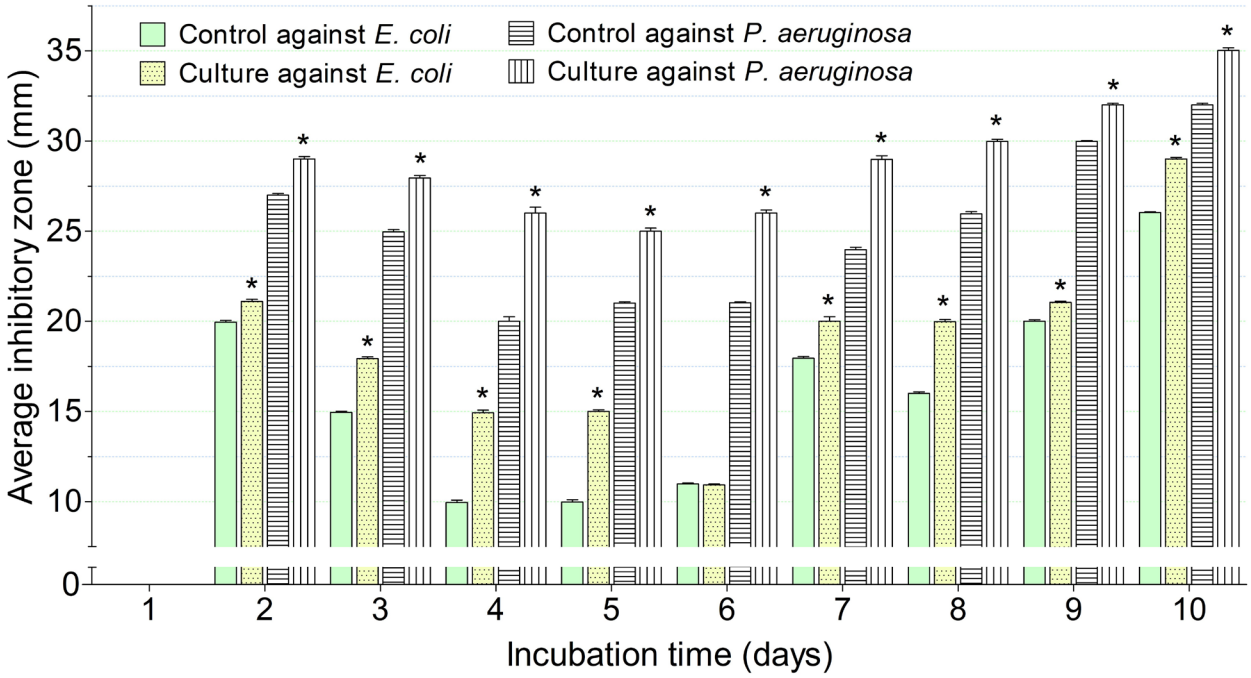

Figure 2. Antibacterial activity of the crude extract from *Streptomyces* sp. previously coculture with *Acanthamoeba polyphaga* for 24 hours. The evaluation of the antimicrobial activity of the crude extract against *Escherichia coli* and *Pseudomonas aeruginosa* was performed by analysis of the formation of inhibitory halos, measured over 10 days. The control used was the extract from *Streptomyces* sp. cultured individually. The assays were performed in triplicates. (*) - Statistically different from the corresponding control ($p < 0.05$).

The antimicrobial effect of CCE prepared after 28 days (672 hours) of cocultivation did not exhibit a statistically significant difference compared to the control ($p < 0.05$). Bactericidal activity of CCE was only observed during the initial phase of the test (days 1 to 4), evident on days 1 and 2 against both bacteria, and exclusively on days 3 and 4 against *P. aeruginosa* (Figure 4c).

Antimicrobial activity of the coculture crude extract after 672 h or period 28 of contact between *A. polyphaga* and *Streptomyces* sp. Evaluation of the antimicrobial activity on 10 days after 168 h, 7th incubation time of coculture (a); 336 h, 14th incubation time (c) and 672 h, 28th incubation time (d). The assessment was performed by formation of inhibitory halos against *E. coli* and *P. aeruginosa* The control used was the extract from *Streptomyces* sp. cultured individually. The assays were performed in triplicates. (*) - Statistically different from the corresponding control ($p < 0.05$).

Evaluation of the amoebicidal activity from coculture crude extract

After 48 h of incubation of the CCE with *A. polyphaga* trophozoites or cysts, no amoebicidal activity was detected, indicating that the CCE from *Streptomyces* sp. was more effective against bacterial cells.

DISCUSSION

Bioactive compounds synthesized by microorganisms hold significant promise as pharmaceutical resources, particularly in the realm of antimicrobials. The imperative to explore this reservoir intensifies amidst the global challenge of escalating resistance to various antimicrobial agents constituting our current

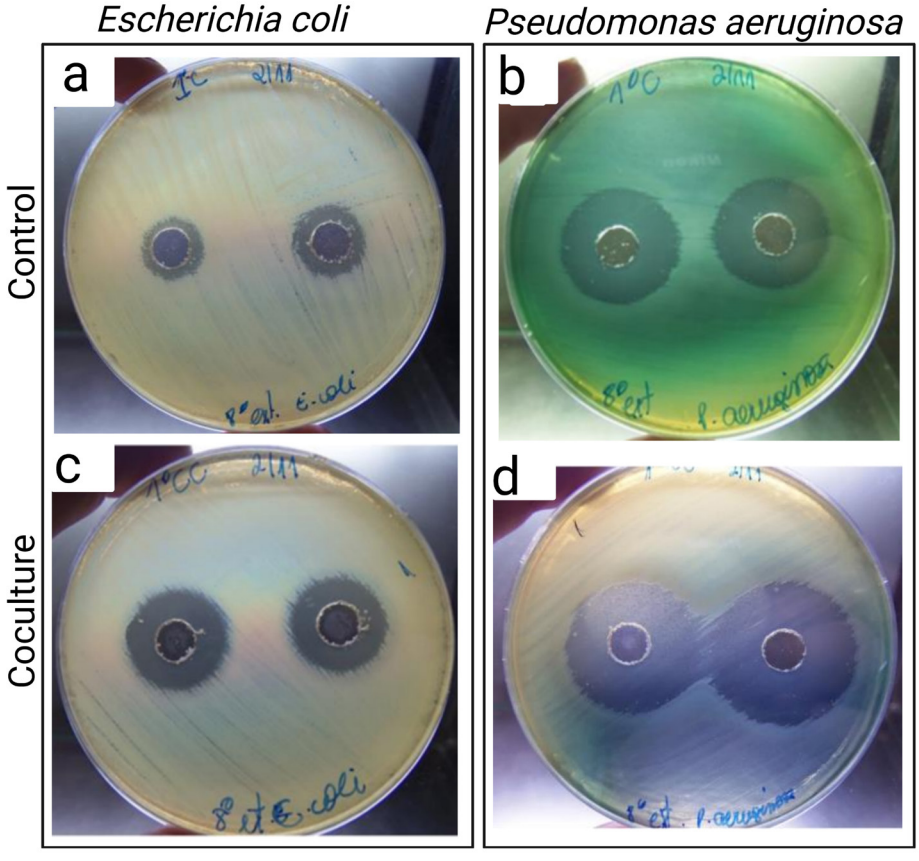

Figure 3. Visualization of inhibition halos by the effect of the crude control extract (a and c) and coculture (b and d), against *Escherichia coli* (a, b) and *Pseudomonas aeruginosa* (c, d). The Figure refers to the eighth day of monitoring the effect of the extract produced through cocultivation of *Streptomyces* sp. and *Acanthamoeba polyphaga* for 24 hours.

pharmacological arsenal. In the present study, we explored the coculture of *Streptomyces* sp. and *A. polyphaga* as an alternative approach to induce the production of secondary metabolites from *Streptomyces* sp., with antimicrobial potential against multidrug-resistant bacteria.

Our findings show that coculture had no negative impact on amoeba viability. The high amoebic viability observed after coculture with *Streptomyces* sp. resembled that reported in a previous study involving the coculture of *A. polyphaga* and *Staphylococcus aureus* (Souza et al. 2017). The maintenance of high amebic viability may be advantageous for bacterial growth and subsequent production of secondary metabolites. *Streptomyces* sp. caused

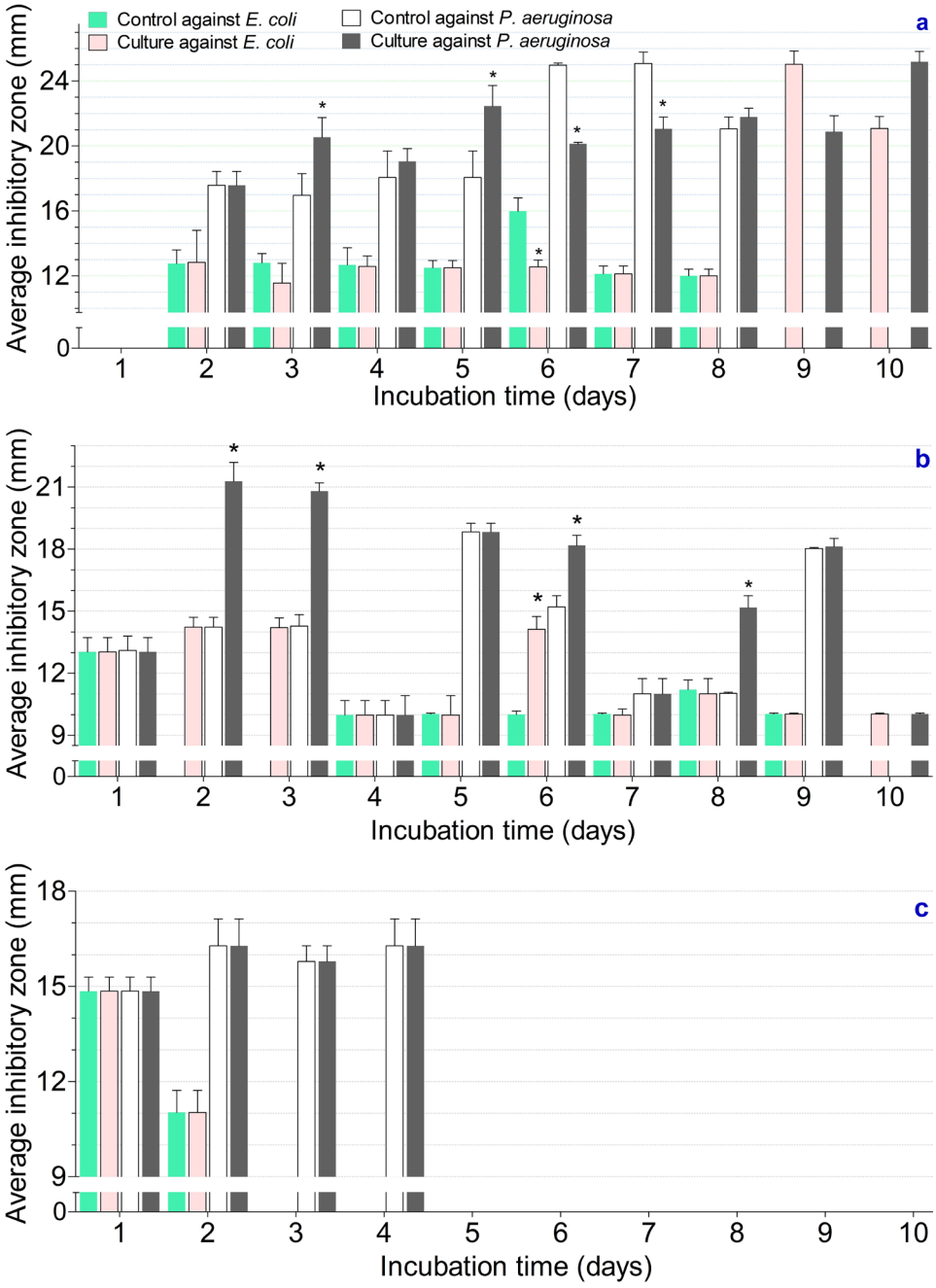

Figure 4. Antimicrobial activity of the coculture crude extract after 672 h or period 28 of contact between *Acanthamoeba polyphaga* and *Streptomyces* sp. Evaluation of the antimicrobial activity on 10 days after 168 h, 7th incubation time of coculture (a); 336 h, 14th incubation time (b) and 672 h, 28th incubation time (c). The assessment was performed by formation of inhibitory halos against *Escherichia coli* and *Pseudomonas aeruginosa*. The control used was the extract from *Streptomyces* cultured individually. The assays were performed in triplicates. (*) - Statistically different from the corresponding control ($p < 0.05$).

a slight decrease in the *A. polyphaga* viability during the coculture assays probably due to the production of the same toxic compounds, as demonstrated with isolated compounds from *Streptomyces sanyensis* (Cartuche et al. 2019a, b), which induced apoptosis in *Acanthamoeba* via the mitochondrial pathway.

The cysts formation due to the scarcity of nutrients and/or by pH decrease caused by metabolic products cannot be discarded. Furthermore, after ingestion/invasion of *Streptomyces* sp. spores the amoebae may have been a food source for development in aerial hyphae (Yli-Pirilä et al. 2006, Lamrabet et al. 2012, Santos-Montañez et al. 2015).

The viability of *Streptomyces* sp. spores was not affected by coculture assays with *A. polyphaga*. In response to environmental changes, the bacteria have developed elaborate genic expression transcriptional regulatory systems that enable fine-tuning of factors that allow for their adaptation and proliferation (Stella et al. 2015).

The high amplification of the growth and development of bacteria in the presence of *A. polyphaga* could have been induced either by reproduction inside the amoebae or by the provision of nutrients owing to the amoebic breakdown (Yli-Pirilä et al. 2006). The lysis of the cells may result from hyphae development within the amoebae. Additionally, *A. polyphaga* may act as a buffer, limiting acidification upon contact with *Streptomyces* sp., thereby facilitating the persistence of bacterial growth (Douesnard-Malo & Daigle 2011).

These results demonstrated that the coculture assays with *A. polyphaga* may be used for the achievement of antimicrobial secondary metabolites from *Streptomyces* sp. in larger quantities compared to bacterium cultured individually. Besides the difficult *in vitro* culture, under traditional conditions (monoculture), only a few compounds have so far been isolated, far less than what is expected based on the genome from *Streptomyces* sp. (Cartuche et al. 2019b, Antunes et al. 2013, Kemung et al. 2018).

The CCE showed antimicrobial activity against *P. aeruginosa* and *E. coli*, indicating that toxic secondary metabolites were produced from *Streptomyces* sp. during the coculture. *P. aeruginosa* and *E. coli* MDRs are Gram-negative bacteria that cause serious infections, such as sepsis especially in patients with compromised immune systems and gastrointestinal diseases, respectively (Dey et al. 2019, Alizade et al. 2019).

Antimicrobial resistance is recognized as one of the greatest threats to public health, and global concern and urgently requires the discovery of novel and more efficient therapeutics drugs. *Streptomyces* spp. represents a significant source for supplying bioactive natural products with clinical and pharmaceutical applications (Sivalingam et al. 2019).

A decline in the activity of CCE from *Streptomyces* sp. was observed against *E. coli* showing a differential effect of these compounds, as already demonstrated in other studies (Duraipandiyan et al. 2016, Hug et al. 2018). The CCE were active especially against *P. aeruginosa* until 32 days after the beginning of the coculture assays. There is a broad spectrum of antibacterial potentials of *Streptomyces* spp. extracts and *P. aeruginosa* may respond more sensitively (Ahmed et al. 2020).

These results suggest a longer time-dependent activity of the CCE compared to extract from control (*Streptomyces* sp. individually) countering the idea that the production of secondary metabolites in bacteria is not always maintained when out of their immediate environment (Carlson et al. 2014).

A. polyphaga induced or optimized the production of different compounds with antimicrobial action from *Streptomyces* sp. The

contact of the two microorganisms may have stimulated the production of some compounds by amoebae that phagocyte the spores and they have triggered the production of different compounds. The interaction between two microorganisms may activate clusters of silent genes (König et al. 2013). However, little is known about the specific nature of the regulation of secondary metabolites in bacteria, as well as the conditions under which bacteria produce a determined antibiotic.

Our work provides the first evaluation of the antimicrobial activity of the CCE from *Streptomyces* sp. against *P. aeruginosa* and *E. coli* after coculture with *A. polyphaga*. The increased antimicrobial effect of the CCE compared to the control was consistent with the high ability of proliferation of *Streptomyces* sp. inside and/or in contact with amoebae during the coculture. Nevertheless, more studies are still necessary to identify the secondary metabolites included in CCE and their interactions with MDR bacteria and, thus, select new targets and/or signaling pathways to be used in future therapeutic approaches.

CONCLUSIONS

The coculture of *A. polyphaga* and *Streptomyces* sp. modulates the production of antimicrobial secondary metabolites by bacteria. The CCE from *Streptomyces* sp. showed a differential and longer time-dependent effect against *P. aeruginosa* and *E. coli*. The extracts were active especially against *P. aeruginosa* until 32 days after the beginning of the coculture assays and require further investigation.

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