



Challenges and perspectives in research on alternatives to animal testing

Galleria mellonella as model for studying virulence and evaluate the efficacy of antifungal agents

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Scenarios

Immunocompromised patients or individuals fitted with medical devices contribute to enhance microbial infection.

Fungi are responsible for many of these processes. These infections are difficult to treat significant morbidity and mortality rates.

-Few antifungal drugs are available, majority presents high toxicity and resistant conditions may appear.

toxicity

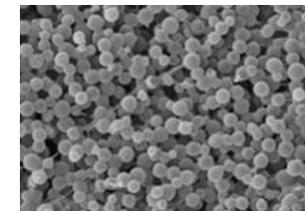


Models to study fungus-host interaction have been developed in order to reduce the use of conventional .

There is a demand of new approaches and new therapies for fungal disease treatment, including fungal biofilm.

Planktonic
X biofilm

Fungal
pathogenesis
virulence





In 1959, Russell and Burch proposed the three R's (Reduction, Replacement and Refinement).

In Brazil there is Arouca Law (Law No. 11.794, 2008) to formalize the ethical of animal use.

The Brazilian National Council for the Control of Animal Experimentation (CONCEA) started the process of reducing animal experimentation in Brazil on July 2014.

New models have been developed and each one has its specific strengths and weaknesses.

***G. mellonella* was first used to study entomopathogenic fungi, and after used as a model for studying human fungal infections.**

In 2000, Cotter et al. showed that this larvae can be killed by *Candida albicans* but not by *Saccharomyces cerevisiae*.

Reasons for using this invertebrate model

Toxicity

Virulence

- Less ethical difficulties for testing "in vivo"

- *G. mellonella* possess defense mechanisms against fungi as mammals

Allows to evaluate the pathogenicity and antifungal efficacy besides toxicity

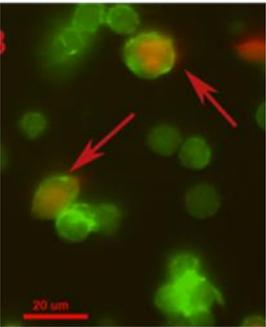
Use a large number of animals for test

Controlled inoculum concentration

Temperatures 25 to 37°C - study of fungi in their natural environment and in the host

phagocytosis

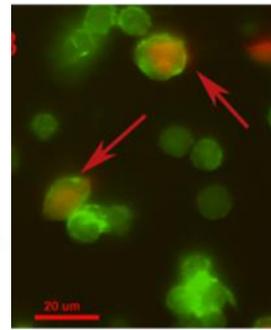
Efficacy of new compounds



Immune system of *G. mellonella*

Cuticle - Mechanical barrier, similar functions to the skin.

Hemolymph - transport of nutrients and molecules similar to blood.



- **Circulating phagocytic hemocytes recognize, engulf and sequester invading microbes.**

- **The phagocytosis is performed by plasmatocytes and granulocytes.**

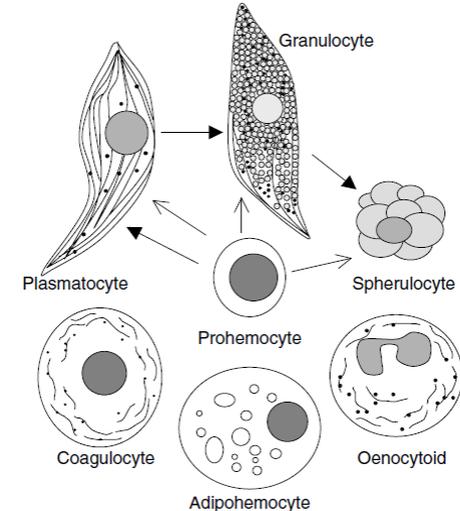
The decrease in the concentration of hemocytes during infection is associated with high virulence.

- **Other cellular responses: formation of nodules and encapsulation.**



- **Humoral responses: coagulation, melanization and production of peptides (gallerimycin, galiomicin, transferrin, and an inducible metalloproteinase inhibitor).**

-proteolytic cascades can be quickly triggered, activating the melanization response (the synthesis and deposition of melanin to sequester pathogens at a wound site), followed by hemolymph coagulation, and opsonization.



FOCUS

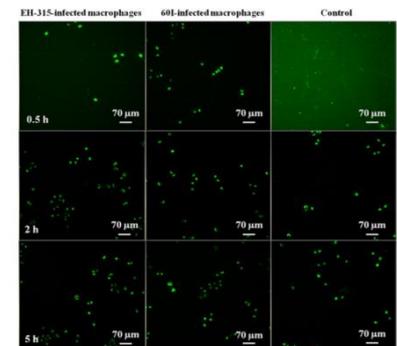
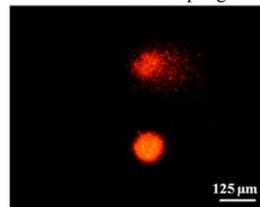
Results of toxicity effects, fungal virulence, in planktonic and biofilm conditions, in the invertebrate hosts *Galleria mellonella*.

Effects of antifungal drugs and substances using alternative animals.

Human lung cells (MRC-5 and A549), liver (HepG2), keratinocytes (NOK), HaCaT (human keratinocytes) and macrophages (AMJ2- C11).



60I-infected macrophages

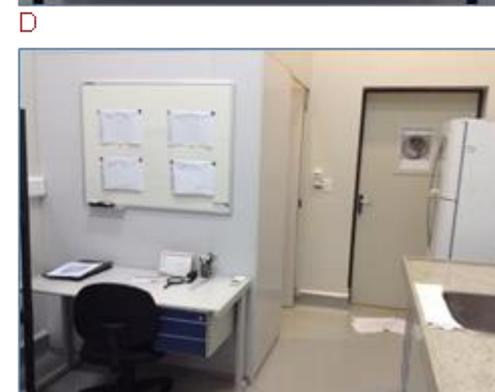
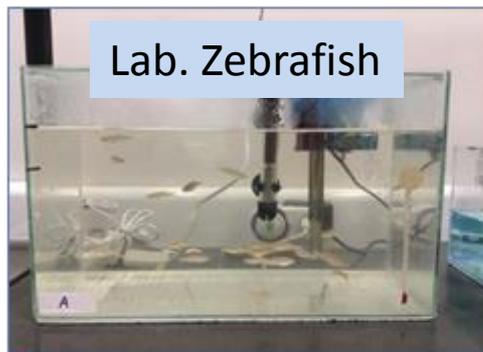




International Center for Development and Validation of Alternative Methods (NIDEVAM)



Lab. *G. mellonella* e *C. elegans*



Antifungal substances and fungal isolates

SCIENTIFIC APPROACH FOR THERAPEUTIC TARGET SEARCH



Chemistry Institute NUBBE and IBILCE-UNESP

Pedalitin and conventional drugs
alkyl gallates series and derived
library peptides obtained by phage display.
Antibodies anti-adhesins

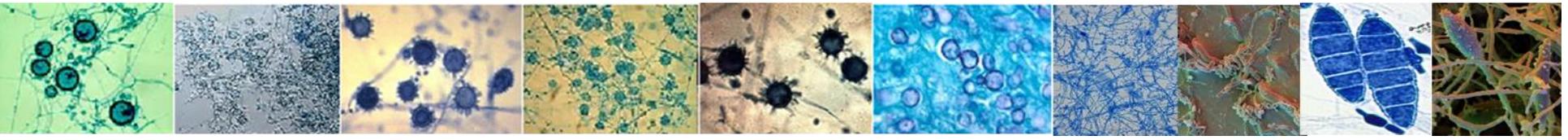
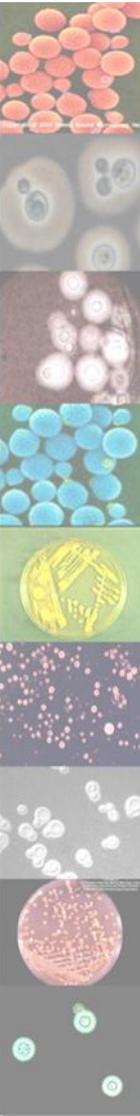
Natural products and their derivatives;
Combination of new antifungal substances and drugs, reporting a synergistic effect;
Substances associated with nanoparticles;
Antibodies and peptides

Fungi

Candida albicans,
Cryptococcus neoformans and
C. gattii

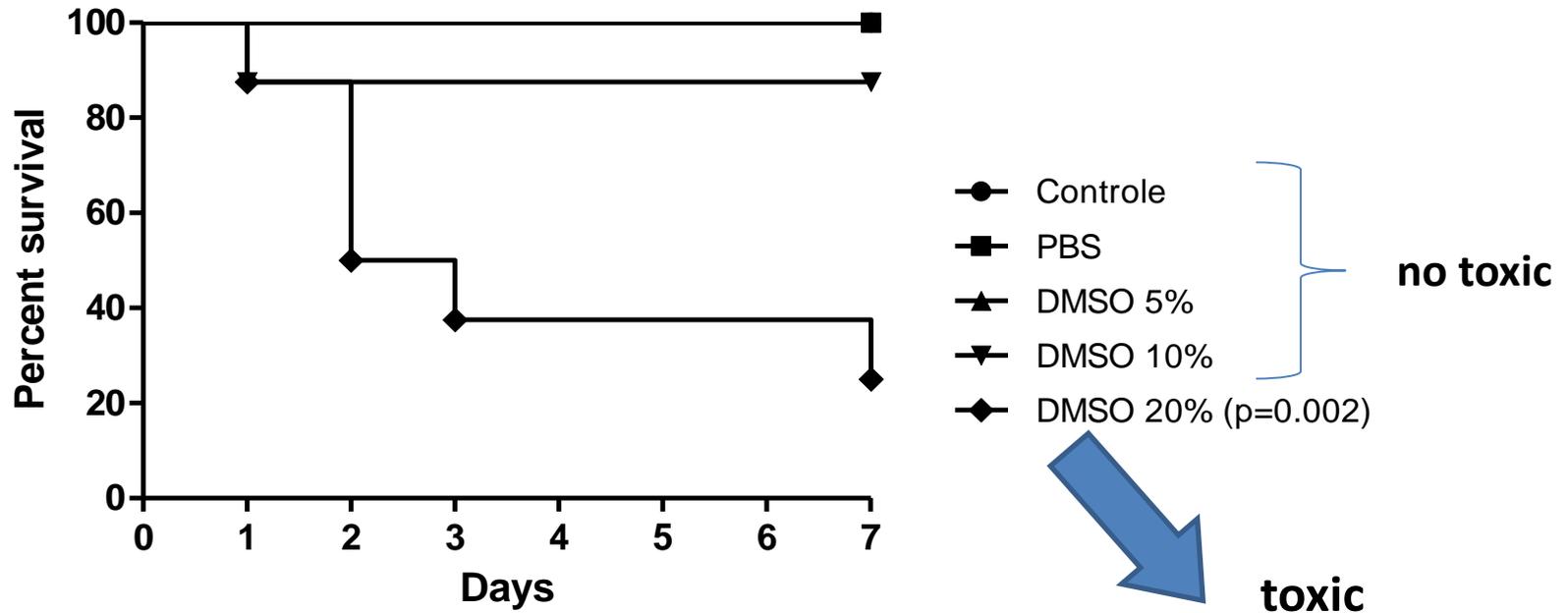
Dimorphic fungi:
Paracoccidioides brasiliensis and
P. lutzii

Some of them were evaluate in both forms: planktonic and biofilm.



DMSO toxicity

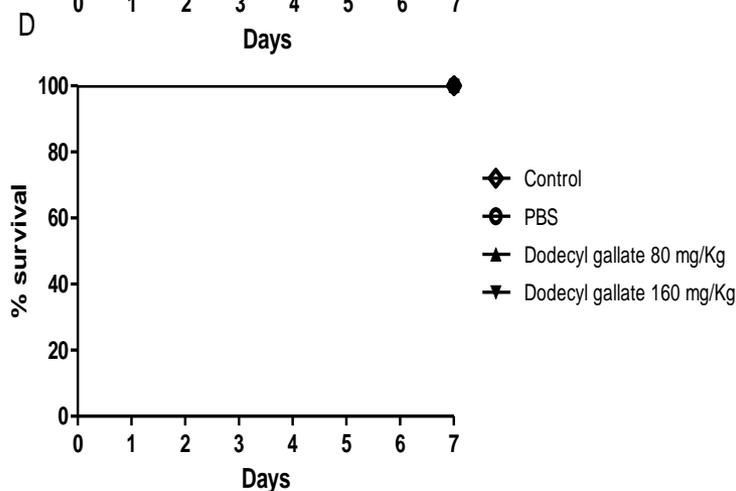
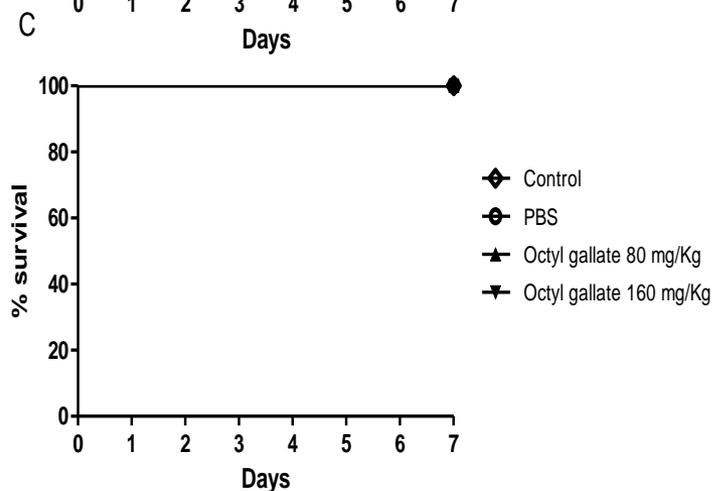
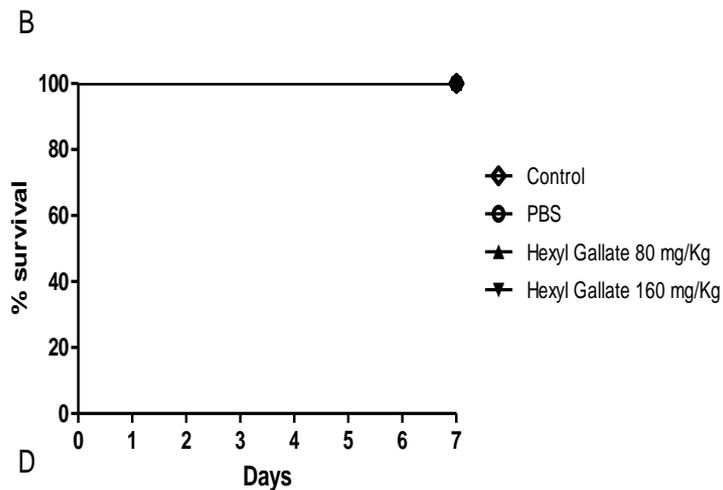
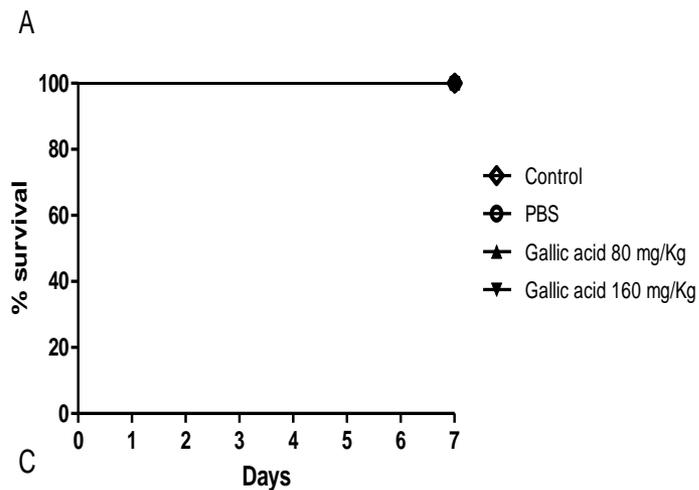
DMSO is used as an industrial solvent for herbicides, fungicides, antibiotics and plant hormones.



Survival of *G. mellonella* after administration of the DMSO solvent.

The concentrations of 5% and 10% were no toxic to the larvae when compared to the control PBS, however the concentration of 20% was toxic to the larvae ($p = 0.002$). $P < 0.05$ was considered statistically significant.

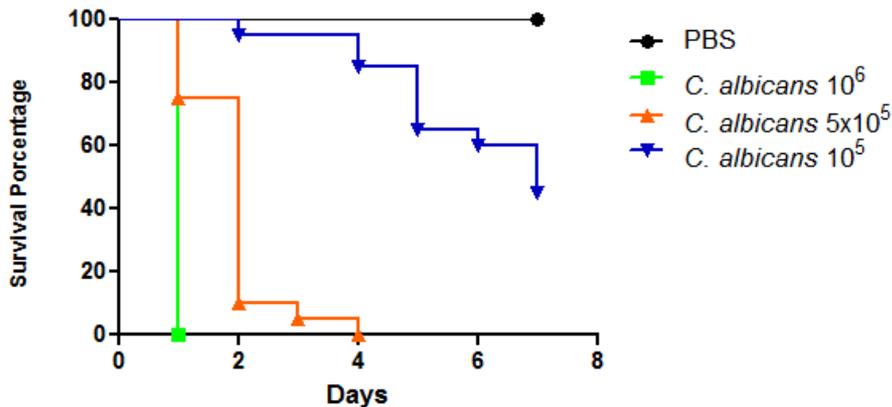
Toxicity of gallic acid and alkyl gallates in *G. mellonella*



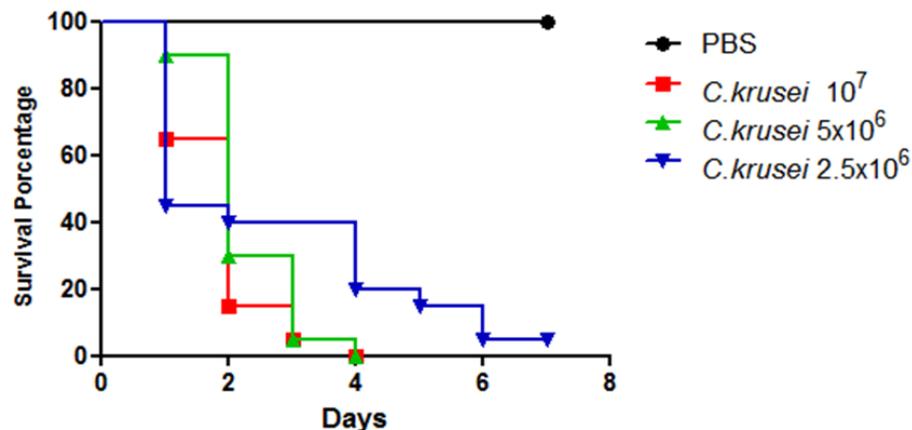
G. mellonella survival after administration of gallic acid and its esters derivatives. There was no killing of *G. mellonella* that received the compounds compared to PBS. A p value <0.05 was considered statistically significant.

G. mellonella infection by Candida species

Survival Curves

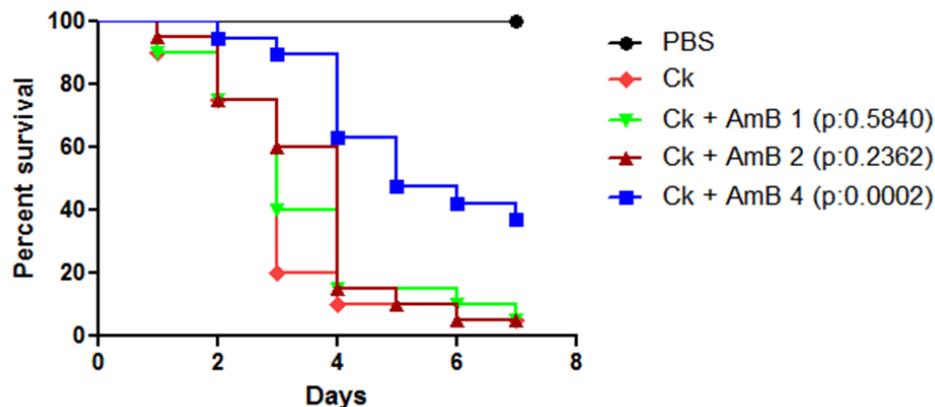


Survival curves of *G. mellonella* infected with different inocula of *C. albicans* and *C. krusei*, incubated at 37°C.



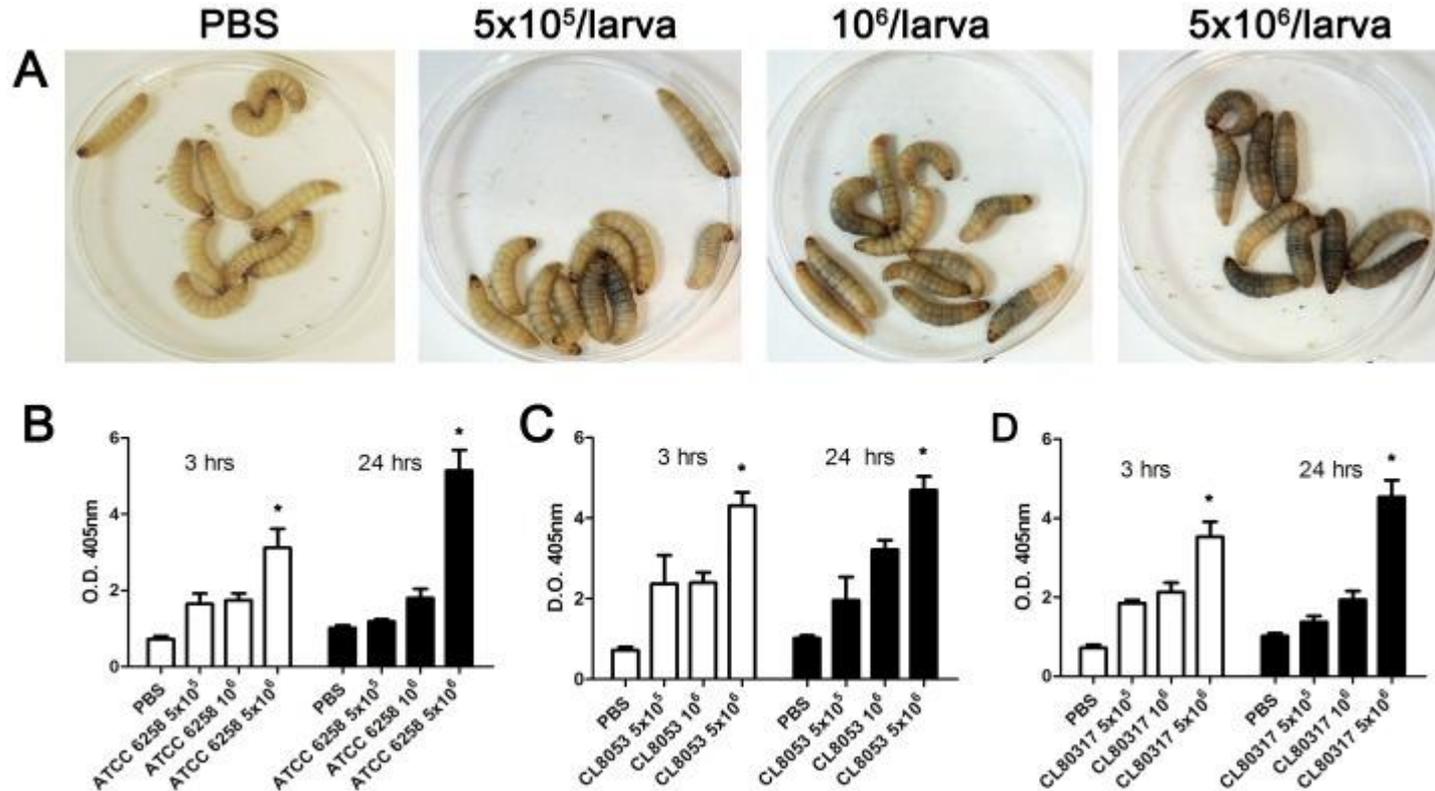
C. krusei is less virulent compared to *C. albicans* (10 X)

C. krusei and *C. albicans* killed *G. mellonella* in a dependent dose-response`.



Efficacy of Amphotericin B during *G. mellonella* infection with *C. krusei*. Amphotericin B treatment efficacy (1, 2, 4 mg/kg)

Melanization of *G. mellonella* infected with *C. krusei*



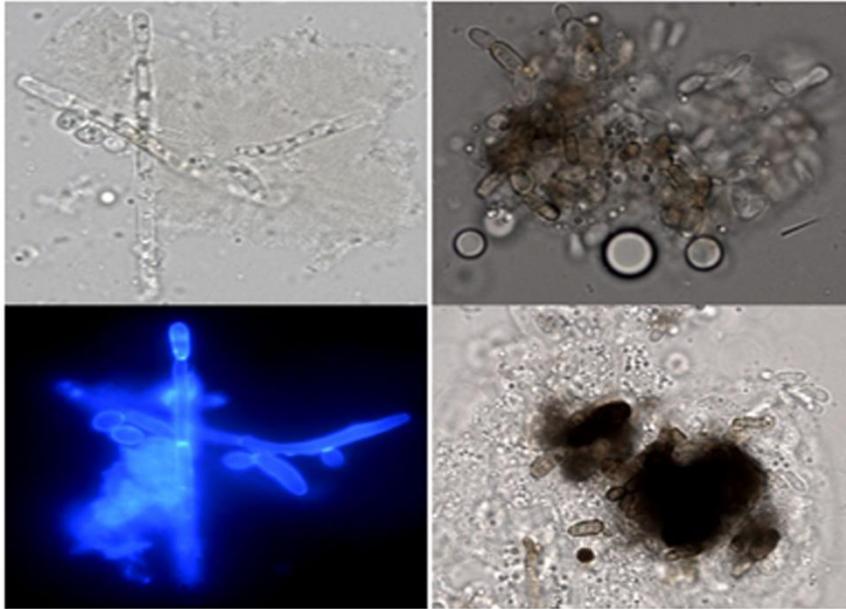
(A) Visual appearance of *G. mellonella* larvae infected with different *C. krusei* doses. (B, C and D) Optical Density (OD) of the haemolymph of *G. mellonella* infected with *C. krusei* ATCC 6258 (B), clinical isolate CL8053 (C) and CL80317 (D) with 5×10^5 , 10^6 , 5×10^6 cells/larva. The different size inoculum reveals dose-response melanization (* $p < 0.05$). All the experiments in this figure were performed at 37°C .

There was a significant accumulation of melanin in the haemolymph (4.3 times compared to the non-infected larvae), and this melanization increased over time (5 times at 24 h).

Filamentation and Hemocytic Density

C. albicans

C. krusei

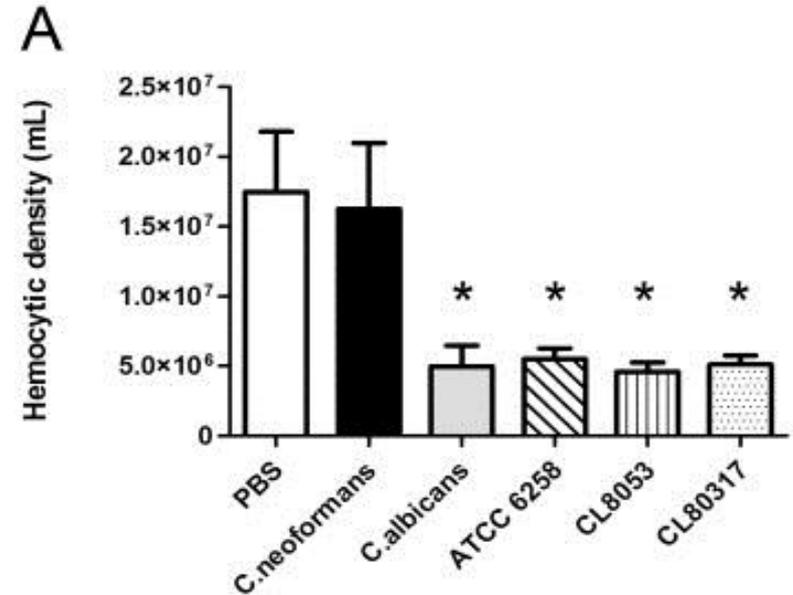


C. albicans produced filaments in the larvae.

Candida krusei also produced filaments, and appeared as clumps of fat body of dark color.

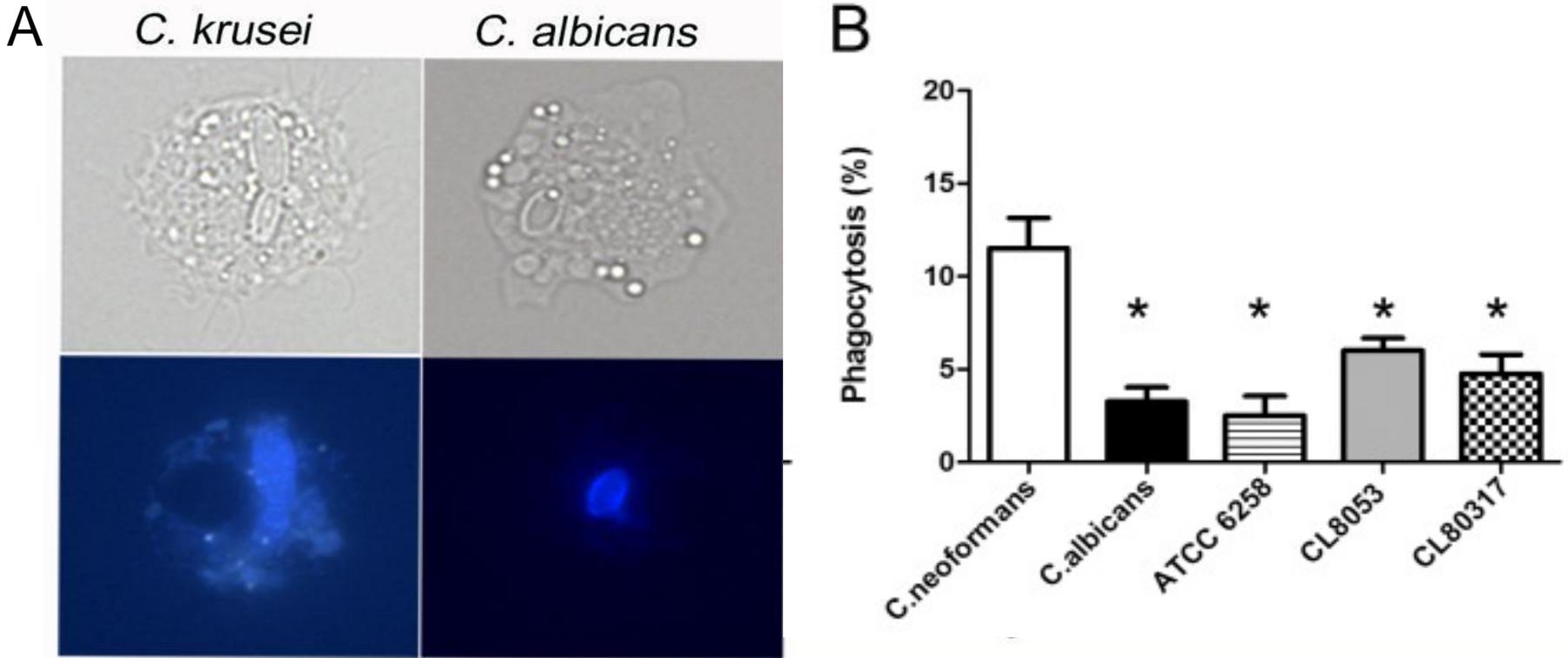
C. krusei produced a decrease in haemocyte density in a similar manner to *C. albicans*.

Changes in haemocyte density during *C. krusei* infection.



The haemolymph of infected larvae with *C. neoformans*, *C. albicans* SC5314, *C. krusei* ATCC 6258, CL8053 and CL80317 clinical isolates and PBS was collected and the concentration of haemocytes was estimated using a haemocytometer.

Phagocytosis



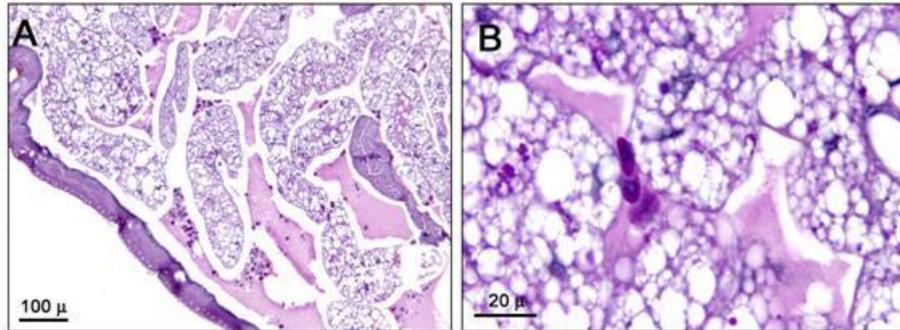
Phagocytosis of *C. albicans* SC5314 or *C. krusei* ATCC 6258 in *G. mellonella*. Calcofluor test (A).

B. Phagocytosis percentage of *C. neoformans*, *C. albicans* SC5314, *C. krusei* ATCC 6258, CL8053 and CL80317 clinical isolates. Asterisks denote differences statistically significant ($p < 0.05$).

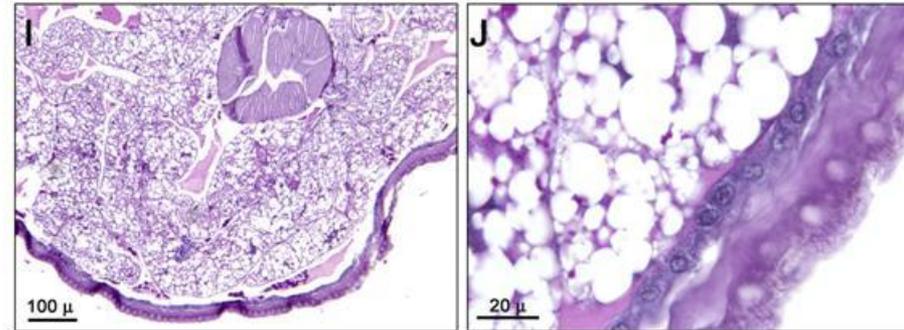
The phagocytosis for all *Candida* strains (*albicans* and *krusei*) was significantly lower when compared to *C. neoformans*

Histopathology

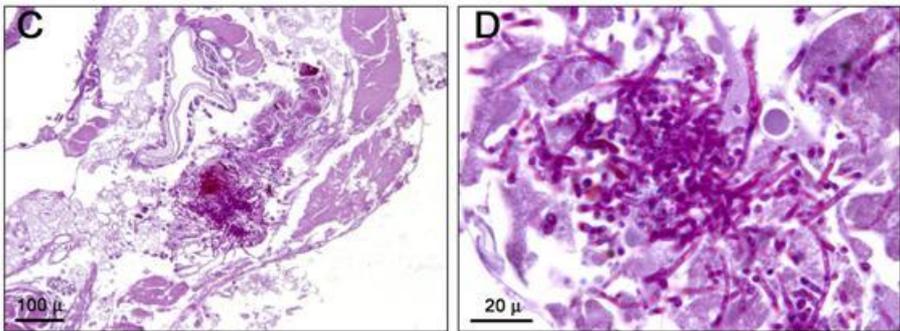
Controle



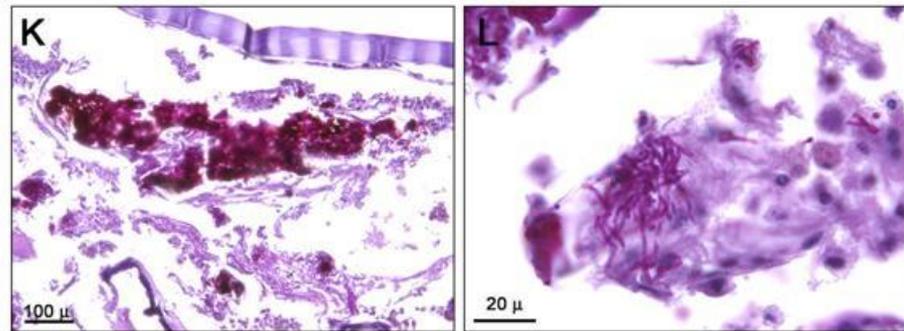
Controle



C. albicans



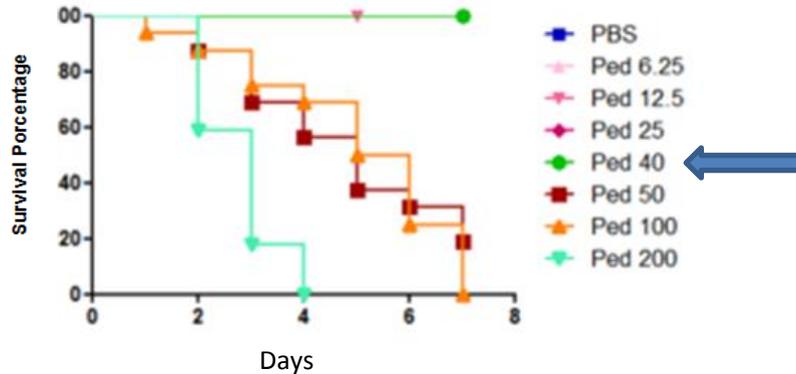
C. krusei



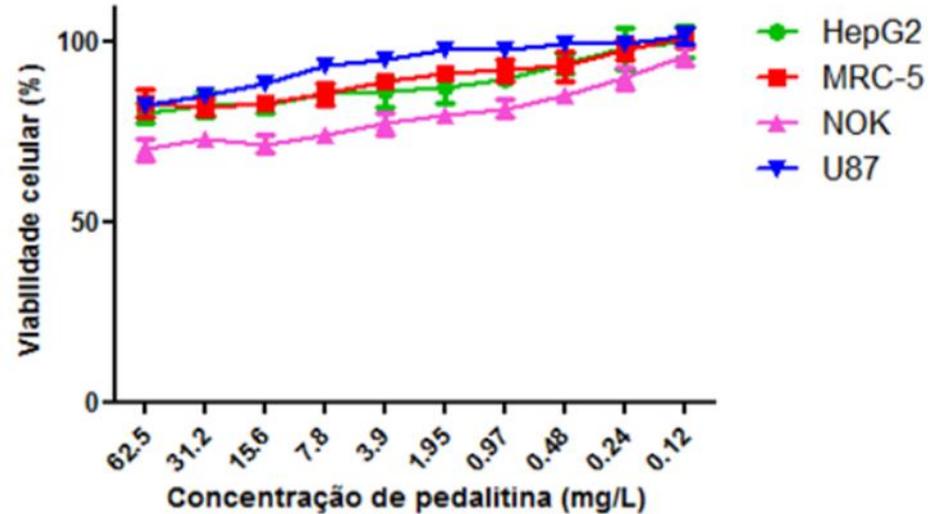
Controls: A,B,I,J. Infected larvae (C, D, K, L)

***Candida albicans* and *C. krusei* were found both in yeast and filament forms**

Toxicity of pedalitin from *Pterogyne nitens* in *G. mellonella*



Toxicity test of pedalitin (6,25, 12,5, 25, 40, 50, 100 e 200 mg/kg) in *Galleria mellonella*.

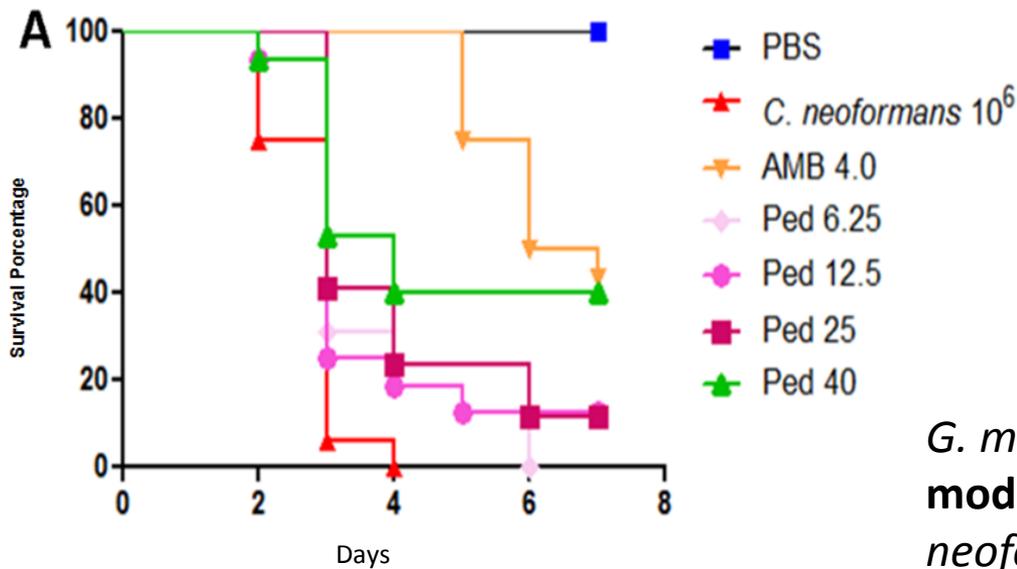


The cytotoxicity was evaluated by the MTT method using HepG2, MRC-5, NOK and U87 cell lines.

The percentage of living cells was greater than 50% for all of the substances tested, thereby indicating that the substances are non-toxic at the concentrations tested in the cell lines.

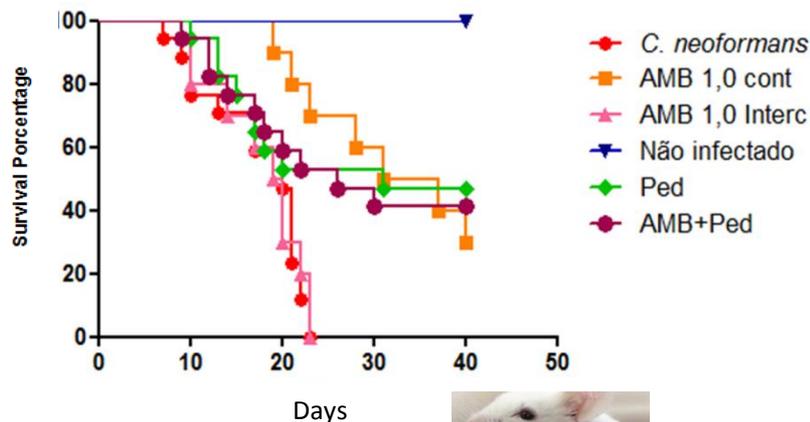


Pedalitin substance and amphotericin B and their activity in vivo against *C. neoformans* using alternative and conventional model



In vivo studies show similarity in the AMB and pedalitin activity, taking into account the fungal burden and the mice survival time of infected with *C. neoformans*.

G. mellonella is convenient and inexpensive model to screening substances against *C. neoformans*.

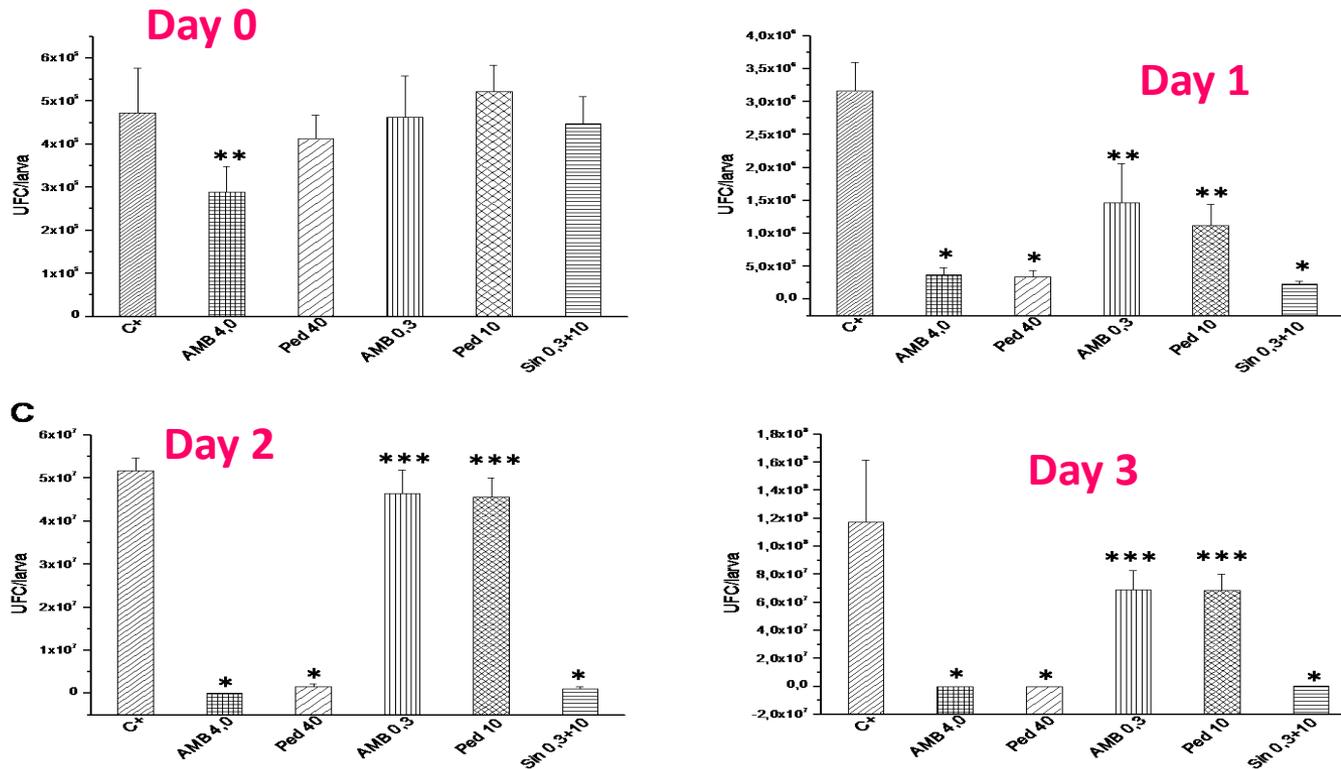


Survival time of mice Balb-C mice infected with *C. neoformans* and treated with amphotericin B (1.0 mg / kg) and the natural substance pedalitin (40 mg / kg).



Sangalli, F et al.

Fungal burden determined in *Galleria* before and after treatment



On day 0, only AMB showed a significant difference compared to the control, while on days 01, 2 and 3 all treatments were different, the most significant being AMB 4.0 mg / kg, pedalitin 40 mg / kg and combination of the two substances. After 72 hours of infection and treatment, AMB, pedalitin and the combination of the two substances showed reduction of over 95% in the number of colony forming units.

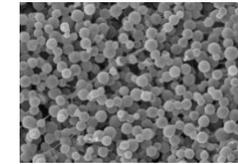
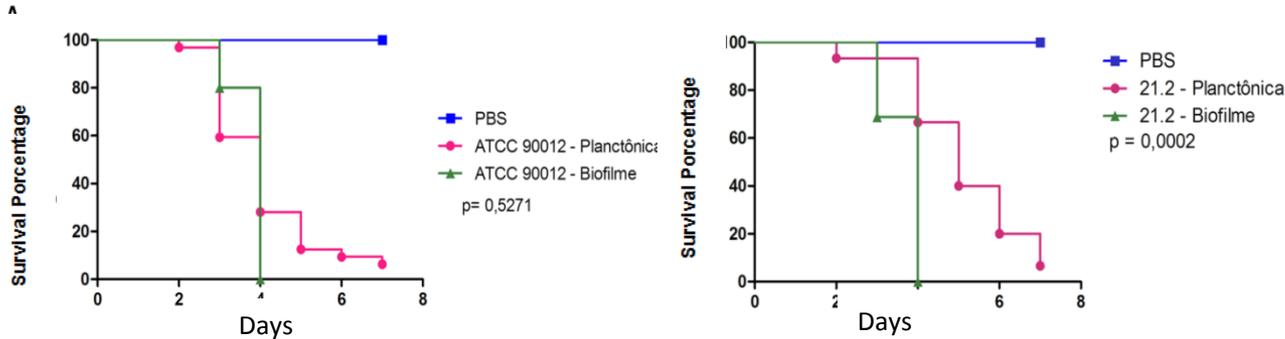
C. neoformans proteins after pedalitin contact

Spot	Massa Molecular	Alteração na expressão	Proteína	Código de acesso
Ped 38	51141	-2,9	Mitochondrial distribution and morphology protein 10	MDM10_LACTC
Ped 41	8938	-2,2	Protein transport protein SSS1	SC61G_YEAST
Proteomic analysis of <i>C. neoformans</i> biofilm resulted in increased expression of proteins related to oxidation-reduction and proteolysis stress response and reduced expression of proteins involved in metabolic processes, transport and translation.				
Ped 27	36269	-3,11	Glyceraldehyde-3-phosphate dehydrogenase	G3P_CRYNE
Ped 18	48759	-3,0	NADP-specific glutamate dehydrogenase 1	DHE4_SCHPO
Ped 35	19842	-2,31	Putative 40S ribosomal protein S4-like (Fragment)	YS4L_PNECA
Ped 07	44571	-4,4	ATP-dependent RNA helicase eIF4A	IF4A_CANAL
Ped 13	22946	-3,7	Peptidyl-prolyl cis-trans isomerase B	PPIB_ASPFU

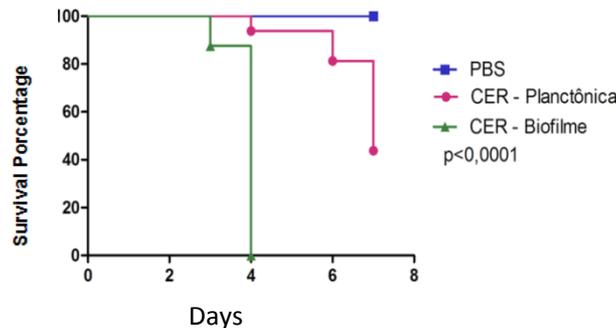
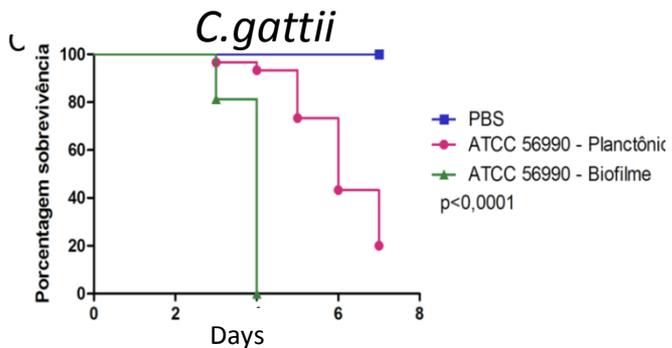
Survival of planktonic and biofilm forms of *C. neoformans* and *C. gattii* in *G. mellonella*

Microbial biofilms play an essential role in several infectious diseases and exhibiting enhanced resistance to antimicrobial drugs.

C. neoformans



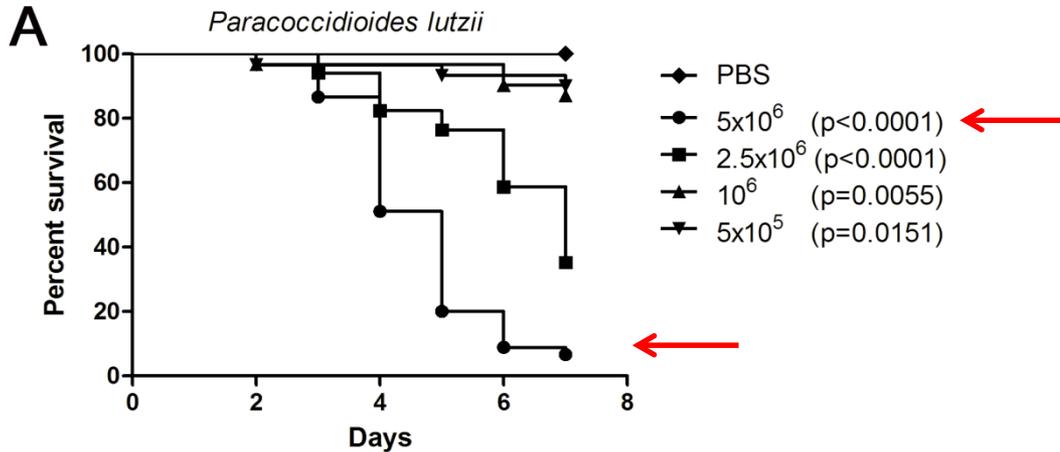
Cells from biofilm formation of two species were more virulent than planktonic cells



Biofilms are AMB and maitenin resistant compared to planktonic cells.

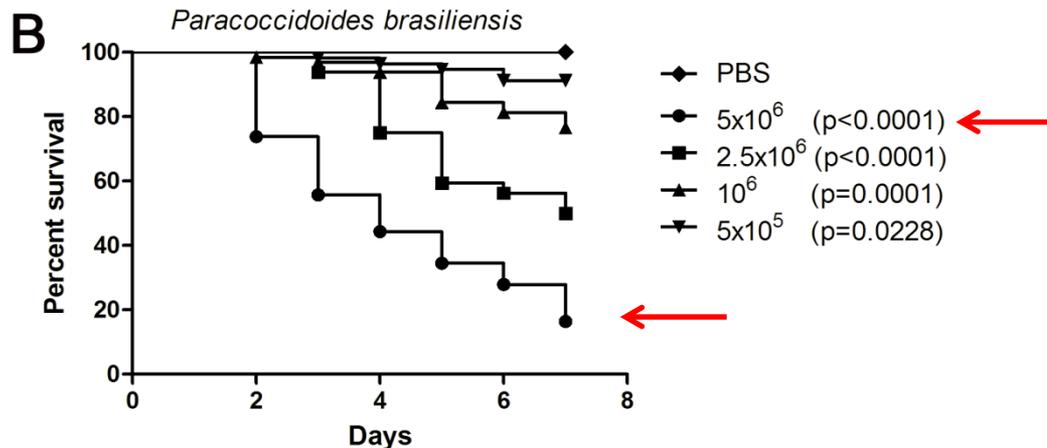
Larvae inoculated with biofilm cells presented less survival

Survival Curves and virulence assay



-Compare the virulence of *P. brasiliensis* and *P. lutzii* in the model *G. mellonella*.

- Evaluate the hemocytes response: hemocytical density, phagocytosis, hemocyte-fungus interaction.

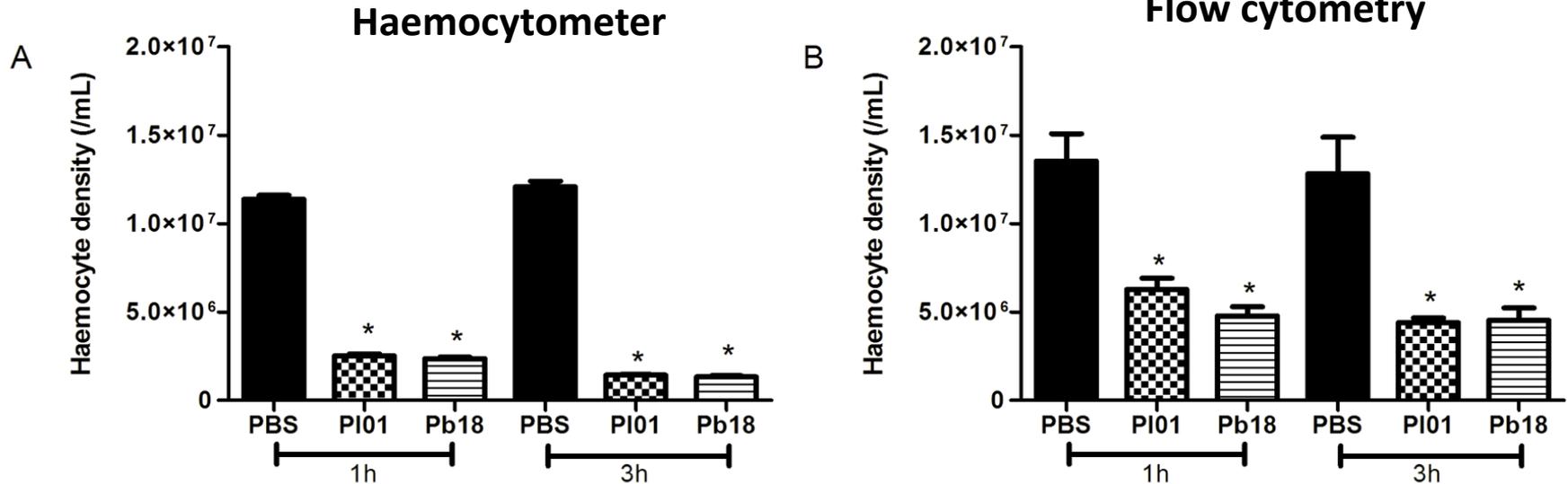


-The death of the larvae is dose-dependent

-Virulence of *P. lutzii* and *P. brasiliensis* is similar in *G. mellonella*

Survival curve of *G. mellonella* infected with *P. lutzii* (A) and *P. brasiliensis* (B) in different concentrations, PBS infected larvae were used as control ($p < 0.05$)

Hemocyte density



There was no statistical difference between species

Haemocyte density, obtained by microscopy (A) and flow cytometry (B), in *G. mellonella* larvae infected with *P. lutzii* (PI01) and *P. brasiliensis* (Pb18) assessed after 1 and 3 hrs. The asterisks indicate statistically significant ($p < 0.05$) relative to the PBS control.

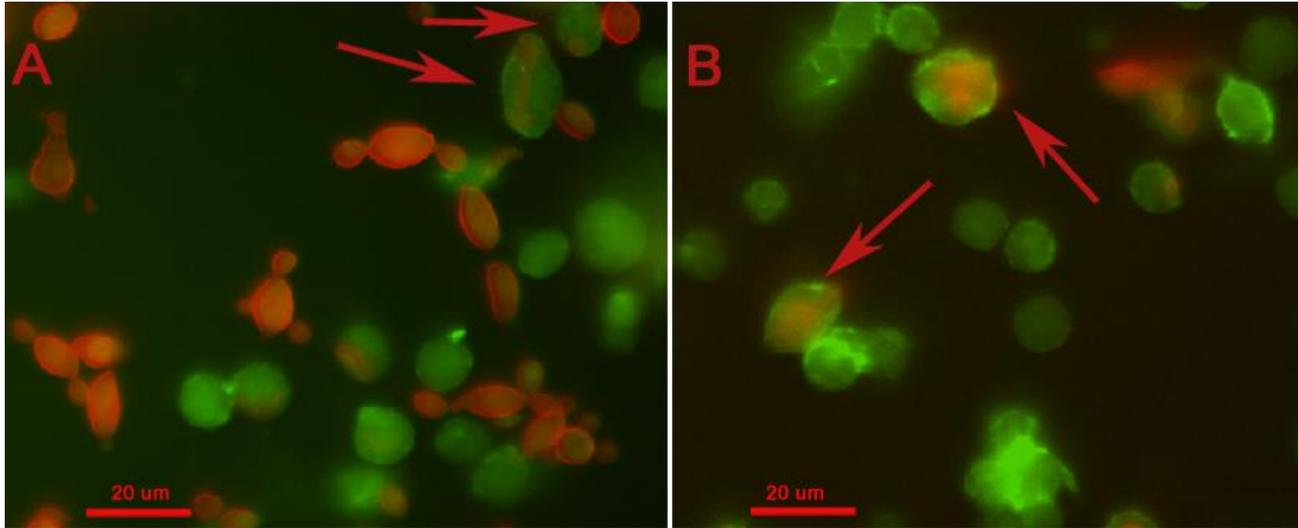
Decrease of 5x in 1 h
Decrease of 8x in 3 h

Decrease of 3 x in 1 and 3 h

***Paracoccidioides* spp infection cause a decrease of the hemocyte density.**

More virulent microorganisms cause a decrease of the number of hemolymph cells
(Bergin *et al.*, 2003)

Phagocytosis assay

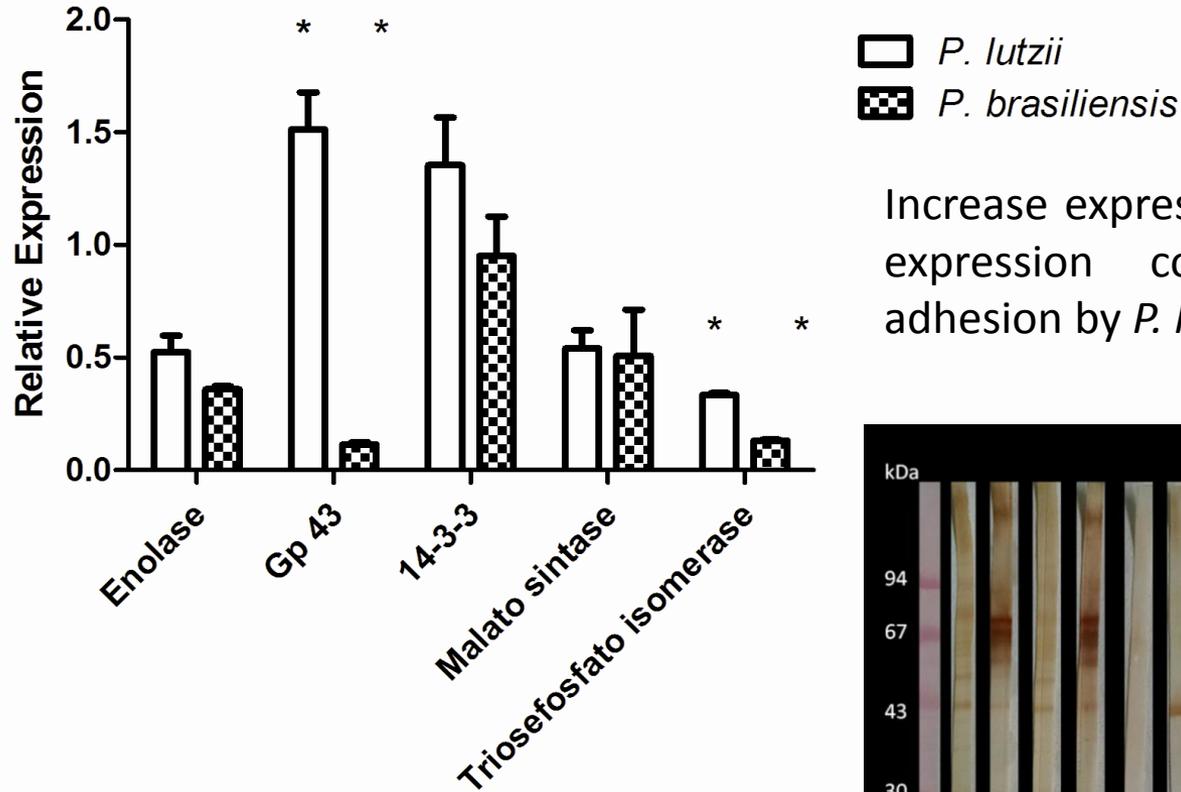


Phagocytosis of *Paracoccidioides* spp by haemocytic cells after 3 hrs of infection with 5×10^6 cells/larva of *P. lutzii* (A), or *P. brasiliensis* (B). Staining: Calcofluor 10 mg/mL for 30 min at 37 °C. Hemolymph collection and hemocytes staining with 0,165 µM Phalloidine.

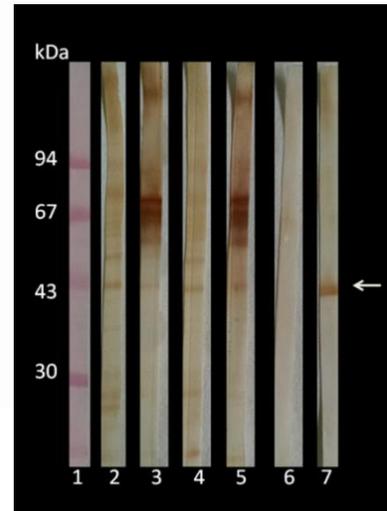
5 % of the hemocyte cells of *G. mellonella* were able to phagocytize *P. brasiliensis* or *P. lutzii*

However, *P. brasiliensis* showed lower interaction to hemocytes in relation to the *P. lutzii* infection, which would indicate a better escape mechanism of the fungus to the phagocytosis.

Paracoccidioides spp gene expression of adhesins by real-time PCR



Increase expression of Gp43. This higher gene expression could explain the potential adhesion by *P. lutzii* to the hemocytes.



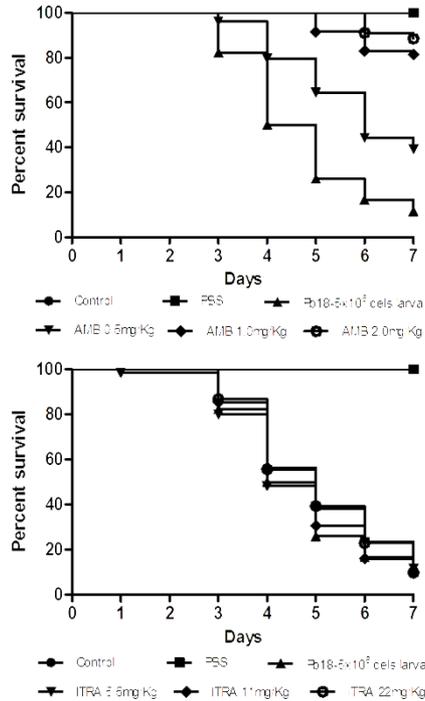
The presence of the 43 kDa fraction was observed in samples taken from *G. mellonella* infected with *P. lutzii* and *P. brasiliensis*

Western blot of *G. mellonella* infected with *P. brasiliensis* or *P. lutzii*. 1) mwm; 2) *P. lutzii* 3) *G. mellonella* infected with *P. lutzii*; 4) *P. brasiliensis* 5) *G. mellonella* infected with *P. brasiliensis*; 6) *G. mellonella* extract infection; 7) gp43 purified. The arrow indicates the Gp43.

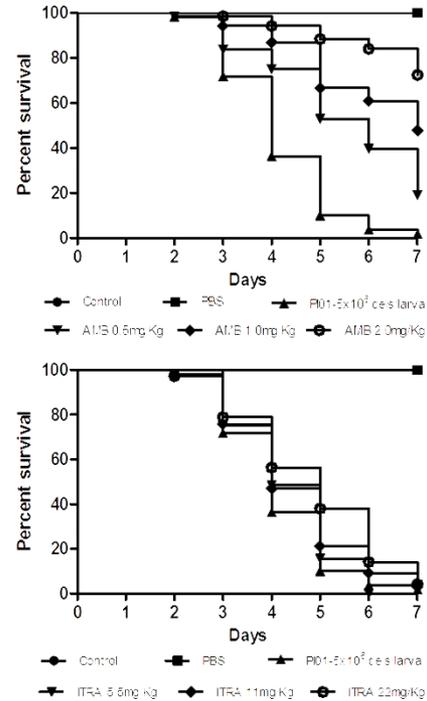
Relative gene expression of enolase, Gp43, 14-3-3, triosephosphate isomerase and malate synthase in *P. lutzii* and *P. brasiliensis*, (*) $p < 0.05$.

Paracoccidioides spp AMB and itraconazole treatment

(a) *Paracoccidioides brasiliensis* (Pb18)



(b) *Paracoccidioides lutzii* (PI01)



G. mellonella infection

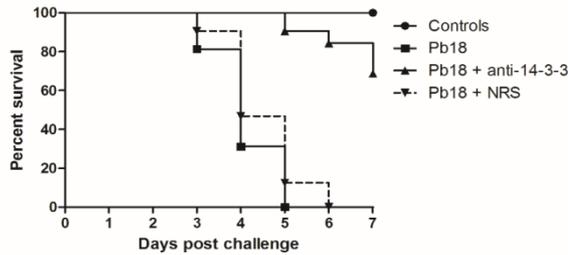
Survival curves of *G. mellonella* infected with 5x10⁶ cells of *P. brasiliensis* (Pb18 - a) and *P. lutzii* (PI01 - b) treated with different doses of amphotericin B (AMB - above) and itraconazole (ITRA - below). A *p* value of <0.05 was considered statistically significant.

***Paracoccidioides* species killed the most of the larvae at the end of 7 days. The treatment with all the doses of amphotericin B (0.5; 1 and 2 mg/Kg) protect the *G. mellonella* from infection. Itraconazole protected the larvae – PI01 at doses of 22 mg/Kg, but not at 11 and 5.5 mg/Kg.**

Importance of adhesins in virulence of *Paracoccidioides* spp.

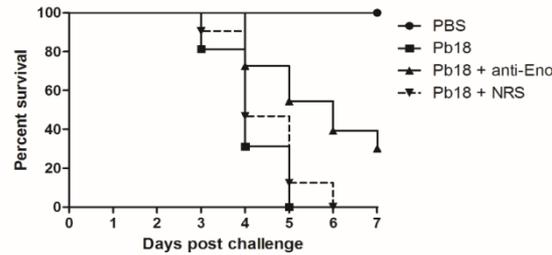
Aims: the role of the adhesins in the virulence of *P. brasiliensis* and *P. lutzii* used *G. mellonella* as model.

P. brasiliensis (Pb18) treatment with anti-14-3-3



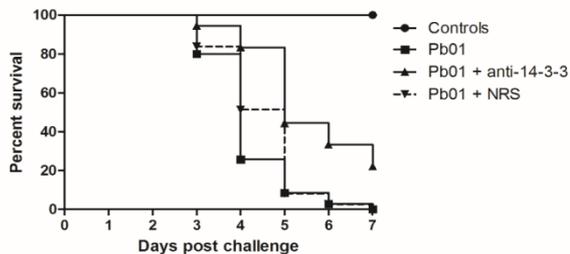
Survival rates of 69%

P. brasiliensis (Pb18) treatment with anti-ENO



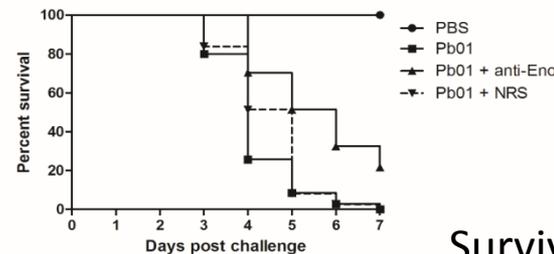
Survival rates of 20%

P. lutzii (Pb01) treatment with anti-14-3-3



Survival rates of 31%

P. lutzii (Pb01) treatment with anti-ENO



Survival rates of 22%

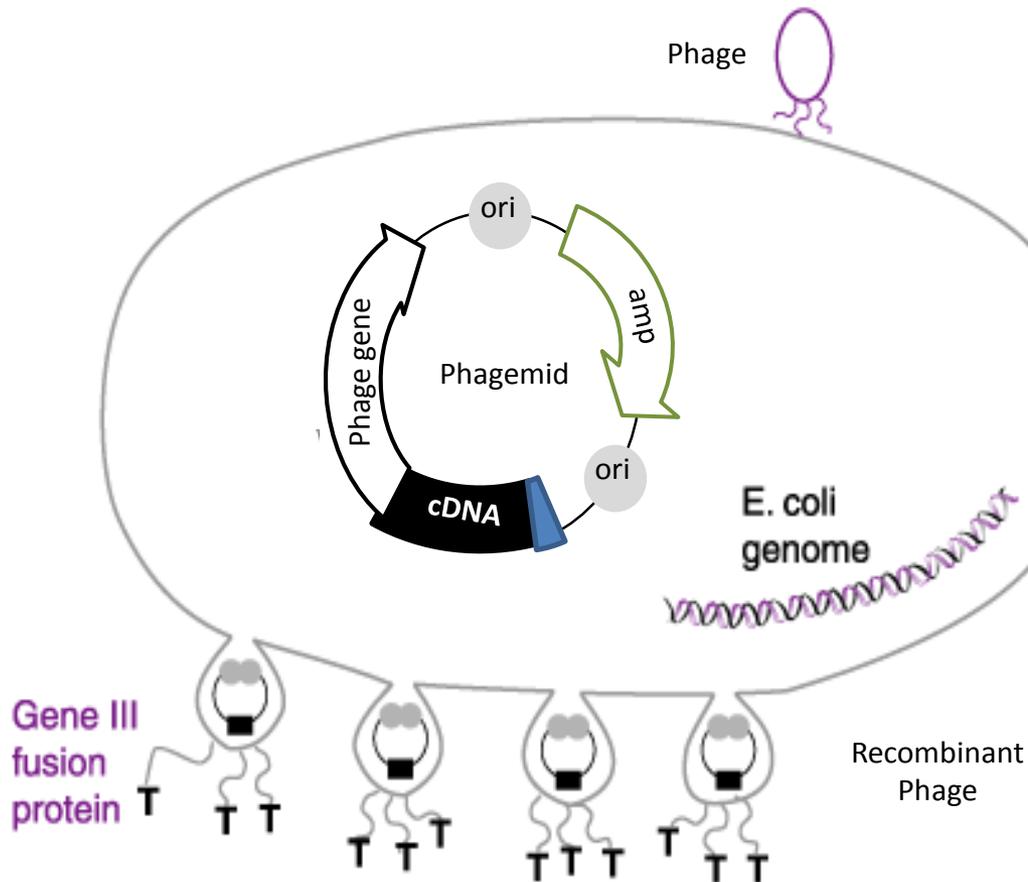
The fungi was tested with two different antibodies of two *Paracoccidioides* spp adhesins (enolase and 14-3-3), and the virulence was evaluated in the *Galleria* model.

Virulence was evaluated after blocking 14-3-3 and enolase adhesins in *G. mellonella*. This blocking led to a reduction in the virulence of *P. lutzii* and *P. brasiliensis*, confirming the relationship between adhesins and virulence.

Anti-adhesive therapy against *Paracoccidioides* spp.

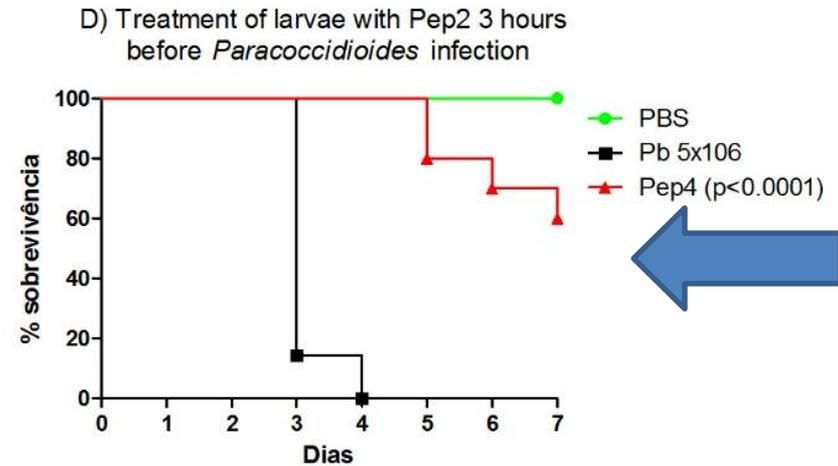
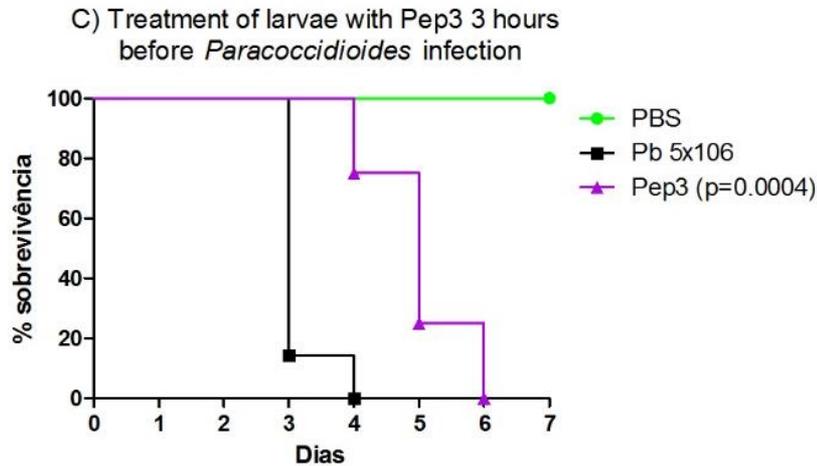
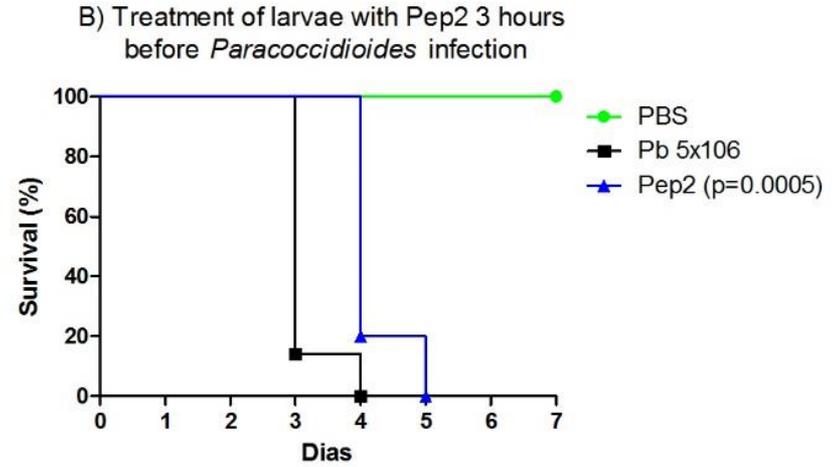
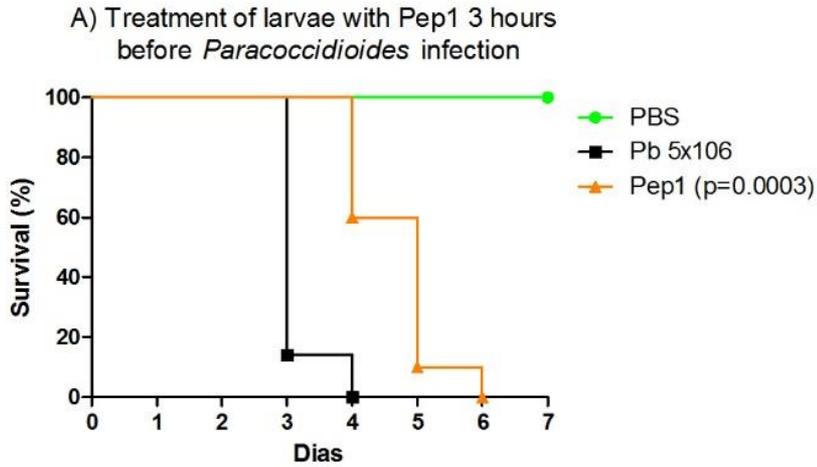
Using a Phage Display system we isolate 4 peptides that strongly binds to *Paracoccidioides* spp. cell wall.

We tested these peptides as possible anti-adhesive therapy agents. To access the peptides influence in *Paracoccidioides* spp. virulence we used the *G. mellonella* model.



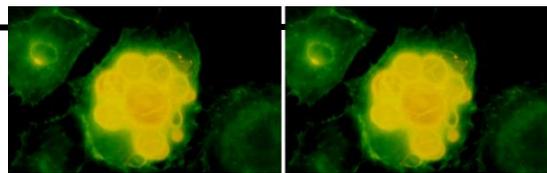
The four selected peptides are in patenting process in INPI. The Phage Display was evaluated in collaboration with Dr. Ricardo José Giordano – USP.

Influence of different peptides in the virulence of *Paracoccidioides lutzii*



These peptides prevent the infection by *P. lutzii* and the results showed a great potential of the peptide 4 to act as an effective molecule to prevent the infection in *G. mellonella*.

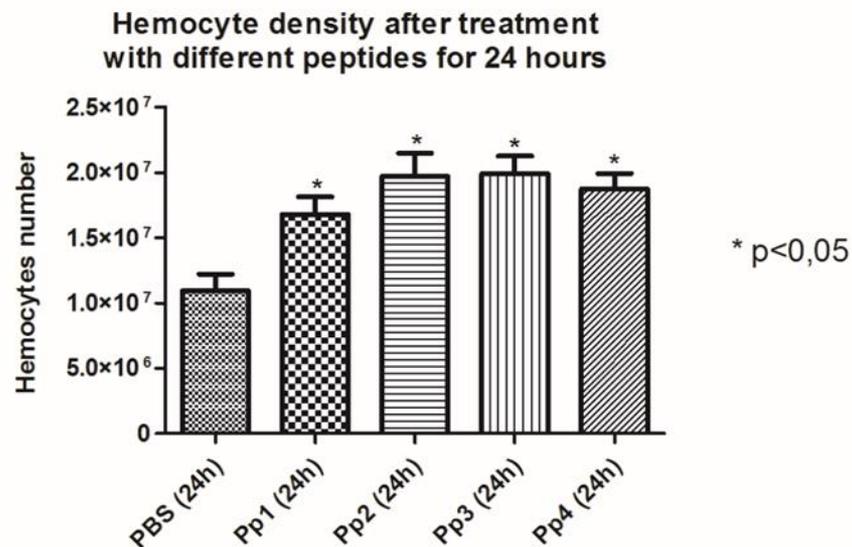
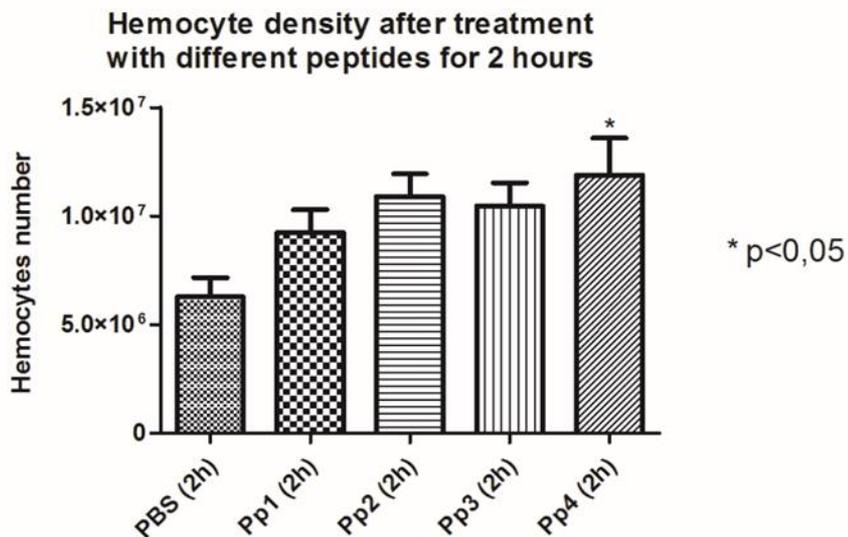
Hemocytes density after treatment of *G. mellonella* with the selected peptides



Hemocytes density after the treatment of *G. mellonella* with the four selected peptides.

All the peptides seems to activate the immune system of the larvae assisting in the defense of the organism against *P. lutzii* infection.

Peptide 4 treatment showed efficiency in activating the immune system of the larvae.



Conclusions

- ***Galleria mellonella* was suitable for virulence analysis in all fungi tested**
- **Quick results when comparing the traditional mouse model (Virulence curves 2 to 8 months)**
- **The overall procedure represents a rational and comprehensive means of evaluating antifungal activity from various perspectives for the selection of initial hits that can be explored in more in-depth mode-of-action studies.**
- **The model of *G. mellonella* is simple and feasible to study virulence, toxicity and efficacy of drugs. It can also be used in screening for verification of dosages that are effective and do not present toxicity to be used later in mammalian model, thereby reducing the number of animals.**
- **Studies using *Galleria* may identify several novel virulence factors, as well as for studying novel protective responses in the host.**
- ***Galleria* tolerates relatively high temperatures,**
The larger size of the *Galleria* larva also allows it to be infected with larger, more controlled doses of the pathogen.

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