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The Effect of Cell-Free Supernatants of Free-Living Amoeba against Some *Staphylococcus* Bacteria: First Findings from Turkey

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ABSTRACT

Objective: Free-living amoeba (FLA) are protozoa living in soil and in natural and man-made water systems. They attract much attention owing to the illnesses associated with them and to their relationships with bacteria. In this study, the effect of cell-free supernatant (CFS) obtained from FLA against *Staphylococcus* was investigated.

Materials and Methods: Environmental FLA strains (A1, A2, A3) were obtained from lake water and swimming pools in Istanbul. *Acanthamoeba castellanii* ATCC 50373 was used as the standard strain. Clinical *Staphylococcus* strains (S1, S2, S3) were obtained from a culture collection at Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology. As standard strains, MRSA ATCC 43300, *S. epidermidis* ATCC 12228, *S. aureus* ATCC 29213 were used. FLA-CFS were obtained by centrifuging and filtering of axenic cultures. Colony counting technique was used to investigate the inhibition activities of FLA-CFS against *Staphylococcus* bacteria.

Results: Against MRSA ATCC 43300 strain, CFSs of *A. castellanii* ATCCC 50373 and A1 showed an inhibition efficiency of 78.36% and 73.47%, respectively. Against S1 strain, CFSs of *A. castellanii* ATCCC 50373 and A2 showed an inhibition of 65.64% and 15.14%, respectively. Against *S. aureus* ATCC 29213, only A1-CFS showed inhibitory effect (44%). It was found that *A. castellanii* ATCC 50373 and A2-CFSs inhibited the S2 strain 26.20% and 9.24% respectively. Against S3 strain, A2-CFS was inhibitory at 33.33%. No FLA-CFS could be inhibitory against *S. epidermidis* ATCC 12228.

Conclusion: It is necessary to devise new studies in which sample numbers are increased when using FLA strains in the inhibition of antibiotic-resistant bacteria.

Keywords: Free-living amoeba, *Staphylococcus* infections, *Acanthamoeba*, antibacterial effect, cell-free supernatant, antibiotic resistance

INTRODUCTION

Free-living amoeba (FLA) are eukaryotic microorganisms living in soil, air, sea water, fresh water and man-made water systems (pools, cooling towers, water pipes, etc.) (1-8). They have two stages in their lifecycle, namely trophozoite and cyst (9). Trophozoites are metabolically active, feeding, mobile forms which transform into cysts. The latter are inactive metabolically and exist in adverse environmental conditions (starvation, temperature changes, pH changes, etc.) (10). Pathogenic FLA for humans are *Naegleria fowleri, Acanthamoeba spp., Balumuthia mandrillaris,* and *Sappinia spp* (5, 11). Along with their pathogenicities, FLA also attracted scientists' attention due to their interactions with other microorganisms in the environments in which they live. In this collective living, they play important roles in the

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regulation of microorganism population and microbial ecology (12, 13): *i*) They use some bacteria, fungi, and algae as food (10, 14, 15). *ii*) Some bacteria which can enter inside FLA find themselves isolated from adverse environmental conditions (antibiotics, disinfectants, etc.) and they find the opportunity to multiply (16-19). After this, they can cause lysis of FLA cells (20). *iii*) They can inhibit some bacteria which does not enter inside FLA cells (non-fagocyting). Very scarce studies in recent years show these abilities (21-24).

Recently, increasing antibiotic resistance has become an important problem in the treatment of bacterial infections. Every year, about 2.9 million infections owing to the presence of antibiotic-resistant bacteria occur and about 36 thousand of them end in death (25). From past to present, antibacterial substances have been isolated from organisms like bacteria, fungi, algae, insects, and plants (26-29).

As is the case all around the world, new antibacterial compounds are also sought after in our country because antibiotic-resistant bacteria cause important problems in the treatment of these infections. However, the number of studies showing the antibacterial effect of FLA is quite limited in the literature (22, 23). So, in this context, this research aims to look for the antibacterial effect of cell-free supernatant (CFS) of FLA strains isolated in Turkish waters against infection-agent *Staphylococcus* bacteria.

MATERIALS AND METHODS

Test Microorganisms

In this study, the antibacterial activity of CFS obtained from four FLA (*Acanthamoeba castellanii* ATCC 50373*,* A1, A2, A3) was investigated against six *Staphylococcus* strains [Methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA ATCC 43300), *Staphylococcus aureus* ATCC 29213 (*S. aureus* ATCC 29213), *Staphylococcus epidermidis* ATCC 12228 (S. *epidermidis* ATCC 12228), Methicillin-resistant *Staphylococcus aureus* S1 (S1), Methicillin-sensitive *Staphylococcus aureus* S2 (S2), Methicillin-resistant *Staphylococcus epidermidis* S3 (S3)] (Table 1). Among the FLA strains used in this study, two of them (A1 and A2) were isolated from swimming pools in a previous study of ours (8), one of them (A3) was isolated from lake water, and *A. castellanii* ATCC 50373 was used as standard strain. Clinical *Staphylococcus* strains (S1, S2, S3) used in our study were obtained from the culture collection at Istanbul University (I.U.) Faculty of Pharmacy, Department of Pharmaceutical Microbiology. MRSA ATCC 43300, *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228 were used as the standard strains.

Bacterial Cultures

MRSA ATCC 43300, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, S1, S2 and S3 strains were cultured in Trypticase soy agar (TSA) at 37°C for 24 hours and used in the experiment.

FLA Cultures

FLA strains (A1, A2) previously isolated and kept in the freezing medium (FM) at -86°C were first brought to room temperature. Petri dishes containing non-nutrient agar (NNA) spread on *Escherichia coli (E. coli*) pre-inactivated (121°C, 15 minutes) were used for the resuscitation of the strains. All Petri dishes were incubated for 3 to 4 weeks at 30°C and they were examined under inverted microscope every day (30, 31). Dense FLA trophozoite-bearing areas in NNA were marked and cut with a sterile lancet, and patched on fresh NNA medium surface (with inactivated *E. coli*) upside-down (2, 32, 33). All Petri dishes were incubated at 30°C.

Table 1. Microorganisms used in antibacterial activity experiments.

Microorganisms (Code)		Source
FLA Strains	*Acanthamoeba castellanii* ATCC 50373	Standard Strain
	A1	Swimming pool isolate
	A2	Swimming pool isolate
	A3	Lake water isolate
***Staphylococcus* Strains**	[a]MRSA ATCC 43300	Standard Strain
	[b]*S. aureus* ATCC 29213	Standard Strain
	[c]*S. epidermidis* ATCC 12228	Standard Strain
	[d]S1	Clinical isolate
	[e]S2	Clinical isolate
	[f]S3	Clinical isolate

a: Methicillin-resistant *Staphylococcus aureus* ATCC 43300, b: Methicillin-sensitive *Staphylococcus aureus* ATCC 29213,
c: Methicillin-sensitive *Staphylococcus epidermidis* ATCC 12228, d: Methicillin-resistant *Staphylococcus aureus,*
e: Methicillin-sensitive *Staphylococcus aureus,* f: Methicillin-resistant *Staphylococcus epidermidis*

First, A3-coded amoeba strain was isolated from Uluabat Lake (Bursa, Turkey) under aseptic conditions, and the water sample in a glass bottle was concentrated by passing through a Sartorius filtering device having a membrane filter (0.22 µm). Then, the filter paper was turned upside down and left on fresh NNA medium having inactivated *E. coli* and incubated at 30°C (4, 34). Petri dishes were examined on an inverted microscope on a daily basis.

FLA-Axenic Culture and FLA-CFS

To obtain FLA-axenic cultures, Pepton Yeast Extract Glucose medium (PYG) containing Page's amoeba saline (PAS) solution (PYG-PAS) was used (11, 35). Antibiotics (0.5 mg/mL penicillin and streptomycin) were added to PYG-PAS to avoid microbial contamination. Amoeba cells in the Petri dishes were collected with 2-3 mL of PAS solution and inoculated into T-25 tissue culture flask containing antibiotic-added PYG-PAS and incubated at 30°C (22, 36). When the mono-layered axenic culture was observed, the PYG-PAS containing antibiotics in the tissue culture flask was discharged and washed with fresh PAS three times, in order to clean the culture from antibiotics (17). After the last washing, fresh PYG-PAS was placed into the flask and incubated for 48-72 hours at 30°C. The FLA-axenic culture thus obtained was subjected to Thoma slide and trypan blue for counting (cell/mL) and vitality determination (37, 38). Cell-free supernatant of FLA (FLA-CFS) was obtained after centrifuging the FLA-axenic cultures (1000 g x 5 min) followed by passing the supernatant through 0.22 µm pore-diameter filters and were used in antibacterial experiments (22, 23).

Antibacterial Activity Experiments

Colony counting method was used to assess the antibacterial effect of FLA-CFSs against *Staphylococcus* bacterial strains (22, 23). Bacterial suspension (10^6 cfu/mL) in phosphate buffered saline (PBS) and FLA-CFS were mixed in a tube (1:10) and incubated for 18-24 hours at 37°C. After incubation, each mixture was diluted further with PBS 10 times to achieve a series of dilutions (10^{-1}–10^{-6}) and spread into Petri dishes containing TSA. All petri dishes were incubated for 24 hours at 37°C and after the period, colonies were counted. In the experiments, PYG-PAS and PBS were used as control groups. All experiments were performed in triplicate.

RESULTS

FLA-Axenic Culture and FLA- CFS

Figure 1 shows the images obtained under inverted microscopic examination (x100) of FLA-axenic cultures.

Each FLA-axenic culture had the following living FLA trophozoite numbers: For *A. castellanii* ATCC 50373, A1, A2, A3, the values are $3x10^5$, $8x10^5$, $5x10^5$, $1x10^5$ cell/mL, respectively. Trypan blue-dyed FLA-axenic cultures had the microscopic view (x100) of undyed/living FLA trophozoites in Figure 2.

Antibacterial Activity

The tested FLA-CFSs showed the highest inhibition effect on the MRSA ATCC 43300 strain among the *Staphylococcus* species tested (Figure 3). *A. castellanii* ATCCC 50373 coded FLA (*A. castellanii* ATCCC 50373-CFS) and A1 encoded FLA-CFS (A1-CFS) inhibited MRSA ATCC 43300 bacteria by 78.36% and 73.47%, respectively. *A. castellanii* ATCCC 50373 and A2-encoded FLA-CFS (A2-CFS) inhibited the S1 strain 65.64% and 15.14%, respectively. Against *S. aureus* ATCC 29213, only A1-CFS showed inhibitory effect (44%). It was found that *A. castellanii* ATCC 50373-CFS and

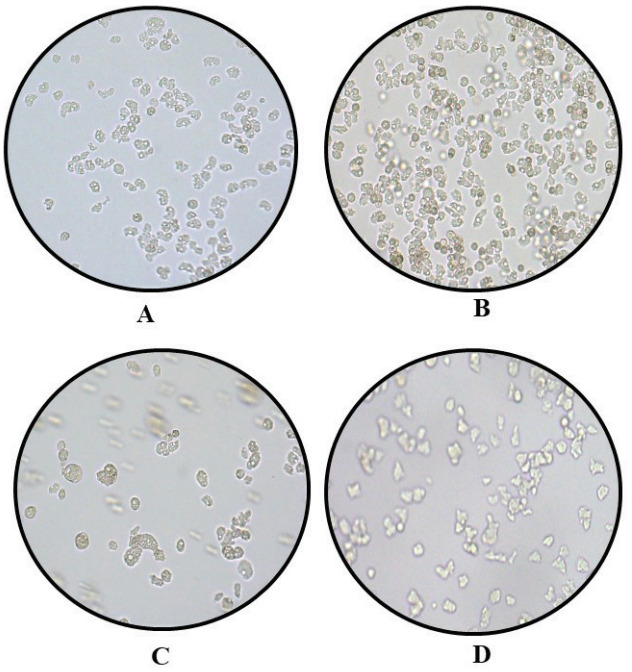

Figure 1. Microscopic images of axenic cultures obtained from each FLA (x100): A) *Acanthamoeba castellanii* ATCC 50373, B) A1, C) A2, D) A3.

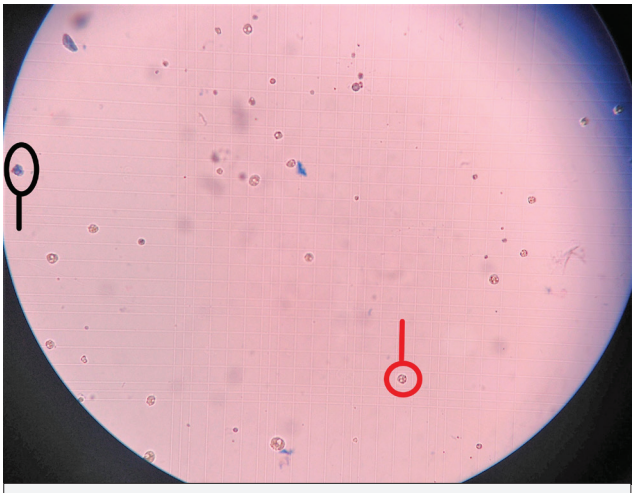

Figure 2. Light microscopic view (x100) of axenic culture of *Acanthamoeba castellanii* ATCC 50373 strain on the trypan blue-dyed Thoma slide; live trophozoite (red labelled) and dead trophozoite (black labelled).

A2-CFS inhibited the S2 strain 26.20% and 9.24% respectively. Although A2-CFS inhibited S3 strain (33.33%), other FLA-CFSs did not show antibacterial effect against this strain. None of the tested FLA-CFSs were found to have an inhibitory effect on *S. epidermidis* ATCC 12228 strain. A3-CFS was found to have no inhibitory effect on any *Staphylococcus* strain tested. However, it was determined that *Staphylococcus* strains tested were inhibited by at least one native FLA-CFS (A1-CFS or A2-CFS), except for *S. epidermidis* ATCC 12228 strain (Figure 3).

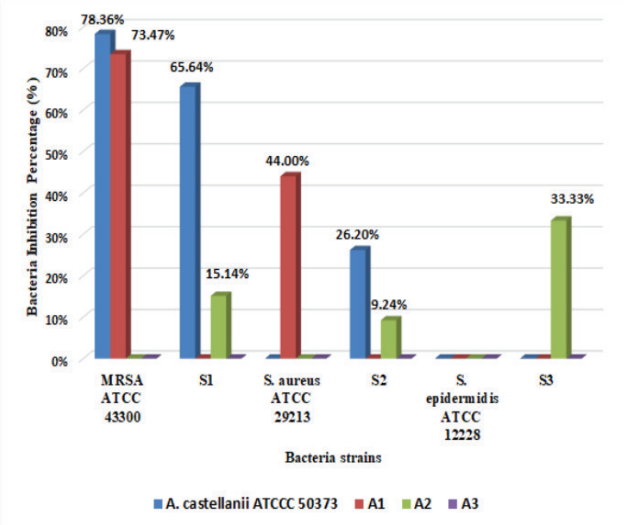

Figure 3. Inhibitory effects of cell-free supernatants (CFSs) against *Staphylococcus* strains.

According to these data, it was understood that FLA-CFS had more inhibitory effect against *S. aureus* than *S. epidermidis* generally. *S. epidermidis* ATCC 12228 strain was not found to be inhibited by any FLA-CFS.

DISCUSSION

FLA have been attracting the attention of scientists for many years due to their role of regulating microorganism populations while living together with bacteria in the same environment. In the literature, FLA-bacteria relationship has frequently centered on the abilities of amoeba phagocyting bacteria, proliferation of bacteria in amoeba and therefore, causing lysis of them (16-19, 39, 40). However, there are few studies with narrow scope about amoeba inhibiting the proliferation of bacteria by secreting antibacterial substances (apart from phagocytosis) (21-24, 41, 42). Increasing the number of studies on this topic would help researchers to understand the FLA-bacteria relationship and would enhance microbial ecologic studies, leading to the discovery of new antibacterial substances for bacteria (especially antibiotic-resistant bacteria), which has been a great burden in these times. It was determined that the FLA tested in our study had an inhibitory effect on *S. aureus* and *S. epidermidis* (except phagocytosis). The highest inhibition against *Staphylococcus* bacteria (65.64%, 78.36%) was detected with *A. castellanii* ATCC 50373-CFS. A native aquatic FLA strain obtained from our waters (A1-CFS) also yielded a similar (73.47%, 44%) inhibition effect. On the contrary, with the other native samples (for A2-CFS) these inhibitions were either low (15.14%, 9.24%, 33.33%) or (for A3-CFS) non-existent not at all (Figure 3). Nevertheless, these data show us that native FLA have the antibacterial potential against *Staphylococcus* bacteria. This study is the first one about the interaction/inhibition of FLA-*Staphylococcus* in which native strains were used. Our studies are ongoing, aiming to investigate more samples to reveal more bacteriostatic or bactericidal effects of FLA isolates against pathogen/potantial pathogen and antibiotic-resistant *Staphylococcus*. If this kind of effect were found, a new, native, and effective antibacterial substance, which would be applied to inhibition of *Staphylococcus* that are causing infections in humans, would be discovered.

Similar to our study, Nakışah and Chandrika reported that clinical and environmental (two sample) *Acanthamoeba* (FLA) lysates showed antibacterial activity against pathogen two *S. aureus* strains (21). Iqbal and co-workers reported that *A. castellanii*-CFS (one sample) was effective against clinical MRSA at 100% whereas it had a bactericidal activity against vancomycin-resistant *E. faecalis* at 8%. However, it did not show a bactericidal effect against *Acinetobacter sp., Pseudomonas aeruginosa (*22). Souza et al. reported that the relationship between a single clinical MRSA isolate and a single *Acanthamoeba polyphaga* ATCC 30461 (FLA) strain yielded that FLA culture lysate supported the growth of MRSA, but the same FLA culture supernatant inhibited the growth of the same bacteria (24). Martin et al. investigated the effects of different genus, kind, and origin of FLA-CFS against *Staphylococcus aureus* (MRSA typed as USA300) and found that *Mycobacterium bovis* biofilms and *A. polyphaga* CCAP 1501/18–CFS sample inhibited *Staphylococcus aureus* to a significant degree (42). As can be seen, there is a very low number of current studies testing a scarce number of strains. However, many more strains (four FLA strains and six *Staphylococcus* strains) were used in our study, thus making it more comprehensive. Moreover, our study used strains different from the standard strains used in other studies, including native strains. For these reasons, we are of the opinion that the inhibitory effect of FLA CFSs on *Staphylococcus* bacteria may vary depending on both FLA strains and *Staphylococcus* strains. The following data obtained from our study (Figure 3) also support this view: i) The tested FLA-CFSs had a greater inhibitory effect against *S. aureus* than *S. epidermidis*. ii) No FLA-CFS had antibacterial activity against *S. epidermidis* ATCC 12228 strain. iii) A3-CFS had no inhibitory effect on any of the Staphylococcus strains tested. iv) *Staphylococcus* strains tested were found to be inhibited by at least one native FLA-CFS (A1-CFS or A2-CFS), except for the *S. epidermidis* ATCC 12228 strain). Nevertheless, new studies are needed in which many more *Staphylococcus* and FLA strains would be used. This would be the only way to shed further light on the subject.

As stated above, since it is thought that the characteristics/abilities (pathogenicity factors) possessed by these microorganisms may play an important role among the factors affecting the relationship between FLA and bacteria living in the same environ-

ment, the subject should be investigated in detail. For example, it is known that the *S. epidermidis* ATCC 12228 strain, which was tested in our study and is avirulent, has molecules called bacteriocin that it releases into the external environment (43). This bacterium thus inhibits some species (eg *S. aureus*) that live in the same environment and are close to it. In our study, the reason why *S. epidermidis* ATCC 12228 strain was not inhibited by any FLA-CFS is perhaps because of these and similar molecules owned by the bacterium. In addition, it was found that the clinical (virulent) *S. epidermidis* strain (S3) tested in our study was inhibited by A2-CFS to a low extent (33.33%). These data suggest that virulent and avirulent *S. epidermidis* strains may be affected in different ways by FLA-CFS. In order to better understand the inhibition effect of FLA-CFS against *S. epidermidis*, new studies are planned in which more strains would be used (both virulent and avirulent *S. epidermidis*).

Souza Gonçalves et al. found that *A. castellanii* secreted different extracellular vesicles under different stress conditions and the CFS of these vesicles contained 69 proteins (41). In the light of this information, it is thought that the amoeba strains used in our study secrete inhibitory molecules against *Staphylococcus* strains, and new studies are planned to find the morphological and biochemical characterization of these possible molecules. Subsequent studies should relate to the purification of FLA-CFS with such activity and the active compound (s) that helped in the discovery of new anti-*Staphylococcus* active compound (s).

CONCLUSION

This is the first study showing the anti-*Staphylococcus* potential of FLA-CFS isolated from Turkey. In order to discover bactericidal FLAs against pathogen/potential pathogen and antibiotic resistant *Staphylococcus* bacteria, new studies using more strains should be planned.

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Conflict of Interest: The authors declare that they have no conflicts of interest to disclose.

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