

## 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

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

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16 **Running title:** Mixed micelles in antifungal protection

17

18 **Key words:** Taurodeoxycholate; polyunsaturated fatty acids; arachidonic acid; azoles;

19 *Candida albicans*; *Aspergillus terreus*

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21

22 **Abstract**

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

41

## 42        **1. Introduction**

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

54    In a murine model of disseminated candidiasis we have previously shown that *Candida*  
55    *albicans* uses the gall bladder as a cryptic reservoir under antifungal therapy (Jacobsen et al.,  
56    2014). Subsequent investigations revealed that bile conferred resistance against commonly  
57    used antifungals such as the echinocandin caspofungin and the polyene macrolide  
58    amphotericin B (Jacobsen et al., 2014). As underlying mechanism of protection we found that  
59    antifungals are trapped in micelles that are formed by conjugated bile salts (Hsieh et al.,  
60    2017). The resulting reduction in the bioavailability of drugs in the biliary system and  
61    possibly also in the intestine might thereby not only prevent the clearance of pathogens from  
62    these host niches, but might also lead to the development of drug resistant strains.  
63    Accordingly, a recent study revealed the emergence of echinocandin resistant *Candida*  
64    species in liver transplant recipients after treatment with caspofungin (Prigent et al., 2017).  
65    Resistant strains were mainly isolated from the digestive system implying that a reduced  
66    bioavailability of drugs might have caused this emergence (Prigent et al., 2017). Furthermore,

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

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

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

90

91        **2. Material and Methods**

92        **2.1 Strains and culture conditions**

93        *C. albicans* (SC5314), *S. cerevisiae* (ATCC 9763) and *C. neoformans* (H99 and 1841) were  
94        pre-cultivated overnight in 20 ml YPD media (per litre: 10 g yeast extract, 20 g peptone, and  
95        20 g glucose) at 30°C and yeast cells were harvested by centrifugation at 4000 × g. After  
96        washing cells twice in phosphate-buffered saline (PBS) cells were suspended and adjusted to  
97        selected cell densities in either YPD or MOPS-buffered RPMI 1640 medium (Sigma)  
98        containing 2% glucose (per litre: 10.4 g RPMI 1640, 34.53 g MOPS, 20 g glucose; pH 6.8;  
99        subsequently defined as RPMI medium). To obtain conidia suspension of *A. fumigatus*  
100        (CBS144-89) and *A. terreus* (SBUG844), conidia were plated on 50 mM glucose *Aspergillus*  
101        minimal media with nitrate as nitrogen source and 2% agar (Gressler et al., 2011). Plates  
102        were incubated at 37°C for 3-4 days (*A. fumigatus*) or 5-6 days (*A. terreus*). Conidia were  
103        harvested in 10 ml sterile PBS and filtered through a 40 µm cell-strainer. After a washing step  
104        in PBS, conidia were diluted to defined concentrations in RPMI medium and used for drug  
105        resistance analyses.

106

107        **2.2 Preparation of antifungals for sensitivity analyses**

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

114

115        **2.3 Preparation of bile solution and lipid solubilisation in conjugated bile salt**

116 Crude porcine bile extract (Sigma, B8631) was solved in either YPD or RPMI medium to  
117 give a final concentration of 12.5% (w/v). Insoluble components were removed by  
118 centrifugation at  $12000 \times g$ . The supernatant was filter sterilised and stored at  $4^{\circ}\text{C}$  in the dark.  
119 Sodium taurodeoxycholate hydrate (Sigma, T0875) and taurocholic acid sodium salt hydrate  
120 (Sigma, T4409) were dissolved at 100 mg/ml in either YPD or RPMI medium, filtered  
121 sterilised and used as stock solutions. The following saturated fatty acids were used for  
122 preparing mixtures with conjugated bile salt: C3 (propionic acid sodium salts, Applichem,  
123 A1931), C4 (sodium butyrate, Aldrich, 303410), C8 (sodium caprylate, Fluka, 71339), C10  
124 (sodium decanoate Fluka, 21490), C14 (myristic acid sodium salt, Fluka, 70140) and C16  
125 (sodium palmitate, Fluka, 76165). Media containing 10 mg/ml taurocholate were  
126 supplemented with the indicated amounts of fatty acids. To solubilise C14 and C16, mixtures  
127 were heated to  $65^{\circ}\text{C}$  for 30-60 min. As a source of phospholipids a soy refined lecithin (Mp  
128 Biomedicals, LLC) was added at indicated concentrations. As sources of polyunsaturated  
129 fatty acids arachidonic acid (Sigma, 10931) and conjugated linolenic acid (Sigma, O5507)  
130 were used that were added to YPD or RPMI media containing 10 mg/ml sodium taurocholate.  
131 For preparation of reconstituted synthetic bile (RSB) the following components were mixed  
132 (per ml of RPMI medium): 30 mg sodium taurocholate, 30 mg sodium taurodeoxycholate, 5  
133 mg lecithin, 10 mg C14, 2.5 mg arachidonic acid and 2.5 mg conjugated linolenic acid  
134 generating an 80 mg/ml RSB stock. Stocks of an equal mixture of tauro- and  
135 taurodeoxycholate (60 mg/ml in total) or RSB lacking phospholipids and saturated fatty acids  
136 (65 mg/ml solubilised solids) were also prepared.

137

## 138 **2.4 Drug resistance analyses**

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

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

153

## 154 **2.5 Determination of critical micelle concentrations (CMC) and rhodamine 6G influx** 155 **analyses**

156 Critical micelle concentration (CMC) was determined as described previously (Hsieh et al.,  
157 2017) by measuring the change of absorbance of eosin Y at 542 nm using a microplate reader  
158 (Multiskan™ Go, Thermo). Bile, conjugated bile salts and mixtures of conjugated bile salts  
159 containing different lipid compositions were tested in serial dilutions and data were analysed  
160 using Microsoft Excel software by plotting the absorbance values against the logarithmic  
161 concentration of the solubilising agent. For rhodamine 6G (R6G) influx assays *C. albicans*  
162 yeast cells were incubated at 30°C in RPMI medium in the presence of 1  $\mu$ M R6G and 1  
163 mg/ml of either bile, conjugated bile salts or mixtures of conjugated bile salts and lipids.  
164 Aliquots of cells were harvested at different time points and washed twice in 50 mM HEPES  
165 buffer pH 7.0. R6G fluorescence was measured in a microplate reader (Fluostar Omega plate



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

168

## 169 **2.6 Statistical analyses**

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

175

## 176 **3. Results**

### 177 **3.1 Taurocholate confers amphotericin B resistance in a variety of fungal species**

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

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

193

### 194 **3.2 Bile, but not its conjugated bile salts protect from azoles**

195 Next, we investigated the efficacy of bile and conjugated bile salts to protect from azoles.

196 While a first-line treatment of *C. albicans* infections recommends the use of echinocandins,

197 fluconazole is still used in clinically stable patients with no previous exposure to azoles

198 (Calandra et al., 2016). Voriconazole is one of the azoles of choice in prophylactic and acute

199 therapy of *A. terreus* infections (Karthaus, 2011; Vehreschild et al., 2007). The use of azoles

200 in the treatment of *A. terreus* infections is of special importance since most *A. terreus* strains

201 exhibit a natural resistance against amphotericin B ( $MIC \geq 1$  mg/l), although the molecular

202 basis for this is not yet well understood (Pastor and Guarro, 2014). Growth of *C. albicans*

203 was inhibited at 0.125  $\mu$ g/ml fluconazole and *A. terreus* was sensitive against voriconazole at

204 a concentration of 0.25  $\mu$ g/ml. Both, *C. albicans* and *A. terreus* tolerated bile,

205 taurodeoxycholate and taurocholate (**Fig. 2**), but only bile conferred resistance against

206 fluconazole for *C. albicans* and voriconazole in case of *A. terreus*. Taurocholate and

207 taurodeoxycholate did not show a protective effect (**Fig. 2**). Therefore, protection against

208 azoles appears specifically mediated by bile and - although a larger number of species and

209 azoles need to be tested - this protection seems largely independent from the type of azole

210 and the fungal species.

211

### 212 **3.3 Phospholipids in conjugated bile salt-mediated antifungal protection**

213 Lecithin (diacylphosphatidylcholine) is the major phospholipid in bile (Hay and Carey,

214 1990). In a previous study we showed that an ethylacetate fraction of bile, mainly containing

215 the lipid fraction of bile was by itself not sufficient for antifungal protection (Hsieh et al.,

216 2017). However, since phospholipids consist of a hydrophilic, zwitterionic phosphocholine  
217 head group and hydrophobic tails from the long fatty acyl chains (Hay and Carey, 1990), the  
218 phospholipid lecithin is likely to form mixed micelles with conjugated bile salts, which may  
219 increase the potency to encapsulate drugs. To test this assumption, we first analysed the effect  
220 of various lecithin concentrations on enhancement of the protective effect of taurocholate  
221 against caspofungin and amphotericin B (**Fig. 3A**). Here, we used a sub-protective  
222 concentration of 10 mg/ml of taurocholate at which *C. albicans* was sensitive against 2 µg/ml  
223 caspofungin and 4 µg/ml amphotericin B. Similarly, the phospholipid lecithin alone, in a  
224 concentration of up to 10 mM, was not protective. However, 2.5 mM of lecithin enhanced the  
225 protective effect of taurocholate and mediated caspofungin and amphotericin B resistance  
226 (**Fig. 3A**). Subsequently, the addition of phospholipids on fluconazole protection was  
227 analysed, whereby the protective effect with either taurocholate, taurodeoxycholate or bile  
228 was tested (**Fig. 3B**). Similar to bile, lecithin inhibited the hyphae inducing effect of  
229 taurocholate and taurodeoxycholate in RPMI medium (Hsieh et al., 2017). While this resulted  
230 in higher growth rates in the absence of drugs, up to 25 mM lecithin did not confer resistance  
231 against the azole. When we analysed the effect of the phospholipid lecithin on the critical  
232 concentration of taurocholate required to form micelles, the CMC value of taurocholate in the  
233 presence of lecithin did not significantly decrease in comparison to taurocholate alone (**Fig.**  
234 **4**). Therefore, we conclude that phospholipids are not facilitating micelle formation, but can  
235 enhance the protective effect of conjugated bile salts by forming mixed micelles. However,  
236 this combination does not protect from azoles and is not sufficient to resemble the protective  
237 effect of bile.

238

### 239 **3.4 Role of saturated fatty acids in antifungal protection**

240 The major proportion of free fatty acids in bile consists of saturated fatty acids (Chatterjee et  
241 al., 2007). Therefore, we investigated a possible contribution of saturated fatty acids towards  
242 antifungal protection. First, we studied growth of *C. albicans* in the presence of varying  
243 concentrations (range 0.1 – 3.2 mM) of saturated fatty acids of different chain length by  
244 simultaneously applying a fixed sub-protective concentration of 10 mg/ml taurocholate. As  
245 shown previously (Otzen et al., 2013), octanoic (C8) and decanoic (C10) acid exhibited a  
246 toxic effect towards *C. albicans*, which was not relieved by the presence of taurocholate.  
247 Propionate (C3) and butyrate (C4) were well tolerated in the concentrations applied, but due  
248 to their high water solubility are unlikely to contribute to micelle formation. No growth  
249 defect was observed by the addition of myristic (C14) and palmitic (C16) acid (**Fig 5A**).  
250 However, despite the presence of 10 mg/ml taurocholate, palmitic acid was not well  
251 solubilised and produced solid particles in the growth media at 37°C. Therefore, we  
252 investigated antifungal protection by the combination of myristic acid with taurocholate (**Fig.**  
253 **5B**). Indeed, when myristic acid exceeded a concentration of 0.8 mM (> 0.18 mg/ml) this  
254 mixture conferred resistance against caspofungin and amphotericin B. However, as with  
255 phospholipids, myristic acid did not confer resistance against fluconazole (not shown).  
256 Furthermore, similar to lecithin, a determination of the CMC value of taurocholate revealed  
257 that myristic acid did not significantly reduce the concentration of taurocholate required to  
258 form micelles (**Fig. 4**). Therefore, similar to phospholipids, saturated fatty acids reduce the  
259 concentration of taurocholate required to confer resistance against amphotericin B and  
260 caspofungin, but are not sufficient for protecting against azoles.

261

### 262 **3.5 Unsaturated fatty acids in antifungal protection**

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

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

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

297

### 298 **3.6 Protective effect of reconstituted synthetic bile**

299 Since all, phospholipids, saturated and unsaturated fatty acids showed some contribution in  
300 enhancement of antifungal protection by conjugated bile salts, we aimed in the reconstitution  
301 of a synthetic bile consisting of a mixture of taurocholate and taurodeoxycholate (30 mg/ml  
302 each), lecithin (5 mg/ml), myristic acid (10 mg/ml), arachidonic acid (2.5 mg/ml) and  
303 linolenic acid (2.5 mg/ml), resulting in a total of 80 mg solubilised solids in 1 ml of water.  
304 This mixture was compared to the protective effect of a mixture of taurocholate and  
305 taurodeoxycholate (30 mg/ml each) containing 2.5 mg/ml of each of the polyunsaturated fatty  
306 acids arachidonic and linolenic acid, resulting in a total of 65 mg/ml solubilised solids. These  
307 mixtures were compared with bile and a mixture of taurocholate and taurodeoxycholate for  
308 their concentration dependent protective effect against azoles and the highly water soluble  
309 antimetabolite flucytosine. Mixtures were adjusted in media to give a final concentration of  
310 10, 5, 2.5 and 0 mg/ml of total solubilised solids and 0.5 µg/ml of fluconazole was added to  
311 test resistance of *C. albicans*. While bile produced the highest cell density after 24 h of  
312 incubation, the complete mixture of all types of fatty acids with conjugated bile salts  
313 (reconstituted synthetic bile, RSB) as well as the polyunsaturated fatty acids in combination  
314 with conjugated bile salts were protective, but with slightly higher efficacy of the latter

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

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

337

#### 338 4. Discussion

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

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

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



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

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

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

382

## 383 **5. Acknowledgements**

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388

389        **6. References**

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466 placebo during induction chemotherapy for acute myelogenous leukaemia (AML). *J*  
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468

469

470 **Figure legends**

471

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

484

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

493

494 **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<sub>600</sub>) 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

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

525

526 **Figure 6: Contribution of polyunsaturated fatty acids on antifungal protection of**  
527 **conjugated bile salts.** Analyses on *C. albicans* were performed in YPD medium. Resistance  
528 of *A. terreus* was tested in RPMI medium. Taurocholate (TC) was used in a concentration of  
529 10 mg/ml. (A) Sensitivity of *C. albicans* against amphotericin B (AMB; 2 µg/ml).  
530 Arachidonic acid (AA; 0.2 mM; 0.06 mg/ml) and conjugated linolenic acid (CLA; 0.2 mM;  
531 0.056 mg/ml) were added in equal concentration. (B) Fluconazole protection of *C. albicans*  
532 by addition of equal amounts of AA (0.8 mM; 0.244 mg/ml) and CLA (0.8 mM; 0.222 mg/ml)  
533 to TC containing media. (C) Protection of *C. albicans* against fluconazole (1 µg/ml) by  
534 addition of different concentrations of either AA or CLA. (D). Voriconazole protection of *A.*  
535 *terreus* by addition of equal amounts of AA (0.8 mM; 0.244 mg/ml) and CLA (0.8 mM; 0.222  
536 mg/ml) to TC containing media. Data were statistically analysed by ANOVA followed by  
537 Tukey's multiple comparison (\*\*p < 0.005, \*p < 0.01). Data represent mean values + SD  
538 from three individual replicates in technical triplicates.

539

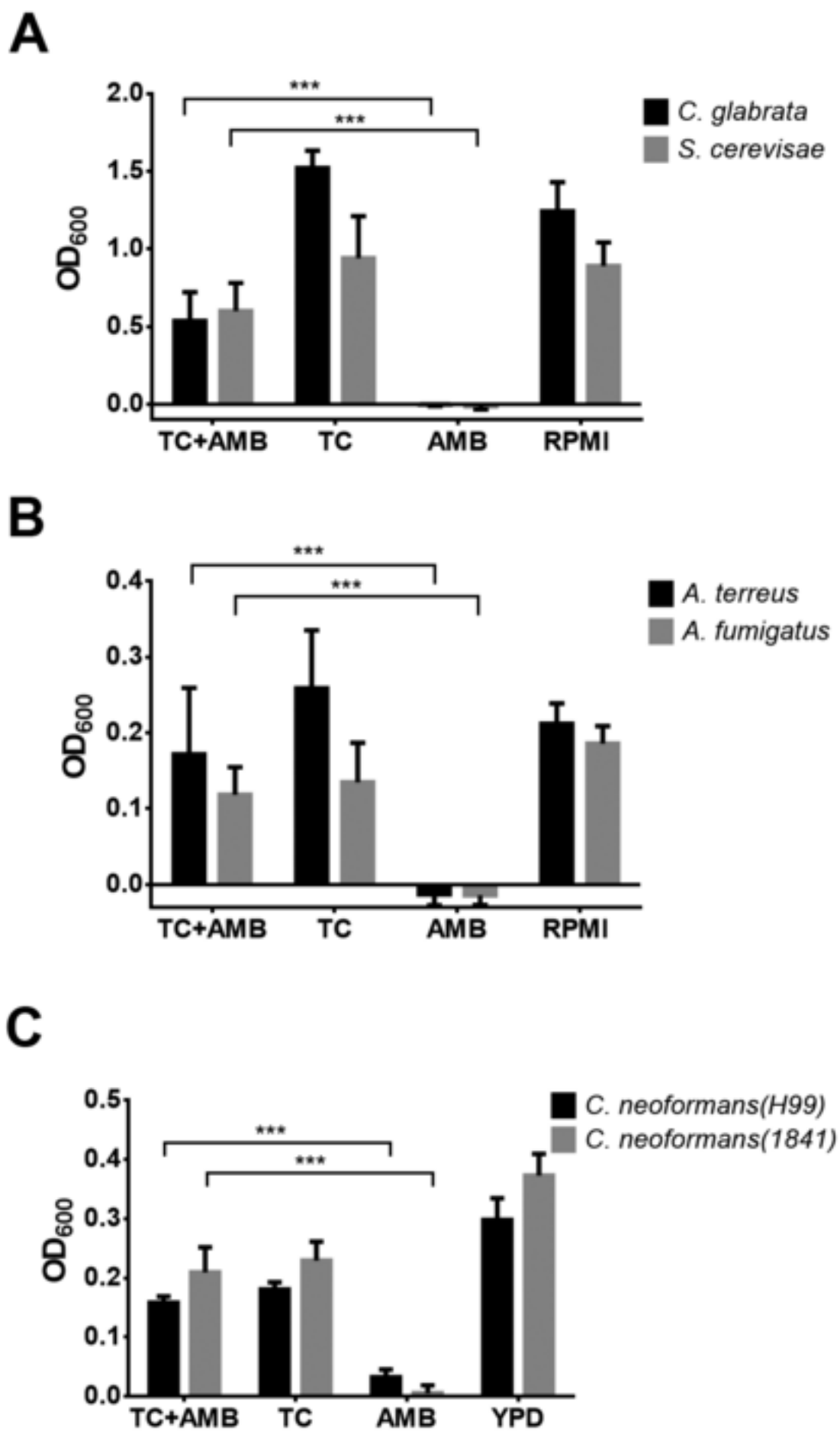
540 **Figure 7: Antifungal protection by reconstituted bile formulations.** RSB = reconstituted  
541 synthetic bile ( 30 mg/ml taurocholate (TC), 30 mg/ml taurodeoxycholate (TDC), 5 mg/ml  
542 lecithin, 10 mg/ml myristic acid, 2.5/ml mg arachidonic acid (AA) and 2.5 mg/ml conjugated  
543 linolenic acid (CLA)); TDC + TC = 30 mg/ml of each compound; TDC + TC + AA + CLA =  
544 30 mg/ml of each conjugated bile salt with 2.5 mg/ml of each polyunsaturated fatty acid. All



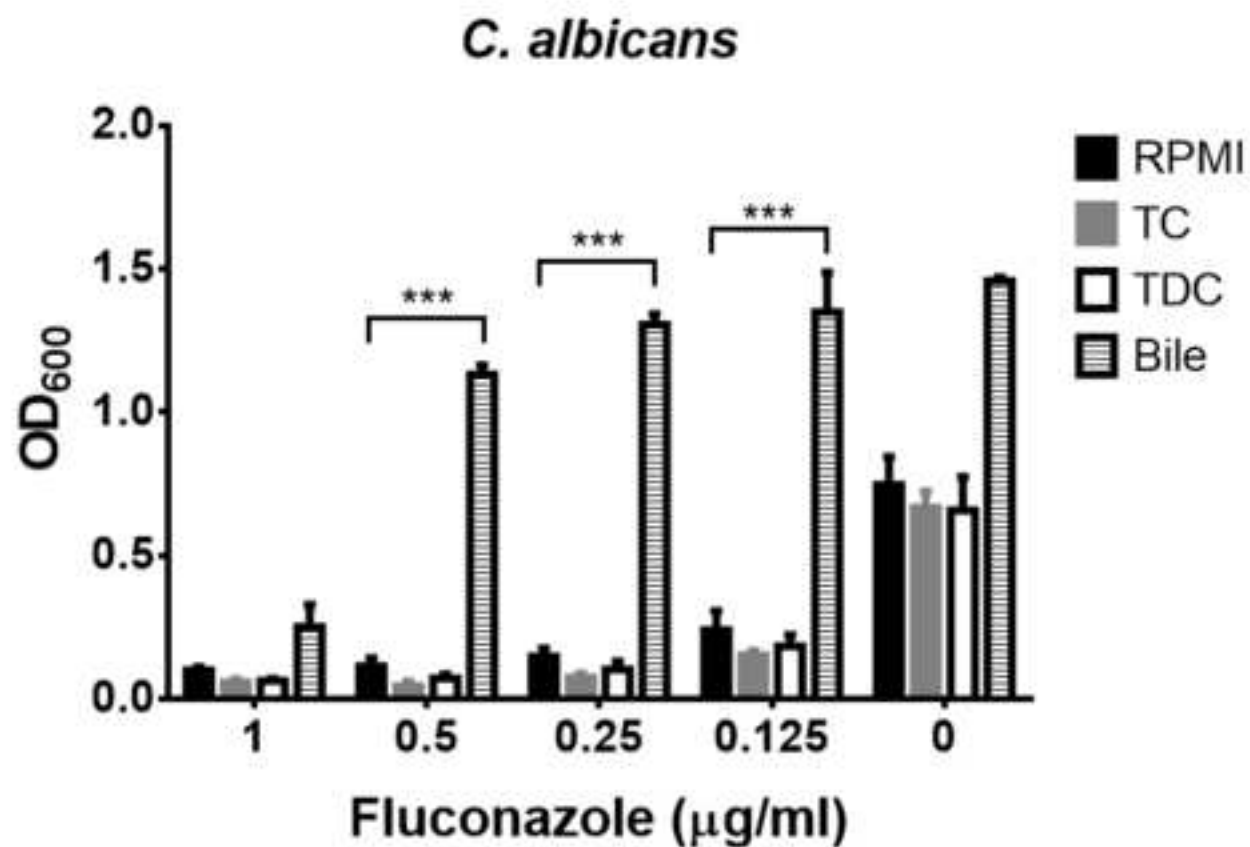
545 solutions were diluted to the respective concentration of solubilised solids as indicated on the  
546 x-axis of the respective panels. **(A)** Comparison of *C. albicans* fluconazole (0.5 µg/ml)  
547 protection. Bile supports growth at a significantly higher level than RSB and the TDC + TC  
548 =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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**A**



**B**

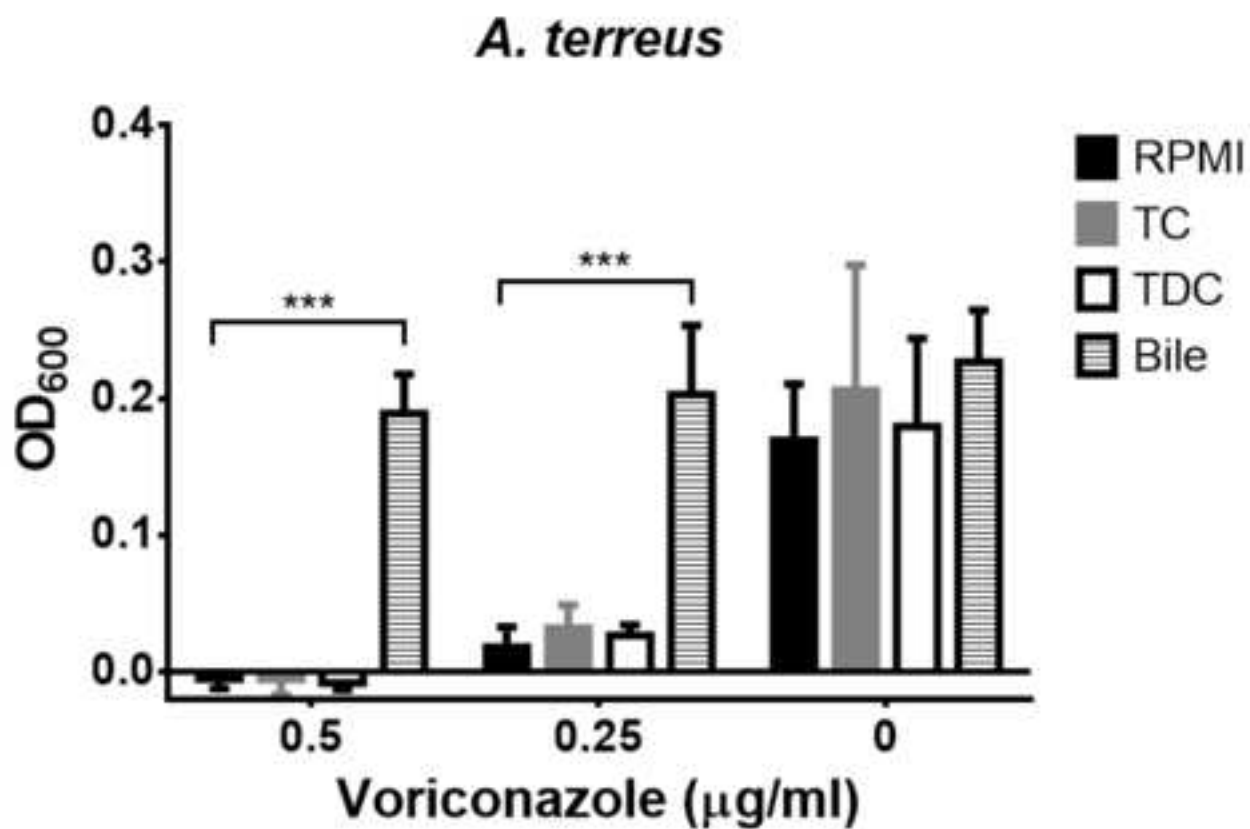
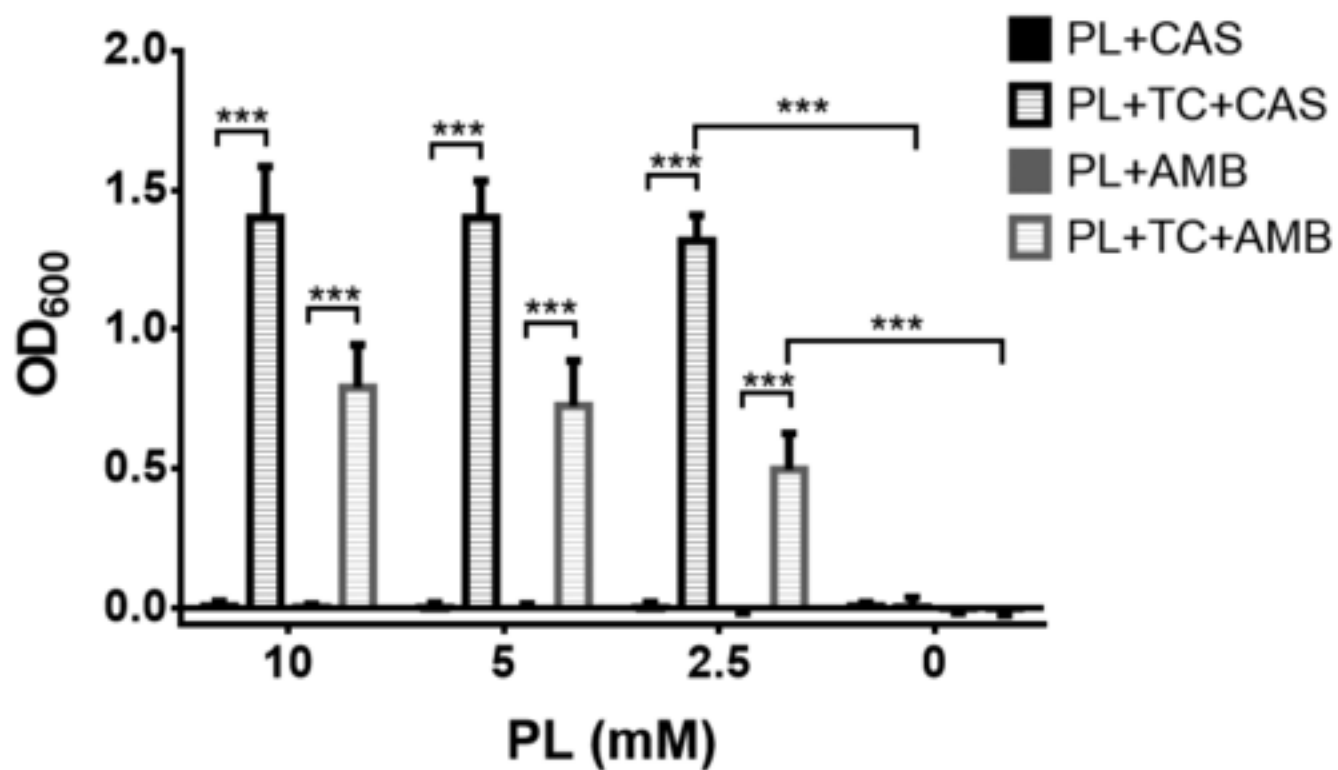


Figure 3  
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**A**



**B**

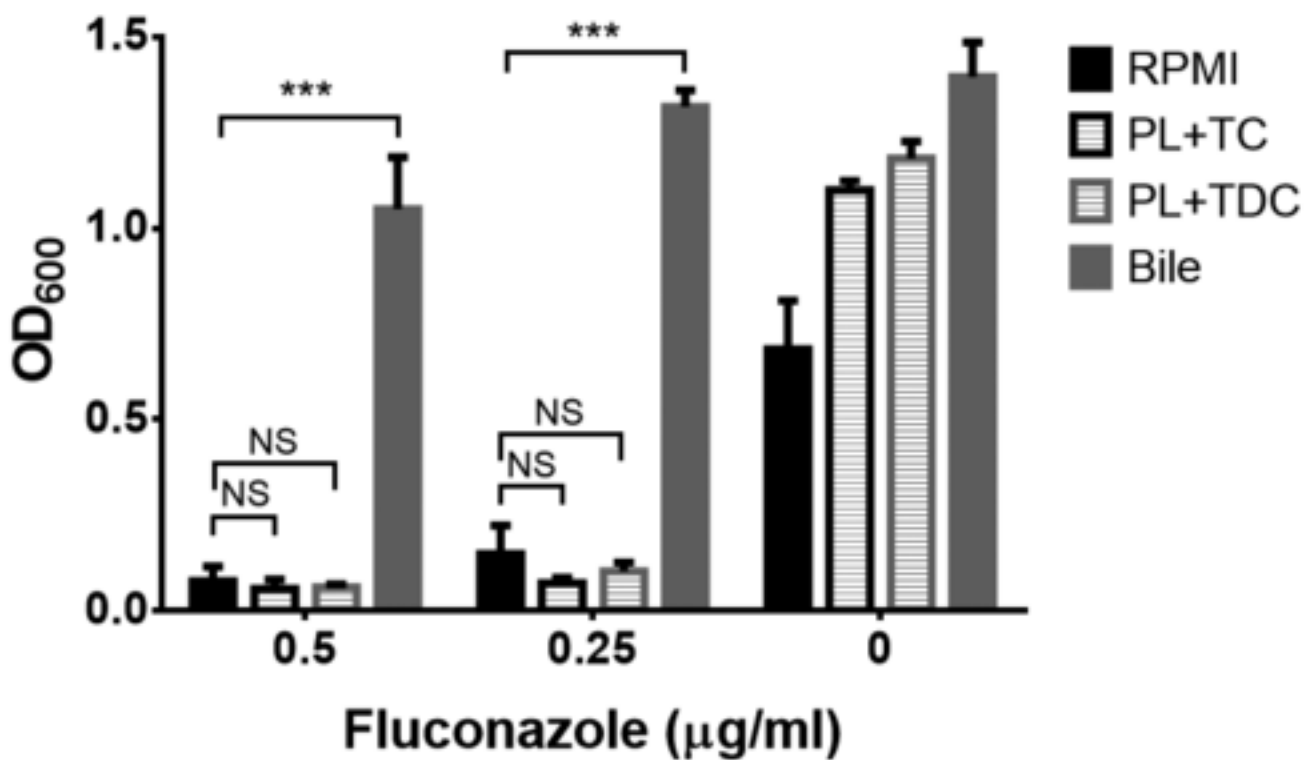


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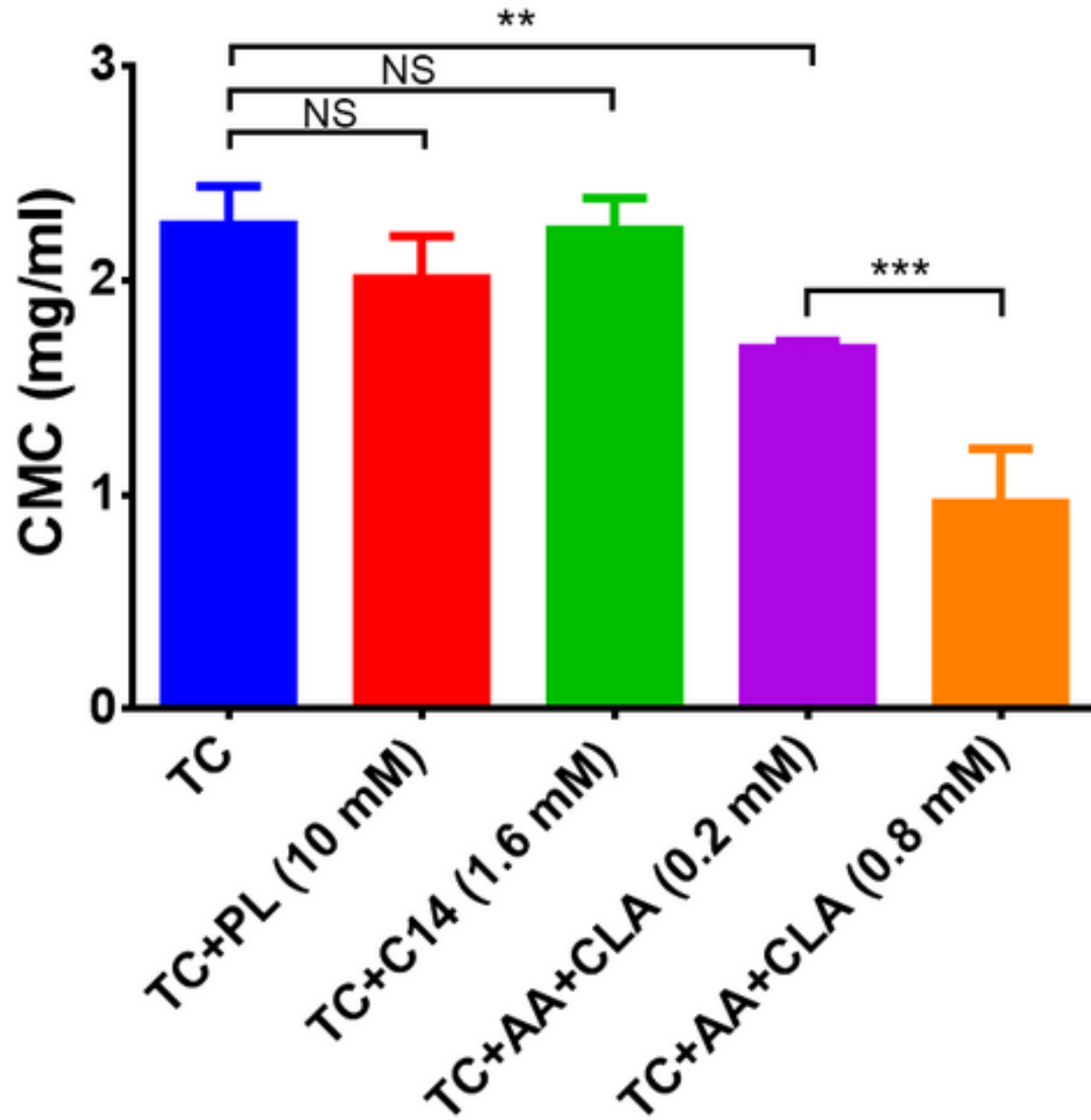
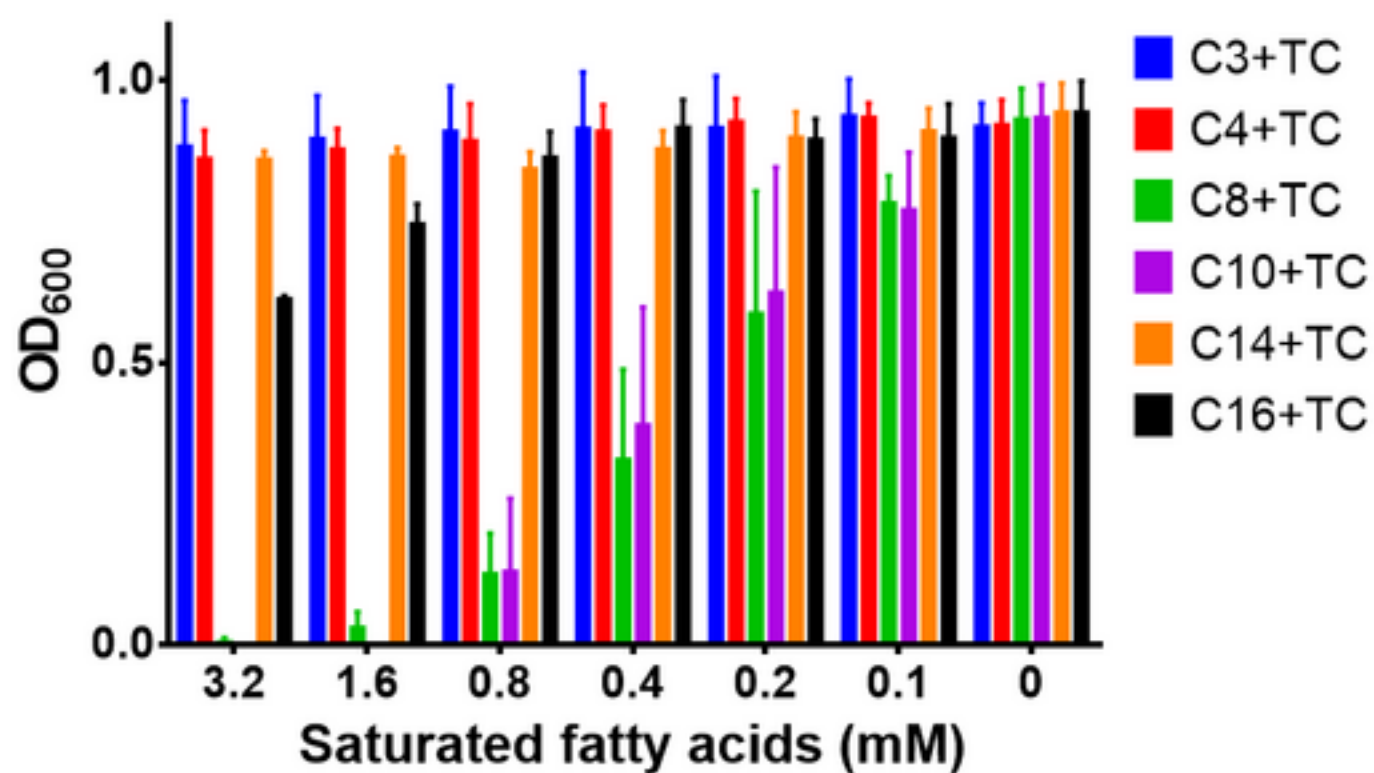


Figure 5  
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**A**



**B**

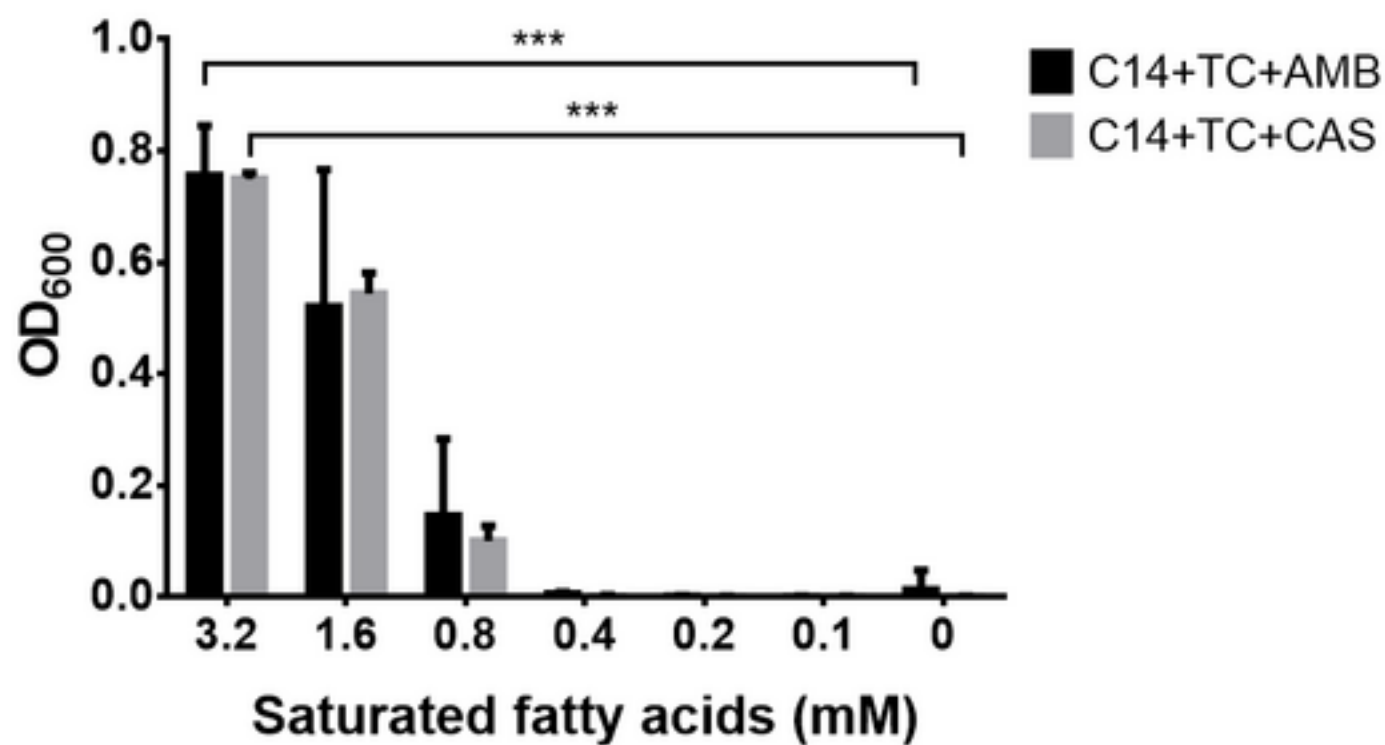


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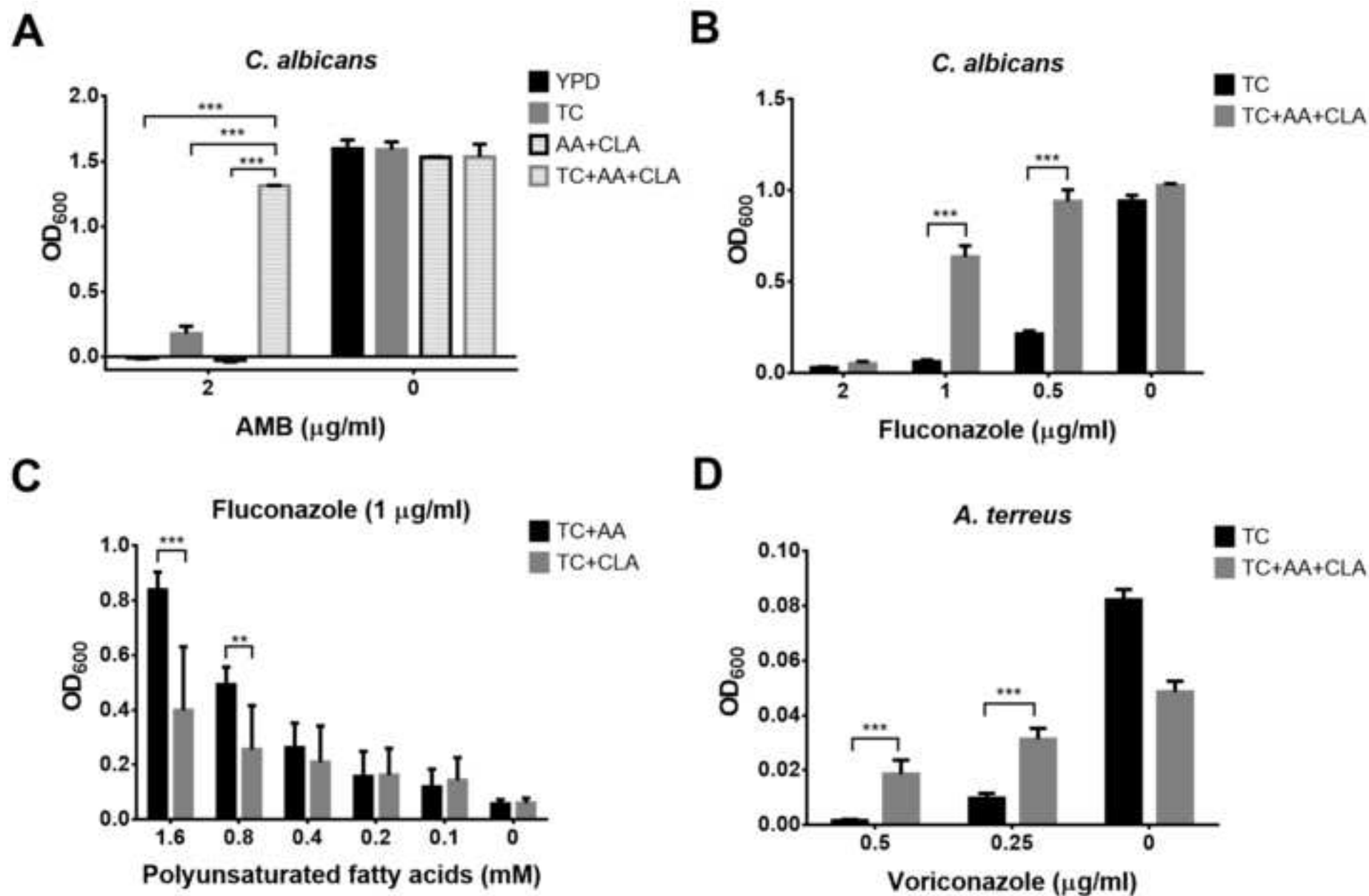


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