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Simulated Vaginal Fluid: *Candida* resistant strains' biofilm characterization and vapor phase of essential oil effect

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Abstract

Introduction: Vulvovaginal candidiasis is a disease that affects millions of women worldwide. Oral formulations, topical creams or ointments are the conventional dosage forms, with an increase in drug administration through vaginal via. The use of simulated biological fluids (e.g. vaginal fluid) in the evaluation of antifungal therapies may better mimic the real biological environments and therefore provide a better understanding of the behavior of the antifungal.

Methods: The main objective of this work was to compare planktonic growth and biofilm formation of *Candida* species, on common growth medium, Sabouraud Dextrose Broth (SDB) and on vaginal simulation conditions, Simulated Vaginal Fluid (SVF), through the optical density determination, colony-forming units and scanning electron microscopy. In addition, under the same conditions this study also evaluated the ability of vapor phase of oregano and white thyme essential oils (VP-EOs), potential alternative treatment, to inhibit biofilm formation and to destroy mature biofilms of vaginal isolates, through the colony-forming units determination.

Results: *Candida* isolates maintained the same biofilm formation capacity and morphology in both media (SVF and SDB). Furthermore, the results obtained in this work related with VP-EOs effect agree with results acquired, previously, with SDB. This means that the effect of VP-EOs is not affected by the SVF medium, and that this fluid allows the dissolution of the volatile and bioactive compounds.

Conclusions: These results can predict the *in vivo* behaviour, suggesting a potential effective application of VP-EOs as prophylactic or therapeutic treatment for biofilm-related vulvovaginal candidiasis.

KEYWORDS: Vulvovaginal candidiasis; Simulated Vaginal Fluid; Biofilm; Vapor phase of essential oils; Vaginal *Candida* isolates.

Introduction

Vulvovaginal candidiasis (VVC) is a very common disease, which affects about 70 % of women at least once during their childbearing years [1,2]. In fact, approximately 138 million women worldwide suffer up to 4 episodes of recurrent VVC (RVVC) per year, due to treatment failure [3]. The most common pathogen is *Candida albicans*; however, the abundance of non-albicans *Candida* species (NAC) has increased over time in VVC infections, particularly in RVVC [4,5]. Among the available antifungals, azoles are the most frequently used drugs in the treatment of VVC. Nevertheless, increasing rates of resistance to azoles class threaten the effectiveness of these agents, in both *C. albicans* and NAC species [6]. Of the NAC species, *Candida glabrata* has shown higher rates of resistance to azoles agents, *Candida krusei* (teleomorph *Pichia kudriavzevii*) is intrinsically resistant to azoles and *Candida guilliermondii,* despite the low incidence of candidemia caused by this organism, has particular clinical significance as it exhibits increased resistance to antifungal agents, compared to other *Candida* species [7,8]. In fact, the biofilm formation, characteristic of this species, leads to high levels of resilience to common antifungal agents, requiring long and intensive therapies [9,10].

Oral formulations, topical creams or ointments are the most conventional pharmaceutical forms [11]. Indeed, drug administration to the vaginal via has gained increasing attention in past decades [11,12]. The vaginal epithelium is usually coated with a surface film of moisture, the vaginal fluid. Vaginal fluid is composed of cervical fluid (contain epithelial glycogen carbohydrates, amino acids, aliphatic acids, and proteins) and small amounts of the secretion from Bartholin's glands (contains a variety of antimicrobial substances, including lysozyme, lactoferrin, fibronectin, polyamines such as spermine and IgA) in the vaginal wall, which serve as a protective barrier against infections [11]. So, the use of simulated biological fluids in *in vitro* evaluation

of antifungal therapies is a promising technique which can allow a better understanding of the antifungal's behavior within biological environments, helping to determine mechanisms of action and potentially predict *in vivo* behavior [13].

Essential oils (EOs) have been suggested as potential alternative treatment, with strong anti-*Candida* effect reported combined with lower side effects, lower toxicity and better biodegradability when compared to available antimicrobial agents [14,15]. So, the administration of EOs through their vapor phase (VP-EOs) can be used for the local release of volatile and bioactive compounds from EOs directly at the local of action (vaginal epithelium). In fact, the VP-EOs has already shown greater activity for the growth of *Candida* sp, avoiding direct contact of the EO with the skin [16]. In this study, two EOs (oregano and white thyme) were selected, due to their characteristics. White thyme EO is recognized for respiratory tract cleansing and calming properties, furthermore, it is described in the literature that it was able to inhibit the growth of NAC species resistant to conventional antifungals [17–19]. In turn, Oregano EO has purifying and stimulating properties and it is, also, described in the literature that it was able to inhibit, *in vitro*, the germination and development of the filamentous form of *C. albicans* and to induce a significant reduction in biofilm formation [19,20].

The most important objective of this work was to compare planktonic growth and biofilm formation of drug-resistant *Candida* species between common growth medium and under vaginal simulation conditions. For this purpose, a synthetic medium with physical and chemical properties very similar to those of vaginal secretions from health women was used. In addition, under the same conditions this study also investigated the ability of VP-EOs to inhibit biofilm formation and to destroy mature biofilms of these vaginal isolates.

Material and Methods

Microorganisms and initial culture conditions

For this study, *C. albicans*, *C. glabrata*, *C. krusei* and *C. guilliermondii* vaginal isolates, recovered from patients and identified as resistant to antifungal agents, belonging to a collection of yeasts created by the *Candida* Research Group of Centre o Biological Engineering were used [21]. *Candida* isolates were stored at –80 ± 2ºC in Sabouraud Dextrose Broth (SDB; Liofilchem) medium with 20 % (v/v) glycerol. The isolates were subcultured from the frozen stock onto Sabouraud Dextrose Agar (SDA; Liofilchem) plates and incubated during 24 h. Then, colonies from SDA plates were used to inoculate SDB for 18 h at 37ºC under agitation (120 rev/min). The cellular suspensions were centrifuged at 5000 g for 10 min at 4 ºC and washed twice with Phosphate Buffered Saline (PBS).

This study evaluated the antifungal activity of two EOs (oregano EO (*Origanum compactum*, florame®, Portugal) and white thyme EO (*Thymus satureiodes;* florame®, Portugal)) with 100% purity. EO's products were stored in the dark at room temperature.

Simulated Vaginal Fluid

Simulated Vaginal Fluid (SVF) was modified from Owen & Katz (1999) and Grazyna J et al., (2008), and consisted of 58 mM NaCl (Biochem), 18 mM KOH (AppliChem), 2 mM $Ca(OH)_2$ (Frilabo), 1.75 mM glycerol (Biochem), 6.7 mM urea (Frilabo), 33 mM glucose (Biochem), and 6.7 g yeast nitrogen base (YNB) l^{-1} (Difco). In addition, natural compounds in the vaginal fluid as acetic acid (17 mM; pKa 54.76) and lactic acid (22 mM; pKa 53.85) were added to maintain the pH at 4.2 [22,23].

Effect of Simulated Vaginal Fluid and Sabouraud Dextrose Broth on vaginal *Candida* isolates

Planktonic growth

Vaginal *Candida* isolates cells from the pre-inoculum were cultivated in SVF or SDB. Cultures of *Candida* cells (1×10^5 cells/mL) were placed in 25 mL Erlenmeyer flasks, maintained at 37 °C with agitation (120 rev/min) and the increase in optical density, at 620 nm, was measured over time using a microtiter plate reader (Thermo Scientific™ Multiskan™ FC, Finland). The results were presented as optical density along 24 h of growth, measured at every 3 hours [24].

Biofilm formation

The biofilm's growth and structure of the vaginal *Candida* isolates (*C. glabrata*, *C. albicans*, *C. krusei* and *C. guillermondii*) was evaluated. For this, the biofilms were developed as described by Stepanovi´c *et al* (2000)., with some modifications [25]. Briefly, *Candida* cellular suspensions were adjusted to 1×10^5 cells/mL, in SDB or SVF, and transferred, under aseptic conditions, to 96-well flat tissue culture plates (polystyrene, Orange Scientific, Braine-L'Alleud, Belgium) (200 µL per well). Culture plates were incubated for 24 h at 37ºC under agitation in an orbital shaker (120 rev/min) conditions, during 24 h and 48 h. Biofilms were analysed through the colony-forming units (CFUs) counting methodology and observed under scanning electron microscopy (SEM) [26,27].

The number of cultivable cells in the biofilms formed in SDB or SVF was assessed using the CFUs counting methodology. Briefly, after 24 h or 48 h, the biofilms were washed with PBS, to remove non-adherent cells, and then scraped from the wells with 1 mL of PBS. The obtained suspensions were serially diluted in PBS and plated onto SDA. The SDA plates were incubated for 24 h at 37ºC and then, the number of grown colonies was counted. The results were presented as colony-forming units per square centimetres (Log (CFU/cm^2)).

In order to examine and compare the biofilm structure of the drug-resistant *Candida* isolates formed in SDB or in SVF, they were observed by SEM. For that, biofilms formed in steel coupons, as described above, for 24 h, were dehydrated with ethanol (using 70 % ethanol for 10 min, 95 % ethanol for 10 min and 100 % ethanol for 20 min) and air dried for 20 min. The samples were kept in a desiccator for at least 24 h. Prior to observation, the coupons were subjected to sputter coated with gold and observed with an S-360 SEM (Leo, Cambridge, MA, USA) [28].

Effect of the vapor-phase of essential oils on biofilms

The effect of the VP-EOs (oregano and white thyme EOs) on biofilm formation and on mature biofilms (24 h-old) of four clinical isolates of *Candida* species (*C. glabrata*, *C. albicans*, *C. krusei* and *C. guillermondii*) was evaluated. Biofilms were developed as described above, with some modifications due to the use of volatile compounds[25]. In order to determine the effect of VP-EOs on biofilm formation, *Candida* cellular suspensions adjusted to 1 x 10^5 cells/mL were transferred to glass wells (1 mL per well) and 25 µL of each EOs (100 %) was discarded on a sterile blank disk, which was placed near the wells (the set was kept inside a glass plate). Plates were incubated for 24 h at 37ºC under agitation in an orbital shaker (120 rev/min). Additionally, biofilms were pre-

formed during 24 h and, after this time, they were incubated in the presence of 25 µL of EOs placed on a sterile blank disk, for an additional 24 h. As control, biofilms were formed without any contact with the VP-EOs during 24 h and 48 h. Biofilms were analysed through determining the number of *Candida* cultivable cells through CFUs counting methodology, as described above.

Statistical analysis

The results obtained in this study were statistically analysed using the Prism software package (GraphPad Software version 6.01). For that, the cell cultivability of biofilms formed in SVF or in SDB, or treated with VP-EOs was compared with that of untreated biofilms using one-way ANOVA and Dunnett's multiple comparisons test. All tests were performed with a confidence level of 95%. For all assays, three independent experiments were carried out (independent pre-inocula) and each analysis was performed in duplicate or triplicate. Statistical significance was assumed at $p < 0.05$.

Result and discussion

***Candida* isolates planktonic growth and biofilm formation in Simulated Vaginal Fluid**

The administration of drugs to the vaginal via has gained increasing attention, highlighting the need for reliable *in vitro* methods to evaluate the performance of new formulations or therapies [29]. Thus, in a first step, the planktonic growth of four *Candida* vaginal isolates under simulating vaginal environment conditions (SVF at pH 4.2) and SDB medium was monitored and compared along 24 hours (Fig. 1). The 4 *Candida* species used (*C. albicans* Ca2, *C. guilliermondii* Cgi1, *C. glabrata* Cg7 and *C.*

krusei Ck1) were selected from a previous study [21], as antifungal-resistant strains. The SDB complex medium (pH 5.6 ± 0.2), which contains pancreatic digest of casein, peptic digest of animal tissue and dextrose, is recommended for yeast isolation, qualitative procedures and for the culture or subculture of fungi from clinical and non-clinical species[30]. The low pH and high concentration of dextrose promotes the growth of yeast while inhibiting bacterial growth [30]. On the other hand, the SVF proposed by Owen and Katz (1999) (with some modification), which was used in this study has been used to simulate the composition of vaginal fluids and to test the diffusion and permeation of new drug delivery systems [23,29]. SVF used as the growth medium consists of YNB with modified concentrations of calcium, potassium, chloride and sodium ions, in agreement with concentrations in the vaginal fluid of healthy premenopausal women during the non-menstrual phase [23,29]. The medium also contained glycerol, urea and glucose, which served as the main source of carbon. SVF was buffered at pH 4.2 using the physiological buffers found in the vaginal fluid, i.e lactic acid and acetic acid [23,29]. In healthy women, *Lactobacillus* spp produces lactic acid, which act as buffer and maintains the pH of vagina between 4.0 and 5.0 (acidic), depending on a woman's menstrual cycle. Hydrogen peroxide (H_2O_2) and bacteriocins are also produced, which resist the overgrowth of pathogenic microbes. During pregnancy, the pH drops (3.8- 4.4) and in post-menopause the pH increases (7.0-7.4) [11].

In planktonic conditions, all *Candida* isolates had a similar growth rate between both culture medium (SDB and SVF) along 24 hours. Despite, the SVF had only a slight effect on the growth rate of *C. albicans* Ca2 (Fig. 1A)*, C. guilliermondii* Cgi1 (Fig. 1B) and *C. glabrata* Cg 7 (Fig. 1C) cells. Moreover, the growth rate of *C. krusei* Ck1with

SDB and SVF was parallel, and after 24 hours the same growth rate was observed (Fig. 1 D). This led us to conclude that the SVF is also an good medium to isolate and culture or subculture the *Candida* species.

After the overgrowth of the *Candida* spp, the colonization of the vaginal epithelium occurs and consequently the transformation from asymptomatic to symptomatic, leading to biofilm formation [31]. So, the biofilm formation (24h and 48h) of *Candida* isolates under conditions simulating the vaginal environment (SVF at pH 4.2) and SDB medium was compared (Fig. 2). Biofilms were analysed through determining the CFUs counting methodology (Fig. 2) and by SEM (Fig. 3).

The biofilm formation capacity of the *Candida* isolates after 24 h is similar between both medium (SDB and SVF), except for *C. glabrata* Cg7 which had a lower capacity to form biofilm in SVF (Fig 2A). Interestingly, in the SVF medium, all *Candida* strains have the same capacity of biofilm formation, while in the SDB medium, some species stand out with greater biofilm formation. Thus, the structure and morphological characteristics of the biofilms formed for 24 h, with SVF and SDB, was observed by SEM (Fig 3). In general, *Candida* isolates maintained the same morphology formed in SVF and SDB, i.e, the species that had more tendency to filament in SDB (Fig. 3A) also filamented in SVF (Fig. 3B), such as *C. guilliermondii* or *C. krusei*. However, in the SDB *Candida* isolates cells formed a multilayer and compact biofilm that covered the entire surface, constituted by network of yeast or hyphal forms (Fig 3A). Differently, in the SVF, the biofilm consisted of a discontinuous multilayer with a lower number of cells. Indeed, the overall architecture of the biofilm can vary depending on the substrate on which it is formed and growth conditions, such as medium composition, pH and oxygen availability, as well as matrix composition [27,32,33]. Although the biofilm

formation is a process present in *Candida* species, it differs significantly from strain to strain, depending on the ability of each strain to present certain characteristics, such as dimorphic growth or producing extracellular polymeric substances [33]. For example, in the case of *C. albicans*, the biofilm characteristic of this strain consists of a two-layer structure composed of a heterogeneous mixture of yeasts, pseudohyphae and hyphae embedded in extracellular polymeric substances, composed mainly of carbohydrates, proteins, phosphorus and hexosamines. On the other hand, the typical biofilm formation of *C. glabrata* is a compact monolayer or multilayer structure of only blastospores, where the cells are embedded within an extracellular matrix composed of high levels of carbohydrates, proteins and hexosamines [27].

Regarding the number of cultivable cells of biofilms after 48h (Fig 2B), the SVF showed lower number of cells in three *Candida* isolates (*C. albicans* Ca2*, C. guilliermondii* Cgi1 and *C. glabrata* Cg 7), resulting in a statistically significant decrease on cell cultivability (p-value ≤0.0001) compared to biofilms formed in the SDB. Despite this, it is considered that the differences found are not very significant, as cell formation and morphology were maintained.

Effect of the vapor-phase of essential oils on biofilms

The bioavailability of vaginally administered therapies depends on the effective dissolution of solid drug particles, if present, in the vaginal fluid prior to absorption, and the degree of deactivation by enzymes present in the fluids [11]. So, to optimize formulations for the vaginal via, it is important to consider several points, specifically the effective dissolution in the vaginal fluid [11,29]. Vaginal fluid is, in fact, the first physical barrier that the administered formulations encounter, and it has been demonstrated that its composition, pH and osmolarity can significantly affect the

delivery of drugs and efficacy [34]. Indeed, vaginal pH affects the degree of ionization of drugs, which can affect their absorption [11]. Considering the characteristics of these fluids and their impact on the effectiveness of the drug formulation, it is crucial to evaluate the effect of therapies with medium that mimics the vaginal environment. Therefore, in this work the effect of VP-EOs on *Candida* species in SVF was evaluated. This approach may allow the reduction of the drug dose needed to produce the pharmacological effect, possibly minimizing systemic side effects and eliminating problems related to first-pass metabolism [35]. So, in order to study the effect of the vapor phase of two EOs (oregano (VP-OEO) and white thyme (VP-WTEO)) on the biofilm formation and pre-formed biofilms of antifungal-resistant *Candida* isolates (*C. albicans* Ca2, *C. guilliermondi* CgiI1, *C. glabrata* Cg7 and *C. krusei* Ck1) in SVF, the number of cultivable cells (Fig. 3) was evaluated.

Generally, the VP-EOs of both oils led to inhibition of biofilm formation and reduction of mature biofilm. The results related to biofilm formation revealed that both EOs had a high inhibitory effect on all tested isolates (Fig. 3A). In fact, VP-OEO was able to completely inhibit the formation of *C. albicans* Ca2 and *C. guilliermondii* Cgi1 biofilms. In addition, a significant reduction, ranging from 1-5 orders of magnitude (Log CFU/mL), in comparison to the absence of VP-EOs was observed in the formation of *C. glabrata* and *C. krusei* Ck1. Moreover, the VP-OEO was also able to completely reduce mature biofilms (24 h) of *C. albicans* Ca2*, C. glabrata* Cg7 and *C. guilliermondii* Cgi1 (Fig. 3B). However, with VP-WTEO only a small reduction was observed to the different species, ranging from 0.2-2 orders of magnitude (Log CFU/mL), both in the formation of biofilms and in mature biofilms (Fig 3). The significant difference observed in the antifungal activity of the two EOs may be a result of the main bioactive compounds of the EOs or due to a synergistic action between the

minor and major compounds [36]. In fact, the antifungal activity of EOs can differ greatly even within the same plant species, according to their origin and chemical profile [37].

Curiously and according to results published recently by our team when the VP-EOs was able to reduce mature biofilms, its impact was higher than on biofilm formation (Fig. 3). The results obtained in this work agree with those results published previously, where SDB was the medium used, this means that the effect of VP-EOs is not affected by SVF as growth medium and that this fluid allows the dissolution of the volatile and bioactive compounds. Inclusive, the results obtained with SVF predict *in vivo* behaviour, suggesting a potential effective application of VP-EOs as prophylactic or therapeutic treatment for biofilm-related VVC. In fact, also Wang et al. (2011) and Pietrella et al. (2011) verified that EO was highly effective in accelerating the elimination of *C. albicans in vivo*, from the vagina of experimentally infected mice, by prophylaxis and therapeutic treatments [38,39].

Conclusion

In the present study, we compared the planktonic growth and biofilm formation of *Candida* species, between SDB and under simulated fluid vaginal (SVF) conditions in order to validate an *in vitro* model. SVF was evaluated as reliable and capable of mimic the vaginal environment and allow the assessment of drug/compound permeation, in this case VP-EOs, intended for vaginal administration. Indeed, this study demonstrates that the SVF medium offers a reliable and predictive tool for mimicking these environments and allow, in fact, to evaluate the behaviour of VP-EOs intended for vaginal administration. Thus, a potential application of VP-EOs as a prophylactic or therapeutic treatment for VVC is suggested. Of the two EOs tested (oregano and white

thyme), VP-OEOs showed the highest antifungal activity. Thus, in the future, studies to analyse the mechanism of action of VP-OEO in *Candida* cells grown in SVF will be important.

Conflict of interest: The authors have no relevant financial or non-financial interests to disclose.

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Figure 1. Simulated Vaginal Fluid (SVF) and Sabouraud Dextrose Broth (SDB) on vaginal *Candida* isolates planktonic cells. Planktonic growth curves of *C. albicans* Ca2 (A), *C. guilliermondii* Cgi1 (B), *C. glabrata* Cg 7 (C) and *C. krusei* Ck1 (D) cells cultivated in SVF and SDB over 24 h.
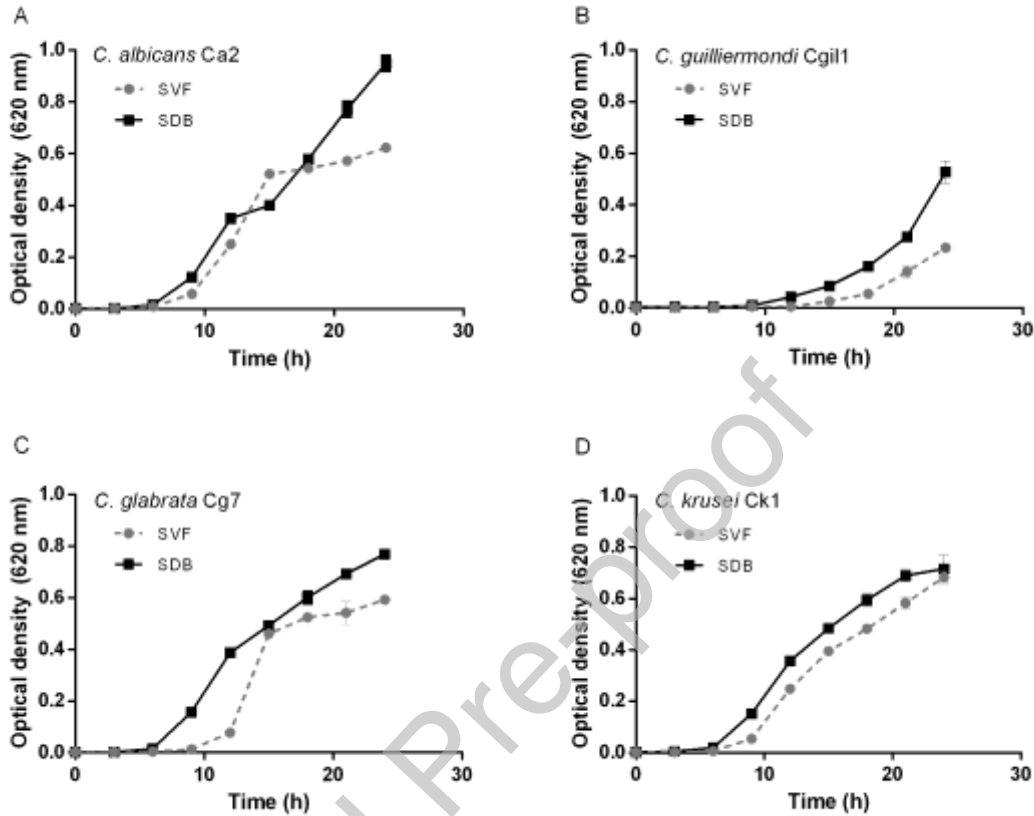

Figure 2. Biofilm formation of antifungal-resistant *Candida* isolates in SVF and SDB. Number of cultivable cells (Log $CFUs/cm^2$) for 24 h (A) and 48 h (B). * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).
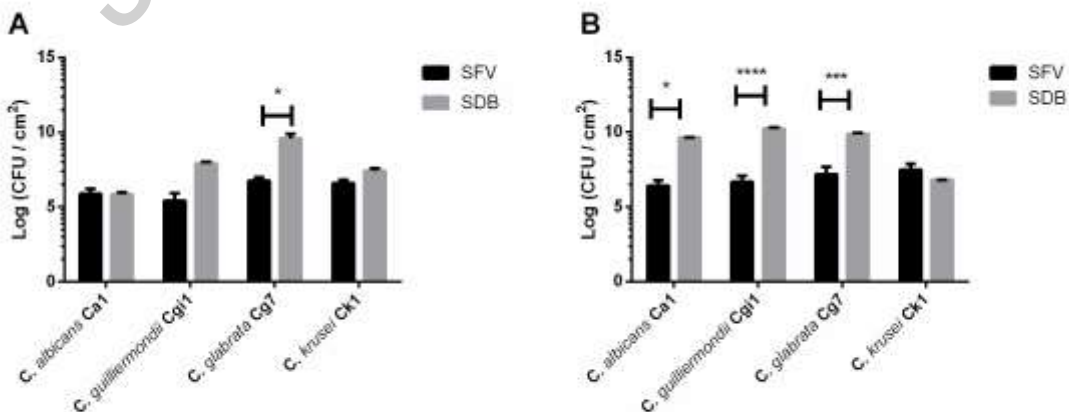
Figure 3. SEM observations of biofilms formation structure (24 h) of antifungal-resistant *Candida* isolates in SVF (A) and SDB (B). Magnification: 5,000×. Scale bar=30 μm.
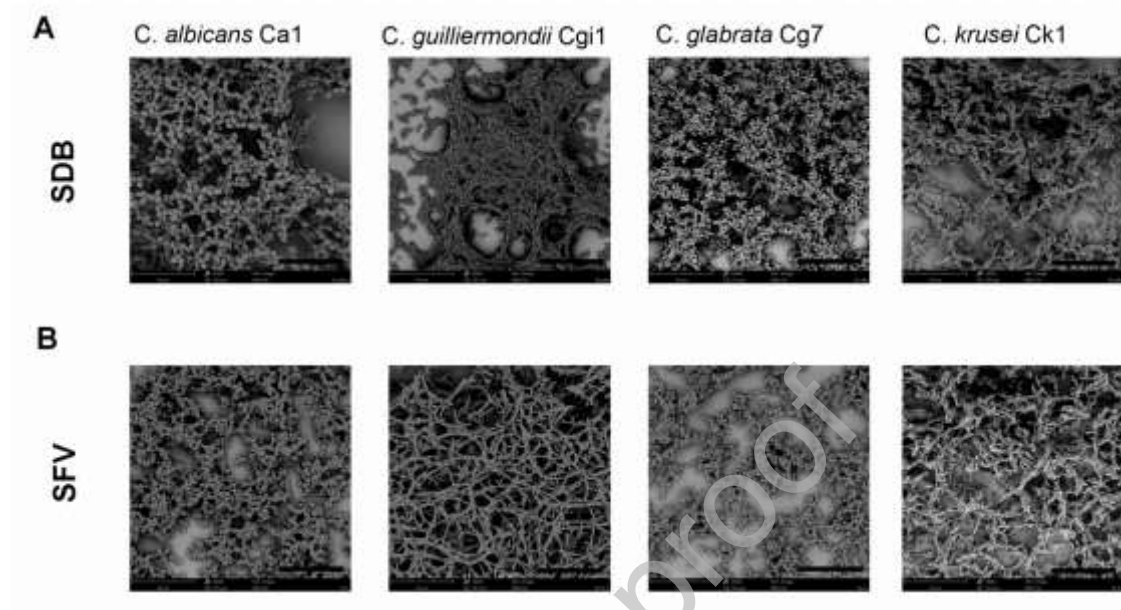

Figure 4. Effect of the vapor phase of essential oils (oregano and white thyme) on biofilm formation (A) and pre-formed biofilms (B) of antifungal-resistant *Candida* isolates. Biofilms of *C. albicans* Ca2, *C. guilliermondii* CgiI1, *C. glabrata* Cg7 and *C. krusei* Ck1 were developed in the absence (control) and presence of the VP-EOs. * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* p< 0.1, ** p< 0.01, *** p< 0.001, **** p< 0.0001).