

Evaluation of antimicrobial action of 3 and 3a IL mixtures

Our previous studies [1] have shown that while the 3 IL, N-(2-Hydroxyethyl)-N, N-Dimethyl-1-Dodecanaminium Bromide, was the most effective antimicrobial IL, 3a, featuring the lipophilic dodecyl benzensulfonate (DBS) as the anion, also displayed preventive antibiofilm activity (De Leo et al. 2021). These findings suggested us that a mixture of 3 and 3a, coupling antibacterial properties of 3 to the antibiofilm activity of 3a, induced by DBS anion, could exert a double function when combined. In order to investigate the bioactivity of 3 and 3a combinations, the MBC of their mixtures at different molar ratio, were investigated.

Materials and Methods

ILs

ILs 3 and 3a were tested at different combination ratios (1:1, 1:3, and 3:1, respectively). ILs were utilized at the original concentration (TQ) or diluted 1:2 or 1:5 and tested on single products and against methanol alone (Table S1). IL solutions in methanol were sterilized by using 0.22 μm disposable Millipore filters.

Table S1. Concentration of IL solutions tested.

ILs	(N)	Initial concentration ($\mu\text{mol/ml}$)	Solvent
3a	0.075	37.5	methanol
3	0.075	37.5	methanol
3:3a (molar ratio 1:1)	0.075	37.5	methanol
3:3a (molar ratio 1:3)	0.075	37.5	methanol
3:3a (molar ratio 3:1)	0.075	37.5	methanol

Microbial strains

The microbial strains used in this study were isolated by the authors (Urzi and De Leo) from stone materials exposed to outdoor environmental conditions. The strains included *Micrococcus luteus* BC657, *Stenotrophomonas maltophilia* BC656, *Cladosporium* sp. MC853, *Aureobasidium* sp. MC875, and *Chlorella* sp. Erc4. All strains are maintained in the culture collection of the MicroABC Laboratory, Department of Chemical, Biological, Pharmaceutical and Environmental Sciences (ChiBioFarAm), University of Messina, Italy.

Before the trial application on the fountain, a possible synergistic effect of the two ionic liquids 3 and 3a was evaluated and methodology used is described as it follows.

Antibiogram tests

Diffusion test in agar medium

The Kirby–Bauer disk diffusion method was used for testing antibacterial susceptibility. Bacterial strains were grown on TSA medium (Tryptone Soy Agar; Condalab, Spain) at 30 °C for 24 h. A bacterial suspension of each strain was then prepared in 4 mL of Mueller–Hinton broth (MHB; Difco, Detroit, MI, USA) and adjusted to a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 cells mL⁻¹). The suspensions were incubated at 30 °C for 2 h. After the incubation period, the suspension was evenly distributed over the surface of MHA (Mueller–Hinton Agar) medium (150 mm diameter plates containing 70 mL of medium were used) using a sterile cotton swab and left to dry for a few minutes (3–4 min). Subsequently, 6 mm disks, previously imbibed with 20 µL of the products at TQ and at 1:2 and 1:5 dilutions, as well as the different combination of 3:3a (1:1, 3:1 and 1:3) and the solvent alone (methanol used as negative control) were applied to the surface of the medium and gently pressed with sterile tweezers.

After 30 min of diffusion at room temperature, the plates were incubated at 30 °C for 24 h for Gram-negative bacteria and 72 h for Gram-positive bacteria. Antibacterial activity was evaluated by measuring the diameter of the inhibition zones around the disks.

The standard protocols for the yeasts (M44-A) and for hyphomycetes (M51-A) of the NCCLS (National Committee for Clinical Laboratory Standards, with slight modification, were used) [2-5].

Cladosporium sp. MC853 was grown in PDA (Potato Dextrose Agar, Condalab, Spain) medium and incubated for about 4-6 days at 26 °C. After the incubation time, the fungal spores were collected using the flooding technique as it follows: . 3 ml of a suspension in 0.85% NaCl supplemented with Tween 80 0.01% were poured into the plate and the surface was gently scraped. Approximately 2 ml were taken from the plate and transferred to two sterile Eppendorf tubes (1 ml in each Eppendorf), the suspensions were washed twice and the pellets were resuspended in 1 ml of 0.85% NaCl saline. A final suspension was prepared in 5 ml of saline solution with a concentration of 5×10^6 spores mL⁻¹. Spore counting was performed in the Thoma-Zeiss counting chamber. Subsequently, 1 ml of the spore suspension was inoculated onto each 150 mm diameter plate containing 70 ml of MHA+ 2 % glucose medium. Using a sterile spatula and a rotating support, the suspension was evenly distributed over the surface. After leaving to dry for a few minutes, the previously prepared 6 mm diameter disks (loaded with 20 µl of biocides at their original concentration (TQ), at 1:2 and 1:5 dilutions and with methanol alone as negative control) were placed on top of the agar surface, and gently pressed with tweezers. Within 15 min after disk placement, the plate was incubated at 26 °C for 72 h. After the incubation period, the diameter of the inhibition zone around each disk was measured.

Aureobasidium sp. MC875 was grown in MEA (Malt Extract Agar, Condalab, Spain) medium and incubated for 72h at 26 °C. After the incubation time, a fungal suspension was prepared in 5 ml in 0.85% NaCl saline with turbidity corresponding to 0.5 McFarland. The final concentration obtained through a Thoma-Zeiss chamber was 4×10^6 cells mL⁻¹

Within 15 min of preparation, 1 mL and 100 µL of the suspension were inoculated into 150 mm and 90 mm diameter plates, respectively, containing 70 mL and 30 mL of MEA medium. Using a sterile spatula and a rotating support, the suspension was evenly distributed over the surface. After being left to dry for about 5 minutes, the disks as described above were placed on the surface of the agar plates, exerting light pressure with tweezers. After 15 min from the deposition of the disks, plates were incubated at 26 °C for 72h. After the incubation period, the diameter of the inhibition halo around each disk was measured.

Spread test in agar was used for the eukaryotic alga as it follows. *Chlorella* sp. Erc-4 strain was inoculated into 20 ml of BG11 liquid medium and incubated for 15 days in daylight and continuous agitation, at room temperature. After the incubation time, 4 ml of the culture was taken and distributed in 4 Eppendorf tubes, 1 ml in each; rinsed twice and then pellets were resuspended in 0.85% NaCl solution. A final suspension of 15 ml was set up, with a concentration of 3×10^6 cells/ml. The count was carried out in the Thoma-Zeiss chamber. Subsequently, 2 ml and 1 ml of the suspension was inoculated into 150 mm and 90 mm diameter plates, respectively, containing 70 ml and 30 ml of BG11 0.9% agar medium. With the help of a sterile spatula and a rotating support, the suspension was evenly distributed over the surface. After leaving to dry for a few minutes, the previously prepared 6 mm diameter disks were placed on the surface of the medium, exerting light pressure with tweezers. Subsequently, the plates were put upside down and incubated at daily light at room temperature.

Results

The results evidence that the strains tested had various sensitivities at the different IL concentrations and combinations. *Chlorella* was sensitive to all the different IL concentrations and formulations, while the other strains were more sensitive at the TQ concentration both alone and in mixture. Methanol did not have any antimicrobial effect. Regarding the antimicrobial activity, IL-3 showed the highest antimicrobial activity, even higher than that of 3a. Since this latter, as previously reported (De Leo et al., 2022).), experiences a peculiar antifouling action, taking into account the good MBC performance of the 3:3a combination, with a 3:1 molar ratio, we decided to exploit this formulation at the T.Q. concentration, for application in situ experiments on stones. The synthesis was already reported [6].

Results are shown in Figures S1 to S5 and in Table S1.

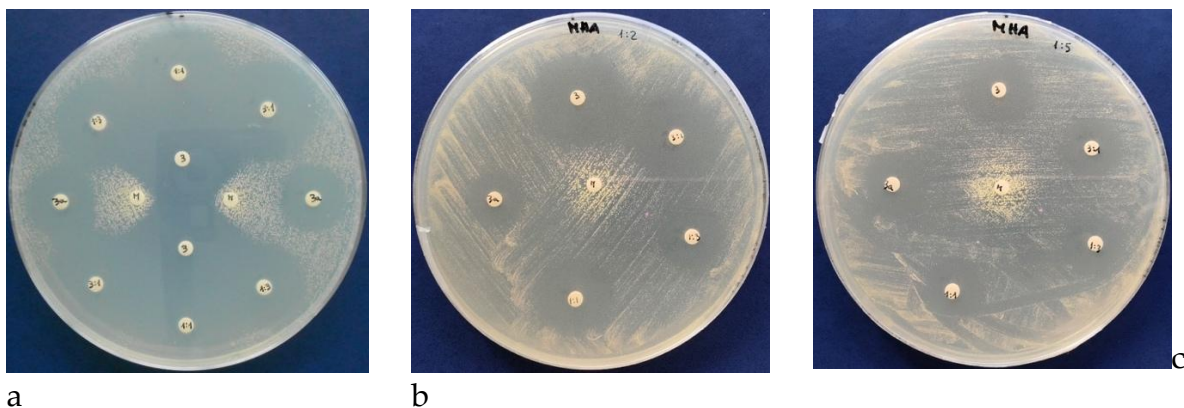


Figure S1 Results of the agar diffusion test for *Micrococcus luteus* BC657 A) at the TQ concentration; B) IL diluted 1:2; C) IL diluted 1:5.

Legend 3 = IL 3; 3a = IL 3a; 1:1, 1:3, 3:1 the ratio used for the combination of 3 and 3a.

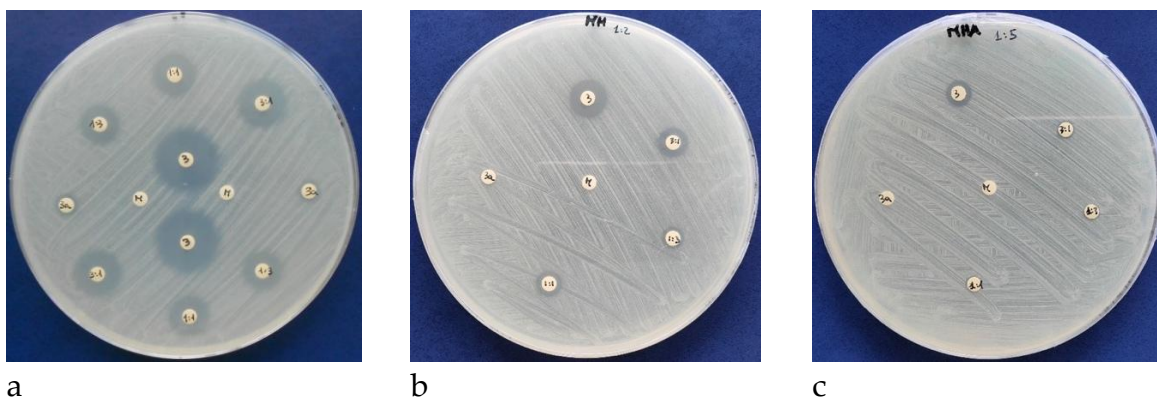


Figure S2 Results of the agar diffusion test of the strain *Stenotrophomonas maltophilia* BC656 A) at the TQ concentration; B) IL diluted 1:2; C) IL diluted 1:5.

Legend 3 = IL 3; 3a = I IL 3a; 1:1, 1:3, 3:1 the ratio used for the combination of 3 and 3a.

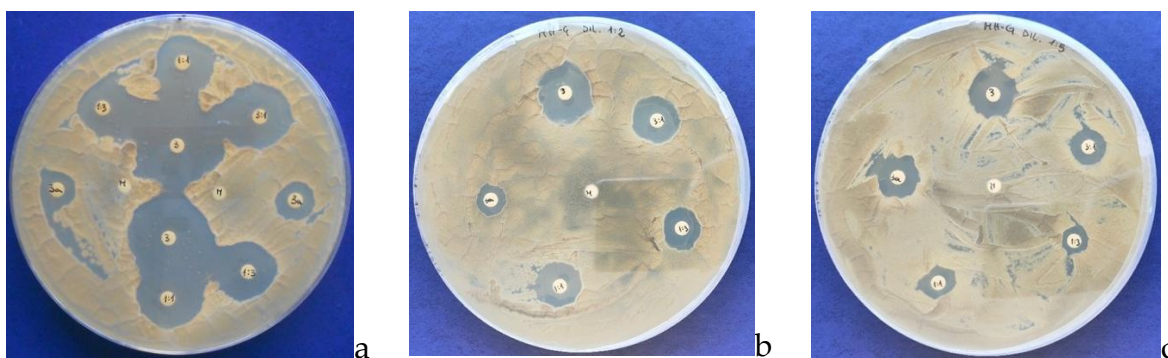
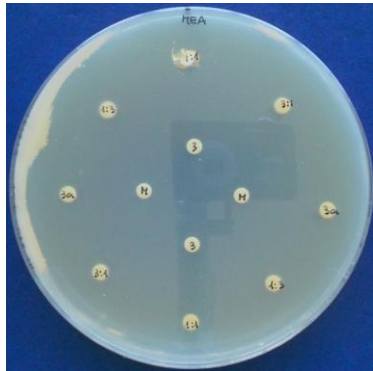
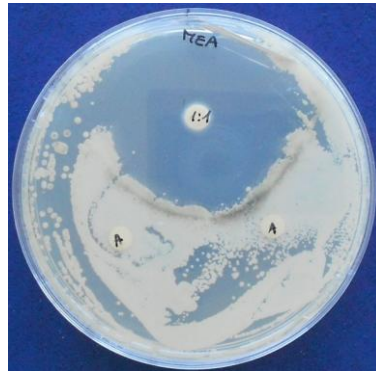


Figure S3 Results of the agar diffusion test of the *Cladosporium* sp. MC853 A) at the TQ concentration; B) IL diluted 1:2; C) IL diluted 1:5.

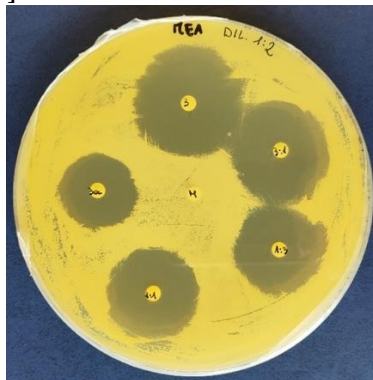
Legend 3 = IL 3; 3a = IL 3a; 1:1, 1:3, 3:1 the ratio used for the combination of 3 and 3a.



a1



a2

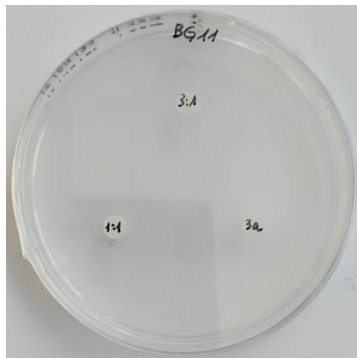


b

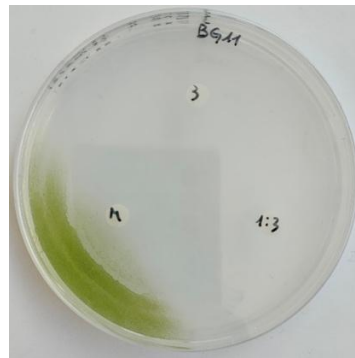


c

Figure S4 Results of the agar diffusion test in agar of strain *Aureobasidium sp.* MC875 a1-a2) at the TQ concentration; b) IL diluted 1:2; c) IL diluted 1:5. Legend 3 = IL 3; 3a = IL 3a; 1:1, 1:3, 3:1 the ratio used for the combination of 3 and 3a.



a1



a2



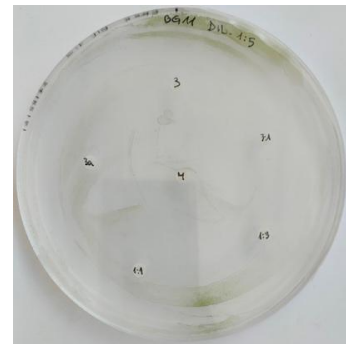
b1



b2



c1



c2

Figure S5 Results of the agar diffusion test of the *Chlorella* sp. Erc 4 strain. a1-a2) at the TQ concentration; b1 and b2) IL diluted 1:2; c1 and c2) IL diluted 1:5. Legenda 3 = IL 3; 3a = IL 3a; 1:1, 1:3, 3:1 the ratio used for the combination of 3 and 3a.

Table S1 Measurements of the diameter of inhibition halos for the tested strains.

Strains	Inhibition diameter (mm)														
	<i>Micrococcus luteus</i> BC657			<i>Stenotrophomonas maltophilia</i> BC656			<i>Cladosporium sp.</i> MC 853			<i>Aureobasidium sp.</i> MC875			<i>Chlorella sp.</i> Erc4		
	concentration			concentration			concentration			concentration			concentration		
ILs	T.Q.	1:2	1:5	T.Q.	1:2	1:5	T.Q.	1:2	1:5	T.Q.	1:2	1:5	T.Q.	1:2	1:5
3	41	39,5	25,5	26,5	14,5	0	36	27,5	20	N.d.	45,5	38	*	*	*
3a	26,5	23	24	9	0	0	16,5	13,5	19,5	34	30,5	23	*	*	*
3:3a ratio 1:1	39	27,5	20,5	16,5	10	0	27	20	15	48	38	31,5	*	*	*
3:3a ratio 1:3	34	25	20	16	9	0	25	18	13	42	36,5	29	*	*	*
3:3a ratio 3:1	41	33	20,5	20	12	7	29	22	16	N. d.	41,5	32,5	*	*	*
M (methanol)	0	0	0	0	0	0	0	0	0	0	0	0	*	*	*

N.d. = * The diameter of inhibition was too large to be measured.

Supplemental materials references

1. De Leo, F.; Marchetta, A.; Capillo, G.; Germanà, A.; Primerano, P.; Schiavo, S.L.; Urzì, C. Surface active ionic liquids based coatings as subaerial anti-biofilms for stone built cultural heritage. *Coatings* **2021**, *11*, 26.
2. Clinical and Laboratory Standards Institute (CLSI). *Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline*, 2nd ed.; CLSI Document M44-A2; CLSI: Wayne, PA, USA, 2009.
3. Clinical and Laboratory Standards Institute (CLSI). *Method for Antifungal Disk Diffusion Susceptibility Testing of Nondermatophyte Filamentous Fungi; Approved Guideline*; CLSI Document M51-A; CLSI: Wayne, PA, USA, 2010.
4. Arendrup, M.C.; Park, S.; Brown, S.; Pfaller, M.A.; Perlin, D.S. Evaluation of CLSI M44-A2 Disk Diffusion and Associated Breakpoint Testing of Caspofungin and Micafungin Using a Well-Characterized Panel of Wild-Type and *fks* Hot Spot Mutant *Candida* Isolates. *Antimicrob. Agents Chemother.* **2011**, *55*, 1891–1895. <https://doi.org/10.1128/AAC.01373-10>.
5. Espinel-Ingroff, A.; Canton, E.; Fothergill, A.; Ghannoum, M.; Johnson, E.; Jones, R.N.; Ostrosky-Zeichner, L.; Schell, W.; Gibbs, D.L.; Wang, A.; Turnidge, J. Quality Control Guidelines for Amphotericin B, Itraconazole, Posaconazole, and Voriconazole Disk Diffusion Susceptibility Tests with Nonsupplemented Mueller–Hinton Agar (CLSI M51-A Document) for Nondermatophyte Filamentous Fungi. *J. Clin. Microbiol.* **2011**, *49*, 2568–2571. <https://doi.org/10.1128/JCM.00393-11>.
6. Luci, M.; De Leo, F.; De Pascale, D.; Galasso, C.; La Russa, M.F.; Lo Schiavo, S.; Ricca, M.; Ruffolo, S.A.; Ruocco, N.; Urzì, C. Surface-active ionic-liquid-based coatings as anti-biofilms for stone: An evaluation of their physical properties. *Coatings* **2023**, *13*, 1669