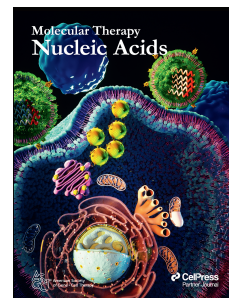


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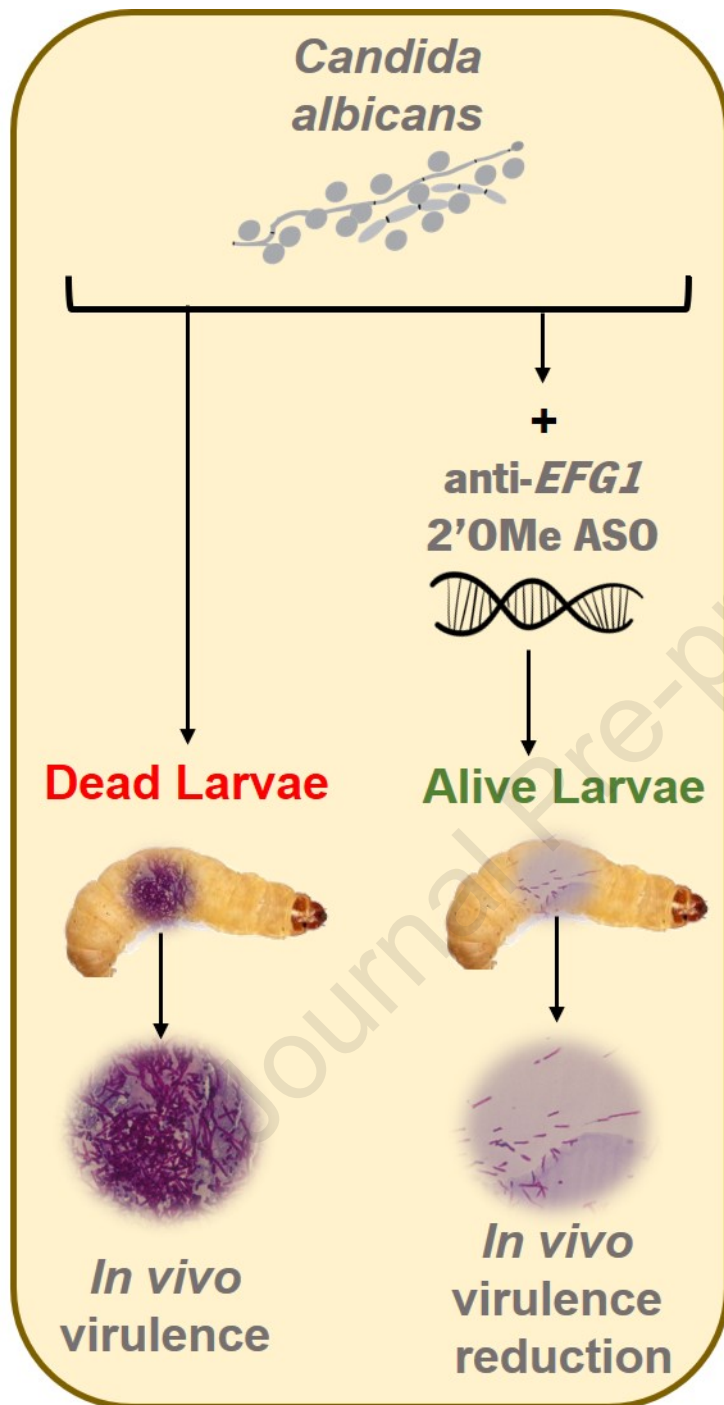
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**Anti-*EFG1* 2'-OMethylRNA oligomer inhibits *Candida albicans*  
filamentation and attenuates the candidiasis in *Galleria mellonella***

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Anti-*EFG1* 2'OMe attenuates candidiasis in *G. mellonella*

**Abstract**

*EFG1* is a central transcriptional regulator of filamentation that is an important virulence factor of *Candida albicans*. This study serves to assess *in vivo* the applicability of the anti-*EFG1* 2'-OMethylRNA oligomer for inhibiting *Candida albicans* filamentation and to attenuate candidiasis, using the *Galleria mellonella* model. For that, larvae infected with a lethal concentration of *C. albicans* cells were treated with a single-dose and with a double-dose of the anti-*EFG1* 2'OMe oligomer (at 40 and 100 nM). The anti-*EFG1* 2'OMe oligomer toxicity and effect on larvae survival was evaluated. No evidence of anti-*EFG1* 2'OMe oligomer toxicity was observed and the treatment with double-dose of 2'OMe oligomer empowered the larvae survival over 24 h (by 90-100%) and prolonged its efficacy until 72 h of infection (by 30%). Undoubtedly, this work validates the *in vivo* therapeutic potential of anti-*EFG1* 2'OMe oligomer for controlling *C. albicans* infections.

**Introduction**

*Candida albicans* remains the most common human fungal pathogen<sup>1</sup> and the most prevalent of all *Candida* species over the world.<sup>1</sup> The pathogenicity of *C. albicans* is dependent of certain virulence factors in which the morphological transition from yeast to filamentous forms is recognized as one of the most alarming.<sup>2-5</sup> *EFG1* gene is one of the most important and well-studied regulator of *C. albicans* filamentation.<sup>6-11</sup> Recently, we applied the antisense technology to project the anti-*EFG1* 2'-OMethylRNA (2'OMe) oligomer, to control *EFG1* gene expression and to prevent *C. albicans* filamentation.<sup>12</sup> The anti-*EFG1* 2'OMe oligomer was designed based on the 2<sup>nd</sup> generation of chemical modifications (2'-OMethyl) to guarantee nucleases resistance, improve RNA-affinity and potency, and to reduce its toxicity.<sup>13</sup> Our *in vitro* work revealed the anti-*EFG1* 2'OMe oligomer's ability to reduce *C. albicans* cells' filamentation (by 80%). Moreover, it was verified that the anti-*EFG1* 2'OMe oligomer keeps the efficacy in different human body fluids.<sup>12</sup> Given these findings, anti-*EFG1* 2'OMe oligomer's *in vivo* validation is crucial. Among the *in vivo* models available, invertebrate models as *Galleria mellonella* have been emerged at the forefront to study fungal pathogenesis.<sup>14,15</sup> The possibilities of pathogens delivery into the larvae, by topical, oral and injection application and is suited to study pathogens at human body temperature makes it a desirable model for the study of fungal pathogenesis.<sup>15,16</sup> Based on *in vitro* promising results,<sup>12</sup> the main goal of this work was to validate *in vivo* the applicability of anti-*EFG1* 2'OMe oligomer for inhibiting *Candida albicans* filamentation and to attenuate candidiasis.

## Results

### Anti-*EFG1* 2'OMe oligomer toxicity

To assess the anti-*EFG1* 2'OMe oligomer toxicity, the *G. mellonella* survival rate was determined through the lactate dehydrogenase (LDH) released and the number of hemocytes was also quantified. For that, *G. mellonella* larvae were injected with two different concentrations of oligomer (40 nM and 100 nM) and the survival evaluated during 96 h. As shown in Figure 1A, no death was observed for both tested concentrations over 96 h. Moreover, the injection of anti-*EFG1* 2'OMe oligomer did not increase the levels of LDH released on hemolymph of larvae after 4 h and 24 h, since the levels of LDH are lower compared to levels released from untreated larvae (injected only with PBS) (Figure 1B). In terms of the total number of hemocytes, there are no evidence of differences between larvae injected with oligomer and the control larvae (Figure 1B). Thus, the anti-*EFG1* 2'OMe oligomer did not reveal toxic effects on *G. mellonella*.

### *Galleria mellonella* survival

To investigate the *in vivo* effects of anti-*EFG1* 2'OMe oligomer on attenuation of *C. albicans* infections a *G. mellonella* larvae model was used, infected with a lethal dose of yeast cells ( $7 \times 10^7$  cells mL<sup>-1</sup>). A first set of larvae was treated with a single-dose (0 h of post infection) of anti-*EFG1* 2'OMe oligomer at 40 nM and 100 nM (Figure 2). To note, the treatment of infected *G. mellonella* with a single-dose of anti-*EFG1* 2'OMe oligomer enhances the survival of larvae over 24 h by 16% with 40 nM ( $P$  value  $>0.05$ ) and by 30% with 100 nM ( $P$  value  $<0.05$ ). Although, no effect was observed in larvae treated with 40 nM of anti-*EFG1* 2'OMe oligomer at 48 h ( $P$  value  $>0.05$ ), the treatment with 100 nM intensified the larvae survival into 17% ( $P$  value  $>0.05$ ). No significant effects were

observed with a single-dose after 72 h of infection for both concentrations tested ( $P$  value  $>0.05$ ).

A second set of infected larvae were treated with a double-dose of anti-*EFG1* 2'OMe oligomer (0 h and 12 h post infection), since the treatments are not usually carried out only with a unique dose (Figure 3A). Results showed that a double-dose of anti-*EFG1* 2'OMe oligomer significantly enhances the *G. mellonella* survival. To note, 90% and 100% of the larvae treated with 40 nM ( $P$  value  $<0.05$ ) and 100 nM ( $P$  value  $<0.001$ ) survived over the first 24 h of infection. An increase on *G. mellonella* survival was also evident at 48 h with a rate of 23% for 40 nM ( $P$  value  $<0.05$ ) and of 50% for 100 nM ( $P$  value  $<0.001$ ). Note that, the administration of a double-dose of anti-*EFG1* 2'OMe oligomer not only was responsible by enhancing the larvae survival but also for prolonging the anti-*EFG1* 2'OMe oligomer effects over 72 h, achieving 30% more on the survival rate with 100 nM of oligomer ( $P$  value  $<0.001$ ). To infer about larvae health, the health index scores were also determined for larvae treated with 100 nM of oligomer. The larvae activity, cocoon formation, melanization and survival were scored, (Figure 3B). As it can be seen, the injection of the larvae with anti-*EFG1* 2'OMe oligomer resulted in high health index scores even after 72 h, with a higher activity and cocoon formation. To assess the effect of anti-*EFG1* 2'OMe oligomer on candidiasis progression and *C. albicans* morphology, the fat body of larvae was fixed, sectioned, stained and evaluated. Figure 4A reveals the quantity and invasiveness progression of *C. albicans* without treatment after 24 h and 48 h of infection. It is evident, that *C. albicans* cells are located mainly in digestive system, around the fat body and tend to organize into clusters with an extensive progression on quantity over the time. *Candida albicans* exhibits predominantly filamentous growth. The images highlight the contrast among the single-

dose and double-dose treatments with the control, exhibiting both an expressive lower quantity of filaments with a significant decrease on fat body area occupied by *C. albicans* cells, with a more pronounced effect on sections of larvae treated with 100 nM of anti-*EFG1* 2'OMe oligomer. The effect of anti-*EFG1* 2'OMe oligomer on *EFG1* gene expression was also determined at 4h and 24 h post infection (Figure 4B). The results revealed no significant differences after 4 h despite a huge reduction in the levels of *EFG1* expression after 24 h post infection comparatively to the levels on untreated larvae ( $P$  value < 0.001).

#### ***Galleria mellonella* immune response**

The *G. mellonella* has an immune system with a high similarity to the mammals, in terms of its ability to produce the antimicrobial peptides, with the ability to eliminate the microorganisms.<sup>17–19</sup> For that, the transcript levels of four encoding peptides with antimicrobial peptides, namely lysozyme, gallerimycin, galliomyacin and inducible metalloproteinase inhibitor (IMPI) were quantified by quantitative real-time PCR (qRT-PCR). The expression levels of AMPs vary according to the peptide, in which lysozyme (Figure 5A) and galliomyacin (Figure 5B) presented higher levels of expression at 4 h and 24 h post infection comparing to the IMPI (Figure 5C) and gallerimycin (Figure 5D), indicating that these AMPs are expressed in a latter response to fight the infection. In the presence of anti-*EFG1* 2'OMe, in general, the levels of AMPs decreased both at 4 h and 24 h post infection ( $P$  value > 0.05), with the exception of IMPI, that interestingly resulted in an increase in the gene expression. No change in gene expression levels of galliomyacin was observed after 24 h (Figure 5 B).

## Discussion

Candidiasis is supported by a series of virulence factors, and one of the most important is the ability of *C. albicans* cells to switch from yeast to filamentous forms. The filamentation is essential for *C. albicans* pathogenicity,<sup>2–5</sup> and it is regulated by a complex network of genes in which *EFG1* is one of the most important virulence determinants.<sup>6,9–11</sup> The anti-*EFG1* 2'OMe was projected to degrade the *EFG1* mRNA by RNase activation. The *in vitro* results demonstrated the ability of anti-*EFG1* 2'OMe to reduce *C. albicans* cells' filamentation (by 80%) and *EFG1* gene expression (by 60%).<sup>12</sup> Taking into account the promising *in vitro* results, the aim of this work was to validate *in vivo* its applicability for inhibiting *C. albicans* filamentation and to attenuate candidiasis, using the *G. mellonella* model. As in other microbiological relevant studies,<sup>17–19</sup> we opted to use the *G. mellonella* model to validate the *in vivo* performance of the anti-*EFG1* 2'OMe oligomer since it is a model that provides a rapid, inexpensive and reliable way to evaluate the nano-drugs effects and toxicity *in vivo*. As in our *in vitro* results,<sup>12</sup> no evidences of *in vivo* toxicity were observed over 96 h (Figure 1). In fact, all larvae stayed alive over 96 h (Figure 1A) with no significant differences in terms of LDH released and in the total number of hemocytes (Figure 1B) on hemolymph of larvae comparatively to larvae injected only with PBS. The infected *G. mellonella* larvae with  $7 \times 10^7$  cells mL<sup>-1</sup> of *C. albicans* cells were treated with a single-dose of anti-*EFG1* 2'OMe oligomer (0 h post infection). It was clear, that the anti-*EFG1* 2'OMe oligomer keeps its performance *in vivo*, once it was observed an increase on larvae survival comparing to untreated larvae. Moreover, with these results it is also clear that the *in vivo* anti-*EFG1* 2'OMe oligomer efficacy is concentration

dependent. In fact, the treatment of infected *G. mellonella* with a single-dose of anti-*EFG1* 2'OMe oligomer enhances the survival of larvae over 24 h (16%), being more pronounced with 100 nM of oligomer (30%) (Figure 2). However, after 48 h of infection the anti-*EFG1* 2'OMe oligomer loses its effectiveness. This result was expected, once in a clinical context, an infection is rarely controlled with a single-dose of antimicrobial and the treatments are not usually carried out over a precise time.<sup>20-23</sup> To mimic that, a double-dose of anti-*EFG1* 2'OMe oligomer was administered (0 h and 12 h post infection) on *G. mellonella* larvae infected with *C. albicans* cells (Figure 3). The results indicate that with a double-dose administration of anti-*EFG1* 2'OMe oligomer it is possible to intensify the molecule efficacy and prolong its effect over the time. In fact, larvae treated with the double-dose of oligomer survived around 90% (with 40 nM) and 100% (with 100 nM) over the first 24 h (Figure 3A). Moreover, an increase on larvae survival was also evident at 48 h (by 50%) and 72 h (by 30%), with more pronounced effect in case of 100 nM of oligomer, with a high health index score. These findings corroborate with the observed on histological images of *G. mellonella* fat body, that evidences a strong decrease on the number of *C. albicans* as filaments and an evident reduction on the extension of area occupied by the *Candida* in tissues from larvae treated with anti-*EFG1* 2'OMe oligomer (Figure 4A). The qRT-PCR assays confirm a huge reduction in the levels of *EFG1* transcripts after 24 h of post infection and treated with the oligomer (Figure 4B), that is in accordance with the decrease in the number of *C. albicans* filaments.

The *G. mellonella* system presents an immune system with a highly similarity to the mammalian immune system, and the ability to release the AMPs is important to fight the infection.<sup>19,24</sup> In general, the expression of AMPs was lower in the presence of 100

nM of anti-*EFG1* 2'OMe oligomer indicating a possible reduction on *C. albicans* infection (Figure 5) when larvae are treated with the oligomer.

Numerous studies have documented the use of AST as biochemical tools for studying human target diseases, and for now there are ten antisense drugs in the market. However, application of AST as anti-*Candida* agents are still scarce and there are one study using the AST to interrupt and efficiently inhibit *C. albicans in vivo* splicing using a PS-modified ASO.<sup>25</sup> Our results reveals that it is possible to synthesize an ASO modified by 2'-OMethyl chemical modification to control a virulence factor of *C. albicans*. Moreover, this study suggests that systemic delivery of anti-*EFG1* 2'OMe oligomer is feasible, devoid of toxicity, and could be a promising treatment strategy for *C. albicans* infections. Therefore, it warrants further studies in other animal models.

## Conclusions

Hereby, the present work confirms that the anti-*EFG1* 2'OMe oligomer is able to inhibits *C. albicans* filamentation and attenuates the candidiasis on *G. mellonella* model. Undoubtedly, this work revealed the *in vivo* therapeutic potential of anti-*EFG1* 2'OMe oligomer for controlling *C. albicans* infections.

## Materials and methods

### Anti-*EFG1* 2'OMe oligomer preparation

The anti-*EFG1* 2'OMe oligomer was designed and synthesized based on the 2<sup>nd</sup> generation of chemical modifications of nucleic acid mimics as described in our recent published works.<sup>12,26</sup> Aliquots of anti-*EFG1* 2'OMe oligomer were prepared in sterile ultrapure water to 4  $\mu$ M and stored at -20 °C for later use. Whenever necessary, oligomer molecules were diluted in PBS to a final concentration of 40 and 100 nM. The lower concentration was selected according to our previous results *in vitro*<sup>12</sup> and 100 nM was used to be tested as an higher concentration.

### *Candida albicans* cells and growth conditions

The *Candida albicans* SC5314, belonging to *Candida* strains collection of the Biofilm group of the Centre of Biological Engineering, was used during these studies. For all experiments, the yeast strain was subcultured on sabouraud dextrose agar (SDA; Merck, Germany) and incubated for 24 h at 37 °C. Cells were then inoculated in sabouraud dextrose broth (SDB; Merck, Germany) and incubated overnight at 37 °C, 120 rpm. After incubation, the cells' suspensions were centrifuged for 10 min, at 3000 g and 4 °C, and washed twice with phosphate-buffered saline (PBS; pH 7, 0.1 M). Pellets were suspended in 5 ml of PBS, and the cellular density was adjusted using a *Neubauer* chamber (Marienfeld, Land-Konicshofem, Germany) to  $7 \times 10^7$  cells mL<sup>-1</sup>.

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230 ***Galleria mellonella* larvae**231 *Galleria mellonella* larvae were reared on a pollen grain and bee wax diet at 25 °C in the

232 darkness and used in a final stage of development with a weight of approximately 250 mg.

233 The larvae were injected into hemolymph via the hindmost left proleg, previously sanitized

234 with 70% (v/v) ethanol, using a micro syringe adapted in a micrometre to control the volume

235 of injection.<sup>20</sup> All experiments were performed in triplicate and in a minimum of three

236 independent assays.

237

238 **Toxicity assays**239 To test the *in vivo* toxicity of the anti-*EFG1* 2'OMe oligomer, 10 larvae of *G. mellonella* were

240 injected with 5 µL of 40 and 100 nM of oligomer prepared in PBS. As control, a set of larvae

241 were injected with the same volume but only with PBS. Larvae were placed in petri dishes

242 and stored in the dark at 37 °C. Larvae's morphology and survival were followed over 4 days

243 and the survival curves were constructed.

244 The LDH activity released from larvae tissues to hemolymph was also evaluated. For that,

245 larvae were sacrificed at 4 h and 24 h after injection and the hemolymph of five larvae was

246 collected into an Eppendorf tube. This assay was performed using the CytoTox-ONE™

247 Homogeneous Membrane Integrity Assay Kit (Promega), according to the manufacturer's

248 instructions. LDH activity was quantified by fluorescence spectrometer evaluation (Cytation

249 3 Cell Imaging Multi-Rode Reader, BioTek) by measuring the NADH disappearance rate at

560 nm excitation and 590 nm emission during the LDH-catalysed conversion of pyruvate to lactate. The value of LDH activity of the larvae injected only with PBS used as a control was subtracted from the LDH activity of the larvae injected with 100 nM of anti-*EFG1* 2'OMe. The levels of LDH released were expressed as relative LDH activity. The total number of hemocytes presented in the hemolymph of larvae were also evaluated. For that, three larvae previously sanitized with 70% (v/v) ethanol, were punctured in the abdomen with a sterile needle and the hemolymph was recovered into a sterile microtube. The hemolymph mixture was diluted 10-fold in sterile PBS and hemocytes were counted with a hemocytometer. The results were presented at logarithm of the concentration ( $\text{Log}_{10}$ ).

#### ***Galleria mellonella* survival assays**

To study the effect of the anti-*EFG1* 2'OMe oligomer on the survival rate of *G. mellonella*, larvae were infected with 5  $\mu\text{L}$  of a lethal dose of *C. albicans* cells ( $7 \times 10^7$  cells  $\text{mL}^{-1}$ ) and randomly allocated to 5 different experimental groups (with a set of 10 larvae). The concentration of *C. albicans* to be injected ( $7 \times 10^7$  cells  $\text{mL}^{-1}$ ) was selected on the basis of the *G. mellonella* lethality results after injection with different concentrations of yeast cells (between  $7 \times 10^7$  cells  $\text{mL}^{-1}$  and  $2 \times 10^8$  cells  $\text{mL}^{-1}$ ) (Figure S1). Two sets of larvae were treated with a single-dose of 40 nM and 100 nM of oligomer (0 h of post infection); two sets of larvae with a double-dose of 40 nM and 100 nM of oligomer (0 h and 12 h of post infection); and a set only with PBS. As control, a set of larvae were injected only with the same volume of PBS. After injections, the larvae were placed in petri dishes and stored in the dark at 37

°C, over 72 h, and consequently, survival curves were constructed. The larvae were considered dead when they displayed no movement in response to touch. The *G. mellonella* health index was also determined for the larvae treated with a double-dose of 100 nM of oligomer, which scores four main parameters: Larvae activity, cocoon formation, melanization and survival.<sup>27</sup>

### Gene expression analysis

The qRT-PCR was used to determine the *EFG1* gene expression on *C. albicans* after the treatment with 100 nM of anti-*EFG1* oligomer. The transcript levels of genes encoding the *G. mellonella* antimicrobial peptides, gallerimycin, galliomyacin, IMPI and lysozyme were also determined to infer about the *G. mellonella* immune response. For that, three larvae treated with oligomer at 4 h and 24 h post-infection and three larvae untreated were cryopreserved, sliced and homogenized in Lysis Buffer reagent.

RNA extraction was performed using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA)<sup>12</sup>. To avoid potential DNA contamination, samples were treated with DNase I (Deoxyrybonuclease I, Amplification Grade, Invitrogen) and the RNA concentration was determined by optical density measurement (NanoDrop 1000 Spectrophotometer Thermo Scientific®). The complementary DNA (cDNA) was synthesized using the iScript Reverse Transcriptase (Biorad) in accordance with the manufacturer's instructions, and qRT-PCR (CFX96, Biorad) was performed on a 96-well microtiter plate using Eva Green Supermix (Biorad, Berkeley, USA). Each reaction was performed in triplicate and mean values of

expression were determined by the  $\Delta C_t$  method. Non-transcriptase reverse (NRT) controls were included in each run. The primers used are presented in Table 1.

#### ***Galleria mellonella* histological fat body analysis**

The histological analysis of *G. mellonella* was performed to study the effect of anti-*EFG1* 2'OMe oligomer on candidiasis progression and *C. albicans* morphology into fat body of larvae. For that, one larva from each group of study were recovered at 24 h and 48 h, to be processed histologically. The fat body was removed, from each larva, through an incision in the midline of the ventral with a scalpel blade. The fat body was placed in 4% (v/v) of paraformaldehyde and stored for 24 h at 4 °C to preserve the structures. The paraffin blocks were cut on sections of 4-5  $\mu$ m, and the sections were stained with periodic acid Schiff (PAS) and haematoxylin-eosin (HE). Tissue sections were viewed and photographed with an OLYMPUS BX51 microscope coupled with a DP71 digital camera (Olympus Portugal SA, Porto, Portugal).

#### **Statistical analysis**

Data are expressed as the mean  $\pm$  standard deviation (SD) of a least three independent experiments. Results were compared using Two-way analysis of variance (ANOVA) using GraphPad Prism 6® (GraphPad Software, CA, USA). All tests were performed with a confidence level of 95%. Kaplan-Meier survival curves were plotted and differences in survival were calculated by using log-rank Mantel-Cox statistical test, all performed with GraphPad Prism 6®.

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## Author Contributions

D. A. and S. S. conceived and designed the study. D. A. and D. M. conducted the experiments. D. A. wrote the manuscript. M. H. and S. S. performed the analysis and read the paper. All authors read and approved the manuscript.

## Conflicts of interest

The authors declare no conflict of interest.

**Keywords:** Antisense oligonucleotides; 2'-OMethyl chemical modification; Candidiasis;

*Galleria mellonella*; Virulence

## REFERENCES

1. Koehler, P., Stecher, M., Cornely, O.A., Koehler, D., Vehreschild, M.J.G.T., Bohlius, J., Wisplinghoff, H., Vehreschild, J.J. (2019). Morbidity and mortality of candidaemia in Europe: an epidemiologic meta-analysis. *Clin Microbiol Infect.* 25(10), 1200-1212.
2. Silva, S., Negri, M., Henriques, M., Oliveira, R., Williams, D.W., Azeredo, J. (2012). *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis*: Biology, epidemiology, pathogenicity and antifungal resistance. *FEMS Microbiol Rev* 36, 288–305.
3. Silva, S., Rodrigues, C., Araújo, D., Rodrigues, M., Henriques, M. (2017). *Candida* species biofilms' antifungal resistance. *J Fungi* 3, 8.
4. Mayer, F.L., Wilson, D., Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence* 4, 119–28.
5. Araújo, D., Henriques, M., Silva, S. (2017). Portrait of *Candida* species biofilm regulatory network genes. *Trends Microbiol* 1, 62–75.
6. Stoldt, V.R., Sonneborn, A., Leuker, C.E., Ernst, J.F. (1997). Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO J* 16, 1982–91.
7. Ernst, J.F. (2000). Transcription factors in *Candida albicans* - environmental control of morphogenesis. *Microbiology* 146(8), 763–74.
8. Ramage, G., Vandewalle, K., López-Ribot, J.L., Wickes, B.L. (2002). The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. *FEMS Microbiol*

- 363 Lett 214, 95–100.
- 364 9. Nobile, C.J., Mitchell, A.P. (2005). Regulation of cell-surface genes and biofilm  
365 formation by the *C. albicans* transcription factor Bcr1p. *Curr Biol* 15, 1150–5.
- 366 10. Nobile, C.J., Fox, E.P., Nett, J.E., Sorrells, T.R., Mitrovich, Q.M., Hernday, A.D.,  
367 Tuch, B.N., Andes, D.R., Johnson, A.D. (2011). A recently evolved transcriptional  
368 network controls biofilm development in *Candida albicans*. *Cell* 148, 126–38.
- 369 11. Connolly, L., Riccombeni, A., Grózer, Z., Holland, L.M., Lynch, D.B., Andes, D.R.,  
370 Gácsér, A., Butler, G. (2013). The APSES transcription factor Efg1 is a global  
371 regulator that controls morphogenesis and biofilm formation in *Candida*  
372 *parapsilosis*. *Mol Microbiol* 90, 36–53.
- 373 12. Araújo, D., Azevedo, N.M., Barbosa, A., Almeida, C., Rodrigues, M.E., Henriques,  
374 M., Silva, S. (2019). Application of 2'-OMethylRNA antisense oligomer to control  
375 *Candida albicans EFG1* Virulence Determinant. *Mol Ther - Nucleic Acids* 18, 508–  
376 17.
- 377 13. Khvorova, A., Watts, J.K. (2017). The chemical evolution of oligonucleotide  
378 therapies of clinical utility. *Nat Biotechnol* 35, 238–48.
- 379 14. Fedhila, S., Buisson, C., Dussurget, O., Serror, P., Glomski, I.J., Liehl, P., Lereclus,  
380 D., Nielsen-LeRoux, C. (2010). Comparative analysis of the virulence of  
381 invertebrate and mammalian pathogenic bacteria in the oral insect infection  
382 model *Galleria mellonella*. *J Invertebr Pathol* 103, 24–9.
- 383 15. Junqueira, J.C. (2012). *Galleria mellonella* as a model host for human pathogens.  
384 *Virulence* 3, 474–6.
- 385 16. Fuchs, B.B., O'Brien, E., Khoury, J.B.E., Mylonakis, E. (2010). Methods for using  
386 *Galleria mellonella* as a model host to study fungal pathogenesis. *Virulence* 1,

- 387 475–82.
- 388 17. Mak, P., Zdybicka-barabas, A. (2010). A different repertoire of *Galleria*  
 389 *mellonella* antimicrobial peptides in larvae challenged with bacteria and fungi.  
 390 Dev Comp Immunol 34, 1129–36.
- 391 18. Moghaddam, M., Tonk, M., Schreiber, C., Salzig, D., Czermak, P., Vilcinskas, A.,  
 392 Rahnamaeian, M. (2011). The potential of the *Galleria mellonella* innate  
 393 immune system is maximized by the co – presentation of diverse antimicrobial  
 394 peptides. Biol Chem 397, 939–45.
- 395 19. Tsai, C.J., Mei, J., Loh, S., Proft, T. (2016). *Galleria mellonella* infection models for  
 396 the study of bacterial diseases and for antimicrobial drug testing. Virulence 7,  
 397 214–29.
- 398 20. Mil-Homens, D., Ferreira-Dias, S., Fialho, A.M. (2016). Fish oils against  
 399 *Burkholderia* and *Pseudomonas aeruginosa*: In vitro efficacy and their  
 400 therapeutic and prophylactic effects on infected *Galleria mellonella* larvae. J  
 401 Appl Microbiol 120, 1509–19.
- 402 21. Vilela, S.F.G., Barbosa, J.O., Rossoni, R.D., Santos, J.D., Prata, M.C.A., Anbinder,  
 403 A.L., Jorge, A.O.C., Junqueira, J.C. (2015). *Lactobacillus acidophilus* ATCC 4356  
 404 inhibits biofilm formation by *C. albicans* and attenuates the experimental  
 405 candidiasis in *Galleria mellonella*. Virulence 6, 29–39.
- 406 22. Rossoni, R.D., dos Santos Velloso, M., Figueiredo, L.M.A., Martins, C.P., Jorge,  
 407 A.O.C., Junqueira, J.C. (2018). Clinical strains of *Lactobacillus* reduce the  
 408 filamentation of *Candida albicans* and protect *Galleria mellonella* against  
 409 experimental candidiasis. Folia Microbiol (Praha) 63, 307–14.
- 410 23. Straarup, E.M., Fisker, N., Hedtjörn, M., Lindholm, M.W., Rosenbohm, C., Aarup,

- V., Hansen, H.F., Ørum, H., Hansen, J.B.R., Koch, T. (2010). Short locked nucleic acid antisense oligonucleotides potently reduce apolipoprotein B mRNA and serum cholesterol in mice and non-human primates. *Nucleic Acids Res* 38, 7100–11.
- 24 Jemel, S., Guillot, J., Kallel, K., Botterel, F., Dannaoui, E. (2020). *Galleria mellonella* for the evaluation of antifungal efficacy against medically important fungi, a Narrative Review. *Microorganisms* 8, 1–19.
- 25 Testa, S., Disney, M., Gryaznov, S., and Turner, D. (2000). Methods and compositions for inhibition of RNA splicing. U.S. WO/2000/055374
- 26 Silva, S., Araújo, D., Azevedo, N.M., Almeida, C., Henriques, M. (2020). Antisense oligomers for controlling *Candida albicans* infections. WO 2020/174366 A1
- 27 Loh, J.M.S., Adenwalla, N., Wiles, S., Proft, T. (2013). *Galleria mellonella* larvae as an infection model for group A streptococcus. *Virulence* 4, 419–28.
- 28 Altincicek, B., Vilcinskas, A. (2006). Metamorphosis and collagen-IV-fragments stimulate innate immune response in the greater wax moth, *Galleria mellonella*. *Dev Comp Immunol* 30, 1108–18.
- 29 Wojda, I., Kowalski, P., Jakubowicz, T. (2009). Humoral immune response of *Galleria mellonella* larvae after infection by *Beauveria bassiana* under optimal and heat-shock conditions. *J Insect Physiol* 55, 525–31.

## Figures Legends

### **Figure 1. Anti-*EFG1* 2'OMe oligomer toxicity evaluation in *Galleria mellonella* model.**

**(A)** Survival curves of larvae injected with 40 nM and 100 nM of anti-*EFG1* 2'OMe oligomer. For each condition, 10 larvae were injected with 40 nM and 100 nM of oligomer and their survival was monitored over 96 h. **(B)** Relative LDH activity released and total number of hemocytes counted after 4 h and 24 h after injection with 100 nM of anti-*EFG1* 2'OMe oligomer. As control larvae were injected only with PBS.

### **Figure 2. Single-dose effect of anti-*EFG1* 2'OMe oligomer on the survival of *Galleria***

***mellonella* infected with *Candida albicans*.** Survival curves of infected larvae were treated with a single-dose of anti-*EFG1* 2'OMe oligomer (0 h post infection). Larvae infected with *C. albicans* cells were treated with 40 nM and 100 nM of anti-*EFG1* 2'OMe oligomer. As control larvae infected were injected only with PBS. \*Significant difference among control and a single-dose of 100 nM of anti-*EFG1* 2'OMe oligomer at 24 h ( $P$  value  $< 0.05$ ).

### **Figure 3. Double-dose effect of anti-*EFG1* 2'OMe oligomer on *Galleria mellonella***

**infected with *Candida albicans*.** **(A)** Survival curves of infected larvae treated with a double-dose of anti-*EFG1* 2'OMe oligomer (0 h and 12 h post infection). Larvae infected with *C. albicans* cells were treated with 40 nM and 100 nM of anti-*EFG1* 2'OMe oligomer. As control larvae infected were injected only with PBS. **(B)** The health index scores of larvae treated with a double-dose of 100 nM of anti-*EFG1* 2'OMe oligomer. Control represents the infected larvae treated only with PBS after 12 h post infection.

\*Significant difference among control and a double-dose of 40 nM of anti-*EFG1* 2'OMe oligomer for all times ( $P$  value < 0.05). \*\*\*Significant difference among control and a double-dose of 100 nM of anti-*EFG1* 2'OMe ASO for all times ( $P$  value < 0.001).

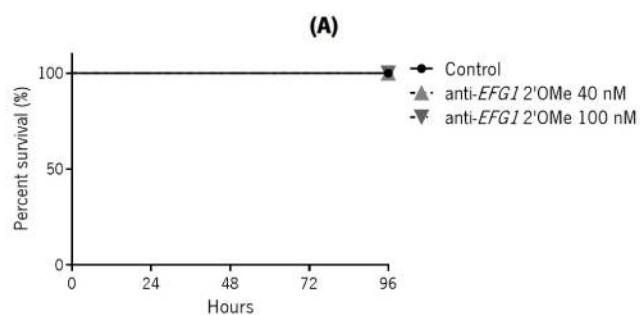
**Figure 4. Anti-*EFG1* 2'OMe oligomer effect on *Candida albicans* cells morphology and progression into fat body of *Galleria mellonella*. (A)** Histological images of larvae infected with *C. albicans* (at 24 h and 48 h) and treated with a single-dose (0 h post infection) and with a double-dose (0 h and 12 h post infection) of 40 nM and 100 nM of anti-*EFG1* 2'OMe oligomer. The larvae sections were labelled with periodic acid Schiff (PAS) coloration. The magnification images were at 400x. **(B)** Levels of *EFG1* gene expression of larvae treated with a double-dose of 100 nM of anti-*EFG1* 2'OMe oligomer evaluated by qRT-PCR and analysed by  $\Delta C_t$  method and normalized to the *CaACT1* mRNA levels after 4 h and 24 h post infection. Control represents the infected larvae treated only with PBS after 12 h post infection. Error bars represent standard deviation. \*\*\*Significant difference among control and a double-dose of 100 nM of anti-*EFG1* 2'OMe oligomer at 24 h post infection ( $P$  value < 0.001).

**Figure 5. Anti-*EFG1* 2'OMe oligomer effect on *Galleria mellonella* immune response.** Levels of gene expression on *G. mellonella* treated with a double-dose of 100 nM of anti-*EFG1* 2'OMe oligomer (0 h and 12 h post infection) of **(A)** Lysozyme, **(B)** Galliomycin, **(C)** Inducible metalloproteinase inhibitor and **(D)** Gallerimycin, after 4 h and 24 h of infection by *C. albicans* SC5314. These results were obtained by qRT-PCR and analysed by  $\Delta C_t$  method and normalized to the *GmACT1* mRNA levels. As control it was used the

*G. mellonella* injected only with PBS after 12 h post infection. Error bars represent standard deviation.

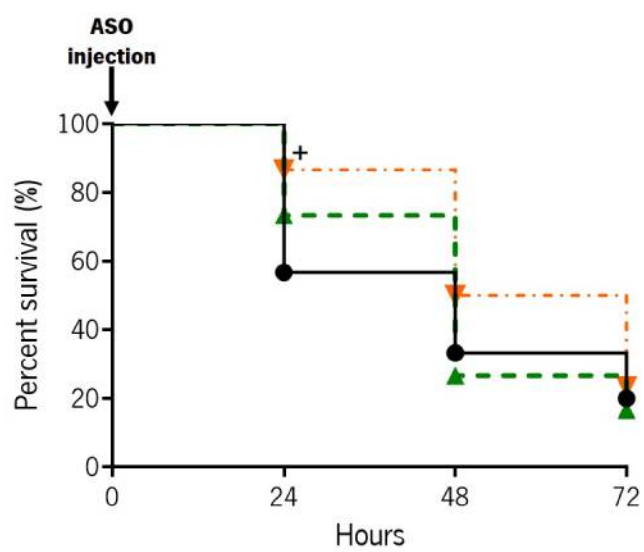
**Table 1.** Primers used for quantitative real time PCR.

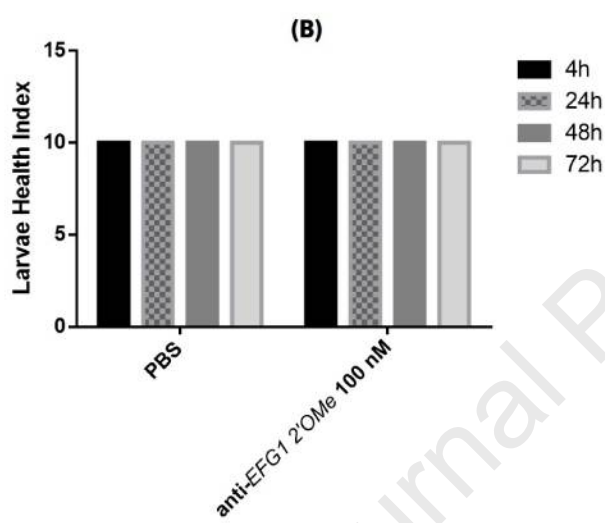
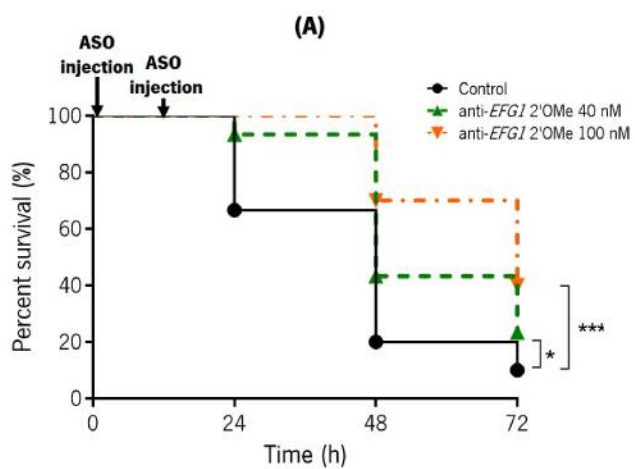
Gene		Sequence (5'-3')	Primer
Name			
<i>Candida albicans</i>	<i>EFG1</i>	TTCTGGTGCAGGTTCCAC	Forward
		CCTGGTTGTGATGCAGGT	Reverse
	<i>ACT1</i>	AATGGGTAGGGTGGGAAAAC	Forward
		AGCCATTTCCATTGATCGTC	Reverse
<i>Galleria mellonella</i>	Actin	ATCCTCACCTGAAGTACCC <sup>28</sup>	P1RT
		CCACACGCAGCTCATTGTA <sup>28</sup>	P2RT
	Lysozyme	TCCCAACTCTTGACCGACGA <sup>28</sup>	P1RT
		AGTGGTTGCGCCATCCATAC <sup>28</sup>	P2RT
	Galliomyeci	TCGTATCGTCACCGCAAATG <sup>29</sup>	P1RT
		GCCGCAATGACCACCTTTATA <sup>29</sup>	P2RT
	IMPI	AGATGGCTATGCAAGGGATG <sup>28</sup>	P1RT
		AGGACCTGTGCAGCATTCT <sup>28</sup>	P2RT
	Gallerimyc	CGCAATATCATTGGCCTTCT <sup>28</sup>	P1RT
		CCTGCAGTTAGCAATGCAC <sup>28</sup>	P2RT
	in		

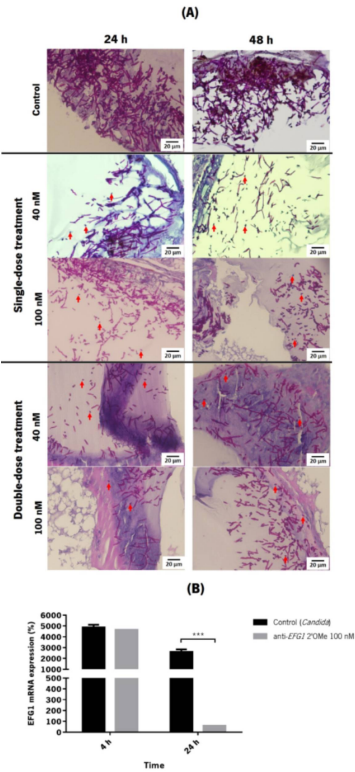


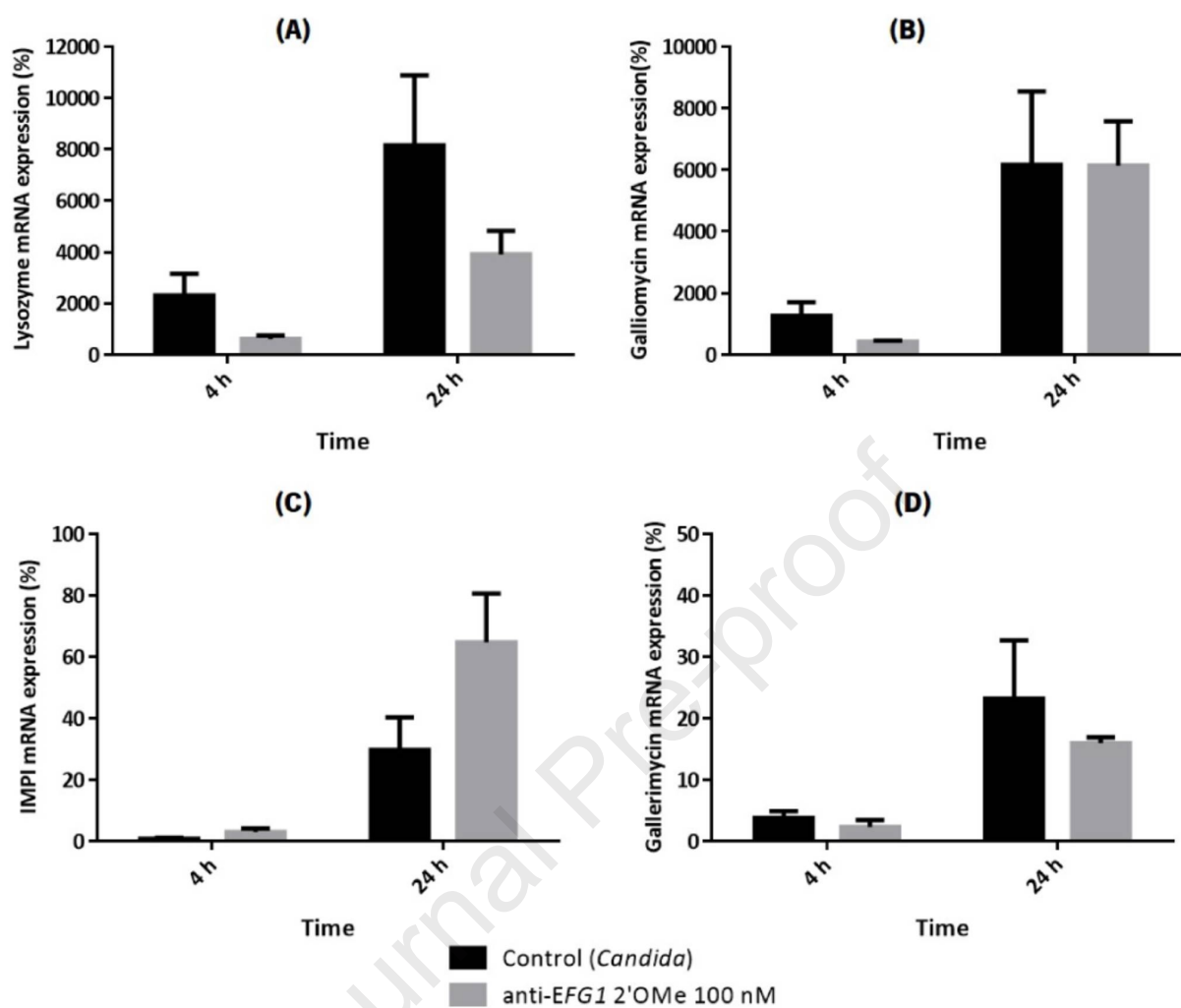
**(B)**

Time	Sample	Relative LDH activity	Number of Hemocytes ( $\log_{10}$ )
4 h	Control PBS	1	$6.79 \pm 0.03$
	Anti- <i>EFG1</i> 2'OMe 100 nM	0.44	$6.75 \pm 0.02$
24 h	Control PBS	1	$6.79 \pm 0.02$
	Anti- <i>EFG1</i> 2'OMe 100 nM	0.83	$6.76 \pm 0.001$









## Highlights

- Administration of anti-*EFG1* 2'OMe oligomer enhances *G. mellonella* survival.
- An anti-*EFG1* 2'OMe double administration prolongs its efficacy over the time.
- The antisense oligomer is a promising strategy to control *C. albicans* infections.