Universidade do Minho
Escola de Ciências

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UMinho | 2019

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Is vaccine efficacy regulated by the gut microbiome?

Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho efetuado sob a orientação de
Professora Doutora Ana Paula Fernandes Monteiro Sampaio Carvalho
e de
Doutor Egídio Manuel Pires Torrado

maio de 2019

Agradecimentos

Ao longo deste percurso e da elaboração deste trabalho, são várias as pessoas a quem gostaria de agradecer.

Em primeiro lugar, gostaria de agradecer à professora Paula Sampaio, por tão bem me ter aceite e acolhido no seu grupo de investigação. Pela sua orientação ao longo de todos estes meses, por todas as vezes em esteve presente, principalmente quando me encontrava mais perdida. Pela sua paciência e preocupação e por todo o conhecimento transmitido.

Ao doutor Egídio Torrado, por me ter acolhido no seu laboratório, permitindo assim a realização de toda a parte *in vivo*.

À Alexandra Correia, parte essencial deste percurso, por todo o apoio prestado ao longo deste trabalho. Pelo seu espírito crítico, rigor científico e disponibilidade.

Um obrigado especial à professora Célia Pais, pela amabilidade que sempre demonstrou ao longo do meu percurso, bem como por todas as suas sugestões.

Ao meu eterno MiniBoss, Augusto Barbosa, ao qual não sei por onde começar a agradecer. Por me ter acolhido como um dos seus "pintainhos" e por me ter ensinado tudo para dar os primeiros passos na realização desta tese de mestrado. Pela companhia durante todo este caminho, por toda a ajuda e todos os bons momentos. Pelas noitadas no laboratório, bem como pelas manhãs que começavam cedo, não esquecendo os dias que exigiam que o almoço fosse a meio da tarde. Por me mostrar o que é ser um verdadeiro investigador, com todo o seu entusiamo, e uma excelente pessoa.

À Margarida Barbosa por ter sido uma constante durante este tempo. Por me introduzir à experimentação animal e me ensinar de uma forma tão paciente tudo o que está relacionado com o processamento dos animais. Por ter sido incansável, sempre curiosa, sempre interessada em todo o trabalho. Por toda a dedicação e empenho.

A todos os elementos da Micro II. Um obrigado especial à Joana Pereira, por ter estado sempre lá quando precisei, e por, tantas vezes, me acalmar e me fazer encarar os problemas de outro ponto de vista. À Rita Alves, pela sua boa disposição inabalável, e à Rita Rocha, pela sua doçura. Por todas as conversas e todas as gargalhadas. À Moli, por ter sido parte essencial do trio dos "pintainhos". Um obrigado, também, à Cátia e à Filipa da Micro I, pela simpatia e disponibilidade sempre que era preciso recorrer ao seu laboratório, e pela ajuda com os Western Blots.

Ao Sr. Luís e à Núria, um obrigado especial cheio de carinho, por todas as vezes em que me presenteavam com um sorriso gigante logo pela manhã, por todas as dúvidas que me tiraram e por toda a ajuda ao longo deste percurso.

Aos meus ninos. Ao João, o farmacêutico da minha vida, por todas as vezes em que me fez rir com o seu jeito de ser. Por estar sempre disposto a ajudar-me no que eu precisar. Por me relembrar que somos a dupla que "combina sempre". À Mimi, pelo seu coração que parece gigante demais para caber dentro da pessoa que ela é. Por todo o carinho, por toda a preocupação. Por todas as conversas. Por ser, efetivamente, uma das melhores pessoas que conheço.

À Marisol, por ser a companheira de todas as horas. Por todas as gordisses, maratonas de séries e confeções de bolos. Por todos os dias de desespero, e por todos aqueles que foram felizes. Por se ter tornado a minha família em Braga e nunca deixar que as coisas "deem ruim" para mim. À Meirinha, por todas as vezes em que me relembra de que existe um caminho e tudo acontece com um propósito. Por dizer sempre que "temos tanto, tanto para estar agradecidos".

Ao Zé Pedro por ser, acima de tudo, a minha âncora, o meu refúgio, o meu melhor amigo, o meu tudo. Por todas as vezes em que me levanta a cabeça e me dá motivação. Por todas as vezes em que me apoia e me diz que tudo vai ficar bem. Por estar sempre comigo, mesmo longe.

Aos meus pais. À minha mãe, por dizer que, embora a vida não seja um mar de rosas, existe sempre forma de nele navegar, com esforço, dedicação e amor. Por ser um dos meus maiores exemplos de força. Ao meu pai, por me alertar que, por vezes, as maiores lições de vida vêm de pessoas que não foram tão afortunadas nessa viagem. Sem eles, nada disto seria possível. Ao meu irmão, que está a crescer rápido demais, pela cumplicidade e motivação que me dá todos os dias.

The work presented in this thesis was performed in the Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal. This work was developed under the scope of the project SECVS 074/2016 and cofounded by the project NORTE-01-0145-FEDER-000013, supported by the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER), through the Competitiveness Factors Operational Programme (COMPETE), and by National funds, through the Foundation for Science and Technology (FCT), under the scope of the project POCI-01-0145-FEDER-007038.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

A eficácia da vacinação é regulada pelo microbioma intestinal?

Resumo

Candida albicans é uma levedura presente no trato gastrointestinal humano, que se mantém maioritariamente num estado comensal, devido ao papel que o restante microbioma tem em controlar a sua colonização e invasão. Porém, alterações na homeostasia intestinal levam a que esta espécie tenha um crescimento descontrolado, revelando o seu potencial de patogénico oportunista. O risco de desenvolver uma candidíase sistémica torna-se particularmente preocupante em pacientes sob o tratamento com antibióticos, pois estes levam a alterações dramáticas no microbiota do trato gastrointestinal. Uma estratégia antifúngica promissora são os sistemas lipossomais. Estudos mostraram que lipossomas catiónicos preparados com DODAB:MO (1:2) carregados com proteínas da parede celular de *C. albicans* (CWSPs) eram estáveis, não citotóxicos e avidamente fagocitados por macrófagos. Através da indução de uma resposta humoral forte e uma resposta Th1/Th17 específica, esta formulação foi capaz de induzir proteção contra uma infeção sistémica letal por *C. albicans*.

Neste estudo, o objetivo foi avaliar o efeito do tratamento com antibióticos na eficácia da vacinação. Os lipossomas preparados, DODAB:MO incubados com CWSP, induziram uma resposta humoral mais baixa e uma tendência para uma resposta celular tipo Th2, conhecida como não-protetora contra infeções sistémicas por *C. albicans*. Contudo, a administração de antibióticos durante a imunização pareceu reduzir esta polarização inicial das células T, protegendo os ratinhos, e levando a uma maior percentagem de sobrevivência neste grupo. Como os lipossomas preparados provaram ser diferentes dos previamente obtidos, analisámos o seu método de preparação, bem como o uso de um único antigénio, Cht3p, na modelação da resposta imune. Os resultados indicaram que alterar o método de preparação dos lipossomas levou à obtenção de lipossomas mais pequenos, que desviou a resposta imune de Th2 para Th1/Th17, e levou a um aumento significativo nos níveis de anticorpos específicos para CWSP. A utilização da Cht3p manteve os tamanhos, mas alterou a carga superficial dos lipossomas, algo que não alterou o tipo de resposta imune, verificando-se a mesma polarização para Th1/Th17. Ainda que Cht3p deva ser encapsulada para induzir uma resposta mais específica, a imunização com DODAB:MO CWSP foi capaz de induzir a melhor resposta humoral, possivelmente devido à presença de várias proteínas que poderiam ajudar a impulsionar o desenvolvimento de anticorpos específicos para a Cht3p.

Em suma, a modulação do microbioma pelo uso de antibióticos desencadeou uma resposta protetora e um controlo melhorado da infeção, sugerindo que a vacinação em pacientes sob o tratamento com antibióticos de largo espectro, em risco de desenvolver infeção sistémica, não é prejudicial e pode até ser benéfica. Além disso, com este sistema lipossomal, o método de preparação tem um grande impacto

na resposta imune, com o tamanho a ter um papel mais importante no desvio da resposta por parte das células T do que propriamente a carga superficial.

Palavras-chave: antibióticos; *Candida albicans*; lipossomas; resposta humoral e mediada por células.

Is vaccine efficacy regulated by the gut microbiome?

Abstract

Candida albicans is a normal colonizer of the human healthy gastrointestinal (GI) tract, persisting in a harmless state most of the time, due to the role of the GI microbiome in controlling its colonization and invasion. However, alterations in the intestinal homeostasis allows this species to overgrow, becoming one of the most important opportunistic pathogens. The risk of developing systemic candidiasis is particularly worrisome in patients undergoing antibiotic treatment, since it will change dramatically the gut microbiome. A promising antifungal strategy is the use of liposomal vaccine delivery systems. Previous studies reported that cationic liposomes prepared with DODAB:MO (1:2) and loaded with *C. albicans* cell wall surface proteins (CWSPs) were stable, non-cytotoxic and avidly phagocytized by macrophages. By inducing a strong humoral response and a specific Th1/Th17, this formulation induced protection against a lethal systemic infection with *C. albicans*.

In this study, our aim was to evaluate the effect of antibiotic treatment in vaccine efficacy. The liposomes prepared, DODAB:MO incubated with CWSP, induced an overall lower humoral response and a bias towards a Th2 response, non-protective against *C. albicans* infections. However, administration of antibiotics during the immunization procedure seems to have reduced this initial T-cell polarization, which protected mice, leading to a higher percentage of survival in this group. Since the liposomes prepared were quite different from the previously reported, we analysed the liposome preparation method and the use of a single antigen, Cht3p in modulating the immune response. Results indicated that changing the liposome preparation method led to smaller liposomes, and this decrease shifted the immune response from a Th2 to a Th1/Th17 response, along with a significant increase in CWSP-specific antibodies. The shift to Cht3p, however, maintained the liposomes size, but changed their surface charge, which did not change the type of immune response, showing the same polarization towards Th1/Th17. It was evident that Cht3p must be encapsulated to induce a better immune response. Nevertheless, immunization with DODAB:MO CWSP still induced the best humoral response, possibly due to the presence of several proteins that could help boost the development of Cht3p-specific antibodies.

In conclusion, microbiome modulation through antibiotic treatment prompted a protective response and an improved control of infection, suggesting that vaccination in patients undergoing broad spectrum antibiotic treatment, that are at risk of developing systemic candidiasis, is not detrimental and could be beneficial. In addition, with this liposome system, preparation method has a huge impact in the immune response, with liposome size having a more prominent role on shifting the T-cell response, rather than surface charge.

Keywords: antibiotics; *Candida albicans*; humoral and cell-mediated response; liposomes.

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List of Abbreviations and Acronyms

% EE	Encapsulation Efficiency
ADS	Antigen Delivery System
Als	Agglutinin-like Sequence
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
BCA	Bicinchoninic Acid Protein Assay
BSA	Bovine Serum Albumin
C. albicans	*Candida albicans*
CBMA	Center of Molecular and Environmental Biology
CFU	Colony Forming Unit
Cht	Chitinase from *C. albicans*
CLR	C-type Lectin Receptors
CR3	Complement Receptor 3
CWSP	Cell Wall Surface Protein
DC	Dendritic cell
DC-SIGN	Dendritic Cell-specific ICAM3 Grabbing Non-integrin
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle Medium
DODAB	Dioctadecyldimethylammonium Bromide
dTMP	Deoxythymidine Monophosphate
DTT	Dithiothreitol
dUMP	Deoxyuridine Monophosphate
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FCyR	FCy Receptor
GALT	Gut-Associated Lymphoid Tissues
GI	Gastrointestinal
GPI	Glycosyl Phosphatidilinositol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV/AIDS	Human Immunideficiency Virus and Acquired Immune Deficiency Syndrome
Hsp	Heat-shock protein
Hwp	Hyphal Wall Protein
ICAM3	Intercellular Adhesion Molecule 3
ICVS	Life and Health Sciences Research Institute
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Int	Integrin-like Protein
kDa	Kilodalton
LNPs	Liposomal nanoparticles
LUV	Large Unillamellar vesicle
M	Microfold
MIC	Minimum Inhibitory Concentration
MLV	Multilamellar Vesicle
MO	Monoolein (monooleoyl-rac-glycerol)
MVV	Multivesicular Vesicle
MW	Molecular Weight
MWM	Molecular Weight Marker
NLR	NOD-like Receptor
NOD	Nucleotide-binding Oligomerization Domain
P. pastoris	*Pichia pastoris*
PAMP	Pathogen-associated Molecular Patterns
PBS	Phosphate-buffered Saline
PDI	Polydispersivity
PI	Propidium Iodide
Pir	Proteins with Internal Repeats
PL	Phospholipase
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene Difluoride
RPMI	Roswell Park Memorial Institute medium
RT	Room Temperature

SAP	Secreted Aspartyl Proteinase
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamid Gel Electrophoresis
SUV	Small Unilamellar Vesicle
TBS	Tris-buffered Saline
TBS-T	Tris-buffered Saline with Tween 20
Th	T Helper
TIR	Intracellular Toll/IL-1 Receptor Homology
TLR	Toll-like Receptor
Treg	Regulatory T cell
YPD	Yeast Extract Peptone Dextrose
ζ-Potential	Zeta Potential

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CHAPTER 1
General Introduction

1. General Introduction

1.1. The role of the gut microbiota in health

Usually, we think about our gastrointestinal microbiology only when there is something amiss with our digestive systems. However, the gastrointestinal (GI) tract is home to a complex community of microorganisms, including bacteria, viruses, and eukaryotes, that exists in symbiosis with the host, providing genetic and metabolic attributes (Antonopoulos *et al.*, 2009). In fact, the collective genome of the gut microbiota exceeds in a large scale the genome of the human body (Lange, Buerger, Stallmach, & Bruns, 2016) and due to its role in human wellbeing, it is often referred to as an additional metabolic organ (Guinane, & Cotter, 2013).

Microbial colonization in the GI tract begins only after delivery, since the babies' intestines are believed to be sterile or with a nearly inexistent level of microorganisms at birth (Bull, & Plummer, 2014). Typically, *Enterobacter* and *Enterococci* are among the early colonizers of the infant's gut. *Bifidobacteria, Clostridia, Bacteroides spp.* emerge later, along with other anaerobic organisms. However, the mode of delivery strongly shapes this first colonization, since in vaginally born infants the maternal microbiota is determinant for the initial composition. Breastfeeding and antibiotic, prebiotic or probiotic use can influence the babies' microbiota as well (Guinane, & Cotter, 2013).

In the first year of life, the gut microbiota composition is quite simple, but throughout the time it evolves and matures according to feeding habits, environment and other endogenous and exogenous factors, leading to a wide variation between individuals (Bull & Plummer, 2014). Figure 1 summarizes some of these factors.

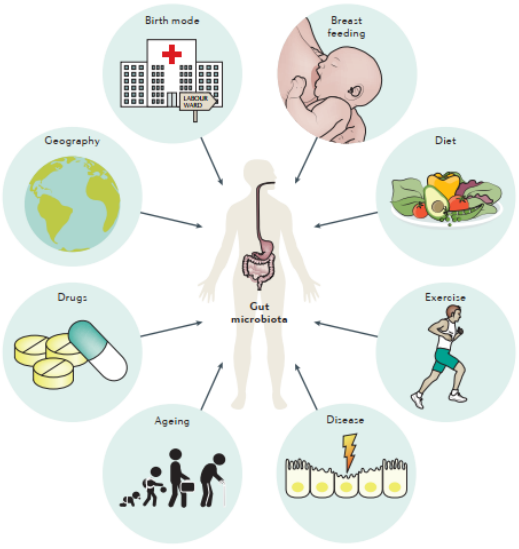

Figure 1. Factors influencing the composition of the human gut microbiota: mode of delivery; shifts in the feeding mode (breast, bottle, introduction of solid food); personal lifestyle factors, such as diet and exercise; disease (obesity, inflammatory disease, etc.); ageing; drug therapy as in the use of antibiotics; geography (from Quigley, 2017).

Our gut microbiota plays a significant role in human health, from host physiology to its metabolism, nutrition and immune function (Guinane & Cotter, 2013). It provides biochemical pathways that we are unable or have a limited capacity to carry on, such as the degradation of non-digestible polysaccharides, like resistant starch. This leads to a recovery of the energy and individual substrates for the host but is likewise needed for bacterial growth and proliferation (Bull & Plummer, 2014). The intestinal flora is involved in microvilli development, as well (Cani & Delzenne, 2009).

Since there is a concerning proximity between host tissues and microbial populations, one of the principal roles of the intestinal microorganisms is to protect the host against colonization by pathogens. Therefore, a balance is needed to maintain valuable strains without allowing overproliferation of the non-beneficial ones. Production of inhibitory molecules and nutrient competition are non-immune derived mechanisms through which this balance can be maintained. However, there are also some processes that rely on innate and adaptative immune response, which is not surprising since the intestinal epithelium is the primary interface between the immune system and the environment. For that reason, the gut microbiota is responsible for the development and education of the immune system, as well (Bull & Plummer, 2014; Kamada, Seo, Chen, & Núñez, 2013).

1.1.1. The Coexistence between the gut microbiota and the immune system

The immune system in the GI tract has to deal with a massive community of microorganisms, not only commensals that continuously grow on our mucosal surface, but also the ones which are

ingested along with the food. Since we rely on the gut microbiota for several functions related to our health, our immune system evolved in order to tolerate microorganisms' presence in the intestinal lumen, while preventing their invasion.

The innate immunity protection is firstly mediated by a stratified mucus layer, originated from goblet cells, along with anti-microbial substances which are produced as an attempt to, along with the mucus, compartmentalize luminal microorganisms, with the aim of minimizing epithelial contact (Abbas, Lichtman, & Pillai, 2014; Hooper, Littman, & Macpherson, 2012). The intestinal epithelium, found underneath, offers the second physical barrier, with a layer of cells that also produce anti-microbial peptides. Intercellular tight-junctions between adjacent intestinal epithelial cells provide an obstacle for the microorganisms to move into the lamina propria and the differential expression of pattern recognition receptors (PRRs) by intestinal epithelial cells, namely toll-like receptors (TLRs) and NOD-like receptors (NLRs), allows the identification of microorganisms that have invaded through the epithelial barrier and the ones that gained access to the cytosol – as well as their products-, respectively. Dendritic cells (DCs), macrophages and intra-epithelial T cells also contribute to maintain this balance (Abbas *et al.*, 2014; Hooper *et al.*, 2012; Prieto, Correia, Pla, & Roman, 2016; Ubeda, Djukovic, & Isaac, 2017).

Regarding the adaptative immunity, it is mediated by a group of subepithelial lymphoid tissues known as Gut-Associated Lymphoid Tissues (GALT). Their main effector cells are the microfold (M) cells and the lamina propria dendritic cells. The first one, present in the epithelial lining, are responsible for the transport of intact antigens and/or molecules as samples to antigen-presenting cells (APCs) in the GALT. DCs, on the other hand, extend themselves between the epithelial cells to collect antigens present in the lumen. Both of these processes can promote protective immune responses. There are also effectors B and T cells, but the humoral immunity in the GI tract is mainly dominated by the secretion of IgA in the lumen, secreted by lamina propria plasma cells. T helper (Th) 17 effector cells, through the secretion of IL-17 and IL-22, enhances epithelial barrier functions, while Th2 cells have their role in the defense against other intestinal parasites. Regulatory T (Treg) cells are also generated to suppress immune responses to commensals and control tissue damage (Abbas *et al.*, 2014, Hooper *et al.*, 2012).

1.2. Antibiotics and gut microbiota

When Alexander Flemming discovered the penicillin, millions of lives were saved, and antibiotics became an important weapon against bacterial assaults and most infectious diseases (Ianiro, Tilg, & Gasbarrini, 2016). Nowadays, however, their use has raised to concerning levels, since a significant

percentage of the population resort to them every day. Therefore, not only are we witnessing an increased exposure to antibiotics, but also their misuse (Becattini, Taur, & Pamer, 2016).

Recent investigations showed that, in fact, antibiotics could be a double-edged sword. Buffie *et al.* (2012) demonstrated that a single dose of clindamycin led to extreme changes in the mouse microbiota composition, conferring long-lasting susceptibility to certain infections. Another study showed that, although the indigenous gut microbiota was known to eventually stabilize a few weeks after the antibiotic treatment, by analyzing the bacterial flora in more detail, loss in diversity and specific taxa was evident, along with the rise of antibiotic-resistant strains (Jernberg, Löfmark, Edlund, & Jansson, 2007). Regarding human studies, Azad and co-workers (2016) aimed to determine the effects of intrapartum antibiotics on the infant microbiota and concluded that there were persistent alterations in the structure and richness of the gut microbiota to at least 3 months of age.

Thus, administration of broad-spectrum antibiotics has recently been linked to an alteration in microbial communities present in the GI tract. Although antibiotics are used to alter the native microbiota, they inevitably cause disturbances in the normal baseline and therefore modify the interactions between the host and the community of microorganisms present in the intestine (Antonopoulos *et al.*, 2009). Even though the healthy microbiome offers some level of resistance to these types of disturbances, alternative stable states resistant to antibiotics and other drugs may arise.

1.2.1. General consequences of the use of antibiotics on gut microbiota

Reduced diversity, loss of major taxa, alterations in metabolic capacity and reduced colonization resistance, which is crucial when pathogens invade, are indicators of a post-antibiotics dysbiosis. This is due to the role that antibiotics have on shifting the gut microbiota to apparent-stable or alternative stable states that can become resilient against external influences (Figure 2) (Lange *et al.*, 2016).

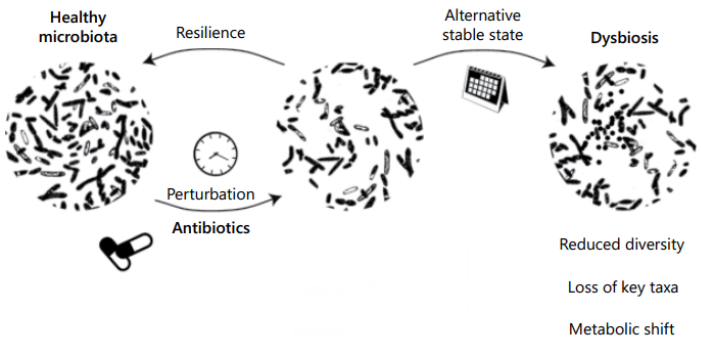

Figure 2. Effects of antibiotics on healthy microbiota. Following a disturbance caused by antibiotics, the gut microbiota is able to offer some resilience, which can restore the communities to their previous equilibrium. However, depending on the severity of the perturbation, the gut microbiota can be shifted to a long-term state of dysbiosis, supporting pathogenesis (Adapted from Lange *et al.,* 2016).

The characteristics of the antibiotics can affect the impact they have on the gut microbiota since they have different spectrums and bacterial targets. Their class, dosage, pharmacodynamics, and pharmacokinetics, among others, influence their behavior and the extension of damage. Also, factors related to the host, as age, lifestyle and the microbiota composition determine the consequences on healthy bacteria and overall communities (Ianiro *et al.*, 2016). Overall, the impact of antibiotic administration on the structure of the protective communities present in the GI tract is massive, enhancing the susceptibility to infections and promoting the expansion of harmful microorganisms, as well as toxin production. (Lange *et al.*, 2016). Under these sensitive conditions, some opportunistic pathogens and pathobionts present in the gut ecosystem might have their opportunity to thrive, so they adapt and show their pathogenic potential (Stecher, Maier, & Hardt, 2013).

1.2.2. Antibiotic treatment and outgrowth of fungi

Most of the studies focus on the effects of antibiotics in suppressing the host bacteria and rarely consider the consequences among fungal communities. However, De Vries-Hospers, Welling, Swabb, & Van der Waaij (1984) reported an increase in yeast populations after the treatment with antibiotics in ten healthy adult volunteers (eight men; two women), and Samonis *et al.* (1993) using the same antimicrobial agents in mice and in humans reported similar results. Based on that, the mouse model proved to be useful in predicting the impact of antibiotics on the level of fungal colonization in human gastrointestinal tract.

Dollive and co-workers (2013) used the murine model, C57BL/6 mice, subjected to a cocktail of four antibiotics to study the gut microbiome over 76 days of treatment and subsequent recovery. They

characterized both the bacterial and the fungal microbiota, analyzing the amount and types of each one. *Bacteriodetes* and *Lachnospiraceae* were the dominant bacterial groups at the beginning, but that changed quickly, and these bacterial communities dropped sharply in abundance upon the debut of antibiotic treatment. With the cessation of the treatment the communities returned nearly to their starting states, although some differences persisted. Multiple studies on the gut microbiome in vertebrates, such as mice and hamsters, showed comparable results (Antonopoulos *et al.,* 2009; Hill *et al.,* 2010; Peterfreund *et al.,* 2012). *Lachnospiraceae* and *Clostridium* returned within one week, *Ruminococcaceae* and other *Firmicutes* increased by proportion two weeks after cessation of the treatment, and *Bacteriodales* did not fully return until the end of the experiment.

Regarding the fungal communities, there were several waves of succession and surprising degrees of fungal variation, with even the control group showing colonization by diverse fungal lineages. Before the treatment with antibiotics, *Eurotiales, Wickerhamomyces* and *Cystofilobasidium* were the dominant fungal groups, with *Candida* appearing in some cages. In the presence of antibiotics, the communities proved to be heterogeneous or dominated by *Clavispora*, *Cyberlindnera*, and *Sporopachydermia*. Nevertheless, it was observed that *Candida,* which began to be more abundant from day six of treatment, was a particularly robust colonist, persisting in the gut through the cessation of antibiotic treatment. Therefore, it was pointed out that *Candida* was favored by the antibiotic treatment.

According to Netea, Brown, Kullberg and Gow (2008), candidaemia has an incidence of 1.1 to 24 cases per 100 000 individuals, and an associated mortality of more than 30%. Therefore, concerns are raised, since the abundance of *Candida* species after the antibiotic treatment may enhance the translocation of yeast cells through the gut mucosa and lead to a systemic infection.

1.3. *Candida albicans* infections and disseminated candidiasis

More than 200 species of *Candida* have been described, although only 10% are known to be responsible for infections. Macroscopically, their colonies are cream-colored to yellowish, with different textures between species. All of them, however, produce blastoconidia with many having dimorphic growing as budding yeast cells, pseudohyphae or true hyphae (Eggimann, Garbino, & Pittet, 2003).

Candida albicans is a normal colonizer of the human healthy gastrointestinal tract, persisting in a harmless state most of the time. This is because the bacteria in the GI tract have a role in controlling its colonization and invasion. However, if there are alterations in the balance of the intestinal homeostasis, as in the modification of bacterial communities using antibiotics, this species become one of the most important opportunistic pathogens, causing superficial and systemic infections (Mason *et al*., 2012). In

fact, there was an increase in the number of reports of candidal infections following the introduction of antibiotics.

As this kind of predisposition factors lead to the overgrowth of *C. albicans*, the immune system becomes overloaded and unable to react to this condition, which allows *Candida* cells to penetrate deeper tissue, enter the bloodstream and thus establish invasive infections (Mavor, Thewes, & Hube, 2005). This turns particularly worrisome in immunocompromised individuals, as in HIV/AIDS, cancer, transplantation and many other conditions that require the use of corticosteroids (Dollive *et al.*, 2013).

1.3.1. From commensalism to dissemination of *Candida*

Candida infection of the GI tract is the first step towards candidal dissemination since it leads to a vicious cycle in which low-level inflammation promotes fungal colonization, and fungal colonization promotes further inflammation (Kumamoto, 2011). In the GI tract, *C. albicans* faces and responds to a broad set of environmental factors, such as pH, levels of nutrients and oxygen and secretions produced there (Kumamoto, 2011). This gives *Candida* the ability to survive and grow not only in the GI tract but also in several distinct sites, with particular environmental pressures. Therefore, given the opportunity, this species can cause disease, which spectrum exceeds that of other microorganisms as *Escherichia coli* (Calderone & Fonzi, 2001).

To originate an invasive infection, *C. albicans* must enter the bloodstream, survive in the blood and then escape to the surrounding organs. It can enter the circulatory system by three main routes. One of them, to which we refer to as the natural route of invasion, involves the yeast cells' penetration in epithelial cells, mainly at the gastrointestinal tract level, rather than *via* skin. The other two are known as iatrogenic routes of invasion, since they are induced inadvertently. One can happen during the use of medical devices such as central venous catheters and cardiovascular devices, through the formation of biofilms on their surfaces, and the other is related to the damage of barriers, when they occur during drug treatment, surgery or polytrauma (Figure 3) (Mavor *et al.*, 2005).

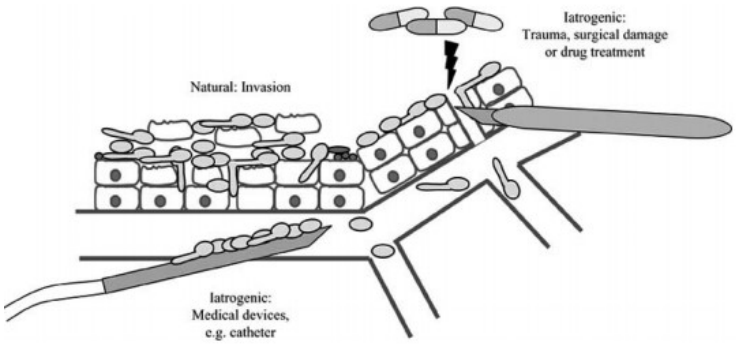

Figure 3. Routes of entry into the bloodstream. The natural route by which *C. albicans* can enter the bloodstream is characterized by invasion mainly through the GI tract mucosa. The other iatrogenic routes are related to the use of medical devices and the damage of body barriers (from Mavor *et al.*, 2005)

Once in the bloodstream, the fungal cells have the opportunity to disseminate throughout the body and infect multiple organs, so regardless of the route of entry, *C. albicans* must ensure that it can colonize the host, penetrate the surface, cause damage to deeper tissues and evade the immune system. To do so, this species expresses several virulence factors, such as adhesion factors, morphogenesis, phenotypic switching, and others.

1.3.2. Virulence factors

The ability to attach to host cells, accomplish colonization, and avoid clearance is a crucial trait of almost all pathogens. *C. albicans* displays adhesins to its cell wall, allowing cells to bind to host cells, host cell proteins or microbial competitors, as well as to blood vessels walls and organs in systemic infections. Some of these biomolecules are Als1p, Als5p, Hwp1p and Int1p. (Calderone & Fonzi, 2001; Mavor *et al.*, 2005).

As discussed above, *C. albicans* can have distinct forms, being considered dimorphic or polymorphic. Budding yeast cells can be induced to form a chain of elongated cells, the pseudohyphae or the true hyphae, and under certain conditions, a fourth growth form known as chlamydospores can occur, although it has rarely been observed *in vivo*. Therefore, morphogenesis is an important virulence factor since it is believed that the yeast cell is better suited for dissemination while hyphae play a major role in tissue and organ invasion (Mavor *et al.*, 2005). The contact to a surface is believed to be the switch that triggers the transition from yeast to hyphae, although other factors such as temperature and pH may influence as well. This contact occurs in either abiotic and biotic surfaces and leads to the formation of biofilms, which consist of *C. albicans*' cells heavily packed and adhered to a surface, surrounded by an extracellular matrix. On certain surfaces, a directed hyphal growth can be observed, a phenomenon

known as thigmotropism. The formation of biofilms is a major virulence attribute, since these complexes can be highly resistant, both to drugs and to the immune system, and, therefore, have the potential to eventually originate systemic infections of tissues and organs (Mayer, Wilson, & Hube, 2013). The switching between multiple morphologies contributes to the plasticity of the organism and is thought to aid survival in different microenvironments and evasion of the immune response (Mavor *et al.*, 2005).

Following adhesion and hyphal growth, *C. albicans* can also express invasins, such as Als3 and Ssa1, to mediate the uptake of the fungus by the host cells (Yang, Yan, Wu, Zhao, & Tang, 2014). To degrade the host surfaces, the secretion of hydrolytic enzymes is required. Not only they aid adhesion, invasion, and destruction of host immune factors, but also nutrient acquisition. Concerning hydrolytic enzymes, the families of secreted aspartyl proteinases (SAP) and phospholipases (PL) are the most representative and have been associated with virulence in *C. albicans*. (Calderone & Fonzi, 2001; Mavor *et al.*, 2005).

Besides these virulence factors, there is a range of fitness attributes that influence *C. albicans* pathogenicity. For instance, heat-shock proteins (Hsp) mediate a robust stress response, acting as molecular chaperones that prevent deleterious protein unfolding and aggregation. There is also an auto-induction of the hyphal formation through uptake of amino acids, excretion of ammonia and coordinate extracellular alkalinization since *C. albicans* can sense and adapt to environment pH and modulate it. Since nutrition is essential for survival and growth, there is a metabolic adaptability that mediates the assimilation of alternative nutrients in dynamic environments. Therefore, there is the uptake of different compounds such as carbon and nitrogen, but also of certain metals, like iron, zinc, and others (Mayer *et al.*, 2013). More recently, a novel virulence factor has been described. Known as “candidalysin”, this cytolytic peptide targets and induces damage to host’s mucosal surfaces, modulating epithelial immunity as well (Da Silva Dantas *et al.*, 2016; Sellam, & Whiteway, 2016). Figure 4 summarizes these mechanisms.

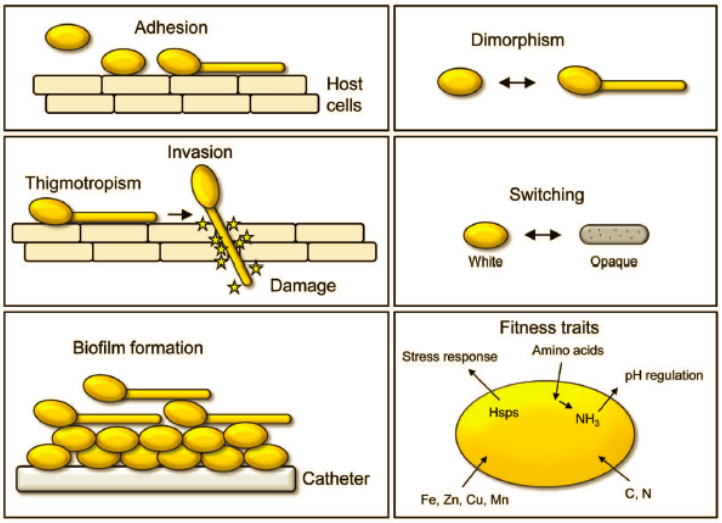

Figure 4. Overview of *C.albicans* virulence factors and pathogenicity mechanisms. Adhesion to host epithelial cells, as a complex and multifactorial process based on the expression of adhesins; yeast-to-hypha transition, also known as dimorphism, favoring the invasion and, consequently, the pathogenicity; thigmotropism following contact sensing; phenotypic plasticity (switching), important for *C. albicans* adaptability to the changing environments during invasion; ability to form biofilms, not only on biotic surfaces, such as the GI tract mucosa, but also on abiotic surfaces, such as catheters, enhancing the resistance to antimicrobial agents and host immune factors; various fitness traits as pH sensing and consequent regulation, stress response mediated by Hsp, metabolic adaptation, metal acquisition, among others (from Mayer *et al.*, 2013).

Understanding the virulence factors and the pathogenicity mechanisms that *C. albicans* uses during infection is one of the firsts steps towards the development of new antifungal therapies and diagnostics.

1.3.3. Fungal Cell Wall and Cell Wall Surface Proteins (CWSPs)

C. albicans is surrounded by a cell wall responsible for its shape and protection against differences in osmotic pressure and physical and chemical aggressions. It makes up approximately 30% of the cell dry weight, being 80%-90% carbohydrates and the rest mainly proteins. Carbohydrates monopolize immune recognition, while proteins have a fundamental role in adhesion and invasion of host cell surfaces. Therefore, the cell wall chemical composition and the assembly of its different macromolecules adjust during cell growth and morphogenesis, and so it mustn't be considered a static structure (Castillo *et al.*, 2008; Gow & Hube, 2012).

Regarding its structure, the polysaccharides are present in three major forms: (i) mannans, O-linked and N-linked; (ii) β - glucans, namely β - 1, 3 glucan and β - 1, 6 glucan; (iii) chitin. Their arrangement in the cell wall is represented in figure 5 (Gow & Hube, 2012).

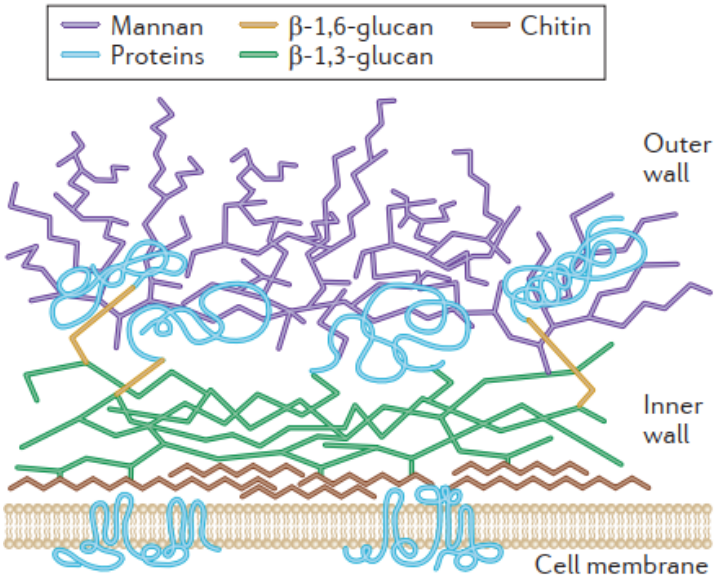

Figure 5. Arrangement of the major components of *C. albicans* cell wall. The inner layer relies on chitin and β - 1, 3 glucan to grant strength and shape. B - 1, 6 glucan acts as a flexible linker to the outer layer, which is mainly composed of O- and N-linked mannans, with other proteins covalently linked. CWSPs, predominantly linked to β- 1, 6 glucan via glycosylphosphatidylinositol (GPI) remnants enrich the outer wall (from Gow & Hube, 2012).

Regarding the inner layer, β – 1,3 glucan is the major polysaccharide and the one responsible for providing a structural backbone essential in the organization of the wall and on creating a stable network around it. Linked to β – 1,3 glucan is the linear polysaccharide chitin whose arrangement contributes to form a stable scaffold for the outer layer. This gives strength and shape to the cell wall. β - 1,6 glucan, since it lacks a regular structure, acts as a flexible linker between the two layers. The outer layer is mainly formed of mannoproteins which determine the cell surface proteins, without influencing its shape (Gow & Hube, 2012; Ibe, Walker, Gow, & Munro, 2017; Ruiz-Herrera, Victoria Elorza, Valentín, & Sentandreu, 2006). Other CWSPs enrich the outer wall and are covalently attached to the skeleton. The most abundant class of CWSPs is linked to β - 1, 6 glucan via glycosylphosphatidylinositol (GPI) remnants. The second class of proteins is linked through alkali-sensitive linkages to β - 1, 3 glucan and are named Pir - proteins with internal repeats (Netea *et al.*, 2008).

CWSPs are overexpressed in human disseminated candidiasis, and as they are very conservative and exposed on the surface of the yeast cells, they become important targets for the host immune response (Xin, Dziadek, Bundle, & Cutler, 2008).

1.3.4. Immune response to *C. albicans* infection

There is a range of host defense mechanisms against fungi that consist of the innate immunity and the adaptative immunity.

Skin and the mucous membranes of the gastrointestinal, genitor-urinary and respiratory tracts are the first line of defense, of the innate immunity. These physical barriers have antimicrobial substances on their surface as well as a microflora of saprophytic microorganisms that block the colonization of pathogenic microorganisms. They provide a rapid recognition of these agents and give non-specific responses (Blanco & Garcia, 2008).

When the physical barriers are passed, fungi have to face cellular barriers. The tasks of recognizing an invading pathogen and activating the host response are achieved through PRRs, that identify certain repeating motifs, pathogen-associated molecular patterns (PAMPs), that can be either cell wall constituents or intracellular components. This enables the control of the infections and contributes to the adaptative immune response, which is antigen-specific and leads to the establishment of immunological memory (Abdul Ghaffar, Kumar Giddam, Zaman, Skwarczynski, & Toth, 2014; Pashine, Valiante, & Ulmer, 2005).

TLRs were the first PRRs discovered to recognize *C. albicans*. They are characterized by an extracellular domain of N-terminal leucine-reach repeats, a transmembrane region and an intracellular Toll/IL- 1 receptor homology (TIR) domain, crucial for signaling. TLRs are expressed both on the cellular surface and in intracellular compartments, so they are able to recognize PAMPs in different cellular locations. As a result, several signaling pathways are activated, resulting in the induction of the activation of transcription factors and the development of an immune response (Abbas *et al.*, 2014; Netea *et al.*, 2008; Stappers, & Brown, 2017; Takeuchi, & Akira, 2010). C-type lectin receptors (CLRs) are the second major PRR family consisting mainly of membrane-bound receptors, comprising a carbohydrate-binding domain, that directly recognize mannans and mannoproteins from the outer layer of *C. albicans* cell wall. Members of Dectin-1 and Dectin-2 family of CLRs are crucial for this recognition since Dectin-1 binds to β - glucan and Dectin-2 recognizes high-mannose oligosaccharides on the hyphal form of *C. albicans.* Dectin-3, mincle, dendritic cell-specific ICAM3 grabbing non-integrin (DC-SIGN), galactin-3, langerin and collectins, among others, are also involved in the recognition of *C. albicans* (Cheng, Joosten, Kullberg, & Netea, 2012; Netea *et al.*, 2008; Stappers, & Brown, 2017). There are also NLRs which are intracellular receptors. The well-characterized inflammasome NLRP3 has been linked with the filamentous state of *C. albicans* (Plato, Hardison, & Brown, 2015).

Circulating monocytes and neutrophils, along with macrophages, are the main cell populations taking part in *C. albicans* recognition. Monocytes express elevated levels of Toll-like receptors on their membranes, and during differentiation into macrophages, they retain the expression of TLRs. Neutrophils show moderate expression of these receptors but strongly express complement receptor 3 (CR3) and Fcγ receptors (FcγRs). DCs are crucial for the antigen processing and presentation, and also express PRRs important for fungal recognition. T- cells have a much lower expression of PRRs, but their activation is essential to develop specific and even protective immunity against *C. albicans* (Netea *et al.*, 2008).

Th1-mediated response, through the production of IFN-γ, has an important role at the activation of phagocytes at the infection site, leading to a regulation of the fungal burden (Romani, 2008; Romani 2011). Associated with the development of a Th2 response, IL-4 emerges as a cytokine related with the promotion of alternative fungal pathways of macrophage activation, and may be required in a late stage of infection rather than in an early one, to counteract the inflammation (Antachopoulos & Roilides, 2005; Mencacci *et al.*, 1998; Romani, 2008; Romani, 2011). The balance of Th1 and Th2 subsets is associated with the outcome of the fungal infection (Mencacci *et al.*, 1998). Treg cells have also an important role. As a consequence of the infection, IL-10 acts in order to keep the inflammation under control, limiting tissue damage and providing a homeostatic environment (Antachopoulos & Roilides, 2005; Romani, 2008; Romani, 2011). Th17 responses, characterized by IL-17 production, arise to end the dichotomy between Th1/Th2 responses, proving that there is more flexibility in T-cells development. Therefore, IL-17, is associated with neutrophils mobilization and antimicrobial peptides, such as defensins, production, which also contribute to the control of the infection, limiting fungal overgrowth (Figure 6) (Conti & Gaffen, 2015; Romani, 2011). Depending on the site of infection, there is a complex relationship between Th17 and Treg responses. Regarding gastrointestinal candidiasis, Treg suppress Th17 responses, diminishing *C. albicans* colonization, which offers protection. While inflammatory Th17/Treg responses are protective at mucosal surfaces, in systemic candidiasis their involvement is not that clear, so it is imperative to have a time-dependent and coordinated Th17/Treg response to get positive results (Pachl *et al.*, 2006). Therefore, the impact of both of these types of responses on disease outcome highlights the importance of understanding their balance (Sampaio, & Pais, 2017; Whibley, & Gaffen, 2014).

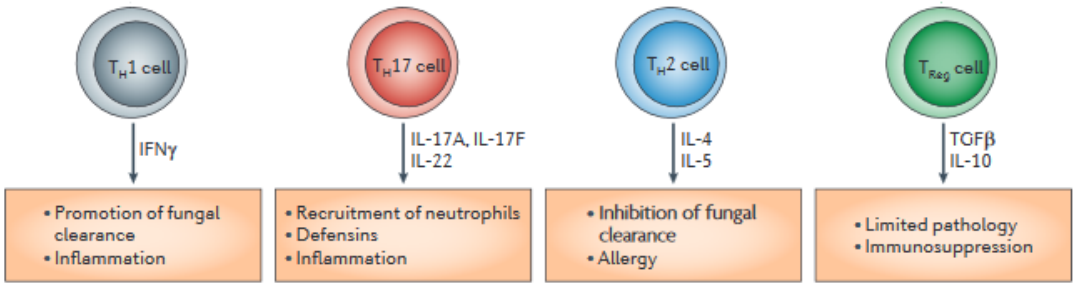

Figure 6 . Overview of T-cell subsets' roles in fungal infections. Th1 cells, mainly through the production of IFN- γ, leads to macrophage activation and stimulation of the antifungal activity of neutrophils against *C. albicans*. Despite their central role in protection, Th17 cells emerge as having a supporting and also a protective role, through mobilization of neutrophils as well as promoting the production of defensins, which lead to the control of the infection. On the other hand, Th2 cells diminish the protective response provided by Th1 cells, by suppressing phagocytic activities. Therefore, a balance is needed between the two types of response for an overall protection. The role of Treg cells is related with the supression of the inflammatory response, coordinating the balance between the other T-cells subsets, while limiting their collateral damage to the host tissues (from Romani, 2011).

Although B-cells contribution to antifungal immunity seems to be smaller when comparing to the other mechanisms, they have also been suggested to have an impact on host defense. Early in life, anti-*Candida* antibodies may be acquired, due to the fact that they are commensal organisms. However, there is little information regarding whether these antibodies are involved in protection or exacerbation of the disease (Cutler, Deepe Jr, & Klein, 2007; Netea, Joosten, Van Der Meer, Kullberg, & Van De Veerdonk, 2015; Sampaio, & Pais, 2017). Nevertheless, the role of antibody-mediated immunity in mediating protection against fungi has been gaining more evidences. Nishiya and co-workers (2016), for instance, evaluated the influence of IgG subclasses of an anti-*Candida* antibody on antibody-mediated resistance to hematogenously disseminated candidiasis in mice and reported that, although some subclasses were more effective than others, all of them conferred protection. Therefore, eliciting protective antibodies through vaccination has been proposed as a new therapeutic approach to improve the resistance to infection (Netea *et al.*, 2015, Sampaio, & Pais, 2017).

Understanding the interactions that exist between the host recognition systems, the immune system and the invading microbial pathogens is essential to develop strategies to fight fungal infections, namely the ones provoked by *C. albicans*.

1.4. Antifungal strategies: liposomal vaccine delivery systems

There are several antifungal agents available for treatment against *C. albicans*. Azoles, the largest class of antifungal drugs, are inhibitors of the lanosterol 14-α-demethylase, an enzyme responsible for the biosynthesis of ergosterol, leading to fungal cell membrane disruption. Echinocandins, on the other

hand, inhibit β - glucan synthesis, by noncompetitive blockage of the enzyme (1,3)-β-D-glucan synthase. This makes the structure of the cell wall compromised and, therefore, more vulnerable to cell lysis. There are also the polyenes, which bind to ergosterol and lead to its disruption. This causes aqueous pores and alters the cell's permeability, which can cause the loss of molecules such as potassium and eventually leads to cell death. Nucleoside analogues inhibit the synthesis of nucleic acids. Flucocytosine, being a pyrimidine analogue, restrain the thymidylate synthase (responsible for the conversion of dUMP in dTMP, leading to an accumulation of the first one. Allylamines, thiocarbonates and griseofulvin are some of others classes of antifungal agents (Spampinato, & Leonardi, 2013). However, there are concerns related to antifungal resistance to these antifungal agents, which in combination in factors such as toxicity, demands the development of novel treatments for *Candida* infections.

Nowadays, vaccines have a significant impact on global health and are routinely used to provide acquired immunity to certain microbial pathogens and to control a variety of infectious diseases. The agent it typically contains is often a surrogate of the disease-causing organism, in their weakened or inactivated forms, its toxins or one of its subunits. However, safety issues arise since reversion of the phenotype or residual virulence can occur (Carneiro *et al.*, 2016). There is also the possibility of allergic and autoimmune reactions, manufacturing drawbacks, and instability (Abdul Ghaffar *et al.*, 2014). A viable way to overcome this problem is the use of subunit vaccines. They are based on recombinant-produced, highly purified pathogen-specific proteins and synthetic peptides, or the injection of plasmids encoding DNA sequences for the microbial antigens that can generate immunity since there is a subsequent production of antigens *in vivo*. Despite having significant advantages, these approaches hold some difficulties because the immunogenicity is insufficient in the absence of an adjuvant, leading to a decrease in the capacity of triggering a long-term immunological memory. Therefore, to guarantee efficient antigen delivery and induction of specific immune responses, it is important to select the appropriate adjuvant or delivery system (Christensen *et* al., 2007). One of the primary roles of a coadjuvant is to recruit and present the antigens to APCs, inducing their activation and their ability to stimulate, enlarge and drive the effector T and B cells' differentiation. The main goal is to promote specific immune responses while eliciting minimal toxicity. Concerning this, liposomes have been used as delivery systems for vaccine antigens in the pharmaceutical industry, reducing degradation and avoiding the clearance of the antigen. (Christensen *et* al., 2007).

1.4.1. Liposomal adjuvants

Liposomes were firstly described by Alec Bangham and associates, and consist on lipid-bilayer membranes which enclose aqueous compartments. Lipids include a nonpolar region, typically comprising one or more fatty acids chains or cholesterol, and a polar region. This polar head comprehends a phosphate group and /or tertiary or quaternary ammonium salts and can have a negative, neutral or positive surface charge (Christensen *et* al., 2007; Henriksen-Lacey, Korsholm, Andersen, Perrie & Christensen, 2011).

Liposomes have a wide range of characteristics that are important to control depending on the application we desire and, consequently, the performance we expect. Regarding the size and the number of bilayers, by the simplest methods of liposomes preparation, we are able to obtain multivesicular vesicles (MVVs), which consist of several vesicles inside a larger one, and multilamellar vesicles (MLVs). MLVs are characterized by various lipidic bilayers that create a multilamellar structure of concentric phospholipid spheres, resembling and onion-like arrangement. By other production methods or even further processing of these vesicles, small unilamellar vesicles (SUVs, d ≤ 200 nm) and large unilamellar vesicles (LUVs, d ≥ 200nm) can be obtained (Figure 7A) (Akbarzadeh *et al.*, 2013; Elizondo *et al.*, 2011; Fielding, 1991). These kinds of arrangements allow the entrapment of different compounds, as shown in the Figure 7B.

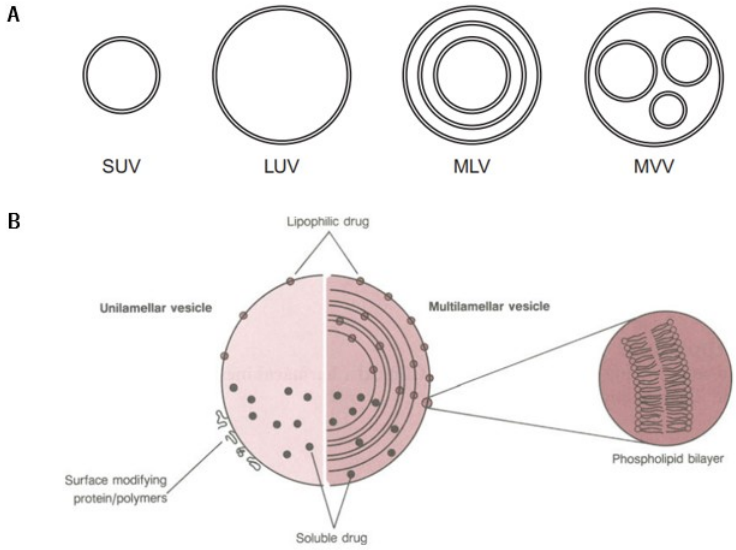

Figure 7. A. Types of vesicles according to lamellarity: small unilamellar vesicles (SUVs); large unilamellar vesicles (LUVs); multilamellar vesicles (MLVs); multivesicular vesicles (MVVs) (from Elizondo *et al.*, 2011). **B.** Overview of a liposomal structure. This kind of arrangement allows the entrapment of both soluble (in the aqueous core) and lipophilic (between bilayers) compounds (from Fielding, 1991).

The surface charge has also a key role in controlling the adjuvant effect of the liposome, being the cationic liposomes the most successful on enhancing the uptake and the effective presentation of antigens by APCs (Christensen *et al.*, 2007). A possible explanation for this is that the positively charged liposome interacts with the negatively charged cell membranes of APCs, thus being more readily uptaken (Abdul Ghaffar *et al.*, 2014).

Within cationic liposomes, those composed by surfactant dioctadecyldimethylammonium bromide (DODAB) have been used as carriers in drug delivery studies, and also as adjuvants in vaccination strategies. Their main advantage is that they require a lower lipid concentration when compared to the levels commonly used in liposomal formulations. Since these preparations can be physically unstable and present some toxicity, neutral molecules can be incorporated to improve the stability without sabotaging their adjuvanticity. Monoolein (MO), when incorporated with DODAB, could act as a stabilizer, because its characteristics decrease the structural rigidity of DODAB, and so increase the lateral mobility of the lipid chain. Therefore, the fusion of the liposomes with cell membranes is improved (Carneiro *et al.*, 2015; Carneiro *et al.*, 2016).

1.4.2. Concerns related to the development of fungal vaccines

The development of new antifungal vaccines has raised as an important alternative to protect patients from fungal infections. However, some points must be taken into consideration, that illustrates concerns regarding *Candida* infections and that will be briefly described. A more detailed explanation can be found in chapter 10 from the book "Immunogenetics of Fungal Diseases" (Sampaio, & Pais, 2017).

One first concern is the fact that the majority of studies is performed in animal models and thus, some limitations may arise. One of them is that mice might not have a *C. albicans* GI flora, and serum natural antibodies against *Candida* as humans do. Thus, vaccines that are very efficient in the animal model could be less efficient on humans. For that reason, the effectiveness of vaccines should be tested in different animal species.

The mechanisms of the *C. albicans* - host interaction in mucosal and systemic infections must be fully understood to select the constituents of the fungus that elicit the appropriate immune response. It is important to understand the balance between protective and pathogenic immunity, crucial in determining the disease outcome, but also the differences between mucosal and systemic infections. One clear example involves Th17 cells and Treg cells, explored in 1.3.4.

Another concern is the choice of the proper adjuvant since a local immunocompetent environment has to be created. This implicates an increased knowledge on their mechanism of action and a selection based on the type of immune response that is needed to be activated. Varying the adjuvant

without considering its mechanism of action may not produce the desired results, and so more studies are required in this field to help in a rational design of vaccines and to inform on adjuvant safety, especially for human use.

Development of autoimmunity is another significant concern. It is important to keep the balance between the immune system in eliminating invading pathogens while maintaining self-tolerance to avoid autoimmunity. Vaccination against *C. albicans* can induce an autoimmune dysfunction by enhancing a chronic inflammation of the mucosa or by developing an adaptative immune response against an epitope similar to a human molecule. That's why the selection of target molecules and epitope mapping analysis should always be taken into consideration. Therefore, the hypothesis of cross-reactions responses against molecules with homology to human ones and the development of autoimmune reactions must be studied to be avoided (Sampaio, & Pais, 2017).

1.4.3. CWSPs as candidates for vaccine development

Because of their exposed location, the proteins present in the fungal wall become good candidates for vaccine development. Additionally, the combination of antigens related to *C. albicans* virulence or biological functions can induce stronger immune responses and so reduce the probability of fungal immune evasion. In this context, Carneiro and co-workers (2015) evaluated the ability of DODAB:MO liposomes to act as protein delivery systems and immunoadjuvants, concluding that those formulations were stable, non-cytotoxic and avidly taken up by macrophages. Their adjuvant activity was also good, inducing strong antibody responses and cell-mediated immunity. The next step was to explore these DODAB:MO-based liposomes loaded with *C. albicans*, aiming the development of an immune protective strategy against *Candida* infections.

Therefore, in 2016, Carneiro *et al.* evaluated the potential of a liposomal antigen delivery system (ADS), composed by DODAB:MO and containing *C. albicans*' CWSPs, in mediating protection against systemic candidiasis. The two formulations, designated ADS1 and ADS2, differed only in the total lipid composition, with ADS1 having seven times more than ADS2. Consequently, ADS1 retained more of the protein added. This formulation was also able to stimulate the maturation of DCs, which are important presenting cells. The stimulation of the expression of the markers related to this maturation was greater than the one achieved with the ADS2 or free CWSPs. They successfully demonstrated ADS1's efficiency in modulating the immune response against the intravenous *C. albicans*, favoring mouse protection and thus revealing a good potential for future vaccine design. Although these results in are promising and reveal good protection, there is still a long way before a vaccine can be routinely established, with some crucial points that need to be solved

CHAPTER 2

Objectives

2. Objectives

The misuse of broad-spectrum antibiotics has been associated with a dramatic change in the composition of the gut microbiome, favoring the outgrowth of fungi. *Candida* species has been identified as one of the most abundant species in the gut after antibiotic treatment, enhancing the probability of a systemic infection in in risk patients under this treatment. Previously, in our group, cationic liposomes prepared with DODAB:MO (1:2) and loaded with *C. albicans* cell wall surface proteins (CWSPs) were able to induce protection against a lethal systemic infection with *C. albicans*.

Therefore, we specifically aimed to:

(I) evaluate the protective effect of immunization against systemic candidiasis through the immunization protocol described by Carneiro *et al*. (2016), while treating the mice with a cocktail of antibiotics containing colistin, streptomycin and ampicillin.

(II) analyze the implications of the liposome preparation method and the use of a single antigen at the immune response level, to develop of a more reproducible and also stable formulation

The work presented was developed as a collaborative project between the Centre of Molecular and Environmental Biology (CBMA) and the Life and Health Sciences Research Institute (ICVS) of the University of Minho, Portugal.

CHAPTER 3

Evaluation of the effect of antibiotics' administration on vaccine efficacy

3. Evaluation of the effect of antibiotics' administration on vaccine efficacy

3.1. Background

Antibiotics have become an important weapon against bacterial assaults and most infectious diseases. However, their misuse has been associated with a dramatic change in the composition of the gut microbiome, favoring the outgrowth of fungi. *Candida* species has been identified as one of the most abundant species in the gut after antibiotic treatment enhancing the probability of a systemic infection.

The most viable way to solve the problems related to the antifungal agents available appears to be the use of subunit vaccines, to promote specific immune responses while eliciting minimal toxicity. Carneiro and co-workers (2015) developed and explored a DODAB:MO lipid-based delivery system loaded with *C. albicans* CWSPs, determining its immunogenicity and, in 2016, assessing its effectiveness in inducing protection.

Therefore, we aimed to evaluate the protective effect of immunization against systemic candidiasis through the immunization protocol described by Carneiro *et al.* (2016), while treating the mice with a cocktail of antibiotics containing colistin, streptomycin and ampicillin.

3.2. Materials and Methods

3.2.1. *Candida albicans* and culture conditions

C.albicans strain SC5314 was used for cell wall surface proteins extraction, while *C. albicans* 124A clinical isolate was used for infection experiments. Both strains were maintained as frozen stocks in 30% glycerol at - 80°C. When needed, yeasts were obtained from a 2-day YPD agar plate (2% glucose, 1% yeast extract, 1% bacto-peptone, 2% agar) incubated at 30°C.

3.2.2. Extraction of yeast cell wall surface proteins and evaluation of membrane integrity

All procedures for CWSPs extraction were performed in a sterile environment and using apyrogenic solutions. CWSPs were obtained from whole intact cells by dithiothreitol (DTT) treatment, as previously described (Insenser *et al.*, 2010). Briefly, *C. albicans* strain SC5314 was inoculated in YPD medium and inoculated overnight at 26°C, 160 rpm, until an optical density of 0.05. The yeast cells were then grown in the same conditions for 17 h. Cells were harvested by centrifugation at 8000 g, 5 min, 4°C and washed twice with 50 mM Tris-HCl buffer pH 7,5. Afterwards, they were resuspended in the same buffer supplemented with 2 mM DTT, incubated at 4°C, 2 h, 150 rpm and centrifuged at 6000 g, 5 min.

To ensure that this method did not disrupted the cell membrane, the yeast pellet obtained was used to assess membrane integrity through cells' incubation with propidium iodide (PI) and analysis by flow cytometry. An aliquot of the cell suspension before treatment was stained for negative control, and boiled cells were used as positive control, since the plasma membrane was completely compromised by boiling. Cells with no staining were used to determine autofluorescence. The yeast CWSPs in the supernatant were concentrated with a 3 kDa Protein Concentrator ultrafiltration centrifugal device supplied by Thermo Scientific at 3760 g, 4°C, washed three times with 50 mM HEPES-buffer solution pH 7,5 and quantified using the Bradford assay. The concentrated proteins were stored at - 80°C in aliquots of 100 μg/mL.

3.2.3. Preparation and characterization of CWSP – loaded liposomes

DODAB:MO based liposomes were prepared through hydration of the thin lipid film and subsequent incubation with CWSPs. Briefly, defined volumes of DODAB (Tokyo Kasei – Japan) and MO (Santa Cruz Biotechnology) stock solutions (prepared at a concentration of 20 mM and using ethanol as a solvent) were mixed in a round-bottom flask. The solvent was removed in a rotary evaporator at 60°C and the lipid film was hydrated with ultrapure water at the same temperature, and vortexed. The created suspension of MLVs of DODAB:MO (1:2) rested for 20 minutes after which CWSPs concentrated at 100 μg/mL were added in equal volumes. The formulation was incubated for 45 minutes at 60°C and then 1 h at RT. For the formulation used, the final concentration of CWSPs was 50 μg/mL and the final total lipid concentration was 888 μg/mL.

Mean size, polydispersity index (PDI) and superficial charge density (ζ-potential) of the liposomes were measured in a Malvern Zetasizer Nano ZS (Malvern, UK) particle analyzer at 25 °C.

3.2.4. Protein encapsulation efficiency

The prepared liposomal nanoparticles (LNPs) were pelleted by ultracentrifugation (100,000 g for 1 h), and the proteins present both, in the pellet and in the supernatant, were submitted to TCA Precipitation Procedure for Interfering Substances Elimination, according to the Pierce™ BCA Protein Assay Kit (Thermo Scientific) manufacturer's instructions.

The Encapsulation Efficiency (%EE) was calculated according to the following formula (3.1):

(3.1)

$$\%\mathrm{EE} = \frac{(\text{Total amount of protein}) - (\text{Free Protein})}{(\text{Total Amount of Protein})} \times 100$$

3.2.5. Mouse Husbandry

Eighty female BALB/c mice, 8–10 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific-pathogen-free conditions at the Life and Health Sciences Research Institute (ICVS), Braga, Portugal. All procedures with live animals were carried out in accordance with the European Directive 86/609/EEC, and were approved by the *Subcomissão de Ética para as Ciências da Vida e da Saúde* (SECVS 074/2016) and the Portuguese National Authority *Direcção Geral de Veterinária*. Mice were divided into four groups, with twenty mice each:

- Control (immunized with 25 mM HEPES-buffer pH 7.5)
- Vac (immunized with DODAB:MO (1:2) liposomes with CWSPs (50:888 μg/mL; CWSP: Lipid))
- Abx (immunized with 25 mM HEPES-buffer pH 7.5 and treated with antibiotics)
- Abx + Vac (immunized with DODAB:MO (1:2) liposomes with CWSPs (50:888 μg/mL; CWSP: Lipid) and treated with antibiotics)

3.2.6. Treatment with antibiotics

Two of the four mice groups were treated with 0,5 mg/mL of colistin, 5 mg/mL of streptomycin and 1 mg/mL of ampicillin (all from Sigma) administered in drinking water. The treatment started 4 days before the first immunization. Colistin was supplied during two weeks, streptomycin was provided until one week before the infection, and ampicillin was given until the day of *C. albicans* administration. The schematic representation of the antibiotic treatment can be found in Annex 1.

3.2.6.1. Minimum inhibitory concentration (MIC) determination

MIC determination was performed as a way to evaluate *C. albicans* susceptibility to the antibiotics used in this work. These assays were performed according to "EUCAST antifungal MIC method for yeasts". Briefly**,** stock solutions of each one of the antibiotics were prepared in water at a concentration 100x higher than the concentration to be tested, and then diluted to a final concentration of 2x in RPMI 1640 supplemented with glucose to a final concentration of 2%, to obtain the working solutions. From there, two-fold dilutions were performed, ranging from 0.5 mg/mL to 0.008 mg/mL in ampicillin, 2.5 mg/mL to 0.039 mg/mL in streptomycin and 0.5 mg/mL to 0.004 mg/mL in colistin, and then plated in flat-bottom plates. The combination of the three antibiotics was also evaluated, and so 3×10^5 cells/mL of the *C. albicans* strain 124A were added to the wells. The plates were incubated at 37°C for 24 h, and the absorbance was read at 530 nm.

3.2.7. Immunization procedures

Twenty BALB/c mice per group, total of four groups, were injected subcutaneously thrice with a 2-week interval, with 200 µL of DODAB:MO (1:2) liposomes with CWSPs (50:888 µg/mL; CWSP: Lipid) or HEPES-buffer 25 mM pH=7.5. A week after the last immunization, serum was collected to evaluate immunological parameters. The schematic representation of the immunization protocol can be found in Annex 1.

3.2.8. *C. albicans* hematogenously disseminated infections

Three weeks following the last immunization, mice were infected with 1×10^5 *C. albicans* yeast form, for the survival experiments. *C. albicans* 124A clinical isolate, used for infection, was inoculated in Winge Medium (0,2% glucose, 0,3% yeast extract) and grown in a shaking incubator for 14 h at 30°C, 200 rpm. Yeast cells were harvested, washed with apyrogenic phosphate-buffered saline (PBS), counted in a Neubauer Chamber and resuspended at the appropriate concentrations. CFU counts on YPD agar plates (37°C, 48 h) were performed to confirm inocula. Infected mice were weighted and monitored to evaluate the progress of the hematogenously disseminated infection.

3.2.9. Kidney fungal burden

Since the kidney is the principal site of infection in the disseminated infection, it was important to determine its fungal burden. To do so, four mice per group were sacrificed at days three and seven following the infection. Mice had both kidneys removed, homogenized in PBS and cultured on YPD agar at 37°C.

3.2.10. Splenocytes intracytoplasmic cytokines determination

At days 49 (before infection), 52 and 56 (three- and seven-days post-infection, respectively) four immunized mice per group were sacrificed and the spleens were aseptically removed, and homogenized in Dulbecco's Modified Eagle Medium (DMEM), by passage through a 70-µm-pore-size nylon cell strainer (BD Biosciences). Red blood cells lysed with 0.15 M ammonium chloride. The remaining cells were counted and plated in round-bottom 96-well plates in DMEM compete medium [cDMEM, DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% HEPES, 1% sodium pyruvate and 1% penicillin/streptomycin (all from Gibco)]. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C for 4 h under stimulation with 20 ng/ml PMA, 200 ng/ml ionomycin and 10 ng/ml brefeldin A (all from Sigma). Then, cells were recovered and incubated with anti-mouse

CD4 APC/Cy7 - conjugated (clone GK1.5) (BioLegend). Following extracellular staining cells were washed, fixed in 2% formaldehyde, washed again and permeabilized with 0.05% saponin in PBS solution. Intracytoplasmic staining was carried out with anti-mouse IFN-γ Pe/Cy7 – conjugated (clone: XMG1.2) (BioLegend); anti-mouse/Rat IL17A FITC - conjugated (clone eBio17B7); anti-mouse IL4 PE – conjugated (clone 11B11) and anti-mouse IL10 APC - conjugated (clone JES5-16E3) (all from eBioscience). Antibody-labeled cells were analyzed in a LSRII flow cytometer (BD Biosciences) data analyzed using FlowJo software (TreeStar).

3.2.11. "*ex vivo*" splenocytes cytokine detection

To assess *in vitro* cytokine production by CWSP-stimulated spleen cells, 5×10^5 cells/well were plated in a flat-bottom 96-well plate and stimulated with CWSPs (final concentration of 20 μg/ml) for 72 h at 37°C and 5% CO_2. The concentrations of IFN-γ, IL-4 and IL-17 in cell culture supernatants were quantified with the corresponding Mouse ELISA Ready-Set-Go kit (eBioscience, San Diego, CA) while IL-10 was quantified using the Mouse IL-10 DuoSet ELISA development system (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

3.2.12. Quantification of CWSP-specific Antibody Isotypes

Specific anti-CWSP immunoglobulins in the collected serum were quantified by an enzyme-linked immunosorbent assay (ELISA). Briefly, polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 μg/ml of CWSPs and incubated overnight at 4°C. All wells were saturated with 2% BSA in TBS-T buffer (10 mM Tris, 150 mM NaCl and 0,05% Tween 20, pH 8) for 1 h at room temperature. Meanwhile, serial dilutions of the serum samples were performed in 1% BSA in TBS-T buffer. Afterwards, they were plated and incubated for 1 h at 37°C. Following the wash, alkaline-phosphatase-coupled monoclonal goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) was added and incubated for 1 h at room temperature. The bound antibodies were detected through p-nitrophenyl phosphate (Sigma) substrate solution addition. The reaction was stopped after 30 min to 1 h by adding 0,1 M EDTA pH 8 solution. The absorbance was measured at 405 nm, subtracting for each well the value of the absorbance at 570 nm. The antibody titers were expressed as the reciprocal of the highest dilution with an absorbance 2x higher than the value of the control (no serum added).

3.2.13. Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) followed by the Turkey test to compare the mean values of different groups, using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Results shown are from three independent experiments with three replicates and differences were considered significant when the p-value was less than 0.05.

3.3. Results

3.3.1. Analysis of membrane integrity after CWSPs extraction

The first task of this work was obtaining CWSPs to incorporate in DODAB:MO liposomes, through a protocol previously described (Insenser *et al.*, 2010). To ensure that the majority of the extracted proteins were from *C. albicans* cells' surface instead of their cytoplasm, membrane integrity after CWSPs extraction using the reducing agent DTT was evaluated. Yeast cells were stained with PI, since this dye cannot cross the membrane of healthy cells, and analyzed through flow cytometry. As shown in the figure below (Figure 8), this analysis confirmed the membrane integrity of the yeast cell (> 99% PI negative), ensuring that the extraction protocol did not compromise the cell membrane and, consequently, the proteins extracted were mainly from *C. albicans* cell surface.

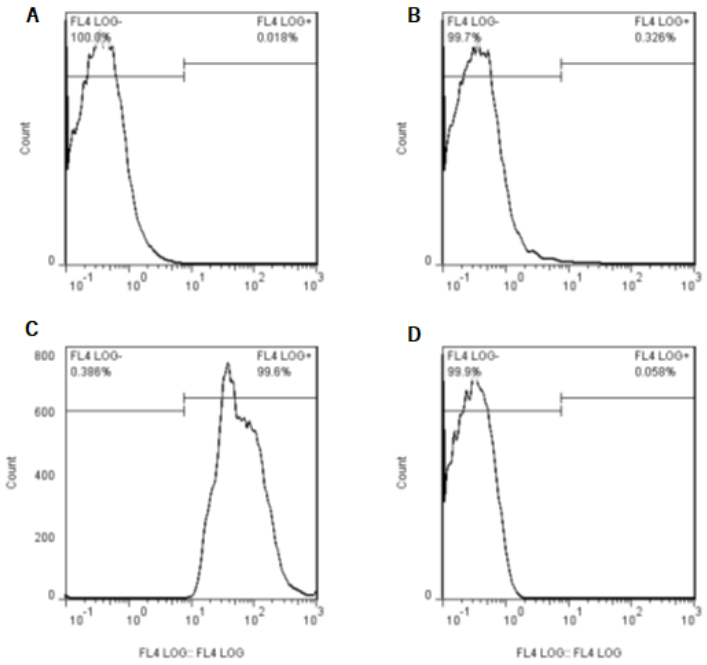

Figure 8. Evaluation of yeast membrane integrity by flow cytometry. **A.** Autofluorescence of *C. albicans* cells. **B.** Membrane integrity before treatment with DTT. **C.** Positive control with non-viable cells (membrane disrupted by heat treatment). **D.** Membrane integrity after treatment with DTT.

3.3.2. Characterization of CWSP-loaded liposomes

Following CWSPs extraction, liposomes were prepared, loaded with the proteins and characterized using a Malvern Zetasizer Nano ZS (Malvern, UK) particle analyzer. The LNPs obtained from DODAB:MO liposomes loaded with *C. albicans* CWSPs had a size around 666.23 ± 91.31 nm with a PDI of 0.36 ± 0.08 These LNPs were negatively charged, presenting a ζ-potential value of -2.30 ± 0.31 mV. This last parameter is essential on predicting the stability of our system. Since the ζ-potential is near to zero, our LNPs will most probably aggregate over time, because the surface charge is not enough to repeal the particles. In what concerns DODAB:MO empty liposomes, they exhibited a ζ-potential value of 53.55 ± 2.73 mV, being much smaller (88.40 ± 2.35 nm) and less polydisperse (0,27 ± 0,04). The %EE was also calculated as previously described, and our formulation presented an %EE of 78,46 ± 1,09% (Table 1).

Table 1. Mean size, PDI and ζ-potential of LNPs.

	Empty Liposomes	DODAB:MO (1:2) CWSP (50:888 µg/mL; CWSP: Lipid)
Mean size (nm)	88.40 ± 2.35	666.23 ± 91.31
PDI	0.27 ± 0.04	0.36 ± 0.08
ζ-potential (mV)	53.55 ± 2.73	-2.30 ± 0.31

3.3.3. Characterization of humoral and cell-mediated response before infection

Humoral and cell-mediated responses before infection were the first evaluated parameters to understand the implication of antibiotics at the immune system and, therefore, vaccine efficacy.

To evaluate the humoral response, we quantified CWSP-specific IgG1 antibody production. Immunization with HEPES-buffer (Control group) induced similar levels of IgG1 as immunization with HEPES-buffer during antibiotic treatment (Abx group). However, the Abx + Vac group was able to induce a higher IgG1 response, comparing to these groups, although not comparable with the group of mice immunized with the ADS without additional treatment (Vac group) (Figure 9A). Of note, during this work, the levels of IgG2a were also quantified, but they turned out to be absent.

Regarding the cell-mediated immune response, CWSPs re-stimulated splenocytes from all groups of mice showed low levels of IFN-γ and IL-10, with the exception of Abx group where no production of IFN-γ was detected. The levels of IL-17A were similar between groups, and only mice immunized with

DODAB:MO CWSP, without additional treatment, were able to produce higher levels of IL-4, differing significantly from the other groups (Figure 9B).

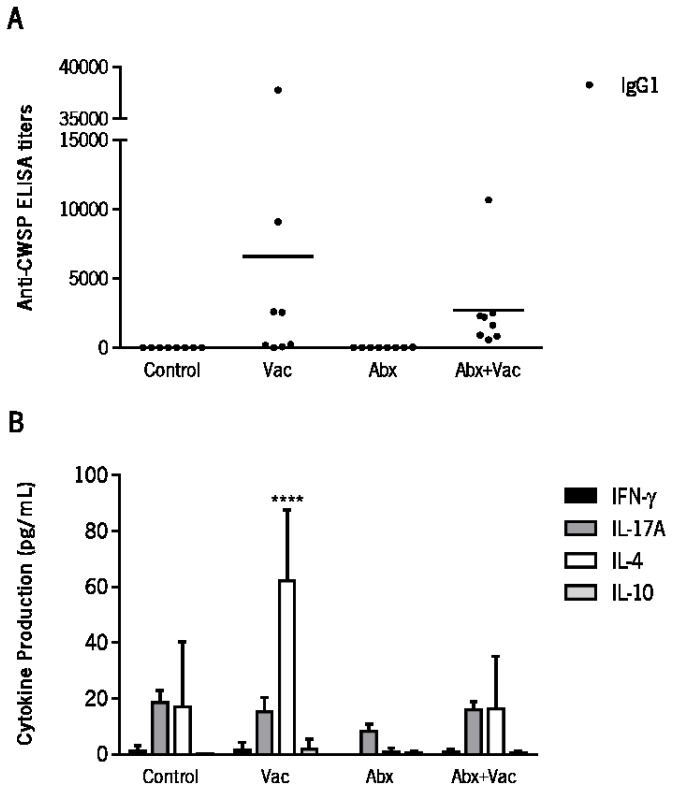

Figure 9. A. Anti-CWSP specific IgG1 response in serum of immunized mice. The antibody titers are expressed as the reciprocal higher dilution with and absorbance 2x higher than the value of control (no serum added). Each point represents an individual mouse, and horizontal lines correspond to the mean value in each group. **B.** Cytokine production from CWSPs re-stimulated splenocytes derived from immunized mice. Results indicate the mean ± SD of four mice per group. The significant difference between Vac group and all the others (Control, Abx, Abx + Vac) is indicated by (****) above the bar (****P < 0.0001).

3.3.4. Survival Curve, kidney fungal burden and MIC determination

Two weeks after the last immunization, all groups of mice were challenged with *C. albicans* cells, from 124A clinical strain, in order to evaluate the protective effect of our formulation and the role of antibiotic treatment in a systemic infection. Mice were weighted and visually monitored for signs of disease during 50 days.

The infection progressed similarly in all groups until 10 days after the challenge, when the animals started to succumb, starting with antibiotic-treated mice, followed by mice immunized with HEPES-buffer pH 7.5. At the end of the experimental period, 37,5% of the mice from Vac group survived the infection, as well as Control mice. None of the Abx mice survived. Despite this, 50% of the Abx + Vac mice were

able to endure, presenting a significant extended survival time compared to mice treated only with antibiotics (P=0.0121) (Figure 10A).

Kidney fungal burden was evaluated and only mice from the Vac group were able to reduce fungal burden from day three today seven following infection, although without statistical significance. Despite this, the fungal burden at day seven post-infection in this group was significantly lower comparing to the Control group (P=0,0053). In fact, when comparing fungal burden at day seven between groups we observe that all of them differ significantly from the control (P=0.0225 in Control vs Abx; P=0.0308 in Control vs Abx + Vac). Although there are no significant differences between mice from Vac and groups treated with antibiotics, the presence of antibiotics seems to induce a more controlled fungal burden at day three after infection (Figure 10B).

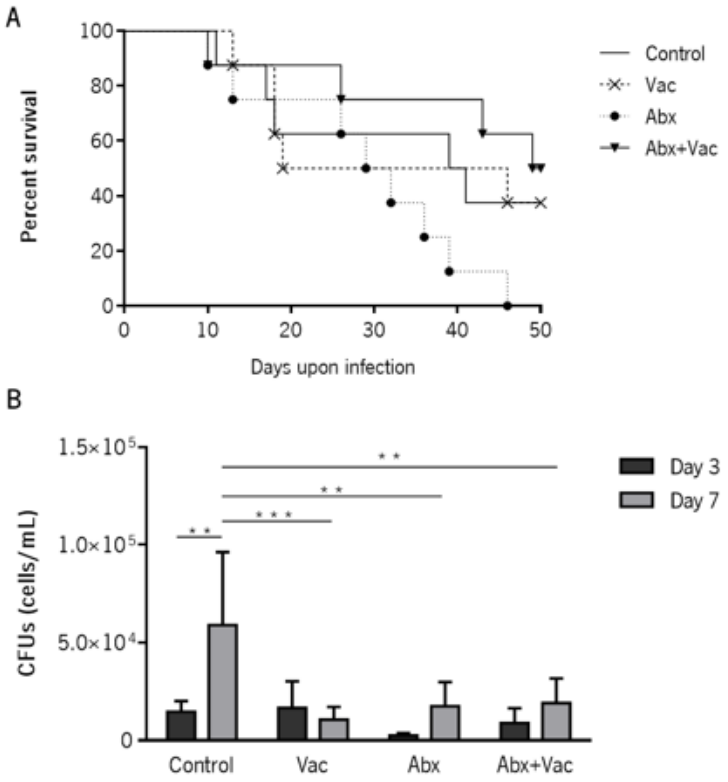

Figure 10. Mice protection after immunization protocol and antibiotic treatment. **A.** Survival rates of immunized mice. Eight mice per group were used, and were sacrificed when reaching humane endpoints. **B.** Kidney fungal burden of mice sacrificed at day 3 and day 7 following *C. albicans* infection. Four mice were used per group and data is displayed as mean ± SD. Significant differences between groups are presented by (*) above the line: (*P < 0,05, ** P < 0,01, *** P < 0,001, **** P < 0,0001).

These results suggest that the antibiotic treatment, when associated with the immunization procedure, has some kind of beneficial role regarding mice overall survival. It also seems that antibiotics might affect fungal burden.

When MIC was determined for each antibiotic, colistin was the only one able to inhibit the growth of yeasts in ≥50% comparing to the antibiotic-free control, at a concentration of 0.5 mg/mL. Neither ampicillin or streptomycin alone, at the concentrations tested, resulted in this kind of susceptibility. Interestingly, when the three antibiotics were combined at a concentration of 0.5 mg/mL of ampicillin, 2.5 mg/mL of streptomycin and 0,25 mg/mL of colistin, we also observed the same behavior obtained with colistin alone. However, it is unlikely that, in this case, colistin has contributed to the results obtained in the *in vivo* experiments, since it was only administered during the first two weeks. Besides that, colistin absorption from the GI tract is insignificant or even absent, following oral administration (Grégoire, Aranzana-Climent, Magréault, Marchand, & Couet, 2017; Guyonnet *et al.*, 2010; Rhouma *et al.*, 2015).

3.3.5. Intracellular cytokine quantification

To achieve protective immunity against *C. albicans*, a coordinate Th1 and Th17 response seems to be decisive. Therefore, the expression of IFN- γ, IL-4, IL-10 and IL-17 in splenic $CD4^+$ T cells collected from mice of the different groups was evaluated through flow cytometry, to figure out if there was a particular type of T cell polarization (Figure 11).

The frequency of splenic $CD4^+$ T cells producing IFN-γ was not majorly altered among groups upon infection. However, within groups, it decreased at day 3 post-infection, comparing to the values observed before the challenge with *C. albicans*, but at day 7, their levels increased in all groups to levels similar to those observed before infection. Having this in regard, it is unlikely that IFN-γ has determined protection.

The detected proportions of $IL\text{-}17^+CD4^+$ T splenocytes seemed to increase in all groups at day 3 post-infection. However, from day 3 to day 7, only mice treated with ADS (either with and without antibiotic treatment) showed a significant increase, and the frequency of these cells were markedly higher, comparing to the other groups. The frequency of $IL\text{-}17^+CD4^+$ T cells remained low in antibiotic treated mice, but when comparing to Abx + Vac, the latter group seems to have a markedly higher proportion at day 7.

Immunization with the ADS, with and without antibiotic treatment, also increased the proportions of splenic $IL\text{-}4^+$ $CD4^+$ T cells, compared to the Control group. However, in antibiotic-treated mice immunized with HEPES-buffer the frequencies of these cells remained similar to the ones observed in the control group. The frequency of IL-10-secreting $CD4^+$ cells was very low and there weren't significative differences between groups.

A higher % of IL-4 secreting cells than of IL-17 was observed, which suggests that a prevailing Th2 response rather than a Th17. These results are consistent with cytokine production before infection (Figure 9B), especially by the immunized group.

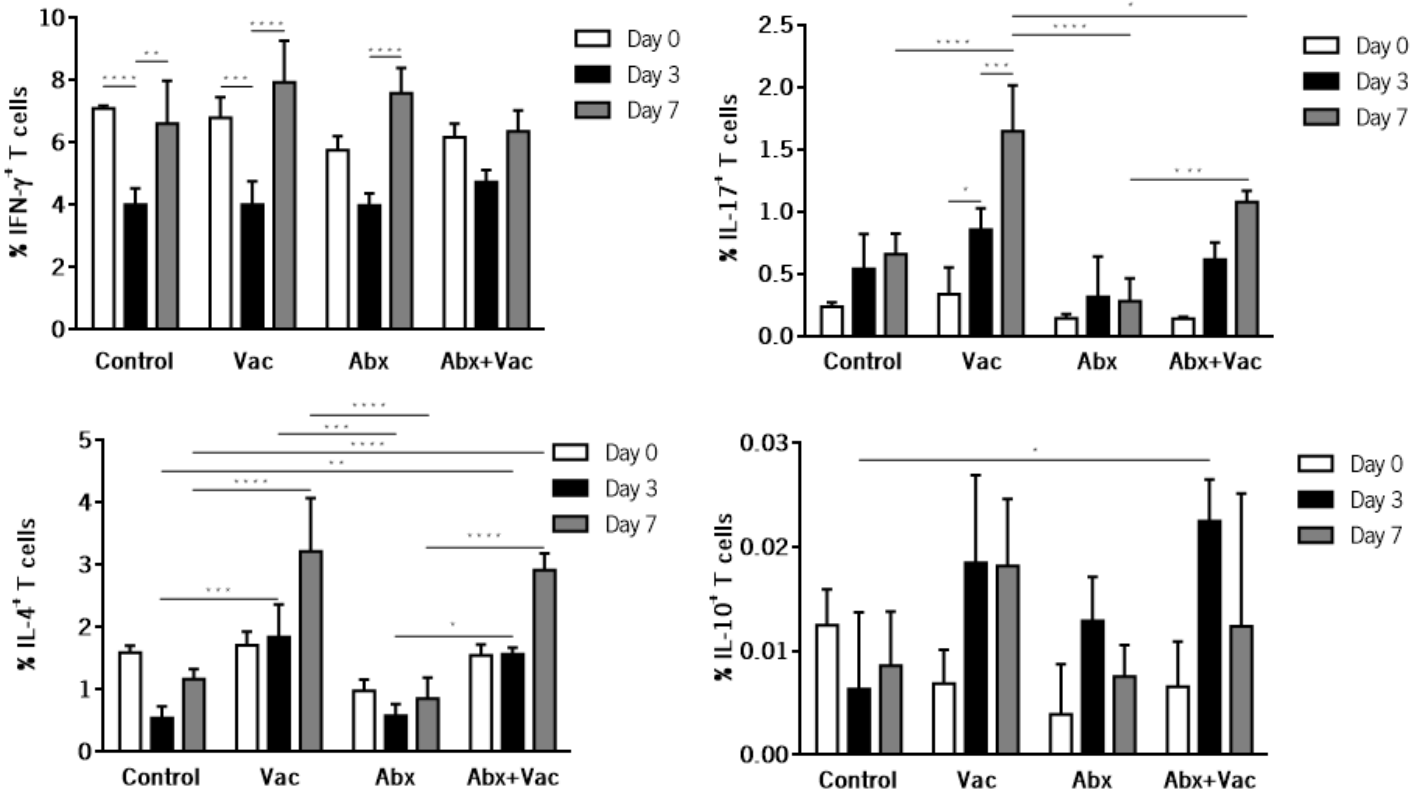

Figure 11. Frequency of IFN-γ+, IL-17+, IL-4+ and IL-10+ cells in splenic CD4+ T cells from immunized mice, isolated at days 49 (before infection), 52 and 56 (three- and seven-days post-infection, respectively). Bars represent mean ± SD of four mice used per group. Statistically significant differences between groups are presented by (*) above the line: (*P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

CHAPTER 4

Evaluation of the liposome preparation method and the use of a single antigen in vaccination strategies against systemic *C. albicans* infection

4. Evaluation of the liposome preparation method and the use of a single antigen in vaccination strategies against systemic *C. albicans* infection

4.1. Background

As shown before, Carneiro *et al.* developed and characterized DODAB:MO (1:2) liposomes, assessing their immunoadjuvant potential and their protective effect in mice challenged with a lethal dose of *C. albicans* cells, enhancing mice survival. However, characterizing and standardizing these formulations is challenging and complicated. Since we work with the crude *C. albicans* CWSP's extract, batch-to-batch variability is a major drawback, which doesn't allow a large-scale production. The liposome preparation is also a challenging step. Results observed in the previous chapter were not as expected, and the characteristics of the liposomes were quite different from the previously reported.

Cht3p emerged as a candidate for use as an antigen when immunoblotting assays performed during Carneiro's work showed that mice immunized with ADS1 produced specific antibodies against this protein. Costa-Barbosa (2017) developed efficient protocols for heterologous production and purification of Cht3p, in its glycosylated and active form, using *Pichia pastoris* as a cell factory, and taking advantage from Cht3p extracellular production in this host. By testing Cht3p with different liposomal formulations, Gouveia-Dias (2019) selected a nanoparticle formulation of 0.0056 P/L weight ratio (final lipid concentration of 888 μg/mL (2 mM)) and 5 μg/mL of Cht3p. This delivery system revealed the best characteristics, being more stable and presenting low cytotoxicity.

Therefore, the efficacy of the vaccine preparation requires the development of a more reproducible and also stable formulation, which will be further evaluated according to two variables: the liposome preparation method, namely the effect of sonication, and the use of a single antigen, Cht3p.

4.2. Materials and Methods

4.2.1. Proteins obtention

C. albicans strain SC5314 was used for CWSPs extraction. The strain was maintained as a frozen stock in 30% glycerol at -80°C and, when needed, yeasts were obtained from a 2-day YPD agar plate (2% glucose, 1% yeast extract, 1% bacto-peptone, 2% agar) incubated at 30°C. CWSPs were released from yeast cells as described in 3.2., through DTT treatment, but were washed with 25 mM HEPES-buffer solution pH 7,5, instead of 50 mM. Following concentration, the proteins were stored at -80°C in aliquots of 100 μg/mL.

Cht3p was obtained using the *P. pastoris* strain created by Costa-Barbosa (2017) and was kindly provided by him. Briefly, the protein was produced in the extracellular environment and its pure form was achieved through ammonium sulfate precipitation followed by dialysis.

4.2.2. Preparation and characterization of CWSP - and Cht3p – loaded liposomes

DODAB:MO - based liposomes were prepared through hydration of the thin lipid film and subsequent incubation with the protein, with some modifications when comparing to which is described in 3.3.

Briefly, defined volumes of DODAB and MO stock solutions (prepared at a concentration of 20 mM and using ethanol for as a solvent) were mixed in a round-bottom flask. The solvent was removed in a rotary evaporator, at 60°C and the lipid film was hydrated with 25 mM HEPES pH 7.5 at 55°C, and vortexed during 15 minutes. The created suspension of MLVs of DODAB:MO (1:2) was placed in a bath sonicator until becoming transparent and slightly bluish.

For the ADSs assembling, equal volumes of CWSPs and Cht3p were added post-lipid film hydration. The final concentration of CWSPs was 50 μg/mL while the final concentration of Cht3p was 5 μg/mL. For both, the final total lipid concentration was 888 μg/mL. The formulations were then incubated for 1 h at 55°C, followed by a brief sonication step in a bath sonicator.

Mean size, PDI and ζ-potential of the liposomes were measured in a Malvern Zetasizer Nano ZS (Malvern, UK) particle analyzer at 25 °C.

4.2.3. Protein encapsulation efficiency

The prepared LNPs were pelleted by ultracentrifugation (100,000 g for 1 hour), and the proteins present both in the pellet and in the supernatant were submitted to TCA Precipitation Procedure for Interfering Substances Elimination, according to the Pierce™ BCA Protein Assay Kit (Thermo Scientific) manufacturer's instructions.

The %EE was calculated according to the same formula indicated at 3.4.

4.2.4. Mouse Husbandry

Eighty female BALB/c mice, 8–10 weeks old, were purchased from Charles River (Barcelona, Spain) and kept under specific-pathogen-free conditions at the Life and Health Sciences Research Institute (ICVS), Braga, Portugal. All procedures with live animals were carried out in accordance with the European Directive 86/609/EEC, and were approved by the *Subcomissão de Ética para as Ciências da Vida e da*

Saúde (SECVS 074/2016) and the Portuguese National Authority *Direcção Geral de Veterinária*. Mice were divided into four groups, with twenty mice each.

4.2.5. Immunization procedures

Twenty BALB/c mice per group, total of four groups, were injected subcutaneously thrice with a 2-week interval, with 200 µL of one of the following preparations: HEPES-buffer 25 mM pH 7.5; Cht3p alone (5 µg/mL); DODAB:MO (1:2) liposomes with Cht3p (5:888 µg/mL; Cht3p:Lipid) or DODAB:MO (1:2) liposomes with CWSPs (50:888 µg/mL; CWSP:Lipid). A week after the last immunization, serum was collected to evaluate immunological parameters. The schematic representation of the immunization protocol can be found in Annex 2.

4.2.6. Splenocytes intracytoplasmic cytokines determination

Three weeks following the last immunization, four immunized mice per group were sacrificed and the spleens were aseptically removed, and homogenized in DMEM, by passage through a 70-µm-pore-size nylon cell strainer (BD Biosciences). Red blood cells lysed with 0.15 M ammonium chloride. The remaining cells were counted and plated in round-bottom 96-well plates in DMEM compete medium [cDMEM, DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% HEPES, 1% sodium pyruvate and 1% penicillin/streptomycin (all from Gibco)]. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C for 4 h under stimulation with 20 ng/ml PMA, 200 ng/ml ionomycin and 10 ng/ml brefeldin A (all from Sigma). Then, cells were recovered and incubated with anti-mouse CD4 APC/Cy7 - conjugated (clone GK1.5) (BioLegend). Following extracellular staining cells were washed, fixed in 2% formaldehyde, washed again and permeabilized with 0.05% saponin in PBS solution. Intracytoplasmic staining was carried out with anti-mouse IFN-γ Pe/Cy7 – conjugated (clone: XMG1.2) (BioLegend); anti-mouse/Rat IL17A FITC - conjugated (clone eBio17B7); anti-mouse IL4 PE – conjugated (clone 11B11) and anti-mouse IL10 APC - conjugated (clone JES5-16E3) (all from eBioscience). Antibody-labeled cells were analyzed in a LSRII flow cytometer (BD Biosciences) data analyzed using FlowJo software (TreeStar).

4.2.7. "*ex vivo*" splenocytes cytokine detection

To assess *in vitro* cytokine production by CWSP- and Cht3p-stimulated spleen cells, 5×10^5 cells/well were plated in a flat-bottom 96-well plate and stimulated with CWSPs (final concentration of 20 µg/mL) or Cht3p (final concentration of 2 µg/mL) for 72h at 37°C and 5% CO_2. The concentrations of IFN- γ, IL-4 and IL-17 in cell culture supernatants were quantified with the corresponding Mouse ELISA

Ready-Set-Go kit (eBioscience, San Diego, CA) while IL-10 was quantified using the Mouse IL-10 DuoSet ELISA development system (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

4.2.8. Quantification of CWSP-specific Antibody Isotypes

Specific anti-CWSP and anti-Cht3p immunoglobulins in the collected serum were quantified by an enzyme-linked immunosorbent assay (ELISA). Briefly, polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated with 5 μg/mL of CWSPs or 5 μg/mL Cht3p and incubated overnight at 4°C. All wells were saturated with 2% BSA in TBS-T buffer (10 mM Tris, 150 mM NaCl and 0,05% Tween 20, pH 8) for 1 h at room temperature. Meanwhile, serial dilutions of the serum samples were performed in 1% BSA in TBS-T buffer. Afterwards, they were plated and incubated for 1 h at 37°C. Following the wash, alkaline-phosphatase-coupled monoclonal goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) was added and incubated for 1 h at room temperature. The bound antibodies were detected through p-nitrophenyl phosphate (Sigma) substrate solution addition. The reaction was stopped after 30 min to 1 h by adding 0,1 M EDTA pH 8 solution. The absorbance was measured at 405 nm, subtracting for each well the value of the absorbance at 570 nm. The antibody titers were expressed as the reciprocal of the highest dilution with an absorbance 2x higher than the value of the control (no serum added).

4.2.9. Western Blot

Extracted CWSPs and the Cht3p, were subjected to 12% SDS-polyacrylamide gel electrophoresis at 20 mA and transferred to Hybond-P Polyvinylidene Difluoride (PVDF) Membrane (Hybond-ECL, GE Healthcare) at 60 mA for 1 h and half. The membranes were blocked with 3% BSA in TBS (w/v) containing 0.05% Tween (v/v) for 3 h at room temperature. After washed, membranes were incubated overnight at 4°C with a pool of antisera (diluted 1:250) from immunized mice. To detect bound antibodies, membranes were incubated with Peroxidase-AffiniPure goat anti-mouse IgG (diluted 1:5000; Jackson ImmunoResearch), for 1h at room temperature, and washed during 15 minutes (4x), with TBS-T. Chemiluminescence detection was performed using the Immobilon ECL detection system (Millipore-Merck) and a Chemi-Doc XRS system (BioRad) or X-ray ortho CP-G films in a X-ray film processor (Curix 60, Agfa Healthcare).

4.2.10. Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) followed by the Turkey test to compare the mean values of different groups, using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Results shown are from three independent experiments with three replicates and differences were considered significant when the p-value was less than 0.05.

4.3. Results

4.3.1. Liposome preparation method: the effect of sonication

The thin-film hydration method for vesicle formation is one of the most common and used techniques. Despite this, there is no control over the vesicle formation, resulting in heterogenous populations that can have associated low encapsulation efficiencies. One way to homogenize the MLVs suspensions obtained is through sonication. The lipid bilayers of the larger vesicles are disrupted, leading to a more homogenous population, resulting in small unilamellar vesicles (SUVs). There are, however, some concerns related to this technique, such as oxidation and degradation of lipids.

As we can observe in table 2, the sonication improved, indeed, the liposomes' characteristics. The liposomes obtained were smaller (249.69 ± 29.34 nm), as a part of a fairly monodisperse suspension (0.20 ± 0.06). The ζ- potential, although more negative (-8.53 ± 0.66 mV), was still not enough to provide stability over time. These results were more consistent with the ones reported by Carneiro *et al.* (2016), which had a mean size of 223 ± 37 nm, a PDI of 0.19 ± 0.015, as well as a ζ- potential around -18.3 ± 1.5.

The % EE obtained was very similar between the experiments. As stated in 4.2., % EE of the liposomes from the first experiment was 78.46 ± 1.09 % and in this second analysis was 71.48 ± 3.35 %.

Table 2. Comparison of mean size, PDI and ζ- potential of DODAB:MO CWSP liposomes between experiments and Carneiro *et al.* (2016).

	1st experiment	2nd experiment	Carneiro *et al.* (2016)
Mean Size (nm)	666.23 ± 91.31	249.69 ± 29.34	223 ± 37
PDI	0.36 ± 0.08	0.20 ± 0.06	0.19 ± 0.015
ζ- potential (mV)	-2.30 ± 0.31	-8.53 ± 0.66	-18.3 ± 1.5

In what concerns results from *in vivo* immunogenic studies the method of liposome preparation has influence, as well. When cytokine production was analyzed, the liposomes from the second experiment expressed high levels of IFN-γ comparing to the control and to the liposomes from the first experiment, suggesting a more inflammatory response. The same happens with IL-17A, although the differences are not statistically significative. In addition, the shift to a Th2 response observed with liposomes from the first experiment seems to be neutralized, since the prevailing cytokines in the second experiment are shift to Th1/Th17 response (Figure 12).

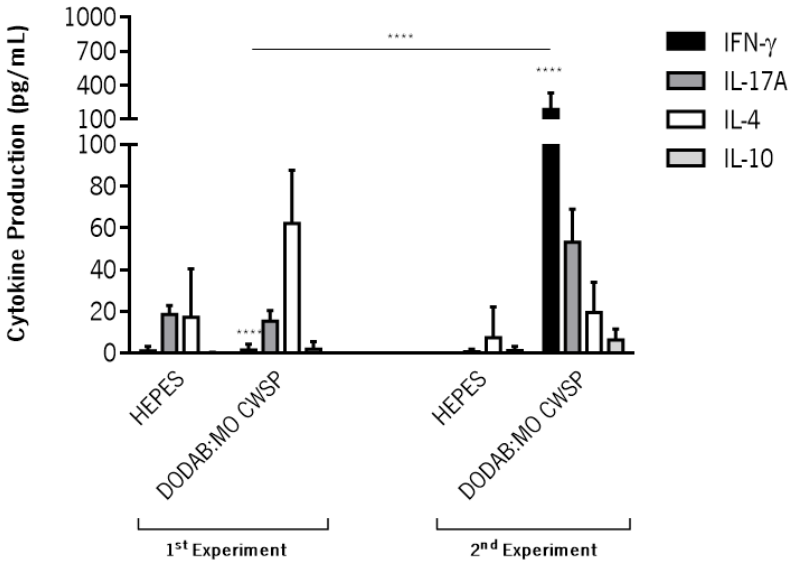

Figure 12. Cytokine production from CWSP re-stimulated splenocytes derived from immunized mice, from both experiments. Results indicate the mean ± SD of four mice per group. The significant differences between DODAB:MO CWSP and control group (HEPES) are indicated by (****) above the bar (****P < 0.0001). The significant difference between the experiments is are presented by (*) above the line: (*P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

The evaluation through flow cytometry of the expression of IFN- γ, IL-17, IL-4 and IL-10 in splenic $CD4^+$ T cells collected from mice, supports the existence of the shift to a Th2 response observed with liposomes from the first experiment and pointed above, since the liposomes from the second experiment induced lower proportions of splenic $IL\text{-}4^+$ $CD4^+$ T cells (P=0.0001). There was also a decrease in the frequency of splenic $CD4^+$ T cells producing IFN-γ regarding these liposomes. Nevertheless, these levels differ significantly from the other cytokines (Figure 13).

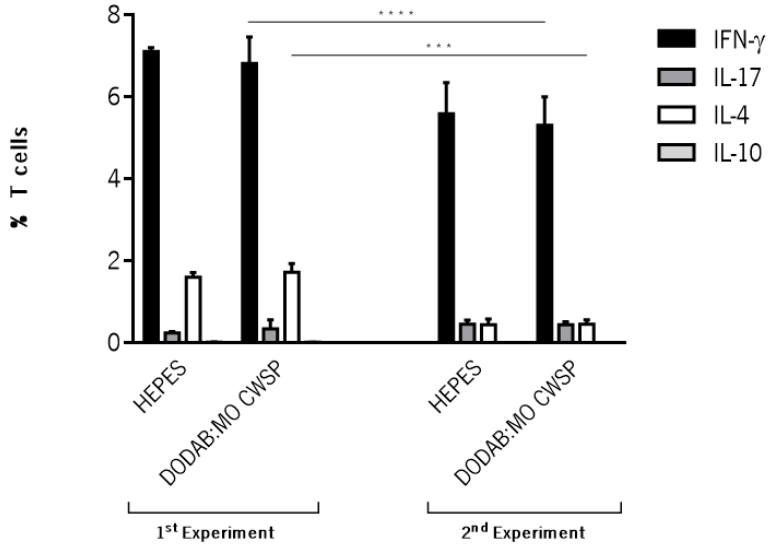

Figure 13. Frequency of $IFN\text{-}\gamma^+$, $IL\text{-}17^+$, $IL\text{-}4^+$ and $IL\text{-}10^+$ cells in splenic $CD4^+$ T cells from immunized mice, isolated three weeks following the last immunization). Bars represent mean ± SD of four mice used per group. Statistically significant differences between experiments are presented by (*) above the line: (*P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

Regarding CWSP-specific IgG1 antibodies, we observed that when liposomes were prepared through hydration of the lipid film with HEPES-buffer and sonication, IgG1 production was significatively greater than the control (HEPES-buffer), and also than liposomes prepared with water and no other technique to reduce the vesicles' size (1^{st} experiment). There were no differences between the latter and the control (Figure 14).

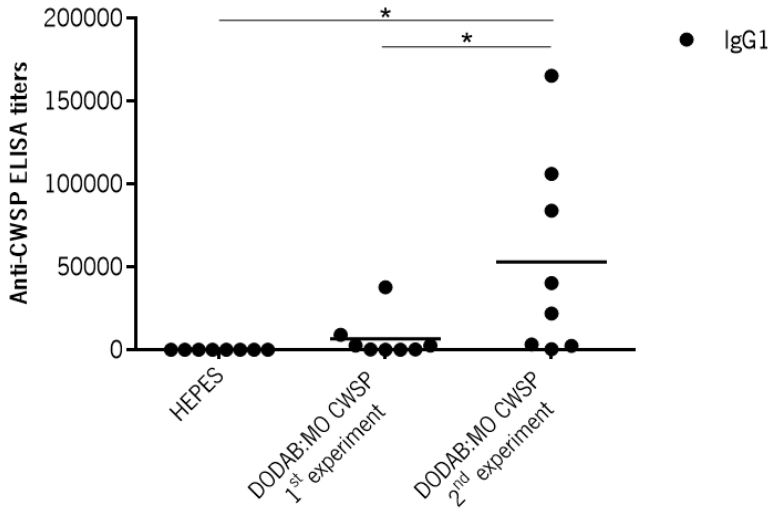

Figure 14. Anti-CWSP specific IgG1 response in serum of immunized mice. The antibody titers are expressed as the reciprocal higher dilution with and absorbance 2x higher than the value of control (no serum added). Each point represents an individual mouse, and horizontal lines correspond to the mean value in each group. Significant differences between groups are presented by (*) above the line: (*P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

4.3.2. The use of Cht3p as an antigen

4.3.2.1. Characterization of CWSP and Cht3p loaded liposomes

According to DLS and ζ - potential measurements, the extracted CWSPs were negatively charged (-11.3 ± 1.61 mV), and were relatively polydisperse (0.38 ± 0.09), with a mean size of 181,5 ± 16,66 nm. DODAB:MO liposomes loaded with the CWSPs, however, showed better polydispersity values (0.20 ± 0.06), with a size of 249.69 ± 29.34 nm and a ζ- potential of -8,53 ± 0,66 mV, as discussed in 5.3.1.

The ζ- potential of Cht3p alone was very similar to the CWSPs' (-9.32 ± 2.10 mV). The PDI values of Cht3p were around 0,54 ± 0,03 and measurements suggested a mean size of 655.8 ± 169.2 nm. Regarding the Cht3p-loaded liposomes, the values were almost identical to the empty liposomes, with a size around 82.94 ± 21.19 nm, PDI of 0.22 ± 0.01 and 51.73 ± 3.96 mV of ζ- potential (Table 3).

Table 3. Composition, mean size, PDI and ζ- potential of proteins and DODAB:MO liposomes.

	Empty Liposomes	Cht3p	DODAB:MO Cht3p	CWSP	DODAB:MO CWSP
Total lipid (μg/mL)	888	-	888	-	888
Protein added (μg/mL)	-	5	5	50	50
Mean Size (nm)	81.84 ± 19.77	655.8 ± 169.2	82.94 ± 21.19	181.5 ± 16.66	249.69 ± 29.34
PDI	0,20 ± 0.013	0.54 ± 0.034	0.22 ± 0.011	0.38 ± 0.09	0.20 ± 0.06
ζ- potential (mV)	55.17 ± 4.18	-9.32 ± 2.10	51.73 ± 3.96	-11.3 ± 1.61	-8.53 ± 0.66

The % EE of DODAB:MO CWSP liposomes, as established in 5.3.1., was 71.48 ± 3.35 %. Regarding DODAB:MO Cht3p liposomes, it was 76,87± 40 %. Gouveia-Dias (2019) reported a % EE of 65.8% when a 100 kDa Protein Concentrator ultrafiltration centrifugal device (Thermo Scientific) was used to evaluate this parameter, instead of ultracentrifugation.

4.3.2.2. Immunological characterization of CWSP- and Cht3p- loaded liposomes

Re-stimulated splenocytes from mice immunized with either DODAB:MO CWSP or DODAB:MO Cht3p secreted IFN-γ and IL-17A in higher levels, while splenocytes from mice immunized with HEPES-buffer or Cht3p alone had much lower production. Importantly, the levels of IFN-γ were significantly higher in mice immunized with DODAB:MO Cht3p than with Cht3p alone ($P < 0.0001$). Regarding, IL-17A levels, no differences were observed in splenocytes of mice immunized with the two DODAB:MO formulations, although its production seemed to be higher than in animals whose immunization was performed with HEPES-buffer or with Cht3p alone. The production of IL-4 and IL-10, remained low in every group of mice, with residual or absent production (Figure 15). Once more, the prevailing cytokines are shift to Th1/Th17 response.

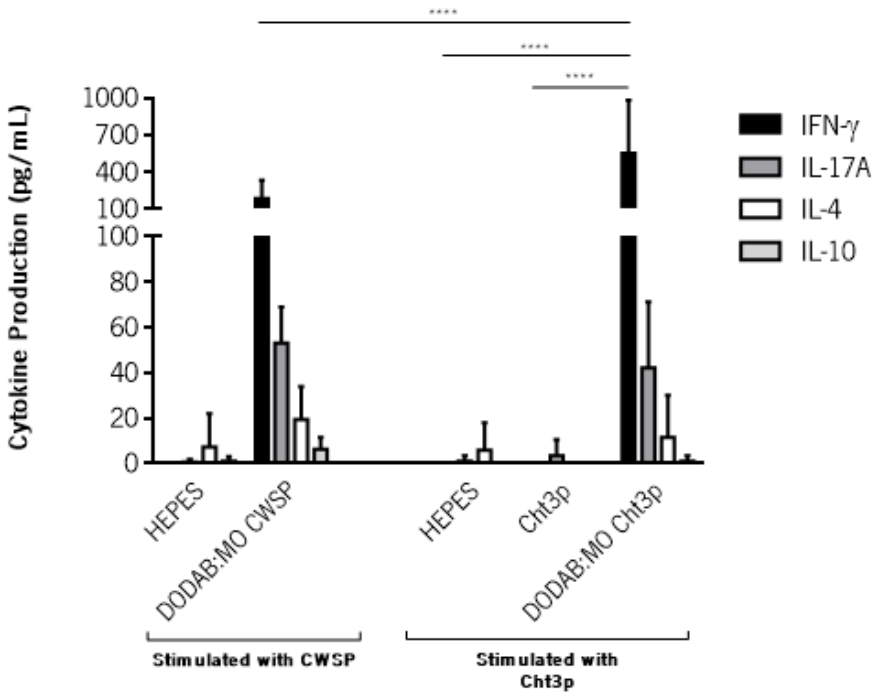

Figure 15. Cytokine production from CWSP and Cht3p re-stimulated splenocytes derived from immunized mice. Results indicate the mean ± SD of four mice per group. Significant differences between groups are presented by (*) above the line: (*P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

The frequency of splenic $CD4^+$ T cells producing IFN-γ is consistent with the results observed during the analysis of the cytokine production. Within groups, the levels of IFN-γ^+ $CD4^+$ T-cells differ significantly from the other cytokines, with $P < 0.0001$, but the differences between groups are small. The group immunized with HEPES-buffer, presented significantly lower levels of IFN-γ^+ $CD4^+$ T-cells than groups where Cht3p was present, either alone ($P = 0.0352$) or encapsulated ($P=0.0156$). When we analyze the group immunized with DODAB:MO CWSP, the levels of IFN-γ^+ $CD4^+$ T-cells are similar to the ones observed in the control group, but lower than the groups immunized with Chtp3 alone ($P=0.0021$) and DODAB:MO Cht3p ($P = 0.0008$). In what concerns the % of IL-17, IL-4 and IL-10 secreting cells, no differences were observed between groups (Figure 15).

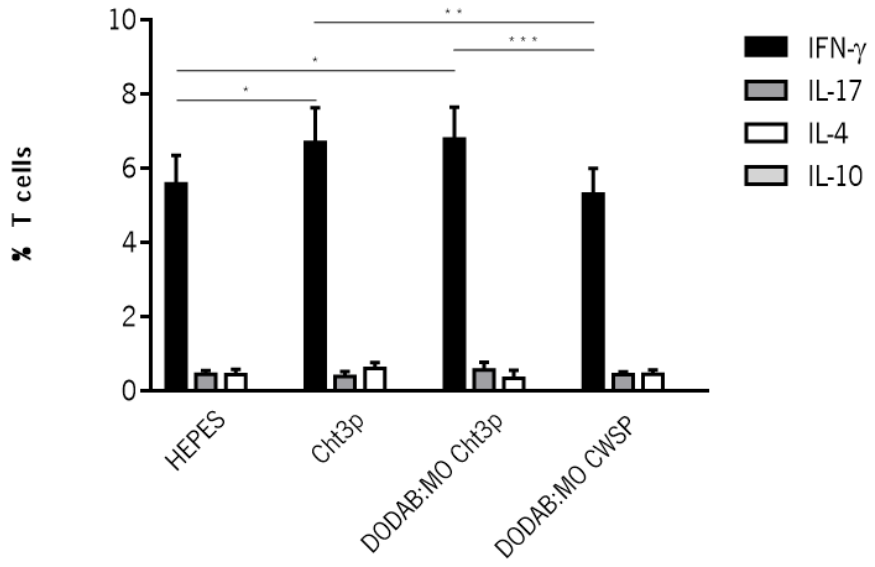

Figure 16. Frequency of IFN-γ$^+$, IL-17$^+$, IL-4$^+$ and IL-10$^+$ cells in splenic CD4$^+$ T cells from immunized mice, isolated three weeks following the last immunization). Bars represent mean ± SD of four mice used per group. Statistically significant differences between groups are presented by (*) above the line: (*P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

As a way to analyze the humoral response levels of Ch3tp-specific IgG1 antibody production in result to the immunization were also assessed. Immunization with Cht3p alone induced similar levels of IgG1 as immunization with HEPES-buffer, but when Cht3p was encapsulated in DODAB:MO liposomes, the IgG1 response was greater. However, CWSP-loaded liposomes still resulted in significantly higher levels of Cht3p-specific antibodies (Figure 17). When the amount of Cht3p present in our batch of CWSPs was analyzed, it turned out that within 50 μg/mL of CWSPs, 8.17 ± 1.13 μg/mL were Cht3p, which corresponds to 16.34% of total protein amount. Therefore, we realized that the lipid-Cht3p formulation prepared in this study carried less amount of this protein (5 μg/mL) than what was delivered with lipid-CWSPs formulation.

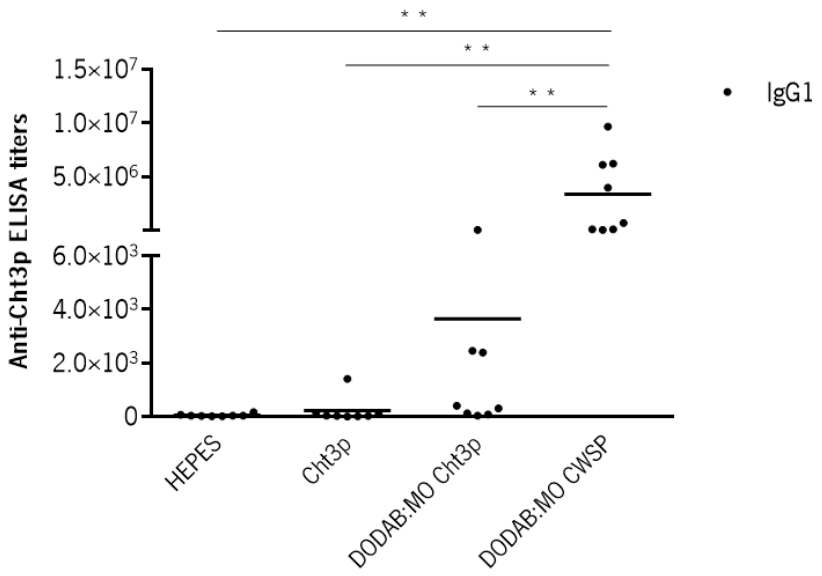

Figure 17. Anti-Cht3p specific IgG1 response in serum of immunized mice. The antibody titers are expressed as the reciprocal higher dilution with and absorbance 2x higher than the value of control (no serum added). Each point represents an individual mouse, and horizontal lines correspond to the mean value in each group. Statistically significant differences between groups are presented by (*) above the line: (*P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001).

4.3.2.3. Analysis of antibody specificities by Western Blotting

To identify to which of the multiple proteins mice had produced antibodies, CWSPs and Cht3p were incubated with serum from immunized animals in a western blot analysis. As we can observe in Figure 18, the serum from mice immunized with HEPES-buffer revealed the presence of a band of approximately 40 kDa, probably recognized by residual antibodies that the mouse already had. Mice immunized with Cht3p alone clearly recognized the recombinant protein however, the reactivity against the native Cht3p was almost undetected within 300s exposition on Film processor. On the other hand, when mice were immunized with the encapsulated Cht3p, the signal was much higher, needing less exposition time on Film Processor (60s) to reveal the band. In addition, we observed that this serum recognized the native Cht3p much better. What concerns the serum of mice immunized with DODAB:MO CWSP, results indicate the presence of antibodies that clearly recognized not only the native Cht3p, as expected, but also the recombinant one.

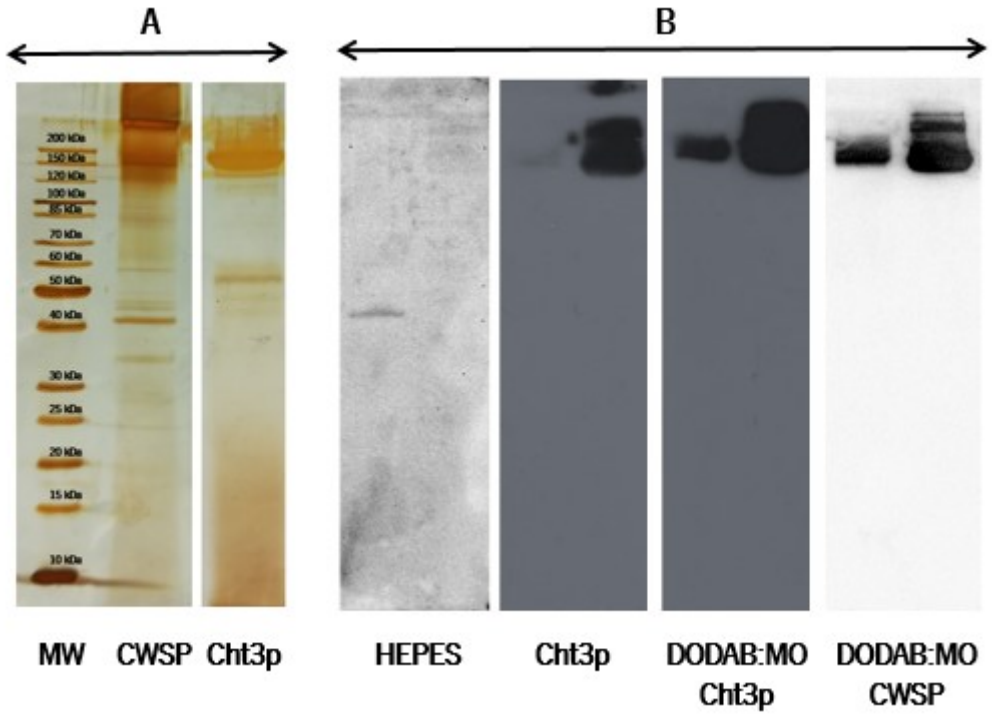

Figure 18. Analysis of antibody specificities by Western Blotting. CWSPs and Cht3p were separated on a 12% SDS-PAGE **(A)**, and transferred onto a PVDF membrane to be probed with serum from mice immunized with HEPES-buffer, Cht3p, DODAB:MO Cht3p or DODAB:MO CWSP **(B)**. PageRuler™ Unstained Protein Ladder (Thermo Fisher Scientific) was used as a molecular weight (MW) marker.

CHAPTER 5
Discussion

5. Discussion

Candida albicans, as a normal colonizer of the human healthy GI tract, remains in a harmless state most of the time. However, administration of broad-spectrum antibiotics leads to alterations in the balance of intestinal homeostasis, allowing this opportunistic pathogen to penetrate deeper tissue, enter the bloodstream and thus establish invasive infections, since the bacteria in the GI tract can no longer control the overgrowth of *C. albicans.*

In our group, a delivery and adjuvant system composed of DODAB:MO liposomes loaded with CWSPs had been developed and explored, revealing a 62,5% protection in a systemic *C. albicans* infection by inducing a specific Th1/Th17 response, along with a strong humoral response (Carneiro *et al.*, 2015; Carneiro *et al.*, 2016). Therefore, our main goal was to test the impact of the microbiome in the protective efficacy of this system. To this end, we chose a cocktail of colistin, streptomycin and ampicillin, a combination that has a high impact on the gut microbiota, since it targets both Gram-positive and Gram-negative bacteria (Candon *et al.*, 2015; Kennedy, King, & Baldridge, 2018).

Using the same methodology, we extracted the CWSPs from the same *C. albicans* strain, confirming the integrity of the cell membrane to ensure that we were extracting cell wall proteins as well. Although the final liposomes had exactly the same composition as the liposomes prepared by Carneiro, the preparation method was slightly different. While Carneiro prepared the empty liposomes as well as the proteins in 25 mM HEPES-buffer, in this study we prepared the empty liposomes in water and the proteins in 50 mM of HEPES-buffer, to obtain a final concentration of 25 mM of HEPES-buffer as well. The reason for this different method was that in previous studies we observed that DODAB:MO liposomes were more stable and less polydisperse when prepared with water than with HEPES-buffer.

The first thing we observed was that the liposomes prepared in the present study were quite different from the ones previously reported, presenting around 666 nm of mean size, while Carneiro obtained liposomes of approximately 223 nm. The surface charge density was also different: approximately - 2.30 mV in the present work contrasting with - 18.3 mV in Carneiro's study. However, since the composition was the same, we proceeded.

After the immunization protocol and before infection with *C. albicans*, the highest production of anti-CWSP antibodies was observed in the Vac group (immunized with DODAB:MO liposomes with CWSPs), followed by the Abx + Vac group (immunization with DODAB:MO CWSP liposomes and orally treated with antibiotics), although this difference was not statistically significant. This result indicated that the immunization protocol induced a humoral response however, the levels of serum IgG1 titers were much lower when compared to what Carneiro and co-workers reported. Regarding cytokine production of

splenocytes after re-stimulation with CWSPs, our results showed a clear increase of IL-4 in the Vac group that was reduced with the introduction of antibiotics during the immunization procedure (Abx + Vac group). Regarding IFN-γ, IL-10, and IL-17A the values were similar between all groups and the control. These results were not as expected since Carneiro pointed to a significant increase in IFN-γ and IL-17A (around 200-400 pg/mL) and an increase of IL 10 (around 100npg/mL), with comparatively low levels of IL- 4 (around 40 pg/mL). In this study, results suggest that the liposomes promoted a bias towards a Th2 profile in comparison with the liposomes prepared by Carneiro and that administration of antibiotics during the immunization reduced the Th2 bias profile.

After infection with *C. albicans*, the Vac group presented the highest frequency of splenic IL- 4 – producing $CD4^+$ T cells, during time, a tendency also observed in the Abx + Vac group. This bias towards IL - 4 production in the Vac group is in agreement with the cytokine production following splenocytes' stimulation with CWSPs before infection. Regarding the frequency of splenic IFN-γ – producing $CD4^+$ T cells there was a significant decrease of these cells from day 0 to day 3 after infection followed by a significant increase to 7 days after infection, but with no significant differences between groups. The frequency of splenic IL- 10 – producing $CD4^+$ T cells was very small. The frequency of splenic IL- 17 – producing $CD4^+$ T cells increased during the seven days of infection, but only significantly for the Vac and the Abx + Vac groups, suggesting that this increase was due to the immunization with DODAB:MO CWSP.

Interestingly, these two groups (Vac and Abx + Vac) were able to control the proliferation of *C. albicans* cells in the kidney, in comparison with the control group, particularly after 7 days post-infection, which is in agreement with the previous study. Surprisingly, the mice from the Abx group (oral administration with antibiotics) were also able to control the infection in comparison with the control. This result led us to analyze the sensibility of the *C. albicans* cells towards these antibiotics at the concentrations used, both alone and in combination. Results showed that growth of *C. albicans* was not affected by streptomycin and ampicillin, as expected, but colistin was able to reduce growth by more than 50%. This sensitivity could explain why the Abx group was also able to control proliferation of *C. albicans* in the kidney , but it has been described that following oral administration, colistin absorption from the GI tract is insignificant or even absent (Grégoire, Aranzana-Climent, Magréault, Marchand, & Couet, 2017; Guyonnet *et al.*, 2010; Rhouma *et al.*, 2015). In addition, the oral administration of colistin was performed only during the first 15 days, ending 35 days before the infection, so the fact that colistin could be the cause of this control in the proliferation *C. albicans* is unlikely but further studies are required to address this issue. However, this initial control in the proliferation *C. albicans* did not protect mice from succumbing to the infection and the Abx group was the only group in which all mice died within 50 days.

It was interesting to observe that 50% of the mice from the Abx + Vac group survived, suggesting that the immunization with DODAB:MO CWSP was able to protect mice treated with antibiotics ($P < 0.05$). Considering mice from Vac group, at the end of the experimental period only 37,5% survived the infection, as well as Control mice. Comparing the Vac group with the Abx + Vac there is no significant difference in the percentage of survival, although the survival was higher in the Abx + Vac group. This is not surprising since, as discussed previously, the presence of antibiotics during immunization seems to reduce the initial Th2 polarization, that is not protective in a systemic infection with *C. albicans*.

Overall, these results indicate that the liposomal system used in this study induced a shift to a Th2 response which was not expected since the previously work reported by Carneiro showed that the liposomal system of DODAB:MO CWSP induced a Th1/Th17 response that resulted in a positive outcome to the systemic *C. albicans* infection. These results are in agreement with the literature since Kong *et al.* (2018) evaluating the antifungal effect of paeoniflorin (PF) in a mouse model of systemic candidiasis, showed that it was detrimental for the host through the inhibition of Th1 and Th17 cells proliferation and the promotion of Th2 cells expression. In a similar way, Shi and co-workers (2018) concluded that inducing Th1-cell differentiation in and early stage of *C. albicans* systemic infection promoted mice survival, avoiding the inflammatory response in later stages, harmful for the host. Therefore, this Th2 response might have been important in a late stage of infection, rather than at the beginning, in order to limit the collateral damage induced by the inflammatory response, along with a Treg response. The introduction of antibiotics in parallel with the immunization protocol was mainly due to the fact that patients at risk of developing a systemic candidiasis are frequently under antibiotics' treatment. However, their use has been associated with a dramatic change in the composition of the gut microbiome, favoring the outgrowth of fungi. Our results seem to indicate that the changes at the gut microbiome by using this cocktail of antibiotics orally administered, had a protective role in the systemic candidiasis by reducing the Th2 response. Wheeler *et al.* (2016) refers that the disruption of commensal bacteria following antibiotic treatment, leads to a general Th2 polarization of the immune system. However, in our study, under a skewing to a Th2 response following immunization, it seems that the change in the composition of the gut microbiome reduced the Th2 response.

Standardizing liposomal formulations is challenging and complicated and since we observed differences in the biophysical characteristics of the liposomes used in this study comparing with the ones prepared by Carneiro, we further evaluated their implications in the type of T cell polarization induced. First, we analyzed the preparation method, that altered biophysical characteristics, and then we evaluated the use of a single antigen.

Regarding the liposome preparation method, since we observed that liposomes prepared in this study were bigger than Carneiro's, we prepared the them with the original protocol, in 25mM HEPES-buffer, and the proteins were also prepared in 25mM HEPES-buffer. Characterization of the new liposomes confirmed mean sizes to be smaller (≈ 249 nm) and similar to what was reported by Carneiro. However, although the surface charge density of the newly prepared liposomes turned out to be more negative (-8.53 ± 0.66 mV) than the first prepared, it was still not comparable to the Carneiro's works (around -18 mV).

Immunization with these new liposomes (2nd experiment) led to a significant increase in CWSP-specific IgG1 antibodies, almost 10x higher than the first liposomes prepared, which was more concordant with Carneiro's work. Surprisingly, the new liposomes induced splenocytes to secrete higher levels of IFN – γ and IL – 17 cytokines, along with lower levels of IL – 4 after re-stimulation with CWSPs, suggestive of a cellular response with a bias toward Th1/Th17 response, as described by Carneiro. These results also suggest that the preparation method, with a main effect on the vesicle size has a key role in the shift between Th responses, proposing that, with this liposomal system, increasing the size will shift from a Th1/Th17 to a Th2 immune response. Considering the literature reports, the results are somehow showing a different trend. Back in 1998, Brewer and co-workers, entrapping ovalbumin (OVA) in lipid vesicles, reported that immunization with vesicles with a mean size ≥ 225 nm induced a Th1-response, while smaller vesicles (≤ 155 nm) showed a shift to Th2, supported by IgG1 production. Later, in 2009, Mann *et al.*, by incorporating bile salts in lipid vesicles and using hemagglutininin as an antigen, produced two formulations. One of them consisted of a single population, ranging from 10 to 100 nm, while the other encompassed two populations, from 60 to 350 nm and from 400 to 2500 nm. Again, the larger vesicles generated an immune response with a bias to Th1, following analysis of the levels of IgG2a in the serum, while the smaller vesicles tended to a Th2 response. Badiee and co-workers (2012), encapsulated rgp63 (a recombinant major surface glycoprotein of *Leishmania*) in liposomes, observing that for small sized liposomes (100 nm), the tendency was also to Th2, accompanied by higher levels of IgG1 in immunized mice, when comparing to immunization with ≥ 400 nm liposomes, which shifted to Th1. The literature on liposomes, therefore, implies that the increase of vesicle size of liposomes is accompanied with a shift from Th2 response to a Th1-biased response. Contrastingly, when taking in to consideration non-liposomal formulations (namely polymeric or gold particles), a stronger Th1 response is induced by smaller particles (10 to 200 nm), while larger particles (200 – 500 nm) shift to a Th2 response (Benne *et al.*, 2016).

In what concerns liposome surface charge, cationic liposomes are often associated with efficient cellular delivery and induction of stronger immune response due to their interaction with the negatively charged membranes of APCs. The liposomes prepared in this study are cationic liposomes, with a surface charge of around 53 mV, however, when incubated with the CWSPs the final surface charge changes to a negative charge of -2.3 mV (1° experiment) to -8.5 mV (2° experiment). Bhowmick, Ravindran, & Ali (2007), for instance, by encapsulating soluble leishmanial antigens (SLA) in negative (- 33.5 ± 3.1 mV), neutral (4.3 ± 0.6 mV) and positively charged liposomes (28.4 ± 1.6 mV), reported that there was a dominance of a Th1 response following BALB/c mice immunization with the positively charged liposomes. Anionic liposomes, on the other hand, are considered weaker adjuvants and the studies examining their effect are still rather conflicting and inconclusive (Badiee *et al.*, 2009; Orr *et al.*, 2013).

It becomes clear that for understanding how size and surface charge influence immune response, there is still a long way, and more studies are needed. In addition, the liposome preparation method must be fully understood and optimized, taking in consideration some concerns related to DODAB stability following sonication that state that bath-sonication leads to a metastable state of DODAB, slowly evolving to a more polydisperse distribution, and making them inappropriate for encapsulation of molecules (Feitosa, & Brown, 1997; Feitosa, Karlsson, & Edwards, 2006).

The *in vivo* immunogenic studies related to the use of Cht3p as an antigen also provided some insights. Although there were almost no differences between groups in what concerns the frequency of IFN-γ^+, IL-17$^+$, IL-4$^+$ and IL-10$^+$ cells in splenic CD4$^+$ T cells from immunized mice, it was evident that, after re-stimulation with Cht3p, splenocytes of mice immunized with DODAB:MO Cht3p produced higher levels of all cytokines than splenocytes from mice immunized with Cht3p alone. These results are similar to what Carneiro *et al.* (2015) reported when comparing free CWSPs with DODAB:MO CWSP. In addition, the profile obtained with DODAB:MO Cht3p was very similar to the obtained with DODAB:MO CWSP (2° experiment), indicating the same polarization towards a Th1/Th17 response.

Regarding the humoral response, immunization with Cht3p alone induced very low levels of Cht3p-specific antibodies, but with the encapsulated Cht3p their levels were higher. Taking this into consideration, it is evident that Cht3p must be encapsulated in order to induce a better immune response. Western Blot analysis also supports this, because mice immunized with Cht3p alone needed more exposition time on the Film Processor to reveal the hybridization with the protein rather than mice immunized with the encapsulated Cht3p.

However, immunization with DODAB:MO CWSP (2° experiment) produced around 1000 times more Cht3p-specific antibodies. This result could be due to the fact that the native protein is much more

efficient in inducing the humoral response than the recombinant, to the presence of other proteins in the CWSPs that could help boost the development of these specific Cht3p response, or even to the amount of Cht3p present in the CWSPs, since in the crude extract of CWSPs (50 μg/mL) there was 8.1 μg/mL of Cht3p, while only 5.0 μg/mL were used in DODAB:MO Cht3p liposomes. Regarding Western Blot analysis we confirmed that anti-Cht3p antibodies induced by DODAB:MO CWSP liposomes were able to recognize the native Cht3p but also the recombinant. However, the anti-Cht3p antibodies induced by DODAB:MO Cht3p liposomes recognized very well the recombinant protein but the detection signal for the native protein was very low. This could be due to the low level of antibody induce by these liposomes. In immunoblotting results, we expected a range of proteins to able to hybridize with the antibodies present in their serum of mice immunized with DODAB:MO CWSP, as Carneiro and co-workers (2015) reported in their immunoblotting assays. Curiously, in our case, the induction of antibodies seems to be mainly towards Cht3p.

Taken together, our results indicate that the preparation method is important in the type adaptive cellular response, with liposomes with lower sizes shifting to a Th1/Th17 response that is more protective than a Th2 response against systemic candidiasis. The surface charge does not seem to affect significantly the type of cellular response since DODAB:MO CWSP with surface charge of -8.5 mV and DODAB:MO Cht3p with surface charge of 51.7 mV presented similar Th1/Th17 response, as well as the DODAB:MO CWSP from Carneiro´s work with a surface charge of -18.3 mV. The shift to a single protein, Cht3p, did not change the type of immune response, what was somehow expected since Cht3p was the main antigenic protein of the bulk of CWSPs, however the humoral response seems to be lower and some doubts about the efficient recognition of the native protein arise.

Although Carneiro's works demonstrated the efficiency of a DODAB:MO-based ADS containing CWSPs on eliciting a humoral and cellular immune response, as well as protecting mice against a *C. albicans* systemic infection, there are still key issues regarding its potential for future vaccine design. The batch-to-batch variability regarding the extracted CWSPs is still a drawback that does not allow the characterization and standardization of this formulation hence the need to explore the potential of Cht3p as an antigen, either by itself and within a multivalent formulation, which will be our main future objectives. In addition, the results regarding the treatment of mice with antibiotics were very interesting since it seems that the microbiome modulation through antibiotic treatment prompted a protective response resulting in improved control of infection, suggesting that this approach helped in the immune response against systemic candidiasis. It also seems that changing the intestinal microbiome with antibiotics helped in the protection. However, the liposomes used in the study were not ideal, once they clearly shifted the

immune response to a non-protective Th2 response. Thus, it is our objective in the future to repeat this analysis, using liposomes that induce a protective response

CHAPTER 6

Concluding Remarks and Future Perspectives

6. Concluding Remarks and Future Perspectives

6.1. Concluding Remarks

In conclusion, the main objectives of this thesis were achieved.

Regarding the administration of antibiotics, we observed that the microbiome modulation through this treatment led to a protective response and in an improved control of the infection, since the initial bias to a Th2 response induced by the liposomes obtained was reduced when mice were immunized with our liposomal system while receiving a cocktail of antibiotics.

In what concerns the liposome preparation method and the use of a single antigen, Cht3p, results indicated that changing the first parameter led to smaller liposomes, from 666 nm to 249 nm, and this decrease shifted the immune response from a Th2 to a Th1/Th17 response, along with a significant increase (10x) in CWSP-specific IgG1 antibodies. The shift to Cht3p, on the other hand, maintained the liposomes size, but changed the its surface charge from -8.53 mV to 51.73 mV, which did not change the type of immune response, showing the same polarization towards Th1/Th17.

6.2. Future Perspectives

Future investigation regarding both the role of the antibiotic treatment on a systemic *C. albicans* infections and the develop of a more reproducible and stable formulation to use in vaccination strategies include:

(I) Determine the changes in the gut microbiome due to the vaccination
- Analysis of the abundance of fungi and bacteria, and the proportional composition of the communities from frozen fragments of the GI tract of mice

(II) repeat the antibiotic treatment, using liposomes that induce a protective Th1/Th17 response

(III) explore the potential of Cht3p as an antigen, either by itself and within a multivalent formulation.

CHAPTER 7

References

7. References

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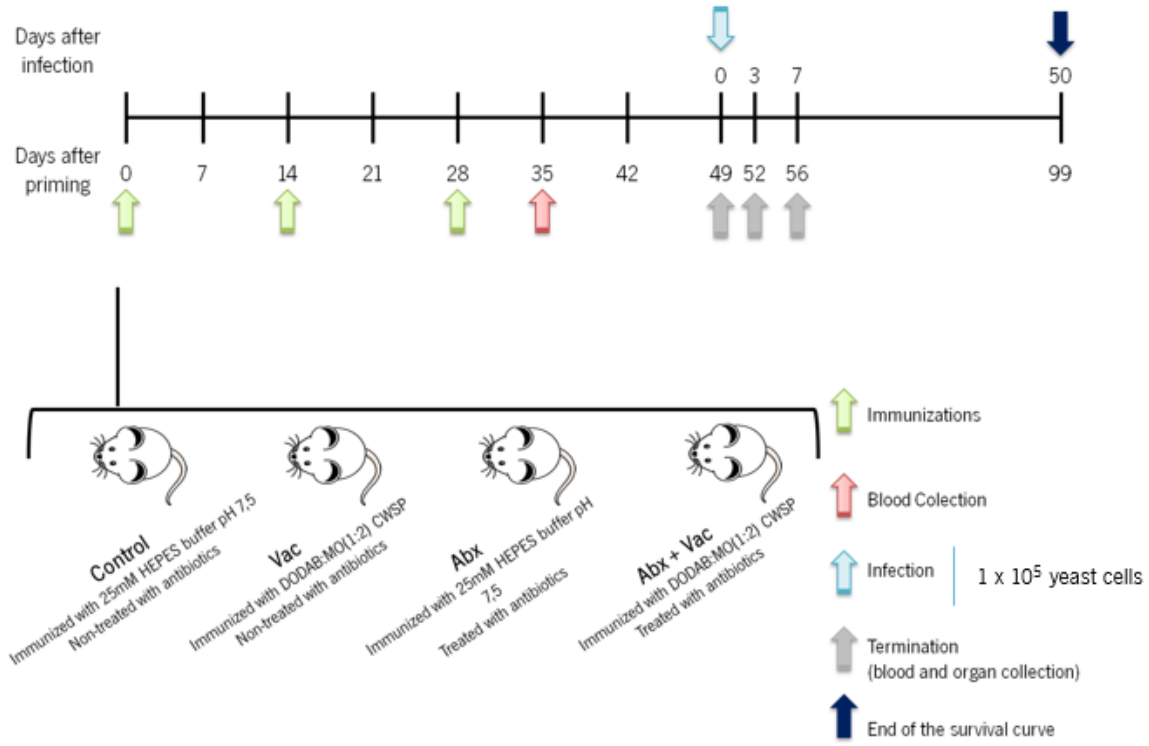
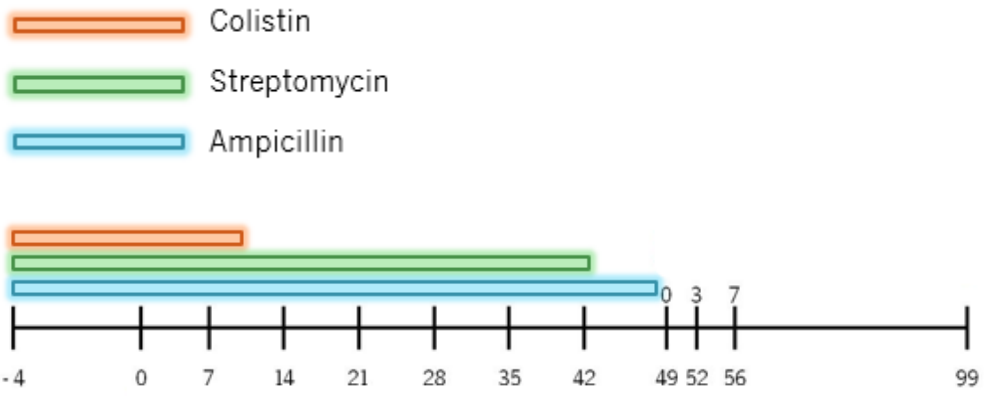
CHAPTER 8

Annexes

8. Annexes

Annex 1: Schematic representation of the immunization procedure and the treatment with antibiotics





Annex 2: Schematic representation of the immunization procedure (evaluation of the liposome preparation method and the use of a single antigen)