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The University of Kent

School of Bioscience

*“Candida albicans Histone
Deacetylase 2 (HDA2) and its role in
morphological switches”*

By:

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MSc in Microbiology

September 2019

Index

Contenido

Abstract	1
Introduction	2
<i>Candida albicans</i> : a dangerous human fungal pathogen.....	2
Yeast to hyphae switch in <i>Candida albicans</i>	2
Hda1 and its role in <i>C. albicans</i> yeast-hyphae switch	5
Available antifungal drugs.....	6
Aim of this project.....	7
Material and Methods	9
1) STRAINS OF <i>C. ALBICANS</i>	9
2) MEDIAS.....	9
3) PROTOCOLS.....	10
3.1) Transformation by electroporation	10
3.2) Transformation for <i>C. albicans</i> by making competent cells.....	13
3.3) Gel agarose.....	14
3.4) Hyphae induction in YPD, RPMI, Spider and Serum agar plates	14
3.5) Hyphae induction in YPD agar plates in a 5% CO ₂ chamber	15
3.6) Hyphae induction in YPD agar plates in hypoxia chamber	15
3.7) Hyphae induction in RPMI and Spider liquid media	15
3.8) Hyphae induction in YPD, RPMI and Spider liquid media in a 5% CO ₂ chamber	15
3.9) Invasion in YPD, RPMI and Spider agar plates	16
3.10) Statistical T-test.....	16
3.11) Hyphal length determination.....	16
Results	17
Attempts to generate a <i>C. albicans hda2</i> Δ/Δ and <i>hda3</i> Δ/Δ double mutant.....	17
Deletion of <i>HDA2</i> does not impact colony morphology in standard growth conditions.....	18
Inducing hyphal morphogenesis in solid medias using Serum, Spider and RPMI medias	20
Serum media	20
Spider media	22
RPMI media	24
Testing lack of O ₂ and increase of CO ₂ (5%) as hyphal inducing conditions during the incubation time	25
Hypoxia.....	26
Increase of CO ₂	27

First trials for hyphal morphogenesis using liquid hyphal inducing medias	28
RPMI liquid media (6 hours growth)	30
Spider liquid media (6 hours growth).....	33
Increasing the time for hyphal morphogenesis in RPMI and Spider liquid media.....	37
RPMI liquid media (24 hours growth)	37
Spider liquid media (24 hours growth)	41
Reaching to the final correct protocol	44
RPMI liquid media (6 hours growth in rich media)	45
Spider liquid media (6 hours growth in rich media).....	48
Measuring the size of filamentation of hyphal cells	51
Spider liquid media (hypha size)	52
RPMI liqui media (hypha size)	54
Incorporation of increased CO ₂ to hyphal inducing liquid medias	56
YPD liquid media (24 hours growth in 5% CO ₂).....	57
RPMI liquid media (24 hours growth in 5% CO ₂).....	58
Spider liquid media (24 hours growth in 5% CO ₂).....	59
Hypha strength and proliferation capacity of <i>C. albicans</i>	60
Discussion	62
Establishment of protocol to test hyphal growth	62
Hda2 and its role in the yeast to hyphae switch	63
Bibliography	65

List of figures, tables and graphs

Figures

- Figure 1. Regulation of the dimorphic transition in <i>C. albicans</i> via multiple signaling pathways.....	5
- Figure 2. Illustration of the <i>Clox</i> System for Multi-Marker gene disruption.....	16
- Figure 3A. Single cell of <i>C. albicans</i>	17
- Figure 3B. Single colony of <i>C. albicans</i>	17
- Figure 4A. High filamentation colony (↑) in YPD.....	18
- Figure 4B. Low filamentation colony (↓) in YPD.....	18
- Figure 4C. Smooth colony in YPD.....	18
- Figure 5A. High filamentation colony (↑) in Serum.....	20
- Figure 5B. Low filamentation colony (↓) in Serum.....	20
- Figure 5C. Smooth colony in Serum.....	20
- Figure 6A. High filamentation colony (↑) in Spider.....	22
- Figure 6B. Low filamentation colony (↓) in Spider.....	22
- Figure 6C. Smooth colony in Spider.....	22
- Figure 7A. High filamentation colony (↑) in RPMI.....	24
- Figure 7B. Low filamentation colony (↓) in RPMI.....	24
- Figure 7C. Smooth colony in RPMI.....	24
- Figure 8. <i>C. albicans</i> cells grown in hypoxia.....	25
- Figure 9. Yeast cell.....	28
- Figure 10A. Pseudohyphal cell.....	28
- Figure 10B. Pseudohyphal cell.....	28
- Figure 11A. Hyphal cell.....	29
- Figure 11B. Hyphal cell.....	29

Tables

- Table 1. Strains.....	8
- Table 2. Medias.....	8

Graph

- Graph 1. Quantification of colony morphology in YPD agar plates.....	18
- Graph 2. Quantification of colony morphology in Serum agar plates.....	20
- Graph 3. Quantification of colony morphology in Spider agar plates.....	21
- Graph 4. Quantification of colony morphology in RPMI agar plates.....	23
- Graph 5. Quantification of colony morphology in YPD agar plates grown in 5% CO ₂ condition.....	26
- Graph 6. Quantification of cells in RPMI liquid media at 2 hours growth.....	30
- Graph 7. Quantification of cells in RPMI liquid media at 4 hours growth.....	30
- Graph 8. Quantification of cells in RPMI liquid media at 6 hours growth.....	31
- Graph 9. Absorbance at 600nm during 6 hours in RPMI liquid media.....	32
- Graph 10. Quantification of cells in Spider liquid media at 2 hours growth.....	33
- Graph 11. Quantification of cells in Spider liquid media at 4 hours growth.....	33

- Graph 12. Quantification of cells in Spider liquid media at 6 hours growth.....	34
- Graph 13. Absorbance at 600nm during 6 hours in Spider liquid media.....	35
- Graph 14. Quantification of cells in RPMI liquid media at 12 hours growth.....	37
- Graph 15. Quantification of cells in RPMI liquid media at 16 hours growth.....	37
- Graph 16. Quantification of cells in RPMI liquid media at 20 hours growth.....	38
- Graph 17. Quantification of cells in RPMI liquid media at 24 hours growth	38
- Graph 18. Absorbance at 600nm during 24 hours in RPMI liquid media.....	39
- Graph 19. Quantification of cells in Spider liquid media at 12 hours growth.....	40
- Graph 20. Quantification of cells in Spider liquid media at 16 hours growth.....	41
- Graph 21. Quantification of cells in Spider liquid media at 20 hours growth.....	41
- Graph 22. Quantification of cells in Spider liquid media at 24 hours growth.....	42
- Graph 23. Absorbance at 600nm during 24 hours in Spider liquid media.....	43
- Graph 24. Quantification of cells for AB54 (WT) and AB347 (<i>hda2</i> Δ/Δ) in RPMI liquid media for 6 hours.....	44
- Graph 25. Quantification of cells for AB66 (WT) and AB629 (<i>hda2</i> Δ/Δ) in RPMI liquid media for 6 hours.....	45
- Graph 26. Quantification of cells for AB216 (WT) and AB525 (<i>hda1</i> Δ/Δ) in RPMI liquid media for 6 hours.....	45
- Graph 27. Absorbance at 600nm during 6 hours in RPMI liquid media.....	46
- Graph 28. Quantification of cells for AB54 (WT) and AB347 (<i>hda2</i> Δ/Δ) in Spider liquid media for 6 hours.....	47
- Graph 29. Quantification of cells for AB66 (WT) and AB629 (<i>hda2</i> Δ/Δ) in Spider liquid media for 6 hours.....	48
- Graph 30. Quantification of cells for AB216 (WT) and AB525 (<i>hda1</i> Δ/Δ) in Spider liquid media for 6 hours.....	48
- Graph 31. Absorbance at 600nm during 6 hours in Spider liquid media.....	50
- Graph 32. Representative size in percentage of hyphal filaments for AB347 (<i>hda2</i> Δ/Δ) compared to AB54 (WT) in Spider liquid media for 6 hours.....	51
- Graph 33. Representative size in percentage of hyphal filaments for AB629 (<i>hda2</i> Δ/Δ) compared to AB66 (WT) in Spider liquid media for 6 hours.....	52
- Graph 34. Representative size in percentage of hyphal filaments for AB525 (<i>hda1</i> Δ/Δ) compared to AB216 (WT) in Spider liquid media for 6 hours.....	52
- Graph 35. Representative size in percentage of hyphal filaments for AB347 (<i>hda2</i> Δ/Δ) compared to AB54 (WT) in RPMI liquid media for 6 hours.....	53
- Graph 36. Representative size in percentage of hyphal filaments for AB629 (<i>hda2</i> Δ/Δ) compared to AB66 (WT) in RPMI liquid media for 6 hours.....	54
- Graph 37. Representative size in percentage of hyphal filaments for AB525 (<i>hda1</i> Δ/Δ) compared to AB216 (WT) in RPMI liquid media for 6 hours.....	54
- Graph 38. Quantification of cells in YPD liquid media in 5% CO ₂ at 24 hours growth.....	56
- Graph 39. Quantification of cells in RPMI liquid media in 5% CO ₂ at 24 hours growth.....	57
- Graph 40. Quantification of cells in Spider liquid media in 5% CO ₂ at 24 hours growth...	58

Abstract

Every year, the human fungal pathogen *Candida albicans* infects millions of people all around the globe, and as time goes by the amount of infections is worryingly increasing due to the incidences of drug resistance and the low availability of antifungal drugs [48]. The main reason why *C. albicans* is a pathogen fungus able to survive through antifungal treatments it is because of its ability to perform morphological switches [34].

In *C. albicans*, the lysine deacetylase Hda1 (class II) is an important regulator of the yeast to hyphae switch [39]. In *Saccharomyces cerevisiae*, Hda1 assembles with two non-catalytic subunits, Hda2 and Hda3 [41]. The role of Hda2 in *C. albicans* biology is still unknown. The aim of this project is to test if Hda2 have also a role on these morphological changes using Hda1 as a positive control.

Genetic manipulation techniques were used for trials to create double mutants (*hda1* Δ/Δ , *hda2* Δ/Δ). Several medias and different conditions were tested to reach the best hyphal inducing template for *C. albicans* with the aim to finally conclude whether if Hda2 does have an influence on these morphological changes.

Higher hyphal induction was detected in RPMI and Spider media. Increase of CO₂ in the environment also increased hyphal morphogenesis. With the results obtained it was then concluded that deletion of *HDA2* has a real impact decreasing hyphal morphogenesis under some conditions and, therefore, making more studies about Hda2 and Hda 3, could be interesting for targeting these genes to create new antifungal drugs.

Introduction

Candida albicans: a dangerous human fungal pathogen

The yeast *Candida albicans* has emerged as a major public health problem during the past two decades [1]. This fungus is a diploid, polymorphic yeast that acts as a prevalent human commensal, growing as an included part of the microflora in the gastrointestinal tract, mouth and genital mucosa. However, when the host immune system is compromised, it can cause mucosal infections and life-threatening disseminated infections [2] acting as the most commonly encountered human fungal pathogen [3].

Although the benefits of *C. albicans* colonization are unclear, the harmful effects are well known: vaginal candidiasis affects 75% of the women at least once during their lifetime (with recurrent forms in 10% of cases) and oropharyngeal candidiasis occurs in 90% of HIV-infected patients with AIDS [1]. *C. albicans* can invade vital organs and cause serious, life threatening systemic infections associated with a mortality rate for invasive candidiasis up to 30-40% [4].

Yeast to hyphae switch in *Candida albicans*

C. albicans has numerous distinctive features not seen in other fungi, one of the most interesting is its capacity to reversibly switch among yeast and hyphal morphology [5]. This capacity to switch between different morphological forms in response to different stimuli is a key for virulence in *C. albicans*. These switches are the responsible of the resistance to drug treatments.

The observation that *C. albicans* is found predominantly in the hyphal form in tissue samples of candidiasis patients suggests that the yeast-to-hyphal morphogenetic switch plays a role in the transition from candidemia to the subsequent tissue invasion [4]. The establishment of candidemia itself might also be aided by the enhanced ability of the hyphal morphology to penetrate the mucous membranes and underlying tissues, and to enter the bloodstream [6]. Furthermore, the hyphae formation in the phagosome was shown to contribute to the

ability of *C. albicans* cells to escape phagocytosis and kill the macrophage [7], [8]. So, virulence of *C. albicans* relies upon its ability to change between yeast and hyphal morphology. Yeast cells are mainly for colonization, early infection and dissemination, while hyphal development is responsible for tissue invasion and chronic infections [9]. Hyphal morphogenesis is a very complex process, and *C. albicans* uses multiple pathways to identify host signals and promote hyphae development.

Indeed, *C. albicans* filamentation can be induced by many environmental cues such as mammalian serum, body temperature, pH, hypoxia and CO₂ concentration which reflects the variety of signals sensed by the fungus in the different microenvironments encountered in the host [4].

The cyclic AMP (cAMP)—protein kinase A (PKA) pathway is the major pathway controlling hyphal growth induced by serum and CO₂ in *C. albicans* [10], [11]. Serum and CO₂ act directly on the adenylate cyclase Cyr1 to activate the catalytic subunit of protein kinase A, Tpk2 [10], [11]. Tpk2 then directly or indirectly phosphorylates the key transcription factor Efg1 [12]–[14]. Efg1 is a negatively auto-regulated transcription factor, which controls morphogenesis via interaction with heat shock factor-type transcriptional regulators Sfl1 and Sfl2, as well as transcription factors Ndt80 and Flo8 [13]–[17].

Sfl1 and Sfl2 antagonistically regulate morphogenesis in *C. albicans* [17]:

- Sfl1 acts via Efg1 and Ndt80 to repress hyphal morphogenesis activators, such as transcription factors Ume6, Tec1, Brg1, and Sfl2, while upregulating morphogenesis repressors, such as transcriptional co-repressors Ssn6 and Nrg1 [18]–[23].
- Sfl2 acts via Efg1 and Ndt80 to express hyphal morphogenesis activators, such as Ume6 and Tec1, while downregulating repressors of morphogenesis, such as the transcriptional co-repressor Nrg1 and Rfg1, as well as Sfl1 [17], [18], [20], [21], [23]–[25].

Nrg1 represses expression of its downstream target Brg1, which directly upregulates Ume6 and the hypha-specific G1 cyclin Hgc1 via interaction with the histone deacetylase Hda1 [24].

Hgc1 then activates the cyclin-dependent kinase Cdc28 to phosphorylate Rga2, a GTPase-activating protein of the central polarity regulator Cdc42 [26], [27]. In addition, expression of Hgc1 is also controlled by Flo8, which functions via interaction with Efg1 [15], [16].

In response to elevated temperature in *C. albicans*, the molecular chaperone Hsp90 is a global regulator of morphogenesis and acts by recruiting the co-chaperone Sgt1 to repress activity of Cyr1 [28]. Hsp90 also inhibits the activity of Cyr1 via direct or indirect interactions with Ras1 [29], [30]. Finally, Hsp90 also controls morphogenesis by directly or indirectly repressing activity of Pho85Pcl1, which phosphorylates the transcription factor Hms1 to activate expression of Ume6 [21], [31].

Under low oxygen condition, Sre1 is cleaved to release the N-terminal transcription factor Sre1N to induce hyphal morphogenesis. Sre1N degradation is accelerated by the prolyl 4-hydroxylase-like 2-oxoglutarate-Fe(II) dioxygenase, Ofd1, in the presence of oxygen [32].

While the transition from yeast to hyphae has been extensively studied in *C. albicans*, the switch from hyphae to yeast still remains poorly understood. Pes1, a *pescadillo* homolog in *C. albicans*, is involved in the hyphae to yeast switch, especially in the budding of yeast from lateral filamentous cells [33].

Detailed analysis of signal transduction pathways in *C. albicans* have identified an intricate network that coordinates signals from diverse environmental cues to modify basic cellular functions, such as cell cycle, membrane and cell wall synthesis, and transport. Ultimately, these modifications allow for *C. albicans* to seamlessly transition between yeast and hyphal growth throughout its interaction with the host [34].

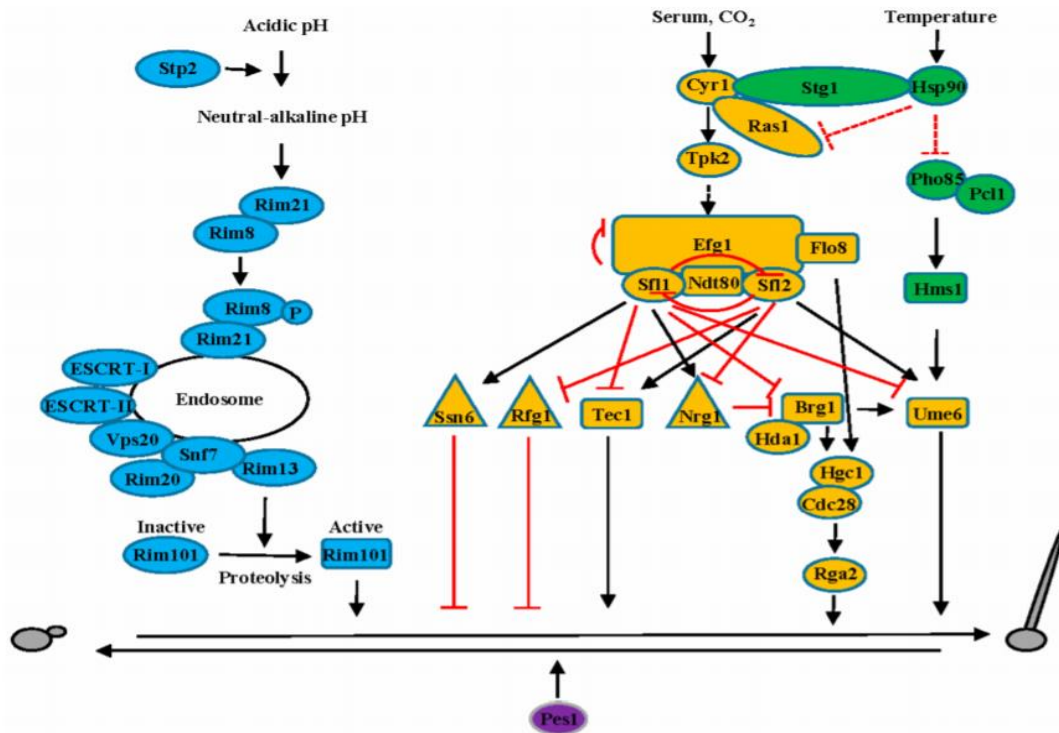


Fig 1. Image from: Morphology Changes in Human Fungal Pathogens upon Interaction with the Host [34]. Regulation of the dimorphic transition in *C. albicans* via multiple signaling pathways. Signaling pathways are illustrated in different colors: cyclic AMP (cAMP)- protein kinase A (PKA) pathway (yellow), Hsp90 pathway (green), Rim101 pathway (blue) and Pes1 pathway (purple). Solid lines or arrows represent direct regulation. Dotted lines or arrows represent indirect or unknown regulation. Positive and negative relationships between components are illustrated in black and red, respectively. Transcription factors are illustrated with a rectangle, transcriptional co-repressors with a triangle, and other proteins with an oval [34].

Hda1 and its role in *C. albicans* yeast-hyphae switch

Protein lysine deacetylases (KDACs), including the classic Zn²⁺-dependent histone deacetylases (HDACs) are enzymes that play critical roles in numerous biological processes, particularly the epigenetic regulation of global gene expression programs in response to internal and external cues [35]. They act as global regulators of gene expression by catalysing the removal of acetyl functional groups from the lysine residues of histones and non-histone proteins [36]. KDACs can favour transcriptional repression by deacetylating lysine residues on histone tails allowing chromatin compaction and/or preventing binding of bromodomain-containing transcriptional activators. KDACs can also activate transcription by deacetylation of non-histone proteins [36]. As a consequence, deletion or inhibition of KDACs often results in the upregulation and downregulation of an approximately equivalent number of genes [37]. There are three different

classes of HDACs (Class I,II, and IV) that share a common active site architecture and a Zn²⁺-dependent catalytic mechanism [38]

In *C. albicans*, the lysine deacetylase Hda1 (class II) is an important regulator of morphological switches. In response to certain stimuli, hyphal morphogenesis is initiated; it requires a rapid but temporary disappearance of the Nrg1 transcriptional repressor of hyphal morphogenesis via activation of the cAMP-PKA pathway, but, maintenance of the hyphal form requires promoter recruitment of Hda1 histone deacetylase under reduced Tor1 (target of rapamycin) signaling [39].

Hda1 deacetylates Yng2, a subunit of the NuA4 histone acetyltransferase module, leading to eviction of the NuA4 acetyltransferase module and blockage of Nrg1 access to promoters of hypha-specific genes [39]. Promoter recruitment of Hda1 for hyphal maintenance happens only during the period when Nrg1 is gone. The sequential regulation of hyphal development by the activation of the cAMP-PKA pathway and reduced Tor1 signaling provides a molecular mechanism for plasticity of dimorphism and how *C. albicans* adapts to the varied host environments in pathogenesis [39]. However, the Hda1 pathway is not required for hyphae elongation in hypoxia or in the presence of elevated CO₂ because of the presence of redundant pathways [32], [40]. As a result, the Hda1-mediated hyphae maintenance pathway contributes, but it is not absolutely required, for virulence in vivo [32]. These results suggest that Hda1 is a good target for antifungal drugs development to be used in combination therapies.

Now HDACs becomes a potential druggable targets, however correctly targeting Hda1 for antifungal drug is flawed because of the high sequence similarities between Hda1 and its human orthologs and consequently turns into high toxicity for the host.

Available antifungal drugs

There are few antifungal drugs effective for the treatment of *C. albicans* infections and their clinical utility is limited by the rapid emergence of drug resistance [48]. The close evolutionary

relationship between fungi and the human makes effective treatment for *C. albicans* infections be hindered by the limited number of potential drug targets [48]. That is why there is an urgent need to develop alternative treatments. Here we study a potential strategy targeting *C. albicans* morphological plasticity.

Aim of this project

In *Saccharomyces cerevisiae*, Hda1 assembles with two non-catalytic subunits, Hda2 and Hda3, essential for Hda1 deacetylation activity both in vivo and in vitro [41]. Interestingly, no metazoan homologous of Hda2 and Hda3 have been identified. Hda2 and Hda3 are similar in sequence and share a similar protein organization with an N-terminal DNA binding domain (DBD) and a C-terminal coil-coil domain (CCD). The DBD domain, is sufficient to bind DNA in vitro. The CCD domains act as a scaffold for the assembly of the Hda1 complex [42].

Studies from the University of Kent demonstrate that Hda1 complex is conserved in *C. albicans* since Hda2 and Hda3 interacts with Hda1 in vivo. Deleting *HDA2* gene may influence on the hyphal morphogenesis since this becomes into a reduced expression of components of the Hda1 complex. In this study we analyze the role of *C. albicans* Hda2 and we pursue to demonstrate how under hyphae-inducing and non-hyphae-inducing conditions, Hda2 regulates at some point the yeast to hyphae switch and in case of positive results propose Hda2 as an attractive target for the development of novel antifungal drugs.

We also hypothesize that using different states of media, such as solid and liquid, may give us a better understanding of how the fungus acts reflecting the human physiology; Solid medium is limited on nutrients once the colonies have grown on the surface for several days which could reflect their infection on several organs. Liquid medium could reflect the human physiology of the blood stream where the fungus is suspended on rich nutrients. Using both medias and having in consideration different parameters of the results such as hyphal lengths of individual cells or the number of hyphal colonies against normal colonies in both WT and mutant strains

may give us a conclusion of the importance of the gene HDA2 in morphological switch of *Candida albicans*.

Material and Methods

1) STRAINS OF *C. ALBICANS*

Table 1. Strains of *C. albicans* used for this project

1. <u>AB66: WT (BWP17)</u>	
2. <u>AB629: <i>hda2</i> Δ/Δ</u>	<i>MTL a/alpha</i> <i>ura3Δ::Δimm434/ura3Δ::Δimm434</i> <i>his1::hisG/his1::hisG</i> <i>arg4::hisG/arg4::hisG</i> <i>hda2Δ/hda2Δ</i>
3. <u>AB54: WT (SN152)</u>	
4. <u>AB347: <i>hda2</i> Δ/Δ</u>	<i>MTL a/alpha ura3Δ-</i> <i>iro1Δ::imm434/URA3-IRO1 his1Δ/his1Δ</i> <i>arg4Δ/arg4Δ leu2Δ/leu2Δ</i> <i>hda2Δ/hda2Δ</i>
5. <u>AB216: WT (SN95)</u>	
6. <u>AB525: <i>hda1</i> Δ/Δ</u>	<i>arg4/arg4 his1/his1 URA3/ura3</i> <i>::imm434 IRO1/iro1::imm434</i> <i>HIS1/his1::TAR-FRT</i> <i>hda1Δ::FRT/hda1Δ::FRT347:</i>

2) MEDIAS

Table 2. Medias used for this project

YPD (1L)	10 g Yeast Extract	20 g Bacto-peptone	20 g Glucose		20 ml Adenine 1M	10 ml Uridine 1M	970 ml H ₂ O (sterile)		20 g Agar
Spider (1L)	20 g Nutrient broth	20 g Mannitol	4 g K ₂ HPO ₄			10 ml Uridine 1M	990 ml H ₂ O (sterile)		27 g Agar
Serum (1L)	10 g Yeast Extract	20 g Bacto-peptone	20 g Glucose		20 ml Adenine 1M	10 ml Uridine 1M	870 ml H ₂ O (sterile)	100 ml Serum	20 g Agar
RPMI (1L)	10,4 g RPMI					10 ml Uridine 1M (only for AB66 and AB629)	990 ml H ₂ O (sterile)		20 g Agar

Only added for agar plates

Autoclaved at 120 °C

Filtered

3) PROTOCOLS

3.1) Transformation by electroporation

3.1.1) PCR product

Preparation of the PCR product (with the brand: PCR Biosystems)

- Plasmid (100 µg/µl)	2 µl
- Oligos 229 (10µM)	1 µl
- Oligos 230 (10µM)	1 µl
- 5x Buffer + dNTP (15mM MgCl ₂ + 5mM dNTPs + enhancers and stabilizers)	5 µl
- HiTaq (2u/µl)	0.25 µl
- H ₂ O (sterile)	15.75 µl
TOTAL	25 µl

Keep diluted oligos and plasmids at -20 °C to be reused. Perform a vortex and put it in the thermocycler.

Times and temperatures at the thermocycler:

- 98 °C	30s	
- 98 °C	30s	30cycles
- 58 °C	30s	
- 72 °C	2,5m	
- 72 °C	7m	

Check by electrophoresis if the pcr product was correctly done. Keep the pcr product at -20 °C to be used.

3.1.2) Transformation for *C. albicans*

Inoculate the strains overnight in 5 ml YPD at 30°C incubator with agitation. The day after transfer 1 ml of the overnight culture to a new flask with 50 ml of YPD. Grow over day until they reach OD 1.5 at 30°C incubator. Transfer the culture to a falcon tube and cold down the falcon centrifuge at 4°C. Centrifuge the culture at 4000 rpm for 2 minutes at 4°C. Discard the supernatant. Wash the culture with 25 ml sterile water by inverting the falcon tube gently. Centrifuge the culture at 4000 rpm for 2 minutes at 4°C. Discard the supernatant. Resuspend the content with 25 ml of TELi and then add 250 µl DTT 1M (DTT should be added fresh directly from the fridge). Incubate the content at room temperature for 1 hour with gentle shaking. Centrifuge the culture at 4000 rpm for 2 minutes at 4°C. Discard the supernatant. Resuspend the content with 25 ml cold sterile water from the fridge. Centrifuge the culture at 4000 rpm for 2 minutes at 4°C. Discard the supernatant. Resuspend the content with 10 ml of cold 1M sorbitol from the fridge. Centrifuge the culture at 4000 rpm for 2 minutes at 4°C. Discard the supernatant. Resuspend the content with 60 µl of cold 1M sorbitol from the fridge. Hold it on ice.

Electroporation:

For each transformation put 40 µl of cells in an eppendorf tube and add 5 µl of the pcr product. Incubate on ice for 5-10 minutes. Transfer the mixture to an ice-cold electro cuvette and place it in an electroporation chamber. Pulse at 1.5 kV, 25 µFD, 200 ohms. Remove the cuvette and immediately add 1 ml of 1M sorbitol (pipet it up and down twice). Transfer this mixture to a 5 ml fresh YPD tube and let them grow overnight at 30°C incubator with agitation. Next day centrifuge the culture at 4000 rpm for 2 minutes (it doesn't matter the temperature). Discard the supernatant. Resuspend the content with 400 µl of sterile water and streak them in YPD plates. Put the plates in the 30 °C incubator for 3-4 days and then check potential positives colonies by PCR.

3.1.2.1) TELi

- 10xTE 10 ml
- 1M LiAc 10 ml
- H₂O (sterile) 80 ml

(Filtered)

3.1.2.2) 10xTE

- 1M Tris 5 ml
- 0,5M EDTA 1 ml
- H₂O (sterile) 44 ml

(Filtered)

3.1.3) NaOH preparation for PCR of a single colony

Select a single colony from the plate and streak it on another YPD plate. From the same selected colony introduce a little of it in an eppendorf containing 40 µl of 0.02M NaOH and mix it until the content is cloudy. Heat the content in a thermal block at 100 - 105 °C for 10 minutes. Cool the content on ice for 10 minutes. Centrifuge the content at 5000 rpm for 2 minutes. Take 1µl of the supernatant to carry out the PCR (or keep it on ice).

3.1.4) Checking the deletion

Preparation of the PCR mix (with the brand: PCR Biosystems)

- ActFW AB165 (10µM) 0,6 µl
- ActRev AB261 (10µM) 0,6 µl
- dNTP (25µM) 0,5 µl
- Taq (5u/µl) 0,13 µl
- Buffer 10x 1,5 µl
(30mM MgCl₂ + enhancers
and stabilizers)
- H₂O (sterile) 10,67 µl
- TOTAL 14 µl

In an eppendorf introduce 1 µl of the NaOH preparation and 14 µl of the PCR mix. Introduce the content in the thermocycler.

Times and temperatures at the thermocycler:

- 94 °C 7m

- 94 °C	45s	30cycles
- 56 °C	1m	
- 72 °C	1m	
- 72 °C	7m	

Once the thermocycler is finished, add 5 µl of running buffer to each 15 µl of the mixtures and then take 15 µl of that and put it to each well in the agarose gel. Also add 7 µl of DNA ladder to each well to see the size of the bands.

3.2) Transformation for *C. albicans* by making competent cells

Making competent cells:

Inoculate overnight the strains in 5 ml YPD at 30°C incubator with agitation. The day after transfer 1 ml of the overnight culture to a new flask with 50 ml of YPD. Grow over day until they reach OD 1.3. Transfer the culture to a falcon tube. Centrifuge the culture at 3000 rpm for 3 minutes at room temperature. Discard the supernatant. Wash the culture with 5 ml sterile water by inverting the falcon tube gently. Centrifuge the culture at 3000 rpm for 3 minutes at room temperature. Discard the supernatant. Resuspend the content with 5 ml of SORB by inverting the falcon tube gently. Centrifuge the culture at 3000 rpm for 3 minutes at room temperature. Discard the supernatant by aspiration. Resuspend cells in 360 µl of SORB and 40 µl of carrier DNA. Make and freeze 7 aliquots of 50 µl of the cells at -80 °C.

Competent cells transformation:

Defrost competent cells at room temperature. Add 5 µl of 100ng/5µl plasmid and 300 µl PEG/TE/LiAc solution to each 50 µl aliquots defrosted. Mix the content by using vortex or pipette. Incubate at 30 °C for 24 hours (if possible, keep mixing it by inverting during incubation time). After the incubation, heat shock it at 44 °C for 15 minutes. Centrifuge the content for 15 seconds at 4000 rpm and decant the supernatant. Resuspend the cell pellet in 350 µl of sterile

water and streak it to YPD plates. Put the plates in the 30°C incubator for 3-4 days and then check potential positives colonies by PCR.

3.2.1) PEG/TE/LiAc

- 1M LiAc 30 µl
- 1M Tris 3 µl
- 0,5M EDTA 0,6 µl
- PEG 240 µl
- H₂O (sterile) 26,4 µl

(Autoclaved)

3.2.2) SORB

- 100mM LiAc
- 10mM Tris-HCL (pH 7.5)
- 1mM EDTA (pH 7.5/8)
- 1M Sorbitol

(Autoclaved)

3.3) Gel agarose

- 0,5 g Agarose
- 50 ml TBE 0,5x (dilute)

Melt the content in the microwave,
then let it cool down.

- Add 2,5µl Ethidium bromide 1M

Let the gel solidify in the appropriate recipient.

(Running conditions: 120 Volts for 20-30 minutes)

3.4) Hyphae induction in YPD, RPMI, Spider and Serum agar plates

Inoculate overnight the strains in 5 ml YPD at 30°C incubator with agitation. The day after transfer 50 µl of the overnight culture to a new tube with 10 ml YPD and place it in the same incubator for around 5 hours till the culture reaches to OD 1. Since *Candida albicans* OD 1 gives around 3×10^7 cells per ml make serial dilutions till getting a 100 cells in 100 ml (for example dilute 50 µl of the culture to 4,95 ml of water, then repeat the same dilution, and finally take

3.2.3) PEG

- 100mM LiAc
- 10mM Tris-HCL (pH 7.5/8)
- 1mM EDTA (pH 8)
- 40% PEG3350 or PEG40000 (both are ok)

(Autoclaved)

3.2.3) Carrier DNA

Denature 10 mg/ml of single stranded DNA at 100°C for 10 minutes, keep it on ice for 5 minutes, and store it at -20°C.

3.3.1) TBE 5X

- 2,7 g of Tris
- 1,37 g Boric Acid
- 0,5M EDTA 1 ml
- H₂O (sterile) 49 ml

(Autoclaved)

1,66 ml of the last dilution to 3,33 ml of water). Streak around 100 theoretical cells (in our example would be using 100 ml of the last dilution) to each plate: YPD, RPMI, Spider and Serum. Incubate the plates at 30 °C for one week. Count how many cells had become high hyphae, low hyphae or not hyphae at all.

3.5) Hyphae induction in YPD agar plates in a 5% CO₂ chamber

Use the same protocol of the section 3.4 but the incubation during the week must be in a 5% CO₂ chamber at 37 °C.

3.6) Hyphae induction in YPD agar plates in hypoxia chamber

Use the same protocol of the section 3.4 but the incubation during the week must be in a hypoxia chamber at 37 °C.

3.7) Hyphae induction in RPMI and Spider liquid media

Inoculate overnight the strains in 5 ml YPD at 30°C incubator with agitation. The day after transfer 50 µl of the overnight culture to a new tube with 10 ml YPD and place it in the same incubator for around 5 hours till the culture reaches to OD 1. Spin the culture at 4000 rpm for 2 minutes at room temperature. Wash the content with 10 ml of sterile water. Transfer 100 µl of each strain to new tubes of 10 ml liquid medias: RPMI and Spider. Incubate the strains at 37 °C incubator with agitation for the amount of hours needed to collect the data (in hour case from each hour to even 24 hours). Count in the microscope how many cells had become high hyphae, low hyphae or not hyphae at all.

3.8) Hyphae induction in YPD, RPMI and Spider liquid media in a 5% CO₂ chamber

Use the same protocol of the section 3.7 but the incubation during the different hours must be in a 5% CO₂ chamber at 37 °C.

3.9) Invasion in YPD, RPMI and Spider agar plates

Inoculate overnight the strains in 5 ml YPD at 30°C incubator with agitation. The day after transfer 50 µl of the overnight culture to a new tube with 10 ml YPD and place it in the same incubator for around 5 hours till the culture reaches to OD 1. Add 150 µl of each strain to a 96-wells plates (using the spots needed) and use a replica platter to sow the strains in each plate. Incubate the plates at 30 °C for 5 days. Take a picture and clean the surface with a glove and some water. Take another picture and put them back to the incubator. Let them grow for some more days till you can see the cells growing again.

3.10) Statistical T-test

To calculate the p-statistic values of the t-tests to eliminate the null hypothesis, Microsoft Excel is used. The number of different types of colonies and cells obtained for each replicate carried out in each experiment is introduced. Since the numerical counts of each replicate have different total colonies/cells numbers, the formula used provided by Microsoft Excel is: “t tests for two samples assuming unequal variances”, to compare the data obtained between WT strains and their mutants.

3.11) Hyphal length measurement

To express the measure of the length of the hyphal form of *C. albicans*, the averages of the WT strains were measured in pictures taken and visualizing them using a program called “XnView”, they were used as a references of 100% of growth, and the mutant strains are represented as the percentages obtained in the graphs over the 100% growth from their WT strains.

Results

Attempts to generate a *C. albicans* *hda2* Δ/Δ and *hda3* Δ/Δ double mutant

Hda2 and Hda3 share a significant sequence similarity and therefore it is possible that have redundant roles. An initial aim of this project was to generate a *hda2* Δ/Δ , *hda3* Δ/Δ double mutant, as single deletion mutant were already generated by members of the lab using the *Clox* system, a genetic system that allow recycling of marker genes as outlined in Fig 2 [43].

The *Clox* system allows for the generation of a homozygous deletion mutant lacking any marker genes and therefore genetically identical to the parental strain except for the deleted gene. This allows for the direct comparison of phenotypes between mutant and parental strains.

Single deletions of *HDA2* or *HDA3* were created using the *Clox* system for gene disruption [43]

using long-oligos PCR, the LAL (loxP-ARG4-loxP) and NAT1-*Clox* (loxP-NAT1-MET3p-cre-loxP) plasmids as templates (Fig 2 as a comparison). During all selections for *Clox* transformants media was supplemented with 2.5 mM methionine and 2.5 mM cysteine to repress the *MET3* promoter and minimize Cre/loxP mediated recombination. *HDA2* and *HDA3* single gene deletions were confirmed by PCR and markers were resolved by allowing Cre expression in media lacking methionine and cysteine as described [43].

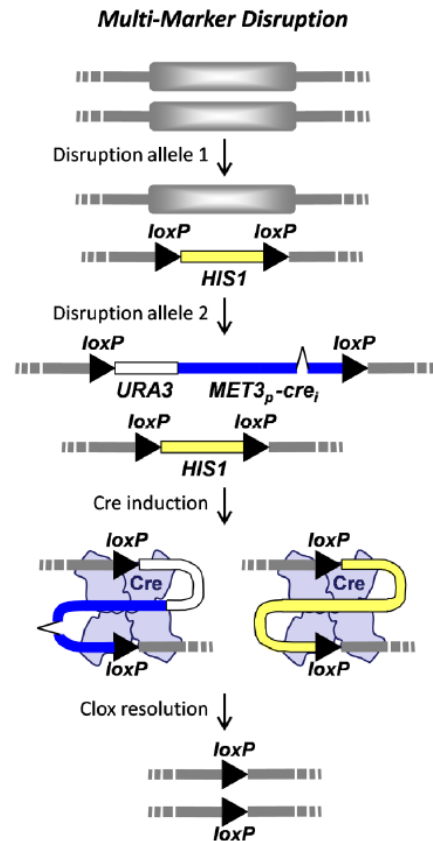


Fig 2. Illustration of the *Clox* System for Multi-Marker gene disruption. In this imagen taken from Shahana S Et Al. [43] it was used LHL (loxP-HIS1-loxP) instead of the one used in our deletion: LAL (loxP-ARG4-loxP), and URA3-Clox (loxP-URA3-MET3p-cre-loxP) instead the one used in our deletion: NAT1-Clox (loxP-NAT1-MET3p-cre-loxP) plasmids

Using the same template, the second deletion for *HDA2* (in *hda3* Δ/Δ) or *HDA3* (in *hda2* Δ/Δ) was not successful even if it was repeated several times for several months.

Due to time restraint, this project focused solely on *hda2* Δ/Δ single mutant to investigate the role of Hda2 in the yeast to hyphae switch.

Deletion of *HDA2* does not impact colony morphology in standard growth conditions.

In the laboratory, *C. albicans* is routinely grown on rich YPD media at 30 °C. Under this growth conditions, WT strains of *C. albicans* grows as a yeast in liquid media (Fig 3A) and form round smooth colonies on solid media (Fig 3B) since YPD is a yeast inducing media [44].

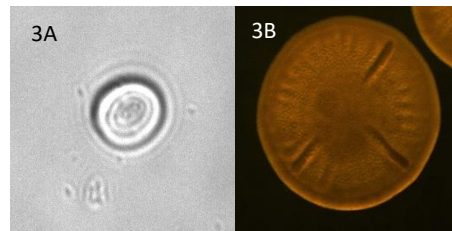
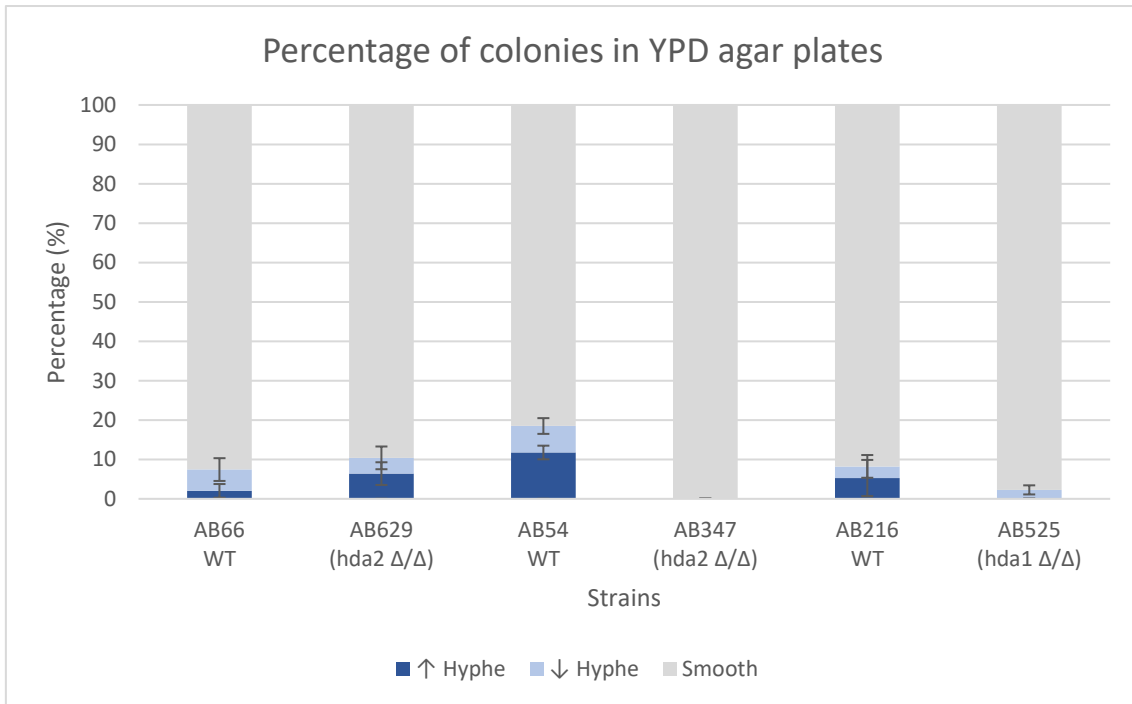


Fig 3A. Picture of a single cell of *C. albicans* (grown in 37°C liquid YPD) in yeast form

Fig 3B. Picture of a single colony of *C. albicans* (grown in 37°C agar YPD) in

The first goal in this project was to test whether deletion of the *HDA2* gene has an impact on *C. albicans* morphology in this same media (YPD agar). To this end, two independent *hda2* Δ/Δ strains (AB347 and AB629) and their original WT strains (AB54 and AB66 respectively) were grown in agar YPD media at 37 °C for 7 days. Around 50 ~ 100 colonies were plated in each YPD agar plates (Protocol 3.4). An *hda1* Δ/Δ strain (AB525) and its original WT strain (AB216) were included as a positive control. The experiment was performed in three biological replicates and results are shown in Graph 1.



Graph 1. Quantification of colony morphology in YPD agar plates

Average in percentage of different colony morphologies observed in three biological replicates, for each strain, grown in YPD agar plates. Error bars represents standard deviation for the three replicates.

The criteria used for determination of the colony morphology was based on the amount of filamentation that could be seen in the colonies grown. Here it is presented some pictures as an example (Fig 4A, Fig 4B and Fig 4C)



Fig 4A. High filamentation colony (↑) **Fig 4B.** Low filamentation colony (↓) **Fig 4C.** Smooth colony

As shown in Graph 1, deletion of *HDA2* did not have a high impact on colony morphology in YPD media, as the vast majority of the colonies were smooth and did not present any filamentation. But it was noticed that hyphal growth was slightly diminished in one of the two *hda2* deleted

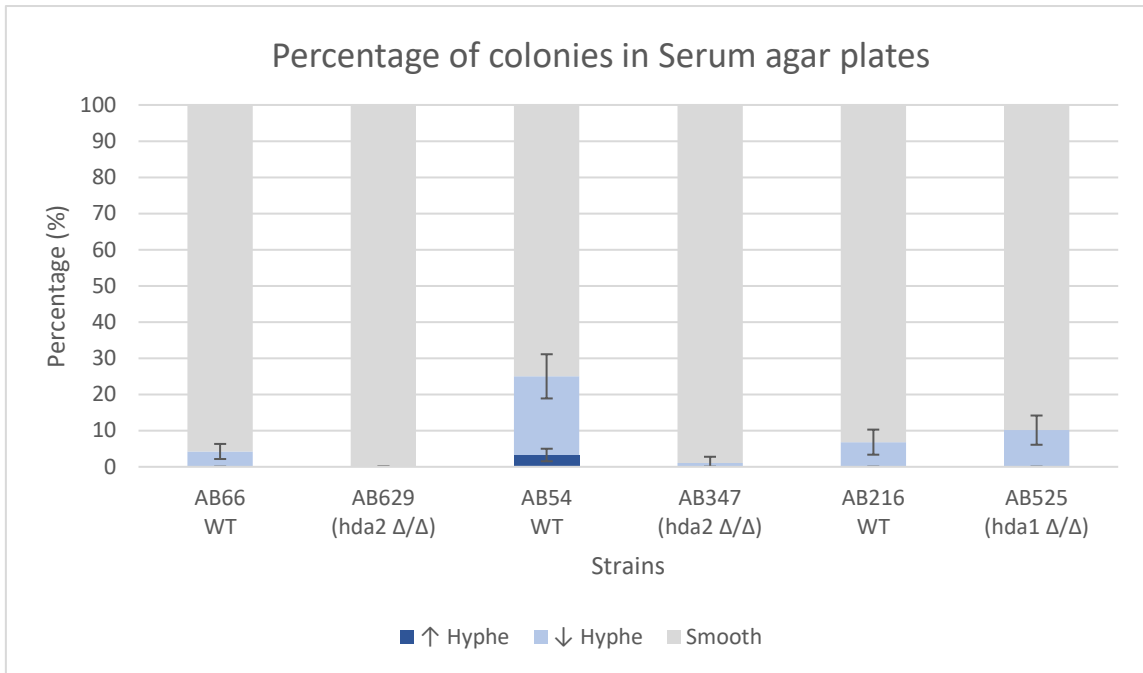
strains (AB347) and in the *hda1* deleted strain (AB525), suggesting that, even though these differences are not statistically significant (all p values between hyphal colonies of WT strains and mutants were higher than 0,05), both genes may have some influence inducing hyphal growth. To test this hypothesis further experiments were carried out in different growth conditions known to strongly induce hyphal formation.

Inducing hyphal morphogenesis in solid medias using Serum, Spider and RPMI medias

Induction of hyphal growth was not strong in YPD. Therefore, new medias were tried in order to increase the hyphal morphogenesis in solid media.

Serum media

The first attempt was done using Serum. This media is very similar to YPD with the differences that 10% of it is filled with Fetal Calf Serum (FCS) heat inactivated. Moreover, this media is known to promote hyphal growth induced by muramyl dipeptides (MDPs) [11]. Therefore, Serum agar plates were prepared and using the same protocol (3.4), the 3 WT strains AB54, AB66, AB216 and their respectively mutants AB347 (*hda2* Δ/Δ), AB629 (*hda2* Δ/Δ) and AB525 (*hda1* Δ/Δ) the same amount of cells (50 ~ 100) were plated in the same temperature (37 °C) during the same time (7 days). This experiment was carried out in order to check if this media could be used as a hyphal inducing media but also to possibly observe differences in hyphal induction between the WT strains and the deleted strains. Three biological replicates were done for each strain but the results shown in Graph 2 proved that this media was not actually useful for its aim.



Graph 2. Quantification of colony morphology in Serum agar plates

Average in percentage of different colony morphologies observed in three biological replicates, for each strain, grown in Serum agar plates. Error bars represents standard deviation for the three replicates.

The reason why these results were obtained are probably because the serum used was not the same as the one reported in the literature [11]. This can be thought by observing how the cells grew in this media (Fig 5A, Fig 5B and Fig 5C). These figures are also used as criteria to determinate whether if the colonies had high filamentation (↑), low filamentation (↓) or if they were smooth colonies.



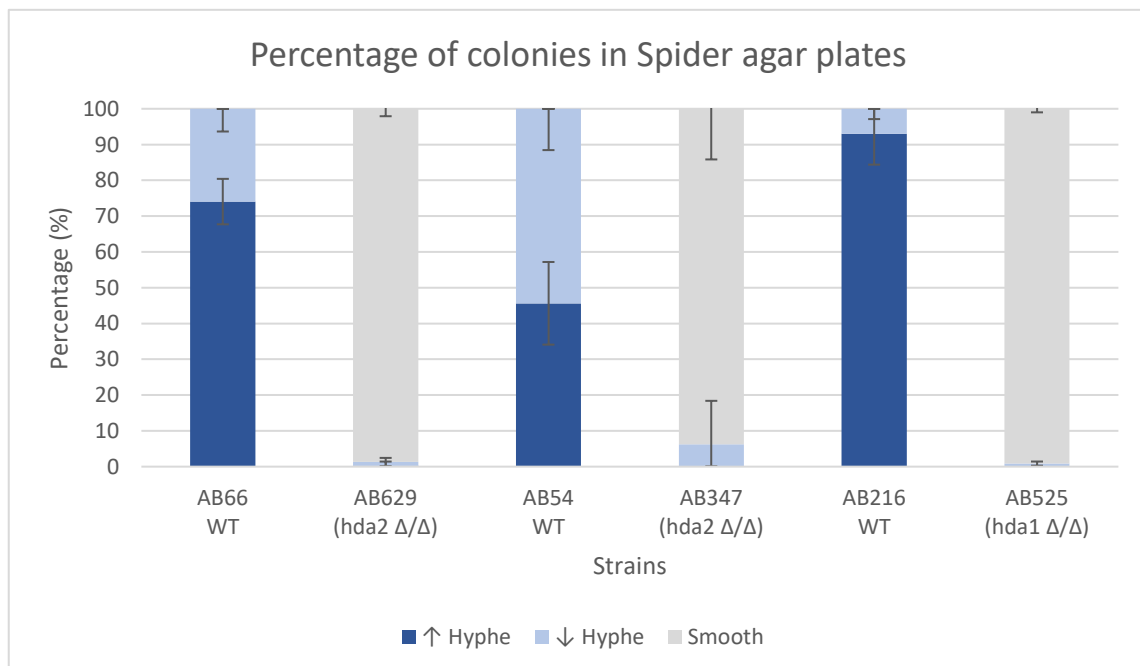
Fig 5A. High filamentation colony (↑) **Fig 5B.** Low filamentation colony (↓) **Fig 5C.** Smooth colony

Observation of colony morphology in the Serum media revealed that the majority of the colonies had a “wrinkled” phenotype and very few of them were filamenting. The cause of this wrinkled

phenotype is still unknown, but it could be due to the lack or not proper nutrients. Since the hyphal induction was not really pronounced (Graph 2) and neither the differences between the WT strains and the mutants, this media was determined as not useful for this study (p values obtained for this experiment were also higher than 0,05 for every comparison between WT strains and their mutants). Therefore, it was eliminated as a possible hyphal inducing media and not used again for further experimentation.

Spider media

The second attempt was done using Spider media. This media it is well known to induce hyphae growth [45]. It was then tested as the second possible hyphal inducing media. Again, agar plates were created, the same two independent *hda2* Δ/Δ strains (AB347 and AB629) and their original WT strains (AB54 and AB66 respectively) were grown in agar Spider media at 37 °C for 7 days. *Hda1* Δ/Δ strain (AB525) and its original WT strain (AB216) were also included as a positive control. Around 50 ~ 100 colonies were plated in each Spider agar plates (Protocol 3.4). The experiment was performed in three biological replicates and results are shown in Graph 3.



Graph 3. Quantification of colony morphology in Spider agar plates

Average in percentage of different colony morphologies observed in three biological replicates, for each strain, grown in Spider agar plates. Error bars represents standard deviation for the three replicates.

For the first time in this study it was possible to observe a strong hyphal induction using Spider media. The results showed an important difference between the WT strains and their mutants.

High filamentation in WT strains reached 45% in the lowest case for AB54, while its mutant, AB347 (*hda2* Δ/Δ), had 0% of high filamentation colonies. A significant value of $p=0,038$ was calculated with a T-test and the hypothesis of the inhibition of high hyphal colonies formation was accepted for these strains.

WT strain AB216 reached a 93% of high hyphal colonies, while its mutant, AB525 (*hda1* Δ/Δ), also had 0% of high hyphal colonies growth. T-test also showed a significant value ($p=0,014$) comparing the results obtained between these two strains.

Finally, WT strain AB66 reached a 74% of high hyphal colonies and its mutant, AB629 (*hda2* Δ/Δ) only had 0,25% of high hyphal colonies, having significant value of $p=0,016$ comparing the results obtained by these strains.

Criteria used for the count of hyphal colonies where the same as the ones used in the previous experiment (YPD and Serum agar plates) and are here presented in Fig 6A, Fig 6B and Fig 6C.

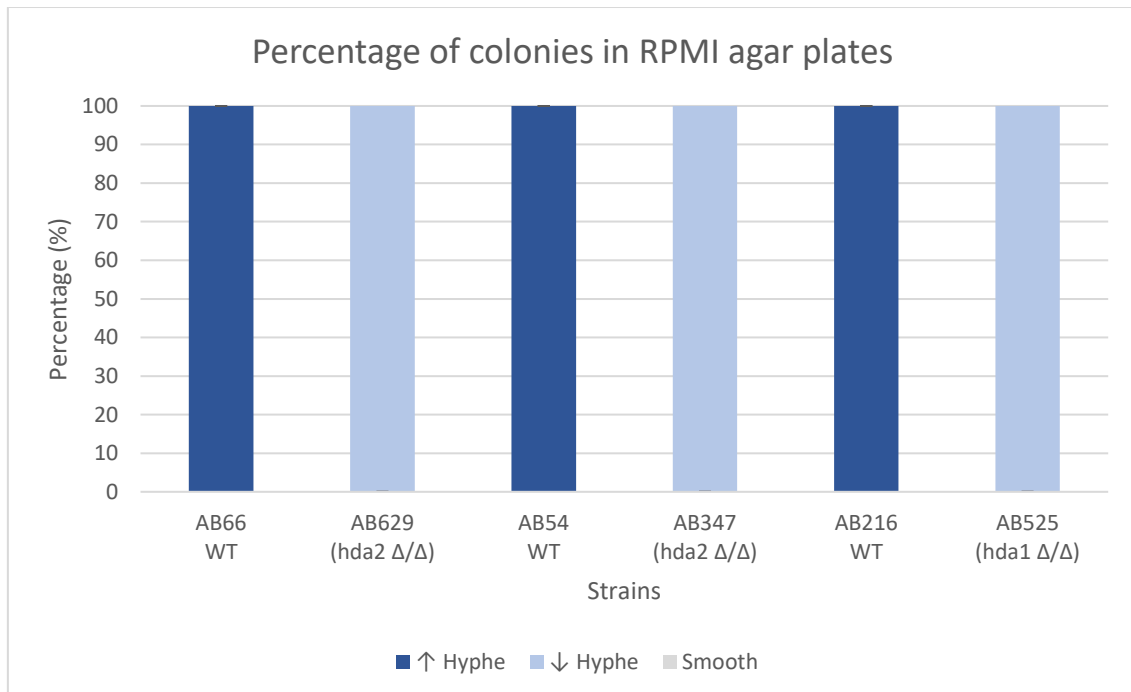


Fig 6A. High filamentation colony (↑) **Fig 6B.** Low filamentation colony (↓) **Fig 6C.** Smooth colony

As it can be seen in these images (Fig 6A, Fig 6B and Fig 6C) the cells had different form and shape but grew healthier than in Serum media. The differences between the non-hyphal and the hyphal colonies were notable. Spider was therefore determined as a useful media for this study and used for further experiments.

RPMI media

The last media tested was RPMI, a media that mimics human physiology conditions and it is also known to induce hyphal morphogenesis [46]. Again, two independent *hda2* Δ/Δ strains (AB347 and AB629) and their parental WT strains (AB54 and AB66 respectively) were grown in RPMI agar media at 37 °C for 7 days. Around 50 ~ 100 colonies were plated in each RPMI agar plates (Protocol 3.4). *Hda1* Δ/Δ strain (AB525) and its parental WT strain (AB216) were also included as a positive control. The experiment was performed in three biological replicates. The results shown in Graph 4 proved that this media was the best for its aim.



Graph 4. Quantification of colony morphology in RPMI agar plates

Average in percentage of different colony morphologies observed in three biological replicates, for each strain, grown in RPMI agar plates. Error bars represents standard deviation for the three replicates.

There was a clear increase of hyphal morphogenesis induction in all strains and, interestingly, the differences between the WT strains and their mutants became so clear. WT strains were able to turn into high filamentation colonies in an amazingly outcome of 100%, while neither of the mutants where able to make the complete switch, staying in 100% of low filamentation colonies (all T-tests showed significant p values between WT strains and their mutants). This experiment proved the importance of this genes (*HDA1* and *HDA2*) to allow *C. albicans* turn into

a complete hypha form under hyphal inducing conditions and proved that cells without these genes does not have the same ability.

Criteria used for the measures of the amount of hyphal colonies were, again, the same as the ones used previously, and are presented in Fig 7A, Fig 7B and Fig 7C, with new different forms and shapes appearing in RPMI media.



Fig 7A. High filamentation colony (↑) **Fig 7B.** Low filamentation colony (↓) **Fig 7C.** Smooth colony

Differences between these 3 types of colonies were very clear and all of them looked healthy, meaning that this media had proper nutrients for the cells to grow and that RPMI could be used as a hyphal inducing media.

Testing lack of O₂ and increase of CO₂ (5%) as hyphal inducing conditions during the incubation time

Results obtained suggest that Hda1 and Hda2 are involved in promoting filamentation growth. However, the mutants showed differences in phenotypes using different medias, suggesting that the proteins are promoted by specific hyphae inducing pathways. To test this hypothesis, hyphal development was tested in two new different conditions also known to induce hyphal morphogenesis using different pathways: Increase of CO₂, and absence of O₂ [32].

These experiments were carried out using a hypoxia chamber for hypoxia condition (absence of O₂) in the first experiment, and another chamber able to perform an environment of increased amount of CO₂ (5%) in the second experiment.

The same 6 strains were used: *hda2* Δ/Δ (AB347 and AB629) and their original WT strains (AB54 and AB66 respectively) and *hda1* Δ/Δ (AB525) and its original WT strain (AB216) as a positive control. Protocol 3.5 (for increased CO₂) and Protocol 3.6 (for hypoxia) were used, and all cells were grown in YPD agar plates at 37 °C for 7 days. Around 50 ~ 100 colonies were sown in each plate.

Hypoxia

Three biological replicates for each strain were done in this experiment, but any of the strains were able to grow in the hypoxia chamber. More repeats were done to make sure there was no human mistake made during the process and other YPD agar plates were used, as controls, with oxygen and having the exact same samples of cells sown in it, to make sure these cells were able to grow in normal condition, but the cells in the hypoxia chamber did not grow in any way (yeast or hyphal). It could only be seen the spots on the agar plates were the cells were added the first day of the incubation but when a sample of each was taken and looked at the optical microscope some cells were bigger than normal and some had a low hyphal induction, but since the cells were not actually able to grow as a colony, results from this experiment wouldn't actually be representative. Here is an example of how the cells looked in the optical microscope at 40x (Fig 8)



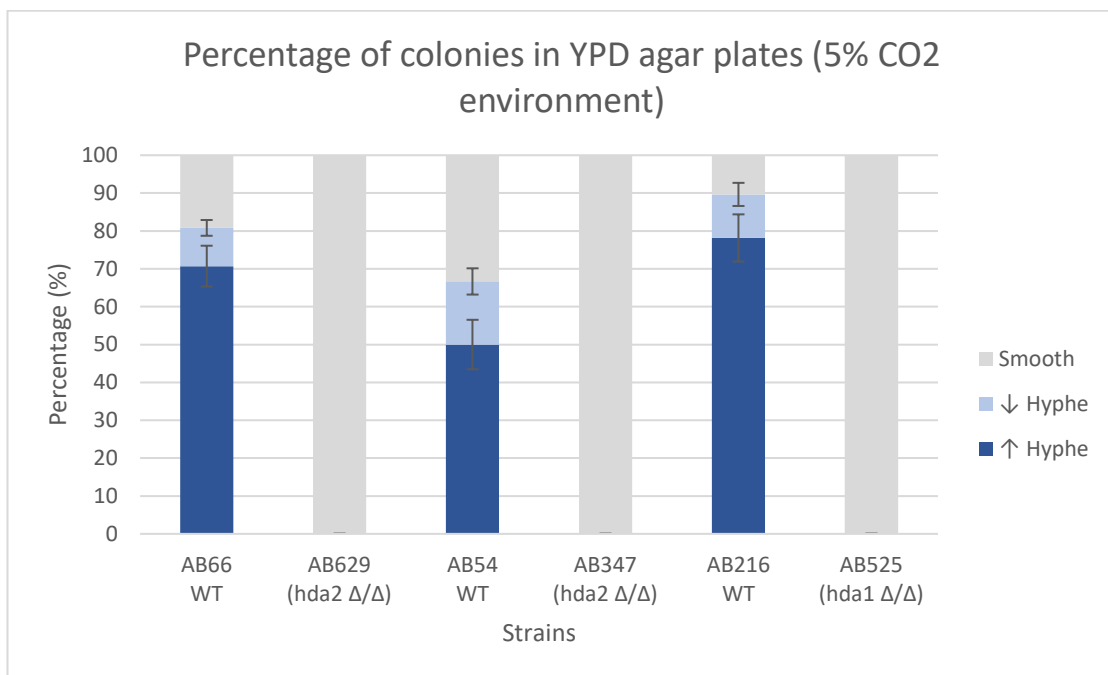
Fig 8. *C. albicans* cells grown in hypoxia chamber in YPD plates at 37 °C for 7 days

Since it is known that these cells can grow without O₂ [32] but our samples were not able to grow as normal colonies in the YPD agar plates, it was then decided not to consider this

experiment as conclusive results. The reason why the cells were not able to grow it is still unknown but due to the restricted time for experimentation in this project, it was decided to move on to the next experiments.

Increase of CO₂

3 biological replicates were also done for each strain. Colonies were counted after the determined time (7 days) using the same criteria for colony characterization as in YPD agar plates. Results are shown in Graph 5.



Graph 5. Quantification of colony morphology in YPD agar plates grown in 5% CO₂ condition

Average in percentage of different colony morphologies observed in three biological replicates, for each strain, grown in YPD agar plates at 5% CO₂. Error bars represents standard deviation for the three replicates.

By the results obtained in Graph 5, we can determine that in WT strains CO₂ strongly induces filamentation.

Comparing these results with the ones obtained in the very first experiment, with YPD agar plates grown in normal concentrations of CO₂ and, considering that YPD is a yeast-inducing media, the results prove that CO₂ has a real capacity to induce hyphal morphogenesis. Increased amount of CO₂ during the incubation may increase the number of cells turning from yeast to

hyphae through a specific genetical pathway [32]. The differences between the WT strains and their mutants are very clear: while the mutants haven't been able to grow any kind of hyphal colony, the WT strains got induced to this morphological change, reaching percentages of 50% (for AB54), 78% (for AB216) and 70% (for AB66) of the total counted colonies. P values obtained with statistical T-test for these results embraced the hypothesis of the increase of filamentation in *C. albicans* due to the increase in CO₂ (p values < 0,05).

First trials for hyphal morphogenesis using liquid hyphal inducing medias

Having established that Hda1 and Hda2 are important for filamentation in solid media, their role in liquid media was tested.

Nutrients are not only essential for cell division, but are also needed in rich conditions for *C. albicans* to make this morphological switch possible. Only in rich media conditions this fungus is able to change from yeast to hypha. In the contrary, cells division is promoted in yeast form, reducing hyphal morphogenesis.

Checking single cells would give a better idea of this yeast-to-hyphal switch, using only the hyphal inducing medias (RPMI and Spider), so the first step was to establish a solid protocol to quantify filamentation in liquid media. In the next following section, it will be discussed the different protocols that were tested to reach the final protocol written in Materials and Methods, Protocol 3.7.

Protocol 1 (First trial)

An overnight culture (~ 16 hours) in YPD liquid media at 30 °C was prepared (yeast inducing conditions). The next day, the culture was centrifugated and washed twice with water. The whole culture was then directly mixed with the hyphal inducing medias (RPMI or Spider) and grown for 6 hours at 37 °C. This procedure is very similar to the final version of the Protocol 3.7

but in this case the difference is the cell density initially used (the amount of cells added to the medias are way higher than the ones latter used in Protocol 3.7).

The same 6 strains were used: *hda2* Δ/Δ (AB347 and AB629) and their original WT strains (AB54 and AB66 respectively) and *hda1* Δ/Δ (AB525) and its original WT strain (AB216) as a positive control. But in the results it is only presented the once obtained from WT strains since the once obtained from the mutants does not give any better information (no hyphal filamentation could be observed in any experiment so all strains had the same results).

Filamentation was quantified at 2, 4 and 6 hours after the inoculation, and the percentage of cells with different morphologies (Fig 9, 10A, 10B, 11A and 11B) were calculated. Cells were counted directly in the optical microscope at 40x (magnification) and in parallel, ODs were measured (Graph 9).

Criteria used to classify the different cells obtained in the cultures and posteriorly in the following sections:



Fig 9. Yeast cell

→ Considered as YEAST CELL

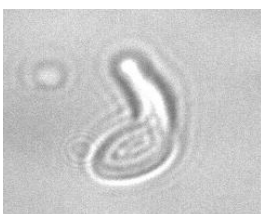


Fig 10A. Pseudohyphal cell

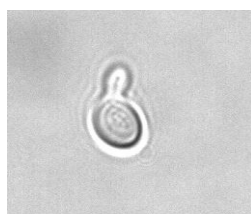


Fig 10B. Pseudohyphal cell

→ Considered as PSEUDOHYPHAL CELL

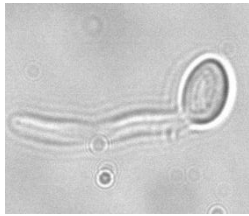


Fig 11A. Hyphal cell

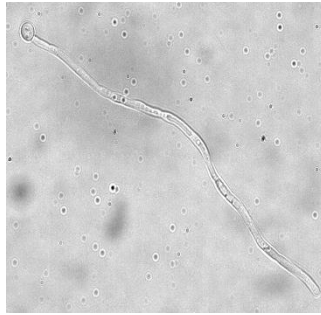


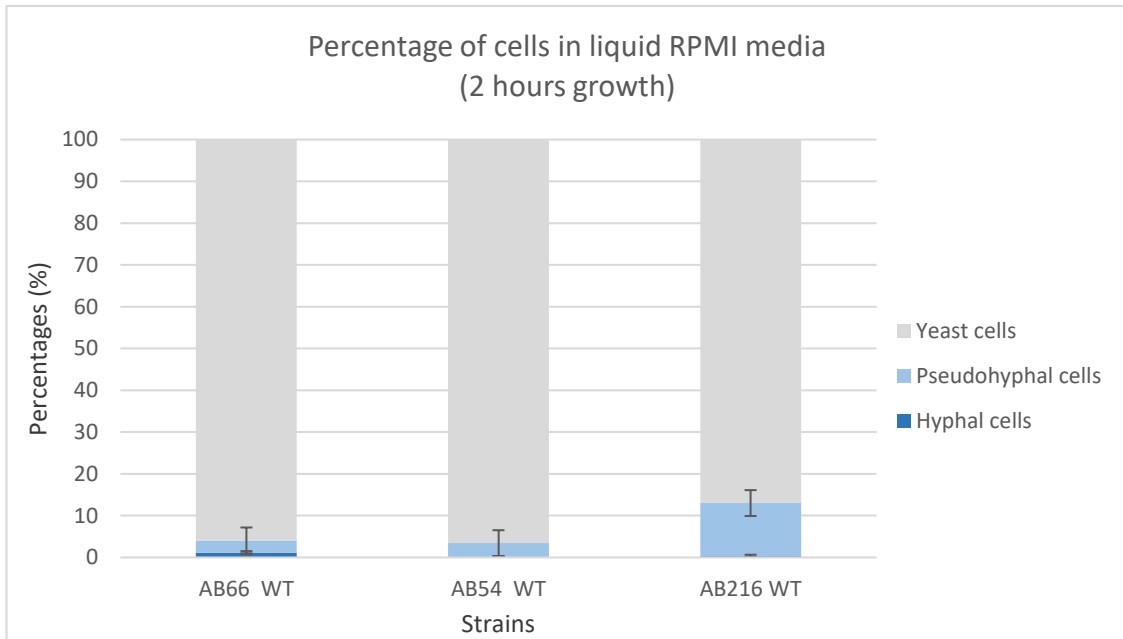
Fig 11B. Hyphal cell

→ Considered as HYPHAL CELL

Figure 9 presents an example of a yeast cell. This kind of cell were the easier ones to identify since all of them have this similar round form. Figures 10 presents 2 different kinds of pseudohyphal cells. Those are the hardest ones to identify since some of these cells may seem to be switching from yeast to hypha, but some are just cell dividing (this is a factor to consider in results). Finally, Figures 11 presents examples of hyphal cells. Hyphal cells can have short to very high filamentation and are easier to identify. All pictures where taken in the Olympus IX-81 microscope with oil immersion objective and a magnification of 100x.

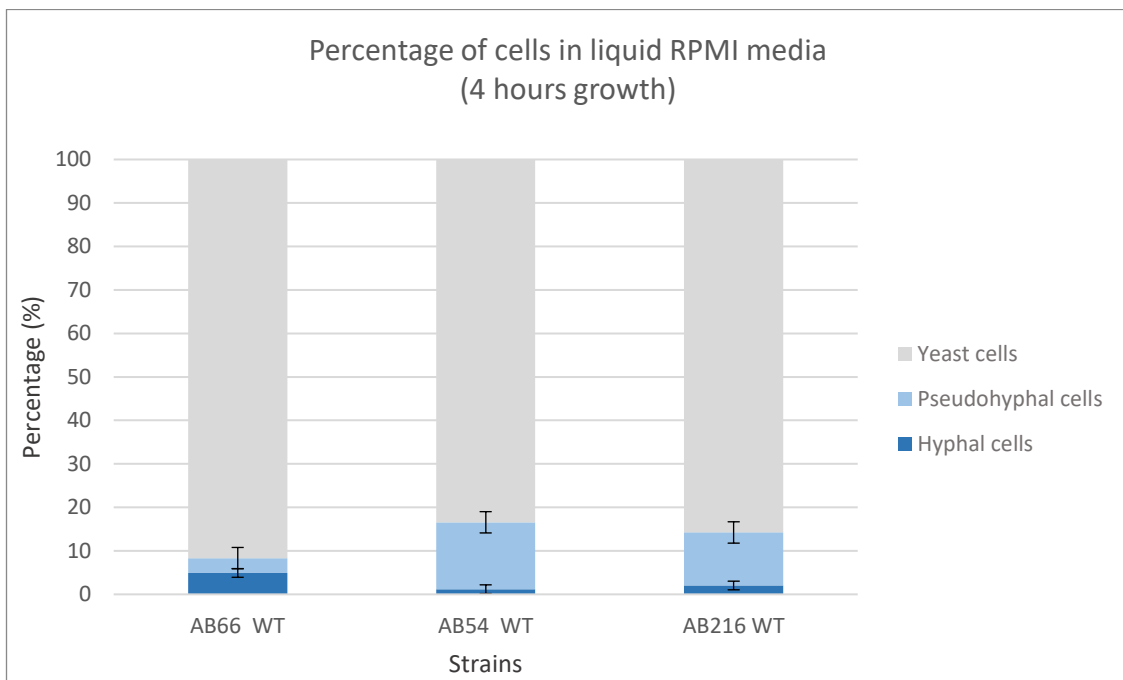
RPMI liquid media (6 hours growth)

The first attempt was done using RPMI liquid media and the results obtained are shown in Graph 6, Graph 7 and Graph 8 (2, 4 and 6 hours of growth time at 37 °C respectively). Three biological replicates were done for each strain. Absorbance of the culture was measured at 600nm to check cell division through this process. Results of absorbance are shown in Graph 9.



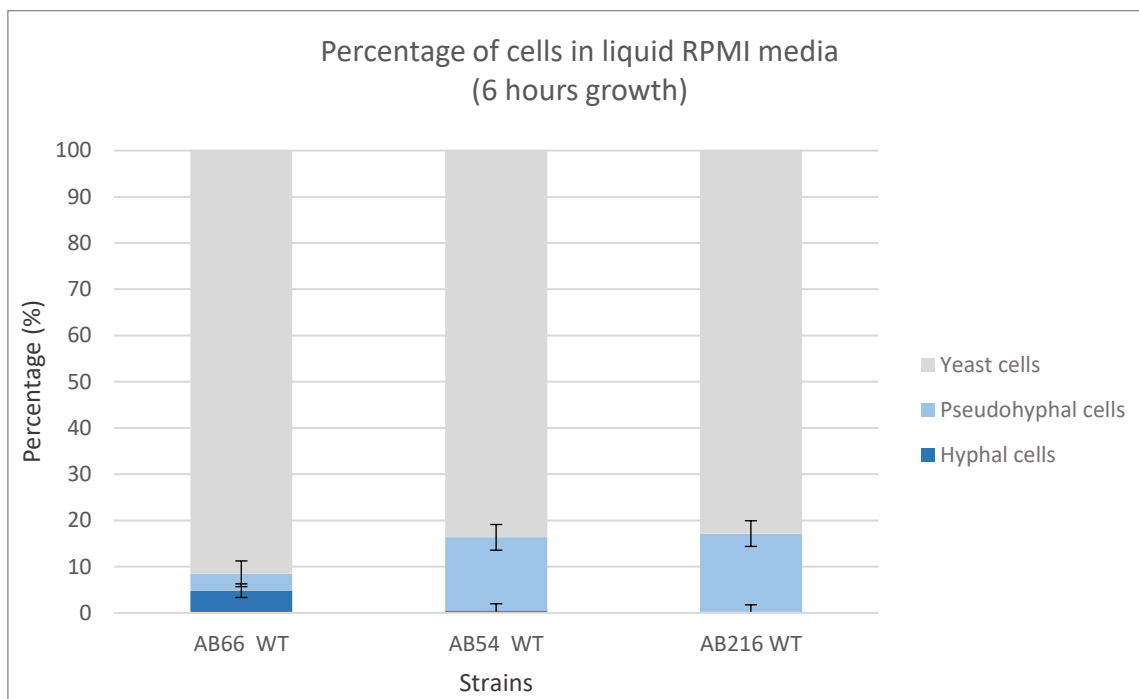
Graph 6. Quantification of cells in RPMI liquid media at 2 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in RPMI liquid media at 2 hours growth. Error bars represents standard deviation for the three replicates.



Graph 7. Quantification of cells in RPMI liquid media at 4 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in RPMI liquid media at 4 hours growth. Error bars represents standard deviation for the three replicates.



Graph 8. Quantification of cells in RPMI liquid media at 6 hours growth

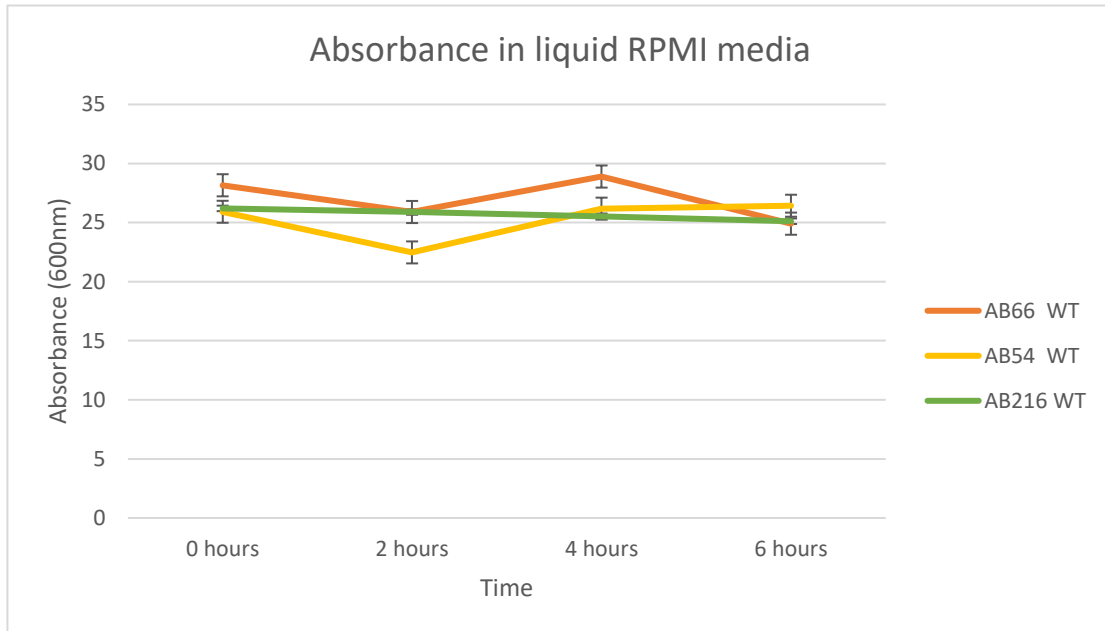
Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in RPMI liquid media at 6 hours growth. Error bars represents standard deviation for the three replicates.

As it can be seen in the results obtained (Graph 6, Graph 7 and Graph 8), this protocol did not lead to a robust hyphal induction, as only the WT strain, AB66, reached a 5% of hyphal cells at 4 and 6 hours since the incubation. WT strains AB54 and AB216 were also able to reach 1% and 2% of hyphal cells respectively at 4 hours growth, but the rest of the strains were not able to even reach a 1% of hyphal cells.

The results obtained had no consistent resolution, and therefore this first protocol used for hyphal induction was not considered effective, but it was useful to realize about the mistake done during the protocol design.

RPMI already proved to be a hyphal inducing media, so which could be the reason for this low hyphal induction? The first thought was about the time used in this first protocol. Maybe the cells did not had time enough to make the morphological change. With the right conditions but

not enough time to switch, *C. albicans* may not be able to change morphologically from yeast to hypha. Considering that, the next protocol was proposed by increasing the time.



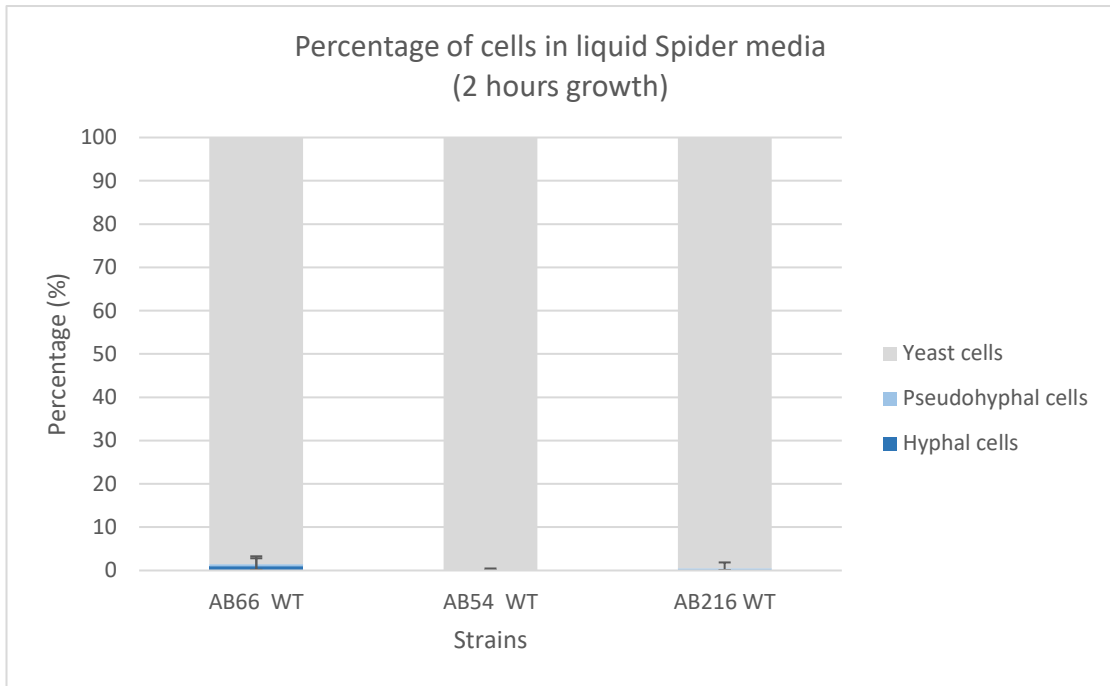
Graph 9. Absorbance at 600nm during 6 hours in RPMI liquid media

Average of absorbances obtained in three biological replicates, for each strain, grown in RPMI liquid media for 6 hours. Error bars represents standard deviation for the three replicates

About the results obtained in Graph 9, measurement of cell density during this time course revealed that none of the strains grow significantly since the ODs were very similar during the 6 hours. The number of cells in the cultures did not increased. This fact gave a new idea of why this experiment in general did not worked as expected (too much initial cell density means less cell division over the time). Considering that, this fact could counter the hypothesis that low hyphal induction in this experiment was not due to the lack of enough time to switch morphologically but to the high cell density initially added in the culture.

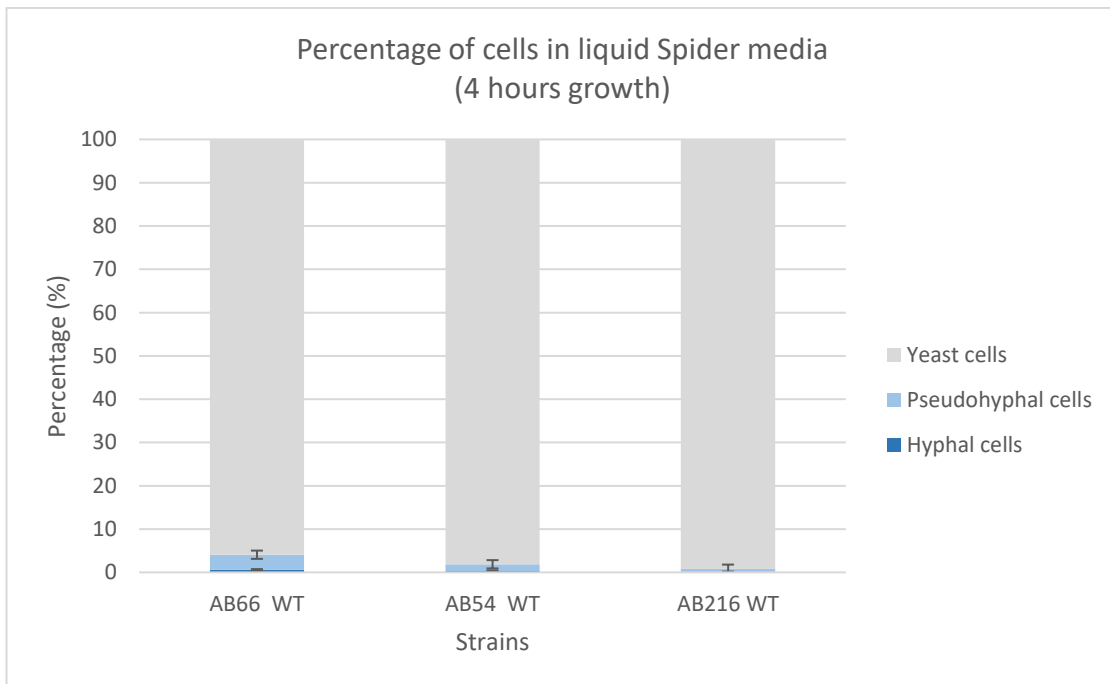
Spider liquid media (6 hours growth)

Using the exact same protocol three biological replicates were done for Spider liquid media. Cells were quantified at 2, 4 and 6 hours from the inoculation and results obtained are presented in Graph 10, Graph 11 and Graph 12 respectively.



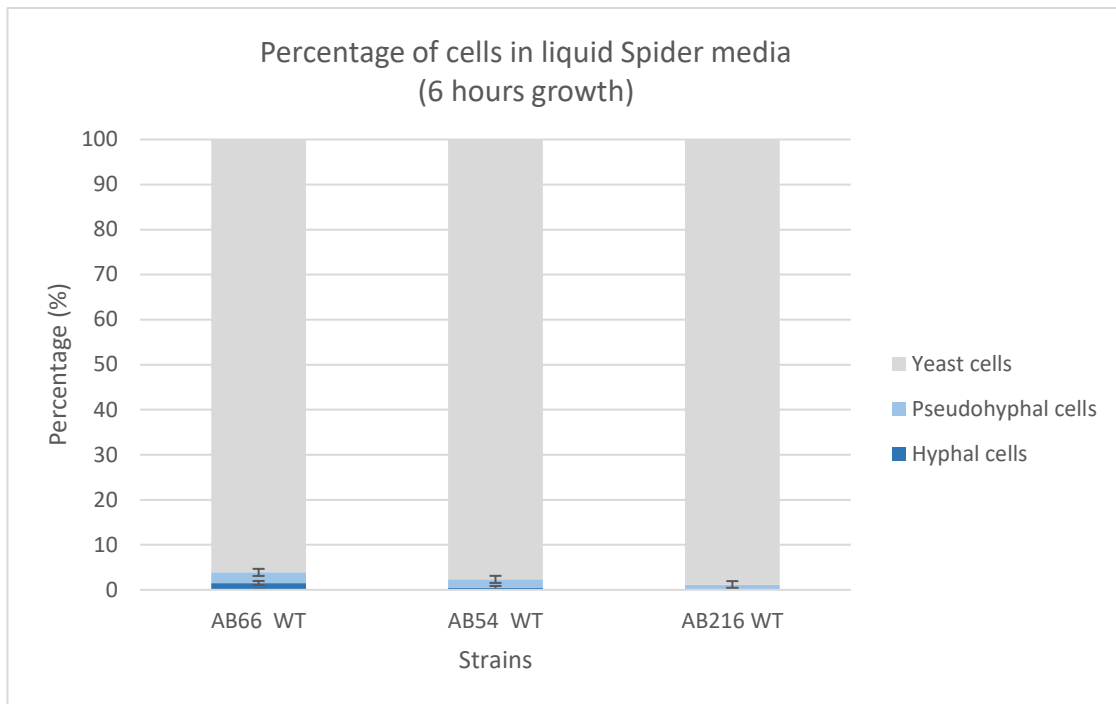
Graph 10. Quantification of cells in Spider liquid media at 2 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in Spider liquid media at 2 hours growth. Error bars represents standard deviation for the three replicates.



Graph 11. Quantification of cells in Spider liquid media at 4 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in Spider liquid media at 4 hours growth. Error bars represents standard deviation for the three replicates.



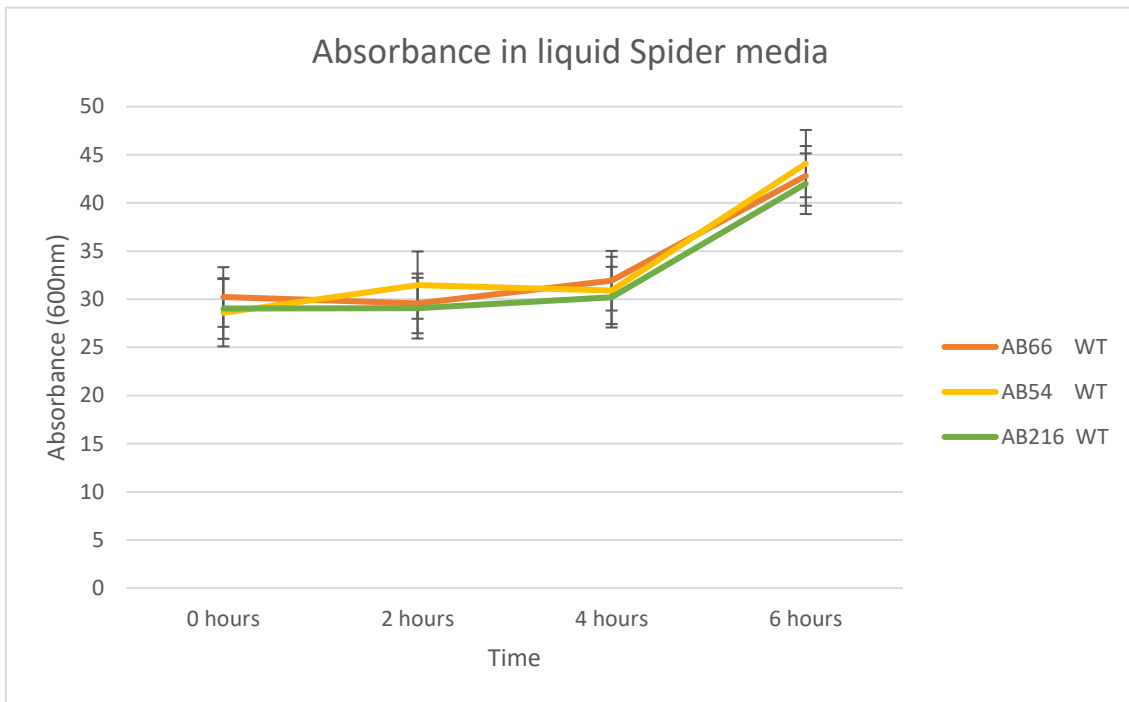
Graph 12. Quantification of cells in Spider liquid media at 6 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in Spider liquid media at 6 hours growth. Error bars represents standard deviation for the three replicates.

Similarly, to what observed in liquid RPMI media, hyphal induction in Spider was very low. Only the WT strain AB66 reached to 1% of hyphal cells at 6 hours growth and 3% of pseudohyphal cells at 4 hours growth.

Poor results obtained in both RPMI and Spider liquid media experiments confirmed a mistake made in the protocol; again, either lack of time or high cell density was blocking the cells to morphologically switch.

Looking to the absorbances obtained in this experiment a conclusion was made (Graph 13).



Graph 13. Absorbance at 600nm during 6 hours in Spider liquid media

Average of absorbances obtained in three biological replicates, for each strain, grown in Spider liquid media for 6 hours. Error bars represents standard deviation for the three replicates

Graph 13 proved that in Spider liquid media, cell division was possible since the ODs increased during the time, meaning that the number of cells in the culture were increasing. This fact made think that the mistake done in the first protocol was about the lack of enough time for hyphal morphogenesis.

If in Spider liquid media the amount of cells were not able to keep increasing (like it happened in RPMI), an initial high cell density would be the most plausible reason of the inhibition of hyphal morphogenesis, since the amount of media used was not being high enough to make either cell division or hyphal switch happen. But since the culture in Spider was able to keep increasing and, therefore, cell division was occurring, the conclusion accepted was that the problem inhibiting hyphal morphogenesis was the lack of time to make this morphological switch.

Increasing the time for hyphal morphogenesis in RPMI and Spider liquid media

As we previously conclude, the hypothetical solution to increase hyphal induction in RPMI and Spider was throw increasing the time of incubation.

In these two new experiments we used Protocol 2

Protocol 2 (Second trial)

An overnight culture (~ 16 hours) in YPD liquid media at 30 °C was prepared (yeast inducing conditions). The next day, the culture was centrifugated and washed twice with water. The whole culture was then directly mixed with the hyphal inducing medias (RPMI or Spider) and grown for 24 hours at 37 °C. This procedure is also very similar to the final version of the Protocol 3.7 but in this case the differences are the cell density initially used (higher) and the growth time (longer).

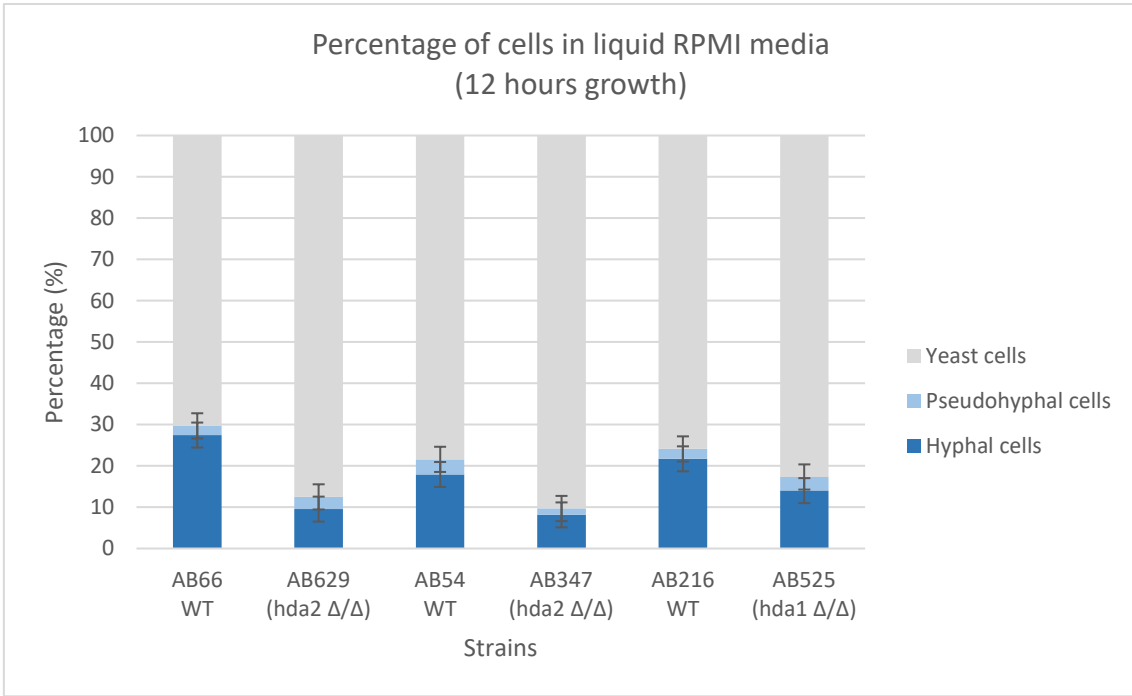
As it can be seen in this protocol, the condition changed was the time of cell growth. Increasing the time of incubation, cells from *C. albicans* would hypothetically have time enough to morphologically change from yeast to hypha.

The same 6 strains were used: *hda2* Δ/Δ (AB347 and AB629) and their original WT strains (AB54 and AB66 respectively) and *hda1* Δ/Δ (AB525) and its original WT strain (AB216) as a positive control.

Filamentation was quantified at 12, 16, 20 and 24 hours after the inoculation, percentage of cells with different morphologies (Fig 8, 9A, 9B, 10A and 10B) were calculated. Cells were counted directly in the optical microscope at 40x (magnification) and in parallel, ODs were measured (Graph 18).

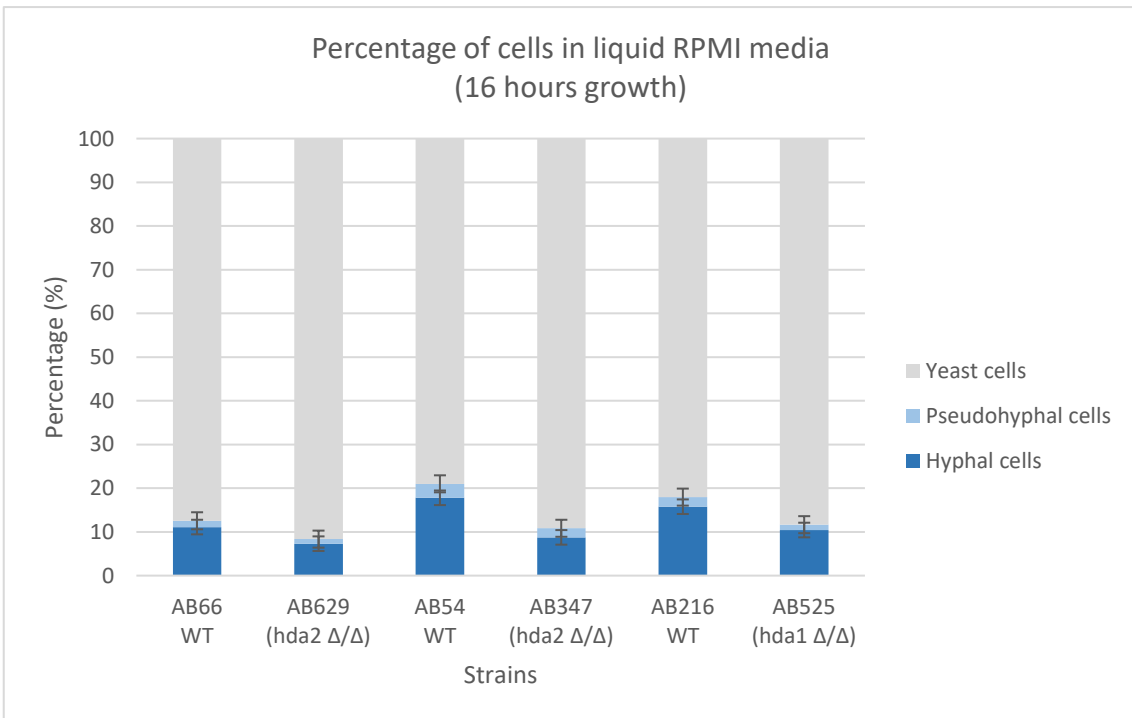
RPMI liquid media (24 hours growth)

Three biological replicates for each strain were carried out. Results for RPMI liquid media are presented in Graphs 14, 15, 16 and 17.



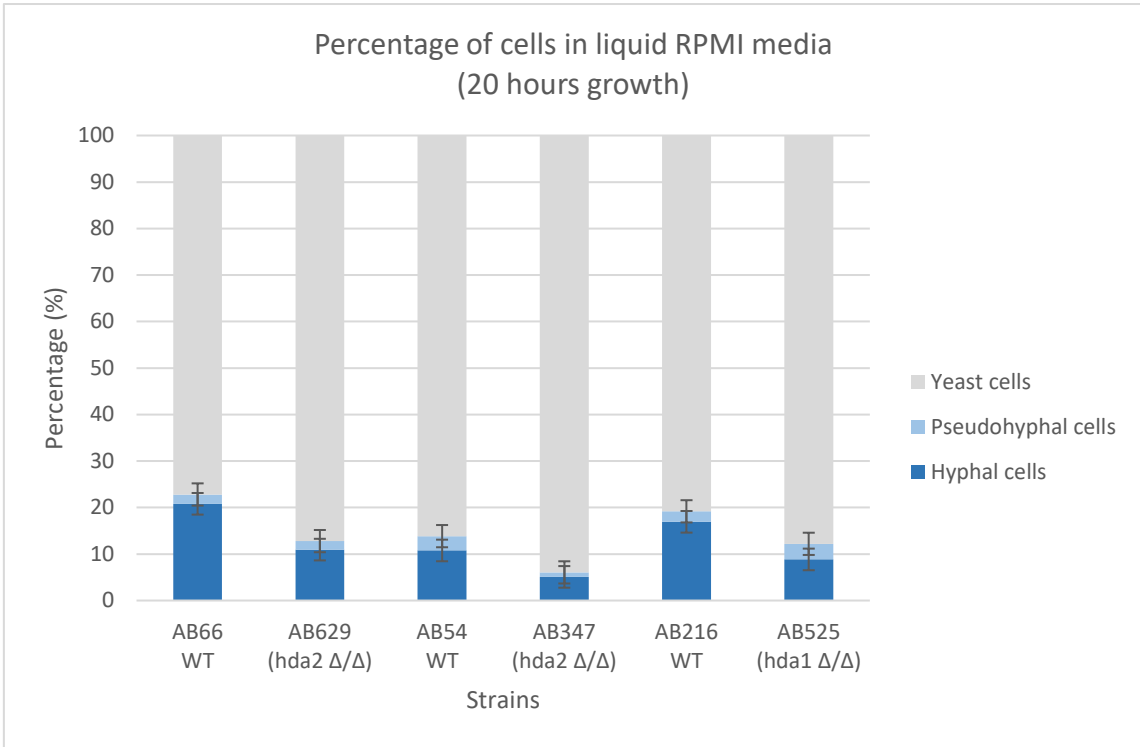
Graph 14. Quantification of cells in RPMI liquid media at 12 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in RPMI liquid media at 12 hours growth. Error bars represents standard deviation for the three replicates.



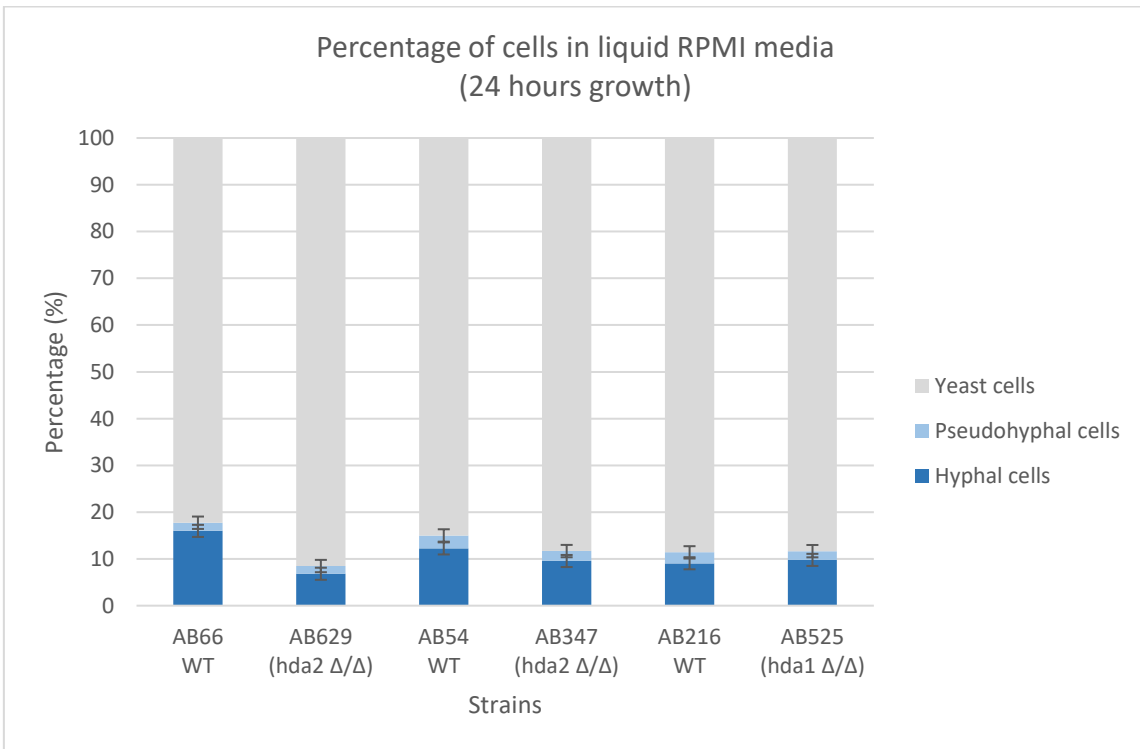
Graph 15. Quantification of cells in RPMI liquid media at 16 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in RPMI liquid media at 16 hours growth. Error bars represents standard deviation for the three replicates.



Graph 16. Quantification of cells in RPMI liquid media at 20 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in RPMI liquid media at 20 hours growth. Error bars represents standard deviation for the three replicates.

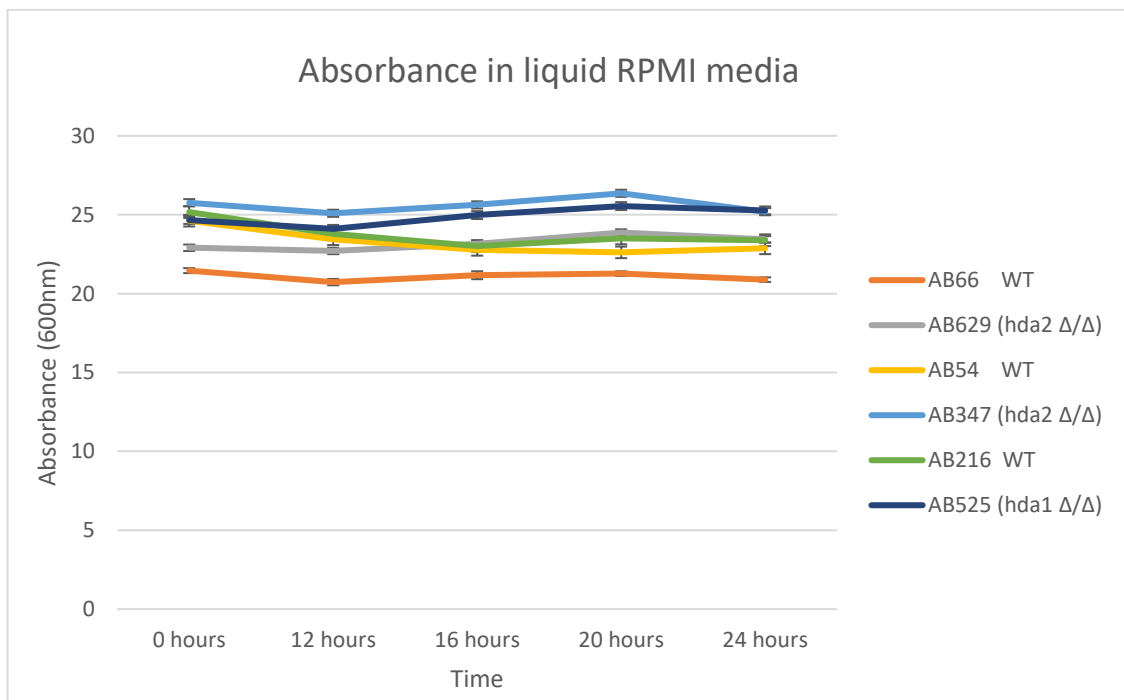


Graph 17. Quantification of cells in RPMI liquid media at 24 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in RPMI liquid media at 24 hours growth. Error bars represents standard deviation for the three replicates.

As the results showed in all these graphs (14, 15, 16 and 17) the problem of hyphal induction was not being solved. It was clear that there was more hyphal growth as WT strains reached percentages of 27% for AB66 and 21% for AB216 at 12 hours (being the highest hyphal cell percentages for WT strains) and mutant strains reached 14% for AB525 (*hda1* Δ/Δ) and almost 10% for AB629 (*hda2* Δ/Δ) (being the highest hyphal cells percentages for mutant strains). However, there was still not enough amount of hyphal cells to consider this as a correct protocol for hyphal induction and the differences in hyphal cells between WT strains and their mutants had no statistical significance (p values $> 0,05$).

About the ODs measured in RPMI liquid media, Graph 18 demonstrate that, again, there was no cell division since the density in the cultures stayed the same during the 24 hours. Which meant that the only left reason for this low hyphal growth was the initial density used in the protocol.

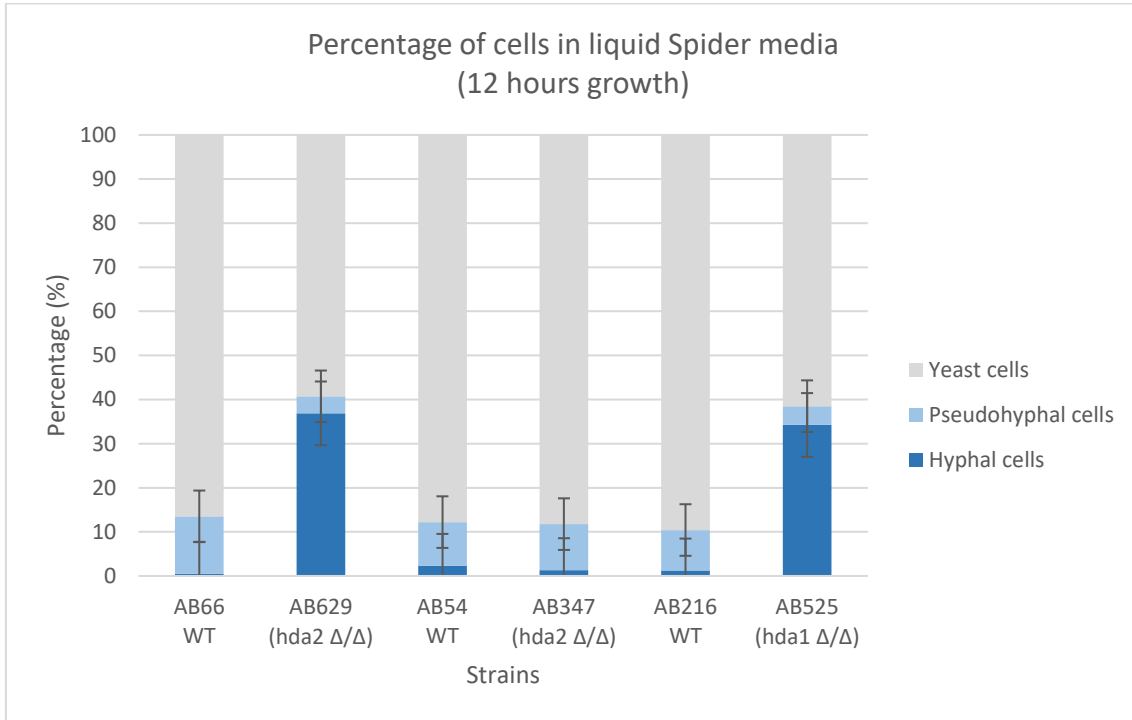


Graph 18. Absorbance at 600nm during 24 hours in RPMI liquid media

Average of absorbances obtained in three biological replicates, for each strain, grown in RPMI liquid media for 24 hours. Error bars represents standard deviation for the three replicates

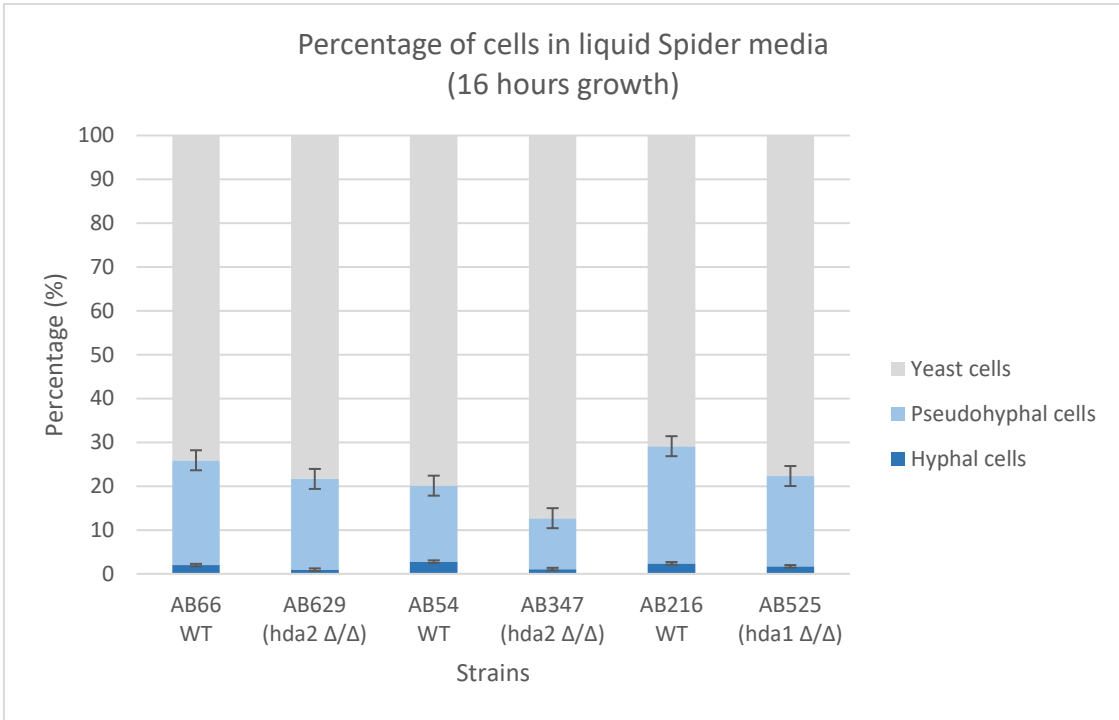
Spider liquid media (24 hours growth)

The same experiment was also repeated in Spider liquid media, measuring hyphal morphogenesis in the same exact hours (12, 16, 20 and 24 hours). Three biological replicates of each strain were grown, and results are presented in Graphs 19, 20, 21 and 22.



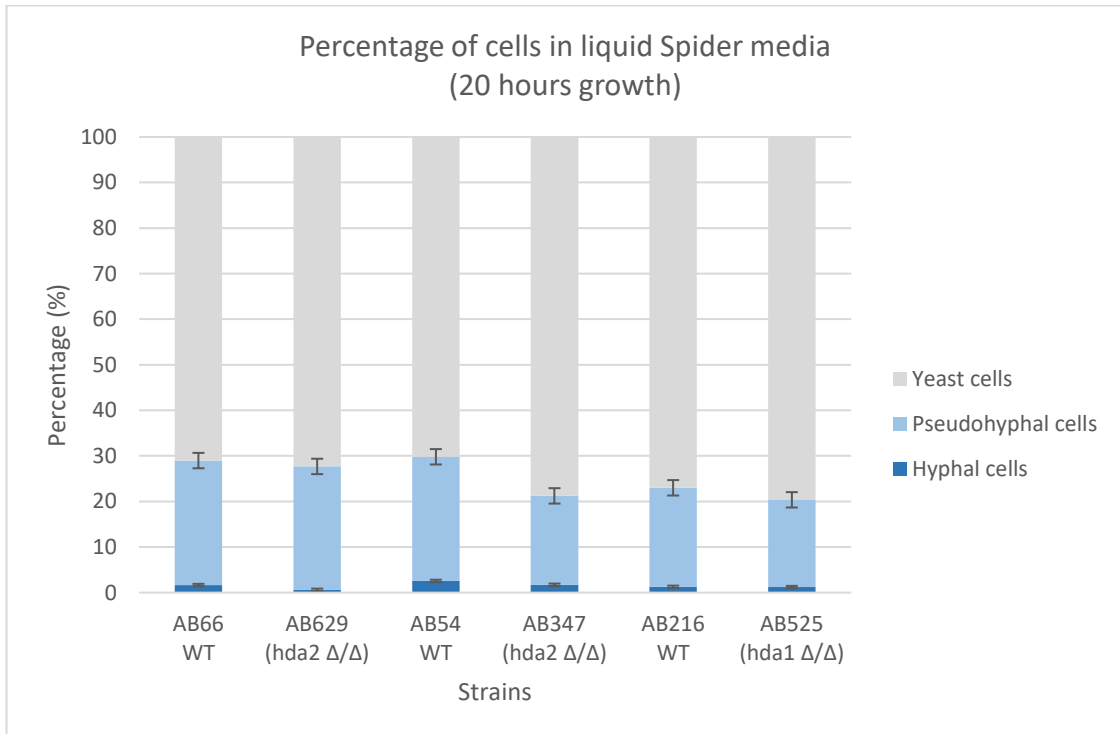
Graph 19. Quantification of cells in Spider liquid media at 12 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in Spider liquid media at 12 hours growth. Error bars represents standard deviation for the three replicates.



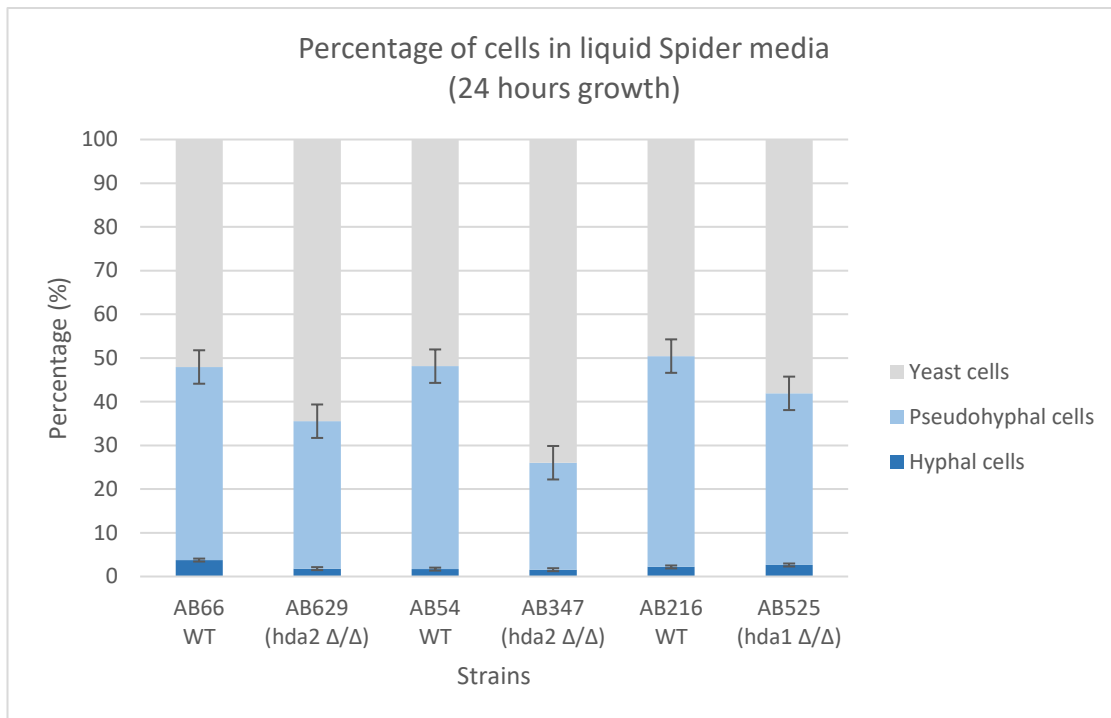
Graph 20. Quantification of cells in Spider liquid media at 16 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in Spider liquid media at 16 hours growth. Error bars represents standard deviation for the three replicates.



Graph 21. Quantification of cells in Spider liquid media at 20 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in Spider liquid media at 20 hours growth. Error bars represents standard deviation for the three replicates.

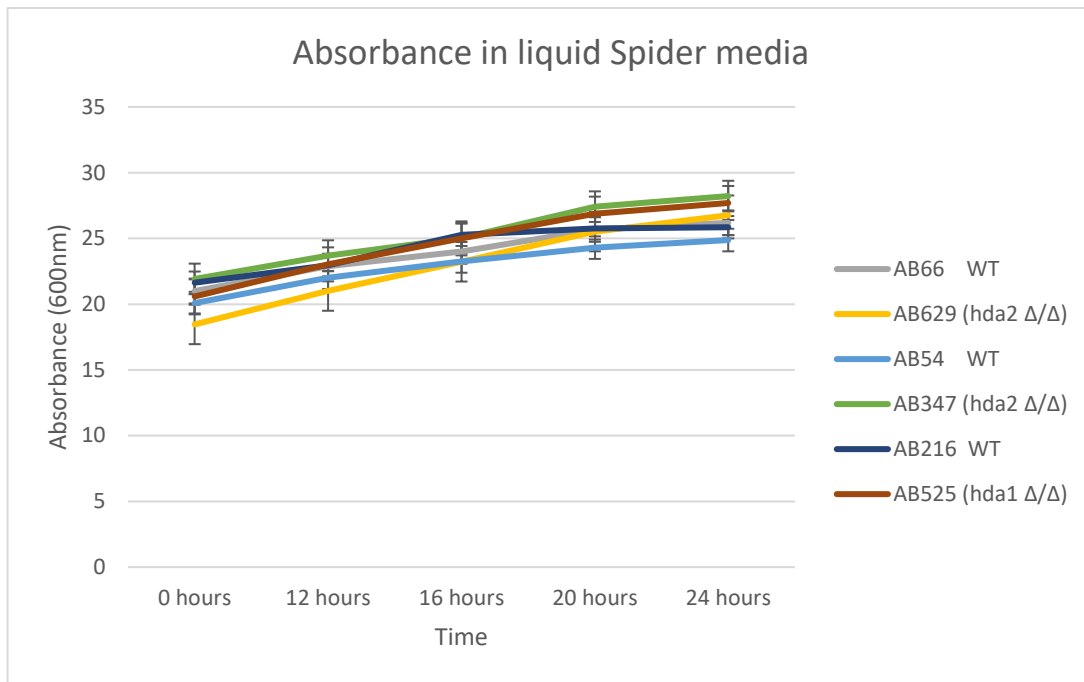


Graph 22. Quantification of cells in Spider liquid media at 24 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in Spider liquid media at 24 hours growth. Error bars represents standard deviation for the three replicates.

In Spider liquid media the percentages of pseudohyphal cells reached higher levels than in RPMI, which meant that cells could start the morphological switch but not complete it. WT strains AB54, AB66 and AB216 reached pseudohyphal cell percentages of 46%, 44% and 48% (respectively) at 24 hours growth, and their mutant strains AB347 (*hda2 Δ/Δ*), AB629 (*hda2 Δ/Δ*) and AB525 (*hda1 Δ/Δ*) reached pseudohyphal cell percentages of 24%, 33% and 39%. In two concrete cases, mutant strains AB629 (*hda2 Δ/Δ*) and AB525 (*hda1 Δ/Δ*) reached hyphal cells percentages of 36% and 34%, but the rest of the results, as expected, had not enough hyphal morphogenesis to consider Protocol 2 as hyphal inducing protocol.

The only conclusion that could be taken from these experiments is that the mistake done in the last protocols was clearly the initial high cell density. Even though in Graph 23 we can see increase of cells during the time, but less pronounced than in experiment “Spider liquid media (6 hours growth)”, it was concluded that both medias were being too much condensed with cells to let them switch morphologically.



Graph 23. Absorbance at 600nm during 24 hours in Spider liquid media

Average of absorbances obtained in three biological replicates, for each strain, grown in RPMI liquid media for 24 hours. Error bars represents standard deviation for the three replicates.

Reaching to the final correct protocol

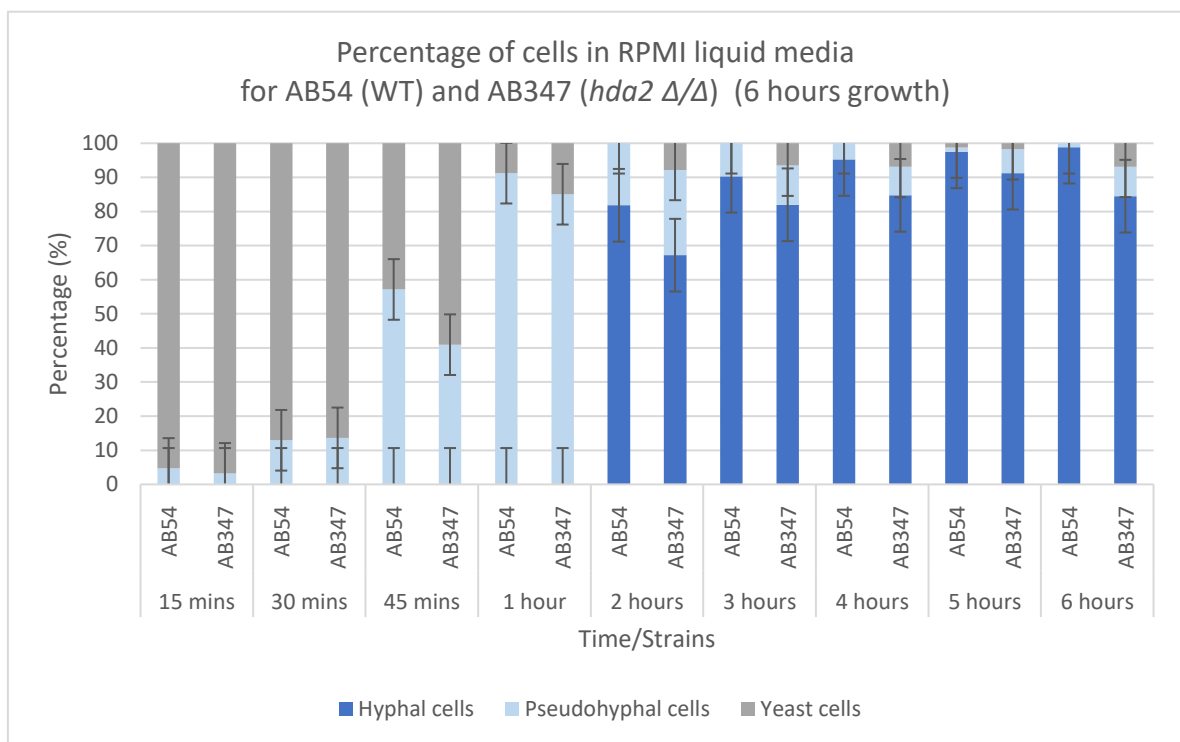
At this moment of the project it was finally completed the right Protocol 3.7. Knowing that the high cell density added in the medias was the problem, the initial concentrations used were highly reduced, in order to get cultures less condense. Instead of using the whole overnight culture, only 100 μ l of this overnight culture was introduced to the 10ml medias after a wash with water (as it is written in the protocol section 3.7). This low amount of cells give them the possibility to grow in a hyphal-inducing media with nutrients and time enough to develop a complete hyphal morphogenesis.

As always, three biological replicates where done for each strain: *hda2* Δ/Δ (AB347 and AB629) and their original WT strains (AB54 and AB66 respectively) and *hda1* Δ/Δ (AB525) and its original WT strain (AB216) as a positive control. Cells where grown at 37 °C for 6 hours, checking hyphal morphogenesis in the very first minutes (15, 30 and 45 minutes) and the following hours (1, 2, 3, 4, 5 and 6 hours). The reason why the cells were checked so early was because hyphal

morphogenesis was observed to happen really soon when the cells have this very rich media conditions.

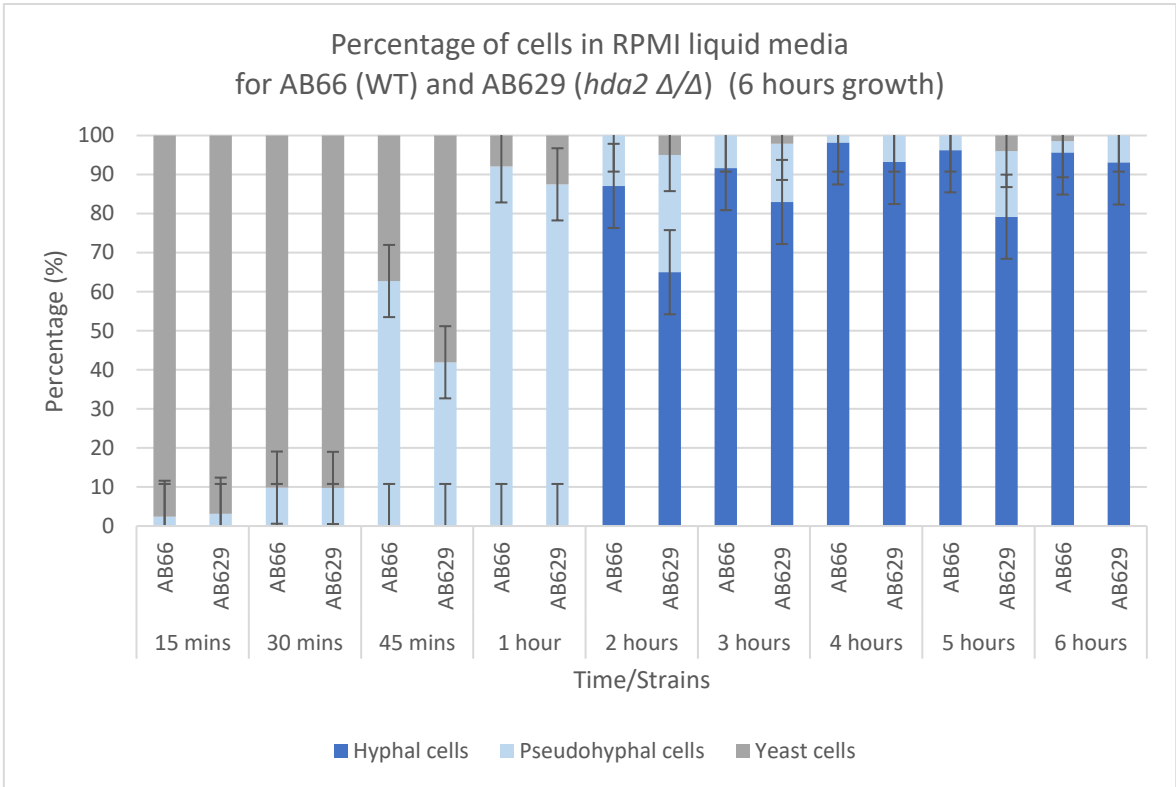
RPMI liquid media (6 hours growth in rich media)

It is first presented the results obtained for RPMI in Graphs 24, 25 and 26, showing the differences in hyphal morphogenesis obtained by each WT strain and its mutanta (*hda2* Δ/Δ or *hda1* Δ/Δ) over the time.



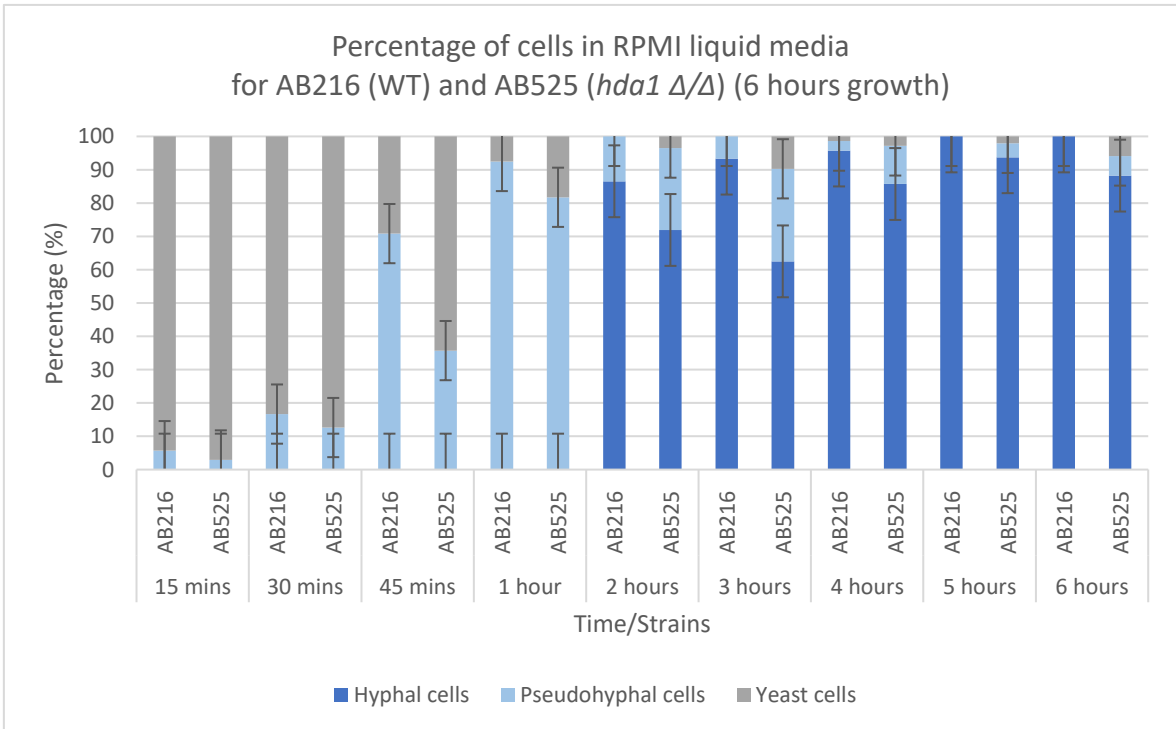
Graph 24. Quantification of cells for AB54 (WT) and AB347 (*hda2* Δ/Δ) in RPMI liquid media for 6 hours

Average in percentage of different cell morphologies observed in three biological replicates, for AB54 (WT) and its mutant AB347 (*hda2* Δ/Δ), grown in RPMI liquid media for 6 hours. Error bars represents standard deviation for the three replicates.



Graph 25. Quantification of cells for AB66 (WT) and AB629 (*hda2* Δ/Δ) in RPMI liquid media for 6 hours

Average in percentage of different cell morphologies observed in three biological replicates, for AB66 (WT) and its mutant AB629 (*hda2* Δ/Δ), grown in RPMI liquid media for 6 hours. Error bars represents standard deviation for the three replicates.



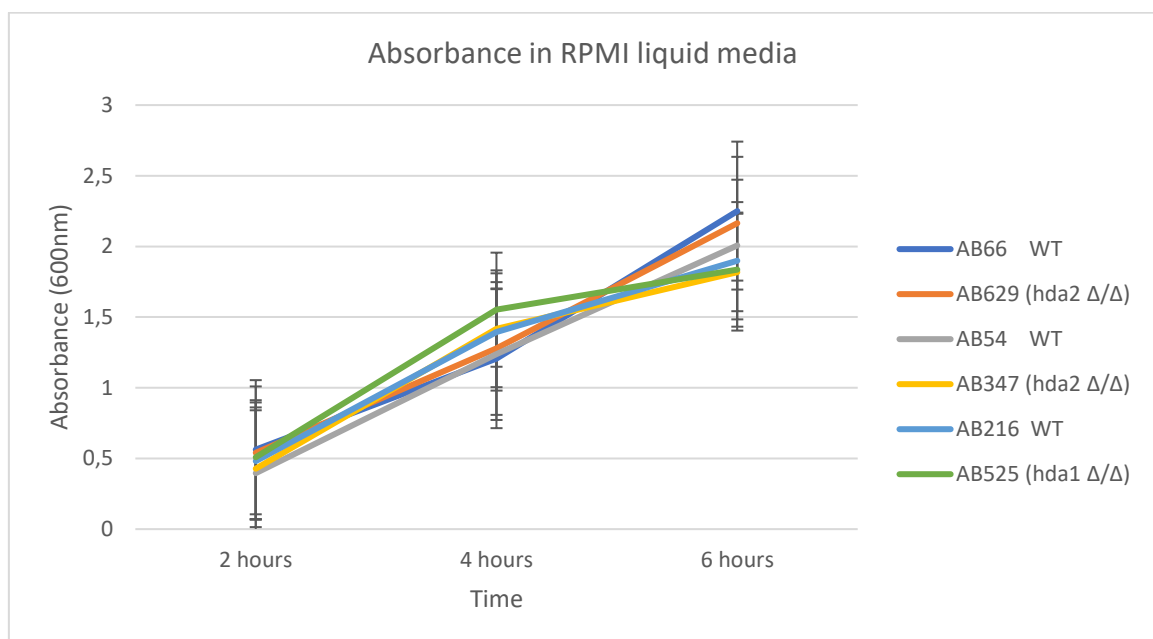
Graph 26. Quantification of cells for AB216 (WT) and AB525 (*hda1* Δ/Δ) in RPMI liquid media for 6 hours

Average in percentage of different cell morphologies observed in three biological replicates, for AB216 (WT) and its mutant AB525 (*hda1* Δ/Δ), grown in RPMI liquid media for 6 hours. Error bars represents standard deviation for the three replicates.

The results obtained for these 3 graphs (24, 25 and 26) proved the ability for *C. albicans* to grow hyphae in rich RPMI media, since all strains got percentages over 60%, reaching in some cases almost a 100%. The problem of hyphal induction was solved, but the expected results on this study were not reached. There is clearly a difference on hyphal morphogenesis between the WT strains and their mutants, it can be observed comparing not only hyphal cells but also pseudohyphal cells, the problem is that the ability to grow hyphae is pretty high in all of them, including the strains missing *HDA2* or *HDA1* genes.

As it was said at the start of the project, the strain selected with *HDA1* deleted (AB525) was used as a positive control, since this deletion is already known to reduce hyphal morphogenesis, but looking at the results obtained, neither strains with *HDA1* or *HDA2* deleted had a high difference in hyphal morphogenesis than their original WT strain, being proved by statistical T-tests that showed no significant values for the differences between these strains (p values $> 0,05$)

Graph 27 presents the increase of cells over the time, and as it can be seen, they increased for the 6 hours growth, meaning that the initial cell density used was then confirmed to be the needed one for both cell division and hyphal induction.



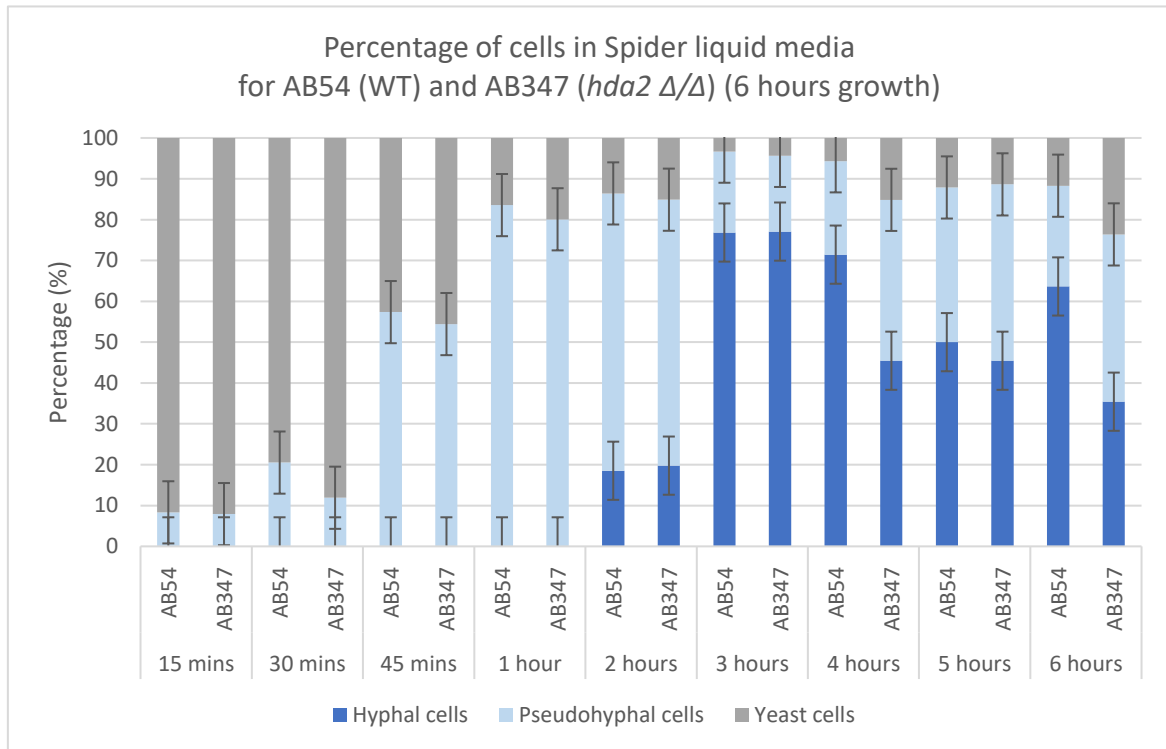
Graph 27. Absorbance at 600nm during 6 hours in RPMI liquid media

Average of absorbances obtained in three biological replicates, for each strain, grown in RPMI liquid media for 6 hours. Error bars represent standard deviation for the three replicates.

Spider liquid media (6 hours growth in rich media)

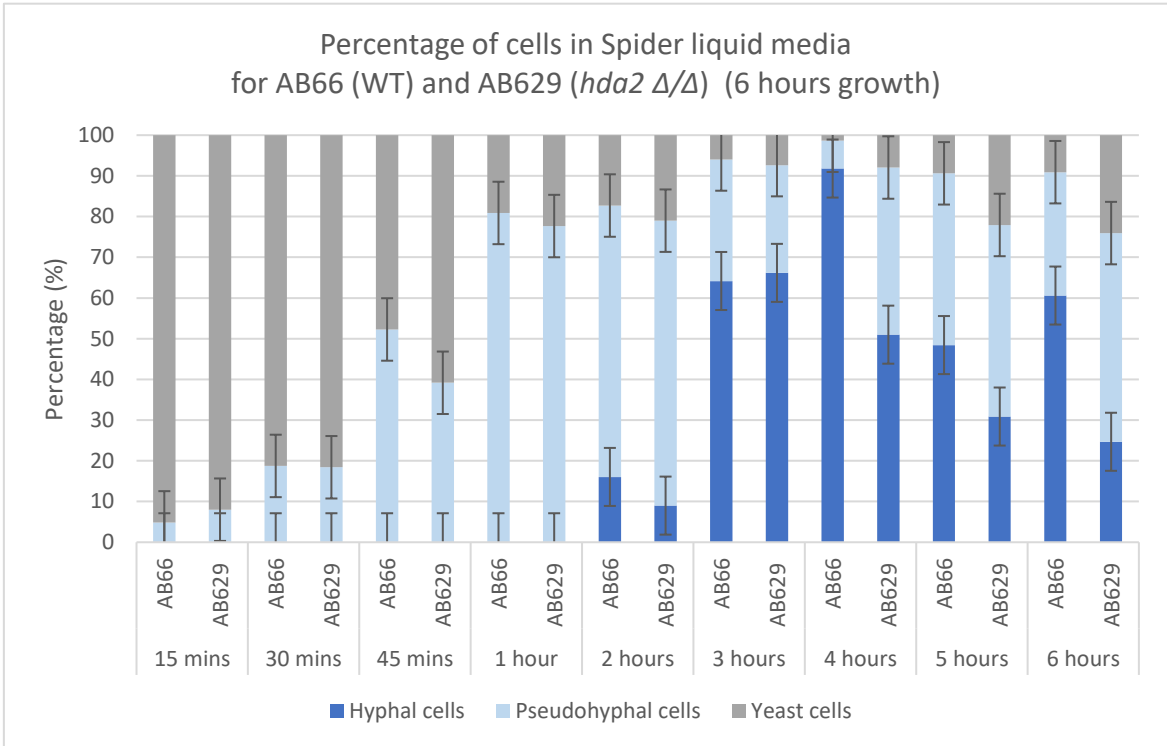
Results from the same experiment done in Spider liquid media are presented in graphs 28, 29,

30. WT strains are compared with their mutants.



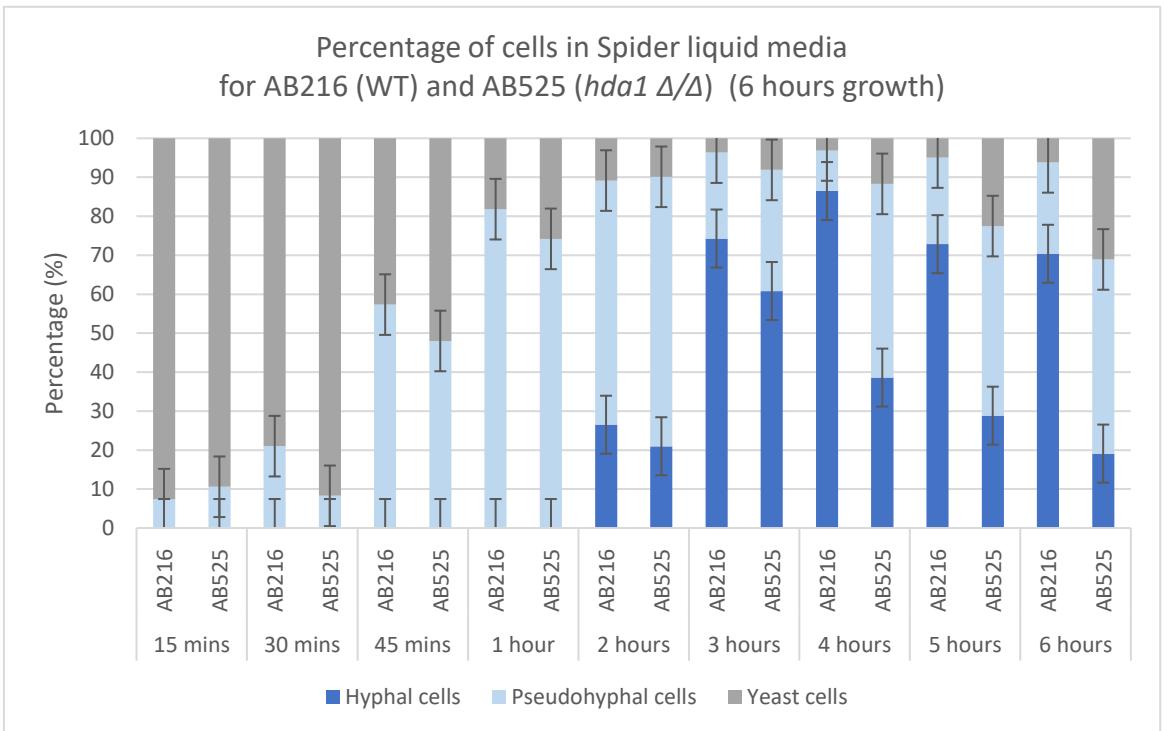
Graph 28. Quantification of cells for AB54 (WT) and AB347 (*hda2* Δ/Δ) in Spider liquid media for 6 hours

Average in percentage of different cell morphologies observed in three biological replicates, for AB54 (WT) and its mutant AB347 (*hda2* Δ/Δ), grown in Spider liquid media for 6 hours. Error bars represents standard deviation for the three replicates.



Graph 29. Quantification of cells for AB66 (WT) and AB629 (*hda2* Δ/Δ) in Spider liquid media for 6 hours

Average in percentage of different cell morphologies observed in three biological replicates, for AB66 (WT) and its mutant AB629 (*hda2* Δ/Δ), grown in Spider liquid media for 6 hours. Error bars represents standard deviation for the three replicates.



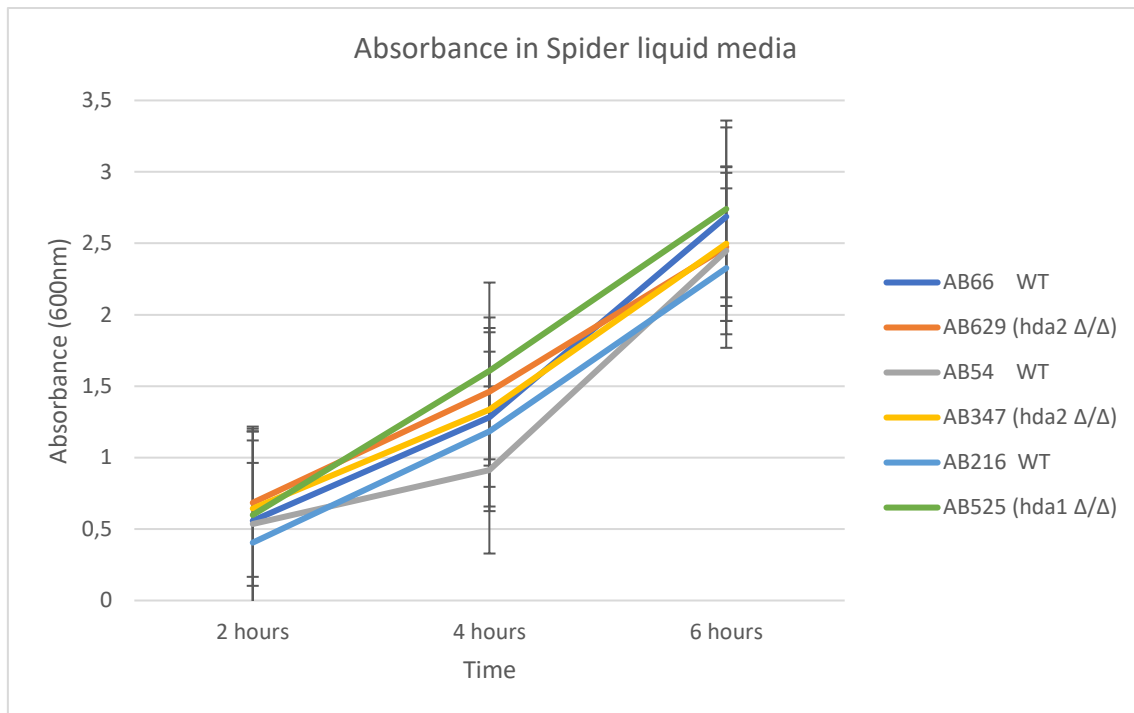
Graph 30. Quantification of cells for AB216 (WT) and AB525 (*hda1* Δ/Δ) in Spider liquid media for 6 hours

Average in percentage of different cell morphologies observed in three biological replicates, for AB216 (WT) and its mutant AB525 (*hda1* Δ/Δ), grown in Spider liquid media for 6 hours. Error bars represents standard deviation for the three replicates.

In this case, results obtained from Spider experiments proved that Spider is not as hyphal inducer as RPMI since the highest percentages of hyphal cells were reached between 3 and 4 hours since the inoculation, but after that, all strains started to reduce the hyphal morphogenesis, while in RPMI the hyphal cells were not reduced over the time. However, the reduction of the percentages of hyphal cells became an advantage rather than a disadvantage.

Comparing WT strains with their mutants it can be seen differences. Generally, this differences are not very clear but if we take a closer look to AB216 (WT) compared to AB347 (*hda1* Δ/Δ), at 4, 5 and 6 hours growth there are differences in percentages of hyphal cells of 48%, 44% and 51% respectively to the hours, having a significance value of $p=0,0025$, $p=0,001$ and $p=0,0004$. That make sense since strain AB347 (*hda1* Δ/Δ) was initially selected as a positive control which was already known to reduce hyphal morphogenesis, but looking to the results obtained with the strains with *HDA2* deleted, the differences in percentages are not that clear, only for AB54 (WT) and its mutant AB347 (*hda2* Δ/Δ) differences in hyphal cells reached 26% at 4 hours growth and 28% at 6 hours growth, but neither of them had a significant value ($p=0,083$ and $p=0,077$ respectively). And, for AB66 (WT) and AB629 (*hda2* Δ/Δ) differences reached to 41% at 4 hours growth and 36% at 6 hours growth, but surprisingly those differences did not have significant value either ($p=0,1743$ and $p=0,0995$ respectively). This is due to the high different number of cells counted in every replicate for each strain, which increases the variance of the sample and therefore T-test proves no significant value considering the percentages in every replicate used (sometimes the average percentage of each strain can be misleading).

Finally, graph 31 shows the increase of cells during the time and, as expected, it had a normal increase very similar to the results obtained in RPMI.



Graph 31. Absorbance at 600nm during 6 hours in Spider liquid media

Bars represent average of three independent biological replicates, error bars standard deviation. For each experiment.

We therefore concluded that deletion of the *HDA2* gene does not impact hyphal growth in liquid Spider and RPMI media

Measuring the size of filamentation of hyphal cells

Another property about *C. albicans* that can be get from this experiment is the differences between the length of the filaments.

It has been reported that deletion of HDA1 influence in reduction of hyphal morphogenesis in terms of quantity of cells but also in terms of elongation of the filament since this deletions disables the strains to maintain hyphal filaments due to the lack of deacetylation of Yng2, inducing the cell to return to its yeast form [47].

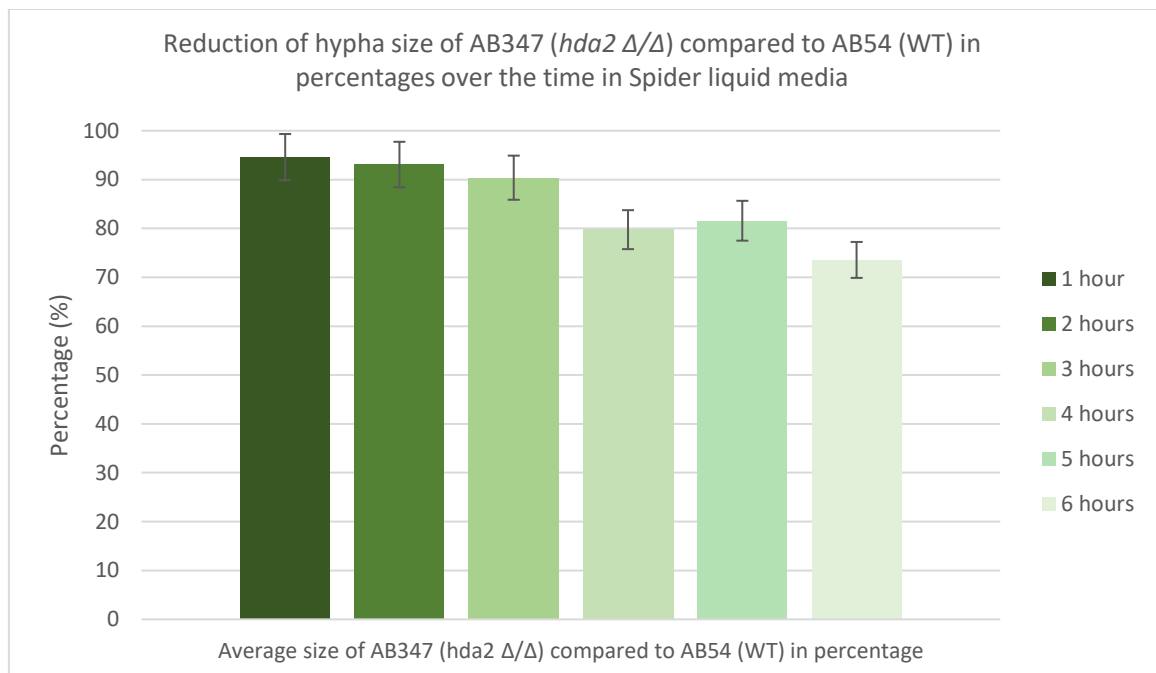
For this section, 3 representative pictures from the same cultures, used in the previous experiments, were taken in an optical microscope with a camera and with the same

magnification (40x). Using an image display program, filamentation was measured. All three biological replicates were counted, and an average was calculated for each strain in each media.

The aim of the following graphs is not quantitative but qualitative. What it is wanted to present here is not the real size of the filaments but how different are the elongations of the filaments between the WT strains and their mutants over the time.

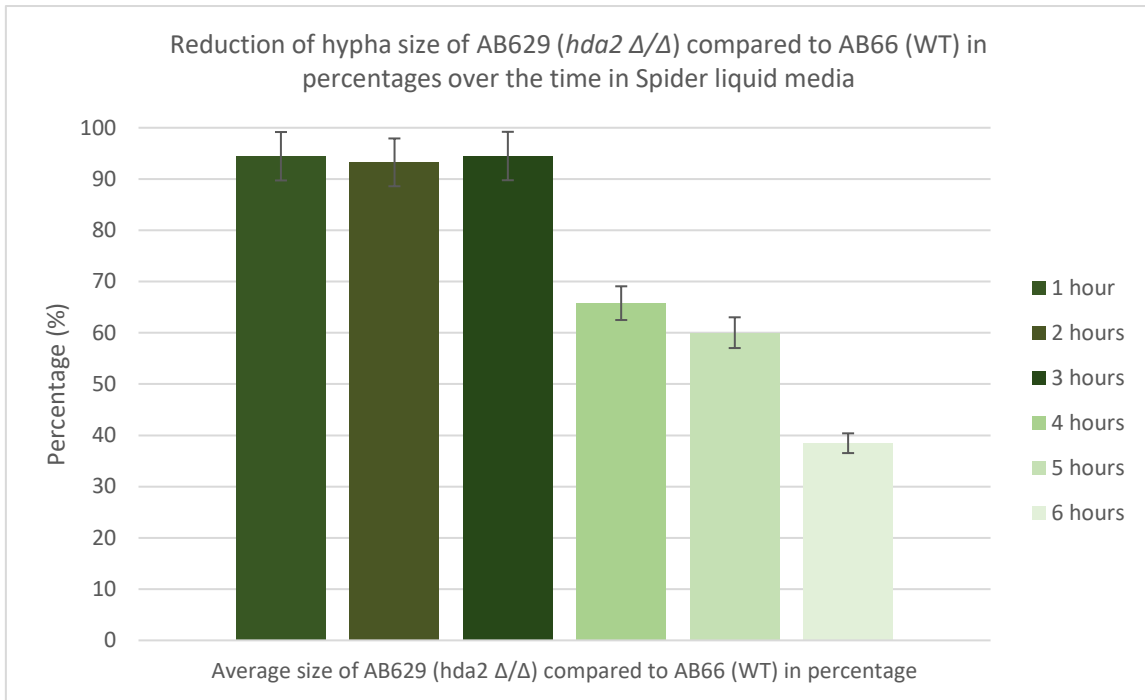
For each graph, it is considered that the 100% represents the average size of the WT strain measured at each hour (1, 2, 3, 4, 5, and 6), while the percentages presented in the graphs represents the reduction in the average size of hypha from mutants compared to their WT strains.

Spider liquid media (hypha size)



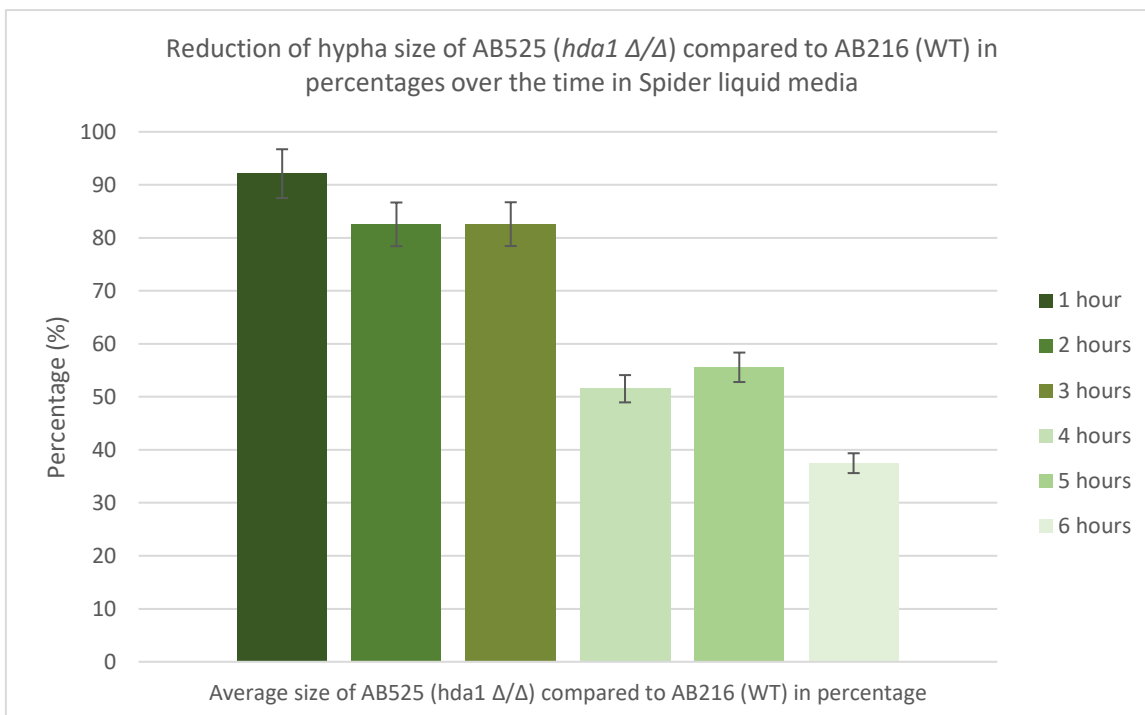
Graph 32. Representative size in percentage of hyphal filaments for AB347 (*hda2* Δ/Δ) compared to AB54 (WT) in Spider liquid media for 6 hours

For each hour, the average size measured for AB54 (WT) represents the 100%, while the average size measured by AB347 (*hda2* Δ/Δ) over the time is presented in the graph. The aim of this graph is to make visible the reduction of the hyphal elongation of the mutant strain over the time.



Graph 33. Representative size in percentage of hyphal filaments for AB629 (*hda2* Δ/Δ) compared to AB66 (WT) in Spider liquid media for 6 hours

For each hour, the average size measured for AB66 (WT) represents the 100%, while the average size measured by AB629 (*hda2* Δ/Δ) over the time is presented in the graph. The aim of this graph is to make visible the reduction of the hyphal elongation of the mutant strain over the time.



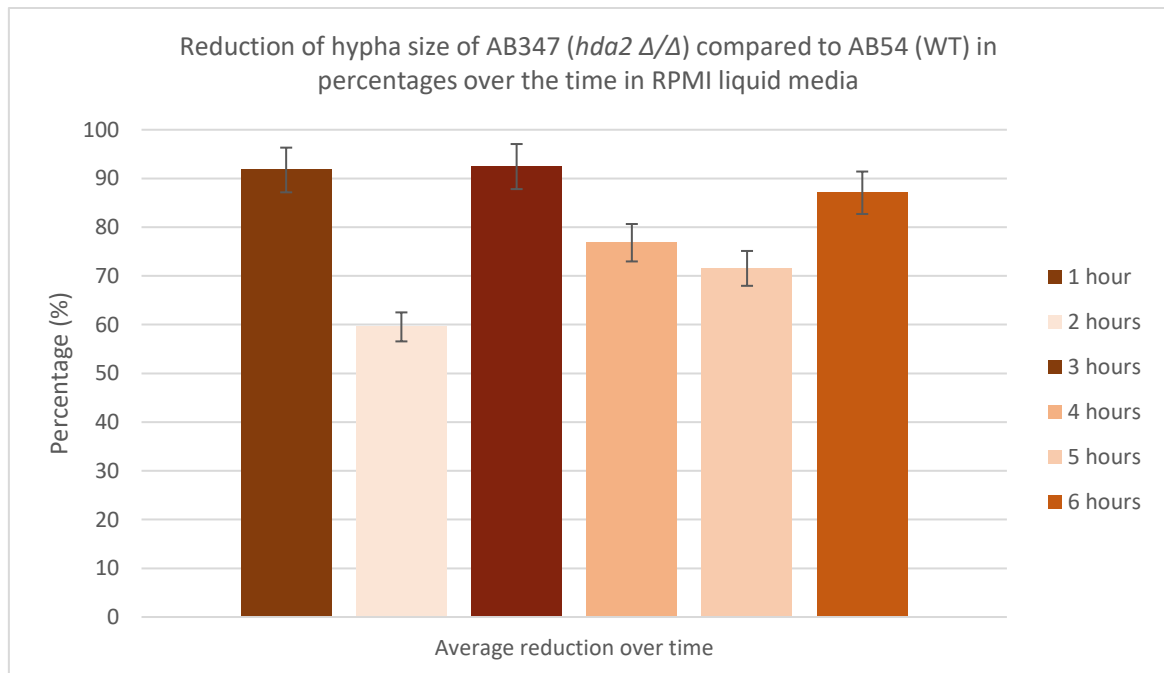
Graph 34. Representative size in percentage of hyphal filaments for AB525 (*hda1* Δ/Δ) compared to AB216 (WT) in Spider liquid media for 6 hours

For each hour, the average size measured for AB216 (WT) represents the 100%, while the average size measured by AB525 (*hda1* Δ/Δ) over the time is presented in the graph. The aim of this graph is to make visible the reduction of the hyphal elongation of the mutant strain over the time.

Results obtained in Spider media proves a clear reduction in the hypha size over the time compared to the WT strains. It's important to consider that every hour, the WT average size of the filament is longer as the time goes by, so the reduction must be every time higher in the mutant strains to see a prolonged reduction.

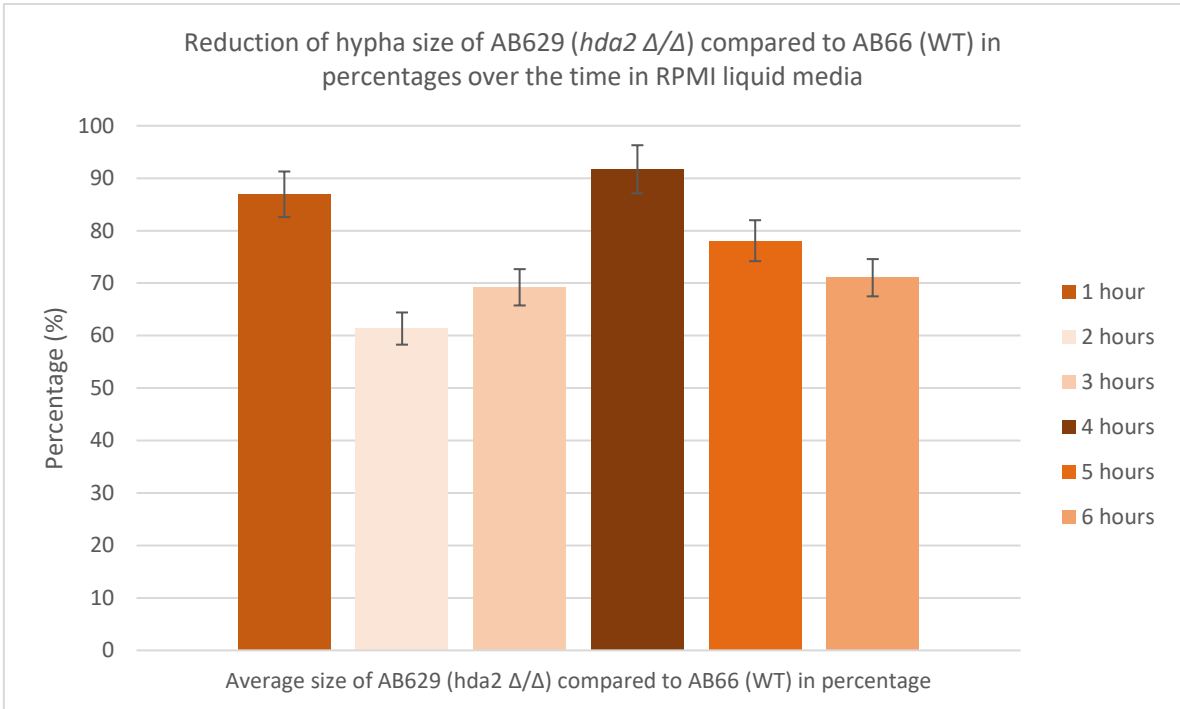
For AB525 (*hda1 Δ/Δ*) and AB629 (*hda2 Δ/Δ*) the reduction of the filament at 6 hours growth is around 60%, and even though the reduction over the time is clearer in AB525 (*hda1 Δ/Δ*), both strains prove a high reduction of the filament length, while strain AB347 (*hda2 Δ/Δ*) only reached at 26% of reduction at its maximum.

RPMI liqui media (hypha size)



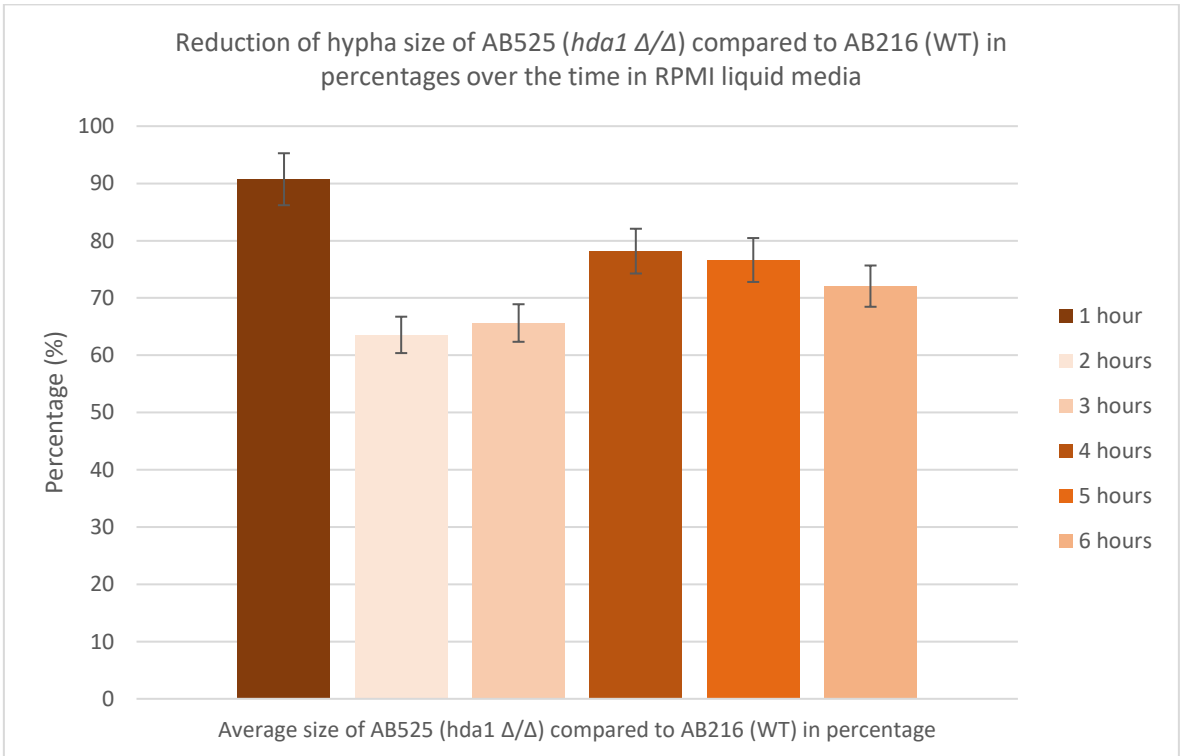
Graph 35. Representative size in percentage of hyphal filaments for AB347 (*hda2 Δ/Δ*) compared to AB54 (WT) in RPMI liquid media for 6 hours

For each hour, the average size measured for AB54 (WT) represents the 100%, while the average size measured by AB347 (*hda2 Δ/Δ*) over the time is presented in the graph. The aim of this graph is to make visible the reduction of the hyphal elongation of the mutant strain over the time.



Graph 36. Representative size in percentage of hyphal filaments for AB629 (*hda2* Δ/Δ) compared to AB66 (WT) in RPMI liquid media for 6 hours

For each hour, the average size measured for AB66 (WT) represents the 100%, while the average size measured by AB629 (*hda2* Δ/Δ) over the time is presented in the graph. The aim of this graph is to make visible the reduction of the hyphal elongation of the mutant strain over the time.



Graph 37. Representative size in percentage of hyphal filaments for AB525 (*hda1* Δ/Δ) compared to AB216 (WT) in RPMI liquid media for 6 hours

For each hour, the average size measured for AB216 (WT) represents the 100%, while the average size measured by AB525 (*hda1* Δ/Δ) over the time is presented in the graph. The aim of this graph is to make visible the reduction of the hyphal elongation of the mutant strain over the time.

In RPMI media, the reduction of the filament of mutant strains changes over the time and does not give a clear idea when the reduction is higher. Still, in all strains with *HDA1* or *HDA2* deleted, the filament average in all hours is smaller than in the WT strains, which again, proves the need of this genes to keep the hypha elongation.

Another fact that can't be presented in these kinds of graphs is that RPMI does not only induces higher amount of cell morphogenesis but also higher elongation of the filamentation to all strains. The average size of the hypha for every strain is higher in RPMI than in Spider.

It was therefore concluded that deletion of *HDA2* gene does also affect in hyphal elongation in both RPMI and Spider.

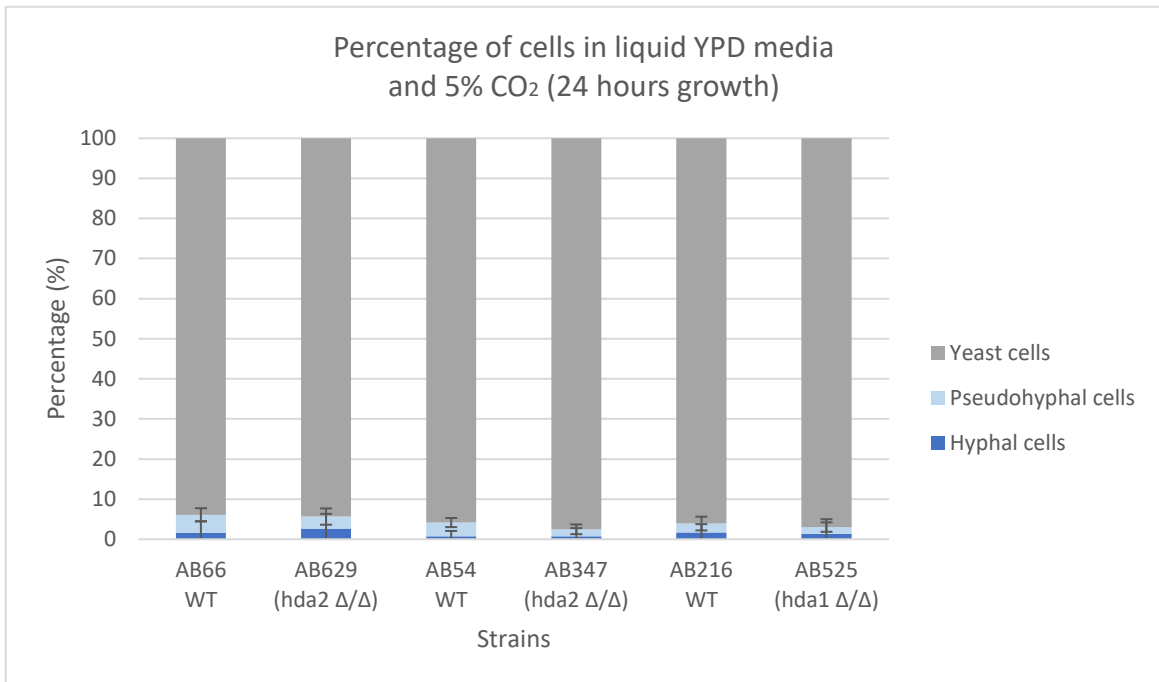
Incorporation of increased CO₂ to hyphal inducing liquid medias

Once the correct protocol proved its capacity to increase hyphal induction and since one of the first experiments, where the augment of CO₂ (up to 5%) in the environment increased the hyphal morphogenesis, the next step was to combine these two conditions, using Protocol 3.8.

Making this experiment it could be possible to maximize the hyphal induction through different pathways at the same time in one last experiment and see if doing that, the differences between the WT strains and their mutants were more notable. For this section we used again the three original medias, including the yeast inducing media (YPD) as a control. This is because in YPD agar plates, increase of CO₂ developed an increase of hyphal morphogenesis, and it was wanted to check if in liquid media it has the same results.

The same 6 strains were used: *hda2* Δ/Δ (AB347 and AB629) and their original WT strains (AB54 and AB66 respectively) and *hda1* Δ/Δ (AB525) and its original WT strain (AB216) as a positive control. Cells were grown at 37 °C and checked at 24 hours. Results obtained are presented in the following graphs.

YPD liquid media (24 hours growth in 5% CO₂)

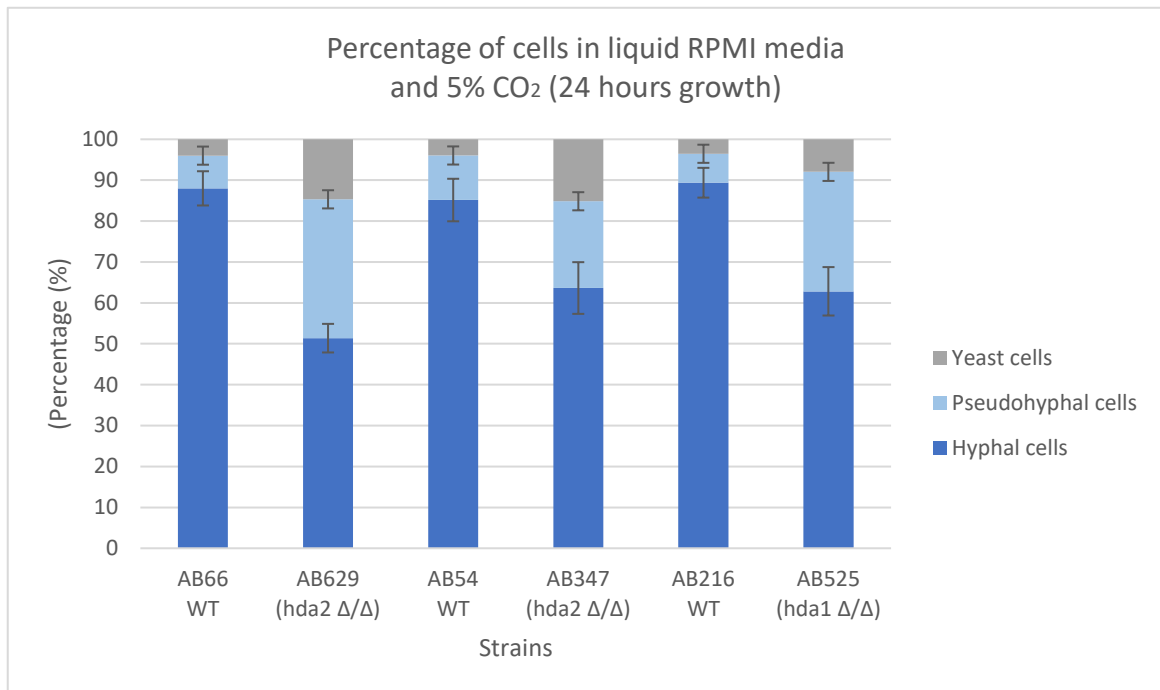


Graph 38. Quantification of cells in YPD liquid media in 5% CO₂ at 24 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in YPD liquid media for 24 hours in 5% CO₂. Error bars represents standard deviation for the three replicates.

Graph 38 demonstrates that increasing CO₂ in the environment does not increase the hyphal morphogenesis in YPD liquid media since the results obtained were very low in percentages of hyphal cells. This may be because the cells are inside the media (not in the surface like they are in an agar plates), so the contact with this gas is not as direct as it is in YPD plates. Therefore, it was concluded that in liquid media the increase of CO₂ does not have the same effect than in agar media.

RPMI liquid media (24 hours growth in 5% CO₂)



Graph 39. Quantification of cells in RPMI liquid media in 5% CO₂ at 24 hours growth

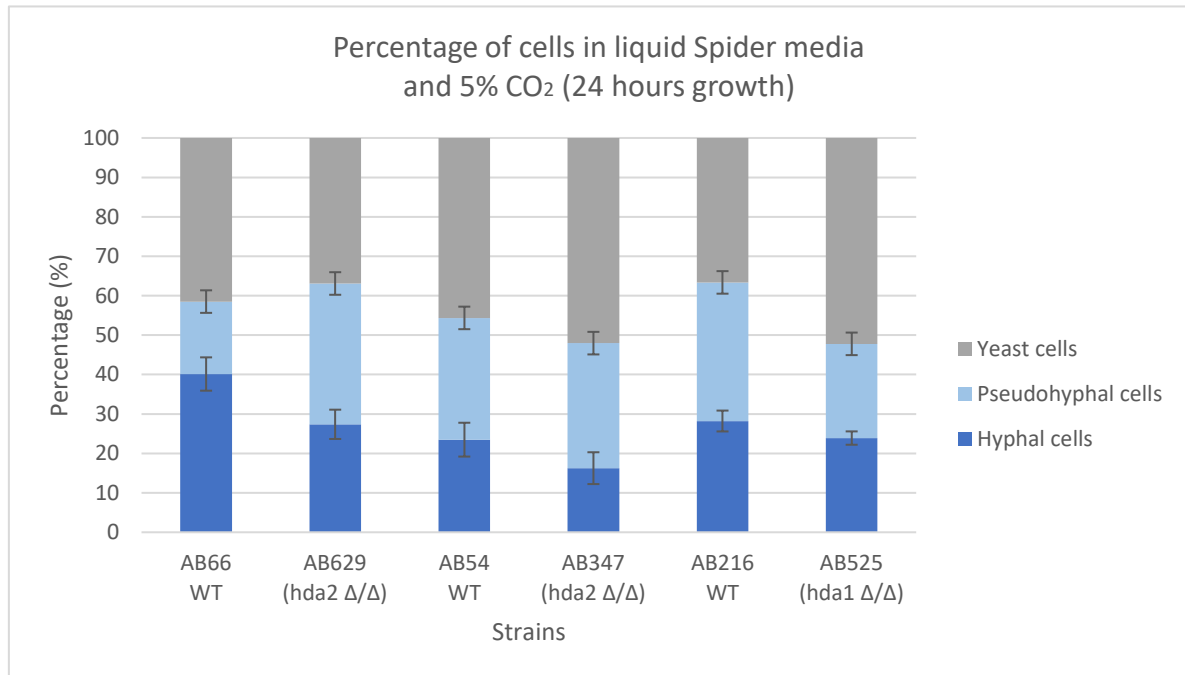
Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in RPMI liquid media for 24 hours in 5% CO₂. Error bars represents standard deviation for the three replicates.

Graph 39 demonstrates that, as we already conclude, RPMI is the higher hyphal inducer media but, in this case, increasing CO₂, the differences between the WT strains and their mutants became more visible. There is a difference around 22% between AB54 (WT) and AB347 (*hda2 Δ/Δ*) having a significance value of $p=0,016$ calculated by T-test. Between AB216 (WT) and AB525 (*hda1 Δ/Δ*) there is a difference of 27% with a significance value of $p=0,002$, and finally this difference expands between AB66 (WT) and AB629 (*hda2 Δ/Δ*) with 37% of less hyphal cells having a significant value of $p=0,0001$. All these differences in the amount of hyphal cells between the WT strains and their mutants prove that *HDA1* and *HDA2* are needed for hyphal induction in these conditions.

Is important also to consider that in this case, cells were checked 24 hours later from the inoculation, which means that during this time, the media was being consumed and cells were losing the initial conditions and stimuli that they had to induce hyphal morphogenesis. Maybe

what increased the differences between the WT strains and their mutants was not the increase of CO₂ but the increase of time when the cells were measured.

Spider liquid media (24 hours growth in 5% CO₂)



Graph 40. Quantification of cells in Spider liquid media in 5% CO₂ at 24 hours growth

Average in percentage of different cell morphologies observed in three biological replicates, for each strain, grown in Spider liquid media for 24 hours in 5% CO₂. Error bars represents standard deviation for the three replicates.

Graph 40 show the results obtained from Spider liquid media in increased CO₂ environment. Disappointingly, the differences between WT strains and mutants became again very unclear. The most feasible explanation for it is that if we look into the experiment “Spider liquid media (6 hours growth in rich media)” it can be seen that in the last hours (5 and 6) generally all strains started to decrease percentages of hyphal cells, meaning hyphal morphogenesis decreased over the time. This explanation taken into this experiment, where the growth of cells was for 24 hours, may explain why the results had very low percentages of hyphal cells and why the differences became unclear and not significant (all p values > 0,05)

Hypha strength and proliferation capacity of *C. albicans*

Finally, two last properties of the hypha formed by *C. albicans* were also wanted to be tested: the differences between WT strains and mutants about the strength of the hypha formed by the fungus and its capacity to proliferate in the media when the complete colony is eliminated from it.

To do that, protocol "3.9" was used. All 6 strains (AB54, AB66, AB216, AB347, AB525, AB629) were sown in agar plates, using the three medias (RPMI, Spider and YPD), and all of them grew at 37 °C to induce hyphal formation.

When the media used was RPMI, once the 5 days passed by, the attempt to clean the surface became practically impossible, and the agar media broke every time during this process for the 3 biological replicates. The hyphae produced in RPMI was then classified as strong and its capacity to proliferate again was impossible to check since the colonies could not be taken from the media. There were no differences observed between the WT strains and the mutants.

For Spider media, the strength and the time used to clean the surface was lower but still not easy. Not any of them were stuck as in RPMI but they were strong attached to the media. The hyphae produced in Spider was then classified as medium and its capacity to proliferate once the time passed by was high. There were no differences observed between the WT strains and the mutants.

For YPD media, there was no need of strength to clean the surface, colonies were easily wiped out and media was cleaned in short time. The hyphae produced in YPD was then classified as weak and its capacity to proliferate was as high as in Spider. There were no differences observed between the WT strains and the mutants.

Structure used:

AB54	AB66	AB216	Water
AB347	AB629	AB525	Water

Example of results:



Discussion

The aim of this project was to test the role of Hda2 in *C. albicans* hyphal formation. In order to do that, a right protocol was needed to be established. During the project, several medias were tested as so different conditions, each one inducing hyphal formation throw different pathways.

Establishment of protocol to test hyphal growth

RPMI has proven to be the best inducing media in solid and liquid state. This media reached the highest hyphal cells percentages and both states. Spider had less hyphal induction in both liquid and solid media, but differences between the WT strains and mutants were clearer in liquid media with significant values obtained with T-test. Serum was not hyphal inducing as expected and YPD was yeast inducing media as already known.

Increase of CO₂ at 5% was confirmed as hyphal inducing condition in solid media while in liquid media could not be confirmed since the results had no significant value. Results about increased CO₂ match with published studies only in solid media [32]. This may be because cells inside the liquid media does not have the same contact with CO₂ than colonies on the surface of the agar plates. Hypoxia environment as hyphal inducing condition could not be tested since the colonies did not grow at this situation. It is known that this fungi can grow in hypoxia and it also known to induce hyphal morphogenesis [32] but in our study, cells were not able to grow. Correct procedure was made during this study so it is not known why this happened.

Protocol optimization in liquid media was done throw modifying different conditions such as time and cell density: For RPMI and Spider liquid media, with low initial cell density in cultures, pseudohyphal cells can be observed in very few minutes and hyphal cells can be observed in less than 2 hours' time. That is because the transcriptional repressor Nrg1 is rapidly reduced in *C. albicans* when hyphal inducing stimuli is detected (increasing the temperature from 30°C to 37°C and putting them in hyphal inducing medias) allowing the cell to morphologically switch [39]. In

the contrary, for YPD, hyphal cells barely appear at any time, since this media is yeast inducing. When the initial cell density is too high in any liquid media, very few hyphal cells can be detected (high condensed medias does not induce hyphal morphogenesis).

Hda2 and its role in the yeast to hyphae switch

Using the results obtained in these studies it has been concluded here that *HDA2* is an important gene for inducing hyphal morphogenesis in both RPMI and Spider solid media. Significant values obtained in Results proved the hypothesis than strains *hda2* Δ/Δ have less hyphal formation than WT strains, as so did the results obtained with increased CO₂ condition in YPD agar plates. In contrast, the hypothesis of the reduction of hyphal induction with mutant strains was not confirmed in liquid RPMI media, which results had no significant value. It's important to remark that after a full revising of this thesis, the corrections suggest that the statistical test used for most of the studies is not completely coherent for multi-sample comparison, but in this case results may not modify the conclusion obtained by it.

In Spider liquid media, at 4, 5 and 6 hours growth, the differences between hyphal cells obtained in each strain proved again the reduction in hyphal cell induction. In liquid media, the increase of CO₂ as hyphal inducing condition was only proved by statistical test in RPMI, while in Spider results had not significant value.

We hypothesize that the differences obtained in the results between the liquid and solid media about the hyphal formation may be for two reasons.

- 1.- The amount of nutrients available in each media. In agar media there are less nutrients available once the colonies are grown for a week. Starvation may be also an inducing hyphal stimuli throw expression of Brg1, a GATA family transcription factor [32]. While in liquid media, counts of cells where done in less than 24 hours when media is still rich.

2.- The difficulty counting the hyphal cells/colonies: while in solid media it is easier to identify a hyphal/not hyphal cells, in agar plates, colonies may be growing filamentation under the visual colony but impossible to observe in the surface.

Another explanation may be that when cells are being counted, results obtained about the behavior of the fungi itself are more representative than when they grow in colonies, since filamentation can be seen in these colonies but that doesn't mean all cells are morphologically switching. Moreover, the number of cells counted for each strain in each experiment is higher than colonies counted in each plate.

Results obtained in our studies matches with the previously hypothesized conclusion: *HDA2* plays an important role in hyphal morphogenesis but is not essential for the yeast-to-hyphal switch because of the existence of redundant pathways.

We also hypothesize that increasing the time of growth of these same experiments may give better differences between the WT strains and mutants about hyphal cells since, in Spider liquid media, the significant values between these strains were obtained at the last hours with the right protocol. We also propose that better studies could be done with double mutant strains: *hda2* Δ/Δ and *hda3* Δ/Δ . Deletion of both genes may have a higher impact reducing hyphal formation in *C. albicans* since there is a reduced expression of the components of Hda1 complex.

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