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# *In vitro* Evaluation of Antimicrobial Lock Technique for inhibition of mixed biofilms non-*albicans Candida* species-bacteria

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**Abstract:**
Non-*albicans Candida* yeasts are incriminated in systemic mycoses, and are often found with bacteria in multi-species biofilms. The management of biofilm-related infections is difficult and new therapeutic solutions are therefore necessary. The aim of this study is to evaluate the effectiveness of the antimicrobial lock technic (ALT) on mixed biofilms non-*albicans Candida* species -bacteria formed on surgical catheters, using ethylene diamine tetra-acetic acid (EDTA) and amphotericin B (AmB) as antimicrobial agents. Mixed biofilms of non-*albicans Candida* species -bacteria, co-isolated from the same medical device, were grown on catheters and incubated with EDTA and AmB lock solution for 1, 3, 5 or 7days. Our results suggest AmB associated to EDTA may be useful ALT agents in the treatment of catheter related infections.

**Keywords:** non-*albicans Candida* species /bacteria, mixed biofilms, antimicrobial therapy, catheter, lock solution, infection.

## 1. Introduction

Fungal biofilms and more particularly *Candida* biofilms are at the origin of infections contracted in hospitals, most often in connection with the use of medical devices [1]. The ability to form biofilms is an important virulence factor for pathogenic microorganisms. Biofilm is a living, dynamic structure, in perpetual remodelling, composed of cells fixed in a self-synthesized matrix of extracellular polymeric substances [2, 3]. Even if we scrupulously respect aseptic techniques, biofilm development is rapid and inevitable on most materials used in human medicine [4]. Many factors govern the biofilm, such as the growth rate and type of microorganisms, the formation of extracellular polymeric substances, the characteristics of the surface (roughness, hydrophobicity)

or the interface (solid, liquid or gas) and the contact time. The formation of a biofilm also depends on the microorganisms that compose it and the relationships that can exist between them [5]. This mode of growth confers certain advantages to its members, including substrate exchange, resistance to antimicrobial drugs, immune system, mechanical and environmental stress, adhesion capacity, nutritional sources, and cellular communication [6]. Due to the heterogeneity of the microorganisms present in the human flora, the biofilms are most often polymicrobial, involving either species of the same genus or species from different kingdoms (such as bacteria and fungi) [7]. Polymicrobial biofilms have a more complicated management, difficult to diagnose, thus requiring complex strategies of poly-chemo-therapeutic treatments [8]. Indeed, the diversity, complexity and different pathogens associated with polymicrobial biofilms can contribute significantly to serious infectious clinical complications [9]. In addition, we often found *Candida* yeasts with bacteria in multi-species biofilms, and research on fungal-bacterial interactions has been growing rapidly for several decades [10].

Currently, the management of catheter-related candidiasis consists of removal and replacement of the infected device and the use of systemic antifungal therapy. However, removal of the infected device is not an adequate solution in the majority of patients [11]. In certain cases, we can consider a different therapeutic approach called "lock therapy". Its consists on the *in situ* filling of the catheter lumen with a small volume of a highly concentrated solution of antimicrobial agent, 100 to 1000 times the minimal inhibitory concentration, left in place for a few hours to a few days. The concentrated solution then acts as a "lock" on the catheter lumen [12, 13]. Numerous studies have been published on the antibiotic lock technique, but there is less data in the literature regarding the efficacy of antifungal locks [14], and no studies on the lock solutions efficacy on polymicrobial yeast-bacterial biofilms. On the other hand, ethylene diamine tetra-acetic acid (EDTA) has proven activity against mono species bacterial biofilms in catheters, with significant reductions in the number of viable biofilms after treatment of hemodialysis catheters for 3 hours and central venous catheters for 21 hours [15]. This agent has many advantages in terms of low cost, ease of use and effectiveness against biofilms [16]. Based on all this data, the aim of the current study was to evaluate the effect of antimicrobial lock technic (ALT) using ethylene diamine tetra-acetic acid and amphotericin B as antimicrobial agents on mixed biofilms formed on surgical catheters.

## 2. Material and Methods

### *2.1.Strains*

Six non-*albicans Candida* yeasts and 11 bacteria strains co-isolated from the same catheter of patients hospitalized at Tlemcen hospital (Algeria) were used (table 1). Only medical devices that had been in place for 48 hours or more were sampled according to the recommendations of Quinet (2006) [17] and Gürcüoglu et al. (2010) [18].

The fungal strains were identified on agar medium*Candida* Chromium agar (Sigma) and Api *Candida*® (BioMérieux). The identification of the bacteria was carried out, after verification of their purity by the study of the macroscopic (aspects of colonies on solid medium), microscopic (mobility and Gram staining) and biochemical characters onApi galleries Api 20E®, Api20NE® and Api Staph® (Bio Mérieux).

Each association (yeast-bacteria) used for forming the mixed biofilm is isolated from the same catheter. All strains were confirmed to be biofilm-forming by using 96-well plate.

**Table 1: Distribution of non-*albicans Candida* species and the co-isolated bacteria strains isolated from the same medical device.**

| Association yeast-bacteria | Yeast strain | Bacteria co-isolated strain |
|---|---|---|
| 1 | *Candida tropicalis* | *Proteus mirabilis* |
| **2** | *Candida tropicalis* | *Providencia stuartii* |
| 3 | *Candida famata* | *Escherichia coli + Pseudomonas aeruginosa + Staphylococcus aureus* |
| 4 | *Candida famata* | *Klebsiella pneumonia+ Proteus mirabilis* |
| 5 | *Candida parapsilosis* | *Proteus mirabilis + Staphylococcus epidermidis* |
| 6 | *Candida glabrata* | *Providencia stuartii + Staphylococcus aureus* |

### *2.2.Locks solutions*

Three locks solutions were freshly prepared dimethyl sulfoxyde (DMSO): (a) stock solution of EDTA (Sigma) at 30mg/mL, (b) a stock solution of amphotericin B (Sigma) at 1mg/mL, and (c) a stock solution of EDTA at 30mg/mL+ amphotericin B at 1mg/mL.

### *2.3.Antimicrobial lock technic (ALT) on mixed biofilms*

According to the protocol of Ko et al. (2010) [19] modified, sterile catheter pieces (1,5cm in diameter) were placed in 10mL of a cell suspension (yeast/bacteria: 50/50) of $1\times10^7$UFC/mL in YNB-Glc. Incubate five days at 37°C with shaking at 75rpm to allow biofilm formation. After 5 days of incubation, the catheters pieces were washed twice with PBS to remove non-adherent cells. The colonized pieces were transferred to YNB-Glc containing the antimicrobial solution. The three locks solutions were evaluated on mature mixed biofilm.-Drug-free YNB-Glc was used as a control for each experiment. The anti-biofilm activity of each antimicrobial solution were assayed after lock periods of 1, 3, 5 or 7days. Antimicrobial lock solutions were replaced every 2 days. Biofilm eradication was evaluated by optical density measurement of viable cells. The catheters pieces were moved into new tubes and washed twice with 10mL of sterile PBS to remove planktonic cell and drug residue. Then, the adherent cells were recovered by high-speed vortexing in 3mL of sterile PBS, followed by sonication using conditions of 120s, 30% cycle, 3.5s pulses. The optical density is then read at 570nm using a spectrophotometer (Specord 200 plus). All experiments were performed three times in duplicate.

To confirm the eradication of mixed biofilm (OD=0) after exposure to the antifungal lock solution, a quantitative evaluation of biofilm removal is carried out. Viable cells are quantified colorimetrically using a 2,3-bis(2-methoxy-4-nitro-5isulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay. XTT solution (SIGMA) was prepared in phosphate-buffered saline (PBS) to a final concentration of 1 mg/mL. A filter-sterilized solution of menadione (SIGMA) (0.4 mM) was also prepared and filtered immediately prior to each assay. A 240 µL solution of XTT was added to each well and incubated for 3 hours at 37°C. After incubation, formazan production was measured by determining absorbance at 490 nm using a spectrophotometer.

### *2.4.Statistical analysis*

Statistical analysis were performed using Graph Pad prism version 8.0.2 (263). The two-way ANOVA was conducted to compare the difference among the three locks solutions using for ALT and duration of treatment. A *p*-value < 0.0001 was considered statistically significant.

An additional analysis is performed to compare the different factors studied. The Tukey post-hoc test is used to compare the mixtures in pairs. Dunnett's t-test is used to treat one group as a control and compare all other groups with it.

## 3. Results

### *3.1.Mixed biofilm formation*

Table 2 display the optical density (OD) measurement of initial viable mixed biofilm of each association after 5 days incubation. OD was 0,89 for *C. tropicalis/P. mirabilis,* 0,75 for *C. tropicalis/P. stuartii*, 0,81 for *C. famata/E.coli/P. aeruginosa/S. aureus*, 0,53 for *C. famata/K. pneumoiae/P. mirabilis,* 0,82 for *C. parapsilosis/P. mirabilis/S. epidermidis* and 0,43 for *C. glabrata/P. stuarttii/S. aureus*. We note that all poly-microbial associations (yeast/bacteria) are able to form multi-species biofilms (DO>0,4 according to Villar-Vidal et al. 2011 [20]).

**Table 2: Ability of co-isolated *non albicans Candida* species/ bacteria strains to form mixed biofilms**

| **Associated strains (non *albicans Candida* yeasts + bacteria strains)** | **DO mixed biofilm after 5 days of incubation** |
|---|---|
| *Candida tropicalis + Proteus mirabilis* | 0,89 |
| *Candida tropicalis + Providencia stuartii* | 0,75 |
| *Candida famata+ Escherichia coli + Pseudomonas aeruginosa + Staphylococcus aureus* | 0,81 |
| *Candida famata +Klebsiella pneumonia+ Proteus mirabilis* | 0,53 |
| *Candida parapsilosis + Proteus mirabilis + Staphylococcus epidermidis* | 0,82 |
| *Candida glabrata Providencia stuartii + Staphylococcus aureus* | 0,43 |

### *3.2.Effect of antimicrobial lock technic (ALT)*

Fig 1 to 6 display the relative ALT effectiveness of the three antimicrobial solutions against mixed biofilms formed by *non albicans Candida* species / *bacteria*. The EDTA solution (30mg/mL) partially reduced (26% to 38%) the mixed biofilms *C. tropicalis/P. stuartii* (Fig. 2), *C. parapsilosis /P. mirabilis/S.epidermidis* (Fig. 5) and *C. glabrata/P. stuarttii/S. aureus* (Fig. 6) after 7 days exposure. However, it halved the mixed biofilm *C.tropicalis/P.mirabilis* (Fig. 1), *C. famata/E. coli/P. aeruginosa/S. aureus* (Fig. 3) and *C. famata/K. pneumoiae/P. mirabilis* (Fig4). Within 7

days exposure, the AmB solution (1mg/mL) halved the mixed biofilms *C. tropicalis/P. mirabilis* (Fig. 1), *C. tropicalis/P. stuartii* (Fig. 2), *C. famata/E. coli/P. aeruginosa/S. aureus* (Fig. 3), *C. parapsilosis/P. mirabilis/S. epidermidis* (Figure. 5) and *C. glabrata/P. stuarttii/S. aureus* (Fig. 6), when it reduced 70% of the biofilm association *C. famata/K. pneumoiae/P. mirabilis* (Fig. 4).

The locked solution EDTA (30mg/mL) + AmB (1mg/mL) showed the most efficiency in treating non-*albicans Candida* species /bacteria biofilms as it eliminated detectable viability removed within 7 days for *C. famata/E.coli/P. aeruginosa/S. aureus* (Fig. 3) and *C. famata/K. pneumoiae/P. mirabilis* (Fig. 4). This antimicrobial solution (EDTA 30mg/mL/AmB 1mg/mL) reduce more than 90% of detectable biofilms of *C. tropicalis/P. mirabilis* (Fig. 1), and *C. parapsilosis/P. mirabilis/S. epidermidis* (Fig. 5), and 85% of detectable biofilms *C. tropicalis/P. stuartii* (Fig. 2) and *C. glabrata/P. stuarttii/S. aureus* (Fig. 6) respectively after 7 days of exposure.

Our results show that EDTA enhance the effectiveness of AmB ALT against mixed biofilm *C. tropicalis/P. mirabilis* (Fig. 1), *C. tropicalis/P. stuartii* (Fig. 2), *C. famata/E.coli/P. aeruginosa/S. aureus* (Fig. 3), *C. famata/K. pneumoiae/P.* mirabilis (Fig. 4), *C. parapsilosis /P. mirabilis/S.epidermidis* (Fig. 5) and *C. glabrata/P. stuarttii/S. aureus* (Fig. 6). As shown in Fig. 1 to 6 we noted a significant difference among the test group at 1day ($p<0.0001$), 3 day ($p<0.0001$), 5 day ($p<0.0001$) and 7 day ($p<0.0001$) of treatment. The OD of mixed biofilm following the combination treatment EDTA+AmB was lower than associated with treatment of EDTA alone ($p<0.0001$), AmB alone ($p<0.0001$) or the broth control ($p<0.0001$).

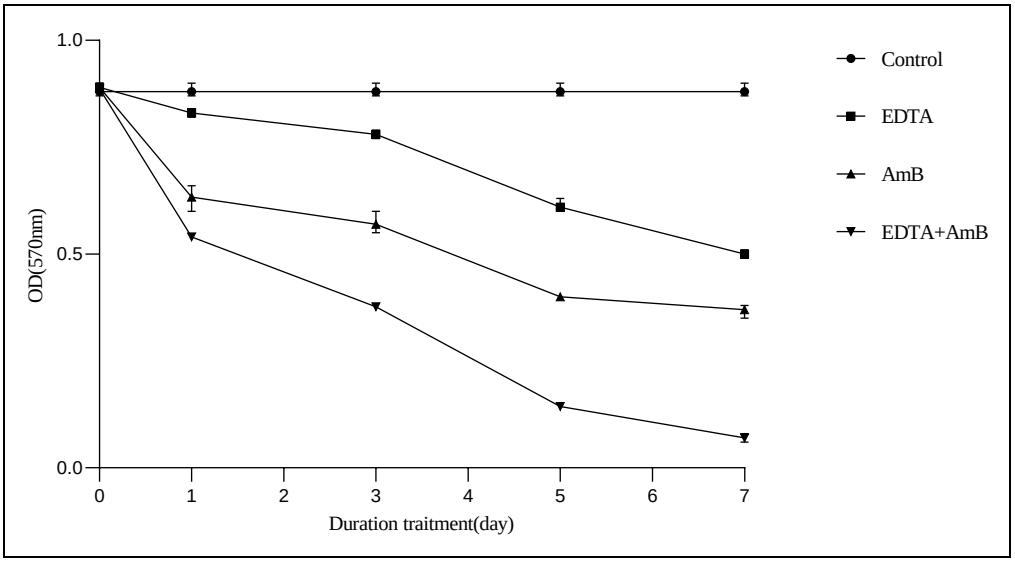


**Figure 1** : Effect of ALT against mixed biofilms formed by *C. tropicalis/P. mirabilis*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control ($p<0.0001$)

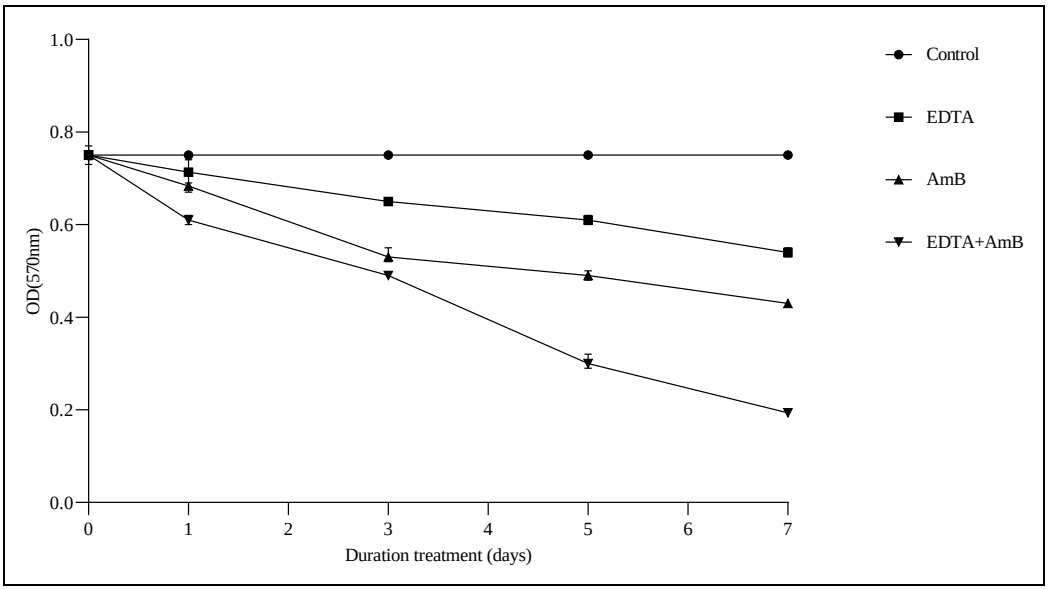


**Figure 2** : Effect of ALT against mixed biofilms formed by *C. tropicalis/P. stuartii*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control (p<0.0001)

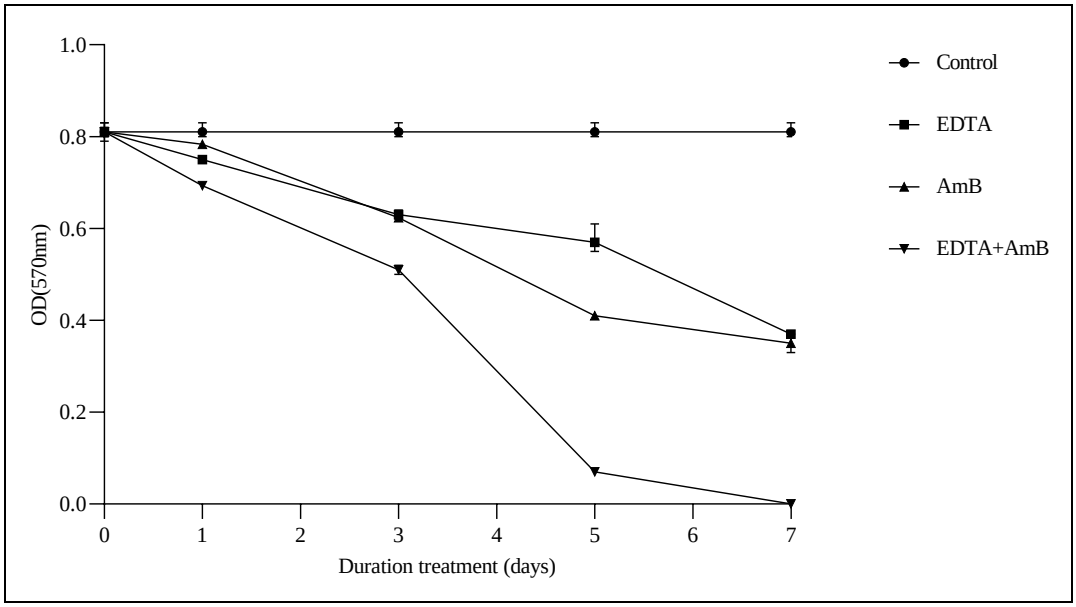


**Figure 3** : Effect of ALT against mixed biofilms formed by *C. famata/E.coli/P. aeruginosa/S. aureus*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control (p<0.0001)

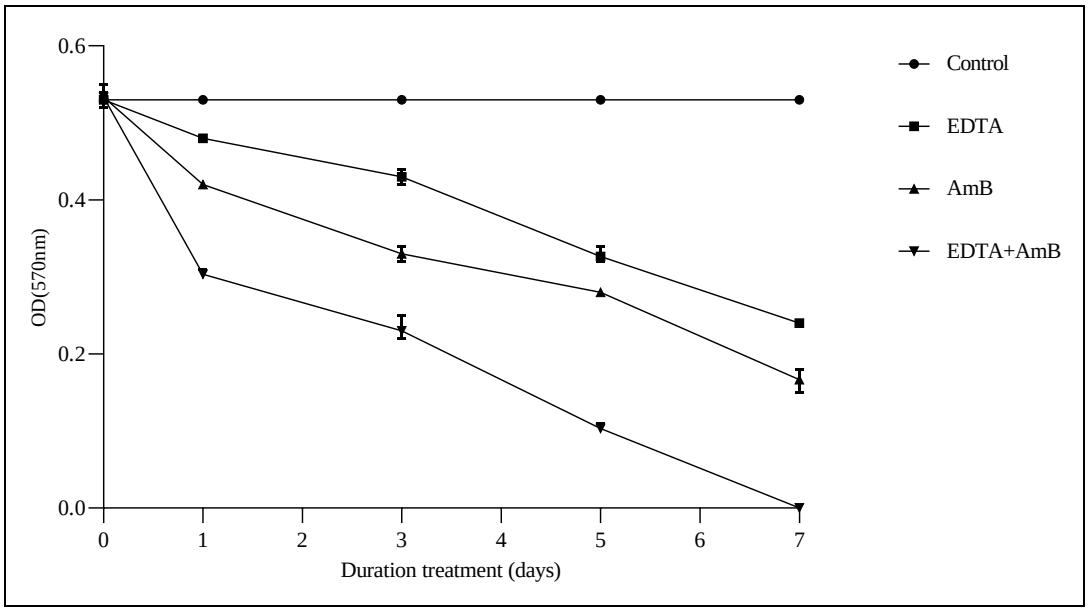


**Figure 4** : Effect of ALT against mixed biofilms formed by *C. famata/K. pneumoiae/P. mirabilis*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control (p<0.0001)

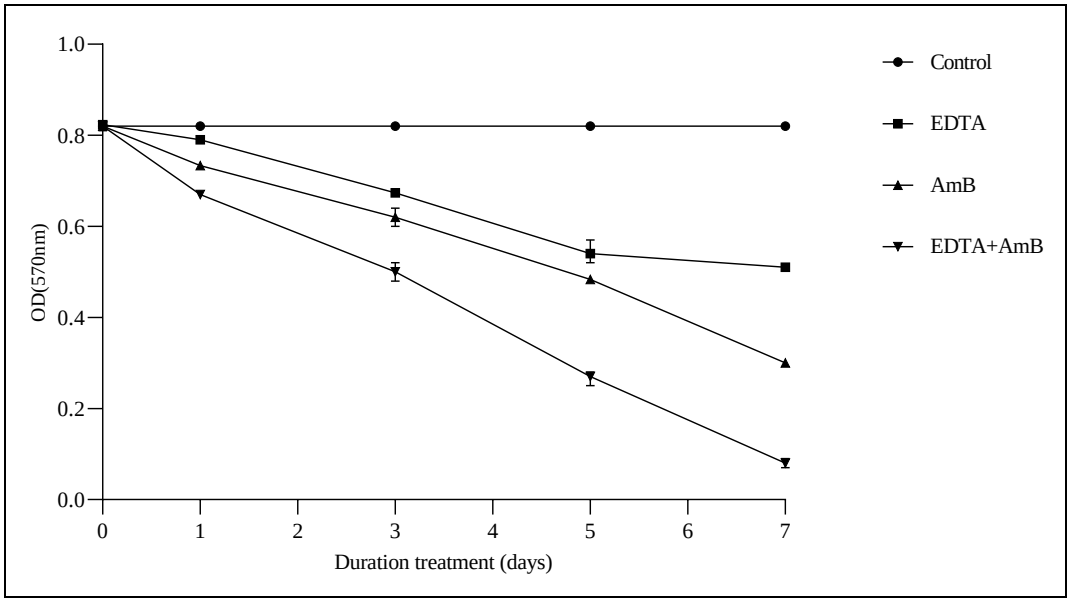


**Figure 5** : Effect of ALT against mixed biofilms formed by *C. parapsilosis /P. mirabilis/S.epidermidis*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control (p<0.0001)

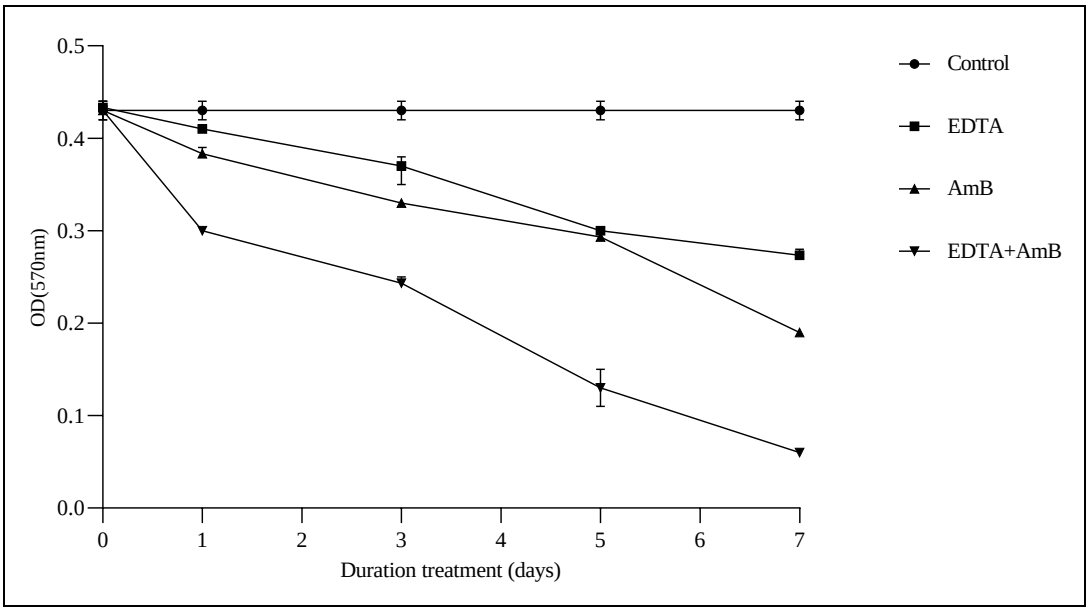


**Figure 6** : Effect of ALT against mixed biofilms formed by *C. glabrata/P. stuarttii/S. aureus*: EDTA+ AmB was significantly more effective than EDTA, AmB or broth control ($p<0.0001$)

## 4. Discussion

The ability to form biofilms on medical devices is an important virulence factor for pathogenic microorganisms [3, 21]. The scientific community is well aware of the importance of biofilms in the contraction of human diseases, due to the increasing number of nosocomial infections linked to medical implants as well as the appearance of new pathogenic species able to form biofilms. Removal of the catheter is the most effective treatment for catheter-related infections, especially in patients with severe sepsis or septic shock. However, the catheter should be retained in patients with stable condition to avoid its replacement [19]. As a conservative strategy, treatment with lock solutions is recommended as an alternative option for catheter-related infections [22]. Although some studies have evaluated the efficacy of the lock solution on *Candida spp* or bacterial biofilms [23, 24, 25], but there are no experimental data on the efficacy of lock solutions on polymicrobial yeast-bacterial biofilms. In the present study, the results obtained show that treatment of catheters with an AmB lock solution (1mg/mL) reduces mixed non-*albicans Candida species* / bacteria biofilms by 40 to 70% depending on the species. Whereas the EDTA lock solution (30mg/mL) only induces a partial reduction (between 25 and 55%) of mixed biofilms *(*non-*albicans Candida* yeasts / bacteria) after 7 days of locking. In contrast, when EDTA is added to AmB to treat the mixed biofilms *(*non-*albicans Candida* yeasts / bacteria) we observe a reduction of more than 60% in biofilm biomass after five days. Furthermore, complete eradication of most mixed biofilms was observed on the 7$^{th}$ day of treatment. This result is in line with the study by Ko et al. (2010) [19], which found that a limited duration of the locked solution, less than five days, using amphotericin B is sufficient to treat fungal colonized catheters, which will decrease the risk of resistance as well as the cost in clinical practice. We also note that the effectiveness of the locked solution seems to depend on the fungal strain and the bacterial species associated with this yeast. On the other hand, it appears that EDTA enhance the effectiveness of AmB ALT against mixed biofilm. This result is in agreement with the study of Raad et al. (2008) [26] who showed that EDTA enhance the

effectiveness of the antifungal activity of AmB lipid complex against *C. albicans* and *C. parapsilosis* mono-specie biofilm. The observed increase in the efficacy of treatment of mixed biofilms with the lock solution combining amphotericin B and EDTA suggests that EDTA functions as a biofilm disruptor, potentiating and enhancing the antifungal activity of amphotericin B against *non-albicans Candida* species present in the biofilm. According to Sen et al. (2000) [27], EDTA inhibits the export of wall mannoproteins required for cell wall formation. EDTA has also been shown to inhibit the formation of *C. albicans* biofilms by inhibiting filamentation [28]. In addition, EDTA disrupts the microbial biofilm through its chelation of calcium, magnesium and iron, which are essential components of the biofilm matrix [29, 30, 31, 32]. It seems that EDTA reinforcing the role of amphotericin B against microorganisms in the poly-microbial biofilm.

## 5. Conclusion

The infectious risk associated with the use of medical devices in hospitals is one of the most frequent complications that constitute a major public health problem and an economic burden. The role of biofilms in these infections is to serve as an infectious reservoir for a variety of bacteria and yeasts. These mixed bacterial-fungal infections may have a correlation with increased frequency or severity of disease. The treatment of these polymicrobial structures is an important issue in the management of the patients surveyed.

Elimination of polymicrobial biofilms *non-albicans Candida* species / bacteria, using amphotericin B-EDTA lock solution would be an inexpensive and easily accessible solution in the hospital setting, particularly for patients requiring the use of indwelling catheters.

### CRediT authorship contribution statement

**Hanane Ziane**: methodology; writing-review and editing. **Lamia Belkherroubi-Sari**: conceptualization, validation, writing-original draft, writing-review and editing, supervision, visualization. **Zahia Boucherit-Otmani**: methodology. **Meriem Benguella-Benmansour:** formal analysis. **Kebir Boucherit** : methodology

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Conflict of interest statement

The authors have no conflicts of interest.

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**Tables and figures:**

**Table 1**: Distribution of non-*albicans Candida* species and the co-isolated bacteria strains isolated from the same medical device.

**Table 2**: Ability of co-isolated *non albicans Candida* species/ bacteria strains to form mixed biofilms

**Figure 1**: Effect of ALT against mixed biofilms formed by *C. tropicalis/P. mirabilis*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control ($p<0.0001$).

**Figure 2**: Effect of ALT against mixed biofilms formed by *C. tropicalis/P. stuartii*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control ($p<0.0001$).

**Figure 3**: Effect of ALT against mixed biofilms formed by *C. famata/E.coli/P. aeruginosa/S. aureus*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control ($p<0.0001$).

**Figure 4**: Effect of ALT against mixed biofilms formed by *C. famata/K. pneumoiae/P. mirabilis*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control ($p<0.0001$)

**Figure 5**: Effect of ALT against mixed biofilms formed by *C. parapsilosis /P. mirabilis/S.epidermidis*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control ($p<0.0001$).

**Figure 6**: Effect of ALT against mixed biofilms formed by *C. glabrata/P. stuarttii/S. aureus*: EDTA+AmB was significantly more effective than EDTA, AmB or broth control ($p<0.0001$).