


## ORIGINAL ARTICLE

# High-throughput assay for effect screening of amphotericin B and bioactive components on filamentous *Candida albicans*

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**Abstract**

**Aims:** The aim of this study was to develop a high-throughput robotic microtiter plate-based screening assay for *Candida albicans*, optimizing growth conditions to replicate the filamentous biofilm growth found in vivo, and subsequently, to demonstrate the assay by evaluating the effect of nutritional drinks alone and in combination with the antifungal amphotericin B (AmB).

**Methods and Results:** *Candida albicans* cultured in a defined growth medium showed filamentous growth in microcolonies, mimicking the morphology of oral mucosal disease (oral candidiasis). Addition of nutrient drinks containing fruit juices, fish oil and whey protein to the medium resulted in changed morphology and promoted growth as free yeast cells and with weak biofilm structures. Minimum inhibitory concentration of AmB on the biofilms was  $0.25 \mu\text{g ml}^{-1}$ , and this was eight-fold reduced ( $0.0038 \mu\text{g ml}^{-1}$ ) in the presence of the nutritional drinks.

**Conclusions:** The established assay demonstrated applicability for screening of antifungal and anti-biofilm effects of bioactive substances on *C. albicans* biofilm with clinically relevant morphology.

**Significance and Impact of the Study:** *Candida albicans* is the causative agent of the majority of fungal infections globally. The filamentous morphology of *C. albicans* and the ability to form biofilm are traits known to increase virulence and resistance towards antifungals. This study describes the development of a plate-based in vitro screening method mimicking the filamentous morphology of *C. albicans* found in vivo. The assay established can thus facilitate efficient antifungal drug discovery and development.

**KEYWORDS**

amphotericin B, antioxidants, biofilm, biofilm eradication, *Candida albicans*, fish oil, high-throughput screening, nutraceutical

Hanne Haslene-Hox and Guro Kruge Nærdal contributed equally.

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## INTRODUCTION

Microbial infections pose a major threat to human health around the world, especially linked to antimicrobial resistant bacteria and persistent fungal infections, and new antimicrobials and alternative treatment strategies are urgently needed (Kainz et al., 2020; Murray et al., 2022). *Candida albicans* is a commensal fungus that is often part of the oral microbiota of healthy people. Concurrently, *Candida* is the causative agent of the majority of pathogenic fungal infections globally and can cause life-threatening systemic infections in humans, that is, human candidaemia, with a mortality rate of 25%–50% (Koehler et al., 2019; Mikulska et al., 2012; Pfaller, 2012). Patients with compromised immune systems, including cancer patients receiving chemotherapy, organ transplant recipients, HIV patients and advanced diabetic patients, are especially susceptible (Cassone & Cauda, 2012; Grossi, 2009; Kainz et al., 2020; Sensoy & Belet, 2011). Moreover, there is a drastic increase in these at-risk groups in the population, due to increased age, and more widespread access to advanced therapies and use of antibiotics (Kainz et al., 2020). Also, corticosteroid use or alteration of the oral microbiota following antibiotic therapies permits a pathogenic transition of *C. albicans* to cause oropharyngeal candidiasis (OPC) (Calderone & Clancy, 2012; Cassone & Cauda, 2012), with the most common form of OPC being acute pseudomembranous candidiasis. Preventive measures that can reduce the burden of fungal infections in these large at-risk groups can have substantial impact on use and effect of antifungal treatment, and overall provide a better quality of life for these patients.

*Candida albicans* is a polymorphic organism and can, among other morphologies, exist as free or adherent ovoid budding yeast cells, also called blastospores, and as filamentous hyphae, where hyphae being the most prevalent form during infections (Jacobsen et al., 2012; Jacobsen & Hube, 2017). Formation of a *C. albicans* biofilm involves three stages: adherence of ovoid yeast cells to a surface, production of extracellular polymeric substances (EPS) and formation of hyphae and maturation into a three-dimensional structure (Cavalheiro & Teixeira, 2018). Biofilms of *C. albicans* that form in oral mucosal disease (oral candidiasis) appear as a specialized pathogenic form of biofilm called microcolonies, or ‘radiating hyphal biofilms’ (McCall et al., 2018). These consist of *C. albicans* cells mainly in pseudohyphae and hyphal form, growing radially outwards from one central mother cell. The hyphae branch out further during growth and reach out towards neighbouring microcolonies to form a web of intertwined hyphae (Citiulo et al., 2012). The hyphal form of *C. albicans* is shown

to be strongly related to both adhesion during growth and virulence of infections (Jacobsen et al., 2012; Mayer et al., 2013; Thompson et al., 2011). Also, the response to drugs varies in cells of different morphology, for example, filamentous *C. albicans* has a greatly increased tolerance to amphotericin B (AmB) compared to ovoid budding yeast cells (Laprade et al., 2016; Niimi et al., 2010). Nevertheless, frequently used assays to evaluate minimum inhibitory concentration (MIC) and drug effects on biofilms in vitro predominately use culture conditions where *C. albicans* is growing as ovoid budding yeast cells (Desai & Mitchell, 2015) and thereby poorly mimicking in vivo conditions. The possibility to recreate clinically relevant morphologies for in vitro experiments are important (Cavalheiro & Teixeira, 2018), thus developing new culturing conditions and robotic screening methods would be beneficial for evaluating realistic drug effects.

Candidiasis is primarily treated by triazoles, echinocandins and AmB (Ben-Ami, 2018), but the prevalence of strains resistant to these drugs are increasing (Grela et al., 2019; Huang & Kao, 2012; Oh et al., 2020; Pfaller, 2012). In addition, *Candida* frequently form biofilms, which make the infection especially resistant to treatment, and contribute to increased mortality (Kainz et al., 2020). AmB is a highly efficient drug with low primary resistance (Ben-Ami, 2018), mainly administered to patients with progressive, potentially life-threatening fungal infections (Laniado-Laborin & Cabrales-Vargas, 2009) due to its severe side effects. Side effects include chronic nephron and hepatotoxicity, seizures, hearing and vision changes, nausea, fever, hypertension or hypotension and hypoxia (Kim et al., 2012; Laniado-Laborin & Cabrales-Vargas, 2009; RxList & Cunha, 2018). Therapeutic strategies to alleviate some of these systemic adverse effects include formulating AmB in various lipid formulations, such as in liposomes, in a ribbon-like lipid complex or a complex with cholesteryl sulphate (Adler-Moore & Proffitt, 2008; Stone et al., 2016). It is also necessary to investigate alternative strategies to improve the efficacy of AmB against *C. albicans*, for example, through synergistic effects with other compounds (Teodoro et al., 2015).

There are different models for mode of action proposed for AmB on fungal cells. AmB binds to ergosterol in the fungal cell membrane and forms pores, leading to ion leakage and cell death (Carolus et al., 2020; Grela et al., 2019; Stone et al., 2016). It is also hypothesized that AmB can adsorb or extract ergosterol from the membrane and thus destabilize the membrane and disrupt important membrane functions (Carolus et al., 2020). AmB in *C. albicans* can also act as a stressor of the fungal antioxidant system (Kim et al., 2012), cause oxidative stress and promote programmed *Candida* yeast cell death (Carolus

et al., 2020; Phillips et al., 2003). Filamentous *Candida* has been shown to be less sensitive to AmB-induced apoptosis than ovoid budding yeast cells (Laprade et al., 2016), and the yeast cell morphology and formation of protective biofilms will impact the effect of AmB. Thus, the present study chose AmB as an example drug for biofilm treatment studies.

There is a pressing need to develop new antifungal treatments, or enhancing the effect of treatment for existing drugs, especially when it comes to treatment of fungal biofilm infections (Cavalheiro & Teixeira, 2018; Laprade et al., 2016). To enable such development, efficient screening methods that replicate clinically relevant fungal growth are needed to evaluate drug effect for single drugs, drug combinations and drugs combined with other active or helper substances or formulations. Many patients at risk for *C. albicans* infections (e.g. cancer patients) also receive medical nutrition in the form of nutritional drinks as part of treatment or disease management. Such drinks contain many health-promoting ingredients and potential bio-actives, such as antioxidants and curcumin (Hewlings & Kalman, 2017). Furthermore, the interplay between nutrient supplements and drugs is important to assess because they can both have deleterious and synergistic effects on treatment (Anadón et al., 2021).

The main aim of the present study was to develop a high-throughput *C. albicans* biofilm screening assay with robotic liquid handling in microtiter plates. The innovative aspect of this study was to incorporate such high-throughput techniques with establishment of cultivation conditions for optimized growth of biofilm in the shape of microcolonies. Microcolonies are the dominating morphology occurring in in vivo oral candidiasis infections (McCall et al., 2018). This enables fast and reliable screening of antifungal treatments in a system that mimic the morphology of *C. albicans* infections in vivo. The assay was demonstrated by evaluating the effect of nutrient drinks containing, among other things, fruit juices, fish oil and whey protein alone or in combination with AmB, to investigate if nutritional drinks could have an impact on fungal biofilms and whether concomitant administration with AmB would give positive, synergistic effects.

## MATERIALS AND METHODS

### Strain and growth conditions

*Candida albicans* ATCC 10231 was used to evaluate biofilm formation. Precultures were made by adding 100 µl from a glycerol stock to 6 ml of tryptone soya broth (TSB;

Oxoid CM0129) in a 50-ml tube. The cultures were incubated over night at 34°C, 1 g at a 45° angle.

### Culture media

In initial experiments, four different culture media were evaluated to identify the culture medium that best facilitates filamentous growth of biofilm as microcolonies, each at three different concentrations: 1×YPD (10 gL<sup>-1</sup> Yeast extract [Oxoid; LP0021], 20 gL<sup>-1</sup> Peptone [Oxoid; LP0037], 20 gL<sup>-1</sup> Dextrose [Difco, 215530; Becton, Dickinson and Company]), 1× Mueller Hinton Broth (LAB114; LabM), 1×M19 without NaCl (pH 6.1, 9.4 gL<sup>-1</sup> Peptone [Oxoid; LP0037], 4.7 gL<sup>-1</sup> Yeast extract [Oxoid; LP0021], 2.4 gL<sup>-1</sup> Beef extract [Difco; 212610], 10 gL<sup>-1</sup> Glucose monohydrate [Merck; 49161]) and 0.5×Def4 (pH 7). The 0.5×Def4 media were based on the 0.5×Def4 reported by Correa et al. (2012) modified as follows: 0.65 gL<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 2.75 gL<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.31 gL<sup>-1</sup> MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.45 gL<sup>-1</sup> citric acid, 10.20 mgL<sup>-1</sup> Fe(III) citrate hydrate, 0.525 mgL<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 2.50 mgL<sup>-1</sup> MnCl<sub>2</sub> · 4H<sub>2</sub>O, 2.10 mgL<sup>-1</sup> EDTA · 2H<sub>2</sub>O, 0.26 mgL<sup>-1</sup> CuCl<sub>2</sub> · 2H<sub>2</sub>O, 0.44 mgL<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O, 0.44 mgL<sup>-1</sup> CoCl<sub>2</sub> · 6H<sub>2</sub>O, 1.30 mgL<sup>-1</sup> Zn acetate · 2H<sub>2</sub>O, 1.0 gL<sup>-1</sup> NaCl, 10 gL<sup>-1</sup> MOPS, 20.0 gL<sup>-1</sup> fructose, supplemented with 147.0 mgL<sup>-1</sup> CaCl<sub>2</sub> · 2H<sub>2</sub>O. YPD, LAB114 and M19 were tested at 1×, 0.5× and 0.25× concentrations, whereas Def4 was tested at 0.5×, 0.25× and 0.125×. The media were diluted in sterile ion-free water prior to use to obtain the desired concentration.

### Nutritional drink formulations

The antifungal drug used was AmB (Biochempartner; BCP09760). Formulations tested alone and combined with AmB were food-grade biocurcumin (BCM-95-WD, 48% pure), a whey protein and fish oil emulsion (WFE) and three nutritional drink formulations (all from SmartFish AS): Curcher (7.5 mg ml<sup>-1</sup> eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA], 0.05 µg ml<sup>-1</sup> vitamin D, 2.5 mg ml<sup>-1</sup> curcumin and other ingredients [whey protein, fibre and fruit juice]); recharge lipid+ (RL+ 10 mg ml<sup>-1</sup> EPA/DHA, 0.015 µg ml<sup>-1</sup> vitamin D and other ingredients [whey protein, fibre and fruit juice]); and a control juice formulation (CJF) with comparable concentrations of whey protein, fibre and fruit juice as the other drinks, but without curcumin, fish oil and vitamin D. Selection of these specific nutrient drink formulations was based on the availability of both complete formulations and formulations excluding specific components (but otherwise with identical composition), to enable the

**TABLE 1** Overview of the active ingredients applied for biofilm inhibitory screening without AmB

Nutritional drink formulation	Content
Recharge lipid+ (RL+)	Fruit juice, whey protein, fibre, 10 mg ml <sup>-1</sup> eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), 0.015 µg ml <sup>-1</sup> vitamin D
Curcher	Fruit juice, whey protein, fibre, 7.5 mg ml <sup>-1</sup> EPA/DHA, 2.5 mg ml <sup>-1</sup> curcumin, 0.05 µg ml <sup>-1</sup> vitamin D
Control juice formulation (CJF)	Formulation with comparable concentrations of whey protein, fibre and fruit juice as the other drinks without curcumin, fish oil and vitamin D
Whey protein and fish oil emulsion without fruit juice (WFE)	20% fish oil, 2.0% whey protein, 1.8% pectin
Curcumin in dimethylsulfoxide (DMSO) diluted in assay media	0.0010–0.52 mg ml <sup>-1</sup> (wv <sup>-1</sup> ), relative to curcumin concentration of 0.0098%–10% (vv <sup>-1</sup> ) Curcher

study of contribution from fish oil, curcumin and fruit juices, separately.

Emulsions of whey protein and fish oil without (WFE) and with 0.1% (w w<sup>-1</sup>) AmB (WFE-AmB) were prepared as follows: whey protein (Arla) was dissolved in deionized water by stirring and then sterile filtered. AmB was dissolved in the whey solution by stirring for 30–45 min at room temperature. Pectin (Trensum Food AB) was dissolved in deionized water, a foam control agent was added (Foamdoctor) and the mixture was heated to 75°C for 10 min, before cooled to a temperature of 40–50°C and mixed with the whey solution. Fish oil stabilized with antioxidants (Orkla Health) was added to the solution and mixed on a magnetic stirrer for 2 min. The solution was treated with a rotor–stator mixer (T25 digital ULTRA-TURRAX®, IKA) for 1 min at 7000 rpm before sonication using an ultrasonifier (Branson Digital Sonifier) for 3 min while cooling on ice. The final composition of the emulsion was 2.0% whey protein, 20% oil, 1.8% pectin and 0.10% AmB. The reference emulsion without AmB was made by the same protocol without addition of AmB.

### Biofilm inhibition assay in microtiter plates

Initial experiments were performed to establish *C. albicans* biofilm for high-throughput screening (HTS) in 96-well microtiter plate format with all liquid handling operations integrated in a robotic liquid handling system that dispense samples and buffers, wash and aspirate liquid from all wells in a plate, with careful control to not touch the well bottom containing the formed biofilms. Such integrations enabled handling of hundreds and thousands of samples by a single operator within a limited period of time. Four culture media (Def4, YPD, LAB114 and M19) were tested at three different

concentrations to evaluate the formation of morphologically relevant biofilms with good growth and surface attachment. Biofilms were grown in sterile, black, uncoated 96-well microplates with a transparent base (BRANDplates®, pureGrade™ S). Based on the initial results, 0.25 × Def4 was selected for subsequent biofilm inhibition assays. Precultures of *C. albicans* were diluted to an optical density at 600 nm (OD) = 0.4 in TSB and further diluted 40× in assay medium. The assay plates with wells containing 120 µl of culture media and active ingredients (quadruple parallels for each condition) were inoculated with 30 µl of the diluted preculture to a final OD of 0.01 in 150 µl using a Beckman robotic system. The assay plates were incubated static at 34°C for 20 h in plastic ziplock bags to prevent evaporation (eight parallels for each condition).

Biofilm inhibition assays were performed to assess the effect of nutritional drink formulations and curcumin without AmB present (Table 1) (four parallels for each condition). The nutritional drink formulations were twofold serial diluted in assay media in concentrations from 10% to 0.0098%. Curcumin was twofold serial diluted in dimethylsulfoxide (DMSO) and subsequently transferred to the assay plate containing media by a Beckman Coulter SCARA robotic system with an integrated Biomek NXP liquid handler, to an assay concentration of 0.52 to 0.001 mg ml<sup>-1</sup> of curcumin in 2% final DMSO concentration. All formulations were added to the culture from start and were present during the entire incubation period. Internal growth references with assay medium with and without 2% DMSO were included on all plates.

To assess the effect of AmB in the presence of the other active ingredients, AmB was twofold serial diluted in DMSO and added to cultures containing a constant concentration of the active ingredients and control wells with only assay medium (0.25 × Def4) (four parallels for each condition). Liquid handling was performed with a Tecan Freedom

EVO-2200 robot system equipped with an MCA384 well pipetting tool. The final AmB concentration in the assays ranged from 8 to  $0.016\ \mu\text{g ml}^{-1}$  with a constant concentration of 2% DMSO. The internal growth references in the assay plates consisted of the same concentration of active ingredients with 2% DMSO and no AmB. Nutritional drink formulations, CJF and WFE were present at 0.2% concentration and curcumin concentration was  $0.01\ \text{mg ml}^{-1}$ . To produce a constant concentration of WFE with variable concentration of AmB, 1% WFE-AmB was twofold serial diluted in 1% WFE (both diluted in assay medium). Similarly, to make a serial dilution of AmB in curcumin, AmB was added to a stock solution of curcumin in DMSO. The solution was then twofold serial diluted in a DMSO solution with the same curcumin concentration and subsequently transferred to the assay plate containing media by the Beckman robotic system.

## Biofilm quantification

The amount of metabolically active yeast cells in cultures after incubation, that is, microbial cell viability, was determined indirectly by measuring adenosine triphosphate with BacTiter GLO™ Microbial Cell Viability Assay (BTG; G8231/G8232). The assay was performed using the Beckman Coulter Biomek NXP and SCARA robotic system. Supernatant (100  $\mu\text{l}$ ) was harvested from assay plates and optical density (600 nm) was measured in a microplate reader (SpectraMax® Paradigm® Multi-Mode Detection Platform). The remaining supernatant in the assay plates was carefully removed from the wells and discarded. Plates were washed once by the robot with 100  $\mu\text{l}$  sterile phosphate-buffered saline (PBS, BR0014G; Oxoid) and BTG reagent (100  $\mu\text{l}$ ) was added. Plates were shaken at 2 g (900 rpm, with 2 mm amplitude) for 30 s on a Quantifoil Instruments GmbH BioShake 3000 elm and incubated in the dark for 15 min. The luminescence was measured in a microplate reader (SpectraMax Paradigm Multi-Mode Detection Platform). The minimum biofilm inhibitory concentration (MBIC) was defined as the concentration at which growth (BTG-signal) in all parallels ( $n = 4$ ) was reduced by 70% compared with the average of controls in the same plate.

## Imaging

Biofilms formed in the microplate wells were visually observed and evaluated using an inverted phase contrast microscope (EVOS FL Auto Imaging system; Life Technologies™).

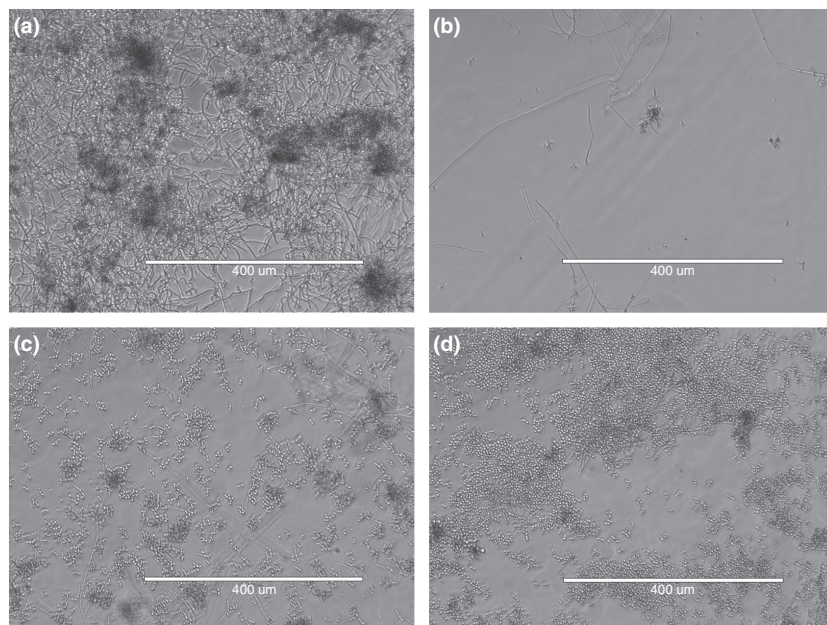
## Statistical analyses

Statistical analysis was performed with GraphPad Prism (version 8.2.1 [441] for Windows; GraphPad Software). Significant changes in the inhibitory effect of different solutions were analysed on pairwise curves, by unpaired multiple *t*-tests, with each row (concentration) analysed individually, without assuming a consistent standard deviation and false discovery rate approach with two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with  $Q = 1\%$  to determine significance.

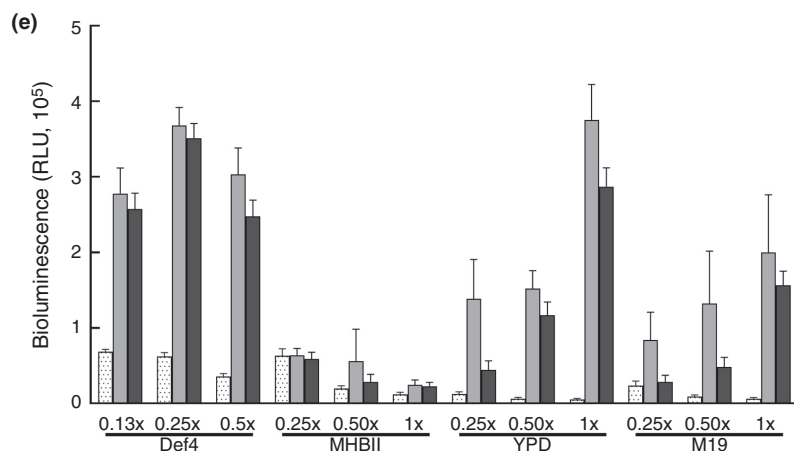
## RESULTS

### Biofilm growth and morphology

The initial experiments performed to establish *C. albicans* biofilm in a HTS format in microtiter plates showed great variation in growth and biofilm structure depending on the assay media (Figures 1 and 2). *C. albicans* grew poorly in LAB114, whereas cultivation in M19 and YPD media promoted high density growth in all medium concentrations in the form of free budding ovoid yeast cells that created a dense layer along the edges of wells after washing with PBS. Cultivation in  $0.125\times\text{Def4}$  and  $0.25\times\text{Def4}$  promoted growth of a filamentous, high-density biofilm that was tightly adhered to the microwell surface (Figure 1a–d). Here, *C. albicans* grew mainly as microcolonies, as previously described by McCall et al. (2018), and we observed the growth of both true hyphae, uniformly shaped, with no constrictions or branches and parallel-sided walls, and pseudohyphae, with constrictions at the septal junctions, branching and several different shapes, ranging from elongated and ellipsoidal yeast cells to long pseudohyphae resembling true hyphae. The hyphae extended from a dense core and branched outwards, and furthermore merged with neighbouring microcolonies resulting in an evenly distributed web across the well bottom (Figure 2). The microcolonies grew large enough to be visible with the naked eye. The biofilm was robust and was not disrupted by washing with PBS (data not shown). Cultivation in  $0.5\times\text{Def4}$  also gave a filamentous, high-density biofilm, but with more free budding ovoid yeast cells and a less robust biofilm. Cultivation of *C. albicans* in  $0.25\times\text{Def4}$  provided the best conditions for biofilm formation with a morphology similar to that observed in vivo and was thus chosen as assay medium for further studies. In addition, several incubation times were tested (selected data shown in Figure 1e, cultivation was tested for 8–48 h) to assess the time needed for confluent biofilm formation and based on these results 20–24 h of incubation at  $34^\circ\text{C}$  was chosen as standard conditions.



**FIGURE 1** (a–d) Selected micrographs of *Candida albicans* biofilm in different culture media after wash with PBS. Scale bars: 400  $\mu\text{m}$ , 10 $\times$  magnification. (a) 0.25 $\times$  Def4; (b) 0.25 $\times$  LAB114; (c) 0.25 $\times$  YPD; (d) 1 $\times$  M19; (e) growth of biofilm over time (8 h [dotted white]; 16 h [grey]; 24 h [dark grey]) determined as adenosine triphosphate measured as bioluminescence (relative luminescence units, RLU) with the BacTiter GLO-assay for four different culture media (Def4, LAB114, YPD and M19), at three concentrations; for LAB114, YPD and M19: 1 $\times$ , 0.5 $\times$ , 0.25 $\times$ ; for Def4, the respective concentrations were halved. Mean  $\pm$  SD error bars given ( $n = 8$ ). PBS, phosphate-buffered saline; SD, standard deviation.



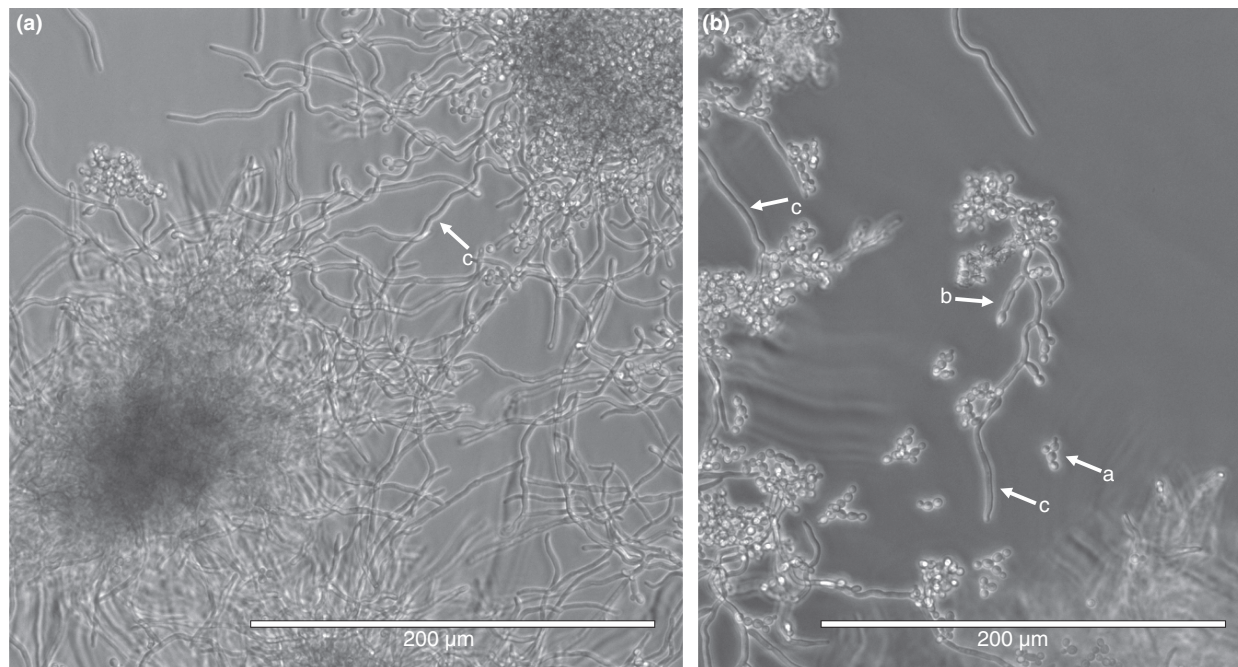
## Biofilm destabilization by nutritional drinks

The established HTS biofilm assay, where *C. albicans* biofilms were grown in 0.25 $\times$  Def4 medium in microplates for 20–24 h, was evaluated by investigating the effect of nutritional drink formulations and curcumin on biofilm formation. No clear inhibitory effect in terms of culture viability (total amount of metabolically active cells) was seen when *C. albicans* was exposed to the active ingredients (Figure 3), although curcumin had effect at concentrations  $>0.07 \text{ mg ml}^{-1}$  (corresponding to curcumin amount in 1.25% Curcher). However, the presence of concentrations as low as 0.02% of the different nutritional drink formulations markedly altered cell adherence and morphology, exemplified in Figure 4 with 0.2% nutritional drink present. The biofilms in the presence of nutritional drink formulations were strongly deformed after washing with PBS (Figure 4b2–e2), showing that both filamentous cells and ovoid yeast cells adhered more loosely to the well plate

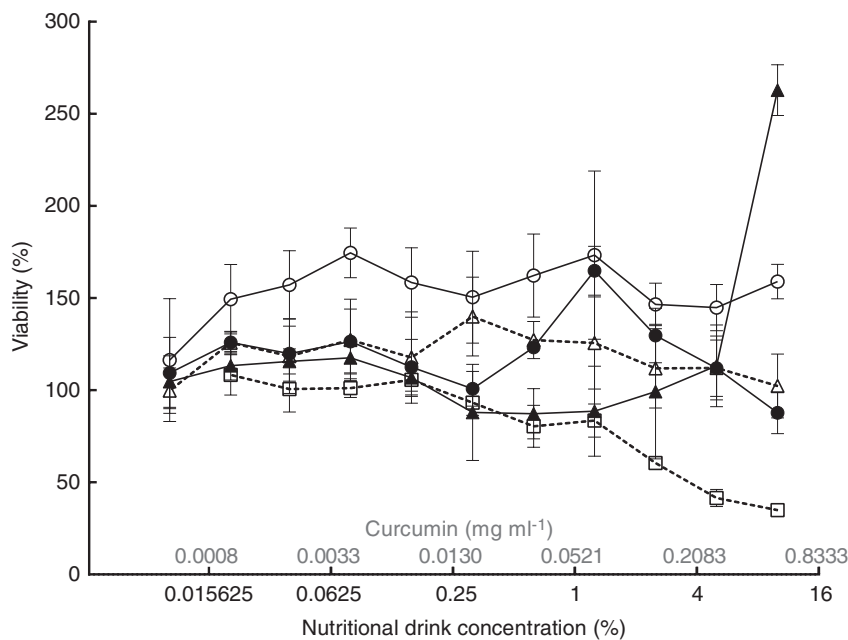
surface. Filamentous growth was observed for all added nutrient formulations, but there were also large amounts of free ovoid yeast cells. This was particularly distinct for RL+ at concentrations  $>1\%$ , where *C. albicans* grew mainly as free ovoid yeast cells, in contrast to the filamentous biofilm formed as microcolonies when cultivated in pure 0.25 $\times$  Def4 (Figure 5). *C. albicans* was also cultivated in the presence of  $0.1 \text{ mg ml}^{-1}$  curcumin, but displayed no clear effects compared to the control (data not shown).

## Biofilm inhibition by AmB

The effect of AmB on biofilm formation was investigated in a HTS format, alone and combined with low concentrations of nutritional drinks and curcumin. The presence of 0.2% nutritional drink formulations in the 0.25 $\times$  Def4 medium had a strong effect on AmB sensitivity (Figure 6a). The MBIC value for AmB (Figure 6b) was found to be  $0.25 \text{ } \mu\text{g ml}^{-1}$  when AmB was added



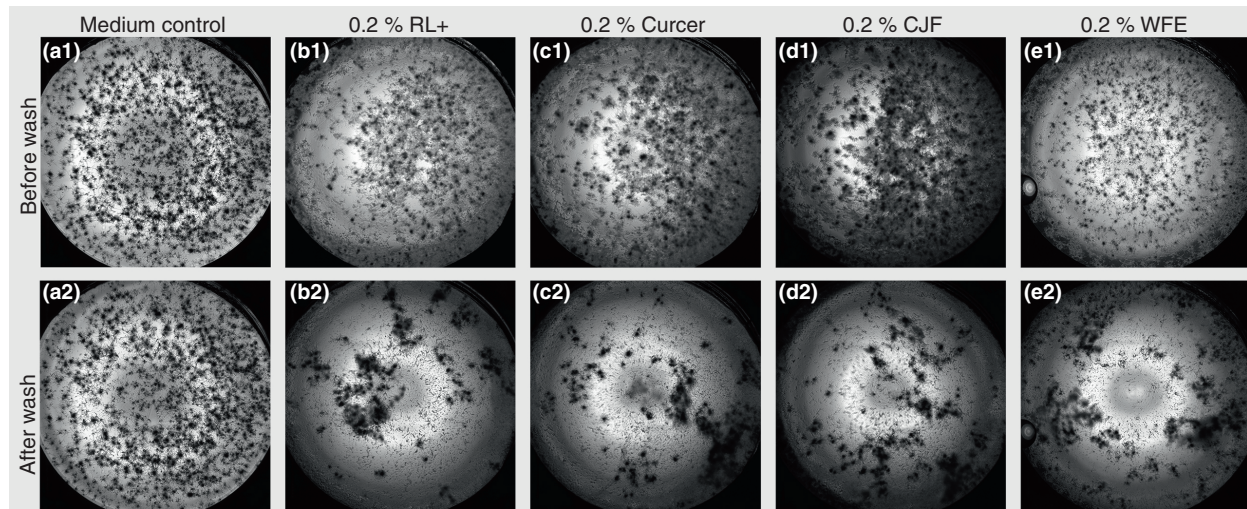
**FIGURE 2** (a) and (b): Micrographs of *Candida albicans* biofilm formed in 0.25× Def4 culture medium after 20 h of incubation showing polymorphism with examples of the different morphologies indicated by arrows: a: budding yeast cells or blastospores, b: pseudohyphae, c: true hyphae. Scale bars: 200 µm, 20× magnification.



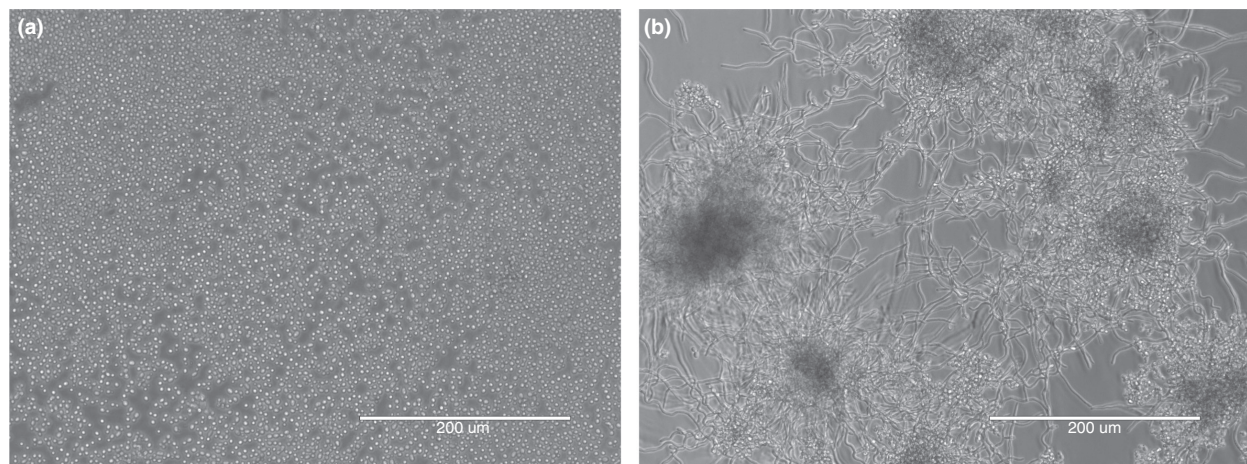
**FIGURE 3** The effect of nutritional drinks and curcumin at different concentrations on biofilm formation. Viability (number of living yeast cells) was calculated as luminescence signal in treated wells compared to signal in growth control with only 0.25× Def4 media. An amount of added nutritional drink formulations are given as percentage of total culture volume (below x-axis) and amount of curcumin as mg ml<sup>-1</sup> (above x-axis). Mean ± SD error bars given ( $n = 4$ ). Recharge lipid+ nutrient drink (filled circle); Curcher nutrient drink (open circle); control juice formulation (CJF, filled triangle); whey protein and fish oil emulsion (WFE, open triangle, dashed line); and curcumin (open square, dashed line). SD, standard deviation.

alone in culture medium. The MBIC value was, however, decreased eightfold to 0.031 µg ml<sup>-1</sup> with 0.2% RL+, Curcher and CJF present in the media, and fourfold decreased in the presence of WFE to 0.063 µg ml<sup>-1</sup>.

Curcumin (0.01 mg ml<sup>-1</sup>) had no effect on MBIC of AmB. RL+ and Curcher showed slightly elevated signal in supernatant, but planktonic growth was marginal for all experiments (data not shown).



**FIGURE 4** *Candida albicans* biofilm integrity observed before and after washing the biofilm with PBS in microplate wells. Well diameter = 7 mm. The free, ovoid yeast cells appeared as grey areas around the dark, hyphal colonies. (a) Biofilm formed with microcolonies in pure  $0.25\times$ Def4 before (a1) and after (a2) wash. (b) Growth of *C. albicans* in  $0.25\times$ Def4 with 0.2% recharge lipid+ (RL+) before (b1) and after (b2) wash. (c) Growth of *C. albicans* in  $0.25\times$ Def4 with 0.2% Curcher before (c1) and after (c2) wash. (d) Growth of *C. albicans* in  $0.25\times$ Def4 with 0.2% control juice emulsion (CJF) before (d1) and after (D2) wash. (e) Growth of *C. albicans* in  $0.25\times$ Def4 with 0.2% whey protein and fish oil emulsion without fruit juice (WFE) before (e1) and after (e2) wash. PBS, phosphate-buffered saline.



**FIGURE 5** Growth of *Candida albicans* in  $0.25\times$ Def4 in the presence (a) and absence (b) of 10% recharge lipid+. Scale bar:  $200\mu\text{m}$ ,  $20\times$  magnification. The presence of high concentrations of recharge lipid+ clearly promotes growth as non-adherent yeast cells (a) in contrast to the morphology of filamentous microcolonies formed in pure  $0.25\times$ Def4 (b). After washing with PBS, most of the cells in micrograph (a) moved to the edges of the well, whereas only minor changes were observed in the biofilm displayed in micrograph (b). PBS, phosphate-buffered saline.

## DISCUSSION

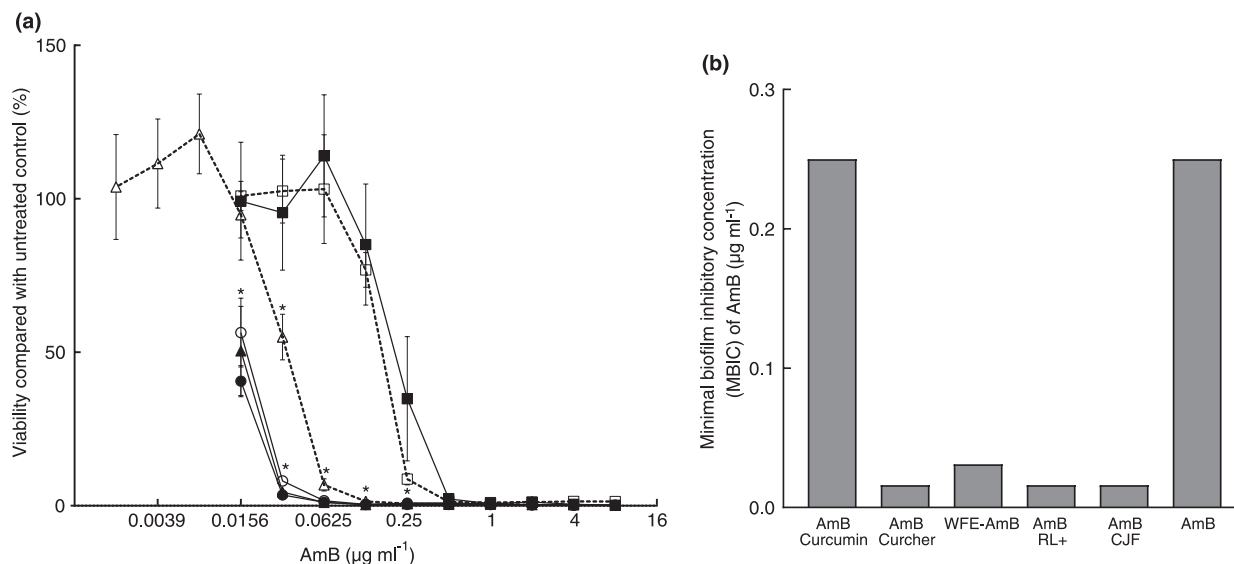
### Growth conditions for filamentous biofilms

As *C. albicans* is known to be most virulent in the filamentous state with hyphae and microcolonies, an in vitro screening system should ideally reflect the same morphology (Laprade et al., 2016). However, the common fungal growth media tested (e.g. YPD, M19) did not yield filaments or biofilm formation. By utilizing the media

that have previously been used primarily for bacteria,  $0.5\times$ Def4 (Correa et al., 2012), we were able to prevent growth of free ovoid yeast cells in the media while stimulating growth of both true hyphae and pseudohyphae (Sudbery et al., 2004). In addition, we found microcolonies formed by filamentous hyphae, in line with virulent forming and pathologically relevant *C. albicans* biofilm (McCall et al., 2018).

Literature have reported that high cell densities, high concentration of nutrients, temperatures below  $30^{\circ}\text{C}$  and  $\text{pH} < 6$  promote growth of free ovoid yeast





**FIGURE 6** The effect of AmB on *C. albicans* when cultivated in pure 0.25×Def4 and in the presence of 0.2% nutritional drink formulations. (a) Viability is shown as percentage of luminescence signal in treated wells compared to control cultures grown in 0.25×Def4 media, 0.2% nutrient solutions and 2% DMSO present. Figure shows mean with SD error bars ( $n = 4$ ). Curcumin concentration is equivalent to the concentration in Curcher. Concentration of AmB is given as  $\mu\text{g ml}^{-1}$ . Recharge lipid+ nutrient drink (RL+ filled circle); Curcher nutrient drink (open circle); control juice formulation (CJF, filled triangle); whey protein and fish oil emulsion (WFE, open triangle, dashed line); curcumin (open square, dashed line); and AmB alone (filled square). \*Denotes a significant difference ( $p < 0.01$ ) between AmB with nutritional drink formulations present for all data points beneath the respective "\*" compared with AmB in culture media alone at the same concentration (filled square). (b) The calculated MBIC values of AmB in the presence of nutrient solutions for nutrient drink formulations (0.2%) and curcumin ( $0.01 \text{ mg ml}^{-1}$ ). MBIC was defined as the concentration where at least 2 of 4 parallel wells showed more than 70% reduction in growth compared with untreated control. DMSO, dimethylsulfoxide; SD, standard deviation.

cells, while low cell density, serum, physiological temperatures, starvation and neutral pH promote hyphal growth (Mayer et al., 2013; Sudbery et al., 2004). High phosphate concentrations (up to 600 mM) also promote pseudohyphal growth (Sudbery et al., 2004). All media tested in the present study have  $\text{pH} > 6$ , were cultivated above  $30^\circ\text{C}$  and had similar sugar content ( $10\text{--}20 \text{ g L}^{-1}$ , glucose [M19], fructose [Def4] and dextrose [YPD]). However, while M19, LAB114 and YPD all contain complex hydrolysates, Def4 does not contain organic nitrogen sources. *C. albicans* is known to prefer ammonia, glutamine and glutamate as nitrogen sources, but has an extensive amino acid metabolism to enable uptake also of other amino acids (Garbe & Vylkova, 2019). Amino acid starvation can cause changes in virulence and filamentous growth in *C. albicans* (Tripathi et al., 2002), in line with results shown here.

The low planktonic growth in Def4 medium also indicated that the cells strongly preferred to grow in adherent biofilm form in this media. Biofilm formation is a known microbial survival strategy when exposed to environmental challenges or stress. Preliminary experiments on increasing inoculation density did not change biofilm formation (data not shown). The biofilm presented was more robust in lower medium concentration (0.25×) compared to higher concentration (0.5×) of Def4, also indicating

that biofilm formation is stronger at lower overall nutrient concentrations, together with the limitation in nitrogen sources. The different media may also influence expression of EPS, which in turn would impact the biofilm formation (Cavalheiro & Teixeira, 2018).

## Disruption of biofilm by nutritional drinks

Challenging the *Candida* biofilms with nutritional drink formulations was based on the assumption that the oral mucosal surfaces are the most directly available place where virulent filamentous *C. albicans* biofilms are problematic (Cassone & Cauda, 2012; Grossi, 2009; Sensoy & Belet, 2011). At the same time, large patient groups with suppressed immune systems, cystic fibrosis patients and patients undergoing chemotherapy have challenges with frequent oral *Candida* infections and are often in need of nutritional supplements (Cassone & Cauda, 2012; Grossi, 2009). If nutritional supplements could have favourable effects on *C. albicans*, this would benefit such patients.

The amount of viable *C. albicans* cells was not affected by exposure to nutritional drinks. However, the addition of even low concentrations of nutritional drink formulations weakened the biofilm integrity, and in some cases

also changed the morphology from filamentous to free ovoid yeast cell growth. Increasing nutrient access is one way to route the cells away from biofilm formation, also reduce attachment (Jacobsen et al., 2012). The nutritional drinks contain whey protein and fruit sugars that the yeast cells may utilize. However, we observed clear effects on biofilm stability of adding nutritional drinks in concentrations down to 0.02%. For a concentration of 0.2% drinks, 0.17 g L<sup>-1</sup> of sugar is added to the 10 g L<sup>-1</sup> fructose supplied from the culture media. The added sugar represents a negligible amount as a growth substrate, that would likely not induce a morphological shift away from biofilm formation. A reduction in pH can favour free growing yeast cells. The pH of the culture media (Def4) remains unchanged at pH 6.4 up to an admixture of 1.3% drink concentration, and up to 10% was needed to bring pH below 6. The effect of the drinks on biofilm stability is thus prominent at a concentration where neither accessible macronutrients for growth nor pH are affected.

Fish oil, vitamin D and curcumin, all known to exhibit antioxidizing effects, are present in the applied nutritional drink formulations. In addition, pomegranate, chokeberry and cherries, which account for the main juice contents of the nutritional drink formulations, all have high amounts of phenols with high antioxidant capacity, especially polyphenols (Kelley et al., 2018; Nowak et al., 2017). Documentation from manufacturer confirms the presence of phenols and stable content of active antioxidants in the drinks comparable to that of berry and berry products (Carlsen et al., 2010). Curcumin, present in Curcher, is a polyphenol with antioxidant and anti-inflammatory effects and has been strongly indicated to help manage conditions like metabolic syndrome, hyperlipidaemia, arthritis, oxidative and inflammatory conditions and anxiety (Hewlings & Kalman, 2017). Curcumin has poor bioavailability, so ingestion of pure curcumin does not promote the health benefits mentioned above. Curcumin therefore needs to be formulated with agents that improve adsorption or slow down elimination to provide the multiple health benefits (Hewlings & Kalman, 2017). Two formulations, WFE and CJF, were prepared to explore how the absence of either the fruit juices or fish oil from the nutritional drinks would impact the biofilm effect of the formulations. In addition, curcumin was evaluated both incorporated in a nutrient drink, and alone.

According to our results (Figure 3), the polyphenol curcumin inhibited yeast cell growth at concentrations >0.07 mg ml<sup>-1</sup>, which corresponds to results in another study where 0.1 mg ml<sup>-1</sup> curcumin resulted in a 50% growth inhibition of *C. albicans* (Shahzad et al., 2014). The different nutritional drinks did not display any anti-candida effects in terms of culture viability, but all the nutritional drink formulations altered the biofilm integrity

and affected cell adherence and morphological changes (Figure 4).

Fish oil, containing omega-3 polyunsaturated fatty acids (*n*-3 PUFA) and other long- and short-chain fatty acids are known to have anti-candida effects, both as growth inhibitors and preventors of biofilm formation, but the effects are highly dose dependant (Chanda et al., 2018; Huang et al., 2011; Huang & Ebersole, 2010; Thibane et al., 2010, 2012). Marine PUFAs can induce apoptosis in *C. albicans* (Thibane et al., 2012). They also inhibit mitochondrial metabolism of *C. albicans* biofilm and cause severe changes to the cell surface (Thibane et al., 2010). Although no changes to culture viability due to fish oil were detected in this study, the concentrations of PUFAs in the nutritional drink formulations, especially the WFE, may very well be enough to cause changes to the biofilm integrity.

Control juice formulation does not contain fish oil and consists mainly of fruit juices. These juices are high in phenols and polyphenols. Several studies describe how phenols and polyphenols inhibit *C. albicans* biofilm through a wide range of mechanisms, for example, inhibit hyphae formation, reduce germ tube formation, have anti-adhesive properties and general anti-biofilm effects. Phenols and polyphenols can also damage the cell membrane and induce apoptotic mechanisms in *C. albicans*, cause metabolic instability, retard the growth as well as reduce its ability to form and maintain biofilm (Evensen & Braun, 2009; Farkash et al., 2018; Shahzad et al., 2014; Teodoro et al., 2015). Effects occur at physiologically relevant polyphenol concentrations and do not appear as polyphenol-specific or strain specific (Evensen & Braun, 2009). Phenols' ability to inhibit planktonic growth is dose dependant and less prominent than anti-biofilm effects (Shahzad et al., 2014). One study shows how polyphenols reduce biofilm without affecting growth of planktonic cells (Farkash et al., 2018), which is in accordance with the results from *C. albicans* culture exposure to RL+, Curcher and CJF in our study. Farkash et al. (2018) describe how the polyphenols affected *C. albicans* morphological switching ability from ovoid cells to hyphae, downregulated three genes important for cell adhesion (*hwp1*, *als3* and *eap1*) and reduced production of EPS. Thus, it is plausible to suggest that both fish oil and phenols present in the nutrient drink formulations can explain the cause for biofilm destabilization seen in our study.

## Biofilm inhibition by AmB

Combined with the knowledge that AmB acts more efficiently on free than filamentous growing yeast cells, the combination of nutritional drinks and AmB was tested,

to demonstrate the use of the screening assay for MBIC determination.

The MBIC for AmB acting alone on *C. albicans* in this study was  $0.25 \mu\text{g ml}^{-1}$ , similar to MIC reported in literature (Pfaller & Diekema, 2007; Rex et al., 1995). However, we observed an eightfold reduction in MBIC for AmB in the presence of very low concentrations of nutritional drink formulations. This shows that the destabilization of the biofilm observed for nutritional drink formulations did indeed enhance the effect of AmB. The inhibition of hyphae formation and change of biofilm structure has also previously been linked to potentiation of a range of antifungals, and in several fungal strains (Tøndervik et al., 2014).

The three formulations containing fruit juice (i.e. RL+, Curcher and CJF) performed similar, and the absence of fish oil in CJF did not impact results negatively. Thus, the fish oil is likely not the main contributor to AmB potentiation. The nutritional drink formulations with fruit juice enhanced the effect of AmB more than the formulation without fruit juice (WFE), although WFE had effect (fourfold decreased MBIC). This may imply that the effect of polyphenols in RL+, Curcher and CJF is greater than the effect of fish oils in WFE. Phenols downregulate important attachment genes and also reduce EPS production, two crucial factors in biofilm formation. Degradation of EPS using hydrolytic enzymes has been shown to reduce biofilm biomass and potentiate the activity of antimicrobials (Ruiz-Sorribas et al., 2022), attesting the importance of impairing EPS production in combating biofilms. In addition, literature shows that phenols can target the oxidative stress response in *Candida* (Kim et al., 2012) and protect AmB from autoxidation, thus prolonging its biological activity (Beggs et al., 1978). Another example of synergism between phenolic acids and antifungal agents describes how AmB together with polyphenols increase the production of reactive oxygen species, leading to apoptosis (Teodoro et al., 2015). The combined effects of phenols can thus explain the observed potentiating effect of fruit juices compared to fish oil.

As curcumin is also a phenol and antioxidant, a potentiation effect of AmB in combination with pure curcumin could be expected, but this was not seen in this study. Previous studies have shown that curcumin can downregulate *als3* and *hwp1* genes, and thus inhibit biofilm formation, but requires concentrations fivefold of that used in our study (0.05 vs. 0.01, Figure 6) (Shahzad et al., 2014). The nutritional drink formulation containing curcumin (Curcher) did not perform better than the nutritional drink without curcumin (RL+); thus, the curcumin did not seem to impact the AmB effect compared with other ingredients.

The present study revealed interesting anti-biofilm effects of nutritional drinks and potentiation of AmB on

*C. albicans* biofilm formation in line with current findings in literature. Further studies can answer whether single ingredients of the applied formulations could provide similar effects, through which mechanism this happens, and whether the tested formulations can also disrupt already formed, and mature, biofilms. This will be important follow-up studies to determine how these findings can be utilized for clinical use. Nevertheless, ingesting nutraceuticals containing both polyphenols and fish oils have demonstrated important complementary, additive and synergistic beneficial health effects regardless of their antimicrobial capabilities (Mendez & Medina, 2021).

A robotic HTS assay for biofilm growth and evaluation in microtiter plates was successfully established to enable the investigation of *C. albicans* biofilm formation and its response and susceptibility to diverse treatments. Cultivation conditions favouring filamentous growth and microcolony formation were established, which are highly relevant for (oral) candidiasis, virulence and increased resistance towards antifungal therapeutics. The assay enabled studies of morphological changes, growth and biofilm formation, and the HTS format facilitated investigation of antifungal effects of a large number of components, concentrations and combinations. The methodological development provides an important foundation for expanding the assays for other biofilm forming fungal strains with high clinical importance, and can be utilized for a broad range of applications, from developing nutritional drink formulations to evaluating drugs for treating fungal biofilms. The assay was demonstrated by investigating the effect of various nutritional drinks alone and in combination with AmB on *C. albicans* filamentous biofilm. Nutritional drinks containing whey protein, fish oil and fruit juices were shown to affect both the biofilm morphology and stability and were furthermore able to eightfold potentiate the effects of AmB on *C. albicans* biofilms. The established assays can be readily translated to screening of other antifungal substances on *C. albicans*.

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#### CONFLICT OF INTEREST

No conflict of interest declared.

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