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Chitosan ameliorates *Candida auris* virulence in a *Galleria mellonella* infection model

Laís Salomão Arias¹², Mark C Butcher¹⁴, Bryn Short¹⁴, Emily McKloud¹⁴, Chris Delaney¹⁴, Ryan Kean³⁴, Douglas Roberto Monteiro², Craig Williams¹⁴, Gordon Ramage¹⁴*, Jason L Brown¹⁴*

¹Oral Sciences Research Group, Glasgow Dental School, School of Medicine, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK, G12 8TA

²São Paulo State University (Unesp), School of Dentistry, Araçatuba, Department of Preventive and Restorative Dentistry, 16015-050 Araçatuba/São Paulo, Brazil

³Department of Life Sciences, School of Health and Life Sciences, Glasgow Caledonian University, Glasgow, UK, G4 0BA

⁴Glasgow Biofilm Research Network

*Authors for correspondence

Gordon.Ramage@glasgow.ac.uk
Jason.Brown@glasgow.ac.uk

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Abstract

*Candida auris* has emerged as a multi-drug resistant nosocomial pathogen over the last decade. Outbreaks of the organism in healthcare facilities has resulted in life-threatening invasive candidiasis in over 40 countries worldwide. Resistance by *C. auris* to conventional antifungal drugs such as fluconazole and amphotericin B means that alternative therapeutics must be explored. As such, this study served to investigate the efficacy of a naturally derived polysaccharide called chitosan against aggregative (Agg) and non-aggregative (non-Agg) isolates of *C. auris* in vitro and in vivo. *In vitro* results indicated that chitosan was effective against planktonic and sessile forms of Agg and non-Agg *C. auris*. In a *Galleria mellonella* model to assess *C. auris* virulence, chitosan treatment was shown to ameliorate killing effects of both *C. auris* phenotypes (NCPF 8973 and NCPF 8978, respectively) *in vivo*. Specifically, chitosan reduced the fungal load and increased survival rates of infected *Galleria*, whilst treatment alone was non-toxic to the larvae. Finally, chitosan treatment appeared to induce a stress-like gene expression response in NCPF 8973 in the larvae likely arising from a protective response by the organism to resist antifungal activity of the compound. Taken together, results from this study demonstrate that naturally derived compounds such as chitosan may be useful alternatives to conventional antifungals against *C. auris*.

Introduction

Fungal diseases are highly prevalent; nearly a billion people worldwide are estimated to have skin, nail and hair fungal infections (1). Of these diseases, healthcare-associated fungal infections are commonplace. Recently, *Candida auris* has gained unprecedented attention due to its emergence as a prolific nosocomial pathogen. Since its first discovery in 2009 (2), the organism has reportedly been identified in over 40 countries on 6 different continents, with a crude mortality rate of 66% associated with *C. auris* candidemia (3). Coupled with the alarmingly high multi-drug resistance profile in *C. auris*, this organism provides a substantial global risk in healthcare facilities and intensive care units (4-6). In addition, the organism has the ability to persist environmentally, with suggestions that its emergence has coincided with climate change based on its particular attributes (7). *In vitro* studies have shown that standard and high-level strategies of disinfection are incapable of completely eradicating *C.
auris off non-porous surfaces (8, 9), whilst cellular aggregates of C. auris can survive for as long as 14 days even following treatment with clinically relevant concentrations of sodium hypochlorite (10). As such, identification of new antifungal therapies is of utmost importance.

Whole-genome sequencing of C. auris originally led to the identification of four geographically and phylogenetically distinct clades of the organism, each containing genetically identical strains with vast (>100,000) single nucleotide polymorphism (SNP) differences between clades (4). Recently, a fifth clade has been proposed, separated from other clades by >200,000 SNPs (11). Within these clades exist C. auris isolates that have two distinguishable phenotypes; aggregative (Agg) and non-aggregative (non-Agg) isolates (12). In the former, a characteristic accumulation of aggregates containing yeast cells attached to daughter cells after budding are visible in vitro, which cannot be physically disrupted. Furthermore, such aggregates have recently been isolated from harvested tissues of murine models infected with C. auris suggestive that such a phenotype can be observed in vivo (13). In vitro, the non-Agg phenotype, which are characterised by sparse, individual cellular entities, form biofilms with greater biomass than Agg counterparts (14). In a Galleria mellonella killing assay, non-Agg C. auris were significantly more virulent than the aggregate-forming isolates, resulting in increased larvae death (12, 14). The identification of these unique C. auris isolates from various clades further complicates antifungal susceptibility testing, particularly given the differences in virulence traits between the two phenotypes.

Chitosan (poly-(β-1→4)-2-amino-2-deoxy-d-glucopyranose) is a naturally occurring, biodegradable, and non-toxic polysaccharide derived from deacetylated chitin (a constituent of fungal cell walls and crustacean exoskeletons), with wide spectrum antimicrobial activity against different microorganisms (15). As such, the antimicrobial polymer compound provides an exciting alternative to conventional antibacterial and/or antifungal therapeutics. Indeed, several studies have investigated the antifungal efficacy of chitosan or derivatives against a range of Candida species including C. albicans and other clinically relevant fungal species (16-20). At this juncture, the aim of this study was to test the ability of chitosan against non-Agg and Agg isolates of C. auris in vitro. Furthermore, chitosan efficacy was then
tested against two candidate *C. auris* isolates *in vivo* (one non-Agg isolate, NCPF 8973, and one Agg isolate, NCPF 8978) in a *Galleria* infection model.

### Methods

#### Microbial growth

Four Agg and four non-Agg isolates of *C. auris* were used in this study were kindly gifted by Dr Andy Borman (Public Health England). The eight isolates and their clades are shown in Table 1. These isolates were taken from various clinical sites (Public Health England National Collection of Pathogenic Fungi [NCPF]) as previously described (12, 21). The isolates were deemed aggregative if they could not be physically disrupted by vigorous vortex mixing or by detergent treatments as previously described (12).

All isolates were stored in Microbank™ beads (Pro-lab Diagnostics, UK) then grown at 30°C for 24-48 h. All isolates were maintained on Sabouraud dextrose (SAB) agar (Oxoid, Hampshire, UK) at 4°C prior to propagation in yeast peptone dextrose (YPD; Sigma-Aldrich, Dorset, UK) medium overnight (16 h) at 30°C, gently shaking at 200 rpm. Cells were pelleted by centrifugation (3,000 x g) then washed two times in PBS. Fungal cells were then standardized to desired concentration after counting using a haemocytometer in appropriate media as described below.

#### Planktonic and sessile susceptibility testing with chitosan

Chitosan used throughout this study was purchased from Sigma-Aldrich (medium molecular weight, 75-85% deacetylated, cat. no. 448877). Chitosan stocks of 1.4 g/l were freshly prepared in 2% acetic acid, constantly stirring (200 rpm) for 24 h at room temperature until complete solubilisation as previously described (22, 23). All subsequent studies were then completed in accordance with the minimum information guidelines specified for planktonic and/or biofilm testing in microplates (24). Where appropriate, Clinical and Laboratory Standards Institute (CLSI) guidelines were followed for all planktonic susceptibility tests (25).

Firstly, the broth microdilution method (25) was used to determine the minimum inhibitory concentration (MIC) of chitosan on planktonic *C. auris* cells (pMICs). In short, yeast cells
were standardized to $1 \times 10^4$ CFU/mL in Roswell Park Memorial Institute 1640 (RPMI) media. These were then inoculated in 96-well round bottom plates (Corning, UK) containing serial double dilutions of chitosan (ranging from 0.68 to 350 mg/l), and pMICs visually determined after 24 - 48 h. For comparative purposes, conventional anti-fungal (Fluconazole, Amphotericin B and Echinocandins; Micafungin and Caspofungin) PMIC testing was also done for all eight isolates as assessed by broth microdilution method in a similar manner as described above.

For biofilm (sessile) MICs (sMICs), the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt [Sigma-Aldrich, UK]) metabolic reduction assay was used, as described elsewhere (26). In brief, biofilms were formed by culturing yeast cells at $1 \times 10^6$ CFU/mL in RPMI media in flat-bottom wells of 96-well plates (Corning, UK) for 24 h at 37°C, prior to treatment for an additional 24 h. The $s\text{MIC}_{50}$ and $s\text{MIC}_{80}$ corresponds to the concentration that resulted in 50% and 80% reduction of XTT readings when compared to the untreated positive control. All MIC tests were carried out on two separate occasions in quadruplicate wells of a 96-well plate. For all experiments, appropriate negative controls minus inoculum were included on each plate in quadruplicate.

**Galleria mellonella infection model**

Two isolates of *C. auris* (one non-Agg isolate, NCPF 8973 and one Agg isolate, NCPF 8978) were selected for *Galleria mellonella* killing assay as previously described (14). *G. mellonella* larvae were infected with these two isolates in the presence and absence of chitosan in a similar manner to as described elsewhere (27-29). In short, ten sixth-instar *G. mellonella* larvae (Livefoods Direct, Ltd., UK) weighing between 200 and 300 mg were selected for each test group. For infection, a 50 µl Hamilton syringe equipped with 26-gauge needle was used to inject *C. auris* into the *Galleria;* 10 µl of *C. auris* ($2.5 \times 10^5$ cells/Larva) were inoculated through the hindmost right proleg of each larva. The infected larvae were placed in sterile Petri dishes and incubated at 37°C for 2 h. After this period, larvae were injected in the last left proleg with chitosan at different concentrations (50, 100 and 200 mg/kg). Larvae inoculated with PBS and the highest dose of chitosan alone (e.g., 200 mg/kg) were also included as controls. The percentage survival of the larvae was monitored every 24 h over 4 days. A larva was considered dead when it displayed no movement in response to touch.
together with a dark discoloration of the cuticle. The experiment was repeated on three
separate occasions with 10 larvae per group.

**RNA extraction and differential gene expression analysis in C. auris**

RNA was extracted from infected and uninfected larvae as follows. Three larvae from each
experimental group were snap-frozen in liquid nitrogen and ground to a fine powder by
mortar and pestle in TRIzol™ prior to bead beating with 0.5 mm glass beads using a BeadBug
microtube homogeniser for a total of 90 seconds (Benchmark-Scientific, New Jersey, USA).
RNA was then extracted using the RNeasy Mini Kit according to manufacturers’ instructions
(Qiagen Ltd, Crawley, UK) and quantified using a NanoDrop 1000 spectrophotometer
[Thermo Scientific, UK]. RNA was converted to complementary DNA (cDNA) using the High
Capacity RNA to cDNA kit (Life Technologies, Paisley, UK) as per the manufacturer’s
instructions. Gene expression of the *C. auris* infected in the larvae was determined using
quantitative polymerase chain reaction (qPCR) from a total of three individual larvae, each
taken from three independent experiments. For qPCR analyses, the StepOne Plus PCR
machine was used with the following PCR thermal profiles; holding stage at 50°C for 2
minutes, followed by denaturation stage at 95°C for 10 minutes and then 40 cycles of 95°C
for 3 seconds and 60°C for 15 seconds. Expression levels of each gene of interest were
calculated using the ΔΔCT method (30), with expression normalized to house-keeping gene
β-actin. qPCR gene expression data was presented as % expression relative to house-keeping gene or Log2 fold change in untreated and treated NCPF 8973 relative to NCPF 8978. All primer sequences used for qPCR are shown in Table 2.

**DNA extraction for calculating fungal burden in Galleria model**

Uninfected and infected *Galleria* larvae were processed as above for RNA extraction before
a back-extraction buffer (50 mM sodium citrate, 4 M guanidine thiocyanate and 1M Tris [pH
8.0]) was used to extract DNA from samples as previously described (27). Colony-forming
equivalents (CFE) of *C. auris* were then determined using ITS gene primers (sequences
shown in Table 2) by qPCR and CFE/mL quantified using a standard curve methodology of
fungal colony forming units (CFU). Briefly, CFUs of *C. auris* NCPF 8973 and NCPF 8978 that
equate to $1 \times 10^3$ to $1 \times 10^8$ cells/mL were determined using a haemocytometer prior to
DNA extraction. DNA extracted from $1 \times 10^3$ to $1 \times 10^8$ cells/mL of *C. auris* was used for
quantitative analyses using qPCR to generate a standard curve. All samples, including standards were run in duplicate for qPCR analyses. Fungal load was calculated from a total of three individual infected larvae each taken from three independent experiments.

**Scanning and transmission electron microscopy**

For scanning electron microscopy (SEM), non-Agg *C. auris* (NCPF 8973) and Agg *C. auris* (NCPF 8978) were grown as described above. Biofilms were then prepared as above by growth in RPMI-1640 medium on 13 mm Thermanox™ coverslips (Fisher Scientific, UK) placed in the bottom of 24-well microtiter plates (Corning, UK) for 24 h at 37°C. After incubation, biofilms were treated with chitosan at 40 or 80 mg/l (diluted in RPMI-1640 medium) for an additional 24 h then prepared for scanning electron microscopy (SEM) as previously described (31). In brief, following incubation chitosan-treated and untreated biofilms were washed three times with PBS prior to fixation in a solution containing 2% glutaraldehyde, 2% paraformaldehyde, 0.15% alcian blue and 0.15 M sodium cacodylate (pH 7.4). Biofilms were then sputter-coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope (JEOL Ltd, Hertfordshire, UK). All SEM images included in this study were captured at 15,000 x magnification.

For transmission electron microscopy (TEM), samples were prepared as follows. Non-agg *C. auris* (NCPF 8973) and Agg *C. auris* (NCPF 8978) were grown in YPD as described above then standardised to 1x10⁸ cells/mL in PBS. A total of 1 mL aliquots of each isolate were transferred to Eppendorfs and treated for an additional 24 hours, planktonically, with chitosan at 40 mg/l diluted in RPMI-1640 medium. Post-treatment, the samples were centrifuged at 13,000 x rpm for 5 minutes, supernatants discarded, and pellet retained for TEM and prepared for imaging as previously described (32). Following preparation, samples were embedded in araldite/Epon 812 resin and sectioned using a LEICA ultracut UCT and DIATOME diamond knife. Samples were imaged on a JEOL1200EX TEM running at 80 kV. All TEM images included in this study were captured at 6000 x or 25,000 x magnification.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism (version 8; GraphPad Software Inc., La Jolla, CA). Two-tailed Student’s t-tests were used to compare the means of two samples or one-way analysis of variance (ANOVA) to compare the means of more than two
samples. Tukey’s post-test was applied to the p value to account for multiple comparisons of the data. Where appropriate, statistical tests on qPCR gene expression data were completed on ΔCT values. Pooled data from three independent experiments of G. mellonella larvae killing assay were assessed using the Kaplan–Meier method, and treatment groups were compared by the log-rank (Mantel–Cox) test. P-values of < 0.05 were considered statistically significant for all tests.

**Results**

Given the well-established drug resistance profile of C. auris (4), we sought to assess the antimicrobial potential of chitosan, a polymer purported to have broad spectrum activity. We assessed the activity of chitosan against a selection of C. auris isolates with different phenotypes, both *in vitro* and *in vivo*. Firstly, the minimum inhibitory concentration for planktonic (PMIC) and sessile (SMIC) cells were determined for a total of eight different isolates of C. auris (four non-Agg and four Agg isolates, as shown in Table 1). All isolates used possessed similar sensitivity profiles to conventional antifungals as based on sensitivity profiles reported previously (Table 1) (12, 14). PMIC values varied between 5-20 mg/l chitosan from all isolates, with the highest PMIC (20 mg/l) observed for two Agg isolates (NCPF 8977 and NCPF 8978). It is noteworthy that planktonic forms of these two isolates have previously been shown to be highly resistant to caspofungin (14). The SMIC<sub>50</sub> and SMIC<sub>80</sub> values also varied between non-Agg and Agg phenotypes of C. auris. For SMIC<sub>50</sub> these values ranged between 10-80 mg/l, and for SMIC<sub>80</sub> from 40-160 mg/l. The highest SMIC<sub>80</sub> was detected in the same two Agg isolates as above (160 mg/l; NCPF 8977 and NCPF 8978, respectively). Interestingly, there was a certain level of heterogeneity observed in the non-Agg and Agg isolates response to chitosan treatment irrespective of aggregative phenotype.

Next, 24-hour chitosan-treated C. auris biofilms were visualised using scanning and transmission electron microscopy (SEM and TEM) to assess the ultra-structure and morphology of the cells after treatment. For these and subsequent studies, one non-Agg (NCPF 8973) and one Agg (NCPF 8978) isolate was selected for analysis. In SEM, at 15,000 x magnification, morphological differences in the sessile C. auris cells were observed after exposure to chitosan. Untreated non-Agg C. auris biofilms displayed singular oval-shaped
yeast cells (Figure 1A), while Agg phenotype of *C. auris* resulted in clusters or “aggregates” of oval-shaped yeast cells (Figure 1B). At both concentrations of chitosan (40 and 80 mg/l), the drug can be seen coating and encapsulating *C. auris* cells in the biofilms (Figure 1C-F). Intriguingly, the higher concentration of chitosan appeared to adsorb to the cell surface and puncture the non-Agg *C. auris* cell, resulting in a deflated appearance likely resulting from cell death (white arrow; Figure 1E). At the same concentration, chitosan can be seen coating the Agg *C. auris*, however, with no obvious change in morphology (Figure 1F). Such a discrepancy in ultra-structure between the two phenotypes may correlate with the differences in MICs as shown in Table 1, whereby 80 mg/l was two times the SMIC$_{80}$ for the non-Agg *C. auris* NCPF 8973.

Further analyses into the interactions between chitosan and *C. auris* were achieved using transmission electron microscopy (TEM). TEM images showed that chitosan particles coated the cells of both *C. auris* isolates (Figure 2). Untreated cells for non-Agg and Agg *C. auris* appear darkened, with dense intracellular material and a thick, uniform cell wall (Figure 2A and 2B). Following treatment with 40 mg/l of chitosan, particles of the compound are seen coating the cell walls/membranes of both isolates (denoted by red arrows; Figure 2C and 2D). At higher magnification, accumulation of chitosan is evident in periphery of the cell, leading to penetration of the cell wall and membrane (white arrows; Figure 2C and 2D, right panels). Interestingly, chitosan-coated cells for both isolates appear transparent with evidence of a loss of cell morphology and/or cell components, potentially resulting from an efflux of intracellular material following penetration of the cell by the compound.

Given the differences in Agg and non-Agg *C. auris* response to chitosan *in vitro*, the efficacy of chitosan was next tested *in vivo* in a *G. mellonella* infection model. Firstly, the virulence of non-Agg and Agg *C. auris* was assessed in the model using a Kaplan-Meier plot to monitor the survival of infected *G. mellonella* larvae over 4 days post-infection. Similar to previous studies, the non-Agg NCPF 8973 was significantly more virulent than the Agg NCPF 8978 isolate. Infection with NCPF 8973 resulted in killing of approximately ~47% of the larvae four days post-infection, while following infection with NCPF 8978 almost 80% of the larvae remained alive (Figure 3). Following treatment with chitosan, the compound ameliorated the killing effects of both *C. auris* isolates in the infection model. After treatment, at the highest concentration, 200 mg/kg chitosan significantly increased the survival rates of the
larvae infected with NCPF 8973 from approximately 55% to 84% (Figure 3A; **** p<0.0001 according to log-rank test). For the Agg phenotype, in comparison to the untreated group, survival rates of Agg C. auris-infected larvae were significantly increased to ~87% from ~75% when treated with 200 mg/kg chitosan (Figure 3B; * p<0.05).

In order to provide mechanistic insights behind the observed protective effect of chitosan in vivo, fungal load and C. auris gene expression was determined in the infected larvae +/- chitosan treatment. Firstly, fungal load in larvae was significantly reduced following treatment with chitosan (200 mg/kg) for both non-Agg and Agg infection models (Figure 4A and B, both * p<0.05 respectively). For gene expression analyses in the fungi, expression of genes related to adhesion (ALS5, HYR3), hydrolytic enzymes (SAP5, PLB1), cell wall, cell membrane and extracellular matrix (ERG2, KRE6, EXG, ENG1) were investigated in relation to the housekeeping gene β-actin. These candidate genes were selected for analyses as previously described; these and similar genes are differentially regulated in early and mature biofilms of C. auris (33). Expression of all genes arrayed (with the exception of SAP5) were upregulated in the non-Agg C. auris isolate when presented as Log2 fold change relative to the Agg isolate (Figure 5). All genes were upregulated in the non-Agg isolate following treatment with the highest concentration of chitosan, compared to the untreated controls (Supplementary Figure 1; white bars). The greatest change in expression were seen for ALS5, changing from ~0.49% average expression in untreated controls, to ~13.02% average expression following treatment with 200 mg/kg chitosan (Supplementary Figure 1B). For the Agg isolate, SAP5 was the only gene upregulated following treatment with the highest chitosan concentration (Supplementary Figure 1C; ~1.18% in the untreated controls compared to ~21.88% for 200 mg/kg). EXG and ENG1 were the most downregulated genes; expression changed from 8.92% and 6.41% in controls compared to 0.98% and 0.27% for 200 mg/kg chitosan-treated. Taken together, following treatment, chitosan appeared to induce a stress-like response in the non-Agg isolate compared to Agg isolate (Figure 5).

Discussion

Given the propensity for C. auris to be resistant to a wide range of azoles, polyenes, and echinocandins (4, 34), alternative treatment methods need to be explored. Here, a naturally derived compound called chitosan was shown effective against Agg and non-Agg isolates of
C. auris both in vitro and in vivo. Recently, the compound was shown to be effective against C. albicans and other Candida species (16-19); the proposed mechanism of action being that positively charged chitosan molecules interact with negatively charged cell membranes leading to release of proteinaceous and intracellular constituents, causing cell death (15, 35, 36). Here we were able to show that Agg and non-Agg C. auris planktonic and sessile cells were susceptible to chitosan treatment in vitro. Using microscopic analyses, the chitosan can be visualised coating the cell surface of the C. auris resulting in an altered morphological phenotype likely arising from cell death. In addition, C. auris fungal load was reduced and its virulence ameliorated in vivo in a Galleria infection model following treatment with the compound. Interestingly, chitosan treatment induced a stress-like gene response in the more susceptible non-Agg isolate infected in the larvae.

C. auris isolates possess unique strain-specific variability in biofilm formation and virulence (10, 12, 14). Two types of C. auris isolates exist; one that forms an aggregative phenotype in vitro, and the second that forms single cell biofilms (12, 14). Therefore, studies must account for these differences in aggregative phenotype when studying the effects of potential therapeutics against C. auris. As such, this study initially tested the susceptibility of four Agg and non-Agg isolates of C. auris to chitosan. Interestingly, C. auris response to chitosan exhibited a level of heterogeneity in the Agg and non-Agg isolates. These observations are in line with a previous study showing variation in planktonic and sessile MICs for a number of C. auris isolates taken from intensive care unit or candidemic patients against a wide range of conventional antifungal therapies such as Amphotericin B, Fluconazole and Caspofungin (37). Given the high level of heterogeneity amongst isolates to different antifungals, future work merits consideration for direct comparative studies testing novel therapeutics and conventional antifungals against isolates with different phenotypes from various clades.

MIC testing results indicated that two isolates, NCPF 8973 and NCPF 8978, had the lowest and highest PMIC and SMIC$_{80}$ of the initial pool, respectively. Therefore, we hypothesized these two isolates would provide accurate representations for the effects of chitosan against the Agg and non-Agg phenotypes. For these two isolates, the PMIC and SMIC values were higher for the Agg phenotype. This finding may be explained by Agg phenotype of C. auris providing a protective barrier to therapeutics. Indeed, we have recently shown that
**Agg C. auris** NCPF 8978 possesses the ability to survive and persist on surfaces in response to sodium hypochlorite treatment, even after 14 days post-treatment. Conversely, the non-Agg isolate **C. auris** NCPF 8973 was susceptible to such treatment (10). Here, it was evident from the SEM imaging that the non-Agg **C. auris** is visibly encapsulated by the chitosan compound leading to an altered morphology at the higher concentration of drug, whilst in the aggregative phenotype, there appears no change in cellular ultrastructure, suggestive of a protective phenotype when cells are present in aggregates. The altered morphology in the non-Agg isolate SEMs likely arose from chitosan-mediated cell death. In agreement, a publication by Ganan *et al* (2019) recently used confocal microscopic imagery to show that an oligosaccharide of chitosan (chito-oligosaccharide) generated from chemical or enzymatic digestion of chitosan was capable of adsorbing to yeast cells of **C. albicans**, subsequently disrupting cellular structure and accumulating in the cytoplasm (17). Indeed, TEM images confirmed that chitosan, albeit at a concentration above the PMIC for both isolates (e.g., 40 mg/l), was visualised coating the organism, penetrating the cell walls resulting in a loss of cellular morphology and efflux of intracellular material. Moving forward, it would be of interest to quantify the level of adsorption and cell wall/membrane damage in the **C. auris** isolates following treatment with the antifungal. However, such techniques as SEM and TEM are qualitative in nature, with no accurate way of quantifying the level of cellular damage.

In the *Galleria* model to assess **C. auris** virulence, the non-Agg **C. auris** NCPF 8973 induced significantly greater killing of the larvae than the Agg isolate, NCPF 8978. This is in agreement with previous studies from our group and others (12, 14), although why such phenomena arise is unknown. It could be postulated that single cellular forms of **C. auris** can disseminate more rapidly *in vivo* than Agg isolates, leading to increased killing rates. Others have shown that phenotypic form of **C. auris** can switch from yeast to filamentous morphology following ‘passage’ through a mammalian body, suggestive that phenotypic state is inducible under certain conditions (38). It would be of interest to assess whether such phenomena occur in *G. mellonella* larvae infected with non-Agg **C. auris**, which could explain its enhanced virulence traits in this model. Nevertheless, we were able to show that chitosan treatment ameliorated the **C. auris** virulence in this infection model, likely arising from reduced fungal load in the larvae. In a similar manner to results presented here,
several research groups have recently reported the use of novel antifungal compounds against *C. auris* in *vitro* and *in vivo* (39-41). However, such *in vivo* studies are generally limited to studying individual isolates or isolates with similar Agg or non-Agg phenotypes. Such Agg or non-Agg characteristics need to be considered particularly given that similar murine infection models have shown that *C. auris* aggregates can accumulate in tissues of infected animals (13). Therefore, we deemed it pertinent to study the effects of possible antifungal therapeutics against different *C. auris* aggregates *in vivo*. However, it must be stated that the *in vivo* observations described here are limited to one non-Agg and one Agg isolate. Therefore, assumptions about the effects of the chitosan on other isolates in similar model systems cannot be made without further studies, particularly given the high level of heterogeneity amongst isolates to the antifungal.

Finally, differential gene expression of the Agg and non-Agg *C. auris* was observed in the *Galleria* model with chitosan treatment. Several candidate genes involved in important virulence pathways associated with biofilm formation and resistance were selected for comparative expression analyses in the isolates. Although such analyses were limited to one isolate for each phenotype, results were indicative of a unique stress-like response in the non-Agg phenotype following treatment with chitosan *in vivo*, evident by an up-regulation in expression of most of the genes arrayed. In a similar manner, others have identified that chitosan can interfere in gene expression in other *Candida* species. In addition to chitosan penetrating the cell wall and membrane of the cell leading to cell death (15, 35, 36), it is postulated that the compound is able to breach the nucleus of the fungal cell interfering with the synthesis of mRNA and translation of proteins (42). An *in vitro* study recently showed that chitosan represses the function of the SAGA (Spt-Ada-Gcn5 acetyltransferase) complex in *C. albicans* by downregulating *ADA2* and associated genes, which are involved in encoding for proteins involved in maintaining cell wall and membrane integrity (16). Conversely, it could be postulated that the stress-like response of NCPF 8973 to chitosan may simply have resulted from an increased susceptibility to the compound (as shown by the *in vitro* MIC tests). Conclusive elucidation of the mechanistic response seen in *C. auris* to chitosan is currently unknown and requires further investigation.

Direct physical interactions between chitosan and the cell wall may provide an alternative mechanism by which the drug affects gene expression in *C. auris*. It is not uncommon for
antifungal therapies to induce such stress-like responses in Candida species, particularly in vitro (43, 44). For example, echinocandins such as Caspofungin which target β-glucan synthesis pathways can exert stress upon the cell wall leading to attenuated efficacy against C. albicans at high concentrations (45). Similar drug resistance profiles have recently been described for C. auris to echinocandins, resulting from a mutation FKS1 gene, which encodes for a 1-3-β-glucan synthase enzyme (46). Interestingly, in our analyses we found an upregulation in genes associated with cell wall component assembly/disassembly and cell separation pathways in the non-Agg C. auris in increasing concentrations of chitosan. In particular, the genes KRE6 (involved in β-1,6-glucan synthesis), EXG and ENG1 (exo-β-1,3-glucanase and endo-β-1,3-glucanase, involved in cell separation) were upregulated in the non-Agg phenotype following treatment of the Galleria larvae with the drug. Such genes have been shown important in virulence of other fungal species. Disrupting the β-1,6-glucan synthesis pathway by targeting KRE6 and a related gene SKN1 reduced growth and biofilm-forming rates of C. albicans, interfered with cell separation and cell wall formation, and attenuated its virulence in a murine model (47). Furthermore, mutation of ENG1 impaired virulence of Histoplasma yeasts in vivo (48). Therefore, it could be postulated that the gene expression profiles observed in study may be indicative of a response by the organism to upregulate cell wall β-glucan synthesis and cell separation in an attempt to promote survival and circumvent the antifungal effect of chitosan.

Similar gene expression responses were not seen in the Agg isolate of C. auris following treatment with chitosan. Such a result is difficult to interpret without further studies on this and other Agg isolates. However, in vitro observations from this study showed that the Agg isolate was more resistant to chitosan treatment than the non-Agg counterpart, which could explain the observed gene expression profile in vivo. As discussed above, the aggregative phenotype may simply provide a physical barrier for chitosan delivery to the cell. Indeed, this aggregative phenomenon may exist both in vitro and in vivo. Ben-Ami and colleagues recently recovered large aggregates of C. auris cells from harvested tissue of a murine model following infection, which could be a strategy used to evade the host response (13). Nevertheless, the clinical implications of aggregation in C. auris remain limited, although such phenomena have been considered for other microorganisms. The formation of aggregates in bacteria such as Pseudomonas aeruginosa enhances tolerance traits such as
antibiotic resistance and/or evasion of the host response (49, 50). It would be of great interest to assess whether such C. auris isolates can form aggregates in Galleria tissues, and whether non-Agg isolates persist as single cells in vivo. If achievable, this could begin to elucidate the resistance mechanisms utilised by C. auris against antifungals.

In conclusion, this is the first study to show that the naturally derived molecule chitosan may be effective against the putative opportunistic environmental yeast, C. auris. We and others have shown here and in previous studies that the aggregative phenotypes of different C. auris isolates dictate the response of the organism to antifungals (8, 9, 12, 14). As such, future studies must continue to investigate these unique aggregative phenotypic traits of C. auris isolates from different clades to fully comprehend the response of such isolates to conventional and novel therapeutics.

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Declaration of interest
The authors declare no conflicts of interest.

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### Tables

| Phenotype | Strain | Clade                  | PMIC [mg/l] | SMIC<sub>50</sub> [mg/l] | SMIC<sub>80</sub> [mg/l] |
|-----------|--------|------------------------|-------------|---------------------------|--------------------------|
| Non-Agg   | *C. auris* 8973* (FluR, AmpI, EchS) | Southern Asian/Indian | 5           | 40                        | 40                       |
| Non-Agg   | *C. auris* 8989 (FluR, AmpI, EchS) | Southern Asian/Indian | 10          | 40                        | 80                       |
| Non-Agg   | *C. auris* 8971 (FluR, AmpI, EchS) | Southern Asian/Indian | 10          | 40                        | 80                       |
| Non-Agg   | *C. auris* 199 (FluR, AmpI, EchS) | Unknown               | 10          | 10                        | 80                       |
| Agg       | *C. auris* 8977 (FluR, AmpI, EchS) | South African         | 20          | 40                        | 160                      |
| Agg       | *C. auris* 8978* (FluR, AmpI, EchS) | South African         | 20          | 80                        | 160                      |
| Agg       | *C. auris* 8983 (FluR, AmpI, EchS) | Southern Asian/Indian | 5           | 40                        | 40                       |
| Agg       | *C. auris* 8986 (FluR, AmpI, EchS) | Southern Asian/Indian | 10          | 20                        | 40                       |

**Table 1** – Planktonic and sessile minimum inhibitory concentrations of chitosan against eight isolates of *Candida auris*. Planktonic (pMIC) and sessile minimum inhibitory concentrations (sMIC) for chitosan against four non-aggregative (non-Agg) and four aggregative (Agg) isolates of *C. auris*. For pMICs, the broth microdilution method was employed. For sMICs, the XTT metabolic reduction assay was used, and the sMIC<sub>50</sub> and sMIC<sub>80</sub> correspond to the concentration that resulted in 50% and 80% reduction of XTT readings when compared to the untreated positive control. All MIC tests were performed on 2 independent occasions, showing identical results each time. For comparative purposes,
conventional anti-fungal susceptibility profiles are shown for all eight isolates as assessed by broth microdilution method and as previously described (12, 14). Abbreviations; FluR; fluconazole resistant, AmpI; Amphotericin B intermediate, EchI or EchS; Echinocandins intermediate or susceptible. * denotes the two isolates selected for microscopic analyses and Galleria mellonella infection model.
| Gene                  | Direction | Sequence (5’–3’)     |
|-----------------------|-----------|----------------------|
| Fungal load quantification | ITS       | TCGCATCGATGAAGAACGCAGC |
| Gene expression studies | Forward | Reverse |
|-------------------------|---------|---------|
| **B-actin**             | GGCTCATCTTGGCTTCCTT | TCTTTTCCCGCTTATTGATATGC |
| **SAP5**                | GGATGCAGCTCTTCTCGGT | CTTCCAGTTTGCGGTGGTG |
| **PLB1**                | TGCCATCTACAACCGCAACC | TCAACGACGACAAGGGAGG |
| **ENG1**                | TGTGAAGGATAGGCTGCTG | GTGCTAGTCACACCACCCAAG |
| **ERG2**                | ACACAAAGCCGAATTGCAAC | GAGAGGCAAGTGGAAGCAGT |
| **ALS5**                | ATACCAGGGTCGGTACGT | CTATCTTCCGGCCTGGGAT |
| **HYR3**                | TCGACTTCCCTGAGCCAAC | AGCTCGAAACAGCAAGCAG |
| **KRE6**                | ATCAGATCGACATGGGCTC | TCAACGCAACGAAAAACGC |
| **EXG**                 | CAACAAAGGCCTCAACTGGG | TTCATCCACCGGACGAGT |

**Table 2 – List of primer sequences used in this study.** Forward and reverse primer sequences for *C. auris* fungal load quantification and gene expression analyses in this study.

**Figure legends**
Figure 1 – Scanning electron microscopic images of chitosan-treated Candida auris. Chitosan-treated 24 h biofilms of non-aggregative (non-Agg) NCPF 8973 and aggregative (Agg) NCPF 8978 C. auris were visualised using scanning electron microscopy (SEM). Untreated non-Agg and Agg biofilms were used as controls and treated in the same way minus chitosan (A and B). Non-Agg and Agg biofilms of C. auris were treated with 40 mg/l (C and D) or 80 mg/l (E and F) for 24 h prior to imaging at 15,000 x magnification. White arrows highlight the encapsulation of C. auris cells by chitosan particles and deflation in cell morphology of the non-Agg NCPF 8973 isolate (E).

Figure 2 – Transmission electron microscopic images of chitosan-treated Candida auris. Planktonic cells of non-aggregative (non-Agg) NCPF 8973 and aggregative (Agg) NCPF 8978 C. auris at 1 x 10⁸ cells/mL were treated with chitosan prior to imaging using transmission electron microscopy (TEM). Untreated non-Agg and Agg controls were used as comparison minus chitosan treatment (A and B). Non-Agg and Agg C. auris were treated with 40 mg/l of chitosan for 24 h prior to TEM imaging at 6000 x and 25,000 x magnification (C and D). Red arrows in lower magnification panels identify the coating of C. auris cell walls with chitosan particles. White arrows in higher magnification panels highlight penetration of C. auris cell wall/membranes by chitosan.

Figure 3 – Survival curves of Galleria mellonella following infection with Candida auris. G. mellonella larvae were infected with 2.5 x 10⁵ cells/larvae of C. auris +/- chitosan treatment and survival rates monitored every 24 hours for 4 days. A total of 3 chitosan treatments were used (50 mg/kg; green line, 100 mg/kg; yellow line and 200 mg/kg; blue line). Control groups received PBS only (black line) or infected with C. auris minus chitosan treatment (red line). The highest concentration of chitosan had no effect on the survival of the larvae minus C. auris infection (data not shown). Data representative of results from three independent experiments with 10 larvae per group are shown in a Kaplan-Meier plot and statistical differences calculated between treatment groups by the log-rank (Mantel–Cox) test. * denote significant differences between highest concentration of chitosan (200 mg/kg) and infected controls minus chitosan treatment (* p < 0.05, **** p < 0.0001).

Figure 4 – Fungal load from Candida auris-infected Galleria mellonella. G. mellonella larvae were infected with 2.5 x 10⁵ cells/larvae of C. auris +/- chitosan treatment. After 24 h, larvae were harvested and weighed prior to DNA extraction. The abundance of C. auris (presented
as colony forming equivalents/g) in the larvae were calculated by quantitative PCR using a standard curve methodology of fungal colony forming units ranging from $1 \times 10^3$ to $10^8$ CFU/mL. Significant differences were calculated using a one-way analysis of variance (ANOVA) with Tukey’s post-test. Significant differences denoted by * (p < 0.05). Data representative of results from three independent experiments.

**Figure 5 – Gene expression profile of Candida auris in the Galleria mellonella infection model.** *G. mellonella* larvae were infected with $2.5 \times 10^5$ cells/larvae of *C. auris* +/- three concentrations of chitosan treatment (50 mg/kg, 100 mg/kg and 200 mg/kg). After 24 h, larvae were harvested for RNA extraction. Gene expression was measured by quantitative PCR, and expression of all genes of interest calculated relative to a house-keeping gene ($\beta$-actin). Data presented as mean values from three independent experiments in a heatmap, calculated as $\log_2$ fold-change of expression in *C. auris* NCPF 8973 relative to *C. auris* NCPF 8978 +/- chitosan treatment.
Log$_2$ fold change in NCPF 8973 relative to NCPF 8978

chitosan (mg/kg)