



Disparate *Candida albicans* Biofilm Formation in Clinical Lipid Emulsions Due to Capric Acid-Mediated Inhibition

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ABSTRACT Receipt of parenteral nutrition (PN) remains an independent risk factor for developing catheter-related bloodstream infections (CR-BSI) caused by fungi, including by the polymorphic fungus *Candida albicans*, which is notoriously adept at forming drug-resistant biofilm structures. Among a variety of macronutrients, PN solutions contain lipid emulsions to supply daily essential fats and are often delivered via central venous catheters (CVCs). Therefore, using an *in vitro* biofilm model system, we sought to determine whether various clinical lipid emulsions differentially impacted biofilm growth in *C. albicans*. We observed that the lipid emulsions Intralipid and Omegaven both stimulated *C. albicans* biofilm formation during growth in minimal medium or a macronutrient PN solution. Conversely, Smoflipid inhibited *C. albicans* biofilm formation by approximately 50%. Follow-up studies revealed that while Smoflipid did not impair *C. albicans* growth, it did significantly inhibit hypha formation and hyphal elongation. Moreover, growth inhibition could be recapitulated in Intralipid when supplemented with capric acid—a fatty acid present in Smoflipid but absent in Intralipid. Capric acid was also found to dose dependently inhibit *C. albicans* biofilm formation in PN solutions. This is the first study to directly compare different clinical lipid emulsions for their capacity to affect *C. albicans* biofilm growth. Results derived from this study necessitate further research regarding different lipid emulsions and rates of fungus-associated CR-BSIs.

KEYWORDS biofilm, parenteral nutrition, infection, *Candida albicans*, catheter, lipid emulsions, *Candida*, fungi, infection control, lipids

Some patients (e.g., preterm neonates, patients with bowel resection, and the critically ill) are dependent on parenteral nutrition (PN) for essential daily caloric needs (1, 2). PN solutions contain electrolytes, micronutrients, and the macronutrients dextrose, amino acids, and lipid emulsions. A central venous catheter (CVC) is typically used for administration due to the high risk of phlebitis associated with the highly osmolality in PN solutions and/or the need for a venous access point for long-term (e.g., over 30 days) PN administration (3). Catheter-related bloodstream infections (CR-BSIs) are a serious complication of CVCs used for the administration of PN and are considered a health hazard (4). Estimates from 2009 suggest that there are approximately 23,000 CR-BSIs among patients in inpatient wards (5). Several clinical and meta-analyses estimate that CR-BSI develops in patients receiving PN at rates ranging from 0.5 to 10 infections per 1,000 catheter days (6–9). Despite recent decreases in CR-BSIs due to various prevention measures, receipt of PN remains an independent risk factor for a CR-BSI in pediatric, adult, intensive care unit, and home infusion therapy patients (10–12). It is unclear which component of PN increases the infectious risk, but macronutrient composition is likely an important factor, as increased parenteral calories can

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increase the risk of CR-BSIs while receiving PN (13). While risk for CR-BSIs caused by any pathogen is elevated while on PN, multiple clinical guidelines illustrate a link between PN administration and *Candida* infections in particular (14, 15).

Candida albicans is a cause of CR-BSIs associated with high morbidity (e.g., central line replacement) and mortality, as fungus-related sepsis has one of the highest fatality rates (up to 45% in some populations) compared to those of other pathogens (16–19). *C. albicans* is able to morphogenetically switch between a yeast and a filamentous hyphal form, an attribute key to its pathogenicity. The yeast form is important for dissemination, and the hyphal form crucial for tissue invasion and biofilm formation (20, 21). Biofilms are highly structured communities of cells that adhere to biotic and abiotic surfaces, including implanted medical devices like CVCs (22, 23). *C. albicans* biofilms typically form as initially adherent yeasts begin to proliferate, establishing a basal layer of attached cells. After early adherence, this complex layer of yeast cells is followed by robust hyphal extension that constructs a dense cellular meshwork. As the biofilm matures, it secretes a polysaccharide-rich extracellular matrix. The mature biofilm can then disperse yeast cells to potentially seed other sites (24). *C. albicans* biofilms exhibit resistance to antifungals at concentrations that are otherwise effective against planktonic *C. albicans* (25). Failure to achieve device sterilization by standard antifungal therapy often necessitates device removal. However, in the case of patients receiving long-term PN and young neonates/children, vascular sites for catheter reinsertion may be limited. Prevention of catheter-related infections and avoidance of catheter removal is a common goal for patients requiring PN.

It is debated which macronutrient in PN solutions increases the chance of fungal infections, although *in vitro* models suggest that biofilm formation may be independently increased with the addition of lipids (26–28). The main goal of lipid therapy in PN is to prevent essential fatty acid deficiency, and Intralipid (based widely on ω -6 fatty acids extracted from soybean oil) has been the most widely used lipid emulsion for the past 20 years (29). Long-term Intralipid administration can result in an altered ω -6/ ω -3 fatty acid ratio and is linked to morbidities such as cholestasis (30). In order to overcome the risks of prolonged lipid administration and reduce bowel inflammation associated with an abnormal ω -6/ ω -3 serum ratio, newer lipid emulsions, such as Smoflipid and Omegaven, have been developed (29). Smoflipid is composed of roughly equivalent levels of soybean oil (ω -6), medium-chain triglycerides (e.g., coconut oil), olive oil (ω -9), and fish oil (ω -3) and approximately resembles the lipid profile common to breastmilk (31–34). Omegaven is composed mainly of ω -3 fatty acids derived from fish oil (29).

There is limited information available as to whether newer lipid emulsions may affect CR-BSIs or impact the biology of *C. albicans*. Therefore, in this study we aimed to determine whether *C. albicans* biofilm formation was significantly altered by cultivation in PN macronutrient solutions containing Intralipid, Smoflipid, or Omegaven. In addition, we further define the role of individual fatty acids present in newer lipid emulsions in *Candida* biomass and biofilm formation.

RESULTS

Determination of minimal medium to support cell growth but not impact biofilm formation. TrophAmine (a mix of essential and nonessential amino acids used in pediatric patients) and dextrose are common components of PN formulations. Therefore, we first wanted to assess which concentrations would be able to support moderate *C. albicans* growth so that lipid supplementation may show a demonstrable effect. Biofilms were grown for 24 h with serial dilutions of 2% TrophAmine and 17.5% dextrose in $1\times$ yeast nitrogen base (YNB) medium (without ammonium sulfate or amino acids), as these starting concentrations are commonly found in typical PN solutions. Because *C. albicans* needs both a carbon and nitrogen source for robust growth, wells contained minimal amounts of either glucose (0.1%) or amino acids (0.02%) of the nontitrated macronutrient. Biofilm growth in these media was compared to growth in RPMI 1640, a widely used cell culture medium for the cultivation of *C.*

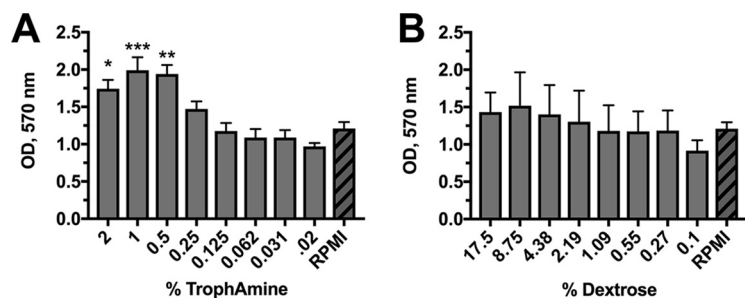


FIG 1 Optimized dosing of TrophAmine and dextrose to identify a minimal growth medium for *C. albicans* biofilm formation. *C. albicans* biofilms were grown for 24 h in 1× YNB medium (without amino acids or ammonium sulfate) containing serial dilutions of (A) TrophAmine or (B) dextrose. Biomass was quantified by the crystal violet method. Data are expressed as the mean ($n = 3$) raw optical density at 570 nm (OD_{570}) and compared to growth in RPMI 1640 medium (hashed bars), which is routinely used for fungal biofilm studies. Error bars represent standard error of the mean (SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, using one-way analysis of variance (ANOVA) with Dunnett’s posttest.

albicans biofilms (35–38). At concentrations above 0.25%, TrophAmine significantly enhanced biofilm growth over that in the RPMI control (Fig. 1A). Similarly, a nonsignificant trend was observed for glucose, in which higher concentrations supported increased biofilm growth (Fig. 1B). We choose the lowest concentrations of TrophAmine (0.02%) and dextrose (0.1%) in 1× YNB (without amino acids or ammonium sulfate) to define the minimal medium used throughout this study.

Smoflipid represses biofilm growth. We next wanted to determine whether various clinical lipid emulsions differentially impact *C. albicans* biofilm growth. In order to answer this, *C. albicans* biofilms were cultivated in the clinical lipid emulsions Smoflipid, Intralipid, or Omegaven and serially diluted in minimal medium. We chose to use minimal medium initially to observe maximal impact that lipids may play in modulating fungal biofilm formation. Both Intralipid and Omegaven significantly stimulated biofilm growth compared to that in the no-lipid control. However, growth in Smoflipid was only marginally and insignificantly increased (Fig. 2A). Qualitative fluorescence microscopy images echoed the quantitative results demonstrating that comparatively little biofilm formed in Smoflipid (Fig. 2B).

Subsequently, we wanted to determine whether similar growth effects could also be observed under more clinically relevant conditions. Therefore, a macronutrient PN solution was made containing 17.5% dextrose and 2% TrophAmine and a range of lipid

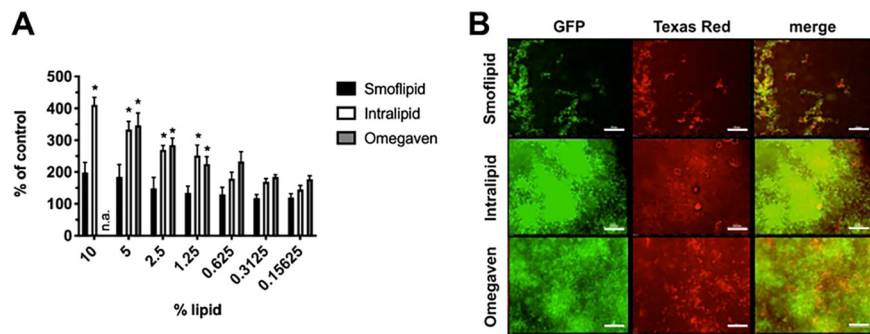


FIG 2 *C. albicans* biofilm formation is differentially affected by growth in clinical lipid emulsions. (A) Biofilms were grown in various concentrations of lipid emulsions (Smoflipid, Intralipid, and Omegaven) in minimal medium (1× YNB, 0.02% TrophAmine, and 0.1% dextrose) for 24 h. Biomass was quantified by the crystal violet method. Data are expressed as the mean ($n = 3$) percentage of the lipid-free controls. Error bars represent SEM. *, $P < 0.05$ using one-way ANOVA with Dunnett’s posttest. n.a., not assessed due to lower starting lipid percentage. (B) Green fluorescent protein (GFP)-expressing *C. albicans* biofilms were cultivated for 24 h on chamber slides using a similar medium and stained with Texas red-conjugated concanavalin A. Representative images ($n = 3$) were captured by fluorescence microscopy. Bar, 100 μ m.

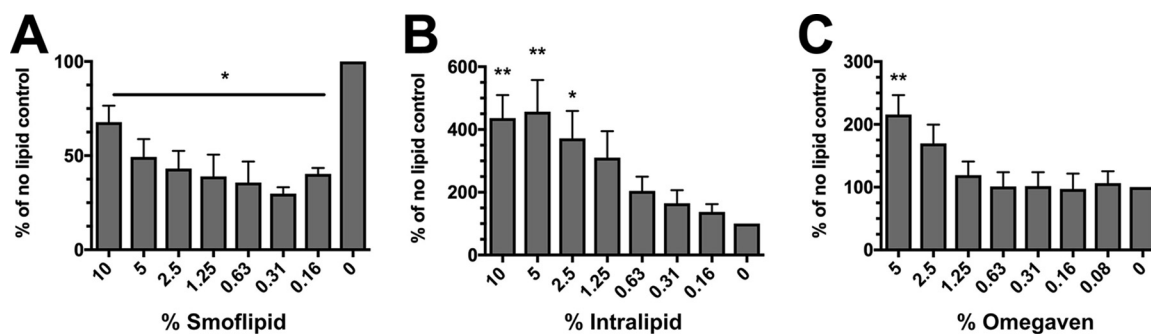


FIG 3 Biofilm formation is modulated by lipid emulsions in a macronutrient PN solution. *C. albicans* biofilms were grown in macronutrient PN solution (1× YNB, 2% TrophAmine, and 17.5% dextrose) containing various concentrations of the lipid emulsions (A) Smoflipid, (B) Intralipid, or (C) Omegaven. Data are expressed as the mean ($n = 3$) percentage of the lipid-free controls. Error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$ using one-way ANOVA with Dunnett's posttest.

emulsion concentrations. Similarly to growth in lipid-supplemented minimal medium, Intralipid and Omegaven both stimulated biofilm growth (Fig. 3A and C). Surprisingly, biofilm formation was not only reduced during growth in Smoflipid, but it was actually repressed (Fig. 3B). Together, these results suggest that Smoflipid fails to augment *C. albicans* biofilm to the same extent as Intralipid or Omegaven under nutrient-deplete and -replete conditions.

Smoflipid does not impair planktonic cell growth. We next wanted to determine whether biofilm repression in Smoflipid was due to reduced cell growth or inhibition of the biofilm process. Thus, we grew *C. albicans* planktonically in 10% (Smoflipid or Intralipid) or 5% (Omegaven) lipid emulsions in biofilm minimal medium or minimal medium alone in planktonic culture. Enumeration of CFU revealed that growth under each lipid condition was similar, suggesting that the biofilm phenotypes observed during growth in lipid emulsions was not linked to growth modulation (Fig. 4).

Smoflipid targets hyphal growth and reduces hyphal length. We next wished to determine whether *C. albicans* hyphal growth is impacted by these lipid emulsions. We used the fluorescent *C. albicans* strain CA14+pKE4-GFP to better visualize hyphae in the opaque lipid emulsions. Fluorescence microscopy revealed that growth in Omegaven yielded hyphal growth similar to that in the control. Growth in Intralipid yielded seemingly robust hyphal growth, and large hyphal aggregates were often observed. Interestingly, Smoflipid appeared to drastically attenuate the capacity of *C. albicans* to undergo the yeast-to-hypha switch (Fig. 5A). Moreover, quantitative assessment of hyphal growth by enumerating the percentage of hyphae per field confirmed this phenotype (Fig. 5B). Further analysis demonstrated that not only was the yeast-to-hypha switch reduced during growth in Smoflipid but that when hyphae do form they are significantly shorter (Fig. 5C). As hypha formation is crucial for robust biofilm

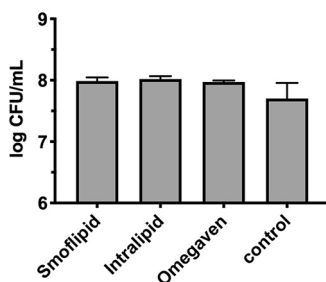


FIG 4 Lipid emulsions do not alter *C. albicans* growth. Planktonic cultures of *C. albicans* were grown in minimal medium supplemented with 10% Smoflipid, 10% Intralipid, or 5% Omegaven. A lipid-free control was also utilized. Data are representative of the mean ($n = 3$) CFU count following serial dilution and growth on yeast-peptone-dextrose (YPD) agar. Error bars represent standard deviation (SD). A one-way ANOVA with Dunnett's posttest was used to assess significance.

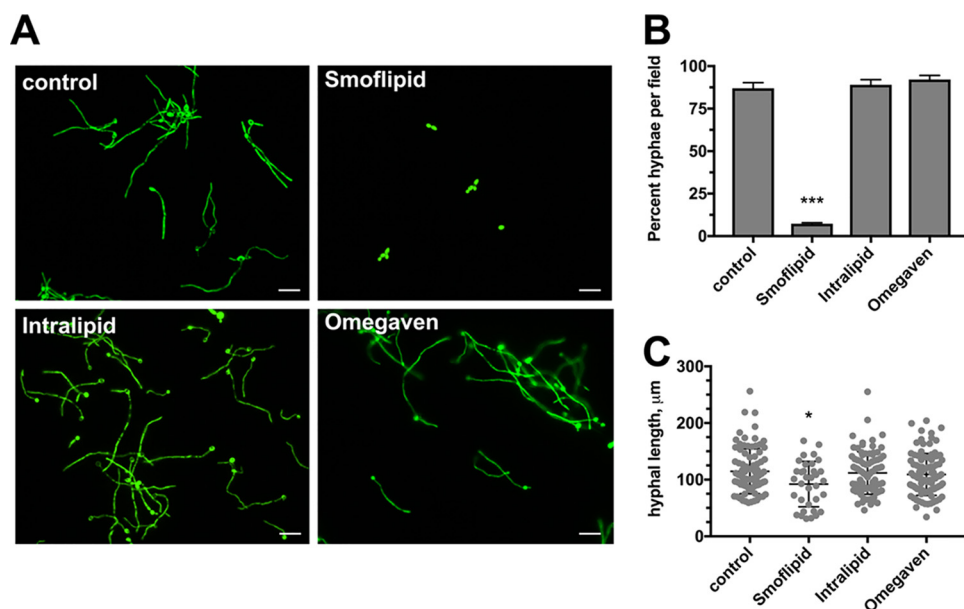


FIG 5 Smoflipid inhibits the yeast-to-hypha transition. (A) *C. albicans* strain CAI4+pKE4-GFP was grown in minimal medium alone (control) or minimal medium supplemented with 10% Smoflipid, 10% Intralipid, or 5% Omegaven for 4 h. Wet mounts (20 μ l) were prepared, and images of 5 nonadjacent fields were captured by fluorescence microscopy. A representative ($n = 3$) of each is depicted. Bar, 30 μ m. (B) Hyphal cells, as well as total cells, per field were enumerated for 10 random images. Data are representative of the mean ($n = 3$) percent hyphae per field. Error bars represent SEM. ***, $P < 0.001$ using a one-way ANOVA and Dunnett's posttest. (C) Using similar images above, hyphal lengths were quantitatively assessed using ImageJ. Data represent the median ($n = 3$) hyphal length \pm SD. *, $P < 0.05$ using a one-way ANOVA and Dunnett's posttest.

formation by *C. albicans*, these results largely explain the observed biofilm growth defect in Smoflipid.

Lipid emulsion fatty acid composition affects hyphal growth. When we compared the composition of the different lipid emulsions, it was observed that Smoflipid, unlike Intralipid and Omegaven, contains caprylic and capric acid (Table S1). In addition, both Smoflipid and Omegaven contain docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), whereas Intralipid lacks these fatty acids. Thus, we determined whether either one of these fatty acids might be responsible for the inhibitory effects on *C. albicans* hyphal growth and biofilm formation observed during growth in Smoflipid. Therefore, we grew *C. albicans* CAI4+pKE4-GFP planktonically in 10% Intralipid (which lacks all of these 4 fatty acids) supplemented with caprylic acid (128.3 mM), capric acid (55.1 mM), DHA (7.4 mM), or EPA (6.9 mM) at concentrations found in 10% Smoflipid used in our assays. Aliquots were taken after allowing sufficient time for hyphal growth and were examined by fluorescence microscopy. DHA and EPA had no effect on hyphal growth, appearing similar to the no-lipid control. However, capric acid completely prevented *C. albicans* hypha formation (Fig. 6A). Like growth in Smoflipid, this effect was not dependent on fungal toxicity, as quantitative CFU counts revealed similar levels of growth in Intralipid supplemented with capric acid to that in Intralipid alone (Fig. 6B). As pH can influence filamentation in *C. albicans*, we wanted to rule out the possibility that addition of capric acid was blocking filamentation simply via a pH-dependent mechanism (39, 40). Therefore, we performed hyphal growth assays in 10% Intralipid solution (pH 5.9 ± 0.1) and 10% Intralipid solutions set to the pH of 10% Smoflipid (pH 5.7 ± 0.1) or 10% Intralipid containing 55.1 mM capric acid (pH 5.3 ± 0.1) to mimic the pH of these conditions. Fluorescence microscopy images and quantitative counts demonstrated no reduction in hyphal growth at these lower pH values, suggesting that pH alone cannot explain reduced hyphal growth phenotypes observed during growth in Smoflipid or capric acid (Fig. S1). Interestingly, while supplementation with caprylic acid demonstrated significant antifungal effects, it also

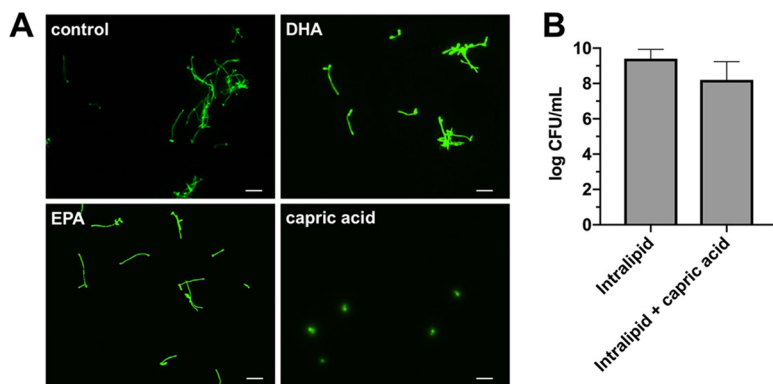


FIG 6 Capric acid impairs *C. albicans* filamentation without inhibiting growth. (A) *C. albicans* strain CA14+pKE4-GFP was grown for 4 h in minimal medium containing 10% Intralipid supplemented with capric acid (55.14 mM), DHA (6.85 mM), or EPA (7.44 mM). Aliquots (20 μ l) were used to make wet mounts, and five nonadjacent fields were captured by fluorescence microscopy. A representative of each is depicted. Bar, 30 μ m. (B) Cells were grown planktonically overnight in minimal medium containing 10% Intralipid alone or supplemented with capric acid (55.14 mM). Growth was assessed by CFU counts following serial dilution plating onto YPD agar. Data are representative of the mean ($n = 3$) count. Error bars represent standard deviation (SD). Significance was assessed using a Mann-Whitney U test.

appeared to completely solubilize the lipid emulsion, which would likely limit its use as a potential lipid supplement in the clinic (Fig. S2).

Capric acid inhibits *C. albicans* biofilm formation. We next wanted to determine whether capric acid supplementation could prevent *C. albicans* biofilm growth. Biofilms in minimal medium both with and without 10% Intralipid were cultivated in the presence or absence of capric acid (55.1 mM). Similarly to hyphal repression, biofilm formation was significantly inhibited compared to the no-capric-acid control (Fig. 7A and B). Qualitative images of biofilms grown in Intralipid with or without capric acid reflected this phenotype. Lastly, we performed dose-response assays to determine the relative potency of capric acid to inhibit biofilm formation in minimal medium, minimal medium containing Intralipid, or macronutrient PN solution containing Intralipid and capric acid. Capric acid up to 13.8 mM was effective at significantly reducing biofilm growth in minimal medium (Fig. 7C). Similarly, capric acid demonstrated efficacy above 3.45 mM in minimal medium containing Intralipid (Fig. 7D). Importantly, capric acid demonstrated significant reduction of biofilm growth in macronutrient PN containing Intralipid at concentrations greater than 1.7 mM (Fig. 7E).

DISCUSSION

Our experiments uniquely illustrated differences between lipid products regarding their effect on *C. albicans* biofilm formation. The repression of biofilm growth noted in Smoflipid was due to inhibition of hypha formation and length. These data have the potential to impact the rate and/or treatment of CR-BSIs in patients receiving PN.

The newer lipid emulsions were introduced mainly to decrease PN-related morbidities like parenteral nutrition-associated cholestasis (PNAC) (2). While much literature exists that correlates PN administration and CR-BSIs, the studies analyzing PN with infection rates or biofilm formation have focused primarily on Intralipid (10, 27, 41). A paucity of information exists on how newer lipid emulsions impact *Candida* biofilm formation and CR-BSI. The differential capacity of lipid emulsions to stimulate fungal growth and biofilm formation may carry important clinical implications for determining which lipid emulsion to use based on individual patient scenarios.

Biofilm formation is believed to be a significant contributor to the pathogenesis of CR-BSIs like those caused by *C. albicans*. Biofilm presence can make infections highly recalcitrant to common antifungal and catheter lock therapies (25, 42). PN solutions typically contain relatively large amounts of glucose (e.g., 10 to 25%) and amino acids (e.g., 2%) (43, 44). Residual solutions remaining in the catheter line, even if further

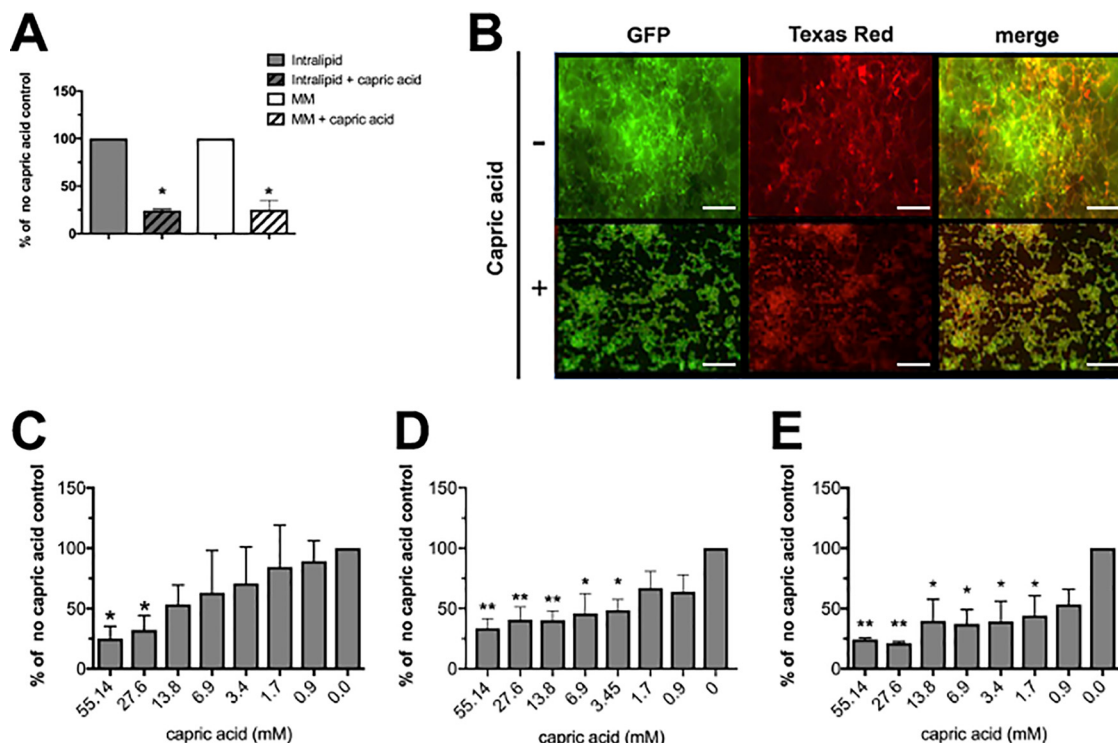


FIG 7 Capric acid represses *C. albicans* biofilm formation. (A) Biofilms (gray bars) were cultivated in minimal medium supplemented with 10% Intralipid (open) or 10% Intralipid containing 55.14 mM capric acid (hashed). Similarly, biofilms were grown in these same conditions (open, no capric acid; hashed, 55.14 mM capric acid) in the absence of Intralipid (white bars). Data represent the mean ($n = 3$) percentage of the capric acid-free control. *, $P < 0.05$ using Student's t test. (B) Representative images of GFP-expressing biofilms grown in minimal medium containing 10% Intralipid with or without capric acid (55.14 mM). Biofilms were stained with Texas red-conjugated concanavalin A and imaged by fluorescence microscopy. Bar, 100 μm . Dose-response assays for capric acid were performed in (C) minimal medium, (D) minimal medium containing 10% Intralipid, or (E) macronutrient PN solution containing 10% Intralipid. Data represent the mean ($n = 3$) percentages of the lipid-free controls. *, $P < 0.05$; **, $P < 0.01$ using a one-way ANOVA and Dunnett's posttest.

diluted, could provide rich nutritional substrates for microbial biofilm growth. Indeed, a study by Herek et al. demonstrated that glucose concentration in PN solutions stimulated *Candida parapsilosis* biofilm formation but did not analyze the impact of protein or lipids (45). Interestingly, Shin et al. showed that high protein and glucose concentrations resembling those found in PN solutions did not promote *C. albicans* biofilm formation, suggesting that factors other than high glucose and protein concentrations in PN emulsions may play a role in exacerbated *C. albicans* pathogenicity or biofilm growth during PN therapy (46). Our data support this finding, as moderately high glucose concentrations (17.5%) had only marginal impact on biofilm growth over that of minimal medium containing only 0.1% of this carbon source. Together, these studies highlight the interspecies heterogeneity in response to growth in PN solutions, and such differences likely span the spectrum of the *Candida* species.

Although the role of glucose and protein content of PN solutions in promoting fungal biofilm formation is unclear, several studies have determined varied effects of lipid emulsion or fatty acid components on *C. albicans* biology. Swindell et al. demonstrated that Intralipid increased *C. albicans* biofilm formation compared to that in non-lipid-supplemented medium (27). Our data are in agreement, given that Intralipid (and also Omegaven) increased biofilm growth in minimal medium and concomitantly with clinically relevant concentrations of glucose and protein. The detailed mechanisms driving augmented hyphal growth in lipid emulsions remain elusive. However, it is clear that lipid and fatty acid composition (e.g., medium-chain varieties) plays a significant role in the process, as Smoflipid uniquely repressed biofilm growth in the presence of protein and dextrose concentrations commonly seen in PN formulations.

There exists a substantial body of literature describing antimicrobial effects of medium-chain fatty acids against a plethora of bacteria and fungi. *C. albicans* appears to be uniquely sensitive to fatty acids with carbon chain lengths of C₈, C₁₀, and C₁₂, represented by caprylic, capric, and lauric acid, respectively (47). As early as the 1960s, Tsukahara demonstrated that caprylic acid was a robust, fast-acting fungicide (48). Treatment of *C. albicans* with caprylic acid at even relatively low concentrations (e.g., 10⁻⁵ M) led to total killing in as little as 10 min of contact time. A study by Bergsson et al. further supported these early observations by demonstrating that capric and lauric acid also exhibited fungicidal effects (49). Closer inspection of *C. albicans* morphology following treatment with these fatty acids by transmission electron microscopy revealed no overall defects in cell size or plasma membrane alternations. However, cellular cytoplasm appeared to be disorganized, which the authors hypothesized could be due to turgor pressure changes induced by fatty acid treatment. Our results are somewhat at odds with those of this study, given we observed no growth inhibitory effect observed during culture in nutrient broth supplemented with lipid emulsions or capric acid. Instead, our results more closely mirror those by several other groups, which also did not report growth modulatory effects of lipid emulsions or capric acid on *C. albicans* (50–53). Importantly, several of these studies did demonstrate reduced hyphal growth and hypha-associated gene expression in the presence of capric acid, similar to our findings (47, 54). Given that hypha formation is required for robust biofilm architecture in this fungal species, fatty acids would also be predicted to indirectly affect this growth modality.

Although the role of capric acid in *C. albicans* growth and hypha formation has been previously established, the link between the presence of capric acid in Smoflipid and reduction of *Candida* CR-BSIs or *Candida* biofilm formation on CVCs used for PN administration has not yet been clearly defined or statistically vetted. That said, an analysis focused on incidence of fungal CR-BSIs using Smoflipid versus other lipid emulsions would be warranted to validate our findings on a broadly relevant scale, where patient status, medication coadministration, and infection control measures could vastly impact outcomes. Regarding this point, various lipid emulsions not only differentially affect microbial agents but also affect host physiology, including modulation of the immune system (55–60). After all, newer lipid products were designed and marketed to be less inflammatory. Interestingly, a small crossover study of 8 healthy volunteers receiving lipid emulsions composed of either long-chain fatty acids (LCFA) only or of a mixture of long-chain and medium-chain fatty acids (MCFA) followed by a placebo period revealed that MCFA-LCFA mixtures altered cytokine expression following *C. albicans* challenge of isolated peripheral blood mononuclear cells compared to that with LCFA and placebo controls (61). Specifically, levels of interleukin 10 (IL-10; anti-inflammatory) were increased and gamma interferon (IFN- γ ; proinflammatory) were decreased resulting in a shift of the Th1/Th2 cytokine balance to a less inflammatory state. This could have potential consequences for protective antifungal responses in localized tissue. However, other clinical evidence, including a randomized clinical trial, suggests that overall infection rate is decreased with the use of lipid products containing fish oil or combinations of fish oil and MCFAs (62–64). Unfortunately, studies assessing infection rates associated with use of different lipid products have not clearly differentiated causative pathogens and were not specific to central line infections. These conflicting data highlight the multifactorial nature of PN and lipid therapy. Thus, the data presented here should be interpreted with caution until further studies are completed if extrapolating to the clinical setting or other microbes.

A current approach to preventing and sometimes treating CR-BSIs involves the use of antimicrobial catheter lock solutions when a line is not in use (e.g., a cycled PN patient). These solutions can sterilize the luminal catheter surface and prevent biofilm growth (42, 65). The use of these solutions in the lumen of catheters for extended periods of time to prevent infections is commonly referred to as antimicrobial lock therapy (ALT). Prevention of catheter contamination could improve sepsis rates associated with catheter use (66). Our results show that capric acid may be useful for

prevention of biofilm development either as a standalone lock solution, when administered within Smoflipid, or as an additive to lipid emulsions lacking this fatty acid (e.g., Intralipid). Caprylic acid has been studied using both *in vitro* and animal models and in conjunction with other agents such as glyceryl trinitrate as a potential lock therapy (67). However, capric acid-based lock solutions have been minimally studied and their efficacy following PN exposure remains unclear. The capacity of capric acid to diminish biofilm production in a common PN macronutrient solution illustrates the relevance of this agent in patients receiving PN via a CVC. A lipid product that both provides essential lipid calories and contains an agent that prevents biofilm formation would have a significant impact on CR-BSIs in patients receiving PN. Collectively, these data suggest another method of CR-BSI prevention and possible treatment. Whether these data may also apply to limit the growth of other prevalent CR-BSIs pathogens (e.g., staphylococci) is among the important questions to be addressed in future studies.

In summary, our results show that there are major differences between PN lipid emulsions and their ability to stimulate biofilm formation. We have demonstrated that Smoflipid significantly inhibits *Candida* biofilm formation *in vitro* by inhibiting hyphal growth, most likely due to capric acid activity. Importantly, we showed that Intralipid and Omegaven both stimulate biofilm formation, which might have important clinical implications for the use of these PN emulsions in patients requiring PN. In addition, use of Smoflipid may help prevent CR-BSIs in this patient population. Moreover, PN-specific or general-use catheter lock solutions could include capric acid to aid in the prevention of *C. albicans* biofilm formation. Future *in vitro*, *in vivo*, and clinical studies are warranted to further evaluate the utility of these findings.

MATERIALS AND METHODS

Strains and growth conditions. Biofilm-forming *C. albicans* prototypical isolate SC5314 and *C. albicans* CA14+pKE4-GFP γ (single *IRO1-URA3* locus restored) were used in this study and are as described previously (68, 69). Both organisms were maintained as frozen stocks at -80°C . Prior to use, *C. albicans* was subcultured onto yeast-peptone-dextrose (YPD) agar (Difco) at 30°C . A single colony was cultured in $1\times$ yeast nitrogen base (YNB) liquid medium containing 0.5% ammonium sulfate and 2% glucose (Difco) at 30°C for 24 h. Following growth, *C. albicans* was washed in phosphate-buffered saline (PBS) by centrifugation, counted on a hemocytometer, and adjusted to 1×10^7 CFU/ml in $1\times$ YNB.

Biofilm growth. For biofilm formation, $100\ \mu\text{l}$ of adjusted *C. albicans* culture in $1\times$ YNB was added to each well of a 96-well cell culture-treated polystyrene microtiter plate (1×10^6 CFU per well). Plates were incubated for 2 h at 37°C in a humidified chamber to allow *C. albicans* to adhere to the surface. After 2 h, nonadherent cells were carefully removed by pipetting and the wells were washed $3\times$ with sterile cell-culture grade distilled water (dH_2O) to completely remove all adherent cells. Various concentrations of Intralipid (Baxter Healthcare Corporation, Deerfield, IL, USA), Smoflipid (Fresenius Kabi, Bad Homburg, Germany) at 10%, 5%, 2.5%, 1.25%, 0.625%, 0.3125%, and 0.15625%, or Omegaven (Fresenius Kabi) at 5%, 2.5%, 1.25%, 0.625%, 0.3125%, 0.15625%, and 0.078125% were added to macronutrient PN solutions consisting of 2% TrophAmine (Braun Medical, Bethlehem, PA), 17.5% dextrose (Baxter Healthcare Corporation), and $1\times$ YNB (without ammonium sulfate or amino acids). In some experiments, $1\times$ YNB (without ammonium sulfate, amino acids, or glucose) was supplemented with TrophAmine (2%, 1%, 0.5%, 0.25%, 0.125%, 0.0625%, 0.03125%, or 0.02%) or dextrose (17.5%, 8.75%, 4.375%, 2.1875%, 1.09375%, 0.546875%, 0.273438%, or 0.1%) in order to define a minimal medium ($1\times$ YNB, 0.02% TrophAmine, and 0.1% dextrose) that supported growth but did not enhance biofilm formation. As a control, biofilms were prepared similarly using $1\times$ RPMI 1640 cell culture medium. Plates were incubated for 24 h at 37°C to induce biofilm formation.

Crystal violet assay for biofilm quantitation. In order to quantify biomass during growth, biofilms were grown as described above and processed for crystal violet staining as previously described (70). Briefly, wells were extensively washed in dH_2O to remove nonadherent cells, stained with 0.1% crystal violet, and repeatedly washed in dH_2O . Bound crystal violet was resolubilized in 95% ethanol, and the absorbance was read at 570 nm on a microplate reader. Wells containing medium alone were similarly stained and used to blank subtract from experimental wells. Wells containing neat or diluted lipid solutions alone did not show significant staining above that of medium-only controls (data not shown). Each condition was conducted using technical triplicates and the data were averaged. Experiments were independently repeated ($n = 3$) and data are represented as the experimental mean + standard error of the mean (SEM). Results were reported as either raw optical density at 570 nm (OD_{570}) nm readings or as percentage of the relative control.

Planktonic growth and quantitative growth assay. *C. albicans* culture was prepared as described above and adjusted to a final concentration of 1×10^6 cells/ml in $2\times$ minimal medium (described above) supplemented with final concentrations of 10% Smoflipid, 10% Intralipid, or 5% Omegaven. Cells were grown at 30°C in a shaking incubator for 20 h at 225 rpm. The following day, cultures were serially diluted

10-fold and plated onto YPD agar by the drop plate method (71). The plates were incubated for 24 h at 37°C, colonies were enumerated, and numbers of CFU/ml were reported as the mean + SEM ($n = 3$).

Quantitative hyphal growth assays. A *C. albicans* strain engineered to constitutively express green fluorescent protein (GFP), CAI4+pKE4-GFP, was grown overnight in 1× YNB, washed in PBS, diluted to 1×10^6 cells/ml as described above in minimal medium containing 10% Smoflipid, 10% Intralipid, or 5% Omegaven, and cultured in a 37°C shaking incubator for 4 h to allow for hyphal growth. Aliquots (20 μ l) were removed, and wet mounts were prepared and then imaged with a Nikon Ni-U fluorescence microscope using a 488-nm laser and GFP filter set. Percent hyphae per field were enumerated using a total of 10 random images per condition. Hyphal length was assessed using ImageJ by calibrating distance to Ni-U scalebars. At least 10 cells per field were enumerated from 10 random fields per condition. Results for qualitative and quantitative microscopy were representative of experimental replicates ($n = 3$).

Hyphal growth assay with fatty acids. CAI4+pKE4-GFP was prepared as described above and diluted to 1×10^6 cells/ml in minimal medium containing 10% Intralipid spiked with a final concentration of 55.1 mM capric acid, 128.3 mM caprylic acid, 7.4 mM EPA, or 6.8 mM DHA. Cultures were grown in a 37°C shaking incubator for 4 h. Aliquots (20 μ l) were removed and imaged as described above.

Measurement and modulation of PN lipid emulsion pH. A standard pH meter was used to assess the pH of solutions of 10% Smoflipid, 10% Intralipid, or 10% Intralipid containing 55.1 mM capric acid prepared in minimal medium. In some cases, the pH of Intralipid was altered by adjusting with 1N hydrochloric acid.

Imaging of *C. albicans* biofilm architecture in lipid emulsions. *C. albicans* strain CAI4+pKE4-GFP was cultured as described above, and 200 μ l of adjusted *C. albicans* culture was added to each chamber of a Permax-coated 8-well chamber slide (1×10^6 CFU per well). Plates were incubated for 2 h at 37°C in a humidified chamber to allow for cell attachment. Nonadherent cells were carefully removed by washing 3× with sterile dH₂O. Smoflipid, Intralipid (10%), Omegaven (5%), YNB minimal medium, or Intralipid supplemented with capric acid (55.1 mM) was added to the wells. Biofilms were allowed to grow for 48 h in total at 37°C in a humidified chamber with the medium replaced after 24 h of growth. After incubation, the supernatant was aspirated, and nonadherent cells were removed by washing with sterile dH₂O. Biofilms were fixed in 4% formaldehyde and stained with concanavalin A-Texas red (50 μ g/ml) for 30 min. Images were captured by fluorescence microscopy (Nikon Ni-U) using GFP and tetramethylrhodamine (TRITC) filter sets.

Image construction and capture. Images were constructed in Microsoft PowerPoint and converted to appropriate format and resolution using Adobe Photoshop. In some cases, images were captured by digital camera.

Statistics and image construction. All experiments were performed in biological triplicate and repeated a minimum of three times. Biomasses from crystal violet staining, CFU counts, and hyphal growth were compared using one-way analysis of variance (ANOVA) and Dunnett's posttest. Differences with a P value of <0.05 were considered significant. All statistical analyses were performed and graphs were composed with GraphPad Prism.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01394-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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B.M.P. and J.S.S. designed the study; H.M.E.W., J.P.F., and M.E.C. collected the data; B.M.P., J.S.S., and H.M.E.W. performed the data analysis and interpreted study results; and B.M.P., J.S.S., and H.M.E.W. wrote the paper. All authors gave approval of the final version to be submitted.

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