Alginate Oligosaccharides Modify Hyphal Infiltration of *Candida albicans* in an *In Vitro* Model of Invasive Human Candidosis

Abbreviated Running Headline: Altering *In Vitro* Hyphal Invasion

Manon F. Pritchard[1], Alison Jack[1], Lydia C. Powell[1], Hina Sadh[1], Philip D. Rye[2], Katja E. Hill[1], David W. Thomas[1].

[1] Advanced Therapies Group, Cardiff University School of Dentistry, College of Biomedical and Life Sciences, Cardiff, UK

[2] AlgiPharma AS, Sandvika, Norway

Correspondence

Manon F. Pritchard, Advanced Therapies Group, Cardiff University School of Dentistry, College of Biomedical and Life Sciences, Cardiff, CF14 4XY, UK

E-mail: pritchardmf@cardiff.ac.uk

Abstract

Aims: A novel alginate oligomer (OligoG CF-5/20) has been shown to potentiate antifungal therapy against a range of fungal pathogens. The current study assessed the effect of this oligomer on *in vitro* virulence factor expression and epithelial invasion by *Candida* species.

Methods and Results: Plate substrate assays and epithelial models were used to assess *Candida albicans* (CCUG 39343 and ATCC 90028) invasion, in conjunction with confocal laser-scanning microscopy and histochemistry. Expression of candidal virulence factors was determined biochemically and by quantitative PCR (qPCR). Changes in surface charge of *C. albicans* following OligoG treatment were analysed using electrophoretic light scattering. OligoG induced marked alterations in hyphal formation in the substrate assays and reduced invasion in the epithelial model ($p<0.001$). Significant dose-dependent inhibition of phospholipase activity in *C. albicans* was evident following OligoG treatment ($p<0.05$). Whilst OligoG binding failed to affect alterations in surface-charge ($p>0.05$), qPCR demonstrated a reduction in phospholipase-B (*PLB2*) and SAPs (*SAP4* and *SAP6*) expression.

Conclusion: OligoG CF-5/20 reduced *in vitro* virulence factor expression and invasion by *C. albicans.*

Significance and Impact of the Study: These results, and the previously described potentiation of antifungal activity, define a potential therapeutic opportunity in the treatment of invasive candidal infections.

Keywords

Virulence, Biofilms, Antimicrobials, Infection, Fungi

Introduction

The incidence of human fungal infections has risen annually with increasing numbers of immunocompromised patients (Miceli *et al*. 2011), in-dwelling prosthetic devices (Chen *et al*. 2011; Silva *et al*. 2012), broad-spectrum antibiotic use and cytotoxic/immunosuppressive therapy. *Candida* species (Pfaller and Diekema 2007) are responsible for >50% of systemic fungal infections (Lass-Floerl 2009) and are the most frequently-reported human nosocomial fungal pathogens. In health, *Candida* exist as harmless commensal organisms on the skin-surface, oral mucosa and gastrointestinal tract (Lass-Floerl 2009). *Candida* are, however, opportunistic pathogens in both local- and systemic infection; the latter being associated with both significant mortality (estimated at 30%) (Lass-Floerl 2009) and high treatment costs (Ramage *et al*. 2005; Leroy *et al*. 2009).

Candida readily form biofilms on epithelial and material surfaces which exhibit resistance to antifungals including polyenes and azoles (Kuhn *et al*. 2002). Once a candidal biofilm is formed *in vivo*, elimination generally demands removal of the substratum which supports the biofilm. However, removal of medical devices is often impossible, due to the patient's underlying medical condition and/or the anatomic location of the biofilm (Ramage *et al*. 2006). Following adhesion and biofilm formation, the ability of *Candida* (especially *Candida albicans*) to undergo morphological alteration and secrete hydrolytic enzymes facilitates invasion and contributes to their pathogenicity (Mayer *et al*. 2013). The invasion of host-tissue in the pathogenesis of candidal infection is a complex combination of physical, mechanical and enzymatic events, which are both host- and microorganism-dependent. Whilst adhesion to host tissues and morphogenesis contributes to candidal virulence (Yang 2003), virulence factor production is also associated with *Candida* invasion (Shimizu *et al*. 1996).

Intact human epithelium acts as a considerable physical and chemical barrier against *Candida* spp. infection. *Candida* spp. possess several discrete mechanisms to increase their dermal pathogenicity, with the expression of surface proteins, e.g. adhesins and invasins facilitating attachment (Nobile *et al*. 2006), and biofilm formation supporting persistence (Finkel and Mitchell 2011). Moreover, yeast-to-hyphal transition facilitates enzymatically-induced tissue invasion (Mayer *et al*. 2013), and thigmotropism (contact sensing) directs site-specific invasion (Kumamoto 2008). *Candida*

species are highly metabolically adaptable, having the ability to adapt to changes in environmental pH and nutritional availability, as well as having a robust stress-response mechanism (Mayer *et al*. 2013).

Secretion of hydrolases is important for the pathogenicity of *C. albicans* and is mediated by three main enzyme classes: secreted aspartyl proteinases (SAPs), phospholipases (PL) and lipases (Mayer *et al*. 2013). Distinct SAPS genes are known to facilitate active penetration of the cross-linked epithelial barrier of the skin (Schaller *et al*. 2000). Increased expression of PL has been associated with antifungal resistance (Ying and Chunyang 2012), whilst secretion of SAPs have been implicated in systemic infections (Sanglard *et al*. 1997).

Previous studies demonstrated the ability of a low molecular weight alginate, OligoG CF-5/20 (OligoG), to modify bacterial biofilm assembly and reduced resistance to antimicrobial therapy (Khan *et al*. 2012; Powell *et al*. 2013; Roberts *et al*. 2013; Powell *et al*. 2014). More recently, the ability of OligoG to inhibit growth and biofilm formation of *Candida* and *Aspergillus* spp. has been demonstrated (Tøndervik *et al*. 2014). These changes were associated with significantly increased sensitivity to antifungal agents and marked decreases in hyphal formation (Tøndervik *et al*. 2014). The extent to which these changes modulate binding to-, and invasion of, mucosal surfaces by fungal pathogens remains unknown.

Candidal adhesion and invasion was investigated using an *in vitro* organotypic keratinocyte model in the presence and absence of OligoG. Substrate assays and real-time PCR were employed to investigate the mechanisms responsible for the observed changes.

Materials and methods

Strains and routine culture media

C. albicans CCUG 39343 (clinical isolate) and *C. albicans* ATCC 90028 (reference strain for antifungal susceptibility testing) and *C. glabrata* ATCC 2001 (non-hyphal producing control) were used in this study. *Candida* were grown at 37°C for 18 h on Sabouraud dextrose agar (SDA, Oxoid) or in liquid

culture using Sabouraud dextrose broth (SAB, Lab M). The alginate oligomer, OligoG CF-5/20, was prepared as previously described (Khan *et al*. 2012).

Growth curves

C. albicans ATCC 90028 was grown overnight in Yeast Nitrogen Base (YNB; BD Diagnostics, Cowley, UK) medium supplemented with 0.5% (w/v) glucose at 37°C. The culture was diluted to 5 x 10^6 cells ml^{-1} and placed in 96-well microtiter plates ± 0.2, 2 and 6% OligoG (w/v) at 37°C for 24 h, with a YNB ± OligoG blank. Optical density was measured at 600 nm (Fluostar Omega plate reader; BMG LABTECH).

Hyphal invasion assay

Yeast Peptone Dextrose (YPD) agar was prepared (Roberts and Fink 1994) ± 0.2%, 2% and 6% OligoG. Plates were dried (1 h) at room temperature prior to use. Overnight cultures of *C. albicans* ATCC 90028 were grown in YPD broth at 37°C whilst shaking. *Candida* (20 µl ≃ $1x10^7$ cells ml^{-1} in YPD medium) were deposited on to the YPD agar surface (n=3) and incubated for 72 h at 37°C, followed by 72 h at room temperature. Hyphal agar invasion was examined after rinsing the colonies from the agar surface with deionized water (Roberts and Fink 1994). Assays were conducted in triplicate in three, independent, experiments. Photographs employed the same focal length. Agar samples were stained using hematoxylin and eosin (H&E) to study hyphal invasion of the epithelium. Light microscopy images (x100 objective lens) of control and OligoG-treated samples were taken.

Reconstituted human epithelium model

Reconstituted human oral epithelium (RHE) tissue was obtained from SkinEthic Laboratories (Nice, France). RHE tissues (n=5) were placed in 6-well tissue culture plates with 1 ml of SkinEthic maintenance medium ± 0.2% OligoG (w/v). *Candida albicans* ATCC 90028, cultured on SDA at 37°C for 24 h, was inoculated into YNB medium supplemented with 0.5% glucose (w/v) for 12 h at 37°C under gentle agitation. The cell-suspension was centrifuged and washed (x3) with phosphate buffered

saline (PBS). Following direct counting using a hemocytometer, 50 µl of 2 x 10^6 cells ml^{-1} was added to the surface of each RHE and incubated for 1, 3, 6 or 12 h at 37°C in a humidified atmosphere with 5% CO_2. Non-infected controls were included in all experiments. The RHE were then rinsed (x2) in PBS to remove planktonic cells and bisected. Half was fixed in 10% (v/v) formalin prior to being embedded in paraffin wax, with the remainder used immediately for LIVE/DEAD® staining (Molecular Probes-Invitrogen, Paisley, UK).

Confocal laser scanning LIVE/DEAD® staining of RHE

RHE samples from each time-point were placed on microscope slides and 100 µl of LIVE/DEAD® stain (containing 25 µmol l^{-1} SYTO® 9 and 15 µmol l^{-1} propidium iodide) applied directly to the tissue, as previously described (Boros-Majewska *et al*. 2015). Treated samples were incubated for 30 min at 37˚C in the dark prior to transfer to clean glass slides. RHE samples were covered with Vectashield® mounting medium and confocal laser scanning microscopy (CLSM) performed (n=3) using a Leica TCS SP2 AOBS spectral confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Periodic Acid-Schiff staining

RHE sections (5 µm) were stained using Periodic Acid-Schiff (PAS) stain to study hyphal invasion of the epithelium. Light microscopic images (x100 objective lens) of control and 0.2%-treated OligoG samples (n=144) were taken (10 images/section), and the depth of invasion ($\geq$3 per image) was analysed by direct measurement, using ImageJ software.

Phospholipase (PL) activity plate assay

Candida albicans ATCC 90028 and *C. albicans* CCUG 39343 (with *C. glabrata* ATCC 2001 used as a negative control) were screened for extracellular PL activity after growth on egg-yolk agar (Samaranayake *et al*. 1984). Experiments employed OligoG in the overnight culture medium (prior to inoculation of the egg-yolk agar) and OligoG incorporated into the egg yolk agar at 0.2, 2 and 6% (w/v) without $CaCl_2$. Static overnight cultures in SAB were diluted 100-fold into fresh SAB ± 0.2%, 2% and 6% (w/v) OligoG for 18 h at 37°C. A standard inoculum of the test *Candida* (5 µl $\simeq$ 10^7 cfu ml^{-1}) was

deposited onto the egg-yolk agar and dried at room temperature. Plates were incubated at 37°C for 3-7 days; the diameter of the precipitation zone around the colony was then measured (n=3) and PL activity (P_z value) expressed as the ratio of the colony diameter to the total diameter of the colony and precipitation zone (Price *et al*. 1982).

Secreted aspartyl protease (SAP) activity plate assay

Candida albicans ATCC 90028, *C. albicans* CCUG 39343, and *C. glabrata* ATCC 2001 were screened for SAP production after growth on modified YNB medium (de Menezes Thiele *et al*. 2008). Briefly, 1.5% agar (Sigma; w/v) and 0.2% glucose (w/v) was autoclaved followed by the addition of filter sterilized 1.17% YNB without ammonium sulfate, 0.2% bovine serum albumin (Sigma; w/v) and 0.01% Vitox (Oxoid; v/v). The inoculum preparation and analysis was identical to that described in the PL activity assay.

Hemolysis plate assay

Sheep-blood SDA, supplemented with 3% glucose, was prepared ± 0.2%, 2% and 6% OligoG. Static overnight cultures of *C. albicans* CCUG 39343, *C. albicans* ATCC 90028 and *C. glabrata* ATCC 2001 were incubated at 37°C in SAB. Cultures were diluted 100-fold into SAB ± 0.2, 2 and 6% OligoG and re-incubated for 18 h at 37°C. The overnight culture was centrifuged at 2,000 *g* for 5 min and re-suspended in water to 1 x 10^7 cells ml^{-1}; a 5µl inoculum was placed on each plate (n=3). Plates were incubated at 37°C (48 h) and hemolysis production calculated by dividing the diameter of the colony by the total colony diameter (including the translucent periphery), to determine the hemolytic index, Hi (Yigit *et al*. 2011; Deorukhkar *et al*. 2014). Controls included *Streptococcus pyogenes* (clinical strain; beta hemolytic), *S. pneumoniae* (ATCC 49619; alpha hemolytic) and *Staphylococcus epidermidis* (ATCC 14990; negative control) grown in Mueller-Hinton broth.

RNA extraction and Real-Time-PCR (qPCR)

RNA was extracted from *C. albicans* ATCC 90028 cultured as described above. Cultures were adjusted to 1.0 x 10^8 cells ml^{-1} in PBS and centrifuged (12000 *g*, 2 min) before being re-suspended in 0.5 ml

RNAlater® and stored at -20°C until required. Cells were pelleted (12000 x *g*, 2 min) and re-suspended in lysis buffer (RLT buffer, QIAgen, Crawley, UK) containing 1% (v/v) beta-mercaptoethanol. Cells were then lysed using a Mini-Bead-Beater-8 for a total of 4 min (at 2 min intervals with 1 min on ice). Resultant supernatants were transferred to fresh tubes, and the cell-debris pelleted by centrifugation. Total nucleic acid was extracted from the supernatant using 500 µl phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich, Pool, UK). Total RNA was recovered from the aqueous layer after DNase I treatment using the RNeasy Mini Kit (QIAGEN). Gel electrophoresis was used to check purity and integrity of the total RNA, and RNA concentration was measured spectrophotometrically (NanoVue, GE Healthcare, Little Chalfont, UK) and standardized to 300 ng ml^{-1}. Reverse transcription reactions for cDNA synthesis were performed using a total RNA template of (300 ng ml^{-1}), 40 µmol of random nonamer primers (PrimerDesign Ltd) and molecular grade water in a final reaction volume of 10 µl. An annealing step of 5 min at 65°C was performed, samples were cooled on ice and added to the extension mix; 4 µl of 4 x NanoScript2 buffer, 1 µl dNTP mix (10 mM), NanoScript2 enzyme at 1.5 µl (Primer Design, Southampton, UK), and 2.5 µl of molecular grade water and a final volume of 20 µl was incubated at 25°C for 5 min and then at 42°C for 20 min.

The primers used in the qPCR analysis were based on previous findings, and are shown in Table 1. Primer specificity was tested on extracted genomic DNA. Regions amplified were secreted aspartyl proteinases *SAP4*, *SAP5* and *SAP6* and phospholipases *PLB1* and *PLB2*, with *ACT1* serving as a reference control for *C. albicans.* PCR was performed in 96-well plates in an ABI 7000 instrument (Life Technologies). Each 25 µl reaction contained 2 µl cDNA, 12.5 µl (x2) of SYBR-Green PCR master mix (PrecisionPlus Mastermix; Primer Design, Southampton, UK), 0.5 µl of each primer (10 mM), made up to 25 µl with molecular grade water. The thermal cycle profile comprised of initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 15s, primer annealing at 58°C for 15s, and primer extension at 72°C for 30s. A final extension at 72°C for 2 min was performed, followed by a final cooling step at 4°C. A dissociation stage at 60°C was used to generate a melting-curve for verification of the amplified product. After qPCR, the threshold was adjusted according to the amplification curves of all evaluated genes. Comparison between groups was based on the cycle

number at which both the target and reference genes reached threshold cycle (Ct) fluorescence. Analysis of relative gene expression was according to the $^{\Delta\Delta}$CT method (Bustin *et al*. 2009).

Candida cell-surface charge analysis

Zeta-potential analyses were performed using electrophoretic light scattering on *C. albicans* 39343 at pH 5, 7 and 9 and at a salt concentration of 0.01 mol l^{-1} NaCl. *C. albicans* was grown overnight in SAB at 37°C, and diluted 100-fold in fresh SAB prior to culture at 37°C for 19 h at 80 rev min^{-1}. One ml of the culture was washed in distilled-water (5,500 x *g*; 3 min). The pellet was re-suspended in 100 µl of 0.01 mol l^{-1} NaCl (pH 5, 7 or 9) and 20 µl added to 1 ml of the buffer ± OligoG (10%) for 20 min; the sample was washed and centrifuged (2,500 x *g*; 6 min). A Zetasizer Nano ZS (Malvern Instruments) and disposable capillary cells (DTS1061 Malvern instruments) were employed and the resultant zeta potential calculated by applying Smoluchowski's model (Wilson *et al*. 2001).

Statistical analysis

GraphPad Prism 3 was used to perform statistical analysis (GraphPad software Inc, California, USA). Measurements of invasion in the PAS-stained RHE images were analysed using a Mann-Whitney test. The data were analyzed by one-way ANOVA followed by a Dunnett Multiple Comparisons test (growth curve, SAP and PL plate assays) and Tukey Kramer multiple comparisons test (real-time PCR data). Data represents the mean in all figures (standard deviation; **Fig. 1.-5**, **S1-S2** and standard error of mean in **Fig. 6**). $p<0.05$ was considered significant.

Results

OligoG reduced candidal growth

Growth curve studies showed that there was no change in the lag- or exponential-phases when *C. albicans* was grown in the presence of OligoG. However, a decrease in optical density was evident at the late-exponential growth phase (>10 h) when samples were treated with ≥2% OligoG (Fig. 1a).

OligoG induced a significant dose-dependent reduction in candidal growth compared to the control (at 18 h), which was maintained throughout stationary phase (Fig. 1b; $p<0.05$).

OligoG reduced *in vitro* candidal hyphal production and invasion

The hyphal invasion agar assay confirmed that treatment with OligoG (>0.2%) induced a marked dose-dependent decrease in candidal invasion (Fig. 2). A visible reduction in the ability of *C. albicans* to penetrate the agar was seen at increasing OligoG concentrations. H&E stained sections of the agar showed that considerable hyphal invasion was seen in the control samples, and interestingly this often led to a cluster of yeast cells penetrating through the agar along with the hyphae. As the concentration of OligoG increased, less hyphae were visible, with reduced yeast cell clustering within the agar, particularly at OligoG concentrations ≥2%. Alternatively, hyphal cells may have reverted to yeast cells.

Longitudinal CLSM studies of the RHE tissues showed candidal hyphal formation did not occur before 6 h (Fig. 3). However, in contrast to the control in which a vital epithelial layer was evident (depicted by viable green cells), hyphal formation was clearly evident at 6 h on the surface of the inoculated epithelial cells. Moreover, at 12 h, there was an unmistakable further increase in hyphal formation, in conjunction with greater epithelial cell death (red cells). This 12 h time point was therefore chosen as optimal for testing the RHE tissues with OligoG.

Fig. 4 shows OligoG-treated RHE sections. An intact epithelium composed of viable (green) keratinocytes in the controls (Fig. 4a), contrasted with increasing numbers of non-viable (red) keratinocytes and abundant hyphal formation in the infected RHE tissues, with a striking decrease in hyphal formation observed in the 0.2% OligoG-treated samples. No change in the ratio of live to dead cells was evident.

Quantification of hyphal invasion was conducted using PAS-stained RHE cross-sectional images (Fig. 4b). PAS images demonstrated intact stratified keratinocytes in the uninfected control. However, in the infected control, the stratified layers were clearly infiltrated with hyphae penetrating at least half way through the keratinocyte layers. Quantification of hyphal invasion of the epithelial surface in the RHE model showed a statistically significant reduction in the depth of hyphal invasion observed in the 0.2% OligoG-treated RHE tissues ($p<0.05$; n=144 measurements of 10 images/section

with ≥3 measurements of depth of invasion/image) compared to the untreated control, with the hyphae seen predominantly on the tissue surface rather than invading the underlying keratinocytes.

Production of the virulence factor phospholipase was reduced in the presence of OligoG

Standard egg-yolk plates showed that phospholipase (PL) production by *C. albicans* ATCC 90028 cells pre-treated with OligoG was significantly decreased, but only at the 5 day time point using 6% OligoG (Fig. 5**;** $p<0.05$). Slight strain variations were evident, with no significance seen following OligoG pre-treatment with strain CCUG39343. However, incorporating OligoG into the egg-yolk plates produced a pronounced dose-dependent effect, with significantly decreased production of PL evident at days 3, 5 and 7 (at ≥2% OligoG; $p<0.05$; **Fig. 5**) for both strains tested.

Analysis of secreted aspartyl protease (SAPs) production showed that treatment of *Candida* with OligoG failed to induce significant differences in hemolytic activity, in either pre-treatment ($p>0.05$; **Fig. S1a**) or following incorporation of OligoG into the agar ($p>0.05$; **Fig. S1b**). Unusually, the two *C. albicans* strains tested (ATCC 90028 and CCUG 39343) produced no SAPs in the plate assays, even in untreated controls after 7 day incubations, suggesting that the assay was not sufficiently sensitive.

OligoG reduced expression of key phospholipase and secreted aspartyl proteinases

Quantitative RT-PCR demonstrated OligoG induced a decrease in key phospholipase and secreted aspartyl proteinase production at concentrations ≥0.2%. However, the data only reached statistical significance ($p<0.05$) for *PLB2*, *SAP4* and *SAP6* expression at 6% OligoG (**Fig. 6**). A dose-dependent decrease in *SAP5* was also evident at 6% OligoG, however this did not reach statistical significance.

OligoG did not alter the surface charge of the candidal cell wall

Zeta-potential measurements demonstrated that the surface charge of *Candida* was increased following OligoG treatment, at pH 5 and 7 (not pH 9). Following thorough washing, these changes were not significantly different from the control in any of these test conditions (**Fig. S2**).

Discussion

This study demonstrated the effect of OligoG on the important human fungal pathogen *C. albicans* and showed the ability of this polymer therapy to modify virulence factor production and invasion of *C. albicans in vitro.* Numerous *in vivo*, *ex vivo* and *in vitro* models of candidal biofilm formation and invasion have been developed. Whilst no single in *vitro* model of *Candida* infection exists, numerous studies have employed this RHE model (de Fraissinette *et al*. 1999) which, although lacking any immunological component, has been extensively used to study *Candida*/keratinocyte interactions/invasion at the gene, protein and cellular level (Schaller *et al*. 2001; Schaller *et al*. 2002; Bartie *et al*. 2004; Jayatilake *et al*. 2005; Malic *et al*. 2007). Although authors have criticized the model (Murdoch *et al*. 2005; Colley *et al*. 2011; Yadev *et al*. 2011) it is still useful as it allows the early events of fungal pathogenesis *i.e.* adhesion to the keratinocyte surface and initial invasion of the insoluble keratinocyte barrier of cross-linked proteins and lipids (Schaller *et al*. 2006) to be studied. Researchers have also shown how strain-dependent invasiveness in the RHE may reflect pathogenicity *in vivo* (Malic *et al*. 2007). It should also be borne in mind that in the early stages of pathogenesis, keratinocytes (via this physical barrier and their expression of cytokines and Toll-like receptors) represent an important element of the dermal innate immune response to fungal and many other pathogens (Mogensen 2009).

Imaging revealed that no candidal hyphal infiltration was evident at <6 h. Beyond 6 h however, adherence and hyphal formation were clearly evident. At 12 h, LIVE/DEAD® staining demonstrated markedly decreased hyphal formation on the surface of the OligoG-treated RHE models, with few actual hyphae visible in contrast to the abundant hyphae present in the untreated control. Whilst these results may appear to contrast with the growth inhibition described in previous studies, these were not however, apparent at early time points ≤12 h; the previously-described differences being evident only at 48 h (Tøndervik *et al*. 2014).

Whilst attachment, proliferation and biofilm assembly are important in colonization of the epithelial surface, invasion is a key pathogenic phenotype in candidal pathogenesis (Bartie *et al*. 2004; Mayer *et al*. 2013). Invasion studies here employed cross-sectional imaging of RHE as previously utilized in virulence studies of clinical *Candida* isolates (Malic *et al*. 2007; Boros-Majewska *et al*.

2014), which revealed that OligoG was not fungicidal (as ascertained by the absence of red-stained, non-vital cells), differential quantification of samples, as previously described by Boros-Majewska *et al*. (2014), could not be employed. Analysis of invasion in the RHE and agar model systems revealed that whilst no differences in fungal viability were evident, OligoG treatment induced markedly reduced invasion in both model systems, with a dose-dependent decrease in hyphal formation being evident in the agar model. Interestingly at high concentrations of OligoG (>6%) in the agar model, addition of the alginate oligomers reduced the structural integrity of the agar. The decreased invasion, therefore, did not reflect the OligoG induced alteration in physical density of the media. The findings in the RHE model were in line with the reduced *Candida* growth demonstrated in the presence of OligoG. It should be noted that the use of higher concentrations of OligoG (>2%) was not possible in this system as higher concentrations impaired both direct visualization and the cellular viability of the epithelial component of the superficial keratinocyte layer, in this (avascular) air-liquid interface model. This effect on keratinocyte viability is a particular feature of this model and has not been observed in the extensive pre-clinical (*in vitro*), animal, and human clinical testing of chronically-inhaled OligoG prior to EMA and FDA approval (Pritchard *et al*. 2016).

Dermal invasion by *Candida* is facilitated by production of a heterogeneous group of hydrolytic enzymes (Schaller *et al*. 2005). SAPs are believed to mediate *Candida* invasion of epithelial cells, with *SAP5* in particular, also strongly associated with proteolytic degradation of E-cadherin found in the intracellular junctions of keratinocytes (Villar *et al*. 2007). Interestingly, as keratinocyte damage is not prevented by pepstatin A (which partially inhibits invasion), other mechanisms are, therefore, clearly important (Naglik *et al*. 2008). *SAPs 1-3* have been shown to have a direct role in the tissue damage of superficial infection whilst *SAPs 4-6* are important for invasion and interaction with components of cellular defense (Schaller *et al*. 2001) and yeast to hypha transition (Naglik *et al*. 2003). A reduction in *SAP4-6* in the presence of OligoG (being significant for *SAP 4* and *6*) could explain the corresponding decrease in hyphal formation and invasion. However, the pathogenicity of SAPs remains contentious (Correia *et al*. 2010) with poor correlation found between individual *SAP* gene-expression and epithelial damage (Naglik *et al*. 2008). *SAP* expression is also dependent on environmental pH (Staib *et al*. 2000), therefore, it is unsurprising that SAP production in *C. albicans* was not detected

phenotypically in this study. The marked reductions in hyphal formation, which were induced by OligoG, were reflected in alterations in the gene expression of *PLB2*, *SAP4* and *SAP6*, evident by qPCR analysis. The lower expression of PLs and SAPs in the RHE model system may reflect their decreased expression in established laboratory isolates, and the reduced sensitivity of the plate-based assay in comparison to the qPCR (Boriollo *et al*. 2009).

The effects of OligoG on hyphal formation and fungal invasion in the RHE model reflected its previously reported reduction of germ-tube formation *in vitro* (Tøndervik *et al*. 2014). *In vivo,* invasion is not only mediated by hydrolytic enzymes, but may be potentiated by synergistic interactions with other bacterial species on the skin or mucosal surfaces e.g. *Staphylococcus* (Zago *et al*. 2015) and *Streptococcus* spp. (Bamford *et al*. 2009). *SAPs 4-6* were investigated due to their involvement in systemic infections (Malic *et al*. 2007). *SAP 4-6* are hyphal-specific; their expression being associated with invasive candidal strains. It has recently been suggested that the importance of SAPs may be an over-stated epiphenomenon in *ex-vivo* systems, reflecting experimental conditions, rather than pathological invasion *in vivo* (Naglik *et al*. 2008). Both *SAPs 1-3* and *SAPs 4-6* subfamilies were reported to be lower when analysed using qPCR in the RHE model, indicating that SAP production needs to be further analysed *in vivo* (Naglik *et al*. 2008).

C. albicans is known to possess up to five extracellular phospholipases (*PLB1-5*) which may also play a role in virulence, possibly via disruption of the host membrane (Mayer *et al* 2013). PL activity represents an important virulence factor in not only *Candida*, but also in a range of other fungi including *Aspergillus* (Alp and Arikan 2008), *Cryptococcus* (Ganendren *et al*. 2006) and bacteria such as *Clostridium perfringens* and *Pseudomonas aeruginosa* (Ghannoum 2000). Candidal PLs are a heterogeneous group of important enzymes associated with invasion (Ghannoum 2000) and expression is localized to the peripheries of the hyphal tips and initial sites of bud- formation (Jayatilake *et al*. 2005) (where they act as a mechanical anchor for yeast survival on the keratinocyte surface). *PL* expression is reduced in non-hyphal forming species e.g. *C. glabrata* (Kantarcioglu and Yucel 2002) and are believed to modify host transduction pathways, perturb cell signaling (Oishi *et al*. 1988) and local immune responses (Soares *et al*. 2010). Whilst *PL* expression has however previously been shown

not to correlate with RHE invasion in this model (Malic *et al*. 2007) it was interesting that the reduced hyphal formation and invasion induced by OligoG was associated with a significant decrease in Plb2.

Studies of the interaction of OligoG with the *Candida* cell wall were deemed important as alterations in pseudomonal bacterial (and biofilm) behavior had previously been shown related to bacterial cell-surface binding and modification of cell-surface charge by long-range forces (which determine pathogen/host cell interactions) (Powell *et al*. 2014). Interestingly, whilst ELS experiments with *P. aeruginosa* showed how alginate oligomers bound tightly to the bacterial cell surface and resist hydrodynamic shear, in *Candida* no such changes were apparent. Treated candidal specimens also failed to demonstrate the "clumping"/aggregation previously observed in *P. aeruginosa* biofilms. These findings reflect the contrasting nature of the yeast cell wall (composed of glucan, chitin and mannoproteins), with that of the lipid-rich pseudomonal outer membrane incorporating both peptidoglycan and lipopolysaccharide (Hawrani *et al*. 2010).

In conclusion, these studies have demonstrated the ability of the alginate oligosaccharide OligoG CF-5/20, to modify virulence factor expression in *Candida* and inhibit hyphal formation and invasion. The observed reduction in hyphal infiltration reflected reductions in hydrolytic enzyme production. These findings demonstrate direct and indirect mechanisms by which OligoG may influence candidal invasion and be of potential utility in the management of fungal pathogens in human disease.

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Conflict of interest

D.W.T. has a consultancy relationship and has, with K.E.H., received research funding from AlgiPharma AS. P.D.R. is the R&D director of AlgiPharma AS. The other authors have no conflicts of interest to disclose.

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589 **Table 1** Primers used for quantitative PCR.

Target gene	Additional information	Forward primer (5' - 3')	Reverse primer (5' - 3')	Reference
SAP4	Secreted aspartyl proteinase	AATGATGTGGGCAAAGAGG	ACACCACCAATACCAACGGT	(Monod *et al*. 1994; Monod *et al*. 1998)
SAP5	Secreted aspartyl proteinase	ATTAATTGATGCGGCTCCAG	ACACCACCAATACCAACGGT	(Monod *et al*. 1994; Monod *et al*. 1998)
SAP6	Secreted aspartyl proteinase	TCCAAACCAACGAAGCTACC	GCAGGAACGGAGATCTTGAG	(Monod *et al*. 1994; Monod *et al*. 1998)
PLB1	Phospholipase B	CAACGAAGCGGTGTTGTCTA	TTGCTGCCAGAACTTTTGAA	(Sugiyama *et al*. 1999; Niewerth and Korting 2001)
PLB2	Phospholipase B	GGCCAGATGGATCAGCTTTA	AAGTTCTGGGCATCACATCC	(Sugiyama *et al*. 1999; Niewerth and Korting 2001)
ACT1	*C. albicans* actin reference gene	TGCTGAACGTATGCAAAAGG	TGAACAATGGATGGACCAGA	(Cavalcanti *et al*. 2015)

590

Figure legends

Figure 1 Growth of *C. albicans* in the presence of OligoG. Growth curves of *Candida albicans* ATCC 90028 (n=3) ± OligoG at 0% (○), 0.2% (□), 2% (●), 6% (■) over 24 h grown in SAB (a) Representative growth curves. (b) Mean optical density at 18 h time point (n=3; *p<0.05).

Figure 2 Agar hyphal invasion model of *C. albicans* in the presence of OligoG. Yeast Peptone Dextrose agar plate assay ± OligoG (0.2%, 2% and 6%) showing hyphal invasion with corresponding light microscopy H&E stained images of cross-sectional agar slices (x20).

Figure 3 Epithelial attachment of *Candida* in the RHE model. Time dependent invasion model showing LIVE/DEAD® CLSM images of RHE samples infected with *C. albicans* ATCC 90028 (2×10^6 cfu ml^{-1}) for 1, 3, 6 and 12 h, scale bar 40 µm.

Figure 4 Imaging and quantification of OligoG treated *C. albicans* hyphal invasion in RHE. CLSM images of RHE samples infected with *C. albicans* ATCC 90028 for 12 h. (a) LIVE/DEAD® staining, scale bar 40µm; (b) Periodic acid–Schiff staining of fixed RHE samples (blue) infected with *C. albicans* ATCC 90028 (purple) and boxplot of hyphal invasion measurements (n=64; *p< 0.05).

Figure 5 Phospholipase production of *C. albicans* in the presence of OligoG. *C. albicans* ATCC 90028 and CCUG 39343 phospholipase plate assay ± OligoG in the overnight broth or agar (0.2%, 2% and 6%) for 3 days (white), 5 days (grey) and 7 days (black) showing actual Pz values (*p< 0.05; n=3). Pz of 1.0 = no phospholipase activity.

Figure 6 Molecular quantification of hydrolytic enzyme production of *C. albicans* in the presence of OligoG. Relative gene expression of phospholipase B (*PLB1, PLB2)* and secreted aspartyl proteinases (*SAPs 4, 5 and 6)* following *C. albicans* ATCC 90028 treatment with OligoG (n=3; *p<0.05).

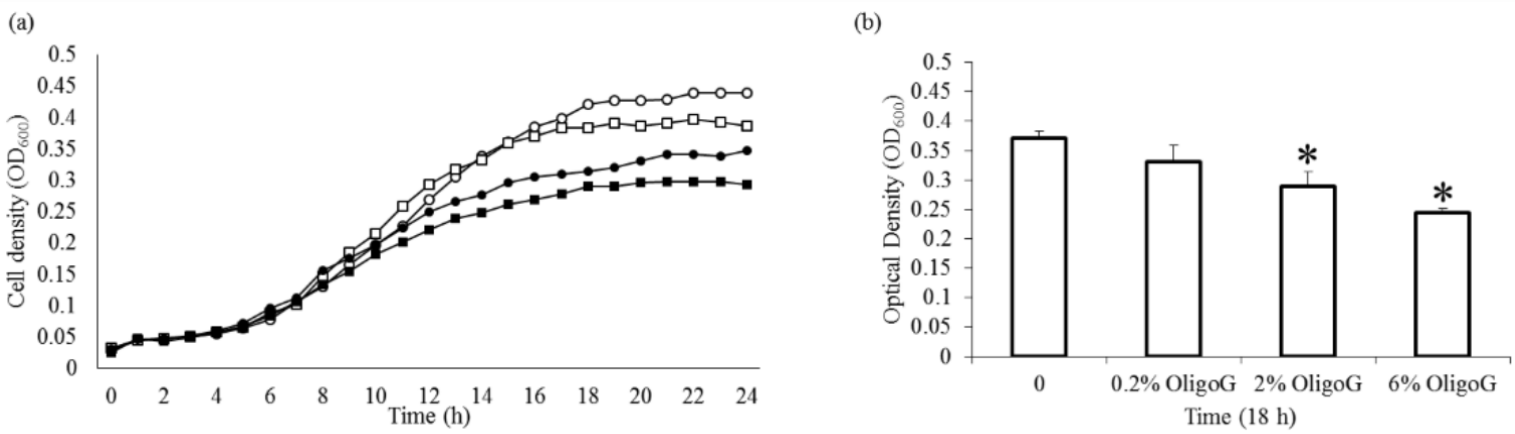
Supporting information

Figure S1 *C. albicans* ATCC 90028 hemolysis agar plate assay ± OligoG 0.2%, 2% and 6% (a) in broth or (b) in agar.

Figure S2 Mean Zeta Potential (mV) values for *C. albicans* CCUG 39343; untreated *Candida* (pre-wash), treated *Candida* with 10% OligoG (pre-wash), untreated *Candida* (post-wash) and treated *Candida* with 10% OligoG (post-wash) at 0.01 mol l^{-1} NaCl, pH 5, 7 and 9. (^ false positive result).

638 **Figure 1**

639



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647 **Figure 2**

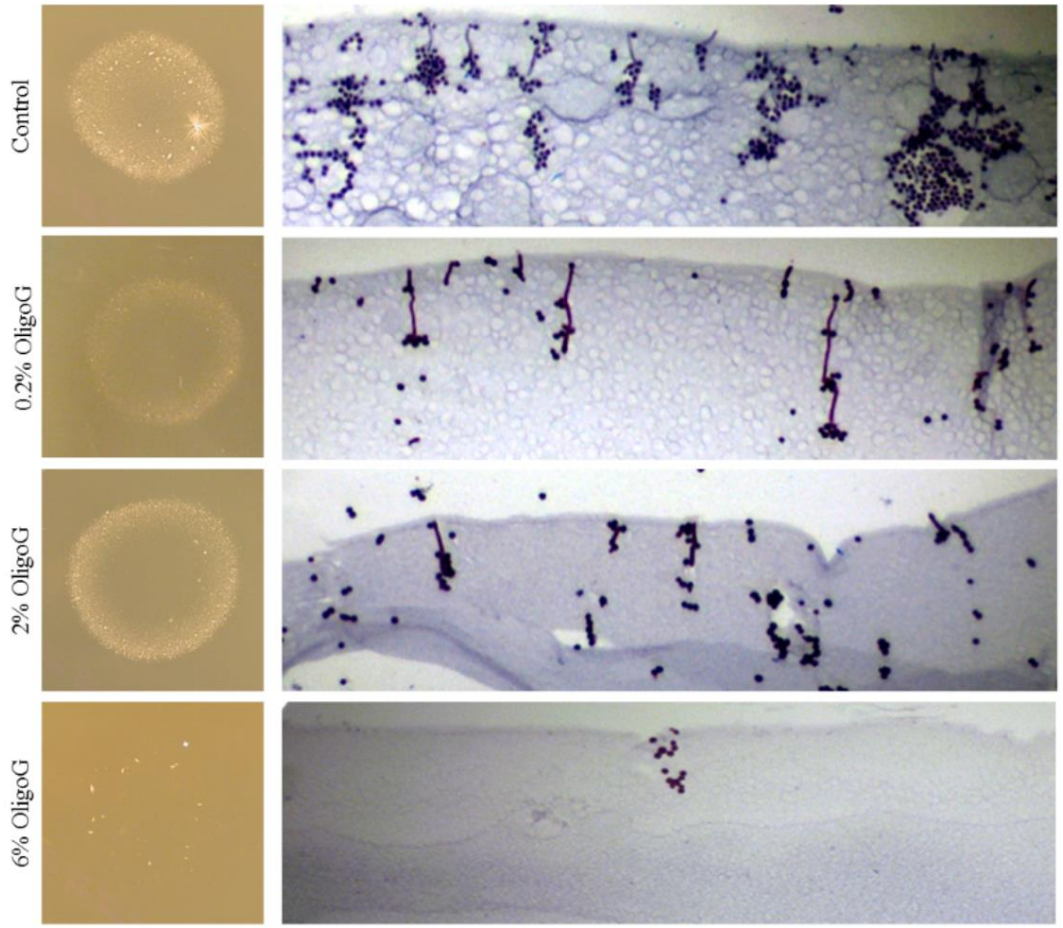

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Figure 3

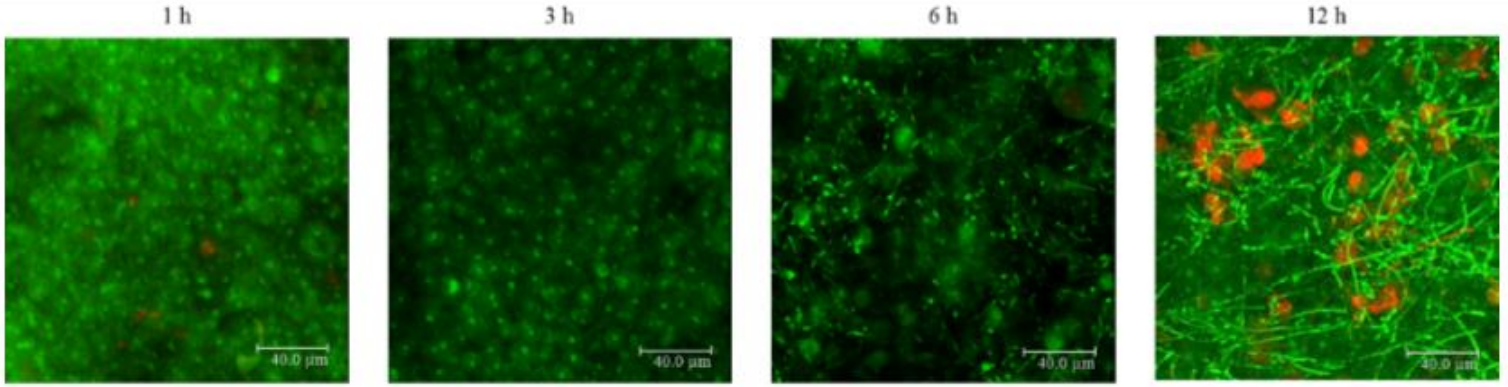


Figure 4

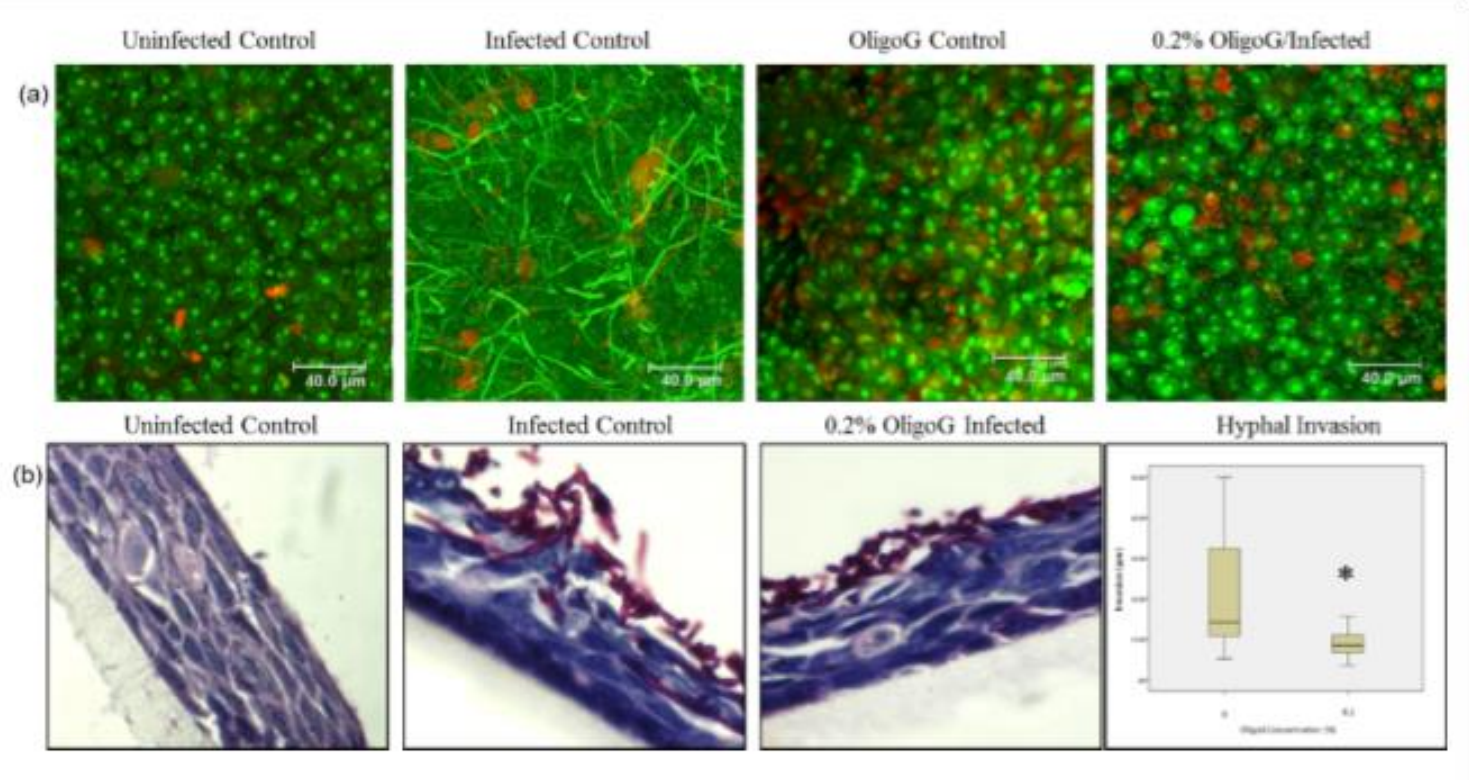
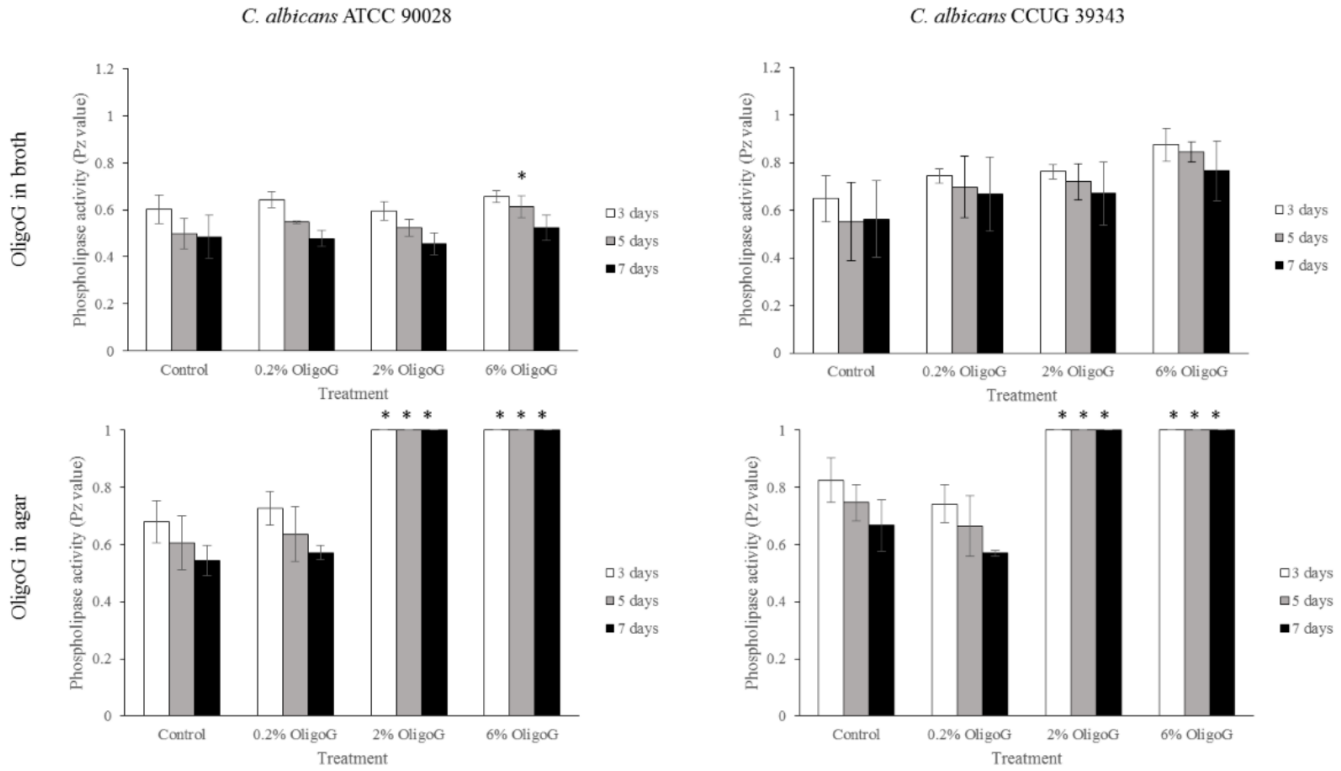
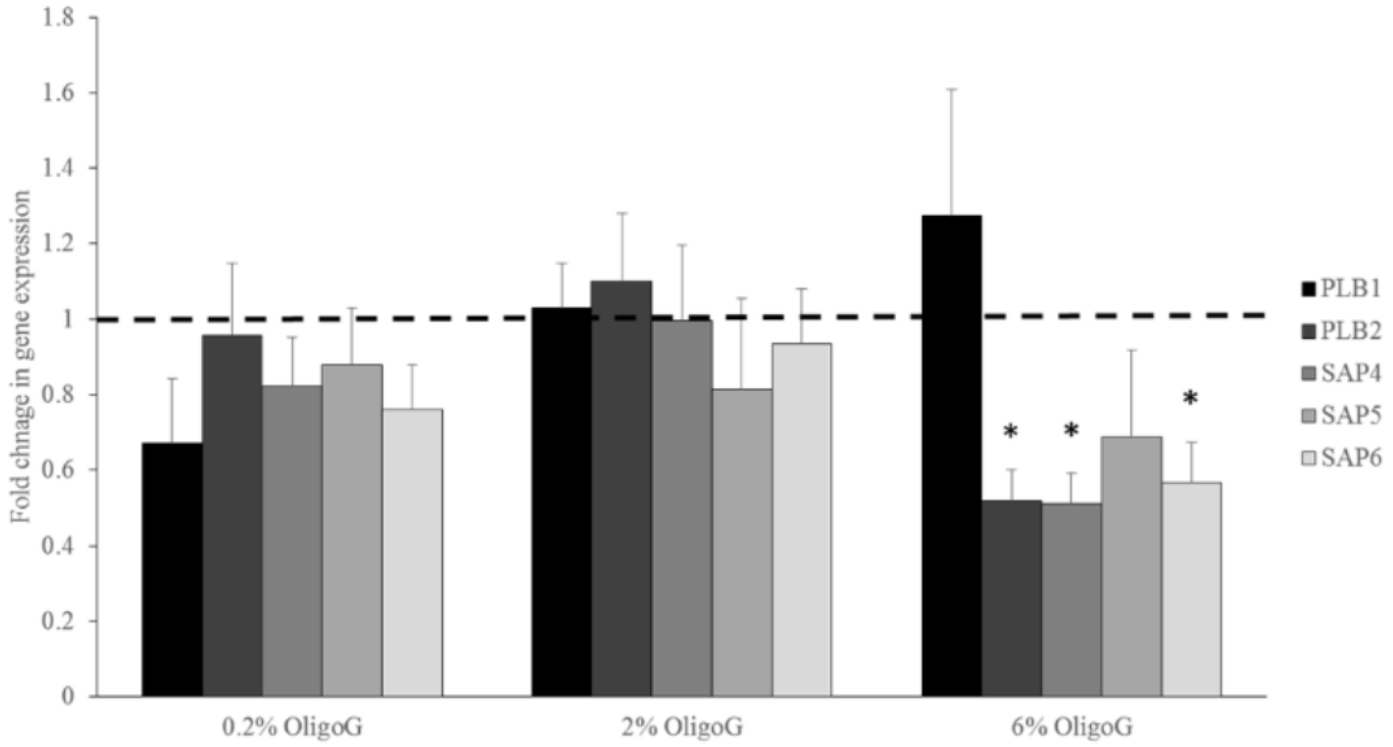

Uninfected Control
Infected Control
OligoG Control
0.2% OligoG/Infected
(a)
40.0 µm
Uninfected Control
Infected Control
0.2% OligoG Infected
Hyphal Invasion
(b)

Figure 5


C. albicans ATCC 90028
C. albicans CCUG 39343
OligoG in broth
OligoG in agar
Phospholipase activity (Pz value)
Control
0.2% OligoG
2% OligoG
6% OligoG
Treatment
3 days
5 days
7 days

669 **Figure 6**



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