Research Paper

Candida species extracellular alcohols: production and effect in sessile cells

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Cell-cell signaling alcohol molecules were recently identified in *Candida albicans* and *Candida dubliniensis* supernatants. To date, it is not known whether these molecules are produced by other *Candida* species and their role in biofilm formation is not fully clarified. Herein, *Candida parapsilosis* and *Candida tropicalis* extracellular alcohols production by planktonic cultures was analyzed by headspace-solid-phase microextraction and gas chromatography-mass spectrometry. Both these *Candida* species extracellular media contained *E*,*E*-farnesol, 1-dodecanol, 2-phenylethanol, and isoamyl alcohol but not *E*-nerolidol, as produced by *C. albicans* and *C. dubliniensis*. Moreover, the ability of these compounds to regulate *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis* sessile cells was assessed by adding the alcohols after 3 h of adhesion and 48 h of biofilm formation. After 24 h, biofilms were analyzed in terms of cellular mitochondrial activity and total biomass. *E*,*E*-Farnesol affected *C. albicans* and *C. dubliniensis*. *E*-Nerolidol and 1-dodecanol elicited *C. parapsilosis* and *C. tropicalis* changes in further biofilm development. *C. tropicalis* was affected by 2-phenylethanol and isoamyl alcohol triggered changes in *C. albicans*, *C. dubliniensis*, and *C. tropicalis* sessile cells. The results demonstrated that almost all of these alcohols are produced by these *Candida* species and also evidence the complexity of biofilm formation.

Abbreviations: HS-SPME: headspace-solid-phase microextraction and gas chromatography-mass spectrometry; XTT: [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide]

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Introduction

Candida albicans is the predominant etiological agent of candidiasis, nevertheless, non-*C. albicans Candida* (NCAC) species have been emerging as significant pathogens [1]. Specifically, *Candida dubliniensis* has been associated with oral candidiasis infections in human immunodeficiency virus infected and acquired immunodeficiency syndrome populations [2]. *Candida parapsilosis* and *Candida tropicalis* are pathogens found in association with candidemia, and are frequently isolated from implanted devices [3, 4].

The success of *Candida* species as pathogens is attributed to survival strategies such as their ability to form biofilms. In fact, biofilms may initiate or prolong infections by providing a source of cells that can colonize local and new sites, and be recalcitrant to antifungal therapy and host immune defences [3]. *C. albicans* biofilm formation is modulated by the extracellular alco-

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hol *E*,*E*-farnesol [5]. However, other extracellular alcohols have been identified in *C. albicans* and *C. dubliniensis* supernatants namely *E*-nerolidol, 1-dodecanol, 2-phenylethanol, and isoamyl alcohol. These molecules were shown to inhibit *C. albicans* and *C. dubliniensis* yeast to filament formation in filamentation inducing conditions [6], but the production and role of these molecules in other *Candida* species has been poorly studied.

This work aimed at investigating the production of extracellular alcohols by *C. parapsilosis* and *C. tropicalis*, and to evaluate the effect of the addition of such alcohols' commercial formulations on *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, and *C. tropicalis* biofilm development.

Materials and methods

Chemicals

E,*E*-Farnesol (purity ≥95%), 2-phenylethanol (purity ≥99%), and isoamyl alcohol (purity ≥99%) were obtained from Sigma Aldrich, and *E*-nerolidol (purity ≥85%) and 1-dodecanol (purity ≥99.5%) were obtained from Fluka. Pure compounds were stored according to suppliers' instructions. *E*,*E*-farnesol, *E*-nerolidol, 1-dodecanol, and 2-phenylethanol for simplicity are referred through the text as farnesol, nerolidol, dodecanol, and phenylethanol, respectively.

Isoamyl alcohol working solutions were prepared by direct dilution in growth medium. All the other alcohols stock solutions were prepared in methanol (VWR) and appropriated dilutions made in growth medium. Stock solutions were prepared freshly before each experiment and appropriated controls were performed in order to ensure compounds stability through the course of the experiments.

Farnesol was already described to regulate biofilm formation of *C. albicans* [5], *C. parapsilosis* [7], and *C. dubliniensis* [8]. In this sense, 150 μmol l^{-1} farnesol was used as a control in this study. The selected concentrations of all the other alcohols were based on previously reported physiological and supraphysiological concentrations in *C. albicans* and *C. dubliniensis* [6]. Specifically, they were used at final concentrations of: 1.5×10^{-3}, 1.5, and 150 μmol l^{-1} for nerolidol; 2×10^{-3} and 2 μmol l^{-1} for dodecanol; 5 and 500 μmol l^{-1} for phenylethanol, 46×10^{-3} and 23 mmol l^{-1} for isoamyl alcohol.

Strains

In this study the following *Candida* type strains were used: *C. albicans* CECT 1472, *C. dubliniensis* CBS 7987, *C. parapsilosis* ATCC 22019, and *C. tropicalis* ATCC 750.

Growth conditions

Candida species were stored at −80 °C and propagated by streaking a loopfull of cells onto Sabouraud dextrose agar medium (Liofilchem) and incubating at 37 °C for 24 h. These stocks were stored at 4 °C for no longer than two weeks.

For all experiments, batches of Sabouraud dextrose broth (Liofilchem) (20 ml in 50 ml flasks) were inoculated with freshly grown *Candida* cells, and incubated at 37 °C with agitation (130 rpm). After 24 h of growth the cells were harvested by centrifugation, washed twice with ultrapure sterile water, and enumerated in a hematocytometer. Before use, in further experiments, 1×10^6 cells ml^{-1} standardized cell suspensions were prepared in RPMI-1640 L-glutamine (Sigma), supplemented with sodium bicarbonate [0.2% (w/v)], and buffered with morpholinepropanesulfonic acid (Sigma) (0.165 mol l^{-1}, pH 7.0).

Preparation of supernatant samples

C. parapsilosis and *C. tropicalis* culture supernatants were prepared by inoculation of 120 ml of a standardized cell suspension in 300 ml flasks, and incubated at 37 °C with shaking at 130 rpm for 24 h. Cell free fractions were recovered by centrifugation and filter sterilized (0.22 μm pore size).

Culture biomass dry weight measurements were performed by filtration of 1 ml of culture through preweighed filters (0.45 μm) and washed three times with ultrapure sterilized water. Filters were dried at 60 °C until constant weight and cell dry weight was determined.

Supernatant alcohol analysis

Supernatant alcohol composition was evaluated by headspace-solid-phase microextraction (HS-SPME) using a carbowax/divinylbenzene-coated fibre combined with gas chromatography-mass spectrometry (GC-MS) as described by Martins *et al.* [6]. In order to increase the sensitivity of the methodology, the samples were analysed in selective ion monitoring acquisition mode: farnesol (*m*/*z* 69, 107 and 161), nerolidol (*m*/*z* 69, 93 and 107), dodecanol (*m*/*z* 69, 83 and 111), phenylethanol (*m*/*z* 91 and 122), and isoamyl alcohol (*m*/*z* 55 and 77). These alcohols were identified by comparing GC retention times and mass spectra with those of pure standard compounds. A control analysis was performed with RPMI medium, and no interfering substances were found in the retention times of those compounds. The GC peak area data were used (1) to compare alcohols' relative abundance in different *Candida* species (determining the peak areas in both species); (2) to estimate

the compound level in the sample (comparing the GC peak area of each alcohol in the sample with that of one standard with a known concentration).

Biofilm formation on the surface of wells of microtiter plates

For the determination of the effect of alcohols on subsequent biofilm development, a rapid and robust method, the 96-well plate model [9] that allows testing of multiple parameters in the same experiment was used. Due to the volatile nature of these alcohols and to avoid possible cross contaminations, each compound was assayed individually in a single microtitre plate.

Candida biofilms were formed on commercially available polystyrene, flat-bottomed, 96-well microtitre plates (Orange Scientific). Standardized cell suspensions (200 μl) were seeded into the wells and the plates were incubated at 37 °C and 130 rpm. After 3 h, the medium was aspirated, and the wells were washed twice with ultrapure sterile water to remove non-adherent cells.

For the study of alcohols' effect on subsequent biofilm formation of adhered cells, each alcohol diluted in RPMI medium (200 μl) was added to the adhered yeast cells. The plates were incubated at 37 °C (130 rpm) for additional 24 h.

To evaluate alcohols' effect on further development of preformed biofilms, RPMI medium was added to adhered cells, and was renewed after 24 h of growth. At 48 h of incubation, the medium was removed, and the wells were washed as described above. Two hundred microlitres of fresh medium containing each alcohol at the desired concentrations were added to each well, and the plates were incubated for further 24 h.

Positive controls for biofilm formation consisted of unchallenged biofilms. Negative background controls for subsequent analysis consisted of non seeded wells filled with RPMI medium. Solvent interferences were discarded including methanol controls. All wells contained at most 0.07% (v/v) of methanol. An agreement of more than 90% in biofilm parameters evaluated (see below) was observed for methanol treated and untreated sessile cells.

For each experiment, at the end of the incubation time, the wells were washed two times with ultrapure sterile water, and the biofilm development was estimated as described below.

Colony-forming units of cells in biofilms

In the assays performed, each alcohol was tested at the same concentrations in all *Candida* species, but the number of cells within biofilms may vary with *Candida* species. To estimate the number of colony forming units (cfu) of sessile cells before alcohol challenge, cells were scraped, after the washing procedure, and resuspended in ultrapure sterile water in four steps: 3×100 μl plus 200 μl, in a total of 500 μl. Suspensions containing the biofilm cells were vortexed for 2 min. Viable counts were obtained by serial dilution in ultrapure sterile water, and plating on Sabouraud dextrose agar medium. After 24 h of incubation at 37 °C, cfu were enumerated. The efficacy of biomass removal was assessed by crystal violet staining (see below) performed in each well.

Assessment of biofilm cells mitochondrial activity

Biofilm cells mitochondrial activity was determined through the quantification of the reduction of [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2*H*-tetrazolium-5-carboxanilide] (XTT) [10]. Briefly, 200 μl of 100 μg μl^{-1} XTT (Sigma) and 10 μg μl^{-1} phenazine methosulphate (PMS; Sigma) were added to each well, containing washed biofilms. Then, the plates were incubated in the dark at 37 °C and 130 rpm for 3 h. After that, cells were allowed to settle, and 150 μl of the supernatant was transferred to a new 96-well plate. Formazan salt formation was measured by absorbance reading at 490 nm (A_{490}) (Bio-Tek Synergy HT, Izasa). Controls were performed to check for interferences between XTT, and the alcohols assayed, and no changes were detected in A_{490} readings. Samples exhibiting very intense color, yielding "offscale" absorbance values, were diluted as necessary before performing a second absorbance reading.

Assessment of biofilm total biomass (crystal violet assay)

Biofilm biomass was quantified using the crystal violet assay [11]. Briefly, after the washing procedure, air dried biofilms were fixed with 200 μl of 99% (v/v) methanol for 15 min. After methanol removal, the plates were left to air dry. Then, the biofilm within the wells was stained with 200 μl of crystal violet (VWR) diluted to 1% (v/v) during 5 min. Afterwards, each well was washed twice with ultrapure sterile water, air dried and destained with 200 μl of 33% (v/v) acetic acid (VWR). The resulting solution was transferred to a new microtiter plate, and the absorbance was measured at 570 nm (A_{570}). Samples exhibiting very intense color, yielding "offscale" absorbance values, were diluted as necessary before performing a second absorbance reading.

Reproducibility and statistical analyses

Biomass dry weight measurements were performed three times. Supernatant alcohol analysis was made

with two replicates, with an agreement of results within ≤15%. Colony forming units were determined from four independent experiments with two technical replicates. Biofilm cells mitochondrial activity and biomass determinations were performed in sets of eight replicates, on at least three separate occasions.

Statistical analysis was performed using GraphPad Prism, version 5.00 software for Windows. When appropriate, data normality was tested by the Kolmogorov-Smirnov method.

The distribution of culture cell dry weight measurements was performed by Mann-Whitney test. The medians of cfu of biofilms subjected to alcohols treatment were compared by Kruskal-Wallis, with a Dunn's post test. Comparison between biofilm biomass and mitochondrial activity means of treated with untreated samples was performed by two-tailed unpaired *t*-test (confidence interval 95%). When the two sample standard deviations were not equal, Welch's correction to Student's *t*-test was used.

Results

Qualitative evaluation of extracellular alcohols in *C. parapsilosis* and *C. tropicalis* supernatants

Candida species planktonic cultures biomass dry weight determined at the end of 24 h were not statistically different (median biomass dry weight of *C. parapsilosis* was 3.5 mg ml^{-1}, and of *C. tropicalis* 3 mg ml^{-1}, $P = 0.18$). Cell free supernatants were analyzed by HS-SPME/GC-MS and the obtained alcohols profile is summarized in Table 1. Nerolidol was not detected in culture supernatants of both species. Farnesol and dodecanol were identified in both culture supernatants, and their concentration was below nanomolar (Table 1). The comparison of phenylethanol and isoamyl alcohol GC peak intensity in the two *Candida* species samples indicates that these compounds levels are approximately 20× and 100× higher in *C. tropicalis*, respectively, and for this *Candida* species their concentration is in the micromolar range (Table 1).

Effect of the addition of synthetic alcohols in *C. albicans*, *C. dubliniensis*, *C. parapsilosis* and *C. tropicalis* biofilm development

The effect of farnesol, nerolidol, dodecanol, phenylethanol, and isoamyl alcohol on *Candida* species was individually evaluated on biofilm development of adhered cells (Fig. 1) and mature biofilms (Fig. 2).

The analysis of the number of culturable sessile cells at 3 h and 48 h revealed that: (i) *C. tropicalis* adhered cell population was higher than that of *C. albicans* (median log cfu of 5.2 vs 3.4, respectively, $P < 0.05$), and (ii) *C. dubliniensis* mature biofilms contained more culturable cells than those of *C. parapsilosis* (mean log cfu of 6.7 vs 5.8, respectively, $P < 0.05$).

Farnesol

Farnesol was used as a control for *C. albicans*, *C. dubliniensis* and *C. parapsilosis*. Results show that 150 μmol l^{-1}, farnesol only reduced *C. albicans* biofilm biomass by 50% (Fig. 1A-II), and *C. dubliniensis* mitochondrial activity by 38% (Fig. 1A-I) plus biofilm biomass by 64% (Fig. 1A-II) when added to the adhered cell population. This alcohol did not affect *C. parapsilosis* biofilm cells (Fig. 1A and 2A). These data is in accordance to previous reports [5, 7, 8]. Additionally, farnesol was tested on *C. tropicalis* sessile cells and no changes were detected on adhered cells (Fig. 1A) and mature biofilms (Fig. 2A) development.

Nerolidol

Nerolidol was assayed at three concentrations (1.5×10^{-3}, 1.5, and 150 μmol l^{-1}) (Fig. 1B and 2B) and showed antibiofilm activity against *C. parapsilosis* and *C. tropicalis* sessile cells.

C. parapsilosis adhered cells treatment with 1.5×10^{-3} and 1.5 μmol l^{-1} nerolidol resulted in a reduction of mitochondrial activity ≥25% ($P < 0.05$) (Fig. 1B-I), whereas

Table 1. Alcohols composition of *C. parapsilosis* and *C. tropicalis* supernatants- alcohols and the corresponding GC peak area ($\times 10^{-5}$) determined in samples and standards are shown.

Compound	Mean GC peak area ($\times 10^{-5}$)[a]		
	Standard (concentration)	*C. parapsilosis*	*C. tropicalis*
Farnesol	99 (12 nmol l^{-1})	2.8	1.5
Nerolidol	6 (4 nmol l^{-1})	n.d.	n.d.
Dodecanol	5.4 (0.06 nmol l^{-1})	3.9	10.9
Phenylethanol	710 (21 μmol l^{-1})	25.9	558.2
Isoamyl alcohol	390 (182 μmol l^{-1})	5.1	523.7

n.d., not detected; [a] with an agreement of results within ≤15%.

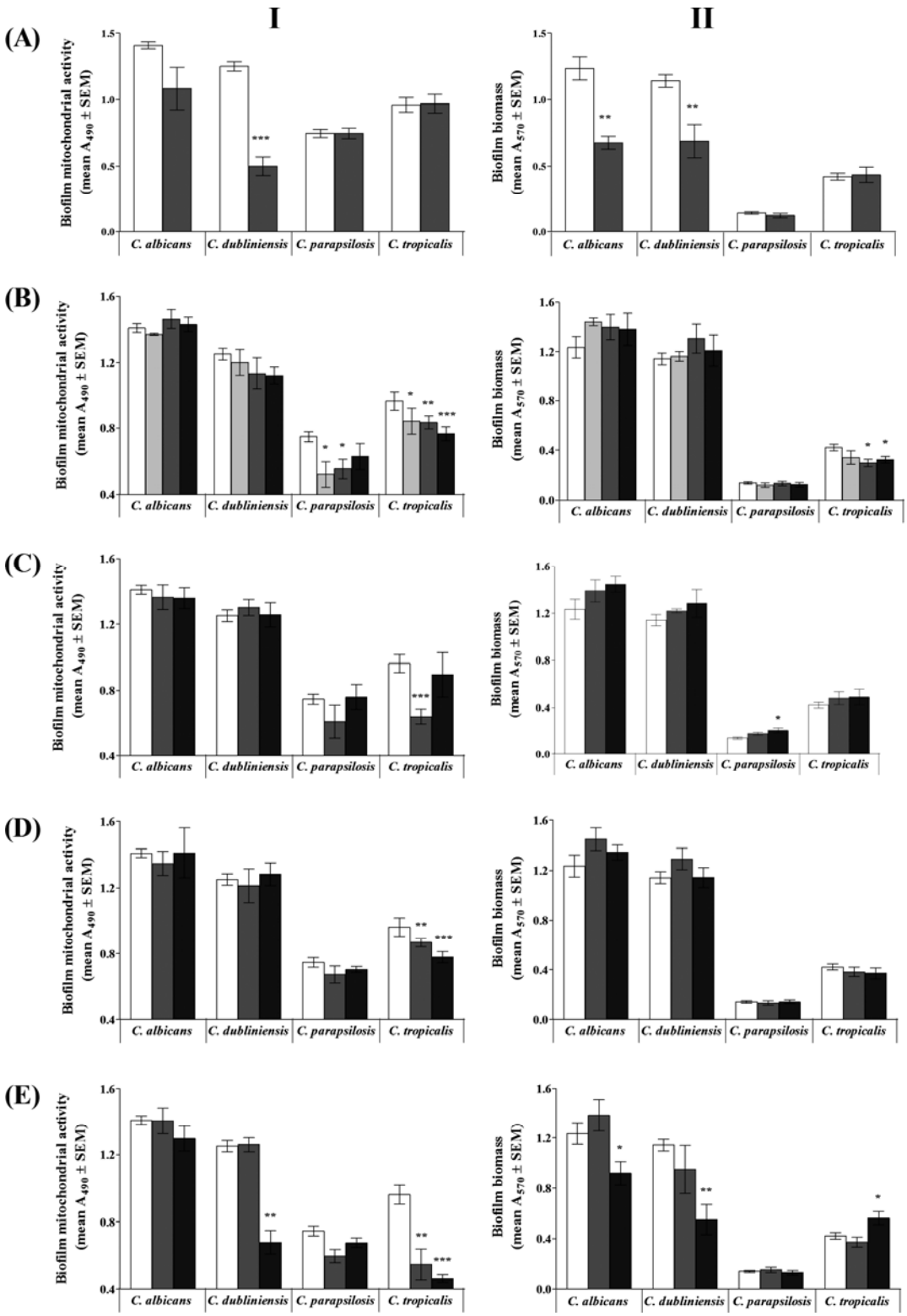

Figure 1. Effect of alcohols on *Candida* species adhered cells subsequent biofilm development. Biofilm mitochondrial activity, assayed by XTT reduction, (A_{490}) (I) and biofilm biomass, assayed by crystal violet staining (A_{570}) (II) were estimated 24 h after exposure to: farnesol (A) [0 (□) and 150 µmol l^{-1} (■)], nerolidol (B) [0 (□), 1.5×10^{-3} (■), 1.5 (■), and 150 µmol l^{-1} (■)], dodecanol (C) [0 (□), 2×10^{-3} (■), and 2 µmol l^{-1} (■)], phenylethanol (D) [0 (□), 5 (■), and 500 µmol l^{-1} (■)], and isoamyl alcohol (E) [0 (□), 46×10^{-3} (■), and 23 mmol l^{-1} (■)]. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ for treated biofilms compared with untreated controls (unpaired *t* test).

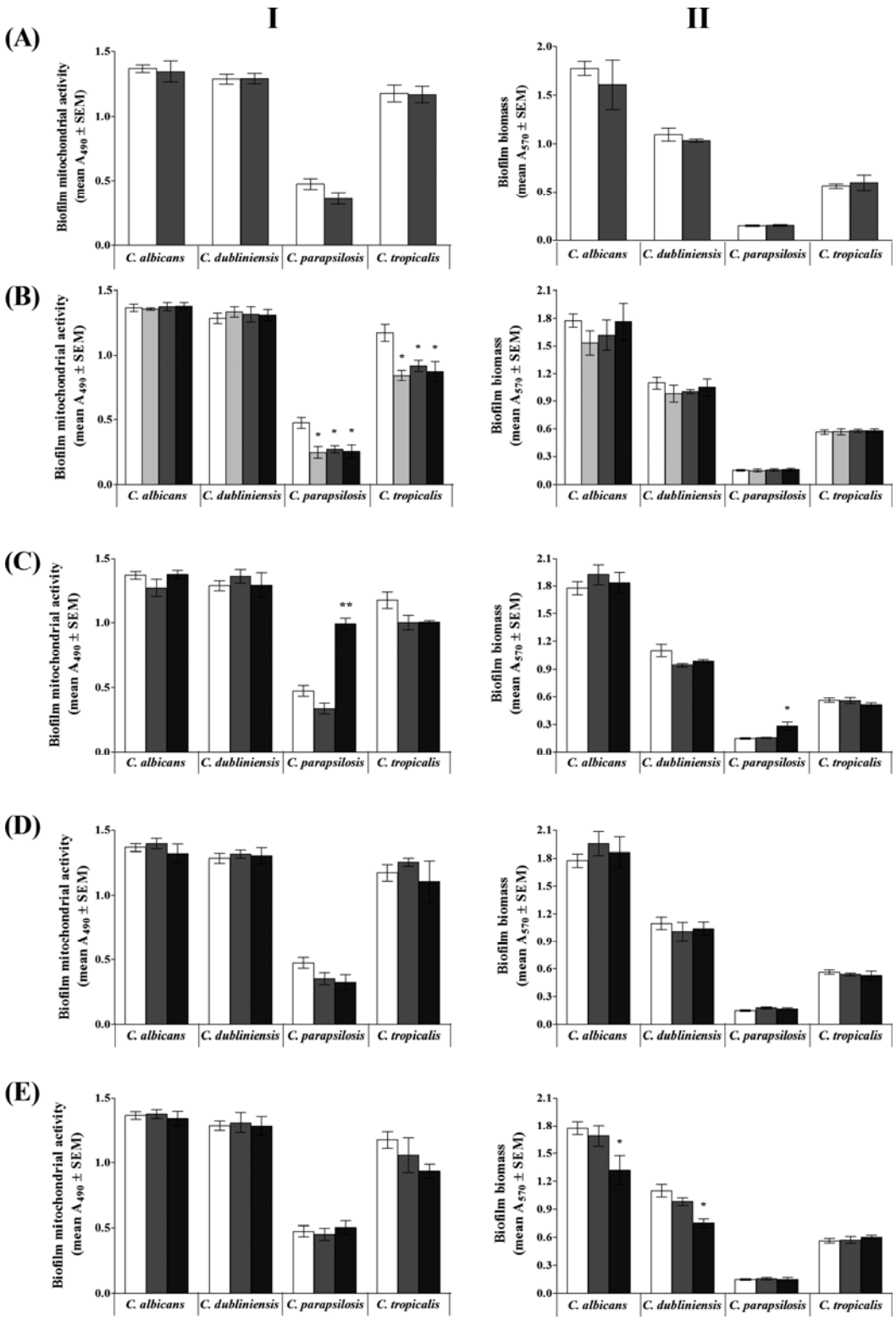

Figure 2. Effect of alcohols on *Candida* species mature biofilms subsequent development. Biofilm mitochondrial activity, assayed by XTT reduction, (A_{490}) (I) and biofilm biomass, assayed by crystal violet staining (A_{570}) (II) were estimated after 24 h exposure to: farnesol (A) [0 (□) and 150 μmol l^{-1} (■)], nerolidol (B) [0 (□), 1.5×10^{-3} (■), 1.5 (■), and 150 μmol l^{-1} (■)], dodecanol (C) [0 (□), 2×10^{-3} (■), and 2 μmol l^{-1} (■)], phenylethanol (D) [0 (□), 5 (■), and 500 μmol l^{-1} (■)], and isoamyl alcohol (E) [0 (□), 46×10^{-3} (■), and 23 mmol l^{-1} (■)]. *, $P < 0.05$, **, $P < 0.01$ for treated biofilms compared with untreated controls (unpaired *t*-test).

mitochondrial activity of mature biofilm cells was significantly decreased independently of the concentration tested (Fig. 2B-I).

Biofilm formation by adhered *C. tropicalis* cells population was hampered by nerolidol. In fact, there was a statistically significant decrease in biofilm cells mitochondrial activity (Fig. 1B-I) and biofilm biomass (Fig. 1B-II) induced by nerolidol. In contrast, the addition of nerolidol (1.5×10^{-3} to 150 μmol l^{-1}) to mature biofilms induced a reduction in mitochondrial activity during further development ($P < 0.05$) (Fig. 2B-I) but did not affect biofilm biomass (Fig. 2B-II).

Dodecanol

The effect of dodecanol was tested at concentrations of 2×10^{-3} and 2 μmol l^{-1} (Fig. 1C and 2C). This compound elicited a probiofilm activity in *C. parapsilosis* but an antibiofilm activity in *C. tropicalis* sessile cells.

Biofilm biomass of adhered *C. parapsilosis* cells was increased by 46% after the 24-h treatment with the highest alcohol concentration ($P = 0.04$) (Fig. 1C-II). Fig. 2C shows that dodecanol (2 μmol l^{-1}) concomitantly increased *C. parapsilosis* mature biofilm cells mitochondrial activity ($P = 0.004$) (Fig. 2C-I), and biofilm biomass ($P = 0.02$) (Fig. 2C-II) by more than 90% during the subsequent development.

C. tropicalis adherent cells 24-h treatment with 2×10^{-3} μmol l^{-1} dodecanol resulted in a significant decrease in mitochondrial activity (33%) (Fig. 1C-I), without changes in biofilm biomass (Fig. 1C-II).

Phenylethanol

Phenylethanol at concentrations of 5 and 500 μmol l^{-1} (Fig. 1D and 2D) showed antibiofilm activity against *C. tropicalis* sessile cells. Specifically, the mitochondrial activity of *C. tropicalis* adhered cells (Fig. 1D-I) (but not mature biofilms – Fig. 2D-I) was significantly reduced in a dose dependent trend upon 24 h exposure to phenylethanol, with a 20% decrease for the highest concentration tested.

Isoamyl alcohol

Isoamyl alcohol concentrations of 46×10^{-3} and 23 mmol l^{-1} used herein (Fig. 1E and 2E) elicited antibiofilm activity against *C. albicans* and *C. dubliniensis* and a heterogenous activity against *C. tropicalis* at early stages of development.

C. albicans adhered cells treatment with isoamyl alcohol 23 mmol l^{-1} resulted in a 26% reduction of biofilm biomass ($P = 0.02$) during the subsequent development (Fig. 1E-II).

Biofilm formation by the adhered *C. dubliniensis* cells population was significantly reduced by 23 mmol l^{-1} isoamyl alcohol in terms of mitochondrial activity (Fig. 1E-I) and biofilm biomass (Fig. 1E-II).

The 24 h challenge of *C. tropicalis* adhered cells with isoamyl alcohol (46×10^{-3} and 23 mmol l^{-1}) induced a significant decrease in cellular mitochondrial activity (Fig. 1E-I). Unexpectedly, upon challenge with the highest isoamyl alcohol concentration assayed, biofilm biomass increased 30% ($P = 0.04$) (Fig. 1E-II).

Discussion

Microbial supernatant media contain a variety of products that comprehend final fermentation products, temporarily released metabolites, secondary metabolites, and proteins [12]. In *Candida* field, a cohort of secreted alcohol molecules has been sparsely identified in culture supernatants, namely, farnesol [13], tyrosol [14], nerolidol, dodecanol, phenylethanol, and isoamyl alcohol [6]. In this study it was shown that *C. parapsilosis* and *C. tropicalis* secrete farnesol, dodecanol, phenylethanol, and isoamyl alcohol but not nerolidol, into the extracellular medium (Table 1). This suggests that, in general, the release of alcohols into the extracellular medium is a common trait of *Candida* species. However, farnesol and dodecanol concentration range in *C. parapsilosis* and *C. tropicalis* supernatant (below nanomolar) (Table 1) was lower than that previously described for *C. albicans* and *C. dubliniensis* (nanomolar) [6]. In contrast, the phenylethanol and isoamylalcohol concentration range detected in *C. tropicalis* supernatant (within micromolar) (Table 1) was similar to that earlier described for *C. albicans* and *C. dubliniensis* [6]. Thus, although it cannot be excluded that the supernatant alcohol profiling may be dependent on experimental conditions [15], and strains used, data on Table 1 suggests a heterogeneity in terms of the amounts of alcohols production among *Candida* species.

It has been suggested that the majority of the molecules secreted by microorganisms modulate physiological functions, although the observed effects are not always derived from a quorum sensing circuit but many times resultant from processes of compound metabolisation or detoxification [16]. This work examined the effect of the addition of commercial formulations of extracellular alcohols in *Candida* species biofilm formation (Figs. 1 and 2), as indicators of a role for secreted alcohols in biofilm development. In an attempt to ascertain the physiological relevance of the alcohols, this study was generally focused on the evaluation of

the effect of physiological and slightly higher concentrations of secreted alcohols [6].

The alcohols treatment induced different types of effects, in terms of mitochondrial activity and biofilm biomass changes for the specific strains evaluated. First, a 'dose-response' effect, which assumes that, as the dose of the effector increases, also does the biological response caused [17], as suggested by dodecanol (Fig. 1C-II, *C. parapsilosis*; Fig. 2C-I and -II, *C. parapsilosis*), phenylethanol (Fig. 1D-I, *C. tropicalis*), and isoamyl alcohol (Fig. 1E-I, *C. dubliniensis*; Fig. 1E-II, *C. albicans*, *C. dubliniensis*, and *C. tropicalis*). Second, alcohols that induced the highest effect at the lower concentrations tested, which resembles the paradoxical effect [18]. An example shown herein include the biofilm development changes induced by dodecanol (Fig. 1C-I, *C. tropicalis*). Third, alcohols that triggered a similar quantitative effect despite the concentration used, as observed for nerolidol (Fig. 1B-II, *C. tropicalis*; Fig. 1B-I and 2B-I, *C. parapsilosis* and *C. tropicalis*), and isoamyl alcohol (Fig. 1E-I, *C. tropicalis*). Fourth, alcohols that induced a simultaneous change in mitochondrial cells activity and in biofilm biomass. Although only observed at alcohol supra-physiological concentrations, examples include: nerolidol (Fig. 1B, *C. tropicalis*), dodecanol (Fig. 2C, *C. parapsilosis*), isoamyl alcohol (Fig. 1E, *C. dubliniensis*), and farnesol (Fig. 1A, *C. dubliniensis*).

Overall, the alcohols assayed showed a moderate antibiofilm activity, except dodecanol in *C. parapsilosis* (Fig. 1C-II and 2C), and isoamyl alcohol in *C. tropicalis* (Fig. 1E-II), that induced a probiofilm activity. However, it cannot be ruled out that these molecules elicit other effects in biofilms such as changes in morphology [19] or biofilm matrix synthesis [20]. The effects elicited by these alcohols did not appear to be compound specific, but rather dependent on the biofilm development stage (Fig. 1 and 2). Specifically, although all the alcohols induced changes in further biofilm formation when added to adhered cells population (Fig. 1), only nerolidol (Fig. 2B-I, *C. parapsilosis* and *C. tropicalis*) and dodecanol (Fig. 2C-I and -II, *C. parapsilosis*) had influence on mature biofilms development.

During the last years the recognition of polymicrobial cultures has been increasing [21]. However, *Candida* species interactions within mixed species biofilms has been scarcely studied [22–24]. The production of diffusible alcohol molecules probably offers a strategy for communication between *Candida* species. As noted, nerolidol was not detected in *C. tropicalis* and *C. parapsilosis* supernatants (Table 1). Interestingly, this compound, even at *C. albicans* and *C. dubliniensis* physiologically relevant concentrations (nanomolar levels) elicited an antibiofilm activity against these specific strains of *C. parapsilosis* and *C. tropicalis* at early (Fig. 1B) and late (Fig. 2B) stages of development. This suggests a competitive advantage for *Candida* species that are nerolidol producers, such as *C. albicans* and *C. dubliniensis* [6]. Additionally, it was also noticed that the levels of isoamyl alcohol and phenylethanol in *C. tropicalis* supernatants are higher than the observed for *C. parapsilosis* (Table 1). The exposure to these compounds did not affect *C. parapsilosis* biofilm but induced a decrease in *C. tropicalis* adhered cells mitochondrial activity at physiologically relevant levels (5 μmol l^{-1} for phenylethanol and 43×10^{-3} mmol l^{-1} for isoamyl alcohol). This suggests that these compounds have an antagonist effect in *C. tropicalis* biofilm development. However, further insights can be achieved by a time course characterization of alcohols production to evaluate possible growth stage specificity, and by determining the profile of alcohols secretion by biofilm cells.

Overall, additional studies to clarify the role of these molecules might include a much higher number of strains of each species, the recognition of compound specific receptors, and evaluation of transcriptional induced changes [12], the use of mutants that do not respond to the molecules and that respond to the molecules but do not increase the molecules production in response to the extracellular stimulus.

In conclusion, this study proved the extracellular secretion of farnesol, dodecanol, phenylethanol, and isoamyl alcohol by *C. parapsilosis* and *C. tropicalis* (as determined by HS-SPME GC-MS), and contributed to shed more light into cell-cell signalling molecules in *Candida* species. Moreover, it was shown that physiological levels of these secreted alcohols do play an effect in *C. parapsilosis* and *C. tropicalis* biofilm development, evidencing the complexity of biofilm formation regulation.

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