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ORIGINAL ARTICLE

Exploring the antimicrobial potential of two haloarchaeal strains belonging to the genera *Halopiger* and *Natrialba* isolated from the Algerian Sahara

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ABSTRACT

Background and Objectives: Halophilic archaea commonly produce antimicrobial peptides (halocins), but only a few studies have been conducted on these molecules. This study explores the antimicrobial potential of two strains belonging to *Halopiger* and *Natrialba* genera, isolated from hypersaline environments in the Algerian Sahara. Antimicrobial compounds produced by these genera have rarely been studied before.

Materials and Methods: The antimicrobial activity of the strains was evaluated, along with the effects of UV radiation and culture conditions on growth and compound production. Stability assays and the effects of extracted compounds on target cells and peripheral blood mononuclear cells (PBMC) were assessed.

Results: The strains exhibited high anti-archaeal activities and cross-domain interactions. Producing extracellular compounds associated with halocin, in the cell-free supernatant (CFS). These compounds remained stable at different temperatures (4°C, 60°C, 80°C, and 100°C) and different pH ranges (4-10 and 5-11), with antimicrobial profiles changed in response to UV light. The active compounds resembled known halocins but displayed unique features suggesting the discovery of new halocins. Additionally, *Natrialba* extracts showed significant activity against PBMC.

Conclusion: This investigation confirms that Algerian saline soils are a promising source of interesting antimicrobial compounds.

Keywords: Archaea; *Halopiger*; *Natrialba*; Extreme environments; Antimicrobial peptides; Peripheral blood mononuclear cells

INTRODUCTION

Halophilic microorganisms are commonly found in hypersaline ecosystems with high levels of NaCl. They make up the natural microbial communities in these environments and are present worldwide (1, 2). The Algerian Sahara has extreme ecosystems with hypersaline soils, such as chotts and sebkhas (3). Haloarchaea are microorganisms that dominate hypersaline environments (4). They belong to the Halobacteria class, which is metabolically diverse and depends on high salt concentrations (5). This class is divided into three orders and six families (6-8).

Halobacteria may reach high densities by secreting halocins, which are antimicrobial peptides or proteins (9, 10). Halocin production's role in interspe-

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cies competition is unclear, but it may help maintain species diversity in extremely halophilic environments (11-13). Halocins are antimicrobials produced by halobacteria, with high and low-molecular-mass types. Microhalocins are more potent than protein halocins (10, 14). They are stable at high temperatures, salt concentrations, organic solvents, and alkaline pH conditions. These properties make them useful for biotechnology applications under extreme processing conditions (15).

Although their production is considered common among halophilic archaea, halocins have been reported to be produced erratically among the halobacteria by strains from the genera *Haloferax*, *Natrinema*, or *Halobacterium*. The study of these antimicrobial compounds from other genera, especially those produced from newly characterized strains, is still unexplored. New haloarcheocins are often discovered alongside new haloarchaeal strains (16).

In the present study, we evaluated the antimicrobial potential of two strains from *Halopiger* and *Natrialba*, affiliated with the *Natrialbaceae* family, isolated from hypersaline environments in the Algerian Sahara. The antimicrobial compounds produced by these genera have rarely been investigated. To the best of our knowledge, since the discovery of the genus *Halopiger* in 2007, no study has focused on exploring the production of their antimicrobial compounds, and only a few studies have been conducted on the genus *Natrialba*. The present investigation explored the antimicrobial and bioactive properties of two strains selected based on the results of our previous study.

MATERIALS AND METHODS

Archaeal strains and antimicrobial activity assays. The archaeal strains used in this study had been isolated and characterized in a previous study (3). Strain G3A was affiliated with *Halopiger djelfamassiliensis* (*Halopiger djelfimassiliensis* corrig.), which was originally isolated from the Algerian soil by our team member (17, 18). The strain *Halopiger djelfimassiliensis* G3A was isolated from EL Golea Sebkha (El Menia) (30°34’59.99’’N, 2°52’59.998’’E) located in the centre of the Algerian Sahara. The strain *Natrialba aegyptia* TSA4 was isolated from Sidi Ameur Sebkha (Bousaâda) (35°12’36.97’’N, 40°10’46.08’’E) located in the northern Algerian Sahara. The re-verification of these strains was performed in the modified DSMZ medium 97 (3). Table 1 presents the haloarchaeal strains used in this study.

The antimicrobial activity of the strains *Halopiger djelfimassiliensis* G3A and *Natrialba aegyptia* TSA4 was evaluated using the agar cylinder method (3). To test if the activity was related to the membrane, we cultured 7-day-old strains in modified DSMZ 97 media, incubated at 40°C for 96 hours, collected supernatant and pellet, and tested them against target strain using agar well diffusion method (19).

Effect of UV radiation on the production of antimicrobial compounds. To examine the effect of UV radiation on antimicrobial production, five Petri dishes of each strain incubated at 40°C for 5 days were subjected to UV irradiation (254 nm) at different time intervals: 30 s, 1 min, 2 min, and 5 min. Antimicrobial bioassays were performed using the agar cylinder technique, as previously described. Different diameters resulting from the zones of inhibition were subsequently measured.

Effect of culture conditions on growth and antimicrobial production patterns. The growth of *Halopiger djelfimassiliensis* strain G3A and *Natrialba aegyptia* TSA4 was monitored in modified DSMZ medium 97 broths incubated at 40°C for 7 d. Samples were taken every 24 hours to determine optical density at 600 nm. Antimicrobial activities against the target strains were measured along the growth curve using the well-diffuse test.

We examined the growth and optimal conditions of the producing strains under different temperatures (4, 25, 30, 37, 40, 55, and 60°C), pH levels from 4.0 to 12.0, and varying NaCl concentrations (5.0%, 10.0%, 12.0%, 15.0%, 20.0%, 25%, and 30%). Additionally, we investigated the production of antimicrobial compounds under the aforementioned conditions.

Assays of stability of the antimicrobial compounds produced in CFSs. The antimicrobial activity of the cell-free supernatant (CFS) was tested against the target strain, followed by testing the effects of temperature (4, 60, 80, and 100°C for 1h and 121°C for 20 min), pH (4, 5, 6, 7, 8, 9, and 10), salinity, and proteolytic enzymes on the activity. To test whether the antimicrobial activity could be maintained, the active CFSs were left for one month at 4°C and then tested. The activities were then compared

Table 1. Haloarchaeal strains used in this study

Strain	Isolation site	Accession number	Reference
Halopiger djelfimassiliensis G3A	ELGolea Sebkha (El Menia)	KT032242	Quadri et al., 2016
Natrialba aegyptia TSA4	Sidi Ameur Sebkha (Bousaada)	KT257568	Quadri et al., 2016
Halorubrum saccharovorum AMR1	Sebkha Ezzmoul (Ain M'lila)	KT032246	Quadri et al., 2016
Halorubrum terrestre AMB 1	Sebkha Ezzmoul (Ain M'lila)	KT032241	Quadri et al., 2016

with those of the control stored at room temperature (25°C) and the activities before and after each treatment were assessed using a well-diffuse test.

Effect of antimicrobial compounds produced in CFSs on target cells. The target strain cells of the most sensitive strains were incubated in a modified DSMZ medium 97 broth, at 40°C, with or without the corresponding CFS for different time intervals (1 h, 3 h, 24 h, 48 h, and 96 h). The cells were observed under the microscope (Carl Zeiss, Germany) to monitor any changes in their appearance. Additionally, the optical density of both the control and treated samples was measured at the same time intervals.

Extraction of antimicrobial compounds and the effect of the active extract on peripheral blood mononuclear cells (PBMC). We used the organic solvent extraction method to extract active compounds from the producing strains (3). The concentrated extracts were tested on PBMC to evaluate their impact on eukaryotic cell models. The viability of isolated and cultured cells was assessed before and after the experiment using trypan blue staining and hemocytometer counting (20). The viability percentage was calculated using the following equation:

$$\%\text{Viability} = \frac{\text{Number of colorless cells}}{\text{Total number of cells}} \times 100.$$

RESULTS

We studied two strains *Halopiger djelfimassiliensis* G3A and *Natrialba aegyptia* TSA4 isolated from hypersaline environments in the Algerian Sahara to assess their potential antimicrobial characteristics. The phylogenetic trees of the two strains are presented in Figs. 1 and 2.

Both strains displayed a wide haloarchaeal activity spectrum. *Halopiger djelfimassiliensis* G3A can inhibit the growth of 19 out of the 46 tested strains, and *Natrialba aegyptia* TSA4 showed significant activity against 17 out of 46 the tested strains. The strain *Halorubrum saccharovorum* AMR1 was the most sensitive one to the antimicrobial compound produced by *Halopiger djelfimassiliensis* G3A, and *Halorubrum terrestre* AMB 1 for the strain *Natrialba aegyptia* TSA4. Based on these results, these strains were selected as target strains for the subsequent tests.

The antimicrobial compounds released by both strains were ineffective towards closely related strains. Interestingly, *Natrialba aegyptia* TSA4 was active against *Halopiger djelfimassiliensis* G3A, indicating that the antimicrobial molecules produced by the two strains are different. Both strains showed significant antimicrobial activity in their culture supernatants, confirming the secretion of bioactive molecules into the extracellular medium.

Results showed that the exposure to UV light caused a change in the antimicrobial profile of the strains. For *Halopiger djelfimassiliensis* G3A, there was a gradual decrease in activity with increased exposure time, while *Natrialba aegyptia* TSA4 showed a decrease in production at 30 seconds but regained stable activity following being exposed for 1 to 5minutes. The results of antimicrobial activities of the two producing strains under UV light exposure are summarized in Table 2.

The two strains displayed similar growth and production characteristics with slight differences. The growth of *Halopiger djelfimassiliensis* G3A increased drastically between 24 to 96 hours (1-4th day) after inoculation, which corresponds to the exponential phase, then started to slow down and was followed by a long stationary phase, which lasted up to 192 h of incubation. The antimicrobial production of this strain was observed from the 3rd day of growth (72 hours) and increased gradually until reaching a maximum value on the 5th day (120 h), then the production decreased thereafter.

On another hand, the growth curve analysis of the strain *Natrialba aegyptia* TSA4 revealed that after

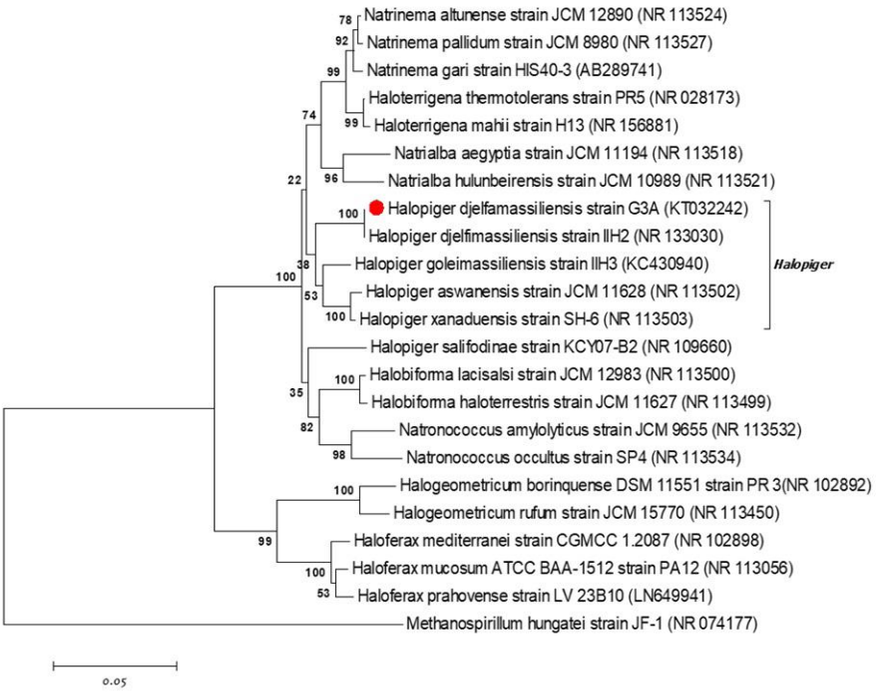

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence comparisons, showing the position of isolated strain and some other related haloarchaeal species. GenBank accession numbers are indicated in parentheses. Sequences were aligned using MUSCLE, and phylogenetic inferences obtained using the MEGA software. Numbers at the nodes are bootstrap values obtained by repeating the analysis 1,000 times to generate a majority consensus tree. *Methanospirillum hungatei* was used as an outgroup.

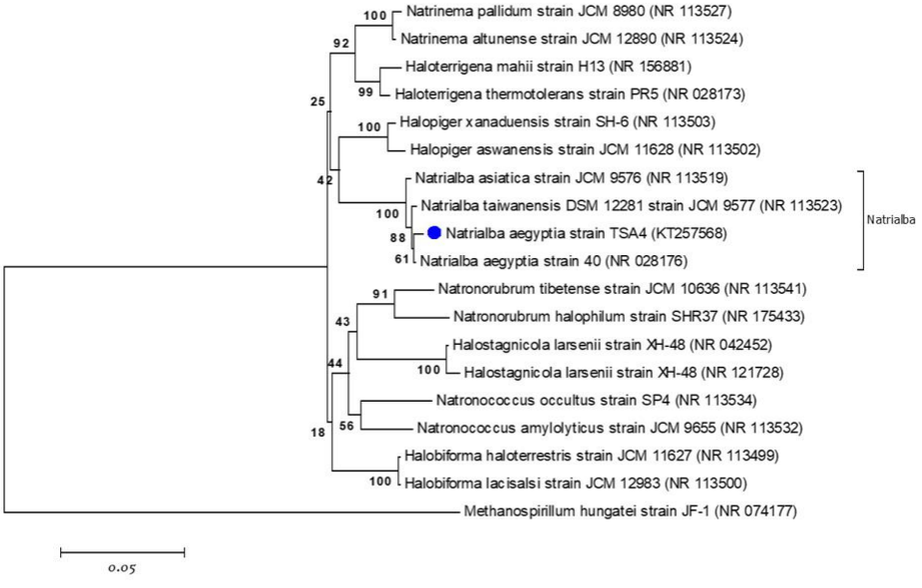

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence comparisons, showing the position of isolated strain and some other related haloarchaeal species. GenBank accession numbers are indicated in parentheses. Sequences were aligned using MUSCLE, and phylogenetic inferences obtained using the MEGA software. Numbers at the nodes are bootstrap values obtained by repeating the analysis 1,000 times to generate a majority consensus tree. *Methanospirillum hungatei* was used as an outgroup.

Table 2. Results of antimicrobial activities of the two producing strains under UV light exposure. The diameters of the zones of inhibition produced by the metabolites on the target strains are given in millimetres (mm)

		UV exposure time			
	Control	**30 sec**	**1 min**	**2 min**	**5 min**
Halopiger djelfimassiliensis G3A active compounds	25	24	18	16	12
Natrialba aegyptia TSA4 active compounds	27	20	25	24	24

a short lag phase, the growth of the strain increased rapidly between the 2nd and 6th days (48-144 h) indicating the exponential phase. Then the strain reached the stationary phase by the 6th day. The antimicrobial activity of the strain *Natrialba aegyptia* TSA4 was detectable throughout the exponential growth, starting the first day (1st - 4th day) until reaching their maximum on the 5th day which corresponds to the beginning of the stationary phase. It, then, started decreasing. The correlation between the growth of the two strains and the production of their antimicrobial compounds indicates that the peak of production occurs during the transition from the exponential phase to the stationary phase. Figs 3 and 4 illustrate the cell growth and antimicrobial production of the two strains against the target strains.

The results showed that the strain *Halopiger djelfimassiliensis* G3A grew at 30-60°C (optimum 55°C), at pH 6-11 (optimum pH 8) and with 15-30% of NaCl (optimum 25%). This strain was found to produce the antimicrobial compounds, against the target strain *Halorubrum saccharovorum* AMR1, in the same growth conditions. Similar culture conditions were required for the growth and maximum production of the strain *Natrialba aegyptia* TSA4 against *Halorubrum terrestre* AMB1. Both growth and production occur at NaCl concentrations of 15-30%, at pH values in the range 5-10, and within the temperature range 30-55°C. Optimal NaCl concentration, pH, and temperature for growth and maximum production were 25%, 7 and 40°C, respectively. We noticed that the same growth optimal conditions supported maximum antimicrobial production for both producing strains against their respective target strains.

For the stability assays, the two supernatants showed similar reactions to the different treatments. The CFS of strain *Halopiger djelfimassiliensis* G3A (S-G3A) was stable at different pH ranges (5-11), and the CFS of strain *Natrialba aegyptia* TSA4 (S-TSA4) at pH varying (4-10). We noticed a decrease in the activity of both strains at acidic and high alkaline pH. Both tested CFSs were found to be stable at 4°C, 60°C, 80°C and 100°C, compared to the activity at room temperature, and complete loss of activity after an autoclave cycle of 121°C for 20 min. We noticed that the molecules remained active after one month at 4°C, showing similar antimicrobial activity as freshly prepared CFSs. And the loss of activity in both supernatants when incubated with proteinase K.

We need to mention that both target strains are

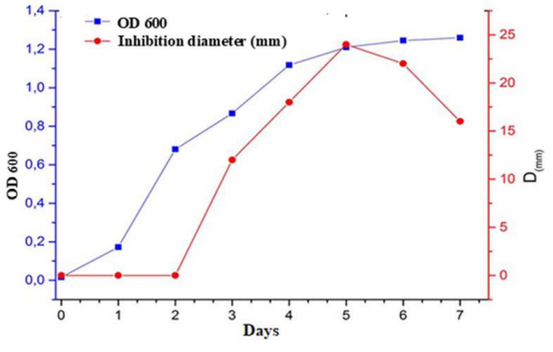

Fig. 3. *Halopiger djelfimassiliensis* G3A cell growth and antimicrobial production against the target strain *Halorubrum saccharovorum* AMR1.

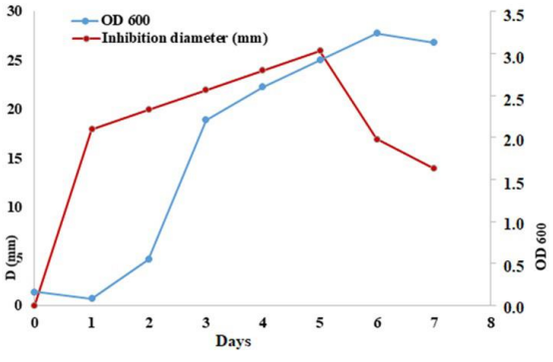

Fig. 4. *Natrialba aegyptia* TSA4 cell growth and antimicrobial production against the target strain *Halorubrum terrestre* AMB1.

motile and characterized by pleomorphic shape. The effect of treatment with the culture supernatants of the producing strains on the growth of the target strains was monitored at different time intervals up to 96 hours using a photonic microscope. The untreated control morphology was stable during the experiment. For the treated cells, we have noticed a slight but quick drop in the number of cells (from 1 H) and a decrease in the motility for both treated sensitive cells. After 24 hours of incubation, we only observed a few remaining cells and cell debris, which could be explained by cell lysis after swelling. As for the optical density, the treated sample showed a considerable drop in growth at 24 h compared to the untreated cells.

The extract obtained from the CFS of the producing strain *Natrialba aegyptia* TSA4 exhibited maximum antimicrobial activity compared to the CFS. However, despite repeating the test for several times, we could not get the active extract from the strain *Halopiger djelfimassiliensis* G3A in the current study.

The test on peripheral blood mononuclear cells (PBMC) was conducted only on the extract of *Natrialba aegyptia* TSA4. We have obtained the following results: for the untreated PBMCs; the viability after culture was 100%. We observed that exposure to increasing concentrations of the crude extract (5 µl and 10 µL) resulted in a concentration decrease in cell viability. The viability of cells was estimated at 52% and 40%, for the PBMCs treated with 5 µL and 10 µL of active extract, respectively.

DISCUSSION

We evaluated the antimicrobial production of two strains, *Halopiger djelfimassiliensis* G3A and *Natrialba aegyptia* TSA4, isolated from hypersaline environments of the Algerian Sahara. These strains were selected based on the interesting results of our previous study, and based on the fact that antimicrobial compounds from these genera have rarely been investigated. Until our study in 2016, there had been no other reports of halocins or any antimicrobial agent from the genus *Halopiger*, and an investigation reported *Natrialba* potential to produce carotenoids (21). Strain G3A is associated with *Halopiger djelfimassiliensis,* isolated from Lake Zahrez Gharbi, Algeria by our team members (17). The discovery of new haloarcheocins is linked to the finding of new haloarchaeal strains. *Natrialba aegyptia* was originally isolated from Egypt (22).

Antimicrobial compounds are produced by halophilic archaea and seem to be a universal feature of halobacteria, but only a few have been characterized and purified to date. Another interesting fact supporting our choice of strains is that the gene encoding of halocin C8 was not found in *H. djelfimassilliensisp* (G3A) (3). This suggests that antimicrobial compounds produced by this strain could be related to another halocin, or even related to a new one. The possibility of getting novel compounds from the new isolates was previously suggested. Both strains demonstrated significant activity against various haloarchaeal strains, each with a different spectrum. A few described halocins are reported with a wide host range of activity such as halocin C8, H1, and H4 (23-26).

The antimicrobial compounds released by both strains were ineffective against closely related strains of the same genera, and hence presumably produced analogous compounds. This is because antimicrobial producers are immune to their toxins due to different mechanisms. Antimicrobial compounds are released in the culture supernatant, similar to halocins H4 and S8. Halocin C8 production was reported in the supernatant and membrane fraction (10).

Halophilic archaea are resistant to UV light due to their robust UV DNA repair systems (27). Studies have been conducted on *Halobacterium* regarding their resistance to UV irradiation. However, no investigations has demonstrated the effect of UV irradiation on the production of antimicrobial compounds produced by halophilic archaea. Our results indicate different reactions in antimicrobial production when exposed to UV rays. Further testing is needed on larger strain collection to see if UV exposure can enhance or deactivate antimicrobial activities.

Our investigation is in tune with the previous studies on the most known halocins, most of the halophilic archaea produce optimum halocin during the transition from exponential to stationary phase, except for the production of the halocin H1, which was found to be maximum at the mid-exponential phase and remains stable till the stationary phase (28). Certain haloarchaea species, such as *Haloferax mediterranei,* exhibit a reduction in halocin production during the stationary phase. Consequently, halocin synthesis is observed to be linked to the growth pro-

cess (23, 29).

The antimicrobial compounds produced by two strains may be related to halocin production, as suggested by the results of culture condition and stability assays. Many halocins are stable in a wide range of pH, including acidic to alkaline pH range. For instance, the Sech7a halocin activity remains stable in a pH range of 2-10 (30).

The results of temperature stability are similar to those reported for halocins C8, S8, and A4, which conserve their activity at 100°C. The activity of the G3A supernatant was higher at 55°C, compared to the untreated control, which tunes in with our results of the culture conditions on the production. Several halocins such as H1, H6, R1, and Sech7a were reported to lose their activity at 121°C. The activity of the two CFSs remains active after one month of storage at 4°C, showing similar antimicrobial activity as freshly prepared CFSs. The antimicrobial activity of HalC8- containing *Natrinema* sp. AS7092 supernatant was shown to remain stable after one year of storage at 4°C and 1 h treatment at 100°C (10, 31). Finally, the loss of activity in both supernatants when incubated with proteinase K, confirmed the proteinaceous nature of the produced compounds.

According to our results, the active compounds have similar features with different known halocins that have been characterized either fully or partially but yet have unique characteristics suggesting that they can be related to new halocins. This theory is highly empowered by the fact that the producing strains genera had not been investigated before, as mentioned above.

The study reveals that antimicrobial compounds produced in CFSs have similar effects on target cell growth and morphology, suggesting a cytocidal nature.

It has been reported that halocins generally kill the indicator organisms by cell swelling followed by cell lysis (24, 30-32). Halocins such as H4, C8, and Sech7a cause swelling followed by lysis of cells in less than 24 h. Other halocins are cytostatic, like in the case of halocins S8 and R1 (24, 30, 31). The only precise mode of action revealed was for the halocin H7, which kills sensitive cells by inhibiting the Na+ / H+ anti-porter (33).

Previous research has demonstrated that peptide halocins are highly stable, including halocin S8, R1, A4, and C8. These halocins resist acids, bases, and organic compounds (24). It is possible that the active extract from the *Halopiger djelfimassiliensis* G3A strain was not obtained because some halocins can lose their activity in the presence of organic solvents.

Only rare reports were done on the effect of halocins on eucaryotic cells. The halocin H6/H7 inhibits the haloarchaeal Na+/H+ antiporter and also inhibits the Na+/H+ antiporter in a dog model. It is not known if halocin H6/H7 inhibits Na+/H+ antiporters in organisms evolutionarily intermediate between haloarchaea and mammals (33). For microhalocins, no study has shown their effects on eukaryotic cells. The finding in the current study confirms the unique characteristics of the active compound produced by the strain.

Our results empower the interest in using active compounds from halophilic archaea for new sources of important bioactive compounds. More recently, an investigation on carotenoids produced by *Natrialba* sp. M6, isolated from Wadi El-Natrun in Egypt, has shown anticancer and antiviral effects. The carotenoid pigment demonstrated notably higher efficacy in eliminating HCV and HBV from infected human blood mononuclear cells compared to currently utilized medications (21).

CONCLUSION

Two haloarchaeal strains affiliated with the *Natrialbaceae* family, isolated and characterized in our previous work on halophilic archaea in the Algerian Sahara, exhibited high anti-archaeal activities and cross-domain interactions. In the current study, we extended our research on their antimicrobial properties and the characteristics of the produced compounds.

All the features propose that the antimicrobial molecules secreted by the two strains could be strongly related to halocin production. The distinct characteristics and affiliations of the strains suggest that the compounds can be considered as new molecules. The active extract of the strain *Natrialba aegyptia* TSA4 showed an important activity on the PBMCs, which had never been reported before for halocins. This result can provide leads for further investigations to develop drugs against auto-immune diseases. Further work will be carried out to fully characterize the composition of the active extracts and their efficacy.

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