Research highlights

- Bile protects various pathogenic fungi from antifungals
- Bile lipids increase antifungal trapping efficiency of conjugated bile salts
- Polyunsaturated fatty acids mediate azole resistance of conjugated bile salts
- Pathogen elimination from biliary system depends on antifungals not trapped in bile

Lipid components of bile increase the protective effect of conjugated bile salts against antifungal drugs

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Abstract

Fungi and bacteria are able to persist in the human gall bladder. Previous studies have shown that bile protects *C. albicans* in this cryptic host niche from antifungals, providing a reservoir for re-colonization of the intestine after discontinuation of antifungal therapy. Bile and conjugated bile salts trap antifungals in micelles, thereby reducing their bioavailability and possibly promoting the development of drug resistance. Here we show that the protective effect of bile and conjugated bile salts is not limited to *C. albicans*, but also observed with other fungi. Interestingly, bile, but not conjugated bile salts conferred resistance of *C. albicans* against fluconazole. Similarly, only bile conferred resistance of *Aspergillus terreus* against voriconazole. To investigate this higher potency of bile in mediating antifungal protection we aimed in a step-wise reconstitution of bile from conjugated bile salts. While phospholipids and saturated fatty acids increased the ability of conjugated bile salts to protect from amphotericin B, both failed to protect from azoles. In contrast, when conjugated bile salts were supplemented with polyunsaturated fatty acids, resistance against azoles increased and the critical micelle concentration of conjugated bile salts decreased to the level of bile. We conclude that polyunsaturated fatty acids are vital in the formation of mixed micelles that exhibit a high potential to trap antifungals. Since bile-mediated protection depicts a general problem in biliary tract infections, reconstituted synthetic bile should be used to investigate drug efficacy in the biliary system.

1. Introduction

In liver transplant recipients biliary tract infections comprise one of the major complications that increase mortality rates and may inforce the requirement for re-transplantation (Moreno and Berenguer, 2006). About 50% of liver transplant recipients encounter at least one episode of viral, bacterial or fungal infection with *Escherichia coli* and *Klebsiella pneumonia* as the main bacterial infections followed by fungi such as *Candida* or *Aspergillus* species (Chiereghin et al., 2017). Furthermore, it has been shown that the bacterium *Listeria monocytogenes* freely replicates in bile implying that the gall bladder acts as a source for excretion of these bacteria (Hardy et al., 2004). In addition, gall bladder provides a reservoir for *Salmonella enterica* serovar Typhi even after antibiotic therapy causing severe chronic infections frequently accompanied by the development of gall stones (Gonzalez-Escobedo et al., 2011).

In a murine model of disseminated candidiasis we have previously shown that *Candida albicans* uses the gall bladder as a cryptic reservoir under antifungal therapy (Jacobsen et al., 2014). Subsequent investigations revealed that bile conferred resistance against commonly used antifungals such as the echinocandin caspofungin and the polyene macrolide amphotericin B (Jacobsen et al., 2014). As underlying mechanism of protection we found that antifungals are trapped in micelles that are formed by conjugated bile salts (Hsieh et al., 2017). The resulting reduction in the bioavailability of drugs in the biliary system and possibly also in the intestine might thereby not only prevent the clearance of pathogens from these host niches, but might also lead to the development of drug resistant strains. Accordingly, a recent study revealed the emergence of echinocandin resistant *Candida* species in liver transplant recipients after treatment with caspofungin (Prigent et al., 2017). Resistant strains were mainly isolated from the digestive system implying that a reduced bioavailability of drugs might have caused this emergence (Prigent et al., 2017). Furthermore,

case studies indicate that also other fungi and especially *Aspergillus* species are able to cause severe cholangitis (Erdman et al., 2002; Garcia-Ruiz et al., 1998). Therefore, it appears of high importance to clear pathogenic microorganisms residing in the biliary system. However, this requires the application of drugs that are not trapped in micelles.

Antifungal protection is strictly dependent on a concentration of conjugated bile salts that is above their specific critical micelle concentration (CMC). In this respect, taurocholate displays a higher CMC value at physiological conditions than taurodeoxycholate and, accordingly, higher concentrations of taurocholate are required to confer resistance. However, while both conjugated bile salts effectively protect against caspofungin and amphotericin B, neither of both compounds protected *C. albicans* against the azole fluconazole (Hsieh et al., 2017). On the contrary, bile strongly reduced the *in vitro* sensitivity of *C. albicans* against fluconazole and enabled persistence in the gall bladder under fluconazole treatment in a murine model of systemic candidiasis (Jacobsen et al., 2014). Since even high concentrations of taurodeoxycholate were not able to confer fluconazole resistance this increased protective effect of bile might be linked to a lower CMC value of bile accompanied by an increased ability to trap antifungals. It has been speculated that the more complex composition of bile including fatty acids and phospholipids leads to mixed micelles that allow a more effective trapping of antifungals (Hsieh et al., 2017).

To address this question, we studied the *in vitro* resistance of several fungal species in the presence and absence of conjugated bile salts to confirm a general mechanism of bile mediated antifungal protection. Subsequently, we analysed the effect of different fatty acid and lipid components either alone or in combination with conjugated bile salts on antifungal protection with a focus on azole resistance.

2. Material and Methods

2.1 Strains and culture conditions

C. albicans (SC5314), *S. cerevisiae* (ATCC 9763) and *C. neoformans* (H99 and 1841) were pre-cultivated overnight in 20 ml YPD media (per litre: 10 g yeast extract, 20 g peptone, and 20 g glucose) at 30°C and yeast cells were harvested by centrifugation at 4000 × g. After washing cells twice in phosphate-buffered saline (PBS) cells were suspended and adjusted to selected cell densities in either YPD or MOPS-buffered RPMI 1640 medium (Sigma) containing 2% glucose (per litre: 10.4 g RPMI 1640, 34.53 g MOPS, 20 g glucose; pH 6.8; subsequently defined as RPMI medium). To obtain conidia suspension of *A. fumigatus* (CBS144-89) and *A. terreus* (SBUG844), conidia were plated on 50 mM glucose *Aspergillus* minimal media with nitrate as nitrogen source and 2% agar (Gressler et al., 2011). Plates were incubated at 37°C for 3-4 days (*A. fumigatus*) or 5-6 days (*A. terreus*). Conidia were harvested in 10 ml sterile PBS and filtered through a 40 μm cell-strainer. After a washing step in PBS, conidia were diluted to defined concentrations in RPMI medium and used for drug resistance analyses.

2.2 Preparation of antifungals for sensitivity analyses

Stock solutions of caspofungin (5 mg/ml; Cancidas, Merck, Germany) and flucytosine (10 mg/ml) were prepared in PBS and filter sterilized. Stock solutions of amphotericin B (4 mg/ml) and voriconazole (16 mg/ml) were prepared in DMSO. Fluconazole at 2 mg/ml was purchased from B. Braun, Germany. All drugs were diluted in the respective media used for resistance analyses. Controls were prepared according to the solvents used for stock solutions of the respective antifungals.

2.3 Preparation of bile solution and lipid solubilisation in conjugated bile salt

Crude porcine bile extract (Sigma, B8631) was solved in either YPD or RPMI medium to give a final concentration of 12.5% (w/v). Insoluble components were removed by centrifugation at 12000 × g. The supernatant was filter sterilised and stored at 4°C in the dark. Sodium taurodeoxycholate hydrate (Sigma, T0875) and taurocholic acid sodium salt hydrate (Sigma, T4409) were dissolved at 100 mg/ml in either YPD or RPMI medium, filtered sterilised and used as stock solutions. The following saturated fatty acids were used for preparing mixtures with conjugated bile salt: C3 (propionic acid sodium salts, Applichem, A1931), C4 (sodium butyrate, Aldrich, 303410), C8 (sodium caprylate, Fluka, 71339), C10 (sodium decanoate Fluka, 21490), C14 (myristic acid sodium salt, Fluka, 70140) and C16 (sodium palmitate, Fluka, 76165). Media containing 10 mg/ml taurocholate were supplemented with the indicated amounts of fatty acids. To solubilise C14 and C16, mixtures were heated to 65°C for 30-60 min. As a source of phospholipids a soy refined lecithin (Mp Biomedicals, LLC) was added at indicated concentrations. As sources of polyunsaturated fatty acids arachidonic acid (Sigma, 10931) and conjugated linolenic acid (Sigma, O5507) were used that were added to YPD or RPMI media containing 10 mg/ml sodium taurocholate. For preparation of reconstituted synthetic bile (RSB) the following components were mixed (per ml of RPMI medium): 30 mg sodium taurocholate, 30 mg sodium taurodeoxycholate, 5 mg lecithin, 10 mg C14, 2.5 mg arachidonic acid and 2.5 mg conjugated linolenic acid generating an 80 mg/ml RSB stock. Stocks of an equal mixture of tauro- and taurodeoxycholate (60 mg/ml in total) or RSB lacking phospholipids and saturated fatty acids (65 mg/ml solubilised solids) were also prepared.

2.4 Drug resistance analyses

Antifungal drug resistance or toxicity of saturated fatty acids was tested virtually as described previously (Hsieh et al., 2017). In brief, dilutions of bile and mixtures of conjugated bile salts

and lipids either with or without antifungals were prepared in either YPD or RPMI medium and transferred to 96-well plates. Fungal yeast cells or conidia were pre-diluted in the respective growth medium and added to a final concentration of 4×10^4 yeasts or 1×10^5 conidia resulting in 200 µl supplemented medium. Plates were sealed with transparent gas permeable moisture barrier seal (4titude) and incubated at 37°C except for *C. neoformans* species that were incubated at 30°C. Plates were rotated every 15 min prior to interval readings of the optical density at 600 nm (OD_{600}). Final end point measurements of plates were performed at the indicated time points. Results were either shown as OD_{600} readings or in percentage of residual growth when normalised against control groups without antifungal treatment. All growth tests were performed in three parallel wells with at least one independent biological replication. Data were analysed by using Microsoft Excel or GraphPad Prism software.

2.5 Determination of critical micelle concentrations (CMC) and rhodamine 6G influx analyses

Critical micelle concentration (CMC) was determined as described previously (Hsieh et al., 2017) by measuring the change of absorbance of eosin Y at 542 nm using a microplate reader (Multiskan™ Go, Thermo). Bile, conjugated bile salts and mixtures of conjugated bile salts containing different lipid compositions were tested in serial dilutions and data were analysed using Microsoft Excel software by plotting the absorbance values against the logarithmic concentration of the solubilising agent. For rhodamine 6G (R6G) influx assays *C. albicans* yeast cells were incubated at 30°C in RPMI medium in the presence of 1 µM R6G and 1 mg/ml of either bile, conjugated bile salts or mixtures of conjugated bile salts and lipids. Aliquots of cells were harvested at different time points and washed twice in 50 mM HEPES buffer pH 7.0. R6G fluorescence was measured in a microplate reader (Fluostar Omega plate

reader, BMG Labtech) at 545 nm excitation and 590 nm emission. Fluorescence was normalised against the cell density at 600 nm.

2.6 Statistical analyses

All bar diagrams show mean values + standard deviation (SD). All experiments were performed in biological duplicates or triplicates with three wells inoculated in parallel. Comparisons between multiple groups were analysed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism (GraphPad Software).

3. Results

3.1 Taurocholate confers amphotericin B resistance in a variety of fungal species

We first studied whether drug resistance mediated by conjugated bile salt is limited to *C. albicans* or depicts a general mechanism of protection in fungi against antifungals. Therefore, we selected the non-pathogenic ascomycete yeast *Saccharomyces cerevisiae* and its pathogenic relative *Candida glabrata*, the filamentous ascomycetes *Aspergillus fumigatus* and *Aspergillus terreus* as well as the basidiomycete *Cryptococcus neoformans* var. *grubii* H99 (serotype A) and *C. neoformans* 1841 (serotype D) for protection analyses against amphotericin B. A concentration of 25 mg/ml taurocholate was tested first for its toxicity, but was well tolerated by all strains, except for the *C. neoformans* isolates that revealed a slight, but significant reduction in growth rate (**Fig.1**). Subsequently, we determined amphotericin B concentrations that suppress growth of the respective species. *S. cerevisiae* and *C. glabrata* showed no growth at 0.5 µg/ml, the two *Aspergillus* species did not proliferate at 1 µg/ml and the two *C. neoformans* strains did not grow in the presence of 2 µg/ml of amphotericin B (**Fig.1**). The combination of taurocholate with the respective amphotericin B concentration

restored growth of all fungal strains (**Fig.1**) indicating that conjugated bile salts mediate fungal protection that is not limited to *C. albicans*.

3.2 Bile, but not its conjugated bile salts protect from azoles

Next, we investigated the efficacy of bile and conjugated bile salts to protect from azoles. While a first-line treatment of *C. albicans* infections recommends the use of echinocandins, fluconazole is still used in clinically stable patients with no previous exposure to azoles (Calandra et al., 2016). Voriconazole is one of the azoles of choice in prophylactic and acute therapy of *A. terreus* infections (Karthaus, 2011; Vehreschild et al., 2007). The use of azoles in the treatment of *A. terreus* infections is of special importance since most *A. terreus* strains exhibit a natural resistance against amphotericin B (MIC $\geq$ 1 mg/l), although the molecular basis for this is not yet well understood (Pastor and Guarro, 2014). Growth of *C. albicans* was inhibited at 0.125 µg/ml fluconazole and *A. terreus* was sensitive against voriconazole at a concentration of 0.25 µg/ml. Both, *C. albicans* and *A. terreus* tolerated bile, taurodeoxycholate and taurocholate (**Fig. 2**), but only bile conferred resistance against fluconazole for *C. albicans* and voriconazole in case of *A. terreus*. Taurocholate and taurodeoxycholate did not show a protective effect (**Fig. 2**). Therefore, protection against azoles appears specifically mediated by bile and - although a larger number of species and azoles need to be tested - this protection seems largely independent from the type of azole and the fungal species.

3.3 Phospholipids in conjugated bile salt-mediated antifungal protection

Lecithin (diacylphosphatidylcholine) is the major phospholipid in bile (Hay and Carey, 1990). In a previous study we showed that an ethylacetate fraction of bile, mainly containing the lipid fraction of bile was by itself not sufficient for antifungal protection (Hsieh et al.,

2017). However, since phospholipids consist of a hydrophilic, zwitterionic phosphocholine head group and hydrophobic tails from the long fatty acyl chains (Hay and Carey, 1990), the phospholipid lecithin is likely to form mixed micelles with conjugated bile salts, which may increase the potency to encapsulate drugs. To test this assumption, we first analysed the effect of various lecithin concentrations on enhancement of the protective effect of taurocholate against caspofungin and amphotericin B (**Fig. 3A**). Here, we used a sub-protective concentration of 10 mg/ml of taurocholate at which *C. albicans* was sensitive against 2 µg/ml caspofungin and 4 µg/ml amphotericin B. Similarly, the phospholipid lecithin alone, in a concentration of up to 10 mM, was not protective. However, 2.5 mM of lecithin enhanced the protective effect of taurocholate and mediated caspofungin and amphotericin B resistance (**Fig. 3A**). Subsequently, the addition of phospholipids on fluconazole protection was analysed, whereby the protective effect with either taurocholate, taurodeoxycholate or bile was tested (**Fig. 3B**). Similar to bile, lecithin inhibited the hyphae inducing effect of taurocholate and taurodeoxycholate in RPMI medium (Hsieh et al., 2017). While this resulted in higher growth rates in the absence of drugs, up to 25 mM lecithin did not confer resistance against the azole. When we analysed the effect of the phospholipid lecithin on the critical concentration of taurocholate required to form micelles, the CMC value of taurocholate in the presence of lecithin did not significantly decrease in comparison to taurocholate alone (**Fig. 4**). Therefore, we conclude that phospholipids are not facilitating micelle formation, but can enhance the protective effect of conjugated bile salts by forming mixed micelles. However, this combination does not protect from azoles and is not sufficient to resemble the protective effect of bile.

3.4 Role of saturated fatty acids in antifungal protection

The major proportion of free fatty acids in bile consists of saturated fatty acids (Chatterjee et al., 2007). Therefore, we investigated a possible contribution of saturated fatty acids towards antifungal protection. First, we studied growth of *C. albicans* in the presence of varying concentrations (range 0.1 – 3.2 mM) of saturated fatty acids of different chain length by simultaneously applying a fixed sub-protective concentration of 10 mg/ml taurocholate. As shown previously (Otzen et al., 2013), octanoic (C8) and decanoic (C10) acid exhibited a toxic effect towards *C. albicans*, which was not relieved by the presence of taurocholate. Propionate (C3) and butyrate (C4) were well tolerated in the concentrations applied, but due to their high water solubility are unlikely to contribute to micelle formation. No growth defect was observed by the addition of myristic (C14) and palmitic (C16) acid (**Fig 5A**). However, despite the presence of 10 mg/ml taurocholate, palmitic acid was not well solubilised and produced solid particles in the growth media at 37°C. Therefore, we investigated antifungal protection by the combination of myristic acid with taurocholate (**Fig. 5B**). Indeed, when myristic acid exceeded a concentration of 0.8 mM (> 0.18 mg/ml) this mixture conferred resistance against caspofungin and amphotericin B. However, as with phospholipids, myristic acid did not confer resistance against fluconazole (not shown). Furthermore, similar to lecithin, a determination of the CMC value of taurocholate revealed that myristic acid did not significantly reduce the concentration of taurocholate required to form micelles (**Fig. 4**). Therefore, similar to phospholipids, saturated fatty acids reduce the concentration of taurocholate required to confer resistance against amphotericin B and caspofungin, but are not sufficient for protecting against azoles.

3.5 Unsaturated fatty acids in antifungal protection

Besides phospholipids and saturated fatty acids bile contains a significant proportion of unsaturated fatty acids, which is in agreement with a special importance of bile salts in

solubilisation and uptake of essential fatty acids such as linolenic and arachidonic acid (Mullins et al., 1998). Fractionation of bile identified unsaturated fatty acids as playing a major role in repression of cholera toxin production in *Vibrio cholera* (Chatterjee et al., 2007; Plecha and Withey, 2015) and either bile, arachidonic or linoleic acid were shown to be effective in inhibiting binding of cholera toxin and *Escherichia coli* enterotoxin to the cell surface receptor GM1 (Chatterjee and Chowdhury, 2008). Therefore, we tested the effect of the polyunsaturated fatty acids arachidonic (20:4) and conjugated linolenic acid (18:3) on their protection from antifungals. A mixture of arachidonic and linolenic acid at a concentration of 0.2 or 0.8 mM of each compound was well tolerated by *C. albicans*, but failed to confer resistance against either amphotericin B (2 µg/ml, **Fig. 6A**) or fluconazole (in a range of 0.5 – 2 µg/ml, not shown). When added at concentrations above 0.8 mM, arachidonic acid, but not linolenic acid, inhibited growth of *C. albicans*. However, when combined with taurocholate even 1.6 mM of arachidonic acid was well tolerated. Furthermore, the mixture of arachidonic acid and linolenic acid (0.2 mM each) in combination with TC conferred resistance against amphotericin B and was also protective against up to 1 µg/ml fluconazole when polyunsaturated fatty acids were added with TC in a concentration of 0.8 mM of each unsaturated fatty acid (**Fig. 6A and 6B**). A single species of these polyunsaturated fatty acids was also able to confer resistance in a concentration dependent manner when combined with taurocholate. In these analyses, arachidonic acid showed a higher protective effect compared to linolenic acid. (**Fig. 6C**). In conclusion, while we cannot exclude an increased drug resistance by cell-wall reprogramming due to changes in the carbon source composition in the medium (Ene et al., 2012), mixed micelles formed from unsaturated fatty acids and conjugated bile salts increase the capability of taurocholate to encapsulate azole drugs. The results were further confirmed by investigating the effect of these polyunsaturated fatty acids on protection of *A. terreus* against voriconazole. While

growth of *A. terreus* was slightly inhibited by a mixture taurocholate containing 0.8 mM of each unsaturated fatty acid, a protection against 0.25 and 0.5 µg/ml of voriconazole was observed (**Fig. 6D**). Unexpectedly, while neither phospholipids nor saturated fatty acids significantly decreased the CMC value, a significant decrease in the concentration of taurocholate required to form micelles was observed in the presence of the polyunsaturated fatty acids (**Fig. 4**). Therefore, these unsaturated fatty acids significantly contribute to the efficiency of bile to confer resistance against azole drugs.

3.6 Protective effect of reconstituted synthetic bile

Since all, phospholipids, saturated and unsaturated fatty acids showed some contribution in enhancement of antifungal protection by conjugated bile salts, we aimed in the reconstitution of a synthetic bile consisting of a mixture of taurocholate and taurodeoxycholate (30 mg/ml each), lecithin (5 mg/ml), myristic acid (10 mg/ml), arachidonic acid (2.5 mg/ml) and linolenic acid (2.5 mg/ml), resulting in a total of 80 mg solubilised solids in 1 ml of water. This mixture was compared to the protective effect of a mixture of taurocholate and taurodeoxycholate (30 mg/ml each) containing 2.5 mg/ml of each of the polyunsaturated fatty acids arachidonic and linolenic acid, resulting in a total of 65 mg/ml solubilised solids. These mixtures were compared with bile and a mixture of taurocholate and taurodeoxycholate for their concentration dependent protective effect against azoles and the highly water soluble antimetabolite flucytosine. Mixtures were adjusted in media to give a final concentration of 10, 5, 2.5 and 0 mg/ml of total solubilised solids and 0.5 µg/ml of fluconazole was added to test resistance of *C. albicans*. While bile produced the highest cell density after 24 h of incubation, the complete mixture of all types of fatty acids with conjugated bile salts (reconstituted synthetic bile, RSB) as well as the polyunsaturated fatty acids in combination with conjugated bile salts were protective, but with slightly higher efficacy of the latter

mixture compared to RSB (**Fig. 7A**). A mixture of the two conjugated bile salts alone did not protect against fluconazole in any of the concentrations tested. Similar to fluconazole resistance mediated towards *C. albicans*, the mixtures also conferred resistance of *A. terreus* against voriconazole with a comparable efficiency of all formulations except for the mixture only containing taurocholate and taurodeoxycholate that was not protective (**Fig. 7B**). This implied that polyunsaturated fatty acids added as additives are sufficient for the protective effect, whereas saturated fatty acids and phospholipids are mainly dispensable. Indeed, a determination of CMC values revealed that a mixture of arachidonic and linoleic acid in combination with unconjugated bile salts showed the same value as that determined for bile (**Fig. 7C**). In summary, these results indicate that reduction of the CMC by addition of polyunsaturated fatty acids appears as the main driving force to enhance the protection of conjugated bile salts against azoles. In contrast, when the same mixtures were tested for protection of *C. albicans* from flucytosine no protection was observed (**Fig. 7D**). Thus, similar to bile, mixed micelles of polyunsaturated fatty acids with conjugated bile salts are not able to inactivate small highly water soluble molecules.

We finally compared bile, a mixture of conjugated bile salts and conjugated bile salts containing solubilised polyunsaturated fatty acids for inhibiting the influx of the model drug rhodamine 6G from *C. albicans* cells, which was measured by the time dependent increase of cellular fluorescence. Both, bile and conjugated bile salts with polyunsaturated fatty acids inhibited drug influx to a similar extent (**Fig. 7E**). This further confirms the essential contribution of polyunsaturated fatty acids to the high efficiency of bile to protect from antifungals.

4. Discussion

Previous studies have shown that *C. albicans* persists under antifungal therapy in the gall bladder of mice (Jacobsen et al., 2014). This protection is mediated by bile and conjugated bile salts that trap antifungals in micelles (Hsieh et al., 2017). However, it remained unclear, by which mechanism bile, but not conjugated bile salts can protect from azoles. Here, we discovered that polyunsaturated fatty acids, which are normal constituents of bile are responsible for mediating this higher protective efficiency of bile compared to conjugated bile salts. Moreover, we also found that antifungal protection is not limited to *C. albicans*, but also protects other pathogenic fungi, and probably pathogenic microorganisms in general from therapeutic drugs. In this respect, a preliminary analysis revealed that bile and reconstituted bile containing polyunsaturated fatty acids conferred increased resistance of *E. coli* against kanamycin, whereby ampicillin remained active (data not shown).

The increase of the protective effect of polyunsaturated fatty acids and especially that of arachidonic acid was surprising, taking into account previous investigations that revealed increased antifungal drug sensitivity of *C. albicans* when cultivated in presence of arachidonic acid (Ells et al., 2009; Mishra et al., 2014). Incorporation of arachidonic acid into the fungal cell membrane provokes an increase in the production of ergosterol and is accompanied by increased membrane fluidity. Although arachidonic acid does not reduce cell viability, this membrane modulation appears to increase susceptibility against antifungals (Ells et al., 2009). Integration of polyunsaturated fatty acids also occurs when *C. albicans* is grown in bile as confirmed by analyses of bile-grown *C. albicans.* Under these conditions cell membranes revealed an integration of arachidonic and linolenic acid (data not shown). Furthermore, in agreement with previous studies, neither of these polyunsaturated fatty acids protected against antifungals (**Fig. 6A**) when used in the absence of conjugated bile salts.

The capacity of conjugated bile salts to solubilise polyunsaturated fatty acids is larger than that for monounsaturated or saturated fatty acids (Smith and Lough, 1976). Thereby,

compared to monounsaturated or saturated fatty acids, polyunsaturated fatty acids efficiently lower the CMC value of glycodeoxycholate (Freeman, 1969). Therefore it can be concluded that arachidonic and linolenic acid increase the efficiency of conjugated bile salts to form micelles, which is obviously accompanied by a greater capability to trap antifungals in these mixed micelles as shown by the reduction of the bioavailability of azole drugs and the high efficiency in preventing rhodamine 6G influx.

Orthotopic liver transplantation produces a significant risk factor for patients to acquire invasive fungal infections with *Aspergillus* and *Candida* species as main causative agents (Pacholczyk et al., 2011; Salavert, 2008). Prophylactic therapy in these patients with polyene macrolides, echinocandins or azoles may decrease the risk for invasive fungal infections as these antifungals may prevent fungal dissemination and establishment of disease outside of the biliary system (Liu et al., 2011). However, our study indicates that these drugs appear ineffective in clearing infections that persist within the gall bladder, biliary duct or even in the intestine, as bile efficiently reduces their bioavailability.

In conclusion, it should be considered to test the efficacy of antifungals in the presence of bile or a mixture of polyunsaturated fatty acids with conjugated bile salts. Drugs that remain active under these conditions as shown here for flucytosine might act as more suitable compounds for prophylactic therapy in high risk patients.

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Figure legends

Figure 1: Analysis of amphotericin B sensitivity of various fungal species in the presence of conjugated bile salts. (**A**) Resistance of *C. glabrata* and *S. cerevisiae* against 0.5 µg/ml amphotericin B. Cells were incubated at 37°C in RPMI medium with or without 25 mg/ml taurocholate (TC). End point OD_{600} was taken for *C. glabrata* after 20 h and for *S. cerevisiae* after 45 h. (**B**) Resistance of *A. fumigatus* and *A. terreus* against 1.0 µg/ml amphotericin B. Cells were incubated at 37°C in RPMI medium with or without 25 mg/ml TC. End point OD_{600} was taken after 20 h. (**C**) Resistance of *C. neoformans* strain H99 and 1841 against 2.0 µg/ml amphotericin B. Cells were incubated at 30°C in YPD medium with or without 25 mg/ml TC and end points were measured after 20 h. (C). The bar diagrams show mean + SD from three independent experiments in technical duplicates. Data were analysed by ANOVA followed by Tukey's multiple comparison (***$p < 0.005$). Taurocholate confers resistance against amphotericin B in all species tested.

Figure 2: Protection of bile and conjugated bile salts against azoles. Fungi were incubated at 37°C in RPMI medium supplemented with either 25 mg/ml taurocholate (TC), 12.5 mg/ml taurodeoxycholate (TDC) or 12.5 mg/ml bile and OD_{600} was taken after 20 h of growth. (**A**) Resistance of *C. albicans* against various concentrations of fluconazole. (**B**) Resistance of *A. terreus* against various concentrations of voriconazole. Results are shown as mean + SD from three independent experiments in technical duplicates. Statistical analyses were performed by ANOVA followed by Tukey's multiple comparison (***$p < 0.005$). Only bile confers resistance against azole treatment.

Figure 3: Analysis of the effect of phospholipids on antifungal resistance. (**A**) YPD

495 medium was supplemented with various concentrations of phospholipids (PL, lecithin) with
496 or without 10 mg/ml taurocholate (TC, sub-protective at this concentration) and resistance of
497 *C. albicans* against amphotericin B (AMB; 4 µg/ml) and caspofungin (CAS; 2 µg/ml) was
498 studied. Growth was evaluated after 20 h incubation at 37°C. Addition of phospholipids (in
499 the form of lecithin) increased the efficacy of TC to protect from both drugs. (**B**) Protective
500 effect of 25 mM PLs with or without 12.5 mg/ml TC or taurodeoxycholate (TDC) on *C.*
501 *albicans* cultivated in the presence of various fluconazole concentrations. PLs do not mediate
502 fluconazole resistance. Data are shown as mean + SD from three independent experiments in
503 technical triplicates. Statistical analyses were performed by ANOVA followed by Tukey's
504 multiple comparison (***$p < 0.005$; NS = not significant).
505
506 **Figure 4: Effects of lipid components on critical micelle concentrations of taurocholate.**
507 Taurocholate (10 mg/ml; TC) was mixed with either 10 mM phospholipids (PL), 1.6 mM
508 myristic acid (C14) or an equal mixture of arachidonic (AA; 0.2 or 0.8 mM) and conjugated
509 linolenic acid (CLA; 0.2 or 0.8 mM). The critical micelle concentration (CMC) was
510 determined from serial dilutions in the presence of Eosin Y. Data represent mean + SD from
511 two independent experiments. (***$P < 0.005$, NS = not significant). Statistical significance
512 was calculated by ANOVA followed by Tukey's multiple comparison.
513
514 **Figure 5: Role of saturated fatty acids in antifungal protection.** C3 = propionic acid, C4 =
515 butyric acid, C8 = caprylic acid, C10 = decanoic acid, C14 = myristic acid, C16 = palmitic
516 acid. In all analyses YPD medium supplemented with 10 mg/ml taurocholate (TC) was used.
517 *C. albicans* cells were grown at 37°C and optical density (OD_{600}) was determined after 20 h.
518 (**A**) Effect of various fatty acids in combination with TC on growth of *C. albicans*. C8 and
519 C10 provoke toxic effects that are not compensated by TC. (**B**) Protective effect of different

C14 concentrations in presence of TC on caspofungin (CAS; 2 µg/ml) and amphotericin B (AMB; 4µg/ml) resistance. At least 0.8 mM myristic acid are required for increasing the protective effect of TC. Bar diagrams show mean values + SD from three independent assays measured in duplicates. Data were analysed by ANOVA followed by Tukey's multiple comparison (***$p < 0.005$).

Figure 6: Contribution of polyunsaturated fatty acids on antifungal protection of conjugated bile salts. Analyses on *C. albicans* were performed in YPD medium. Resistance of *A. terreus* was tested in RPMI medium. Taurocholate (TC) was used in a concentration of 10 mg/ml. (**A**) Sensitivity of *C. albicans* against amphotericin B (AMB; 2 µg/ml). Arachidonic acid (AA; 0.2 mM; 0.06 mg/ml) and conjugated linolenic acid (CLA; 0.2 mM; 0.056 mg/ml) were added in equal concentration. (**B**) Fluconazole protection of *C. albicans* by addition of equal amounts of AA (0.8 mM; 0.244 mg/ml) and CLA (0.8 mM; 0.222 mg/ml) to TC containing media. (**C**) Protection of *C. albicans* against fluconazole (1 µg/ml) by addition of different concentrations of either AA or CLA. (**D**). Voriconazole protection of *A. terreus* by addition of equal amounts of AA (0.8 mM; 0.244 mg/ml) and CLA (0.8 mM; 0.222 mg/ml) to TC containing media. Data were statistically analysed by ANOVA followed by Tukey's multiple comparison (***$p < 0.005$, **$p < 0.01$). Data represent mean values + SD from three individual replicates in technical triplicates.

Figure 7: Antifungal protection by reconstituted bile formulations. RSB = reconstituted synthetic bile (30 mg/ml taurocholate (TC), 30 mg/ml taurodeoxycholate (TDC), 5 mg/ml lecithin, 10 mg/ml myristic acid, 2.5/ml mg arachidonic acid (AA) and 2.5 mg/ml conjugated linolenic acid (CLA)); TDC + TC = 30 mg/ml of each compound; TDC + TC + AA + CLA = 30 mg/ml of each conjugated bile salt with 2.5 mg/ml of each polyunsaturated fatty acid. All

545 solutions were diluted to the respective concentration of solubilised solids as indicated on the
546 x-axis of the respective panels. (**A**) Comparison of *C. albicans* fluconazole (0.5 µg/ml)
547 protection. Bile supports growth at a significantly higher level than RSB and the TDC + TC
548 =AA + CLA mixtures. Only the mixture of TDC + TC does not protect. (**B**) Voriconazole (1
549 µg/ml) protection of *A. terreus*. TDC + TC are not protective. No significant difference in the
550 protection efficacy is observed with the other mixtures compared to bile. (**C**) Comparison of
551 critical micelle concentrations of bile, TDC + TC and TDC +TC + AA +CLA. (**D**) Protection
552 of *C. albicans* against flucytosine (4 µg/ml). No protective effect is observed. (**E**) Rhodamine
553 6G (R6G) influx assay of *C. albicans* cultivated in the presence of 1 µM R6G in RPMI
554 medium supplemented with 1 mg/ml of bile or reconstituted bile formulations. RPMI
555 medium with R6G but without other supplements served as control. All data represent mean
556 values + SD from at least two independent experiments and measured from technical
557 duplicates. Statistical analyses were performed by ANOVA followed by Tukey's multiple
558 comparison (***$p < 0.005$, **$p < 0.01$, NS, = not significant).
559

Figure 1

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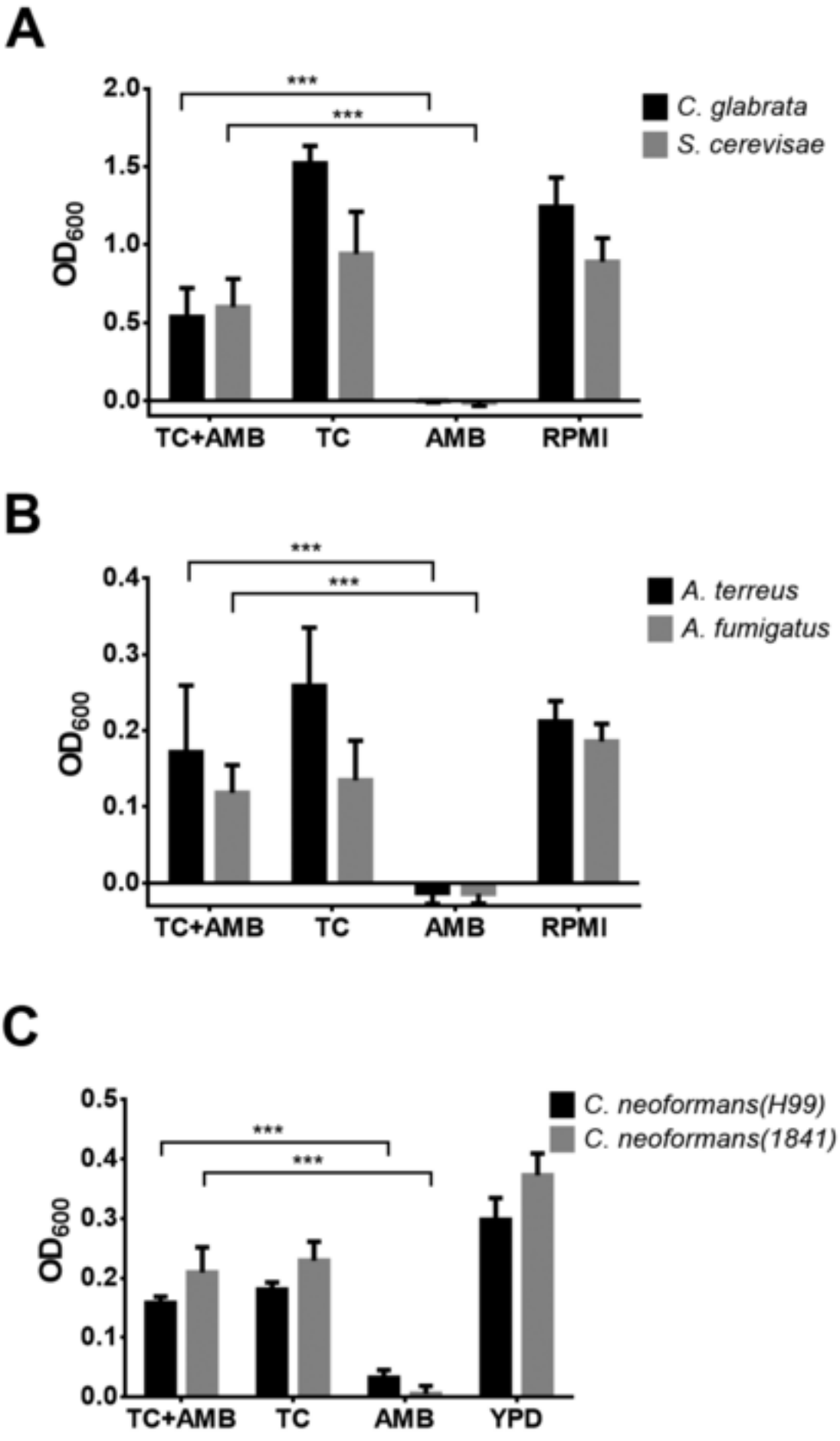

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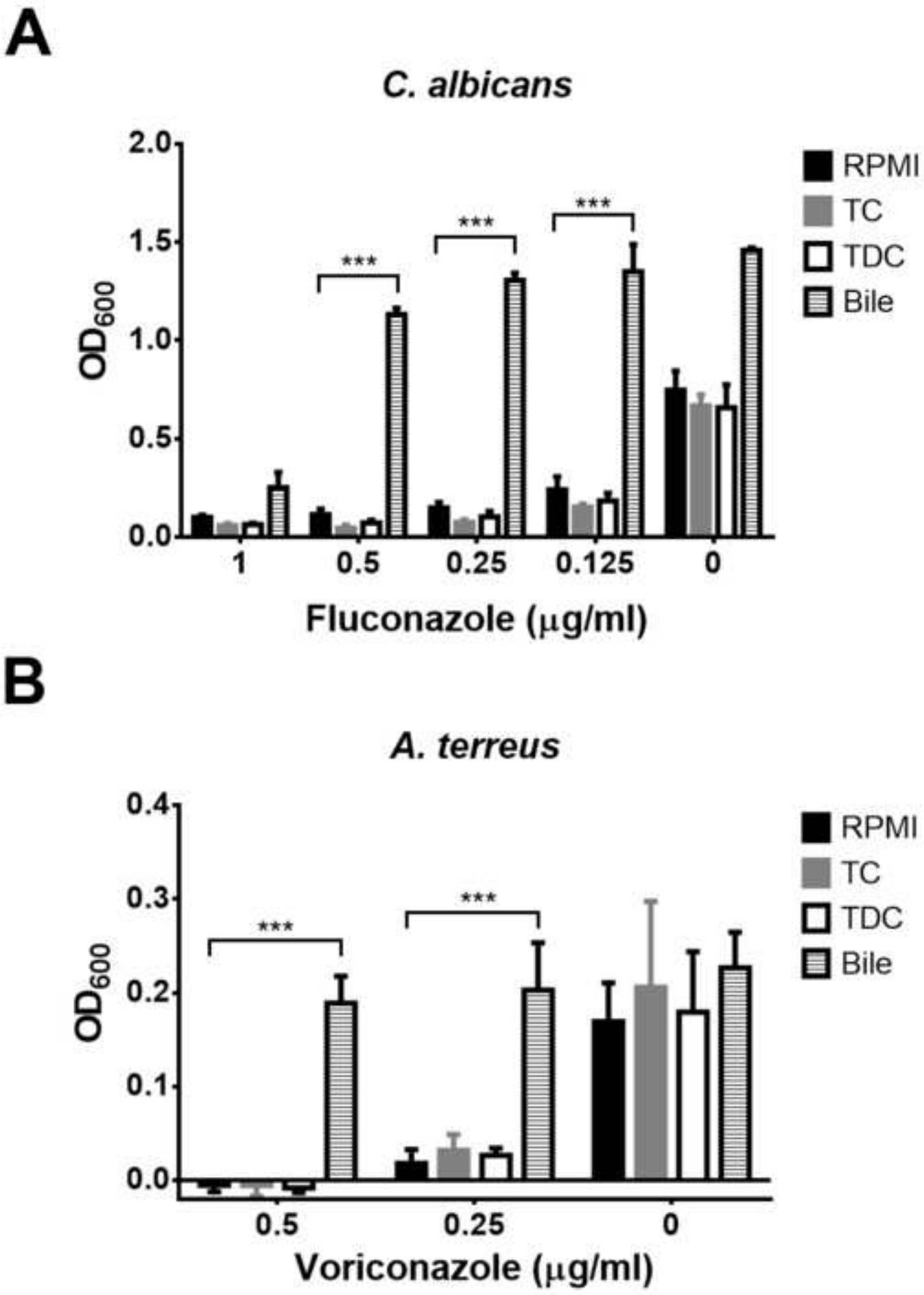

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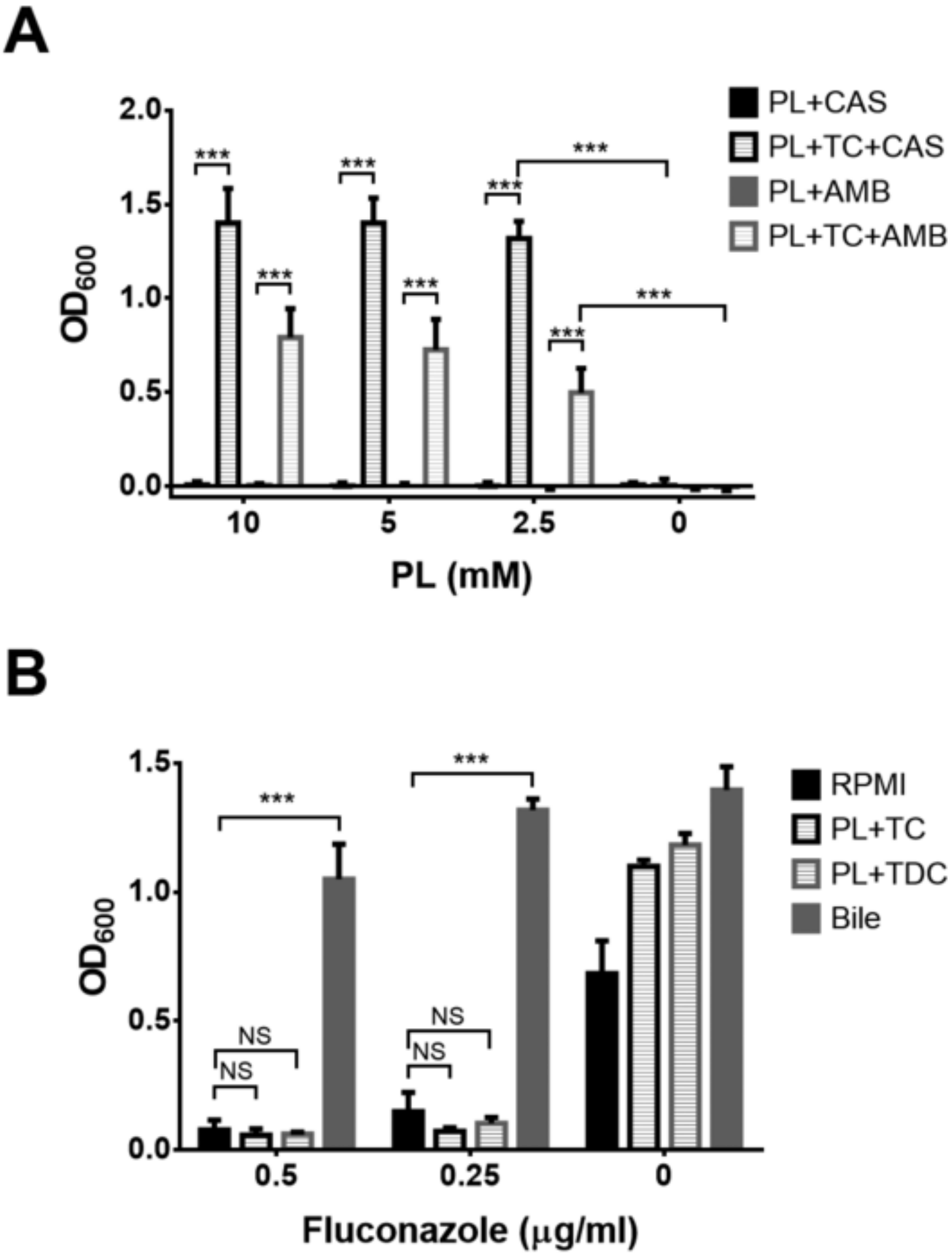

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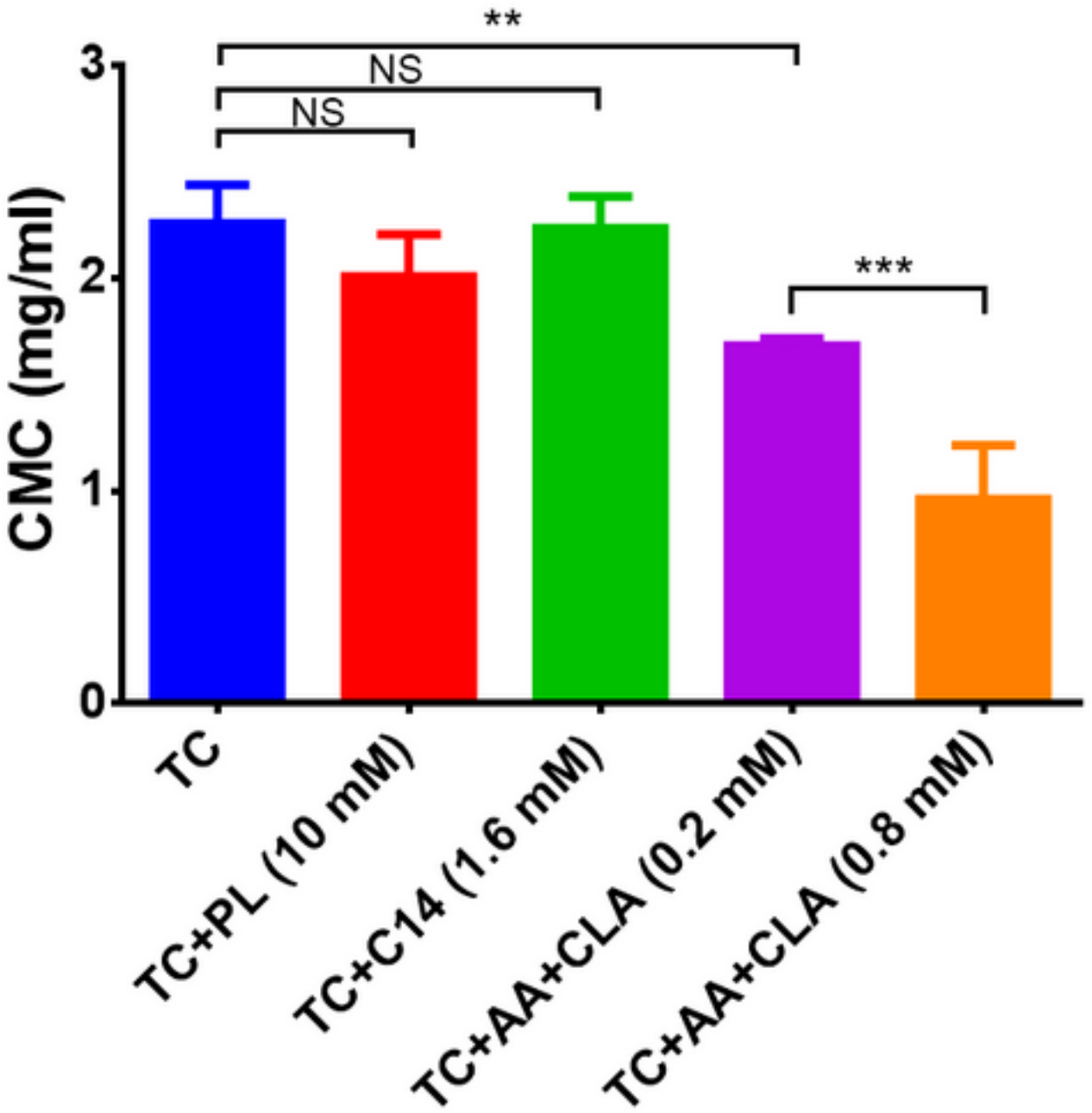

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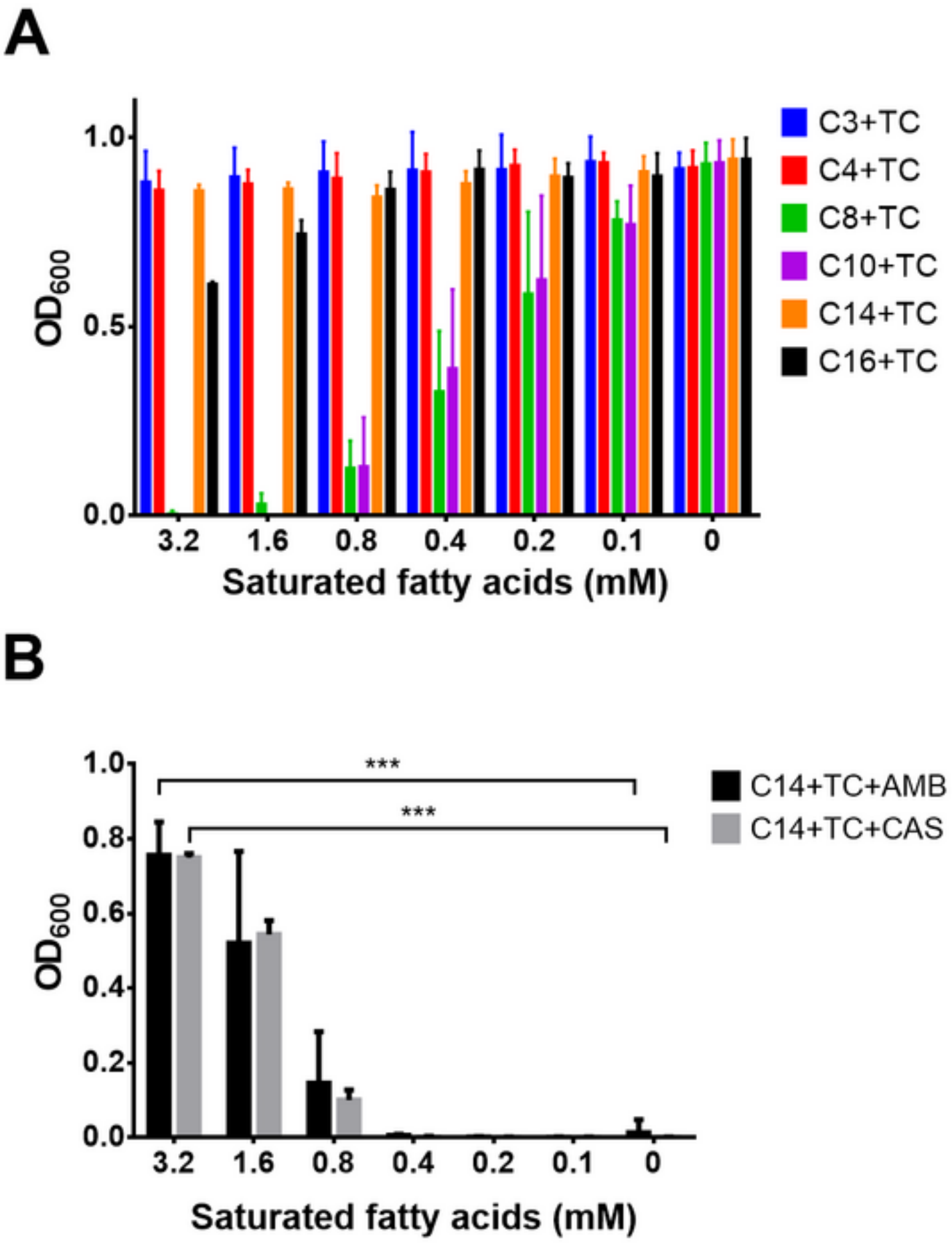

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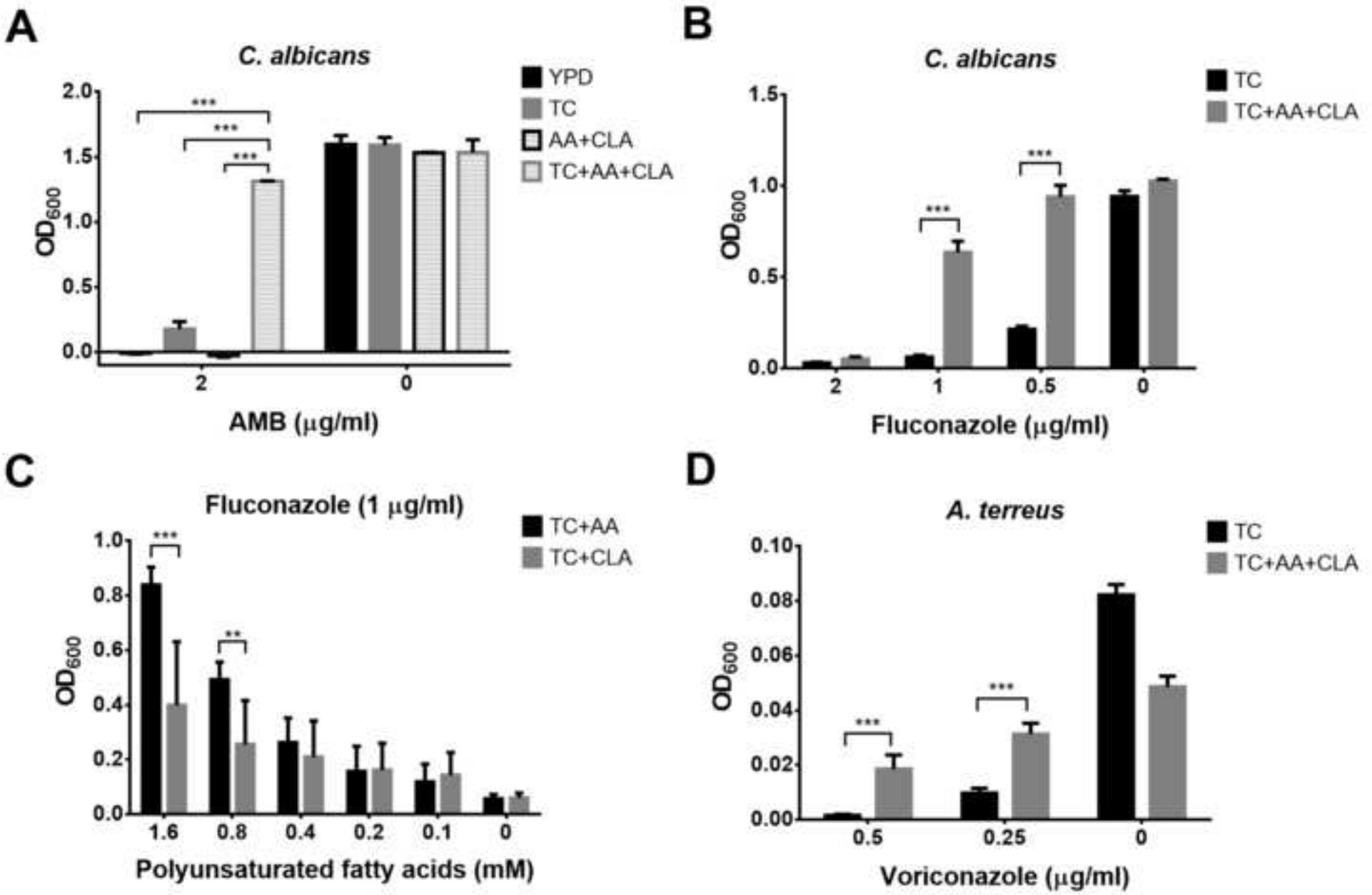

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